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Optimization and Characterization of Gentamicin Loaded Chitosan Microspheres for Effective Wound Healing

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Abstract

In the present study, Box-Behnken experimental design was employed to statistically optimize the formulation parameters of gentamicin sulphate loaded chitosan microspheres for maximum entrapment and controlled release. The independent variables selected were ***concentration of*** *drug (X_1), cross linking agent (X_2) and chitosan concentration (X_3) whereas dependent variables studied were entrapment efficiency (Y_1) and mean diameter (Y_2) of microspheres. The quantitative effect of the formulation parameters at different levels on drug entrapment and microsphere size could be predicted using polynomial equations. For estimation of coefficients in the approximating polynomial function, the least square regression method was applied. The information about the model reliability was verified by using the analysis of variance (ANOVA). A formulation comprising of 10mg gentamicin, 2% (w/v) tripolyphosphate and 3% (w/v) chitosan, was identified for maximizing entrapment (80.04±2.24%) and* ***minimizing*** *particle size to optimum level (18.64±0.61µm). The optimal microsphere preparation was subsequently characterized in terms of morphology, release kinetics, and antimicrobial activity. Scanning electron microscopy confirmed the smooth spherical microspheres in the size range of 11.42±0.56µm to 26.34±0.72µm. Kinetic models revealed that drug release followed non-Fickian pattern.* ***Drug*** *bioactivity was found to remain intact after microencapsulation. Thus, experimental design methodology could efficiently be applied for characterization and optimization of parameters affecting drug entrapment to obtain the maximum amount of information in a short period of time with the fewest number of experiments.*

Keywords: *Gentamicin; chitosan; Box-Behnken Design; Microspheres*

INTRODUCTION

Chitosan is a natural linear biopolyaminosaccharide derived from chitin by alkaline deacetylation.[1,2] It is the most abundant polymer found in nature after cellulose, being a structural component of shellfish, insects and the cell walls of bacteria and mushrooms.[3,4] Chitosan derived from chitin possess a tissue cell growth function serving as a favorable medium for cell attachment and proliferation. This promotes rapid dermal regeneration leading to accelerated wound healing.[5] Moreover, it has many other useful and advantageous biological properties in the application as a wound dressing, namely biocompatibility, biodegradability, hemostatic activity, anti-infectional activity that accelerate wound healing.[6,7]

Wound healing is a complex multifactorial process that results in contraction and closure of wound and restoration of a functional barrier.[8] It is important to keep the wound free of infection as bacterial bioburden causes delayed or impaired healing inhibiting natural healing process. Moreover, during infection the bacterium is localized intracellularly making the treatment difficult with antibiotics.[9] Thus there is a need of delivery vehicles that allow localized and controlled delivery of antibiotics for preventing microbial infections by intracellular pathogens as well as have in built natural healing property of dermal regeneration. Chitosan microspheres are the most widely studied drug delivery systems for the controlled release of drugs viz., antibiotics, antihypertensive agents, anticancer agents, proteins, peptide drugs and vaccines.[10-12] These are effective for systemic as well as for local therapy.[13] Liposomes and microspheres were previously developed for this aim, showing promising results.[14,15] However, several drawbacks were described related with its low

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encapsulation efficiencies and stability problems.

Gentamicin sulphate is a hydrophilic aminoglycoside antibiotic with short half-life of 2-4 hrs[16] used in the treatment of serious microbial infections. While gentamicin microspheres have been prepared but based on literature cited there exists a lack of studies regarding statistical optimization of formulation parameters to enhance both the entrapment and controlled drug release of gentamicin from chitosan microspheres.[15]

Designing controlled-release formulations with the minimum number of trials is very crucial for pharmaceutical scientists.[17] The response surface method has been commonly used for the optimization of formulations with various kinds of drugs in the development of controlled-release formulation design.[18-20]

In this research cross-linked chitosan microspheres were developed by the suspension cross-linking method using response surface methodology combined with Box-Behnken design to encapsulate and release hydrophilic antibacterial agents such as gentamicin in a controlled manner. Variables selected were gentamicin concentration (GM) (X_1), tripolyphosphate concentration (TPP) (X_2) and chitosan concentration (CC) (X_3) and the response variables were the mean diameter (MD) **as** Y_1 and the entrapment efficiency (EE) **as** Y_2 of microspheres. The levels for these variables were determined from the preliminary trials. Furthermore, selected formulation with maximum entrapment was evaluated for *in-vitro* release studies and antimicrobial activity.

MATERIALS AND METHODS

Materials:

Gentamicin sulphate (GM) (RS Spectra chemicals Ltd., Ahmednagar, India) and Chitosan (with 80% degree of deacetylation and viscosity of 16 mPa) **(Chemchito natural products, Chennai, India)** were received as gift **samples**; *P. aeruginosa* MTCC 424 **was obtained** from IMTECH, Chandigarh, India. All other chemicals used in the study were of analytical grade.

Methods:

Preparation and characterization of the microspheres

Chitosan microparticles were prepared by modification of the method reported by Akbuga and Durmaz (1994) and Akbuga and Bergisadi (1996).[21,22] Chitosan solution of varying concentration (1, 2 and 3%w/v) was prepared in 1%w/v lactic acid solution. GM (5, 10 and 15mg) was dispersed **in 5ml** of this solution and mixed well. This mixture was added to the oily phase (100ml of paraffin oil containing 2% w/v of sorbitan sesquioleate) to form water-in-oil (w/o) emulsion. This dispersion was stirred for 1 hour at 250 rpm (Laboratory stirrer, 1NL-2116, Remi Motors Ltd., Mumbai, India) after the addition of sodium tripolyphosphate (TPP) (2, 4 and 6% w/v) dropwise. The **synthesized microspheres of crosslinked chitosan** were **obtained** as a suspension in the oily continuous phase after 2.5 h. All batches were prepared at least three times. The microspheres were isolated by vacuum filtration (0.45 μm, PFTE membrane filters), washed with equal volume of n-hexane and freeze dried (Heto power dry LL 3000 Lyophilizer). The full factorial design and layout with coded and actual values of variables for each batch and responses are shown in Table No. 1. The trials were performed in random order.

Experimental design

A Box-Behnken experimental design was employed to statistically optimize the formulation parameters of GM microsphere preparation for maximum entrapment, **optimum diameter** and controlled drug release. The Box-Behnken design was specifically selected since it requires fewer treatment combinations than other design in cases involving three or four factors. The Box-Behnken design is also rotable and contains statistical "missing corners" which may be useful when the experimenter is trying to avoid combined factor extremes. This property prevents a potential loss of data in those cases. Generation and evaluation of the statistical experimental design was performed with the STAT-EASE, design expert, 7.0.3. A design matrix comprising of 16 experimental runs was constructed. An interactive second order polynomial model was utilized to evaluate both the response variables:

$$Y_i=b_{0+}b_1X_{1+}b_2X_2+b_3X_3+b_4X_1X_1+b_5X_2X_2+b_6X_3X_3+b_7X_1X_1+b_8X_1X_3+b_9X_2X_3 \text{ - (1)}$$

where b_0–b_9 are the regression coefficients, X_1–X_3 **are** the factors studied and Y_i is the measured response associated with each factor level combination.

Morphological characterization of microspheres

The particle size was measured directly by optical

microscopy using a compound microscope (Erma, Tokyo, Japan) on 300 microspheres.[23] **Mean diameter (MD) were calculated**. The morphology and surface appearance of microspheres were examined by scanning electron microscopy (SEM) (Leo, VP-435, Cambridge, UK). **Microspheres were mounted on the standard specimen mounting stubs and were coated with a thin layer (20nm) of gold by sputter coater unit (VG Microtech, UK).** Photomicrographs were observed at 211X magnification operated with an acceleration voltage of 15kV and working distance of 21mm was maintained.

***Entrapment efficiency* (EE)**

A weighed quantity of microspheres was extracted with dimethylformamide for 24h, centrifuged at 6000 rpm for 30 min and filtered (0.2 µm nylon filters, Whatman, UK). The filtrate was analyzed for gentamicin content using UV-Vis spectrophotometer (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan). Derivatization **was** done with o-phthaldialdehyde and absorbance wavelength **was** 339nm for UV-Vis spectrophotometer.[24]

The **entrapment** efficiency was calculated from the following expression:

$$\%\text{Entrapment efficiency} = \frac{\text{Total amount of drug - free amount of drug}}{\text{Total amount of drug}} \times 100 \text{---(2)}$$

Results were expressed as mean (±SD) of 3 experiments.

The measured responses are shown in Table No. 1.

In vitro release studies

A weighed quantity of GM microspheres was suspended in isotonic phosphate buffer (pH 7.4, 50ml, 37°C). The dissolution medium was agitated **employing paddle** at 50rpm and maintained at a constant temperature of 37°C± 0.5°C in a water bath.[21] Samples were periodically removed at predetermined time intervals up to 24 hrs and the volume was replaced immediately by fresh phosphate buffer. The samples withdrawn were centrifuged (3000rpm, 15 minutes, at room temperature). The supernatant was analyzed for gentamicin content using UV-Vis spectrophotometer at absorbance wavelength of 339nm (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan) by derivatization with o-phthaldialdehyde.[24] Results were expressed as mean (±SD) of 3 experiments.

Antimicrobial efficacy of GM microspheres:

The GM released from chitosan microsphere samples were tested for activity according to the Ph. Eur. Suppl. 2000 by the diffusion method 2.7.2 using *P. aeruginosa* as test organism. Firstly, aliquots of pure gentamicin at different concentrations were evaluated for inhibition diameters and calibration curve was plotted. The antimicrobial activity of drug-loaded microspheres were evaluated by collecting samples (*in vitro* release aliquots) from the microspheres at different time intervals (0.5, 1, 2, 4, 8, 12 and 24hr) and **testing** against *P. aeruginosa.*

Molten agar media was transferred to sterilized petridishes and allowed to solidify. The plates were swabbed with the culture of the microorganism. Wells equidistant from one another were made in the solidified medium using a sterilized well borer. The solutions (100µl) collected (*in vitro* drug release aliquots) were filtered through sterilized Millipore membrane filters (0.45µm) and carefully filled into the wells. Samples were allowed to diffuse for 2hr at room temperature. The plates were then incubated for 18hr at 37±0.5ºC. The diameter (mm) of zone of growth inhibition surrounding each agar well was measured using a **vernier** caliper. Concentration of GM obtained by inhibition diameters was then compared with the concentration obtained from release method to evaluate the effect of **entrapment** on antimicrobial activity.[9] Each experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Morphology and surface characteristics of the microspheres were determined using scanning electron microscopy (**Leo, VP-435, Cambridge, UK**). TPP is a non-toxic and multivalent anion that can form cross-links by ionic interaction between positively charged amino groups of chitosan and multivalent negatively charged TPP molecules.[25,26] The TPP was selected **because of its** non toxic and stability improving nature. The yields of microspheres were upto 50% (most of the formulations had yields of more than 50%), which **reflect** good efficiency of the preparation method.

For the response surface methodology involving Box-Behnken design, a total of 16 experiments were performed for three factors at three levels each. Table No. 1 summarizes the experimental runs, their factor combinations and the levels of experimental units used in the study as well as the entrapment and mean diameter obtained for each factor combination. In order to determine the levels of factors which yielded maximum

entrapment, mathematical relationships were generated between the dependent and independent variables.

For estimation of coefficients in the approximating polynomial function (equation 1) applying uncoded values of factor levels, the least square regression method was used. A suitable polynomial equation involving the individual main effects and interaction factors was selected based on the estimation of several statistical parameters such as the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS) provided by the design expert software 7.0.3.

The resultant equations for both responses Y_1 and Y_2 are presented below (full model):

$$Y_1(MD)=18.71+0.75X_1+2.83X_2+3.39X_3+0.25X_1X_2+0.70X_2X_3+0.82X_1X_2+0.69X_1^2+0.76X_2^2 \text{ - (3)}$$

$$Y_2(EE)=7.30+1.66X_1+5.81X_2+8.89X_3+0.12X_1X_2+0.13X_2X_3+0.79X_1X_3+3.88X_1^2+3.94X_2^2+2.51X_3^2 \text{ - (4)}$$

As presented in table No. 2, the quadratic model was selected as a suitable statistical model for optimized formulation with maximum entrapment because it had the smallest value of PRESS (165.46 for EE). PRESS is a measure of the fit of the model to the points in the design. The smaller the PRESS statistic is, the better the model fits to the data points.[27] The model showed a statistically insignificant lack of fit as shown in table No. 2. From the p-values presented in table No.2, it **was** concluded that for both responses the cross product contribution (2FI) of the model was not significant indicating the absence of interaction effects.

EE and MD of STP microspheres showed R^2 values of equation 3 and 4 to be 0.9232 and 0.9897 (Table No. 3), respectively; indicating good fit and it **could be** concluded that the second order model adequately approximated the true surface. For estimation of significance of the model, the analysis of variance (ANOVA) was applied. Using 5% significance level, a model is considered significant if the p-value is less than 0.05. The results of multiple regression analysis and analysis of variance (ANOVA) **test** are also summarized in Table No. 3.

All the **formulations** prepared with in the experimental design yielded smooth spherical microspheres with size in the range of **11.42- 26.34** (Fig. 1). GM at medium level (X_1, 0), TPP at low level (X_2, -1) and chitosan at high level (X_3, +1) yielded microspheres with highest drug entrapment (79.56%) **and** 20.61μm mean diameter.

In Table No. 3, factor effects of the Box-Behnken model associated p-values and standardized main effects (SME) values for both responses are also presented. A factor is considered to influence the response if the effects significantly differ from zero and the p-value is less than 0.05. Coefficient signs also give an indication of the effect produced (Table No. 3). A positive sign indicates a synergistic effect, while a negative sign represents an antagonistic effect of the factor on the selected response. SME values were calculated by dividing the main effects by the standard error of the main effects. The large SME of CC (X_3) for both responses studied indicated that the chitosan concentration was the main influential factor on the drug entrapment as well as size of microspheres. This was further investigated by the study of ANOVA. The breakup of source sum of squares (Source SS) in ANOVA indicated that the contribution of factor X_3 (CC) (SSY_1–127.12; SSY_2–631.55) is much higher than factor X_1 (GM) (SSY_1-4.47; SSY_2-22.08) and X_2 (TPP) (SSY_1-63.90; SSY_2-214.76) for optimizing the drug entrapment as well as the mean diameter of microspheres. The interaction terms X_1X_2, X_2X_3, X_1X_3 and the polynomial terms X_1X_1, X_2X_2 and X_3X_3 indicated insignificant values of individual source sum of squares. In addition, three dimensional response plots were presented to estimate the effects of the independent variables on responses by keeping one factor at constant level (3D response graphs for effect on entrapment are shown) (Fig. 2-4).

On the basis of above results, factor X_3 (CC) **was** found to be the main influential factor on the entrapment and microsphere size. It exerted positive effect on entrapment and particle size, also supported by the positive coefficient in the fitted model equation 3 and 4. This significant increase **might be** because of the increase in viscosity of the droplets. The increase in the entrapment efficiency with an increase in the concentration of chitosan is in accordance with previous reports.[28,29]

The particle size of microspheres increased with increase in X_3 (CC) which **might** be due to the fact that increase in the concentration of polymer **increases** the cross-linking, and hence the matrix density of the microspheres

resulting in **increased** particle size of the microspheres.[30] GM concentration (X_1) also exerted positive effect on both responses but it was not significant at higher concentration of the drug (Table No. 3). Increase in size **might** be because of the increase in viscosity of the droplets present in the internal phase caused by the increase in drug concentration as explained by Denkbas et al.[31]. However, it appears that this **apply** only at the lower level of GM concentration. Although further increase in drug concentration might be increasing the viscosity of the droplet, it does not result in significant change in mean diameter of microspheres.

TPP concentration exerted negative effect on the entrapment efficiency and positive effect on MD of microspheres as indicated by sign of coefficients in table No. 3. The decrease in entrapment with increasing TPP concentration could be due to the increased binding of the main groups of the drug to the added TPP.[32] TPP exerted positive effect **(MD increased with the increase in TPP concentration)** on MD of microspheres as in the presence of sufficient amount of TPP, fusion of smaller microspheres occur giving rise to chitosan microspheres of considerable larger size. The multifunctional TPP acts here to facilitate inter-microparticles binding of the chitosan.

Using the model generated with both responses (Equation 3 and 4), the optimization tool in the experimental design software was used to identify a formulation with a maximum entrapment. It predicted a maximum entrapment of 80.04 and optimum MD of 18.64µm with a formulation comprising of 10mg GM concentration, 2% (w/v) TPP concentration and 3% (w/v) chitosan concentration. To confirm the validity of the model, three batches of microspheres were prepared using this formulation and entrapment was determined. The actual experimental entrapment obtained was 79.56±3.37%. The predicted response and residual value performed at optimal values investigated in this study was found to be 80.05% and -0.49 respectively, validating the model generated in this study.

In vitro release behavior of microspheres exhibiting maximum entrapment (GC11) and pure drug (GM) was investigated in phosphate buffer (pH 7.4) for 24h (Fig. 5). An initial burst of 23.4±2.68% was observed in the first hour due to the drug located on or near the surface of microspheres. At the end of the 24h test period the **microspheres** showed 90.57±4.06% drug release. In order to investigate the release mechanism of present drug delivery system, the release data of prepared GM loaded chitosan microspheres in phosphate buffer (pH 7.4) were fitted to classic drug release kinetics models. The release rates were analyzed by least square linear regression method. Release models such as first order model, Higuchi model and Ritger-Peppas empirical model were applied to the release data (Table No. 4).[33,34] The coefficient of determination (R^2) of equation for release of GM from GC11 microspheres in phosphate buffer was 0.9741 signifying first order release pattern. The value of coefficient of determination (R^2) in Higuchi equation was found to be 0.9866 which indicates the integrity of chitosan gel and diffusion-controlled release. Substituting the release values in Ritger-Peppas equation, the value of coefficient of determination was about 0.9807. The value of n obtained was found to be 0.4680 indicating non-Fickian release as $n = 0.43$ indicates Fickian (case I) release; > 0.43 but < 0.85 for non-Fickian (anomalous) release; and > 0.89 indicates super case II type of release. Non-Fickian refers to a combination of both diffusion and erosion controlled-drug release.[35] This result was attributable to the sustained release of drug signifying mixed type of release pattern. These results are consistent with those obtained by Govender et al. who studied the release pattern of tetracycline, potent antibiotic, from microspheres prepared using chitosan for maximum bioadhesivity and controlled drug release.[36] Dhawan and Singla also reported the similar non Fickian release for nifedipine loaded chitosan microspheres prepared by emulsification solvent evaporation method.[37]

Although, GM is very stable in aqueous buffers over a wide pH and temperature range.[16] But, we conducted a bioassay to determine the effect on the antimicrobial activity of the encapsulated drug. The assay measured growth inhibition of *P. aeruginosa* MTCC 424 on molten agar media. **The activity of entrapped gentamicin was calculated by studying zone of inhibition.** Inhibition diameter versus the log of GM concentration (3-30µg/ml) of standard solutions closely correlated (R^2-0.9186). The inhibition zones of MS incubation media corresponded almost similar to the GM concentrations

expected from the amount of MS and the actual drug loading (Fig. 6). Thus, GM fully retained its biological activity upon encapsulation with negligible amount of loss may be due to processing steps during experimentation or adsorption to the vessel wall.

CONCLUSION

The optimized formulation for gentamicin sulphate was obtained with 2% GM concentration, 2%w/v TPP concentration and 3%w/v chitosan concentration using response surface methodology based on a Box-Behnken design. It was found that the observed responses were close to the predicted values for the optimized formulation. Microencapsulation didn't affect the bioactivity of entrapped drug as determined by antimicrobial assay. In conclusion, topical controlled release delivery system utilizing natural polymer i.e. chitosan for GM was successfully developed. **Further parameters for dosage form designing can be identified for optimum formulation in terms of desirable long-term stability and to study the therapeutic effects of these particles *in vivo*.**

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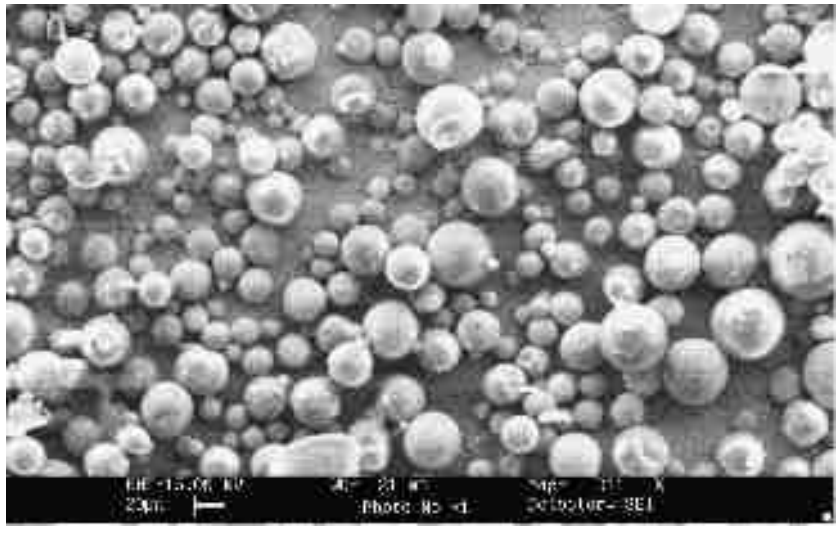
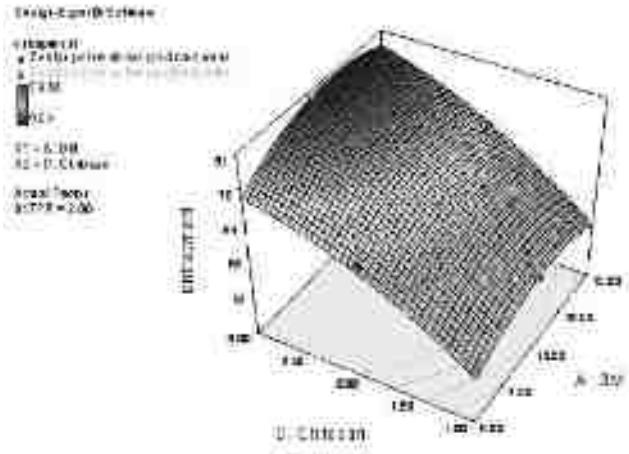


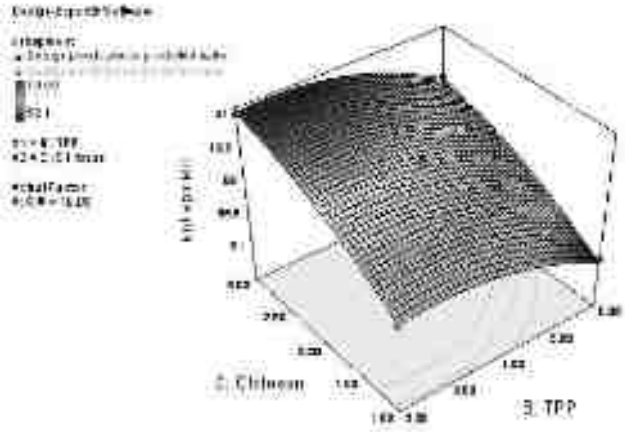
Scanning electron micrographs of GC11 chitosan microspheres

Fig. 2



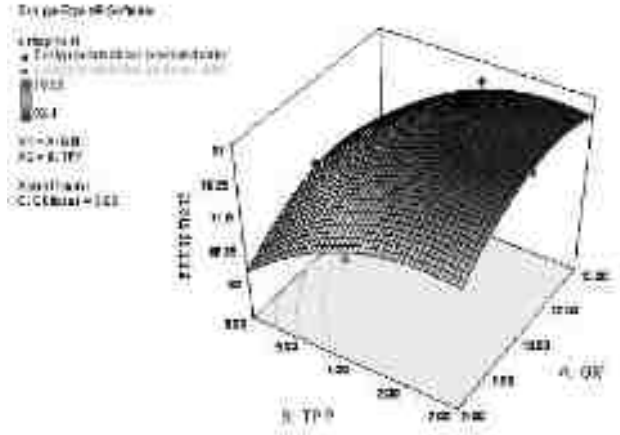
3D surface curve for the effect of selected variables (X1, X3) on the entrapment of microspheres (X2, -1).

Fig. 3



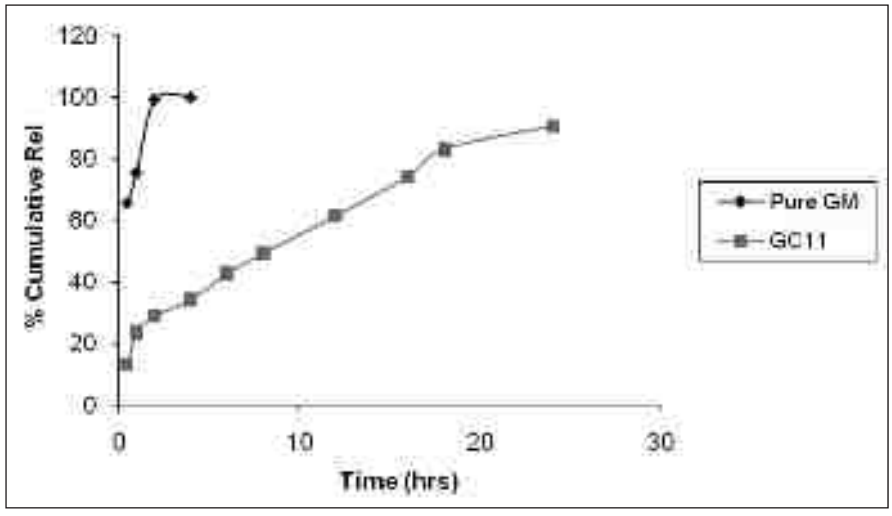
3D surface curve for the effect of selected variables (X2, X3) on the entrapment of microspheres (X1, 0).

Fig. 4



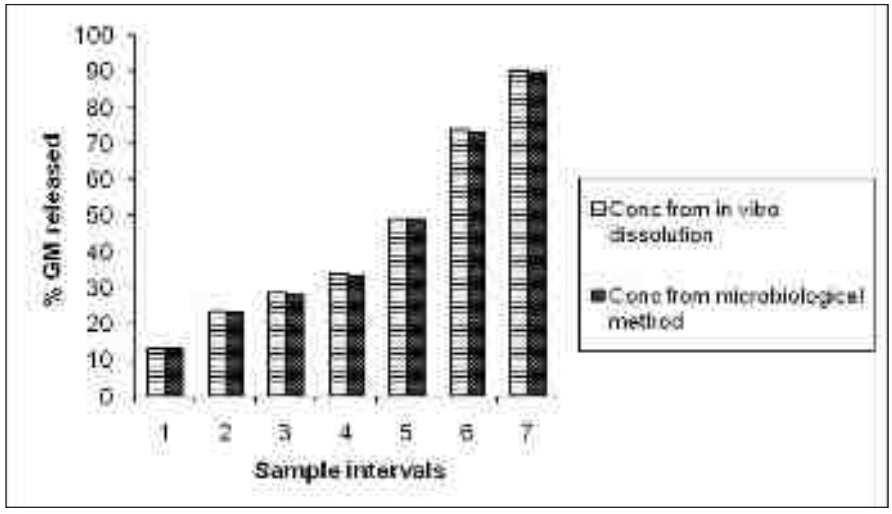
3D surface curve for the effect of selected variables (X1, X2) on the entrapment of microspheres (X3, +1).

Fig. 5



In vitro release profiles of GM from GC11 microspheres.

Fig. 6



Comparison of concentration of GM as determined from antimicrobial assay and drug release studies.

Table No. 1 Full factorial experimental design layout with coded levels and actual values of variables

Formulation code	X_1 GM (mg)	X_2 TPP(%w/v)	X_3 Chitosan(%w/v)	Y_1 MD[a] (µm)	Y_2 EE[b](%)
Gc1	15(+1)[c]	4(0)	3(+1)	22.54 78.36	
GC 2	10(0)	2(-1)	1(-1)	11.42	61.66
GC 3	10(0)	6(+1)	3(+1)	26.34	69.79
GC 4	5(-1)	4(0)	1(-1)	15.31	55.04
GC 5	10(0)	6(+1)	1(-1)	14.35	52.4
GC 6	15(+1)	6(+1)	2(0)	24.45	59.62
GC 7	15(+1)	4(0)	1(-1)	15.55	58.89
GC 8	10(0)	4(0)	2(0)	18.84	71.97
GC 9	5(-1)	4(0)	3(+1)	19.03	71.36
GC 10	15	2(-1)	2(0)	16.98	70.58
GC 11	10(0)	2(-1)	3(+1)	20.61	79.56
GC 12	10(0)	4(0)	2(0)	18.79	72.56
GC 13	5(-1)	2(-1)	2(0)	16.36	69.61
GC 14	10(0)	4(0)	2(0)	18.46	72.10
GC 15	10(0)	4(0)	2(0)	18.74	72.58
GC 16	5(-1)	6(+1)	2(0)	22.84	58.15

[a]MD-Mean diameter; [b]EE-Entrapment efficiency [c]Value in parenthesis indicates coded levels of the variables

Table No. 2 Summary of results of a) model analysis b) lack of fit c) R-square analysis for measured responses

Source	(MD)[a] Y_1		(EE)[b] Y_2	
	Sum of squares	P>F	Sum of squares	P>F
a) Model analysis				
Mean vs total	5647.90	-	72123.13	-
Linear vs. mean	195.49	<0.0001	868.39	<0.0001
2FI vs. linear	4.88	0.6823	2.61	0.9845
Quadratic vs. 2FI	10.86	0.3761	147.35	0.0006
Cubic vs. quadratic	17.49	0.0006	10.31	0.0078
Residual	0.087	-	0.29	-
Total	5876.70	-	73152.08	-
b) Lack of fit				
Linear	33.23	-	160.27	-
2FI	28.35	0.0010	157.66	0.0006
Quadratic	17.49	0.0007	10.31	0.0004
Cubic	0.087	-	0.29	-
Pure error				
c) R-square analysis	Adjusted R-square	PRESS	Adjusted R-square	PRESS
Linear	0.8180	70.37	0.8049	235.14
2FI	0.7929	148.10	0.7441	388.27
Quadratic	0.8080	279.98	0.9742	165.46
Cubic	0.9981	-	0.9986	-

[a]MD-Mean diameter; [b]EE-Entrapment efficiency * - Not applicable

Table No. 3 Regression analysis data and ANOVA for measured responses with standardized main effects of the factors on the responses and associated p-values

	Y_1 (MD[a])				Y_2 (EE[b])			
Coefficients	**Full Model**	**Reduced Model**	**p-value**	**SME[c]**	**Full Model**	**Reduced Model**	**p-value**	**SME[c]**
b_0	18.71	18.79	-	-	72.30	67.14	-	-
b_1	0.75	0.75	0.2629	0.872	1.66	1.66	0.0123	3.53
b_2	2.83	2.83	0.0034	4.639	-5.18	-5.18	< 0.0001	-11.02
b_3	3.39	3.99	0.0006	6.540	8.89	8.89	< 0.0001	18.91
$b_1 b_2$	0.25	-	0.7822	0.290	0.12	-	0.8570	0.181
$b_2 b_3$	0.70	-	0.4446	0.953	-0.13	-	0.8542	1.196
$b_1 b_3$	0.82	-	0.3763	0.813	0.79	-	0.2809	-0.196
b_1^2	0.69	-	0.4516	0.802	-3.88	-	0.0011	-5.878
b_2^2	0.76	-	0.4080	0.883	-3.94	-	0.0010	-5.969
b_3^2	-1.29	-	0.1828	-1.5	-2.51	-	0.0092	-3.803
R_2	0.9232	0.8544	-	-	0.9897	0.8440	-	-
Significance	0.0099	<0.0001	-	-	<0.0001	<0.0001	-	-
F	8.01	23.47	-	-	64.03	21.63	-	-

[a]MD-Mean diameter; [b]EE-Entrapment efficiency [c]Standardized main effects (SME) were calculated by dividing the main effect by the standard error of the main effect

Table No. 4 Release behavior of GM from GC11 in phosphate buffer (pH 7.4)

S.no	Kinetics		GC11
1.	First order	k	0.0870
		R^2	0.9741
2.	Higuchi	k	18.25
		R^2	0.9866
3.	Ritger-Peppas	n	0.4680
		R^2	0.9807

[a]k, Release rate constant; R^2, coefficient of determination; n, release exponent

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