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Research Article

Design, Optimization and Characterization of Metformin Hydrochloride Loaded Biodegradable Microspheres using Box Behnken design for Local Delivery in Periodontitis

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ABSTRACT

In the present study, metformin hydrochloride-loaded microspheres were prepared for filling into the periodontal pockets with or without grafts for the treatment of periodontitis. For this purpose, chitosan was chosen as a polymer and used at different drug/polymer ratios in the preparation of microspheres by emulsion cross-linking method. Optimization was carried out by implementing a three-factor, three-level Box–Behnken design. Mathematical models were generated by regression analysis for responses of particle size (PS) and entrapment efficiency (EE). The experimental design took into account the preparation of optimized formulation with maximum %EE and minimum PS at optimum process conditions for the microsphere formulation by reducing chemical use and formulation time, in an economical way. The optimized formulation was selected on the basis of the desirability function and was further evaluated with respect to the particle size, entrapment efficiency, *in-vitro* drug release, differential scanning calorimetry (DSC), fourier transform infrared (FTIR) spectroscopy and surface morphology studies. The results of release studies were evaluated kinetically and statistically. Particle size and entrapment efficiency of the selected batch were found to be in the range of 40.2 to 59.6 µm and 85 to 95%, respectively. The DSC studies revealed molecular dispersion and conversion of the drug into an amorphous form. Surface morphology of microspheres was analyzed by scanning electron microscopy (SEM) and found to be spherical in shape with a smooth surface. The mean particle size, EE, and *in-vitro* drug release of the optimized batch were found to be 51.4 ± 4.8 µm, 96.5 ± 1.42%, and 79.8 ± 3.1%, respectively. The release kinetics showed that the release followed the Peppas model, and the main mechanism of drug release was diffusion. These sustained-release chitosan microspheres could be a promising drug delivery system for local delivery of metformin hydrochloride in the treatment of periodontitis.

INTRODUCTION

Metformin hydrochloride, a second-generation biguanide, is used as a hypoglycemic agent in treating type 2 diabetes mellitus.[1] Use of metformin hydrochloride for the treatment of diabetes is known but it has also shown osteogenic activity. It has shown a dose-dependent increase of cell proliferation in two osteoblast-like cells (UMR106 and MC3T3E1). It has also shown an increase in type-I collagen production in both cell lines and stimulated alkaline phosphatase activity in MC3T3E1 osteoblasts.[2] Periodontitis is a set of inflammatory diseases affecting the periodontium, i.e., the tissues that surround and support the teeth. It destroys the attachment apparatus of the teeth which forms the periodontal pocket and normal osseous anatomy i.e. it causes progressive loss of the alveolar bone around the teeth.[3] There are two approaches for treating periodontitis, viz. anti-infective and regenerative treatments. The current study is based on the approach

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of regenerative treatment. As periodontitis involves progressive loss of alveolar bone, bone regeneration is required to treat the disease. Conventional therapy for bone regeneration involves use of bone grafts. These bone grafts are solid in nature and are placed at the site to assist bone regeneration. Chitosan microspheres containing metformin hydrochloride can be used along with or in place of bone grafts as a regenerative therapy for periodontitis.

Microspheres allow the controlled release of drug at the target site.[4] Microspheres can be used to achieve the desired release profile by choosing a polymer of appropriate molecular weight and the right method of preparation or by varying copolymer concentration. As they have small particle size and hence large surface-to-volume ratio, they can be used for controlled delivery and hence may be used for sustained release of the metformin hydrochloride for the treatment of periodontitis. Chitosan is a biodegradable natural polymer with various properties like biocompatibility, high charge density, non-toxicity, and mucoadhesion. Various techniques like interactions with anions, thermal cross-linking, chemicals, solvent evaporation technique, etc can prepare chitosan microspheres.[5]

Box Behnken Design (BBD) requires fewer treatment combinations and helps statistically evaluate the effect and interaction among variables. BBD involves the construction of response surface plots and polynomial equations and thus helps optimize the process with fewer experimental runs.[6]

The aim of this study was to find the optimal formulation parameters i.e. drug to polymer ratio, surfactant concentration, and rotation speed for the preparation of metformin hydrochloride loaded chitosan microspheres with sustained drug release by using Box Behnken design.

Materials and Methods

Materials

Metformin hydrochloride was received as a gift sample from USV, Mumbai, India. Chitosan (85% deacetylation, with Molecular weight of 10000 Da) was obtained from Annlab Fine Chemicals, Mumbai. Glacial acetic acid, light liquid paraffin and glutaraldehyde were obtained from Research Lab Fine Chem Industries, Mumbai. Span 80 was obtained from Molychem Industries, Mumbai.

Preparation of Microspheres[7]

The metformin hydrochloride-loaded chitosan microspheres were prepared by emulsion cross-linking method. Box Behnken design was used for the optimization of process parameters.

Chitosan was dissolved insufficient amount of 1% glacial acetic acid. The drug was dissolved in an inappropriate amount of distilled water, which was mixed with a chitosan solution to obtain an aqueous phase. This aqueous phase was added drop-wise into the oily phase i.e. light liquid paraffin containing span 80 as surfactant using a needle. The w/o emulsion formed was stirred for 40 minutes and 1.5 mL, and glutaraldehyde solution was added to it at 10, 20, and 30 minutes intervals. The microspheres formed were separated by centrifugation at 5000 rpm for 30 minutes and washed with chloroform to remove traces of light liquid paraffin and were air-dried.

Experimental Design and Statistical Analysis

While developing a complex formulation if traditional experimental methods are used it requires more effort, time, and materials. Hence various experimental designs are being used which require less efforts and provide estimates of the relative significance of different variables.[8-12]

The independent variables (Table 1) selected in the study were drug: polymer (A), surfactant concentration (%) (B) and speed of rotation (RPM) (C) while dependent variables were entrapment efficiency (%) and particle size (µm). The levels of the factors were selected on the basis of preliminary studies (Table 2). The concentration of polymer was varied from 100 to 300 mg, surfactant concentration varied between 1 to 2% while the rotation speed of homogenizer was varied from 3000 to 4000 rpm. Design Expert 7.1 software was used to get a mathematical model for each dependent variable and then it was analyzed statistically.

Selection of the Optimized Formulation

Based on the data obtained from the experimental runs, the design expert software suggests combinations of the independent variable that should be used to give out the best batches with desired entrapment efficiency and particle size than actually preparing and evaluating them. The optimized formulation of microspheres was evaluated for particle size, entrapment efficiency, *in-vitro* drug release, scanning electron microscopy (SEM), DSC and FTIR studies.

Evaluation of Formulation

Particle Size[13]

Particle size was determined by optical microscope (Micron, Optik). The eyepiece was fitted with a micrometer scale which was used to determine the particle size. The microspheres were dispersed on a microscope slide. A microscopic field is scanned and at least 100 particles were examined in all measurements. The average particle size

Table 1: Independent variables and their selected levels for microsphere formulation

Factors	*Levels*		
	-1	*0*	*+1*
(A) Drug: Polymer	1:1	1:2	1:3
(B) Surfactant concentration (%)	1	1.5	2
(C) Speed of rotation (rpm)	3000	3500	4000

of the microspheres was expressed as the volume surface diameter (µm) and the standard deviation was calculated for each batch of microspheres.

Drug Loading and Entrapment Efficiency[14,15]

Spectrophotometric methods were used for the determination of drug contents. Microspheres equivalent to 10 mg of metformin HCl were weighed, crushed, and then dissolved in 0.1M HCl at room temperature. The samples were diluted to 200 µg/mL. The solution was centrifuged at 13000 rpm for 15 minutes and then analyzed at 233 nm using a UV-visible spectrophotometer for metformin HCl. The drug loading and Entrapment efficiency (%) were calculated using the following equations.

$$\text{Drug loading (\%)} = \frac{M_{actual}}{\text{Weighed quantity of powder of microsphere}} \text{ X } 100$$

$$\text{Entrapment efficiency (\%)} = \frac{M_{actual}}{M_{theorical}} \text{ X } 100$$

Mactual is the actual drug content in the quantity of powder of microspheres and $M_{theoretical}$ is the theoretical amount of drug in microspheres calculated from the quantity added in the preparation process.

In-vitro Drug Release[16]

In-vitro drug release from the formulations was studied using phosphate buffer saline pH 7.4 as a medium. In brief, the drug loaded microspheres were suspended in 1-mL PBS with pH 7.4 in a dialysis bag (molecular weight cut off 10000 D) and sealed. The bag was then suspended in a beaker containing 50 mL PBS with sodium azide 0.5% as a preservative and stirred at a constant speed of 50 rpm at 37 ± 0.5°C. Further, the aliquots of 3 mL each were withdrawn at the intervals of 1, 2, 3, 4, 5, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192 and 240 hours and equal volume of buffer was added after each withdrawal. The sample was then filtered using a 0.45 µ membrane filter and further analyzed by UV spectrophotometer at 233 nm. Each experiment was performed in triplicate.

Table 2: A Box Behnken experimental design layout

Formulation code	*Coded level (Independent variable)*		
	A	*B*	*C*
F1	-1	-1	0
F2	+1	-1	0
F3	-1	+1	0
F4	+1	+1	0
F5	-1	0	-1
F6	+1	0	-1
F7	-1	0	+1
F8	+1	0	+1
F9	0	-1	-1
F10	0	+1	-1
F11	0	-1	+1
F12	0	+1	+1

Release Kinetics

Release kinetics of the *in-vitro* release studies was determined by fitting data into the following four mathematical models where M_t/M_∞ is the fraction of drug released at time t. k_0, k_1, k_H, and k are the zero-order release constant, first-order release constant, Higuchi constant, and Korsmeyer- Peppas constant, respectively. In the Power-law model, n is the release exponent and is indicative of the drug release mechanism. The k0, k1, kH, k, and n values were determined by fitting the release data into respective equations. W_0 is the initial amount of drug in pharmaceutical dosage forms. W_t is the amount of drug remaining in dosage forms at the time 't'

Zero order $\frac{Mt}{M\infty} = k_0\, t$

1st order $\frac{Mt}{M\infty} = 1 - \exp(k_1 t)$

Higuchi model $\frac{Mt}{M\infty} = K_H \; t^{1/2}$

Korsmeyer Peppas $M_t / M_\infty = kt^n$

Hixon-crowell $W_0^{\frac{1}{3}} - W_t^{\frac{1}{3}} = kt$

Scanning Electron Microscopy (SEM) Studies

SEM images metformin HCL loaded microspheres were taken. Microspheres were sputtered with gold ions for 5 to 6 minutes. The samples were observed for surface characteristics.

Differential Scanning Calorimetry (DSC)

The thermal behavior of the drug was studied using DSC. The accurately weighed sample was placed onto aluminum pans and then they were hermetically sealed with aluminum lids. A scanning rate of 10˚C/min was maintained over a temperature range of 50 to 350˚C to obtain thermograms in the environment of liquid nitrogen (flow rate – 60 mL/min.).

Fourier Transform Infrared (FTIR)

Microspheres were mixed with potassium bromide in a ratio of 1:100 in a mortar to prepare pellet. Pellet was prepared using hydraulic press under the pressure of 10 tonnes. FTIR spectrum was obtained by scanning the sample on the FTIR spectrometer (Perkin Elmer Spectrum BX2 USA) over the 400-4000 cm^{-1} wavelength range.

Results and Discussion

Preparation of Metformin HCl Loaded Chitosan microspheres

In the present study, metformin HCl loaded microspheres were prepared by emulsion cross linking method using chitosan as a bioadhesive carrier and glutaraldehyde as a cross linking agent. The method resulted in consistent production of smaller size microspheres (<60 µm) with narrow size distribution and good entrapment efficiency.

Experimental Design and Statistical Analysis

Box Behnken design was selected as it requires fewer treatments in comparison with full factorial designs. Three factors at three levels were used and 12 batches were prepared. All the 12 batches were evaluated for particle size (µm) and % entrapment efficiency (%EE). The particle size (µm) and %entrapment efficiency results showed variation between 40.2 to 59.6 µm and 85 to 95%, respectively (Table 3). Hence it can be said that results depend upon the selected independent variables.

Response variables Y_1 and Y_2 were fitted with a quadratic model. The positive sign represents a synergistic effect of the factors on the response, whereas antagonist relationship is represented by the negative sign. Since the values of r^2 are quite high for both the responses (0.9693 for particle size and 0.8997 for %EE), the polynomial equations form excellent fit to the experimental data and are highly statistically valid.

Practically obtained dependent variables were subjected to multiple regressions to give a quadratic equation. The equation obtained for particle size results was as follows.

$$PS = +46.38+4.55*A+1.20*B-0.91*C$$
$$+4.32*A*B+5.54*A*C+0.21*B*C+3.85*A^2-2.47*B^2$$

For particle size, the correlation coefficient was found to be $R^2 = 0.9693$. The Model F-value of 11.86 states that the model is significant. The probability that a "Model F-Value" this large could occur due to noise is only 3.33%. The *p-value* obtained was 0.0,33 which is less than 0, indicatinges that model terms were significant. Signal to noise ratio greater than 4 is desirable. Our ratio of 10.552 indicates an adequate signal.

The equation obtained for % entrapment efficiency was as follows:

$$EE = +88.81+0.89*A+0.42*B+0.46*C+0.40*A*B+4.82*A*C$$
$$+0.30*B*C$$

Table 3: Values of Entrapment Efficiency and particle size of microspheres as per Box Behnken design

Batch no.	*Entrapment Efficiency (%)*	*Particle size (µm)*
F1	87 ± 0.89	44.54 ± 2.69
F2	89.4 ± 0.76	46.28 ±3.82
F3	86.5 ± 1.27	40.6 ± 2.91
F4	90.5 ± 1.53	59.6 ± 3.12
F5	94.3 ± 1.82	52.43 ± 4.26
F6	85.0 ± 0.83	49.18 ± 3.65
F7	85.0 ± 1.42	40.2 ± 4.14
F8	95.0 ± 1.08	59.1 ± 2.87
F9	87.1 ± 1.74	45.3 ± 2.43
F10	87.9 ± 0.77	45.0 ± 1.92
F11	88.0 ± 1.91	42.4 ± 2.28
F12	90.0 ± 1.63	42.92 ± 1.08

The correlation coefficient value obtained was R2 = 0.8997 and F value was found to be 7.47which, which shows that the model is significant. The probability that a "Model f-value" this large could occur due to noise is only 2.16%. The *p-value* obtained was 0.0216, which is less than 0.5, indicating that model terms were significant. Signal to noise ratio greater than 4 is desirable. Our ratio of 9.843 indicates an adequate signal.

Three-dimensional response surface plots for each response parameter were constructed (Figs 1 and 2). It helps in studying the effects of formulation parameters on dependent variables graphically.

Optimization and Validation

Amongst numerous solutions suggested by the Design Expert software, four with the highest desirability values were prepared. The selected factor combinations with desired responses were selected and actually prepared as batch M_1, M_2, $M_3$and M_4. Table 4 compares the predicted and experimental values of the selected responses. The low value of % error reflects the predictive ability of the selected design.

Evaluation of Microspheres

Particle Size

Particle size of the twelve microsphere formulations varied in the range of 40.2 to 59.6 µm. The mean particle size for batches M_1, M_2, M_3 and M_4 was found to be 43.57, 54.04, 51.4 and 52.64, respectively.

Drug Loading and Entrapment Efficiency

Entrapment efficiency for batches M_1, M_2, M_3 and M_4 was found to be 91.2, 88.35, 92.5 and 90.94, respectively.

In-vitro Release Studies

In-vitro release study was performed in phosphate buffer saline pH 7.4 (Fig.3). *In-vitro* release for batches M_1, M_2, M_3 and M_4 on the 10^{th}day was found to be 86.83, 82.68, 79.8, and 86.29%, respectively.

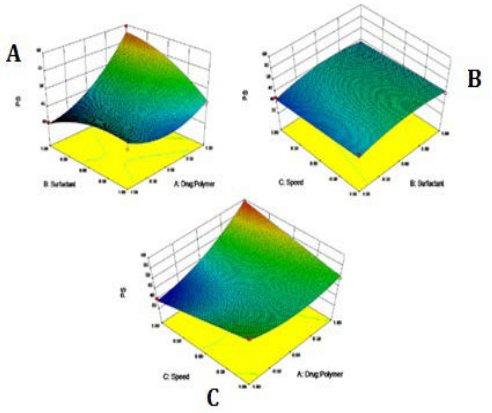

Fig. 1: Three dimensional graphs showing effect of A- Surfactant concentration and drug: polymer, B- Speed and Drug: polymer, and C- Surfactant concentration and Speed on Particle size

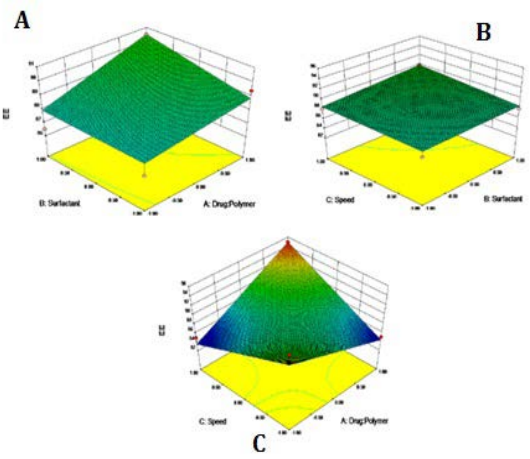

Fig. 2: Three dimensional graphs showing effect of A- Surfactant concentration and drug: polymer, B- Speed and Drug: polymer, and Surfactant concentration and Speed on Entrapment efficiency

Drug release kinetics was analyzed by fitting the drug release data into different mathematical models (Fig. 4). The values of regression coefficients obtained by fitting data into models are listed in Table 5.

Zero-order, first-order, Peppas model, and Hixon Crowell model were used to fit the data of drug release profiles. The best-fit release profile was the one which was having the highest R^2 value. The mechanism of drug release was obtained by fitting the data to the Korsmeyer Peppas equation.

Hence from the results of particle size, entrapment efficiency, and *in-vitro* release study, batch M_3 was selected as optimized formulation and further characterized by SEM, DSC, FTIR studies.

Scanning Electron Microscopy (SEM)

Microspheres were found spherical in nature with slight agglomerations. In addition, micropores were observed on the surface of microspheres at higher magnifications (Fig.5).

Differential Scanning Calorimetry

DSC studies indicated a sharp endothermic peak at 235.8°C corresponding to the melting of metformin HCl. The peaks broadening as well as a change in relative intensities were observed due to dilution of drug in physical mixtures of drug with chitosan (Fig. 6).

Fourier Transform Infrared Spectroscopy

The FTIR spectrum of metformin hydrochloride and the polymers were recorded. The FTIR spectrum of the drug loaded microspheres was recorded to study the change that may have occurred in the formulation during the process. The FTIR spectrum of the drug, polymer, and formulation is shown in Fig. 7.

N- H stretch observed in drug at 3372 cm^{-1} was retained in the formulation IR. The peak at 2924 cm^{-1} indicates C- H stretch which was observed in chitosan and was retained in the formulation. N- H bend was observed at 1567 cm^{-1} which was observed in FTIR of formulation also.

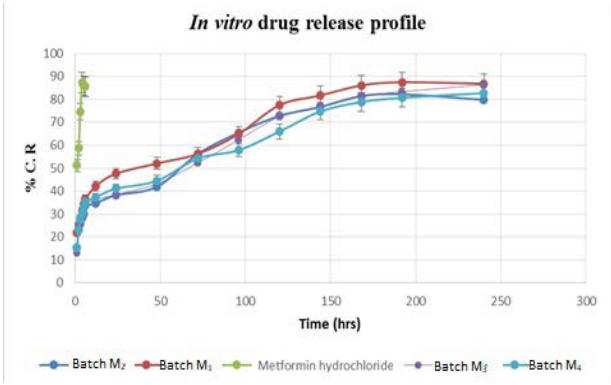

Fig. 3: *In-vitro* drug release in phosphate buffer saline pH 7.4 (n=3)

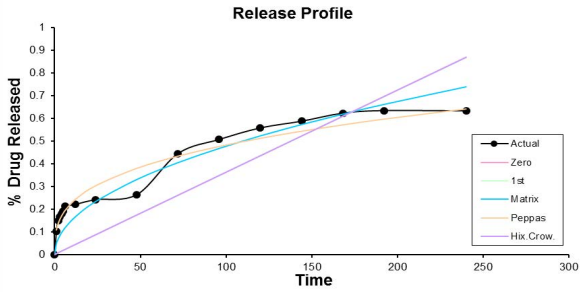

Fig. 4: Kinetic model fitting for drug release data of optimized batch M_3

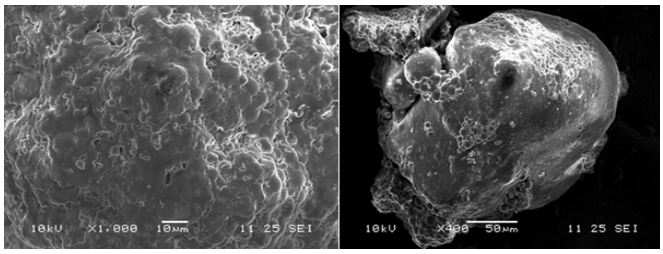

Fig. 5: SEM images of metformin hydrochloride loaded microspheres

Table 4: Comparison of experimental results with predicted responses

Batch No.	*Drug to polymer ratio/ Surfactant Concentration/ Stirring Speed*	*Experimental Particle size (µm)*	*Predicted Particle size (µm)*	*%Error*	*Experimental %EE*	*Predicted %EE*	*%Error*
M_1	0.1/0.8/0.1	43.57	46.5	6.3	91.2	89.38	-2.03
M_2	0.4/0.9/0.4	54.04	50	-8.08	88.35	90.75	2.64
M_3	0.6/0.6/0.6	51.4	53.4	3.7	92.5	91.85	-0.70
M_4	0.8/0.9/0.8	52.64	57.6	8.61	90.94	93.85	3.10

Table 5: Mathematical models for drug release kinetics for batch M_3

S. No.	*Kinetic model*	*Regression coefficient*
1	Zero order	0.7246
2	1st order	0.7262
3	Peppas	0.9773
4	Hixon Crowell	0.7257

Discussion

The objective of this study was to prepare microspheres of metformin hydrochloride by emulsion cross-linking method and to optimize the effects of formulation variables on response parameters. Values of response variables at selected factors were predicted using a mathematical equation obtained from BBD. There is a possibility of getting optimum formulation with ranges in between the selected levels of independent variables.

Results of the particle size analysis study clearly indicate that polymer and surfactant concentrations have a significant effect on particle size. As polymer concentration increases particle size increases while as surfactant concentration increases, particle size decreases. An increase in particle size when polymer concentration is increased can be related to increase in viscosity and cross-linking in the formulation. Speed of rotation has a negative effect on the particle size. The decrease in particle size was observed as the speed of rotation was increased.

The polymer concentration and speed of rotation showed a positive effect on the %entrapment efficiency. This can be related as an increase in polymer concentration increases the viscosity and cross-linking of the formulation, leading to more entrapment of the drug in the polymer. Another reason for high entrapment efficiency can be the higher solubility of metformin hydrochloride in the dispersed phase. Here dispersed phase is aqueous in nature and metformin hydrochloride is completely soluble in water. The drug might remain in the dispersed phase as cross-linking occurs immediately after the addition of glutaraldehyde. The insolubility of metformin hydrochloride in light liquid paraffin i.e. continuous phase can be one of the reasons for high entrapment efficiency of the drug as the drug does not diffuse in continuous phase during the formation of microspheres. But increase surfactant concentration decreased the entrapment efficiency. This can be interpreted as an increase in surfactant concentration, decreasing particle size, and decreasing entrapment efficiency.

Slight higher drug release was observed in batch with lower polymer concentrations. In contrast, slightly more sustained release effect was observed in batch containing higher polymer concentration i.e. batch C. Initially higher release was observed in all the batches which was further sustained with time. Constant release was observed on 9th and 10th day. This concludes that the formulation gives sustained release for 10 days. A low standard deviation in release data indicates the reproducibility of the results.

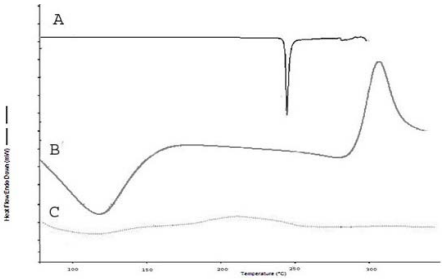

Fig. 6: DSC thermogram of A) metformin HCl B) chitosan and C) optimized batch M_3

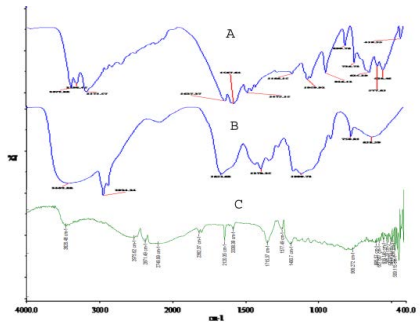

Fig. 7: FTIR spectra of A) metformin HCl B) chitosan and C) optimized batch M_3

In DSC thermogram studies, the peak disappeared for the drug-loaded chitosan microspheres, indicating that the drug was molecularly dispersed inside the chitosan matrix. It can be concluded that the excipients and drug did not interact with each other. Also, the drug didn't form a complex with the excipients as the endothermic peaks remained unchanged in position (Fig. 6). Similarly, the results of FTIR studies indicated that there is no interaction between the drug and the excipients.

Conclusion

The present research proposed metformin hydrochloride loaded biodegradable microspheres for filling periodontal pockets with or without grafts. Microspheres were prepared by the emulsion cross-linking method and evaluated for particle size, entrapment efficiency, and *in-vitro* release. All measurements were found to be in an acceptable range. It was concluded that metformin hydrochloride-loaded biodegradable microspheres could effectively treat periodontitis through local delivery. However, clinical data are still needed to evaluate the risk: benefit ratio.

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