Research Paper

PREPARATION AND CHARACTERIZATION OF SERRATIOPEPTIDASE CONTAINING MICROSPHERES

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ABSTRACT

In the study we formulated the serratiopeptidase loaded cholic acid conjugated and unconjugated chitosan mucoadhesive microspheres. It was observed that the conjugated microspheres show better permeation rate than unconjugated microspheres. Moreover the permeation enhancer increased the % cumulative drug permeation through the mucosal membrane from 69.37 % to 74.39 % for unconjugated microspheres and conjugated microspheres respectively.

KEYWORDS Serratiopeptidase, cholic acid, chitosan mucoadhesive microspheres

1. INTRODUCTION

Microspheres can be defined as solid, spherical, free flowing, smooth surface particles ranging in size from 1 to 1000 µm. They are made up of polymeric, waxy or other protective materials that are biodegradable, synthetic polymers and modified natural product (starch, gums, proteins, fats and waxes). Microspheres improve effectiveness of drug therapy over conventional therapy.

A number of drugs which have been encapsulated into microspheres including anticancer drugs, antibiotics, antidiabetics, NSAIDS, hormones, proteins\peptides and tissues etc(Burgers, 1988).

The most important characteristics of microspheres are the microphase separation morphology which endows it with a controllable variability and degradation rate and also drug release. Advantage of microspheres based carrier is that they could be injected into the body in a suitable vehicle.

2. MICROSPHERES BASED ON BIODEGRADABLE POLYMERS

Biodegradable polymers have been applied as carriers for controlled delivery of low molecular weight drugs as well as bioactive proteins (Pekark, 1994). Biodegradable microspheres can be prepared from certain synthetic as well as natural polymers**.** An important requirement of such polymers is that the degradation products should be non-toxic because such products eventually enter systemic circulation or results in tissue deposition. Long term toxicological evaluation of the degradation products therefore is important in determining the clinical suitability of such carriers. The release rates of the drugs from biodegradable polymers can be controlled by a number of factors such as:

- Biodegradation kinetics of the polymers (Cicek, 1995)
- Physicochemical properties of the polymers and drugs (Abraham, 2003)
- Thermodynamic compatibility between the polymers and drugs (Abraham, 2004)
- Shape of the devices (Chen, 2001)

Biodegradable polymer particles (e.g., microspheres, microcapsules and nanoparticles) are highly useful because they can be administered to a variety of locations *in vivo* through a syringe needle. A variety of drugs, regardless of their molecular weights and water solubility, can be loaded into the biodegradable microparticles using different manufacturing techniques. Examples of biodegradable polymers used in microparticle preparation include polyesters, polyanhydrides, poly (orthoesters), polyphosphazenes and polysaccharides.

3. MATERIAL AND METHODS

All the chemicals were procured from HiMedia Lab., Mumbai, Merk India, Mumbai and CDH, New Delhi.

4. FORMULATION AND EVALUATION OF MICROSPHERES OF SERRATIOPEPTIDASE

The known amount of drug (serratiopeptidase) was mixed with chitosan solution in the preparation of microspheres as per method reported by Thanoo et al. 1992.

4.1 OPTIMIZATION OF PROCESS VARIABLES

The microspheres were prepared using modified method reported by Thanoo et al, 1992. A 4% w/v solution of chitosan was prepared in 5% aqueous acetic acid. The known amount of drug (serratiopeptidase) and 0.5% aprotinin (protease inhibitor) were mixed with chitosan solution. This solution was dispersed in 37.5 ml of liquid paraffin (1:1 ratio of light and heavy paraffin) containing 0.15 g of span 80 in a 100 ml beaker. This dispersion was stirred using stainless steel half moon paddle stirred at speed (1000-4000 rpm)for a 2 minute in ice bath and glutraldehyde saturated toluene solution (1 ml-4 ml) was added and stirred up (1-4 hr) (Patrick et al. 2010). After the stipulated stirring time, the microspheres were centrifuged washed several times with hexane and acetone. The microspheres were then freeze dried in lyophillizer.

The optimization of process variables have been done on the basis of shape and surface morphology, size morphology, size distribution, drug entrapment and swellability.

4.2 CHARACTERIZATION OF PREPARED SYSTEM

Various microsphere systems were characterized for following attributes:

4.2.1 Shape and surface morphology

SEM of microspheres system

Some formulations were also viewed under the microscope and SEM (scanning electron microscopy) photographs of different microspheres system are shown in photomicrograph no.1.

4.2.2 Size and Size Distribution

Microspheres were obtained from emulsion technique were studied microscopically for their size distribution studies using calibrated ocular eyepiece.

Effect of stirring time and glutaraldehyde concentration on the average particle size were studied in table 4.1 and represented graphically in fig. 4.1

Table 4.1: Effect of glutaraldehyde concentration and stirring time on average particle size

SR NO	Formulation Code	Average particle Size (um)
L	DGIT	41.637
2	DG2T	33.519
3	DG3T	29.478
4	DG4T	26.387
5	DGT1	43.173
6	DGT2	36.391
7	DGT3	31.573
8	DGT4	27.313

Where D- drug, G-glutaraldehyde, T-time

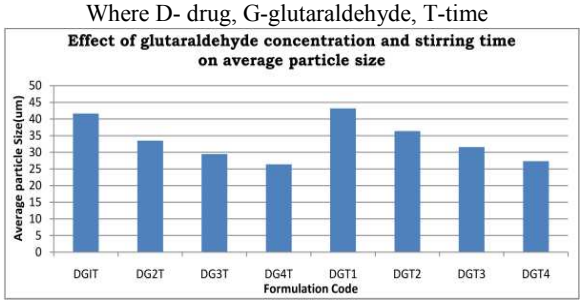

Figure 4.1: Effect of glutaraldehyde concentration and stirring time on average particle size

4.2.3 Percentage Drug entrapment /drug content

Determination of Superficial Drug

Weighed quantity of drug loaded microspheres was taken and suspended in 5 ml distilled water, one drop of tween 80 was added and then pH was adjusted to slightly acidic side. The suspension was shaken gently on wrist hand shaker for 5 min. The suspension was then centrifuged at 3000 rpm for 5 min. The supernatant was analyzed for drug content spectrophotometrically at 280 nm using UV/visible spectrophotometer.

Determination of entrapped drug

Microspheres obtained from the above washing were digested overnight in 5 ml of 0.5 M acetic acid and 5 ml NaOH and pH adjusted to slightly acidic side. The digested homogenate was diluted with PBS (pH 7.4) and centrifuged for 5 min and the drug content in supernatant was determined spectrophotometrically at 280 nm using UV/visible Spectrophotometer (Table 4.2 and Fig. 4.2) (Meenakshi, 1998).

Table 4.2: Effect of glutaraldehyde concentration and stirring time on Percent entrapped drug

Sr. No	Formulation code	Surface drug (%)	Drug entrapped (%)
1	DG1T	27.4 (±0.76)	71.06 (±0.49)
2	DG2T	27.1 (±1.53)	71.5 (±1.35)
3	DG3T	25.8 (±0.46)	71.9 (±0.78)
4	DG4T	22.1 (±1.37)	72.5 (±0.98)
5	DGT1	39.36 (±1 .61)	56.16 (±1.27)
6	DGT2	34.38 (±2.54)	61.25 (±1.31)
7	DGT3	29.53 (±0.38)	68.37 (±1 .73)
8	DGT4	28.17 (±0.41	70.56 (±0.85)

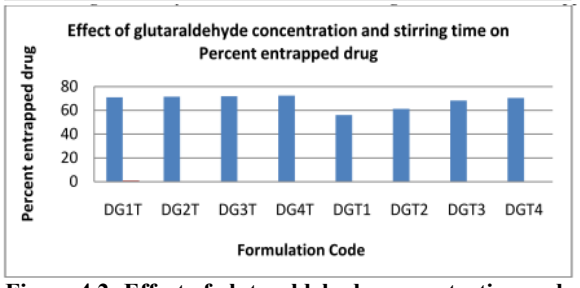

Figure 4.2: Effect of glutaraldehyde concentration and stirring time on Percent entrapped drug

4.2.4 Swellability

The swellability of the microspheres in physiological media was determined by swelling them in PBS Ph7.4 fluid. Accurately weighed amount of microspheres was immersed in little excess of PBS (pH 7.4) and kept for 24 hrs.

The swelling ability of chitosan is influenced by extent of cross–linking induced. The degree of swelling is little affected by the pH or ionic strength of media. The formula used for calculation of degree of swelling is as follows:

$$a = W_s - W_o / W_o$$

where

a= degree of swelling., W_s= wt of microspheres after swelling.

W_o= wt of microspheres before swelling.

Degree of swelling of various microspheres system is reported in Table 4.3 and presented graphically in fig 4.3 (Thanno et al.,1990).

Table 4.3: Effect of glutaraldehyde concentration and stirring time on Degree of Swelling

Formulation code	Degree of swelling
DG1T	0.8913
DG2T	0.8607
DG3T	0.7326
DG4T	0.6310
DGT1	0.8873
DGT2	0.8562
DGT3	0.8551
DGT4	0.8334

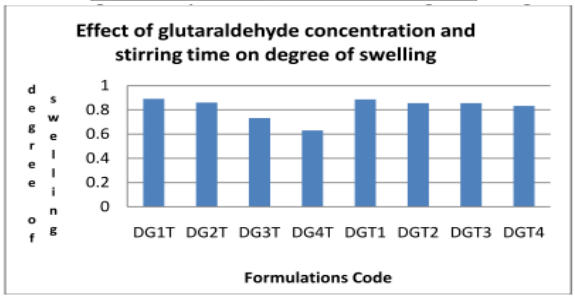

Figure 4.3: Effect of glutaraldehyde concentration and stirring time on degree of swelling

4.2.5 Evaluation of Mucoadhesion

Mucoadhesion of different microspheres system was assessed using the method reported by Mumtaz and Ching (1995) with little modification**.**

Preparation of mucosal membrane

For the purpose, she goat intestinal mucosa obtained from local slaughter house was used. It was cut and opened vertically. The intestinal mucosa was separated from the wall by scrapping off the connective tissue and placed in the Ringer's solution to prevent drying and to provide nutrition. After half an hour mucosa was removed and washed properly, cut into pieces of 5cm x 2cm and placed in well-aerated Ringer's solution.

Measurement of Mucoadhesion

A strip of intestinal mucosa was mounted on a glass slide. The microspheres (accurately weighed) in dispersion form was placed on it. This glass slide was incubated for 15 minutes in a desiccator at 90% RH to allow the polymer to interact with the membrane. Phosphate buffer saline (pH 7.4) previously warmed to 37±0.5°C was circulated over the microspheres and membrane at the rate of 1 ml/min. with the help of peristaltic pump. Washings were collected at different time intervals and microspheres were collected by centrifugation followed by drying at 50°C. The particles were collected and freeze dried at -40°C for 48 hr after that stored at 4°C.

The weight of microspheres washed out was taken and the observations are reported in table 4.4 and fig. 4.4.

Table 4.4: Effect of glutaraldehyde concentration and stirring time on percent mucoadhesion

Formulation code	Initial weight (g)	Weight of microspheres leached out after				% mucoadhesion
		1 h	2 h	3 h	4 h	
DG1T	0.1023	0.0067	0.0083	0.0099	0.0153	85.92
DG2T	0.1016	0.0056	0.0087	0.0103	0.0154	84.82
DG3T	0.1023	0.0069	0.0079	0.0109	0.0293	71.38
DG4T	0.1036	0.0086	0.0093	0.0117	0.0316	70.23
DGT1	0.1008	0.0049	0.0061	0.0089	0.0128	87.65
DGT2	0.1013	0.0052	0.0069	0.0083	0.0173	83.72
DGT3	0.1028	0.0073	0.0087	0.0099	0.0278	73.7
DGT4	0.1031	0.0067	0.0086	0.0112	0.0334	67.58

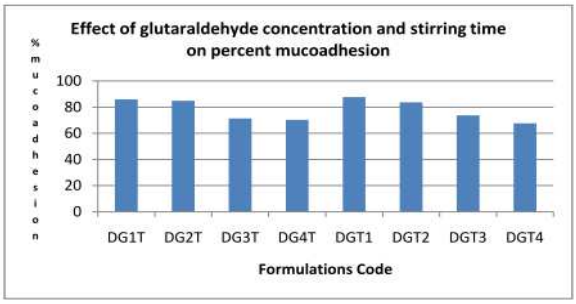

Figure 4.4: Effect of glutaraldehyde concentration and stirring time on percent mucoadhesion

4.3 OPTIMIZATION OF CONJUGATION

Degree of conjugation was optimized by taking three batches of particulate system with three different concentrations of ligands that are being conjugated. The degree of conjugation was determined by estimating unconjugated carrier concentration using colorimetry.

4.3.1 Optimization of cholic acid conjugated system

Microspheres and cholic acid were taken in 2:1, 1:1, 2:3 ratio respectively. These were dispersed in 10 ml of distilled water. This was cooled to 4°C and freshly prepared aliquots of EDAC [1 ethyl] 3(3-dimethylamino propyl) carbodiimide] (10 mg/ml, 100 µL) was added and reaction was allowed for 24 hr at 4°C.

4.3.2 Estimation of unconjugated cholic acid

Resultant conjugated system was separated by centrifugation (at 5000 rpm for 5 min) and filtered through 0.45 µm membrane filter. This resulted in separation of unconjugated and soluble part of cholic acid for removal of adsorbed portion of cholic acid; system was washed 2-3 times with distilled water.

Washing and supernatant were collected in 100 ml volumetric flask and final volume was made up to 100 ml with distilled water. Cholic acid content was determined by spectrophotometerically and further by Gregory and Pascoc test.

Table 4.5: Optimization, Development and Characterization

S No	Ratio taken MP:CA	Absorbance	Corresponding concentration of cholic acid (µg/ml)	Total concentration of unconjugated cholic acid	% conjugation
1	2:1	0.0613	1.795	0.0605	96.63
2	1:1	0.201	54.976	2.474	95.5
3	2:3	1.913	53.931	20.493	62.0

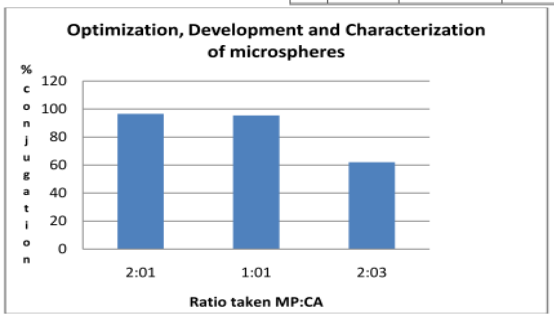

Figure 4.5: Optimization, Development and Characterization

Degree of conjugation was determined by measuring degree of cross Linking before and after conjugation. Degree of cross-linking was determined by method reported by Lowery (1990).

It is done by estimating free amino group of protein presented by measuring absorbance at 440 nm therefore this method can be used to estimate number of amino group presented at the surface of particle before and after conjugation.

4.3.3 Degree of Conjugation

10 mg of conjugated microspheres were dispersed in 10 ml of distilled water. Absorbance is measured at 440 nm. This measure the number of amino group presented before conjugation at the surface of particle.

Unconjugated Microspheres	Conjugated Microspheres	% Conjugated
0.8103	0.4139	49.025

4.4 CHARACTERIZATION OF CONJUGATED MICROSPHERS

4.4.1 Size and size distribution

Microspheres obtained from emulsion technique were studied microscopically for their size and size distribution using calibrated ocular eyepiece.

Effect of stirring time and glutaraldehyde concentration on the average particle size was studied in table 4.6 and represented graphically in fig 4.6.

Table 4.6: Effect of glutaraldehyde concentration and stirring time on average particle size in the case of conjugated microspheres

Sr no	Formulation code	Average particle size(µm)
1	DGITC	42.013
2	DG2TC	34.675
3	DG3TC	29.587
4	DG4TC	27.456
5	DGTIC	43.678
6	DGT2C	36.986
7	DGT3C	32.058
8	DGT4C	28.135

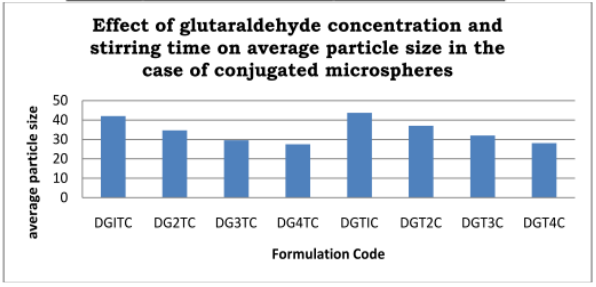

Figure 4.6: Effect of glutaraldehyde concentration and stirring time on average particle size in the case of conjugated microspheres

4.4.2 Percentage Drug Entrapment/Drug Content

Determination of Surface Drug

Weighed quantity of drug loaded conjugated microspheres were taken and suspended in 5 ml distilled water, one drop of Tween 80 was added and then pH was adjusted to slightly acidic side. The suspension shaken gently on wrist hand shaker for 5 min. The suspension was then centrifuged at 3000 rpm for 5 min. The supernatant was analyzed for drug content spectrophotometrically at 280 nm using UV/visible spectrophotometer.

Determination of Entrapped Drug

Microspheres obtained from the above washing were digested overnight in 5 ml of 0.5 M acetic acid and 5 ml of 0.5 M NaOH and pH adjusted to slightly acidic side. The digested homogenate was diluted with PBS (pH 7.4) and centrifuged for 5 min and the drug content in supernatant was determined spectrophotometrically at 280 nm using UV/visible Spectrophotometer (table 4.7 and fig 4.7).

(Meenakshi, 1998).

Table 4.7: Effect of glutaraldehyde concentration and stirring time on percent Entrapped drug in the case of conjugated microspheres

S no	Formulation code	Surface drug (%)	Drug entrapped (%)
1	DG1TC	27.4 (±0.76)	68.05 (±0.34)
2	DG2TC	27.1 (±1.54)	68.51 (±0.54)
3	DG3TC	26.8 (±0.42)	69.50 (±0.52)
4	DG4TC	23.1 (±1.32)	70.35 (±0.75)
5	DGTIC	36.36 (1.65)	55.62 (±0.32)
6	DGT2C	33.38 (±2.54)	60.01 (±0:23)
7	DGT3C	27.53 (±0.33)	67.36 (±0.37)
8	DGT4C	29.17 (±0.42)	68.18 (±0.62)

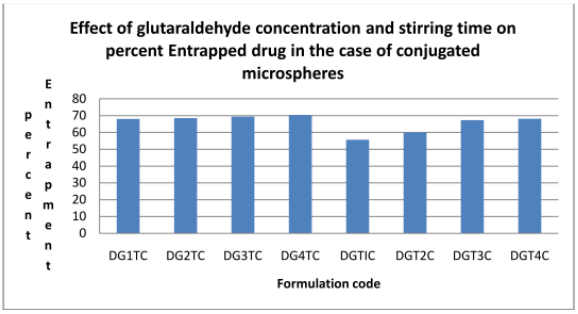

Figure 4.7: Effect of glutaraldehyde concentration and stirring time on percent entrapped drug in the case of conjugated microspheres

4.4.3 Swellability

The swellability of the microspheres in physiological media was determined by swelling them in PBS pH 7.4 fluid. Accurately weighed amount of microspheres was immersed in little excess of PBS (pH 7.4) and kept for 24 hrs.

The swelling ability of chitosan is influenced by extent of cross linking induced. The degree of swelling is little affected by the pH or ionic strength of media. The formula used for calculation of degree of swelling is as follows:

$$A = W_s - W_{o/} Wo$$

Where a= degree of swelling

Ws =wt of microspheres after swelling

Wo = wt of microspheres before swelling.

Degree of swelling of various conjugated microspheres system are reported in table 4.8 and presented graphically in fig. 4.8 (Thanno et al.,1990).

Table 4.8: Effect of glutaraldehyde concentration and stirring time on degree of swelling

Formulation code	Degree of swelling
DG1TC	0.8823
DG2TC	0.8515
DG3TC	0.7012
DG4TC	0.6132
DGT1C	0.8602
DGT2C	0.8481
DGT3C	0.8392
DGT4C	0.8378

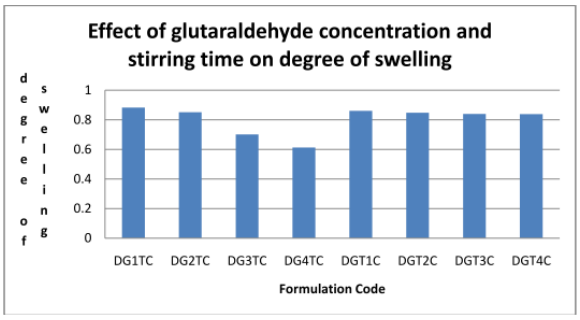

Figure 4.8: Effect of glutaraldehyde concentration and stirring time on degree of swelling

4.4.4 Evaluation of Mucoadhesion

Mucoadhesion of different microspheres system was assessed using the method reported by Mumtaz and Ching (1995) with little modification.

Measurement of Mucoadhesion

A strip of intestinal mucosa was mounted on a glass slide. Conjugated microspheres (accurately weighed) were placed on it. This glass slide was incubated for 15 minutes in a desiccator at 90%RH to allow the polymer to interact. Phosphate buffer saline (pH 7.4) previously warmed to 37±0.5°C was circulated over the microspheres and membrane at the rate of 1 ml/min. with the help of peristaltic pump. Washings were collected at different time intervals and microspheres were collected by centrifugation following by drying at 50°C. The weight of microspheres washed out was taken and the observations are reported in table 4.9 and fig 4.9.

Table 4.9: Effect of glutaraldehyde concentration and stirring time on percent mucoadhesion in the case of conjugated microspheres

Formulation code	Initial weight (g)	Weight of microspheres leached out after				% mucoadhesion
		1 h	2 h	3 h	4 h	
DGITC	0.1067	0.0057	0.0078	0.0099	0.0167	84.36
DG2TC	0.1058	0.0048	0.0069	0.0087	0.0178	83.19
DG3TC	0.1071	0.0061	0.0083	0.0113	0.0321	70.23
DG4TC	0.1054	0.0064	0.0081	0.011	0.0354	68.56
DGT|C	0.1081	0.0071	0.0092	0.0116	0.0151	86.63
DGT2C	0.1059	0.0049	0.0074	0.0105	0.0193	81.78
DGT3C	0.1063	0.0052	0.0078	0.0108	0.0333	69.23
DGT4C	0.1072	0.0061	0.0086	0.0115	0.0402	63.27

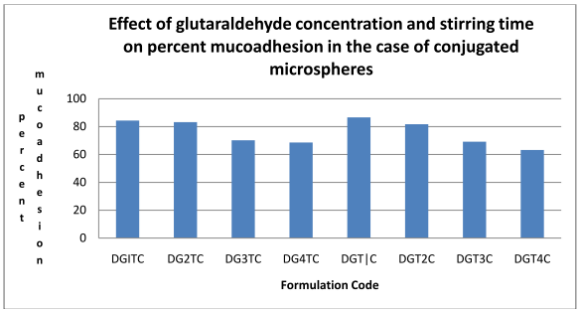

Figure 4.9: Effect of glutaraldehyde concentration and stirring time on Percent mucoadhesion in the case of conjugated microspheres

4.5 IN *VITRO* RELEASE STUDIES

In-vitro release profile provides the most sensitive and reliable information of in vivo availability of drug.

In vitro release kinetics indicates the release pattern and availability of drug under simulated condition. The drug release from different microsphere systems was determined using treated Modified diffusion cell. The diffusion cell was clamped in 50 ml SGF/SIF in beaker maintained at 37°C±2°C on hot plate cum magnetic stirrer.

The aliquots of sample were withdrawn at regular time interval and same volume of fresh media was added to replace the withdrawn sample. The samples were analyzed spectrophotometrically at 280 nm after suitable dilution using U/V visible spectrophotometer**.**

Finally the cumulative % in-vitro release was calculated from the data obtained and presented in the table 4.10 and 4.11.

Table 4.10: Cumulative % in vitro drug release

Time (H)	Effect of Glutaraldehyde concentration on Cumulative % drug release							
	SGF				SIF			
	1ml (DG1T)	2 ml (DG2T)	3 ml (DG3T)	4 ml (DG3T)	1 ml (DG1T)	2 ml (DG2T)	3 ml (DG3T)	4 ml (DG3T)
1	12.3	11.1	7.3	6.4	31.9	29.2	21.3	13.3
2	13.1	13.8	11.1	11.6	34.6	38.6	32.3	23.9
3	19.3	21.3	18.4	13.1	45.6	42.8	38.2	36.9
4	28.7	25.6	23.9	18.3	48.4	50.3	43.3	41.1
5	29.2	26.3	27.6	21.7	55.3	52.4	47.9	48.3
6	33.5	28.1	28.9	26.5	63.5	59.3	57.6	55.8

Table 4.11: Cumulative % in vitro drug release

Time (h)	Effect of Stirring time on Cumulative % in vitro drug release							
	SGF				SIF			
	1 hr (DGT1)	2 hr (DGT2)	3 hr (DGT3)	4 hr (DGT3)	1 hr (DGT1)	2 hr (DGT2)	3 hr (DGT3)	4 hr (DGT3)
1	12.3	13.6	9.8	7.2	33.6	31.3	23.2	5.6
2	17.1	17.8	14.3	11.6	39.3	31.9	31.5	11.9
3	25.3	23.1	16.2	15.1	47.3	3403	41.6	19.3
4	29.2	27.9	19.6	17.4	51.8	38.1	44.6	26.1
5	32.2	29.3	23.8	19.1	58.1	49.2	51.3	35.6
6	35.3	31.2	24.9	20.1	64.9	58.1	55.3	47.8

4.5.1 *Drug release studies*

Weighed amount of drug loaded conjugated and unconjugated microspheres was suspended in 50ml SGF/SIF and was taken in a Modified diffusion cell.

The diffusion cell was kept at 37±2 °C on hot plate cum magnetic stirrer. The aliquots of sample were withdrawn at regular time intervals and same volume of fresh media was added to replace the withdrawn samples.

The samples were analyzed spectrophotometrically at 280 nm after suitable dilution.

Finally the cumulative % in-vitro release was calculated from the data obtained and presented in the table 4.12 and 4.13.

Table 4.12: Cumulative percent drug release in the case of conjugated microspheres

Time (h)	Effect of Glutaraldehyde concentration on Cumulative % drug release							
	SGF				SIF			
	1 ml (DG1TC)	2 ml (DG2TC)	3 ml (DG3TC)	4 ml (DG1TC)	1 ml (DG1TC)	2 ml (DG2TC)	3 ml (DG3TC)	4 ml (DG4TC)
1	6.3	5.3	4.13	3.21	25.3	21.6	18.2	11.3
2	9.4	6.03	3.8	3.39	31.1	26.9	21.5	16.9
3	10.06	7.85	4.1	4.37	38.3	29.3	24.6	24.3
4	12.93	8.73	7.9	6.75	43.2	36.1	31.6	28.1
5	15.61	13.8	9.3	8.35	48.2	35.6	36.3	31.2
6	21.51	18.54	16.71	13.31	51.7	46.31	43.29	39.27

Table 4.13: Cumulative percent drug release in the case of conjugated microspheres

Time (h)	Effect of Stirring time on Cumulative % in vitro drug release							
	SGF				SIF			
	1 hr (DGT1C)	2 hr (DGT2C)	3 hr (DGT3C)	4 hr (DGT1C)	1 hr (DGT1C)	2 hr (DGT2C)	3 hr (DGT3C)	4 hr (DGT4C)
1	12.6	9.7	5.6	3.8	28.2	25.2	18.9	10.3
2	14.53	10.9	6.3	4.6	36.2	29.5	21.8	11.2
3	16.1	12.3	8.9	7.3	39.2	34.6	25.7	14.6
4	21.6	14.6	10.3	9.5	42.3	37.9	29.8	21.3
5	25.4	17.8	13.4	10.6	48.4	46.7	38.6	35.5
6	29.76	25.63	17.89	16.32	56.92	51.75	43.13	38.53

4.5.1.1 *Drug permeation through mucosal membrane*

For ensuring *in vivo* drug permeability through mucosal membrane was performed (Kimley, 1996).

Preparation of mucosal membrane

For this purpose female goat intestinal mucosa obtained from local slaughter house was used. It was cut and opened vertically. The mucosal layer was separated from the wall by scrapping off the connective tissue and placed in the Ringer's solution to prevent drying and to provide nutrition. After half an hour mucosal membrane was removed and washed properly cut into pieces of required size and placed in well aerated Ringer's solution.

Procedure

The mucosal membrane was placed on the modified diffusion cell. Donor compartment was filled with the weighted quantity of microspheres suspended in PBS

(pH 7.5) while the receptor compartment is maintained by PBS (pH 7.4) at 37±1°C. Aliquots of sample were withdrawn at regular time intervals. An equal volume of media was replaced immediately after withdrawal of samples. The samples were analyzed spectrophotometrically at 280 nm. The cumulative % of drug permeate through mucosal membrane was then calculated from the data obtained and is presented in table 4.14.

Table 4.14: Percent Cumulative Permeation

Hours	% Cumulative Permeation	
	DGT	DGTC
0.5	8.56	9.32
1	15.88	17.34
2	22.82	24.66
3	28.65	31.09
4	35.96	39.00
5	45.28	48.39
6	52.79	57.76
7	59.52	64.86
8	69.37	74.39

5. RESULTS AND DISCUSSION

The effect of various process variables such as glutaraldehyde concentration and stirring time were optimized. It was observed that these variables influenced the size, shape and size distribution, swellability as well as percent mucoadhesion of the microspheres. Hence, these parameters were optimized to prepare microspheres of small size with sufficient swellability and narrow size distribution. Time of stirring and cocentration of glutaraldehde were also optimized for maximum drug entrapment. It was observed that on increasing the glutaraldehyde concentration (1-4 ml), the drug entrapment efficiency of cross-linked microspheres was found to increase from 71.06 to 72.5% and on increasing the stirring time (1-4 h), the drug entrapment efficiency of cross-linked microspheres was found to increase from 56.16 to 70.56 % with increase in concentration of toluene saturated with glutaraldehyde, showing good solubility in oil medium. This could be explained due to uniformly availablility of cross-linking agent around the surface of the droplets.

Cross-linked chitosan microspheres were prepared by emulsion method reported by Thanoo et al (1992). Various formulations were studied microscopically for size and its distribution and the effects of glutaraldehyde concentration and stirring time on the particle size of the microspheres was observed.

On increasing the glutaraldehyde concentration (1-4 ml), the average particle size decreased from 41.637 um to 26.387 um and with increase in stirring time (1-4 h), the average particle size decrease from 43.173 µm to 27.313 µm. The shape of the particles was seen microscopically and the micro photographs were taken in dry condition, suggesting spherical shape of microspheres. Smooth and cross-linked surfaces are clearly indicated in scanning electron micrograph.

The swellability was observed by the method reported by Thanoo et al. (1990). The maximum swelling (.8913) was observed with the microspheres (DG1T) with less cross-linking (less quantity of glutaraldehyde) and swellability decrease from .8913 to .6310 with increasing the cross-linking density from (1-4 ml). This could be due to increasing the cross-linking of -OH groups of the polymer with the cross-linking agent. As on increasing the stirring time (1-4 hr) the swelling ability is decreased from (.8873-.8334)

Mucoadhesion study of different products was carried out by method reported by Mumtaj and Ching (1998). On increasing the concentration of glutaraldehyde (1-4 ml), the % mucoadhesion decrease from 85.92% to 70.23% and on increasing the stirring time (1-4 h), % mucoadhesion decreased from 87.65 % to 67.58%.

Quantitative evaluation of conjugated is done by determining the concentration, from that amount of conjugated ligand was calculated.

For optimizing the conjugation, microspheres and cholic acid were taken in 2:1, 1:1 and 2:3 ratios. The total concentration of conjugated cholic acid was found to be 0.1795 mg, 54.76 mg and 53.93 mg respectively, therefore degree of conjugation at 2:1, 1:1 and 2:3 ratio was found to be 96.63%, 95.5% and 62% respectively. Therefore optimum level for cholic acid conjugation was in 2:1 ratio.

It was observed that in the case of conjugated microspheres, on increasing the glutaraldehyde concentration (1-4 ml), the drug entrapment efficiency of cross linked microspheres was found to increase from 68.05% to 70.35% and on increasing in stirring time (l-4 h), the drug entrapment efficiency of cross linked microspheres was found to increase from 55.62% to 68.18%.

In the case of conjugated microspheres, on increasing the glutaraldehyde concentration (1-4ml), the average particle size decreased from 42.013 µm to 27.46 µm. With increase in stirring time (1-4 h), the average particle size decreases from 43.678 µm to 28.135 µm. In case of conjugated microspheres the swellability also decreases from (.8823- .6132) with increasing the cross linking density As on increasing the stirring time the swelling ability is decreased from (.8602-.8378) In case of conjugated microspheres on increasing the concentration of glutaraldehyde (1-4 ml), the % mucoadhesion decreases from 84.36 % to 68.56 % and on increasing the stirring time (1-4 h), % mucoadhesion decreased from 86.63 % to 63.27%.

The microspheres were assessed to establish the release kinetics of Serratiopeptidase. Thus, in order to carry out the release profile studies, modified diffusion cell was selected to support the formulation. Simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 7.5) were taken as diffusion medium. The in vitro release profile of various microspheres was studied .In case of unconjugated microspheres, the cumulative % in vitro release decreases from 33.5 to 26.5 % with increasing glutaraldehyde concentration (1-4 ml) in the presence of SGF (pH 1.2) and cumulative % in vitro release decreased from 63.5% to 55.8% with increase in glutaraldehyde concentration (1-4 ml) in the presence of SIF (pH 7.4).The cumulative % *in vitro* release decreased from 35.3% to 20.1% with increasing stirring time (1-4 h) in the presence of SGF (pH 1.2) and cumulative % *in vitro* release decreased from 64.3% to 47.8% with increasing stirring time (1-4 h) in the presence of SIF (pH 7.5).

In the case of conjugated microspheres the cumulative % in vitro release decrease from 21.51% to 13.31% with increasing glutaraldehyde concentration (1-4 ml) in the presence of SGF (pH 1.2) and % cumulative In vitro release decrease from 51.7% to 39.27% with increasing glutaraldehyde concentration (1-4 ml) in the presence of SIF (pH 7.4).

The cumulative % *in vitro* release decreased from 29.76% to 16.32% with increasing stirring time (1-4 h) in the presence of SGF (pH 1.2) and cumulative % *in vitro* release decreased from 56.92% to 38.53% with increasing stirring time (1-4 h) in the presence of SIF (pH 7.5).'

In the study of drug permeation through mucosal membrane unconjugated optimized microspheres and conjugated optimized microspheres were taken. The she goat intestinal mucosal membrane was used for in vitro drug permeation and the effect of permeation enhancer on drug permeation was also observed.

It was observed that the conjugated microspheres shown better permeation rate than unconjugated microspheres. Moreover the % cumulative drug permeation increased through the mucosal membrane from 69.37 % to 74.39 % for unconjugated microspheres and conjugated microspheres respectively.

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