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Gastroretentive drug delivery system of levofloxacin hemihydrate: Development and evaluation of mucoadhesive microspheres for the treatment of *H. pylori* infection

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Abstract---The aim of the studies to formulate and develop mucoadhesive microcapsules of levofloxacin hemihydrates by using the mucoadhesive polymers for the treatment of *H. pylori* infection. Methods: Microspheres were prepared by Ionotropic gelation technique. To achieve the mucoadhesive and extended-release property in this study, sodium alginate, chitosan and calcium chloride for formulating levofloxacin hemihydrate microcapsules. *In vitro* drug release and *in-vitro* mucoadhesiveness. Results: To achieve the mucoadhesive and extended-release property in this study, mucoadhesive polymer, such as chitosan was used. Sodium dioctyl sulphosuccinate (DOSS) used as surfactant. The percentage yield for levofloxacin hemihydrate loaded microspheres was found to be in the

range of 32.11±1.25% to 87.28±1.67%. Drug content of the levofloxacin hemihydrate loaded microspheres varied from 20.21±1.01% to 30.77±1.88%. The encapsulation efficiency of the prepared microspheres varied from 51.77±1.33% to 78.54±1.55%. The mean diameter of levofloxacin hemihydrate loaded microspheres were found to be in the range of 135.5±3.64 µm to 448.59±5.44µm Mucoadhesive property of the prepared microspheres varied from 45.81±1.32% to 82.97±0.82%. A significant decrease in the rate and extent of drug release was observed with the increase in sodium alginate concentration in beads. Regard to release kinetics, the data best fits in the Higuchi model and showed zero order release with a non-Fickian diffusion mechanism. Based on the mucus turnover rate and dissolution time, best formulations were selected. No remarkable changes were observed in drug content, mucoadhesiveness and *in vitro* drug release in stability studies.

Keywords---mucoadhesive polymers, levofloxacin, microcapsules, *In-vitro* drug release.

Introduction

Helicobacter pylori (H. Pylori) are the causative agent of chronic gastric infections, and it has been estimated that at least half of the world's population is infected. A recent meta-analysis on the global prevalence of *H. pylori* infection has shown an overall prevalence of 44.3% [1]. Socio-economic status, together with the level of urbanization and sanitation conditions, likely reflects the differences of *H. pylori* prevalence from country to country [2]. After it has transited to the gastric lumen, *H. pylori* localizes to specific locations such as the antrum and corpus, where it is well adapted to survive in acidic conditions and establish persistent infection [3]. Once infection is established, several gastro-duodenal complications such as gastritis, gastric ulcer, duodenal ulcer, dyspeptic symptoms, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) B-cell lymphoma may develop [4].

Materials and Methods

Materials

Levofloxacin hemihydrate was purchased from Gold sun Pharmaceuticals limited, Mumbai, Chitosan was purchased from Nalinc Pharmaceuticals limited, Mumbai, sodium alginate and calcium chloride was purchased from Nice Chemical, Bangalore Hydrochloric acid and sodium dioctyl sulphosuccinate was purchased from Qualigens fine chemicals, Mumbai. Acetic acid was purchased from SD Fine chemicals, Mumbai; Methanol was purchased from Merck specialties Pvt. Ltd. Mumbai.

Formulation of mucoadhesive microspheres

Chitosan (CS) and sodium alginate (ALG) microcapsules of levofloxacin hemihydrate were prepared by ionotropic gelation technique.[5] Weighed quantity of micronized levofloxacin hemihydrate powder was suspended thoroughly in the ALG solutions (1-3% w/v) in de-ionized water containing 0.075%w/v sodium dioctyl sulpho succinate (DOSS) as surfactant by vigorous stirring for 10 minutes. The ALG- levofloxacin hemihydrate mixture was directly sprayed using syringe into the calcium chloride solution (2.0% - 4%w/v) containing Chitosan (CS) (1.0% w/v, previously dissolved in acetic acid solution (0.5% v/v). The microcapsules were allowed to harden for 90 minutes before washing them twice with distilled water and dried at 37°C in an oven overnight and the final dried mass was recorded.

Table 1
Formulation of mucoadhesive microsphere

Formulation code	Sodium Alginate (% w/v)	Chitosan (% w/v)	Calcium chloride (% w/v)	Levofloxacin hemihydrate (% w/v)
LCAM1	1	1	2	2.5
LCAM 2	2	1	2	2.5
LCAM 3	3	1	2	2.5
LCAM 4	1	1	3	2.5
LCAM 5	2	1	3	2.5
LCAM6	3	1	3	2.5
LCAM 7	1	1	4	2.5
LCAM 8	2	1	4	2.5
LCAM 9	3	1	4	2.5

Evaluation of mucoadhesive microspheres
Determination of percentage yield of microcapsules [6]

Prepared microcapsules were collected and weighed accurately using a digital balance. The percentage yield of prepared microcapsules was calculated by using the formula mentioned below:

$$\text{Percentage yield of microcapsules} = \frac{\text{Weight of microcapsules of obtained}}{\text{Total weight of drug and polymers}} \times 100$$

Determination of drug content and encapsulation efficiency [7]

The drug content of the microcapsules was measured by extraction method. Accurately weighed 5 mg of mucoadhesive microcapsules were crushed in to a powder using mortar and pestle. The crushed microcapsules were placed in 100 mL of 0.1 N HCl (pH 1.2) and stirred for 2 hours using magnetic stirrer (100 rpm) at 37 ± 0.5°C. The samples were then filtered to obtained clear solution and analyzed for the drug content UV.

$$\text{Drug content in microcapsules} = \frac{\text{Weight of drug in microcapsules}}{\text{Weight of microcapsules}} \times 100$$

$$\text{Encapsulation efficiency} = \frac{\text{Actual drug encapsulated}}{\text{Theoretical drug encapsulated}} \times 100$$

Particle size analysis [8, 9]

Particle size of the drug, excipients and prepared microcapsules were measured by using laser based particle size analyser (780 Accusizer, Particle sizing systems Inc, USA).The particles were dispersed inn-Hexane, and suspended mechanically by magnetic stirring during the analysis.

Shape and surface characterization

The shape and surface characteristics of the microcapsules were observed under a Scanning Electron Microscope (SEM). HITACHI-SEM MODEL S – 450 model scanning electron microscope was used for the study. The prepared microcapsules were placed directly on to the SEM sample holder by using double-sided fixing tape and coated with gold film (thickness 200 nm) under reduced pressure (0.001 torr) and photographed.

***In vitro* evaluation of mucoadhesiveness** [10]

A periodic acid/Schiff (PAS) colorimetric method reported by Mantle and Allen[166] was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the Levofloxacin hemihydrate mucoadhesive microcapsules and its effect on the assessment of mucoadhesive behaviour of prepared mucoadhesive microcapsules. Two reagents were prepared. Schiff reagent contained 100 mL of 1% basic fuchsin (pararos aniline) aqueous solution and 20 mL of 1 M HCL. Sodium meta bisulphite (0.1 g) was added to every 6 mL of Schiff reagent before use, and the resultant solution was incubated at 37°C until it became colourless or pale yellow. Periodic acid reagent was freshly prepared by adding 10 µl of 50% periodic acid solution to 7 mL of 7% (V/V) acetic acid solution. Standard calibration curve were prepared from 2 mL of mucin standard solutions (0.25, 0.5, 0.75, and 1 mg/2 mL).

After adding 0.2 mL of periodic acid reagent, the samples were incubated at 37°C for 2 hours in a water bath. Then, 0.2 mL of Schiff reagent was added at room temperature. Thirty minutes later, the absorbance of the solution was recorded at 555 nm in calibration a UV spectrophotometer (Spectronic 20D). Triplicate samples were run. All the samples were determined with the same procedure. The mucin content was calculated from the standard calibration curve. As comparison, the mucoadhesive potential of microcapsules was also assessed with the above procedure. Each experiment was performed 3 times and standard deviation noted.

Adsorption of Mucin on Chitosan Microcapsules [10, 11]

Mucin aqueous solution with different concentrations (0.025, 0.05, 0.1, 0.2, and 0.5 mg/mL) were prepared. Levofloxacin hemihydrate mucoadhesive microcapsules (20 mg) were dispersed in the above mucin solutions, vortexed, and shaken at room temperature.[167]then, the dispersions were centrifuged at 4000 rpm for 2 minutes, and the supernatant was used for the measurement of the free mucin content. The data obtained were interpreted using Freundlich or Langmuir equations describing the adsorption isotherms:

$$C_{ads} = KC_e^n$$

$$C_{ads} = \frac{aC_e}{b + C_e}$$

Where Cads is the concentration of mucin adsorbed at equilibrium and C_e is the concentration of free mucin at equilibrium. Values of different constants were obtained from the graphs of the above equations. For the Langmuir equation, $1/_{Cads}$ was plotted against $1/_{Cfree}$ to get the constants and for the Freundlich equation, log $_{Cads}$ was plotted against C_{free} to get the constants. The mucin adsorption is estimated using the Equation

$$\text{Mucin adsorption (\%)} = \frac{\text{Total mass of mucin} - \text{free mucin}}{\text{Total mass of mucin}} \times 100\%$$

Compatibility studies [12, 13]
Fourier-Transform Infrared Spectrophotometry (FTIR)

Infrared red spectra for pure Levofloxacin hemi hydrate polymers, blank microcapsules, Levofloxacin hemihydrate mucoadhesive microcapsules were obtained on a FTIR-[Shimadzu (84005)] spectrophotometer using the potassium bromate disk method. 200mg potassium bromate was used for the analysis of 2mg of sample. The scanning range was set into 450–4000 nm.

Differential Scanning Calorimeter (DSC)

The thermal analysis of pure drug, formulations and blank microcapsules were carried out using Universal V4.2E TA instruments, to evaluate possible drug-polymer interaction. 3mg of sample was accurately weighed and placed in a 40µLaluminium pan and sealed with a punched lid. A temperature range of 10–300^{o}C was scanned using a heating rate of 10^{o}C min^{-1}. A nitrogen purge of 50mL min^{-1} was used in the oven.

***In vitro* dissolution studies [14]**

In vitro drug release from mucoadhesive microcapsules was analyzed by using USP dissolution test apparatus 2 (Paddle) with 100 rpm (Disso 2000, Labindia).

Predetermined quantities of microcapsules were placed in bowel. 900 mL of 0.1 N HCl (pH 1.2) was used as the dissolution media. Dissolution studies were conducted at 37°C±0.2°C. Samples were taken at suitable time intervals and replaced with the same quantity of fresh dissolution medium. Collected samples filtered through 0.45µm syringe, absorbance was measured spectrophotometrically (Shimadzu UV/Visible spectrophotometer 2100; Tokyo, Japan) at 293 nm.

Kinetics of drug release [15-17]

In order to know the drug release mechanism and *in-vitro* drug release kinetics various kinetic models were used. Zero order, first order, Higuchi's Peppa's models were used in this study and regression coefficient values (R^2) was calculated and analyzed.

Accelerated stability testing according to ICH Q1A (R2) [18-20]

The optimized formulation (LM 6) were stored in a stability chamber (Remi CHM-10 S®, India) at 40 ± 2°C and humidity of 75 ± 5% RH for 6 months and examined for the drug content, mucoadhesiveness and *in vitro* drug release 0, 30, 90, and 180 days. The zero time samples were used as controls.

Statistical analysis [21]

The data obtained from the production yield, encapsulation efficiency, particle size, *in vitro* release studies and *in vivo* studies of microcapsules were analyzed statistically by one-way ANOVA using Graph pad Prism software (Graph pad Software) and $P < 0.05$ was considered statistically significant.

Result and Discussion
Percentage Yield

Table 2
Percentage yield of Levofloxacin hemihydrate loaded mucoadhesive microspheres

S. No	Formulation code	Percentage yield (Mean of three values ± SD)
1	LCAM 1	32.11±1.25
2	LCAM 2	46.51±1.87
3	LCAM 3	58.41±1.09
4	LCAM 4	55.74±2.15
5	LCAM 5	70.61±2.11
6	LCAM 6	80.27±1.89
7	LCAM 7	61.22±1.87
8	LCAM 8	77.64±1.22
9	LCAM 9	87.28±1.67

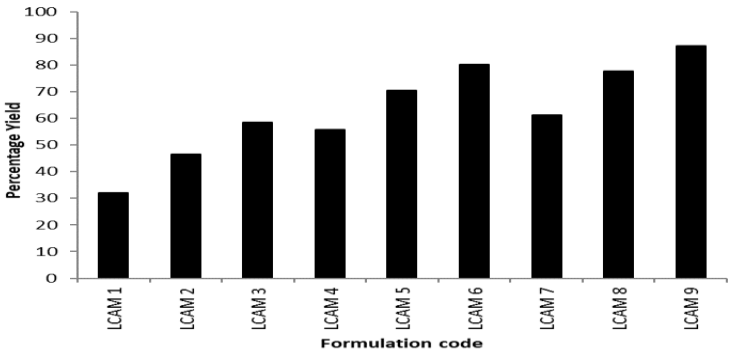

Figure 1. Percentage yield of Levofloxacin hemihydrate loaded mucoadhesive microspheres

The percentage yield for levofloxacin hemihydrate loaded microspheres was found to be in the range of 32.11±1.25% to 87.28±1.67%. The microspheres yield increased with increase in the concentration of sodium alginate and calcium chloride [$P< 0.05$]. It is evident from Table 5 that decreasing the polymer concentration has resulted in a decrease in the percentage yield. This effect can be explained by the fact that as the concentration of alginate decreases the quantity of polymer become insufficient to cover levofloxacin hemihydrate particles completely.

Drug content

Table 3
Drug content of levofloxacin hemihydrate loaded mucoadhesive microspheres

S. No	Formulation code	Theoretical drug content (%)	Practical drug content (%) (Mean of three values ± SD)
1	LM1	55.60	20.21±1.01
2	LM 2	45.45	22.08±1.33
3	LM 3	38.46	22.27±1.52
4	LM 5	55.60	25.08±1.59
5	LM 5	45.45	26.48±1.35
6	LM 6	38.46	27.21±1.24
7	LM 7	55.60	28.42±1.47
8	LM 8	45.45	29.07±1.63
9	LM 9	38.46	30.77±1.88

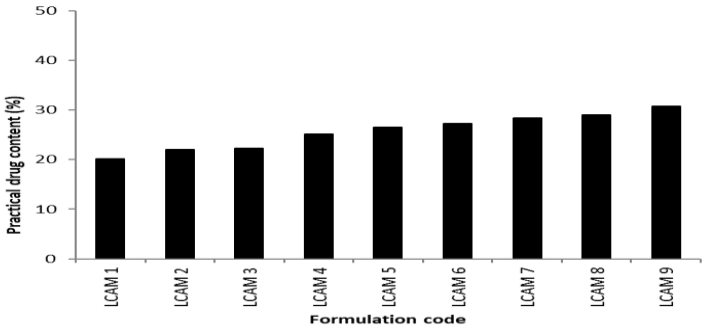

Figure 2. Drug content of levofloxacin hemihydrate loaded mucoadhesive microspheres

Table 4
Encapsulation efficiency of Levofloxacin hemihydrate loaded mucoadhesive microspheres

S. No	Formulation code	Percentage drug loaded (Mean of three values ± SD)
1	LCAM 1	51.77±1.33
2	LCAM 2	60.22±0.55
3	LCAM 3	68.54±1.03
4	LCAM 4	60.25±0.97
5	LCAM 5	68.78±1.82
6	LCAM 6	73.11±1.49
7	LCAM 7	68.55±1.21
8	LCAM 8	75.98±1.84
9	LCAM 9	78.54±1.55

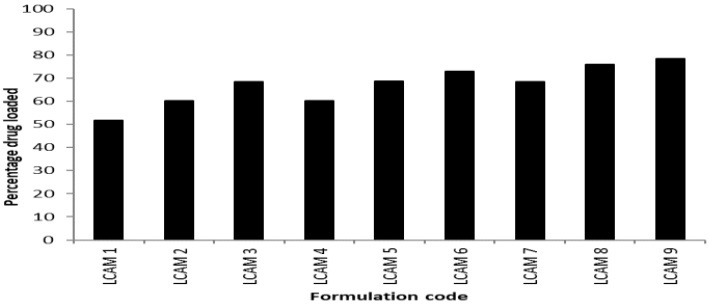

Figure 3. Encapsulation efficiency of Levofloxacin hemihydrate loaded mucoadhesive microspheres

Drug content of the levofloxacin hemihydrate loaded microspheres varied from 20.21±1.01% to 30.77±1.88%. The encapsulation efficiency of the prepared microspheres varied from 51.77±1.33% to 78.54±1.55%. It was observed that drug loading was found to be directly proportional to polymer concentration. The encapsulation efficiency increased progressively by increasing the increase in the concentration of sodium alginate and calcium chloride [$P< 0.05$]. Higher loading

efficiency was obtained as the concentration of alginate increased. This may be attributed to the greater availability of active calcium binding sites in the polymeric chains and consequently, the greater degree of crosslinking as the quantity of sodium alginate increased.

Table 5
Particle size distribution of Levofloxacin hemihydrates loaded mucoadhesive microspheres

S. No	Formulation code	Particle size(µm) (Mean of three values ± SD)
1	LCAM 1	135.5±3.64
2	LCAM 2	217.68±5.87
3	LCAM 3	287.24±5.41
4	LCAM 4	229.22±4.55
5	LCAM 5	314.28±4.23
6	LCAM 6	397.55±3.28
7	LCAM 7	284.37±4.98
8	LCAM 8	357.23±5.01
9	LCAM 9	448.59±5.44

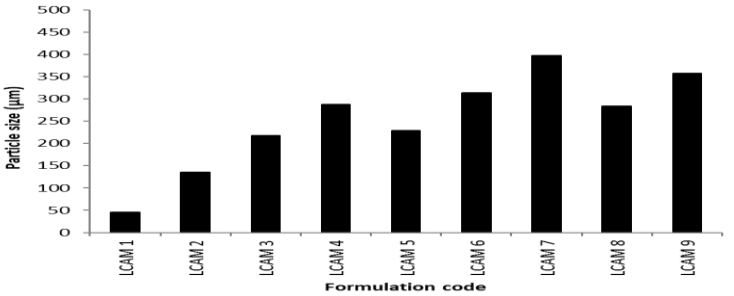

Figure 4. Particle size distribution of Levofloxacin hemihydrate loaded mucoadhesive microsphere

Viscosity of polymer solution is one of the most important factors related to formulation of microcapsules. Fragment formation was observed when low concentration of sodium alginate and calcium chloride was used at 1 % and 2%w/v, respectively, whereas maximum sphericity was observed at when high concentration of sodium alginate and calcium chloride was used at 3 % and 4 %w/v, respectively. The mean diameter of levofloxacin hemihydrate loaded microspheres was found to be in the range of 135.5±3.64 µm to 448.59±5.44µm. The results revealed that the increase in the concentration of sodium alginate increase the size of the beads based on the fact that sodium alginate binds more calcium chloride by cross linking. These observations are in accordance with the research study which described that higher viscosity resulted from increase in the alginate concentration causes development of larger microspheres and greater drug entrapment due to high degree of crosslinking. The polymer concentration increases, the particle size also improves, which could be due to increase in the viscosity of drug and polymer ratio and thickness of polymer.

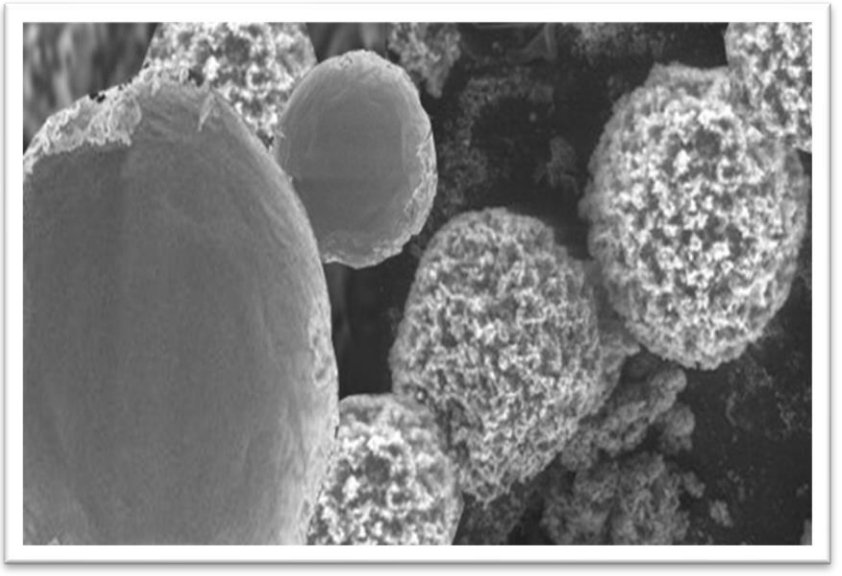

Figure 5. SEM photograph of formulation LCAM 5

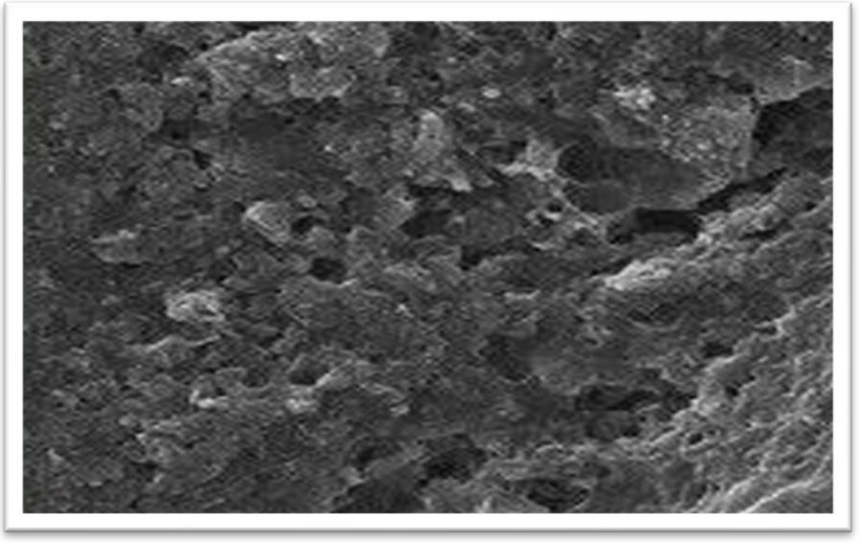

Figure 6. SEM photograph of formulation LCAM 5
(Surface View)

In vitro evaluation of mucoadhesiveness

Table 6
Mucoadhesiveness efficiency of levofloxacin hemihydrate loaded mucoadhesive microspheres

S. No	Formulation code	Mucin Adsorption (%) (Mean of three values ± SD)
1	LCAM 1	45.81±1.32
2	LCAM 2	55.34±1.99
3	LCAM 3	65.71±1.32
4	LCAM 4	55.03±1.87
5	LCAM 5	68.58±1.23
6	LCAM 6	79.41±0.86
7	LCAM 7	62.90±1.41
8	LCAM 8	73.11±1.59
9	LCAM 9	82.97±0.82

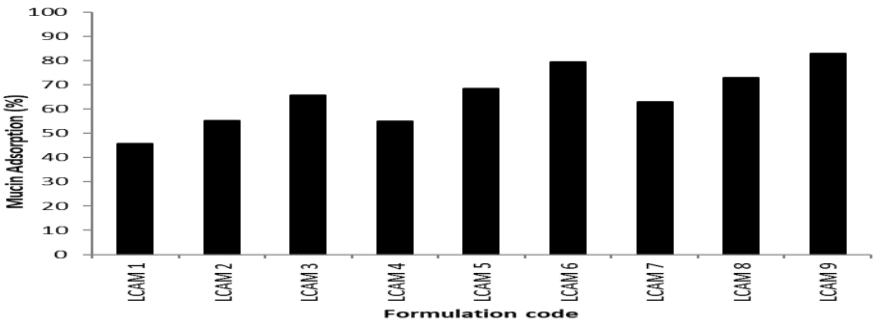

Figure 7. Mucoadsiveness of Levofloxacin hemihydrate loaded mucoadhesive microspheres

The *in vitro* mucoadhesiveness study revealed that all the batches of prepared microspheres had good mucoadhesive property. Mucoadhesive property of the prepared microspheres varied from 45.81±1.32% to 82.97±0.82%. A proportional rise in mucoadhesive strength of the formulation was observed with increase in the proportion of concentration of sodium alginate and calcium chloride. It was noted that mucoadhesive property higher when polymer concentration was reached higher levels. Similarly, Nagda *et al.* also reported that as polymer concentration was increased, it leads to increased mucoadhesion.

Compatibility studies

Figure 8. FTIR Spectra of Levofloxacin hemihydrate

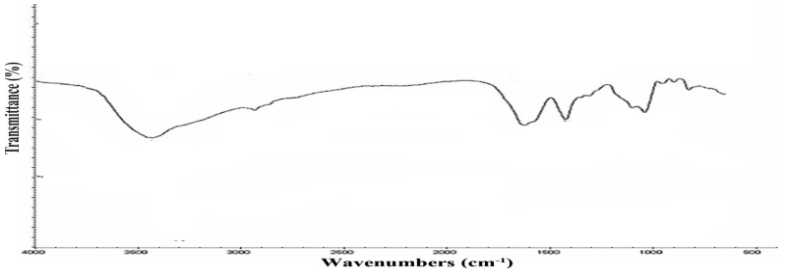

Figure 9. FTIR Spectra of Blank mucoadhesive microcapsules

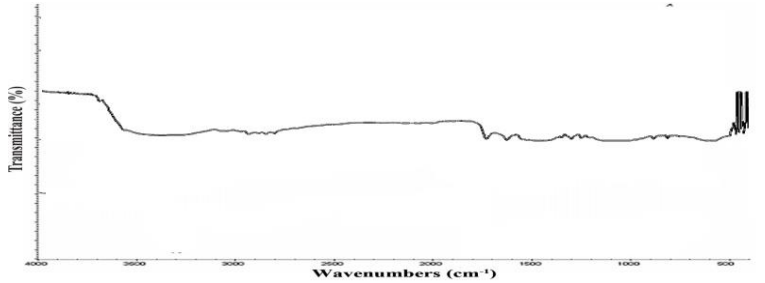

Figure 10. Characteristic IR bands of Levofloxacin hemihydrate in mucoadhesive microspheres

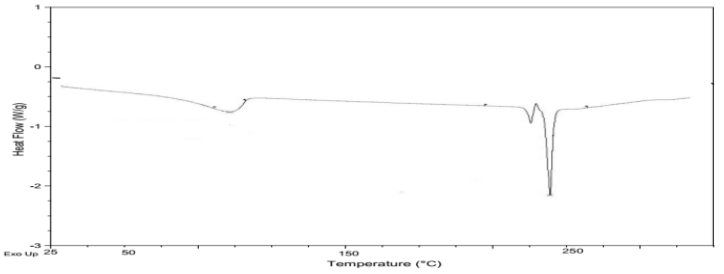

Figure 11. DSC Spectra of Levofloxacin hemihydrate

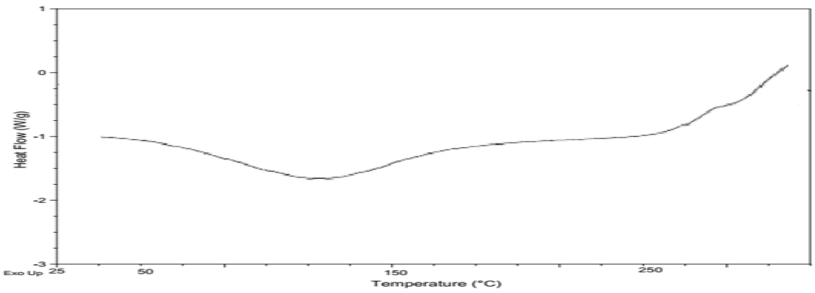

Figure 12. DSC Spectra of Sodium alginate

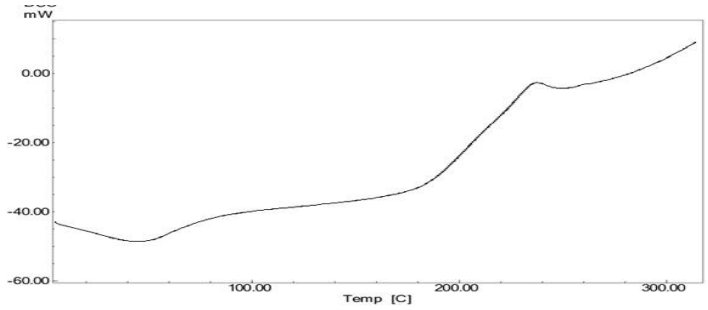

Figure 13. DSC Spectra of chitosan

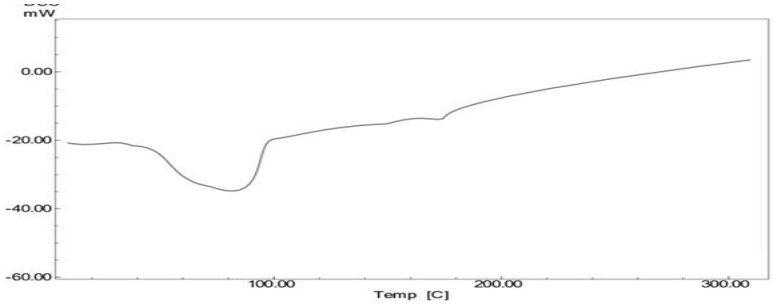

Figure 14. DSC Spectra of blank microspheres

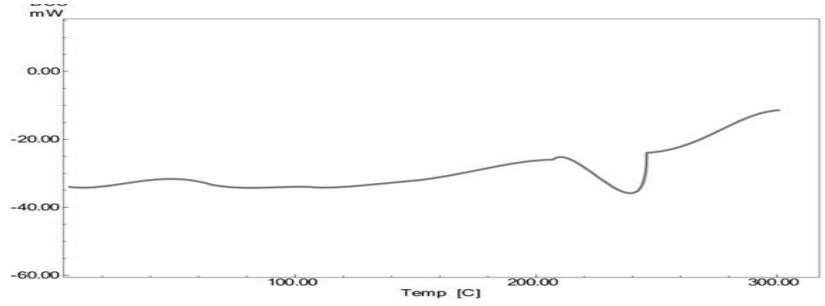

Figure 15. DSC Spectra of Levofloxacin hemihydrate loaded mucoadhesive microspheres [LCAM5]

***In vitro* dissolution studies**

Table 7
In vitro release profile of Levofloxacin hemihydrate loaded mucoadhesive microspheres

Time (hrs)	LCAM1	LCAM2	LCAM3	LCAM4	LCAM5	LCAM6	LCAM7	LCAM8	LCAM9
1	0	0	0	0	0	0	0	0	0
2	54.67	46.22	41.22	47.24	38.22	33.98	33.41	28.33	25.87
3	81.65	71.87	64.82	66.04	56.21	50.88	53.55	43.98	41.22
4	99.51	93.24	85.01	84.21	65.99	61.55	68.87	55.88	49.23
5		99.88	95.22	99.42	76.24	69.04	84.28	65.24	57.19
6			99.66		87.68	79.24	99.68	75.87	65.55
7					99.65	86.21		87.21	73.87
8						95.98		99.74	87.22

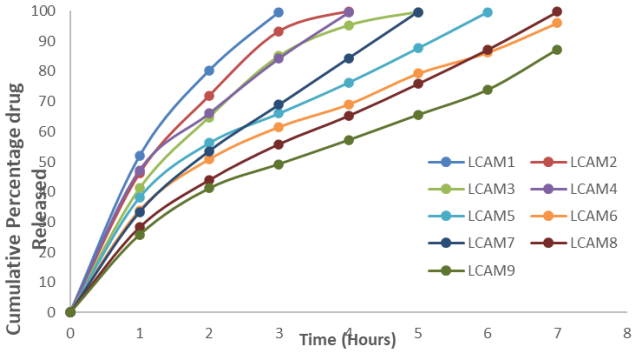

Figure 16. Drug release pattern of various formulations of levofloxacin Hemihydrate

In vitro release kinetics

Table 8
In vitro release kinetic data of Levofloxacin hemihydrate loaded mucoadhesive microspheres

F Code	Zero order plot		First order plot		Higuchi plot	Korsemeyerpeppa's plot	
	K_0	R^2	K_1	R^2	R^2	n	R^2
LCAM1	19.4289	0.9957	-0.4582	0.8178	0.9913	---**	---**
LCAM 2	18.243	0.9911	-0.59788	0.7878	0.9971	---**	---**
LCAM 3	13.211	0.9981	-0.2042	0.9253	0.9911	0.6186	0.9991
LCAM 4	19.574	0.9925	-0.5478	0.7784	0.9987	---**	---**
LCAM 5	15.447	0.9971	-0.6845	0.7257	0.9908	0.5381	0.9964
LCAM 6	14.369	0.9960	-0.2978	0.7875	0.9982	0.6421	0.9909
LCAM 7	20.281	0.9985	-0.6187	0.7921	0.9965	---**	---**
LCAM 8	12.348	0.9801	-0.1841	0.9854	0.9944	0.5841	0.9955
LCAM 9	10.212	0.9946	-0.0975	0.9125	0.9908	0.5245	0.9981

* Insufficient data points to apply kinetics due to rapid release profiles
* Insufficient data points to apply Korsmeyer-Peppas equation up to 70%.
K_0 – Zero order rate constant
K_1 – First order rate constant
R^2 – Regression coefficient
n - Diffusion exponent

Accelerated Stability Studies

[Tested according to ICH Q1 A (R2)]

Table 9
Accelerated stability data of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LCAM5)

S. No	Time (days)	Mucoadhesive strength (Mean ± SE) (n=3)	Drug content (%) (Mean ± SD) (n = 3)	Drug release (%) (Mean ± SD) (n =3)
1	Before storage (0 day)	68.74±1.45	26.27±1.43	99.62±1.87
2	30 days (After storage*)	68.01±1.92	25.94±1.84	99.82±1.84
3	90 days (After storage*)	67.98±1.74	25.68±1.52	99.64±1.13
4	180 days (After storage*)	67.14±1.84	25.80±1.01	98.03±1.51
P - Value		0.0345	0.0387	0.0411

*Storage at 40°C and 75% RH [n = 3].

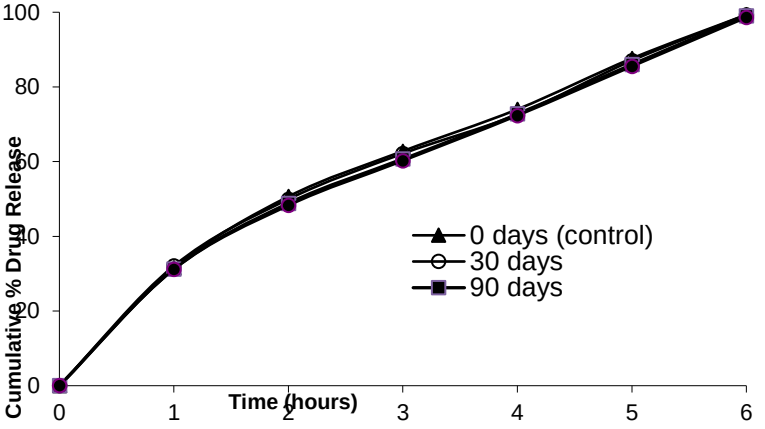

Figure 17. Accelerated stability data of Levofloxacin hemihydrate loaded mucoadhesive microspheres (Formulation LCAM5)

Conclusion

The aim of the present research work was to formulate and evaluate mucoadhesive microspheres of levofloxacin hemihydrate using the mucoadhesive polymer (Chitosan and Sodium alginate) for *H. pylori* eradication. The normal mucus turnover rate is 4–6 hours in rats and likely similar values in humans. The mucus turnover rather than the mucus-polymer interaction that controls the presence of mucoadhesive formulations through the GIT. Based on the mucus turnover rate and dissolution time, formulations LCAM 5 [formulations consisting of 2% w/v Sodium alginate, 1% w/v chitosan and 3% w/v Calcium chloride] were selected as best formulations. Accelerated stability studies were conducted for formulation LCAM 5. From all of the experiments performed, it can be concluded that the developed mucoadhesive polymers can be successful in the effective for treatment of *H. pylori* infection.

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