

Studies on the Preparation and *in vitro* - *in vivo* Evaluation of Mucoadhesive Microspheres of Glycyrrhetic Acid Isolated from Liquorice

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Abstract

The present work deals with the formulation and evaluation of mucoadhesive microspheres containing glycyrrhetic acid. Mucoadhesive microspheres were prepared by single phase emulsification method and heat stabilization method. Chemical stabilization appeared as fast and simple technique for producing glycyrrhetic acid loaded microspheres. Microspheres prepared by this method produced sufficient production yield, high drug encapsulation efficiency and reproducibility. Among the different formulations, microspheres with drug - polymer ratio of 1:1 (C1) was found to have the maximum mucoadhesive property as well as *in vitro* and *in vivo* drug release property. Therefore, it could be considered as the best formulation.

Key words: Mucoadhesive, microspheres, glycyrrhetic acid, chemical stabilization, polymer, liquorice

Introduction

The evolution of drug delivery systems has indicated microspheres as an important part of novel drug delivery systems. Microspheres are solid and spherical particles that range in size of 1-1000 μm . They are made of natural or synthetic polymers. With enormous applicability of microspheres, they are becoming a realistic approach to drug delivery. However, the success of microspheres as a drug delivery system is limited due to their short sustaining time at the site of absorption. This limitation can be overcome by incorporating a mucoadhesive to the microspheres, which would be extending the sustaining time of microspheres at the site of absorption by providing an intimate adhesive contact of the drug delivery system with the absorbing membranes (Shukla and Tiwari, 2012; Sinha *et al.*, 2004; Sivadas *et al.*, 2008).

Mucoadhesive microspheres have efficient absorption and enhanced bioavailability of the drugs due to a high surface to volume ratio, a much more intimate contact with the mucus layer and specific targeting of drugs to the absorption site. The concept of mucoadhesion is most widely utilized in novel drug delivery systems. The mucoadhesion is the adhesive interactions between

polymers and mucus or mucosal surfaces. It refers to the state in which two surfaces, at least one biological in nature gets held together in close contact by interfacial forces for an extended duration of time (Chun *et al.*, 2005b; Chun *et al.*, 2005a; Soane *et al.*, 1999; Sun *et al.*, 2009).

Among the serious health problems of global concern, gastric hyperacidity and ulceration of the stomach mucosa are very common. Peptic ulcer diseases (encompassing gastric ulcer and duodenal ulcer) are induced by several factors, including stress, smoking, nutritional deficiencies, and ingestion of non-steroidal anti-inflammatory drugs. Peptic ulcer results due to imbalance between offensive (acid, pepsin and *Helicobacter pylori*) and defensive factors (mucin, prostaglandin, bicarbonate, nitric oxide and growth factors) (Hall, 2010). Mostly two main approaches are utilized for treating peptic ulcer. The first deals with reducing the production of gastric acid and the second with supporting gastric mucosal protection (Laurence *et al.*, 2011). Commonly available anti-ulcer drugs such as H_2 receptor antagonists, proton pump inhibitors and cytoprotectants have many side effects and therapeutic limitations (Tripathi, 2008; Brenner and

Stevens, 2009). Therefore, natural substances having anti-ulcer properties are preferred. Several plants and herbs have been used to treat gastrointestinal disorders, including gastric ulcers. One of the indigenous plants, *Glycyrrhiza glabra* Linn. (family: Leguminosae) is known to possess components that have antipeptic-ulcer activity (N and Chopra, 2006; Khare, 2007). The roots and rhizomes of *G. glabra* known as licorice is one of the most extensively researched medicinal plants and has a history of consumption for the past 6000 years (Mukherjee *et al.*, 2010). Glycyrrhetic acid is a beta-amyrin type triterpenoid compound having several activities like antiulcer, antibacterial, antifungal, antiviral, anticancer etc. Therefore, this paper deals with preparation and evaluation of mucoadhesive microspheres of glycyrrhetic acid for the treatment of peptic ulcer.

Materials and Methods

Materials: Crude Licorice was purchased from Meerut market during the month of October (2011) and authenticated by Dr. Anjula Pandey, Principal Scientist, National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The dried of licorice (*G. glabra*) was used for extraction of glycyrrhetic acid as ammonium salt and properly identified. A pure drug sample of glycyrrhetic acid (CAS Number 471-53-4) was purchased from Sigma Aldrich, USA as a reference compound. All other reagents and chemicals were of analytical grade and purchased from approved chemical vendors only. These reagents and chemicals include bovine serum albumin (BSA), glutaraldehyde, carbopol, liquid paraffin, acetone, silica gel, chloroform, ammonium hydroxide and hydrochloric acid.

Preparation of microspheres:

Chemical stabilization method: Mucoadhesive microspheres of glycyrrhetic acid ammonium were prepared by single phase emulsification method. Bovine serum albumin and carbopol 934P were used and glutaraldehyde was used as chemical cross linker. Prescribed amount (1500 mg) of the drug and polymers were dissolved in water. The mixture was added in a beaker containing liquid paraffin and shear was applied (100 rpm) to prepare primary emulsion at 15°C. Glutaraldehyde was added drop wise for cross linking of polymer at surface. After 6 hours microspheres were

separated by centrifugation. At last after washing with acetone thrice, microspheres were dried under vacuum. In this way four microsphere formulations with drug polymer at the ratio of 1:1, 1:2, 1:3 and 1: 4 were prepared and named as C1, C2, C3 and C4.

Heat stabilization method: In heat stabilization method, heat (raise in temperature condition) was applied for final rigidification and stabilization of the glycyrrhetic acid loaded microspheres. Bovine serum albumin and carbopol 934P were used. When heat was applied it denaturated the protein at surface. Prescribed amount of drug and polymers were dissolved in water and the mixture was added in a beaker containing liquid paraffin followed by application of shear (100 rpm) to prepare primary emulsion at 15°C. Consequently when heat was applied, linear increase in temperature up to 70°C causes rigidification at the surface. Afterwards 6 hrs centrifugation was done to separate the microspheres. Finally washing was done with acetone thrice and microspheres were dried under vacuum. Similarly, four microspheres formulations in the drug polymer ratio of 1:1, 1:2, 1:3 and 1: 4 were prepared and named as H1, H2, H3 and H4.

Characterization of microspheres

Particle size analysis, uniformity index and elongation ratio: Prepared microspheres were analyzed by optical microscopy to measure its size and shape. At first size was measured in an optical microscope (LEICA DM IL, Switzerland) with a calibrated ocular micrometer by randomly selection around 50 microspheres. Length, width and size of the microspheres were measured. The average size of the microspheres from each batch was expressed as the mean diameter (μm) \pm standard deviation (SD). The shape of the microspheres was estimated by measuring the elongation ratio (ER) which is the quotient of length to breadth of the microspheres. $ER = 1.1 < ER < 1.15$, and $ER > 1.15$ represent a perfect spherical and non-spherical shape, respectively (Das and Ng, 2010).

In order to determine the Uniformity Index (UI), following formula was applied

$$UI = D_w/D_n$$

Where, D_w and D_n are weight average diameter and number average diameter, respectively, and are calculated as follows:

$$D_w = \sum NiDi^4 / \sum NiDi^3, D_n = \sum NiDi / \sum Ni$$

Where, Ni is the number of particles with Di diameter.

Where, UI= Uniformity index (below 1.2 = monodisperse, above 1.2 = broad particle size distribution).

Scanning electron microscopy (SEM): Scanning electron photomicrographs of drug-loaded microspheres were taken. A small amount of micro-spheres was spread on aluminium stub. Afterwards, the stub containing the sample was placed in the scanning electron microscopy (SEM) chamber. A scanning electron photomicrograph was taken at the acceleration voltage of 30 KV, chamber pressure of 0.6 mmHg (Hardenia *et al.*, 2011).

Drug loading and encapsulation efficiency: Samples from each batch of microspheres were dissolved in a phosphate buffer solution (pH 7.4) and the actual drug content was determined by UV spectrophotometer (Model- 1601, Shimadzu, Japan). Encapsulation efficiency was calculated from the ratio of actual to theoretical drug content and expressed as a percentage (Yadav and Jain, 2011).

Percentage yield: The yield of production was calculated as the amount of microspheres obtained with respect to the theoretical content of microsphere. The calculation of percentage yield was done by using the following formula:

$$\text{Yield (\%)} = \text{Amount of microspheres obtained} / \text{Theoretical content} \times 100 \text{ (Yadav and Jain, 2011).}$$

Swelling index: A known weight (50 mg) of microspheres was placed in a glass vial containing 10 ml of distilled water at 37 ± 0.50 °C in incubator with occasional shaking. The microspheres were periodically removed, blotted with filter paper and their changes in weights were measured during the swelling until equilibration was attained. Finally, the weight of the swollen microspheres was recorded after a period of 3 hours, and the swelling ratio (SR) was then calculated from the formula (Shivanand *et al.*, 2010).

$$\text{Swelling Index} = W_e - W_o / W_o$$

Where, W_o = Initial weight of the dry microspheres,

W_e = weight of the swollen microspheres at equilibrium swelling in the media.

The studies were carried out in triplicate.

Mucoadhesion study: The mucoadhesive property of the microspheres was evaluated by *in vitro* adhesion testing method known as wash off method. Piece of goat stomach mucosa 1×1 cm was tied on to a glass slide using a thread. Approximately 100 microspheres were spread on to the wet rinsed tissue specimen and the prepared slide was hung on to one of the grooves of a USP tablet disintegrating test apparatus. The disintegrating test apparatus was operated whereby the tissue specimen was given up and down movements regularly in the beaker of the disintegrating apparatus, which contained the gastric fluid (pH 1.2). At the end of 30 min, 1 hr and thereafter at hourly intervals up to 4 hrs, the number of microspheres still adhering to the tissue was counted (Hardenia *et al.*, 2011).

$$\text{Percent mucoadhesion} = (\text{weight of adhered microspheres} / \text{weight of applied microspheres}) \times 100.$$

In vitro drug release: The drug release study was carried out using USP paddle type apparatus at 37 ± 0.5 °C with 100 RPM using 900 ml of 0.1N HCL solution as a dissolution medium. Five millilitre of aliquot was withdrawn at a pre-determined time intervals, up to 12 hrs. The medium was replaced with 5 ml of fresh buffer each time. The absorbance was measured by UV spectrophotometer at the wavelength of 291 nm and % cumulative release of the formulations was calculated (Shivanand *et al.*, 2010).

In vivo studies: *In vitro* studies and evaluation of the investigated microspheres were further approved by *in vivo* study. *In vivo* study was conducted in accordance to the guidelines prescribed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSCEA), India. Male albino rats of both sexes were selected and divided with three groups comprising minimum of 3 rats. All the animals were housed in standard cages. Group A was considered as Control group (positive control) administered with 80% ethanol. Group B and Group C were administered with pure famotidine (standard) and mucoadhesive microspheres C1, respectively. Peptic ulcer was induced by administering (80% ethanol) as necrotizing agent as well established for producing gastric lesions. All animals were administered by gastric intubation method. Assuming the gastric emptying time of fasted rats as 30 minutes pure glycyrrhizic acid and formulation C1 was administered 30 minutes

before the administration of the necrotizing agent. Rats were sacrificed thereafter under ether anesthesia 1 hr after treatment with ulcerogenic agent or necrotizing agent. Stomachs of the rats were excised and opened along the greater curvature. Excised stomach parts were washed with normal saline and lesions were quantified using a dissecting microscope with square grid eye piece to assess the formation of ulcers. Ulcerated as well as total areas were measured as mm² for each stomach and ulcer index were calculated using the following formula:

$$\text{Ulcer Index} = [\text{Ulcerated area/Total stomach area}] \times 100.$$

Results and Discussion

Preparation, drug loading and encapsulation efficiency of microspheres: Glycyrrhetic acid loaded microspheres were produced with high drug encapsulation efficiency. Chemical stabilization technique was generally characterized by high drug encapsulation efficiency. The yield of the production obtained in the range from 95 to 99 % (Table 2) for both chemical stabilization and heat stabilization techniques.

Characterization of microspheres

Particle size analysis & Scanning Electron Microscopy (SEM): The particle size of each microsphere formulation coded (C1 to C4 & H1 to H4) are reported in Table 1. Particle sizes of the microsphere formulations ranged from 2.87 to 7.23 μm . All the microspheres were found to be spherical to non-spherical and none were found to be perfect spherical. SEM micrographs of the microsphere formulations coded C1 to C4 reported in Figure 1. The microspheres exhibited irregular shape and crumpled surface. They seemed to be hollow microspheres, which collapsed during the preparation process. The micrographs belonging to the formulations H1 and H4 are shown in Figure 2. These microspheres exhibited spherical shape and smooth surface. The results showed that the drug/polymer ratio affected the morphological characteristics of the spray dried microspheres extensively. As the polymer ratio increased, more spherical microspheres with smoother surface were obtained.

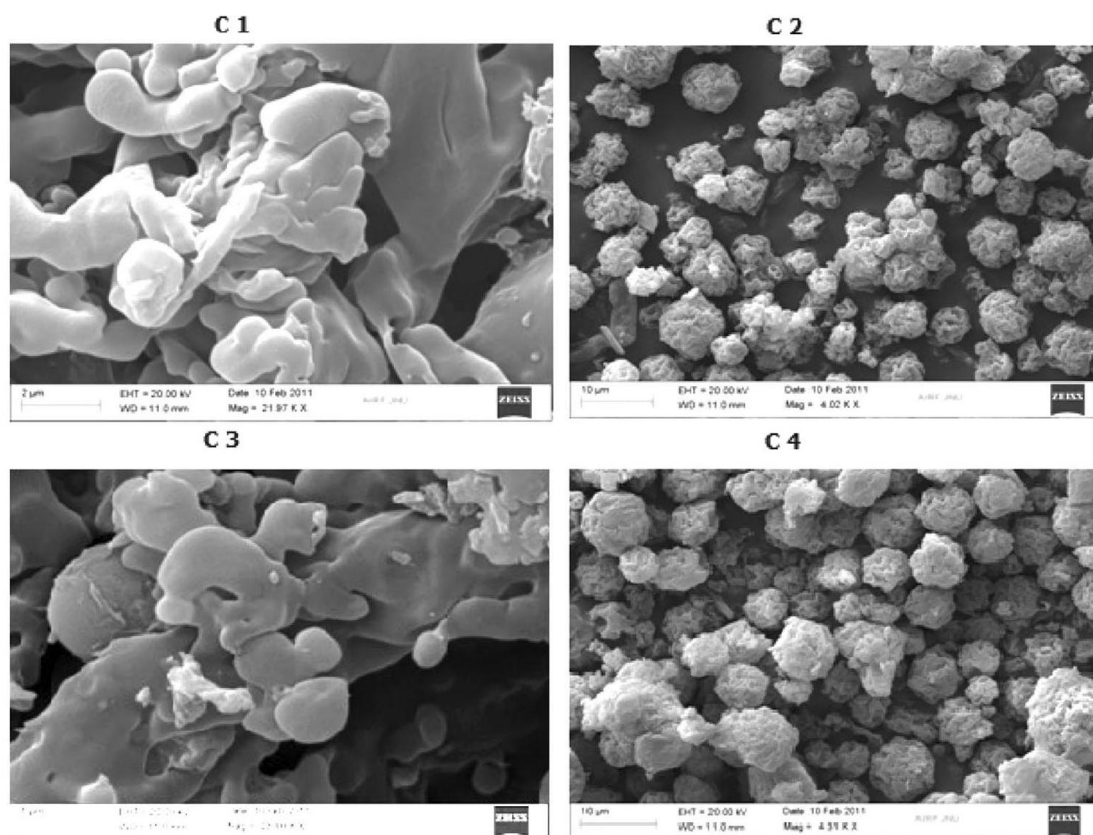


Figure 1. SEM Photograph of formulations C1, C2, C3 and C4.

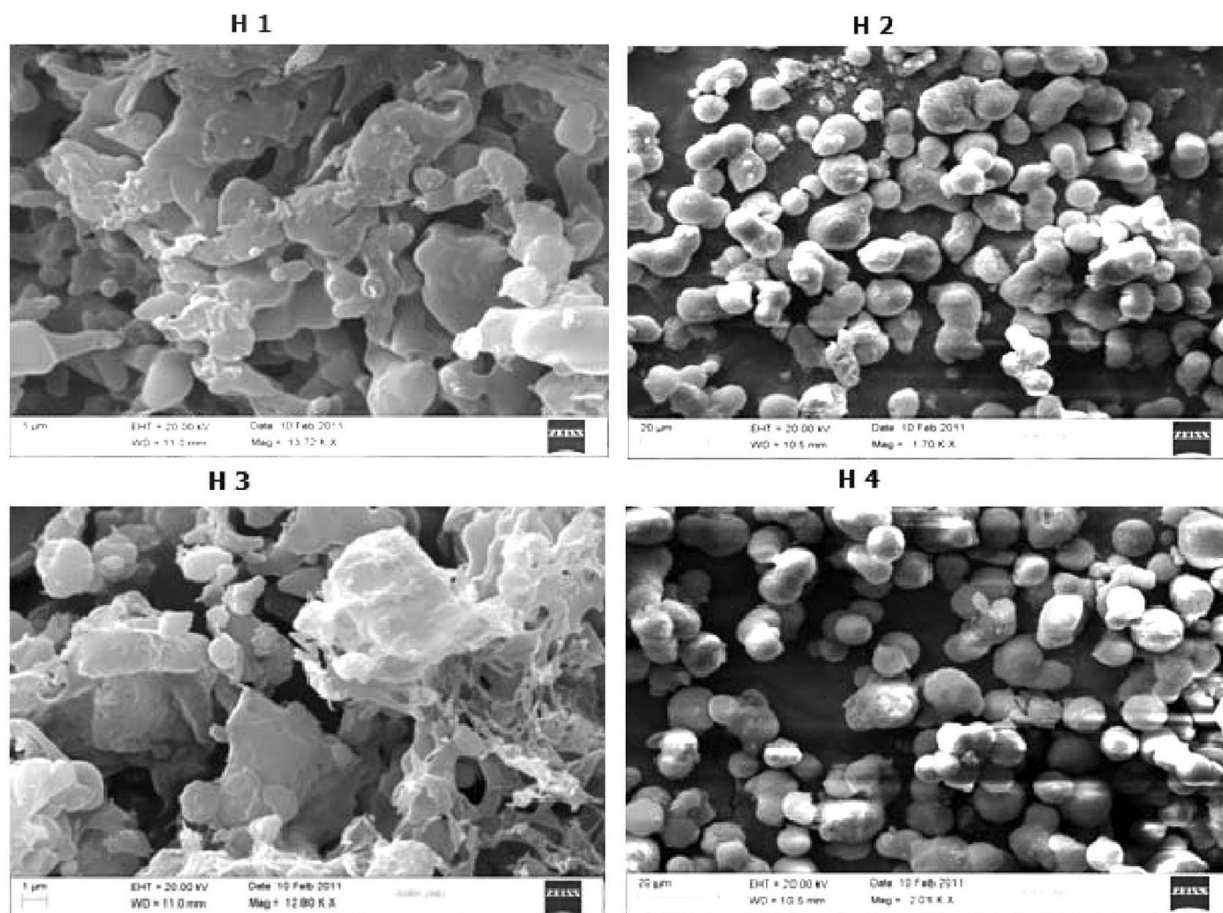


Figure 2. SEM Photograph of formulations H1, H2, H3 and H4.

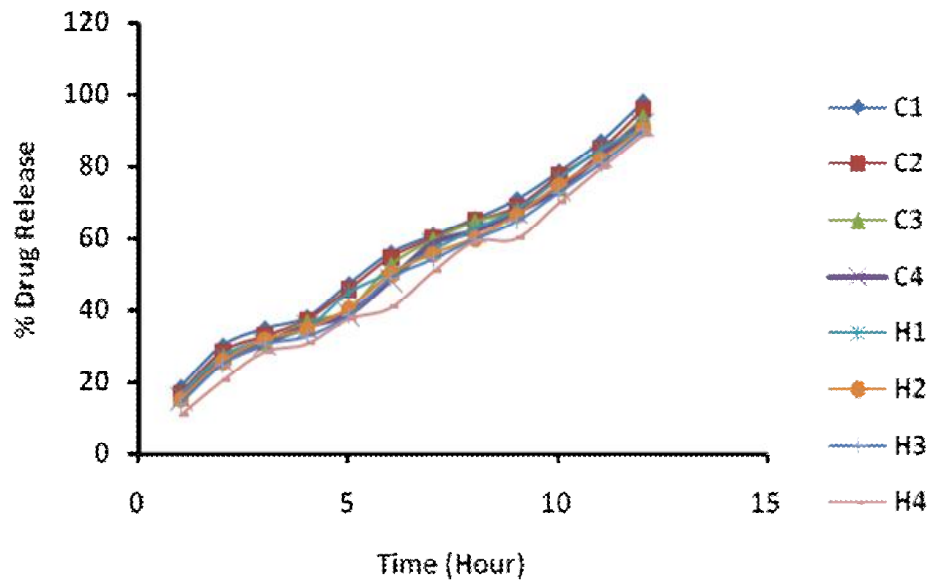
Percentage yield and swelling index: Percentage yield and swelling index of the microspheres are shown in table 1. The results clearly showed that all the formulations had sufficient swelling property as well as percentage yield. Formulation C1 and C2 had the highest swelling and percentage yield, respectively.

Mucoadhesive property and in vitro drug release: Percentages of mucoadhesion shown by microspheres are given in Table 2. The results showed that the microspheres had good mucoadhesive properties and could adequately adhere to stomach mucosa. The results also showed that the microspheres prepared by chemical stabilization method had better mucoadhesive property than those prepared by heat stabilization method. Since the highest percentage (76 ± 1.92) was obtained with C1 formulation, it was considered to be with good mucoadhesive property.

In vitro release profiles of glycyrrhetic microspheres are shown in Figure 3 and Table 3. The

percentage of the drug released was not the same for all formulations. The formulations coded C1 (drug/polymer ratio 1:1) and C4 (drug/polymer ratio 1:4) were found to be the highest and the lowest release profiles, respectively. This result showed that drug/polymer ratio affected the release rate of the drug. As the polymer amount increases, the release rates of drugs decrease.

In vivo study: The microsphere C1 was found to be good protection against gastric ulcers as observed *in vivo* studies. Mucoadhesive properties as well as the protective property of glycyrrhetic acid made this formulation effective against peptic ulcer. Its histopathology (Figure 4) and ulcerative index shows that formulation C1 shows a significant decrease in ulcer index ($P < 0.001$) as compared to the standard group. Presence of ample mucin and hence favoring the mucoadhesive property of the investigated microspheres C1 makes it a successful delivery system for treating peptic ulcer.

Figure 3. *In vitro* drug release at pH 1.2.**Table 1. Particle size, uniformity index, elongation ratio and shape of microspheres.**

Formulation	Particle size (μm) \pm SD	UI	ER	Shape
C1	6.32 \pm 0.12	1.321	1.23 \pm 0.06	Non spherical
C2	5.39 \pm 0.17	1.0989	1.08 \pm 0.09	Spherical
C3	4.38 \pm 0.12	1.3097	1.18 \pm 0.11	Non spherical
C4	6.21 \pm 0.19	0.89	1.11 \pm 0.09	Spherical
H1	2.87 \pm 0.09	1.898	1.21 \pm 0.08	Non spherical
H2	6.04 \pm 0.12	1.005	1.09 \pm 0.04	Spherical
H3	3.89 \pm 0.09	0.99	1.11 \pm 0.10	Spherical
H4	7.23 \pm 0.08	0.9933	1.06 \pm 0.09	Spherical

Where UI= Uniformity index (below 1.2= monodisperse, above 1.2= broad particle size distribution), ER= Elongation ratio (ER=1, Perfect Spherical / 1<ER<1.5= Spherical / ER>1.15= non spherical)

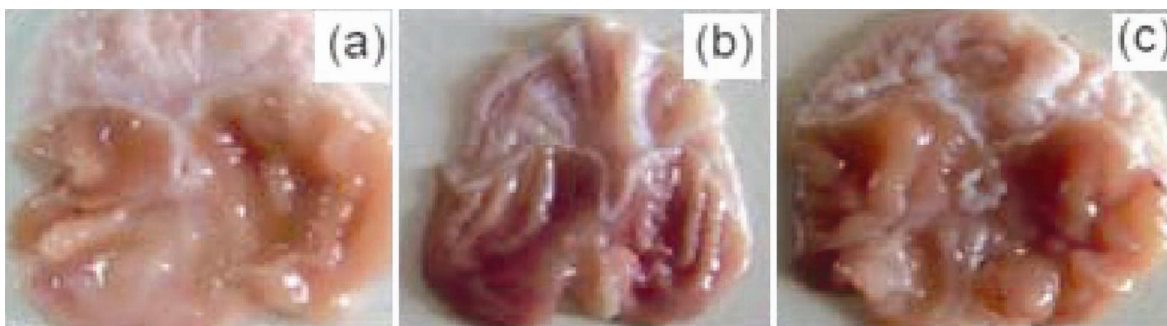
Table 2. Percentage yield, encapsulation efficiency, loading capacity, swelling index and % mucoadhesion.

Formulation	Percentage yield	EE	LC	Swelling index	% Mucoadhesion after 10 hr
C1	98.33 \pm 0.84	98.33	50.12	0.98 \pm 0.13	76 \pm 1.92
C2	99.0 \pm 1.12	99.0	50.34	0.23 \pm 0.02	64 \pm 1.23
C3	96 \pm 0.74	96.0	33.34	0.97 \pm 0.12	70 \pm 1.42
C4	96.33 \pm 1.08	96.33	34.78	0.19 \pm 0.1	56 \pm 1.4
H1	97.33 \pm 0.82	97.33	52.34	0.98 \pm 0.2	67 \pm 2.1
H2	97.66 \pm 0.94	97.66	49.56	0.23 \pm 0.2	54 \pm 1.43
H3	95.57 \pm 1.05	95.57	26.66	0.97 \pm 0.1	60 \pm 1.28
H4	95.67 \pm 0.89	95.68	32.67	0.19 \pm 0.11	49 \pm 13

Where, EE= Encapsulation efficiency, LC= Loading capacity

Table 3. *In vitro* drug release at pH 1.2.

Formulation	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	8 hr	9 hr	10 hr	11 hr	12 hr
C1	18.57	30.14	34.89	37.99	47.31	55.98	60.87	65.45	70.89	78.56	87	98
C2	16.99	28.56	32.88	37.41	45.89	54.86	60.21	65.01	68.99	77.55	85	96
C3	15.78	27.41	31.11	36.88	40.29	52.76	59.76	64.89	68.01	74.34	83.46	94
C4	15.41	25.66	30.77	35.56	38.99	48.54	58.57	62.11	67.45	73.69	83.01	92
H1	16.76	27.44	32.11	35.11	45.11	50.65	56.98	62.90	68.31	76.77	85.11	91
H2	15.42	25.89	31.99	34.89	40.56	50.12	55.76	60.18	66.56	74.89	82.11	90.67
H3	14.11	24.98	30.56	32.79	38.57	48.99	54.31	59.87	64.89	72.90	80.90	90.10
H4	10.99	20.61	28.67	30.88	37.78	40.89	50.87	59.45	60.23	70.11	79.77	89

Figure 4. *In vivo* studies demonstrating effectiveness of C1.

Conclusion

Chemical stabilization was found to be a fast and simple technique for producing glycyrrhetic acid loaded microspheres where they produced sufficient yield, high drug encapsulation efficiency and reproducibility from batch to batch. Owing to the observed high encapsulation efficiency and sufficient production yield, it can be concluded that chemical stabilization method was a simple and suitable technique for producing glycyrrhetic acid loaded microspheres. All the microspheres were at a suitable size and had good mucoadhesive property for administration.

It was concluded from the studies that the microspheres prepared by chemical stabilization and having drug:polymer ratio of 1:1 (C1) was best with respect to the *in vitro* drug release and mucoadhesive properties. The microspheres with drug:polymer ratio of 1:1 had the maximum mucoadhesive property as well as the drug release property, therefore, it was considered as the best formulation. Further *in vivo* studies also confirmed its potential property evaluated by *in vitro* studies. Thus *in vivo* study also revealed the mucoadhesive

microspheres as effective drug delivery system for treating peptic ulcer.

Declaration of interest

The authors declare that there is no any conflict of interest.

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