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Research Article

Design and characterization of Methotrextate Mucoadhesive Microspheres

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ABSTRACT
Present investigation was to formulate and evaluate mucoadhesive microspheres of Methotrexate using chitosan as a mucoadhesive polymer, prepared by simple emulsification phase separation technique using glutaraldehyde as a crosslinking agent. Nine preliminary trial formulations of mucoadhesive microspheres were prepared using different volume (20 mL to 60 mL) of glutaraldehyde (25 % v/v aqueous solution) as crosslinking agent and different crosslinking time of 1 to 3 hr. Polymer to drug ratio was kept constant (5:1).All the nine formulations were subjected for evaluation of drug content and drug entrapment efficiency, particle size, percentage swelling index, percentage mucoadhesion, in vitro drug release study. From these nine preliminary trial formulations F4, F5 and F6 were selected as optimized formulations based on percentage mucoadhesion, sphericity of microspheres, swelling index and in vitro drug release. The drug polymer compatibility studies were carried out using Fourier transform infrared spectroscopy. The morphology of microspheres were characterised by scanning electron microscopy. The optimized formulation were spherical in shape and free flowing in nature with 72.5 %,70.1 % and 66.2 % mucoadhesion after 1 hour and optimum drug entrapment efficiency of 62.08 %, 69.78 % and 73.26 % with the slow release of 93.57 % , 86.27 % and 78.29 % up to 12 hours. From above three optimised formulations F5 was selected as best formulation based on mucoadhesion, drug content and in vitro drug release. As the concentration of glutaraldehyde increased, the mucoadheasiveness decreases showing more crosslinking between drug and polymer.

Keywords: Methotrexate, Chitosan, Mucoadhesive Microspheres, In vitro studies, Stability studies.

INTRODUCTION

Development of multiparticulate systems had gained much fame over the single unit systems for oral drug delivery applications as per reports of last few decades[1]. It has proved to be a potential system due to numerous reasons viz., predictable gastric emptying, reduced risk of toxicity, reduced dose dumping, reduced local irritation, reduced inter-intra subject variability, increased bioavailability, and improved stability. Multiparticulate system mostly used for oral routes includes microspheres, beads, granules, nanoparticles, microparticles, etc., that ensure forunique release profiles, uniform drug dispersion and absorption into the gastrointestinal (GI) tract. The system developed for colonic targeting were able to pass through the upper GI tract easily, while reaches colon region at predictable time and retained at ascending for longer period of time[2,3,4]. Colorectal cancer is the third mainly common cancer in the world, with nearly 1.8 million novel cases diagnosed in 2018, and has deprived prognosis when metastasized to lymph nodes or distant organs[5]. In Europe, it is the second mainly common cancer and in the United States, the third most common form of cancer and second-leading cause of deaths[6, 7]. In fact, colorectal cancer is responsible for a high rate of morbidity and mortality according to global cancer statistics[8]. Colorectal cancer manifests as cancerous growths in the colon, rectum and appendix. Colorectal cancer is the second most ordinary cancer killer overall and third most common cause of cancer-related death in the United States in both males and females. Oral colon-specific drug delivery system is more advantageous over conventional cancer chemotherapy as it is unproductive in delivering drugs to the colon due to absorption or degradation of the active ingredient in the upper gastrointestinal tract. CDDS as an effective and safe therapy for colon cancer provides therapeutic concentrations of anticancer agent at the site of action and spare the normal tissues, with reduced dose and reduced duration of therapy. The effective focused on delivery of medication to the colon by means of the gastrointestinal tract requires the security of a medication from debasement and discharge in

the stomach and small digestive system and afterward guarantees unexpected or controlled discharge in the proximal colon[9].In pH controlled discharge frameworks, the distinctive pH of human GIT is abused by covering the measurement structure with pH subordinate polymers which stays accordingly in the upper GIT and debase in the digestive organ where the pH is high i.e. Subsidiaries of acrylic acid and cellulose are the for the most part utilized pH-subordinate polymers. On the activity of polymers and their solvency at different pH condition, delivery frameworks have been intended to pass on medications at the specific target site. Most regularly utilized pH-subordinate polymers are methacrylicacid copolymer (i.e., Eudragit L100 and S100), which disintegrate at pH 6.0 and 7.0 separately[10,11]. These polymers don't break down in stomach and intestinal pH because of hydrogen holding between the hydroxyl gatherings of the carboxylic moiety and the carbonyl oxygen of ester bunches in the polymer particles. Be that as it may, they breaks up in the colon as a result of the ionization of their carboxyl practical gatherings and discharges the medication in the colon[12].It is possible to modify the polymer characteristics by using the combination of Eudragit S100 and L100 in varying ratio[13,14,15]. The addition of Eudragit L100 to S100 in varying ratios altered the pH at which the polymer solubilized to produce formulations with high accuracy. Methotrexate (MTX) is used as anti-cancer drug, acting as a dihydrofolate reductase inhibitor. It is used in the colo-rectal cancer[16]. High-portion MTX is settled for the treatment of strong tumors and leukemias [17,18] while low-portion regimens are generally utilized in the treatment of immune system diseases[19,20,21] and as of late, as immunosuppressive agent in organ transplantation[22].

MTX was introduced to clinics over six decades ago and is one of the most widely used and studied anticancer agents. Its administration however, has the potential of severe side effects, including neurologic toxicity, renal failure due to tubular obstruction by crystal deposits of MTX and its primary metabolite, 7-hydroxy-methotrexate (7-OH-MTX), myelosuppresion, and mucositis. The effectiveness of HDMTX therapy has been greatly enhanced by the observation that patients at high risk of serious toxicity may be detected by monitoring serum MTX concentrations. Therefore, the routine monitoring of drug serum concentrations is important in guiding leucovorin rescue and is considered to be imperative for both patient safety and evaluation of therapeutic concentrations of MTX. The objective of the present investigation was to formulate and characterize the microspheres of MTX using polymers Eudragit S 100 and sodium lauryl sulfate for colon targeting.

MATERIALS AND METHODS

Materials

MTX was acquired from Khandelwal Laboratory Pvt. Ltd. Mumbai. Eudragit S 100 was acquired from Evonik Degussa India Pvt. Ltd. Mumbai. DCM (Dichloro methane) and SLS (Sodium lauryl sulfate) were acquired from HiMedia Laboratory Pvt. Mumbai, Maharashtra (India).All other reagents and chemicals used were of analytical grade. Triple distilled water was generated in house.

Preformulation Studies

The preformulation studies of drug was carried out by physical examination i.e., colour, texture, odour etc. The solubility of the drug was determined by taking small quantity of drug (aprox. 10 mg) in the 10 ml volumetric flasks separately and added the 10 ml of the solvent (water, ethanol, methanol, 0.1N HCL, 0.1N NaOH, chloroform and 7.4 pH buffer) Shake vigorously and kept for some time. Note the solubility of the drug in various solvents (at room temperature).Melting point was determined by placing small quantity of powder into a fusion tube. That tube was placed in the melting point determining apparatus (Chemline) containing castor oil. The temperature of the castor oil was gradual increased automatically and read the temperature at which powder started to melt and the temperature when all the powder gets melted[23].Quantitative estimation of drug was performed by determination of λ_{max} of methotrexate. Accurately weighed 10 mg of drug was dissolved in 10 ml of phosphate buffer pH 7.4 solution in 10 ml of volumetric flask. The resulted solution 1000μg/ml and from this solution 1 ml pipette out and transfer into 10 ml volumetric flask and volume make up with phosphate buffer pH 7.2 solution. Prepare suitable dilution to make it to a concentration range of 10-30μg/ml. The spectrum of this solution was run in 200-400 nm range in U.V. spectrophotometer (Shimadzu UV1800, Japan). A graph of concentration Vs absorbance was plotted.

FTIR spectroscopy

The concentration of the sample in KBr should be in the range of 0.2% to 1 %. The pellet is a lot thicker than a liquid film, consequently a decrease concentration in the sample is required (Beer's Law). For the die set that you'll be the

usage of, about 80 mg of the mixture is wanted. Too excessive of an attention causes typically difficulties to obtain clean pellets. This pellet keeps into the sample cell and scanned between 4000-400 $c.m^{-1}$ and IR spectra are obtained[24].

Process variables

There are many procedure factors, which could influence the arrangement and properties of the microspheres, were recognized and considered. The technique for planning was in like manner advanced. These are the procedure factors of microspheres arrangement were chosen for optimization of plan

- Concentration of polymer.
- Stirring rate.

Total 9 formulations were designed on the basis of these variables. The formulation code and respectable variables used in the preparation of microspheres are given in Table 1. The effects of these variables were observed on particle size, % yield, % drug entrapment and % drug release. The procedure adopted in the optimization of the variables was follows,

- Concentration of polymer: To optimize the formulation, varying concentration of drug polymer i.e. 1:1, 1:2 and 1:3 were taken by keeping drug and emulsifying agent constant.
- Stirring rate: Stirring rate for the preparation was optimized by keeping the microsphere at different stirring speed i.e. 900, 1200 and 1500 rpm, while keeping all the parameters constant as described in the procedure for the preparation of microspheres.

Fabrication of Eudragit microspheres containing drug

Mucoadhesive microspheres of Methotrexate were prepared by simple emulsification phase separation technique were chitosan was used as a polymer and glutaraldehyde was used as cross linking agent80. Chitosan (1 gm) was dissolved in 100 mL of 1% v/v aqueous acetic acid solution. Five hundred milligrams of drug was dispersed in the polymer solution. In batches F1 to F9 the polymer to drug ratio was kept constant at 5:1. The resultant mixture was extruded through a syringe (No. 20) in 100 mL of liquid paraffin (heavy and light 1:1 ratio) containing 0.5% span 80 and stirring was carried out using a propeller stirrer at 1000 rpm. After 15 min, glutaraldehyde (25% v/v aqueous solution) was added and stirring was continued. The amount of cross-linking agent and cross-linking time were varied in batches F1 to F9 from 20, 40 and 60 mL and 1 to 3 hr respectively as shown in Table 1. Microspheres thus, obtained were filtered and washed several times with petroleum ether (80:20) to remove traces of oil. They were finally washed with water to remove excess of glutaraldehyde. The microspheres were then dried at room temperature (at 25 ºC and 60% RH) for 24 hr.. Formulations with different drug to polymer ratios were prepared as shown in table 1.

Table 1: Composition of various formulation of microsphere

Formulation codes	Volume of glutaraldehyde (mL)	Cross linking time (hr)	Drug : Polymer ratio
F1	20	1	1:5
F2	20	2	1:5
F3	20	3	1:5
F4	40	1	1:5
F5	40	2	1:5
F6	40	3	1:5
F7	60	1	1:5
F8	60	2	1:5
F9	60	3	1:5

Percentage yield

The prepared microspheres with a size range of 1µm to 1000µm were collected and weighed from different formulations. The determined weight was separated by the aggregate sum of all non-unpredictable parts which were utilized for the planning of the microspheres.

$$\% \text{ Yield} = \frac{\text{Actual weight of product}}{\text{Total weight of drug and polymer}} \times 100$$

Percentage drug entrapment

Percent drug entrapment determination is the most important parameter to study the efficiency of the process. Percent drug entrapment of all the batches prepared was determined by using

spectrophotometer to study the effect of various variables.[27] An accurately weighed 100 mg microspheres containing MTX were washed with specific amount of methylene chloride. At that point microspheres were dissolved in 20 ml of ethanol. The arrangement was sifted with a whatman paper (# 40) and 1 ml of this arrangement was around weakened to 10 ml utilizing ethanol and examined spectrophotometrically at 258 nm utilizing UV-spectrophotometer (Shimadzu UV1800, Japan).

$$\% \text{ Drug Entrapment} = \frac{\text{Calculated drug content}}{\text{Theoretical drug content}} \times 100$$

Measurement of mean particle size

The particle size determination of prepared multiparticulate system was performed by optical microscopic method. The size of microsphere was measured. The mean of 100 microspheres was noted as particle size. All the readings of particle size were the mean of three trials ± S.D. The eyepiece micrometer was previously calibrated with a standard stage micrometer. The prepared microsphere was taken on the clean glass slide and the size of the particles was determined by utilizing eyepiece micrometer.

Differential scanning calorimetry

The possible interaction between MTX and Eudragit S 100 during the processing of microspheres is assessed by carrying out the thermal analysis of pure drug along with excipients. The stability of a formulation depends upon the compatibility of the drug with excipients. It is of significance to detect any possible physical (or) chemical interaction, since it can affect the bioavailability and stability. DSC is quick and solid techniques to screen medicates excipients similarity and give most extreme data about the conceivable cooperation. Thermal analysis dose not replace stability test, but is valuable tool at the preformulation stage. DSC in combination with short time stress tests is recommended for easy evaluation and interpretation of DSC curves. The ratio of drug excipients used in the study is subjected to the discretion of the formulator. However, Van Dooren recommends ratio of 1:5 for diluents, 3:1 for binders or disintegrates, 5:1 for lubricants and 10:1 for colorant etc. In DSC an interaction is concluded by elimination of endothermic peak(s), appearance of new peak(s), change in peak shape and its onset, peak temperature/melting point and relative peak area or enthalpy. DSC examination was conducted for the optimized formulation, pure drug, SLS and the polymer using DCS instrument (DSC-4000, Perkin Elmer). Samples (2-5 mg) were weighed and hermetically sealed in flat bottomed aluminum pans. These samples were heated over a temperature range of 50-400^{0}C in an atmosphere of nitrogen (50 ml/min) at a constant rate of 10^{0}C/min, with alumina being the reference standard.[28]

X-Ray diffraction

In order to determine the physical state of drug i.e. amorphous or crystalline nature in formulation, XRD was done. The sharp peak determines the crystalline nature of the drug. To characterize the physical state of MTX, Polymers and formulations, X-ray diffraction analysis was performed in an X-ray diffractometer (Rigaku X-ray diffractometer). The characteristic X-ray diffraction spectra of pure drug and formulation were presented in Fig: 6. The powder drug were recorded using Ni-filter, anode material Cu, K-alpha radiation (1.54060 and 1.5443 Å), scan type continuous, a voltage of 30Kv, a current of 15 mA, scan speed 40 min^{-1} over the 0° to 90° diffraction angle (2ϑ) range and the count range 2000cps. The stability of a formulation depends upon the compatibility of the drug with excipients. It is of significance to detect any possible physical (or) chemical interaction, since it can affect the bioavailability and stability. XRD is a fast and reliable method to screen drug-excipients compatibility and provide maximum information about the possible interaction.

Shape and surface morphology

From the formulated batches of microspheres, formulations (SS 3b) which showed an appropriate balance between the percentage drug releases was examined for surface morphology and shape using scanning electron microscope (Jeol, Japan).[29]Sample was fixed on carbon tape and fine gold sputtering was applied in a high vacuum evaporator. The acceleration voltage was set at 10KV during scanning. Microphotographs were taken on different magnification (1000 and 2500X) was used for surface morphology.

In-Vitro Drug Release

The prepared microspheres were evaluated for *in vitro* drug release by using USP II Basket type dissolution test apparatus. A weighed quantity of formulation (equivalent to 30mg) was filled in capsule and kept in basket of dissolution apparatus with dissolution media (900 ml) at 37±0.2°C. Samples were withdrawn at different time interval and compensated with same amount of fresh dissolution medium.[30] Volume of sample withdrawn was made up to 5ml by media. The samples withdrawn were assayed

spectrophotometrically at 258 nm for percent of release of MTX using UV visible spectrophotometer.

Drug release kinetic data analysis

Several kinetic models have been proposed to describe the release characteristics of a drug from matrix. The following five equations were zero-order, first-order, Higuchi's, Hixon and Korsemeyer-Peppas equation used to determine the mechanism of drug release.[31]

Equation 1, the zero-order model equation (Plotted as cumulative percentage of drug released vs time); Equation 2, the first-order model equation (Plotted as log cumulative percent Drug remaining Vs time); Equation 3, Higuchi's square-root equation (Plotted as cumulative percentage of drug released vs square root of time); Equation 4, Hixon equation (Plotted as percentage cube root of drug remaining vs time); and Equation 5, the Korsemeyer-Peppas equation (Plotted as Log cumulative percentage of drug released vs Log time).

Formulation Studies

Preparation of mucoadhesive microspheres

Mucoadhesive microspheres of Methotrexate were prepared by simple emulsification phase separation technique were chitosan is used as a polymer and glutaraldehyde is used as cross linking agent[80].Chitosan (1 g) was dissolved in 100 mL of 1% v/v aqueous acetic acid solution. Five hundred milligrams of drug was dispersed in the polymer solution. In batches F1 to F9 the polymer to drug ratio was kept constant at 5:1. The resultant mixture was extruded through a syringe (No. 20) in 100 ml of liquid paraffin (heavy and light 1:1 ratio) containing 0.5% span 80 and stirring was carried out using a propeller stirrer at 1000 rpm. After 15 min, glutaraldehyde (25% v/v aqueous solution) was added and stirring was continued. The amount of cross-linking agent and cross-linking time were varied in batches F1 to F9 from 20, 40 and 60 mL and 1 to 3 hr respectively. Microspheres thus, obtained were filtered and washed several times with petroleum ether (80:20) to remove traces of oil.

They were finally washed with water to remove excess of glutaraldehyde. The microspheres were then dried at room temperature (at 25 ºC and 60% RH) for 24 hr.

Evaluations

Drug content and Percentage Drug Entrapment Efficiency

The drug content was determined spectrophotometrically at 306.4 nm after appropriate dilution with 0.1N HCl .The drug content and drug loading efficiency of various mucoadhesive microspheres formulations are given in **Table 2**.

Table 2: Drug content and Percentage Drug Entrapment Efficiency of mucoadhesive microspheres of methotrexate

SL.NO	Formulation Code	Absorbance	Drug content* (mg)	%Entrapment efficiency*
1	**F1**	0.236	21.61 ± 1.9	43.22 ± 1.2
2	**F2**	0.286	26.19 ± 2.7	52.38 ± 1.8
3	**F3**	0.327	29.94 ± 1.2	59.89 ± 1.0
4	**F4**	0.339	31.04 ± 1.9	62.08 ± 1.2
5	**F5**	0.381	34.89 ± 2.4	69.78 ± 1.5
6	**F6**	0.400	36.63 ± 3.2	73.26 ± 2.0
7	**F7**	0.370	33.88 ± 2.2	67.76 ± 1.9
8	**F8**	0.389	35.62 ± 1.3	71.24 ± 0.9
9	**F9**	0.404	36.99 ± 1.4	73.99 ± 1.1

* Average of three determinations

Methotrexate mucoadhesive microspheres prepared by simple emulsification phase separation technique, in which glutaraldehyde used as a cross linking agent varied from 20 to 60 mL and stirring time varied from 1 to 3 hrs. Entrapment efficiency in F1 to F3 formulation was found to be not good indicating less cross linking between the drug and polymer. Formulation F4 to F9, it shows 62.08 ± 1.2 to 73.99 ± 1.1 % entrapment efficiency indicating more entrapment of drug with the polymer because of more volume of glutaraldehyde shows more drug entrapment.

Particle Size

The particle size of the microspheres was determined by using optical microscopy method. Approximately 10 microspheres were counted for particle size using a calibrated optical microscope. Mean particle size of mucoadhesive microspheres of methotrexate shown in Table 3.

Table 3: Mean Particle Size of mucoadhesive microspheres of methotrexate

SL.NO	Formulation Code	Mean particle size* (µm)	Sphericity of Microspheres
1	F1	46.08 ± 1.7	Very irregular
2	F2	39.98 ± 1.5	Very irregular
3	F3	29.90 ± 1.8	Very irregular
4	F4	31.43 ± 1.6	Spherical free flowing
5	F5	26.09 ± 1.5	Spherical free flowing
6	F6	24.87 ± 1.4	Spherical free flowing
7	F7	29.75 ± 1.5	Spherical free flowing
8	F8	23.50 ± 1.6	Spherical free flowing
9	F9	19.99 ± 1.2	Spherical free flowing

* Average of three determinations

The particle size of F1 to F3 varied between 46.08 ±1.7 to 29.90 ±1.8 µm showing very irregular sphericity and F4 to F9 varied between 31.43 ±1.6 to 19.99 ±1.2 µm with spherical in shape and free flowing in nature . This was agreeing with the finding that there was a decrease in the particle size with increase in the glutaraldehyde volume and stirring time. Formulations with the more glutaraldehyde volume and more stirring time showed less particle size.

Swelling Index

For estimating the swelling index, the microspheres (~100 mg) were suspended in 5 mL simulated gastric fluid (pH 1.2). The particle size was monitored by microscopy technique every 1 hr using an optical microscope .The increase in particlesize of the microspheres was noted for up to 10 hrs and the swelling index was calculated. Swelling index of different formulations of mucoadhesive microspheres of Methotrexate shown in Figure 1.

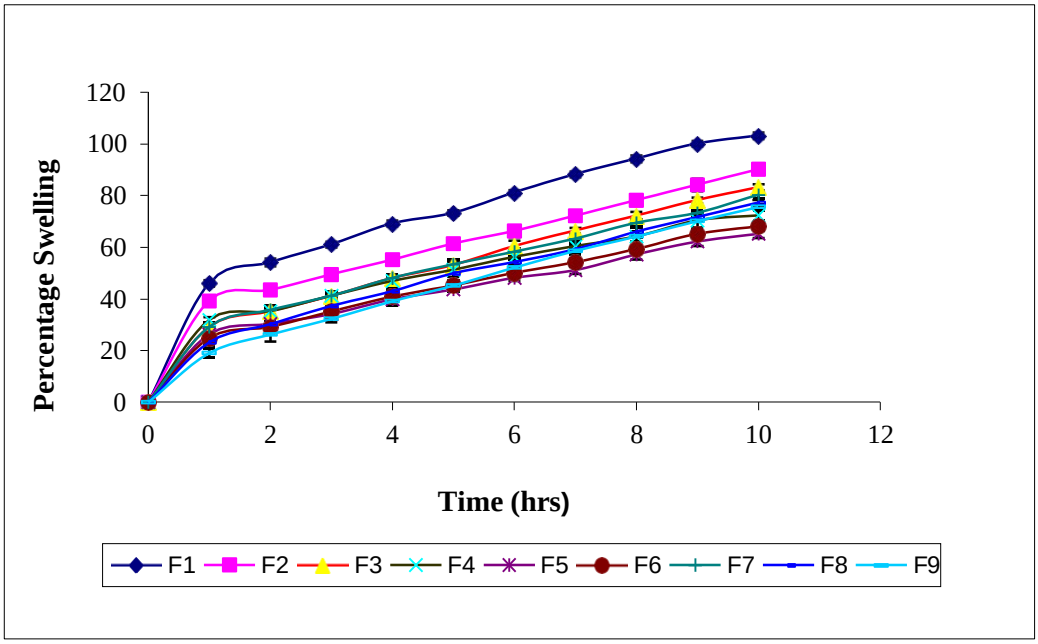


Figure 1: Swelling behaviour of mucoadhesive microspheres formulation (F1-F9)

The swelling behaviour of prepared mucoadhesive microspheres, formulations F1 to F3 ranging from 103.13 ± 1.31% to 83.01 ± 1.22%, F4 to F6 swelling index varied from 72.23±2.13% to 65.13±1.32% and for F7 to F9 varied from 90.12±1.78% to 72.23±2.13%.These results indicated that there was no significant effect of volume of the cross linking agent, glutaraldehyde on swelling properties of microspheres but stirring time directly affect the swelling properties of microspheres. As the stirring time increases swelling index decreases.

In vitro wash-off test

The mucoadhesive property of the microspheres was evaluated by *in vitro* wash- off test. A 1x1 cm piece of rat stomach mucosa was tied onto a glass slide ("3*1") using thread. Microspheres were spread (~50 mg) onto the wet rinsed tissue specimen and the prepared slide was hung onto one of the groves of a USP tablet disintegrating test apparatus. The disintegrating test apparatus was operated whereby the tissue specimen was given regular up and down movements in the beaker of the disintegration apparatus, which contained the At the 1 hr and 10 hr, the weight of microspheres still adhering onto the tissue was calculated. *In vitro* wash off (% mucoadhesion) of different formulations shown in **Table 4** and **Figure 2**, **3** for 1hr and 10 hr respectively.

Table 4: In vitro wash off (% mucoadhesion) of different mucoadhesive microspheres of Methotrexate

SL.NO	Formulation	Mucoadhesion (after 1hr)*	Mucoadhesion (after 10hr)*
1	F1	81.5 ± 0.75	18.5 ± 0.86
2	F2	75.8 ± 0.91	12.5 ± 1.03
3	F3	68.2 ± 1.40	7.5 ± 1.68
4	F4	72.5 ± 0.89	17.4 ± 0.98
5	F5	70.1 ± 1.45	16.8 ± 1.90
6	F6	66.2 ± 1.76	12.4 ± 1.99
7	F7	61.7 ± 1.32	12.3 ± 1.39
8	F8	54.9 ± 1.10	10.1 ± 1.58
9	F9	41.7 ± 1.47	5.4 ± 1.87

* Average of three determinations

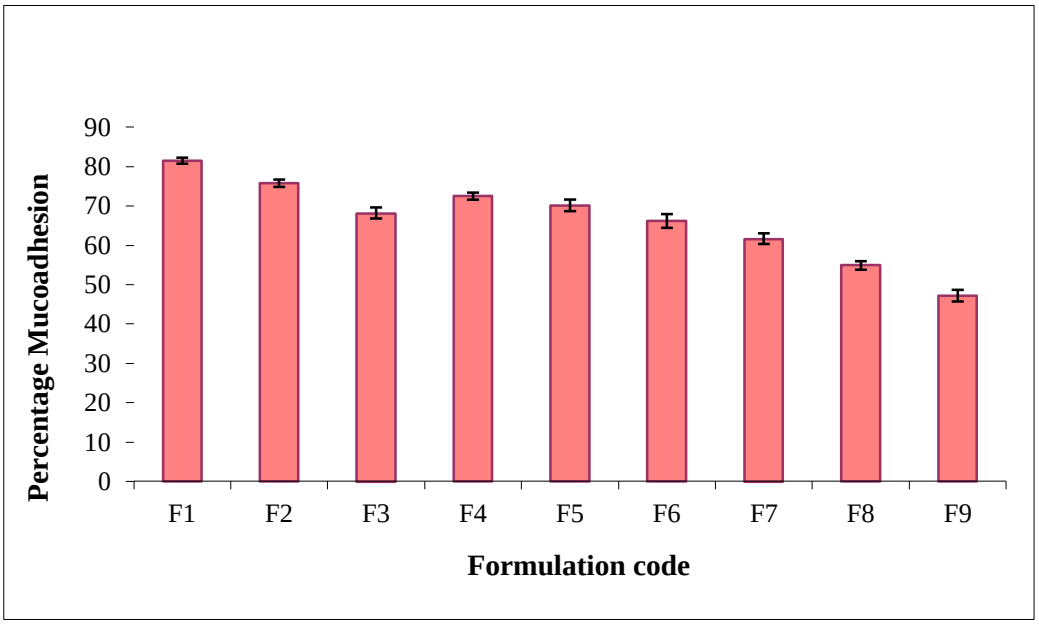


Figure 2: Mucoadhesion data of formulations (F1 to F9) after 1hr

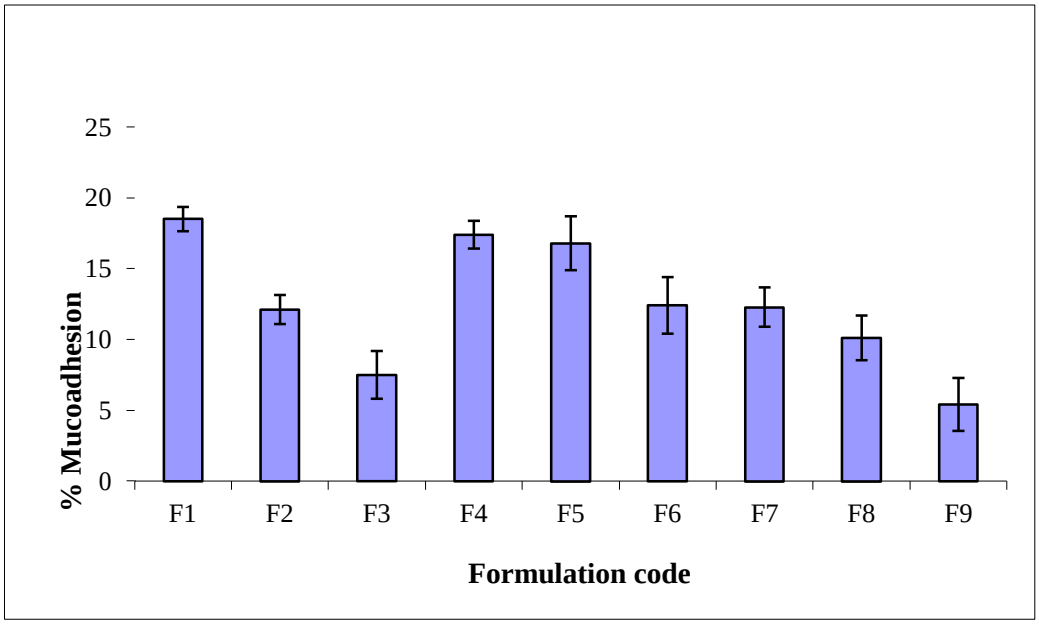


Figure 3: Mucoadhesion data of formulations (F1 to F9) after 10hr

In-vitro wash off test shows the mucoadhesive property of the prepared microspheres, were F1 to F9 showed mucoadhesion after 1hr ranging from 41.7 ± 1.47 to 81.5 ± 0.75 and after 10 hrs it ranges from 18.5 ± 0.86 to 5.4 ± 1.87.This result indicated that with increase in crosslinking agent and stirring time shows decrease in mucoadhesion.

In vitro drug release study

The *in vitro* drug release data for various methotrexate mucoadhesive microspheres formulations are given below in the Table 5.

Table 5: 19 Cumulative percentage drugretained

Time	Cumulative percentage drugretained								
(hr)	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	76.27	79.24	81.21	82.19	85.16	87.15	84.18	88.14	90.11
2	69.98	72.29	75.26	78.23	77.23	82.19	80.21	86.15	87.14
3	62.03	64.02	67.98	69.96	72.26	77.22	76.23	81.19	83.15
4	56.06	57.05	61.02	63.99	66.96	71.26	69.94	77.21	79.19
5	44.14	49.09	54.05	58.02	60.01	66.95	64.96	72.24	75.21
6	38.80	43.10	48.07	50.06	55.02	59.99	59.98	66.93	67.93
7	29.83	33.81	36.79	44.07	49.02	54.01	53.99	60.96	62.95
8	20.86	26.81	28.82	38.74	43.03	49.01	48.99	55.97	57.97
9	11.87	18.82	21.82	31.74	37.69	43.02	43.99	48.99	51.99
10	4.52	11.81	16.79	24.75	28.72	36.69	38.66	45.98	48.97
11	0.13	5.78	11.75	12.79	19.74	29.69	33.65	40.97	46.59
12	-0.31	3.36	6.71	6.43	13.72	21.71	30.61	35.63	41.93

In vitro drug release profile of all the mucoadhesive microspheres formulations shown in **Figure 4**.

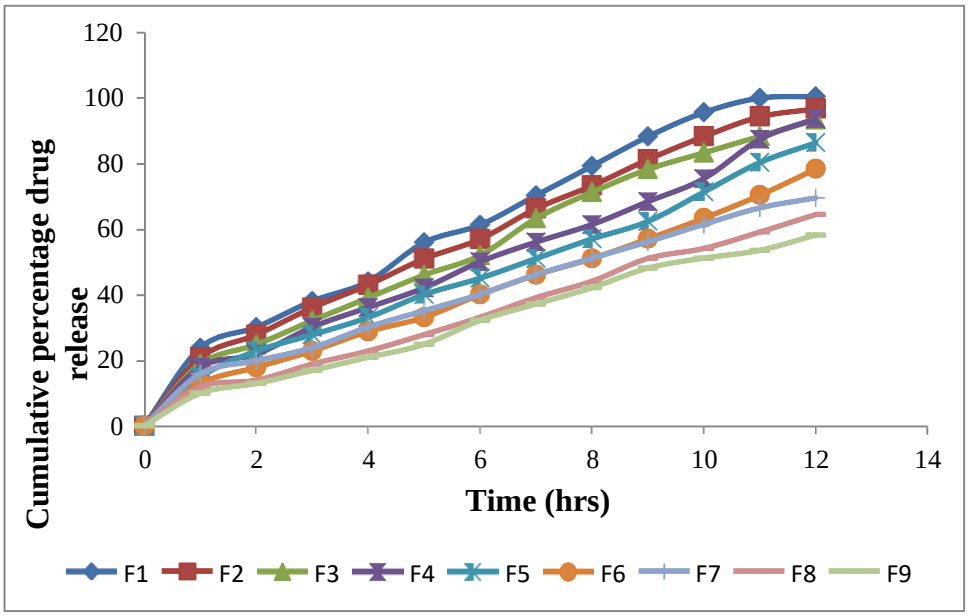


Figure 4: *In vitro* drug releases of all mucoadhesive microspheres formulations of Methotrexate

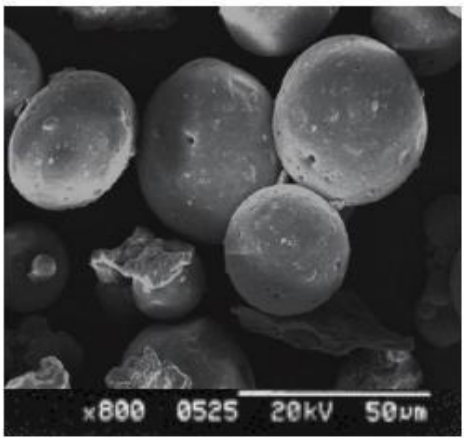
In vitro drug release studies were carried out and the percentage drug dissolved at different time interval was calculated .The *in vitro* drug release is highly dependent on the stirring speed and volume of cross linking agent .Increase in stirring speed, decrease the drug release from the microspheres similar way increase in the volume of cross linking agent decreases the release of drug from microspheres. The result of cumulative percentage drug release of all formulations after 12 hrs for formulations F1, F2, F3, F4, F5,F6, F7, F8 and F9 was found to be 100.31%, 96.64%, 93.29% ,93.57%, 86.27%, 78.29%, 69.39%, 64.37% and 58.07% respectively.

From these nine formulations F4, F5, F6 selected as optimized formulations based on drug entrapment efficiency, *in vitro* drug release and mucoadhesion property. The result of above formulation F4, F5, F6, indicates that 40 mL glutaraldehyde was an optimum volume for cross linking to produce a spherical in nature with good mucoadheasiveness and good drug entrapment efficiency microspheres.

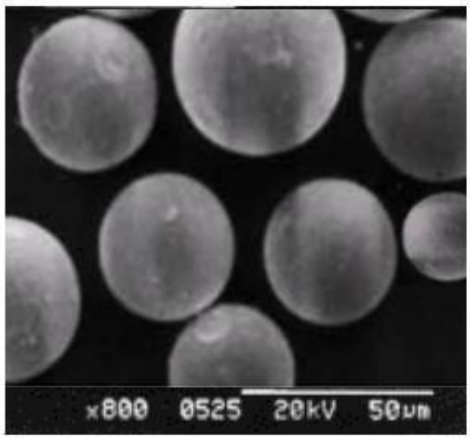
Scanning Electron Microscopy

Scanning Electron Microscopy of selected microspheres formulations (F4, F5, F6) shown in **Figure 5**.

Scanning Electron Microscopy of F4



Scanning Electron Microscopy of F5



Scanning Electron Microscopy of F6

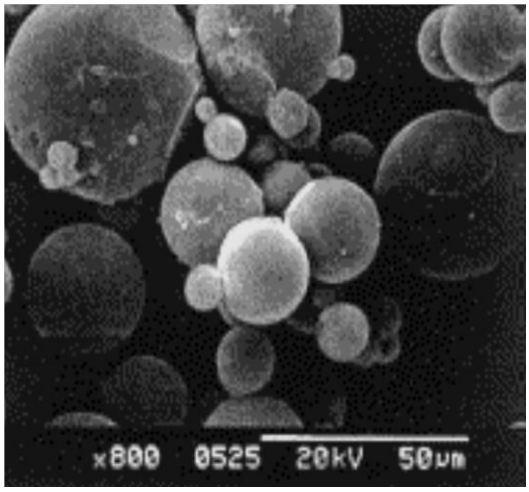


Figure 5: Scanning Electron Microscopy of selected microspheres

Scanning electron photomicrograph was taken at the acceleration voltage of 20 KV, chamber pressure of 0. 6 mm Hg, magnification original x 800.SEM of selected microspheres was found to be free flowing, spherical in shape.

Release kinetics

Table 6: kinetics data obtained from *in-vitro* release profile for methotrexate mucoadhesive microspheres.

Kinetic Profile of various formulations	First order equation		Zero order equation		Korsemeyers-Peppas		Higuchi equation	Release mechanism
	k	r^2	k	r^2	n	r^2	r^2	
F1	0.414	0.6583	8.1296	0.9773	0.6404	0.9776	0.9681	Non fickian
F2	0.254	0.8942	7.6946	0.9837	0.6591	0.9871	0.9699	Non fickian
F3	0.204	0.9330	7.4783	0.9870	0.6917	0.9812	0.9599	Non fickian
F4	0.187	0.8527	7.1510	0.9902	0.6972	0.9729	0.9415	Non fickian
F5	0.144	0.9145	6.6006	0.9903	0.7056	0.9845	0.9434	Non fickian
F6	0.113	0.9453	6.0384	0.9945	0.7453	0.9805	0.9352	Non fickian
F7	0.094	0.9866	5.4110	0.9859	0.6405	0.9731	0.9644	Non fickian
F8	0.082	0.9791	5.1270	0.9940	0.7425	0.9619	0.9329	Non fickian
F9	0.072	0.9890	4.7631	0.9920	0.7752	0.9725	0.9369	Non fickian

The kinetics investigations of the release profile gave us useful insight into the drug release rate and mechanism of drug release. All the formulations dissolution datawas subjected to regression analysis and were fitted to various kinetic models, such asZero order, First order, Higuchi square root and Korsemeyers- peppas.

The r^2 value of zero order of all above 9 formulations ranges from 0.9773 to 0.9945 this indicated that the drug released from the microspheres by zero order rate kinetics.

The dissolution data of all formulations were subjected to Higuchi and Korsemeyers-peppas model, the r^2 value of all formulations for Higuchi model lies between 0.9329 to 0.9699 .This suggested that the Higuchi diffusion plot of all formulations were fairly linear.

Korsemeyers-peppas plot was designed by taking a log % CDR on y-axis and log time on x-axis. The plot was found to be linear and r^2 value ranging from 0.9619 to 0.9871. The n-value of Korsemeyers-peppas ranges from 0.6402 to 0.7752; this indicated that the released of the drug from the microspheres was by non-fickian diffusion mechanism.

So, optimised formulations F4, F5 and F6 released the drug by zero order rate and non-fickian diffusion mechanism.

From these three optimised formulations F5 was the best formulation having spherical free flowing microspheres in nature with 70.1% mucoadhesion, drug entrapment efficiency 69.78% with the slow release of the drug 86.27% upto 12 hrs.

From this we conclude that 40 mL of glutaraldehyde was an optimum amount for cross linking to produce a spherical microsphere with good mucoadheasiveness and good drug entrapment efficiency.

CONCLUSION

The study of mucoadhesive microspheres as a system for controlled release was conducted based on the feasibility of microspheres by using chitosan as a polymer and glutaraldehyde as a crosslinking agent prepared by simple emulsification phase separation technique.

The results revealed that the two variables glutaraldehyde volume and stirring time significantly affected the percentage mucoadhesion, drug entrapment efficiency, particle size, swelling index. As the concentration of glutaraldehyde increases, mucoadhesion decreases .The best formulation exhibited an optimum percentage mucoadhesion 70.1% and optimum drug entrapment efficiency 69.78% .The mucoadhesive microspheres of methotrexate could sustained the drug release for more than 12 hrs.

The results provided in the study confirmed that the objective of achieving a controlled release system which will improve bioavailability and also improves the patient compliance was succeeded. This concludes that this technique is quite suitable to form Methotrexate mucoadhesive microspheres as a potential drug delivery system for achieving controlled release with good stability.

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