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DoE approach for development of localized controlled release microspheres of Vancomycin for treatment of septic arthritis

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Abstract

Background: Septic arthritis is a worse condition of RA that is associated with significant morbidity and mortality. Septic arthritis develops due to direct introduction or invasion of pathogens. The objective of the present study was to formulate Vancomycin hydrochloride-loaded microspheres (VMS) based on Box–Behnken design (BBD) and evaluate its efficacy against septic arthritis. The intraarticular administration of optimized Vancomycin hydrochloride-loaded microspheres (OVMS) can reduce dose size, dosing frequency and systemic exposure with local targeted delivery.

Results: OVMS was further characterized for its drug–polymer compatibility using differential scanning calorimetry and Fourier transmission infrared spectroscopy. In vitro antibacterial activity was determined using the cup–plate method and in vivo anti-arthritic efficacy was evaluated by gross examination of septic arthritis. DSC and FTIR studies exhibited no interaction or incompatibilities between the drug and polymer. SEM images revealed that OVMS were spherical. It followed the first-order release rate according to Fick's law. The micromeritic properties indicated good flow property of OVMS. The zone of inhibition by OVMS was 1.5 cm against *S. aureus*. In vivo antibacterial study revealed that OVMS was significant in reducing septic arthritis and bacterial load, i.e., 110.1 CFU/ml in comparison with the control group (850 CFU/ml).

Conclusions: Thus, OVMS may be used as an effective formulation for the treatment of septic arthritis as compared to marketed IV vancomycin injection after clinical studies.

Keywords: Vancomycin, Microspheres, Arthritis, Sepsis, Micromeritics, Intraarticular injection

Background

Rheumatoid arthritis (RA) is an autoimmune disease that affects 0.5–1% population across the world [1]. It is an inflammatory condition of the synovial membrane due to destruction of cartilage and bone at joints [2, 3]. Septic arthritis is a worse condition of RA that is associated with significant morbidity and mortality. Septic arthritis develops due to direct introduction or invasion of pathogens [4]. The pathogenesis of this condition is based on interaction with the immune system of host and adherence of pathogens. The infestation of bacteria at joint space destroys joint within days. The most common bacteria causing infection at a joint is *Staphylococcus aureus* [5]. The mortality rate ranges from 3 to 25%. The risk factors for septic arthritis include earlier rheumatic disease, low socioeconomic status, ulcers in the leg, diabetes, previous operations, alcohol abuse, viral infection and corticosteroid administration [6]. Antibiotics are the mainstay drugs for the treatment of septic arthritis. However, the choice is based on the likelihood of the organisms.

Aminoglycosides are the drug of choice for septic arthritis but it should be taken cautiously due to their side effects including nephrotoxicity and ototoxicity; however, vancomycin is preferred over aminoglycosides due to its good renal clearance [7]. Vancomycin having

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good penetration ability at joints and tissues at high concentrations but an increase in dose is proportional to toxicity. The dose of the drug can be reduced by formulating an optimized and suitable dosage form that is effective at a low dose [8]. Microspheres are multiparticulated novel drug delivery systems composed of proteins or polymers that are biodegradable in nature with particle size less than 200 µm [9]. Various techniques are used to prepare microspheres that provide multiple options to enhance the efficacy of a drug with reduced toxicity [10].

The traditional pharmaceutical formulations have been developed for antibiotic preparation by changing a variable at a time approach. This method is difficult to prepare an ideal formulation because the independent variable is not considered. So, statistical tools are developed as "design of experiments" to select the most influential factors in the experiment and get their optimum values [11]. The design of experiments involves the selection of an appropriate combination of factors and the levels to be tested in which Box–Behnken design (BBD) is one of them. BBD helps in optimization with factorial designs and analysis of the response. BBD is an efficient, powerful and systematic tool that shortens the time to develop a formulation and increases research output [12].

Therefore, in the present study, Vancomycin microspheres were prepared as controlled release drug delivery system based on BBD against septic arthritis for intraarticular administration. It was characterized for particle size, shape, entrapment and release. Drug–polymer complexity was determined using DSC and FITR studies. The free-flowing properties of microspheres were determined using micromeritics studies. In vitro and in vivo antibacterial activity of prepared Vancomycin hydrochloride-loaded microspheres (VMS) were evaluated against *S. aureus*.

Methods

Drugs, chemicals and reagents

Chitosan from shrimp shells (Degree of deacetylation ~ 75%), Glutaraldehyde as a cross-linking agent, calcium chloride as a gelling agent and Vancomycin HCl as an active pharmaceutical ingredient were purchased from Sigma-Aldrich, USA. All other chemicals used were of analytical grade and distilled water was used throughout the experiment.

Preparation of Vancomycin-loaded chitosan microspheres (VMS)

VMS were prepared by a chemical denaturation method using glutaraldehyde as a cross-linking agent. Briefly, 0.5 mg of chitosan was weighed and dissolved in 40 ml of acetic acid (2%) in which drug (Vancomycin HCl) dissolved in 10 ml of acetic acid (2%) was added. Calcium chloride ($CaCl_2$) was added dropwise (0.05 ml) with a syringe with continuous stirring at 1500 rpm for 10 min; further, glutaraldehyde (2 ml) was added dropwise while stirring at the same speed. It was allowed for curing (40 min) to settle the microspheres, then filtered and dried in air for 24 h [13].

Experimental design for optimization

The Vancomycin HCl microspheres (VMS) were prepared using "Box–Behnken experimental design". It was used statistically to optimize the formulation for the maximum in vitro drug release, percentage entrapment efficiency and minimum particle size. A twelve run Box–Behnken design (VMS1-VMS12) with three factors and three levels including three triplicates at the center point were employed. The amount of drug (X_1), the quantity of $CaCl_2$ (X_2) and stirring speed (X_3) were chosen as independent variables varied at three different levels of the low, medium and high $(-1, 0, +1)$. The dependent variables estimated were particle size (Y_1), entrapment efficiency (Y_2) and drug release (Y_3) [14]. The levels and composition of VMS are shown in Table 1.

Characterization of prepared Vancomycin HCl microspheres (VMS)

Determination of percentage yield

The percentage yield of VMS was determined by weighing accurately the dried microspheres and calculated by using the following formula:

$$\text{Percentage yield} = \left(\text{Practical yield}/\text{Theoretical yield}\right) \times 100\%$$

where

$$\text{Practical yield} = \text{Weight of dried microspheres}$$
$$\text{Theoretical yield} = \text{Weight of drug} + \text{weight of the polymer}$$

Particle size analysis

The particle size of all formulations was determined using an optical microscope (Olympus microscope) with the help of stage micrometer and eyepiece micrometers. The microspheres (100 approx.) were selected randomly and then the mean particle size was measured [15]. The experiment was performed in triplicate and average values were noted.

Determination of entrapment efficiency

An accurately weighed quantity of microspheres was crushed into powder in a motor and pestle and then added to 100 ml of distilled water and mixture was kept aside for 48 h. Further, the solution was filtered and drug content was estimated by using UV–Visible

Table 1 Composition of various microspheres prepared as per Box–Behnken design (BBD)

Formulation	Drug concentration (X_1)		Calcium chloride concentration (X_2)		Stirring speed (X_3)	
	Coded	Actual (mg)	Coded	Actual (mg)	Coded	Actual (rpm)
VMS 1	−1	100	0	200	−1	500
VMS 2	+1	300	0	200	+1	1500
VMS 3	+1	300	0	200	−1	500
VMS 4	0	200	−1	100	+1	1500
VMS 5	−1	100	+1	300	0	1000
VMS 6	−1	100	−1	100	0	1000
VMS 7	0	200	+1	300	+1	1500
VMS 8	−1	100	0	200	+1	1500
VMS 9	+1	300	+1	300	0	1000
VMS 10	0	200	−1	100	−1	500
VMS 11	+1	300	−1	100	0	1000
VMS 12	0	200	+1	300	−1	500

VMS Vancomycin HCl microspheres

spectrophotometer (Shimadzu UV, 1801) at 280 nm [16]. The experiment was performed in triplicate and average values were noted. The drug entrapment efficiency was calculated by using the formula below:

$$\text{Entrapment efficiency} = \left(\text{Experimental drug content}/\text{Theoretical drug content}\right)\times 100\%$$

In vitro drug release profile of VMS

All the formulations of prepared VMS, i.e., VMS1 to VMS12 were evaluated for drug release profile using phosphate buffer (pH 7.4 containing 2% tween-80) as the release medium. Briefly, microspheres were suspended in dissolution medium (50 ml) in a beaker and stirred at 50 rpm on a magnetic stirrer (Remi, Mumbai) in a thermostat bath at 37 °C. The samples (2 ml) were withdrawn at different time intervals and centrifuged (Remi, R-8c) at 5000 rpm. The supernatant was isolated to record the absorbance at 280 nm using UV–Visible spectrophotometer. The procedure was performed in triplicate and average values were noted [17]. In order to investigate the mechanism of drug release from all the twelve formulations, different mathematical equations were used, namely Zero-order equation: $Q = Q_0 - K_0 t$ (Q = Amount of drug released at time "t", Q_0 = Amount of drug released initially (often considered zero), $K0$ = Zero-order rate constant), first-order equation: Log C = Log$C0 - Kt/2.303$ ($C0$ = Initial concentration of the drug, C = Concentration of drug at time "t", K = First-order constant), Higuchi-equation: $Q = Kt_{1/2}$ (K = Constant reflecting design parameters of the system), Korsmeyer–Peppas equation: $Mt/M\alpha = Kt^n$ ($Mt/M\alpha$ = Fractional release of drug, n = Release exponent indicative of mechanism, K = Kinetic constant characteristics of the drug/polymer system).

Statistical analysis of data

Data obtained from all formulations were analyzed using Box–Behnken experimental design (BBD) with software Design Expert[R] Version 12 and used to generate the study design and the response surface plots, polynomial models, including linear, interaction and quadratic terms were generated for three response variables using the software. Also, analysis of variance (ANOVA) was used to identify significant effects of factors on response regression coefficients. The F test and P values were also calculated using the software. The relationship between the dependent and independent variables was studied using contour plots and response surface plots. Using the desirability approach and graphical optimization a new optimized formulation (OVMS) with desired responses was prepared. To validate the chosen experimental design, the experimental values of the responses were quantitatively compared with predicted values and the relative error (%) was calculated using the following equation

$$\%\ \text{Relative Error} = [(\text{Predicted value} - \text{Observed value})/\text{Predicted value}] \times 100\% \quad (1)$$

Characterization of optimized Vancomycin HCl microspheres (OVMS)

Surface morphology of OVMS

Scanning electron microscope (SEM) (JSM-IT 500, Japan) was used to determine morphological characteristics of OVMS. OVMS were mounted on SEM stub and scanned under high vacuum with an electron beam. The images were formed through SEM due to the emission of electrons from the samples.

Drug–polymer compatibility studies

Differential scanning calorimetry (DSC) Differential scanning calorimetry (DSC) analysis of OVMS was performed on DSC-60 (Shimadzu Corporation, Japan). Briefly, the samples of pure drug and optimized formulation were heated in sealed aluminum pans under airflow at the rate of 30 ml/min. The scanning was performed at the rate of 10 °C/min from 35 to 250 °C. DSC thermograms were obtained for pure drug and optimized formulation by measuring the heat flow as a function of temperature [16].

Fourier transmission infrared spectroscopy (FTIR) Fourier transmission infrared spectroscopy (Agilent Cary-630) was used for recording FTIR spectra of the pure drug (Vancomycin HCl), blank microphones and OVMS. The scanning range was between 400 and 4000 cm^{-1}.

Micromeritic studies

Optimized microspheres were characterized for their micromeritic properties like bulk density, tapped density, Carr's index, Hausner's ratio and angle of repose to find its flow properties. **Bulk density** was calculated by taking accurately weighed (w) quantity of prepared microspheres into a 10-ml graduated cylinder. The initial volume was measured without disturbing the cylinder that was noted as bulk volume (V_b) and it was calculated by using the formula, bulk density $= W/V_b$. For **Tapped density,** weighed (w) quantity of microspheres was taken in a graduated cylinder and was tapped 100 times, then the volume of microspheres noted (V_t). The tapped density was calculated by using the formula, tapped density $= W/V_t$. **Compressibility index or Carr's index** evaluates the flow property of powder by comparing the bulk density and tapped density, calculated by using the following formula, Carr's index = (Tapped density − Bulk density)/Tapped density × 100%. **Hausner's ratio** gives an indication of the degree of densification which was calculated by a formula, Hausner's Ratio: Tapped density/Bulk density. **Angle of Repose** for microspheres was determined by the fixed funnel method. The spheres were allowed to flow through a funnel fixed to a stand at a definite height. The angle of repose (θ) was calculated by measuring the height (h) and radius (r) of the formed pile of microspheres and calculated by a formula, angle of repose: $\theta = \tan^{-1}$ (h/r) [18].

In vitro antibacterial activity of OVMS

In vitro antibacterial activity was estimated against *Staphylococcus aureus* using the cup and plate method by measuring the zone of growth inhibition of microorganism. Briefly, the nutrient agar medium was prepared and sterilized by autoclave at 121 °C for 20 min. The sterilized nutrient agar medium was then poured into sterile Petri plates and allowed to solidify. It was followed by transferring *S. aureus* to the Petri plates and swab with cotton for uniform distribution of bacteria in the Petri plates. Further, the disks were made and the marketed drug (Vancomycin) and OVMS were placed on the solidified agar medium. The Petri plates were incubated at 37 ± 1 °C for 24 h. The zone of inhibition was noted using antibiotic zone reader to determine the antibacterial activity of OVMS [19].

In vivo antibacterial activity of OVMS

Experimental protocol

In vivo efficacy of prepared optimized microspheres and marketed IV infusion formulation (Vancomycin) were evaluated on female Wistar rats weighing 150–200 g. The protocol was approved by IAEC with approval no. CPCSEA//1677/PO/Re/S/2019/IAEC/23. The animals were divided into three groups each containing six animals. Group 1 was a diseased control group that was infected with *S. aureus* and not received any treatment, Group 2 was reference group received an intravenous injection of marketed IV infusion formulation (equivalent to Vancomycin HCl animal dose) and Group 3 was tested group received an intraarticular injection of sterile optimized Vancomycin HCl microspheres (OVMS) suspended in the sterile water for injection (equivalent to Vancomycin HCl animal dose, i.e., 13 mg/kg). Injections were given daily to Group 2 and on alternate days to Group 3 up to 12 days.

Induction of sepsis

Sepsis was induced in animals by intraarticular injection of *S. aureus* suspension (0.2 ml, 1×10^3 CFU/knee) in physiological saline. The animals were observed after 3 days of induction for septic arthritis by observing swelling and erythema at the knee joint. The animals were observed after treatment at regular intervals of 3, 6, 9 and 12 days for the following changes:

Gross examination of septic arthritis

The animals were labeled and monitored their limbs at regular intervals (0, 3, 6, 9 and 12 days). The diameter of

the control and sepsis joint was measured by a thread and increase in the diameter of the knee joint was the indication of septic arthritis. The intensity of arthritis was evaluated by clinical scoring, i.e., 0 for normal without swelling (diameter less than 4.5 mm), 1 for mild swelling and erythema (diameter between 5 and 8 mm), 2 for moderate swelling and erythema (diameter between 9 and 14 mm) and 3 for marked swelling and erythema (diameter more than 14.5 mm). The final arthritis index values were tabulated as mean scores from all the animals in each group [20].

Determination of bacterial load

Synovial fluid was collected from infected joints of all animals and diluted in sterile normal saline solution. It was plated on a nutrient agar plate and the results were expressed as the number of bacterial colonies (CFU/ml) of the fluid.

Histopathological examination

The knee joints of all animals were removed after sacrifice the animals on day 13th and fixed in paraformaldehyde (4%), then decalcified and embedded in paraffin. The tissues sections were prepared (7 µm) and stained with hematoxylin and eosin. The sections were examined under a fluorescence microscope and observed for bacterial infection, inflammation in synovial membrane and synovial lining severity of synovitis, synovial hypertrophy and loss of matrix to confirm the effect of optimized and reference formulations.

Statistical analysis

Data were expressed as mean ± SD ($n = 6$), and statistical difference was analyzed using two-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test using graph pad prism version 8 software.

Results

Design and optimization of Vancomycin HCl microspheres (VMS)

The factors studied were the amount of drug (X_1), the hardening agent $CaCl_2$ concentration (X_2) and the stirring speed (X_3). The preliminary studies also provided a set of the levels for each formulation variable. The responses of dependent variables selected were the particle size of the microspheres (Y_1), the entrapment efficiency (Y_2) and the percent drug released for 24 h (Y_3). Table 2 shows the responses of the design. The design was appropriate to study the response surfaces. A total of 12 experimental runs are shown in Table 1. The experimental runs were randomized to maximize the effect of unexplained variability in the observed responses due to extraneous factors. All the dependent variables were estimated in triplicate.

Contour and surface response plots analysis

The contour and response surface plots, which indicate the effect of the drug, hardening agent and stirring speed on particle size, entrapment efficiency and drug release of microspheres were obtained and shown in Figs. 1, 2 and 3, respectively.

Particle size (Y_1)

The particle size (Y_1) of all the prepared microspheres is as shown in Table 2. The range of particle size of all the formulation was from 4.44 to 20.28 µm. The respective

Table 2 Different responses of Vancomycin HCl microspheres employed in Box–Behnken experimental design

Formulation code	Percentage yield (%)	Particle size (Y_1, µm)	Entrapment efficiency (Y_2, %)	Drug release (Y_3, %)
VMS 1	87.5 ± 0.12	18.26 ± 0.31	33.17 ± 0.32	68.04 ± 0.31
VMS 2	92 ± 0.20	12.92 ± 0.65	45.94 ± 0.12	74.10 ± 0.45
VMS 3	90 ± 0.40	4.44 ± 0.64	79.92 ± 0.42	88.02 ± 0.42
VMS 4	85.2 ± 0.31	5.02 ± 0.41	80.42 ± 0.44	84.03 ± 0.61
VMS 5	88.8 ± 0.12	10.23 ± 0.51	58.05 ± 0.51	80.29 ± 0.53
VMS 6	84.2 ± 0.32	14.20 ± 0.32	42.19 ± 0.23	74.30 ± 0.65
VMS 7	85 ± 0.4	6.29 ± 0.61	52.06 ± 0.11	82.02 ± 0.72
VMS 8	86.25 ± 0.51	20.28 ± 0.42	27.12 ± 0.61	69.50 ± 0.21
VMS 9	92.7 ± 0.26	11.50 ± 0.22	50.16 ± 0.32	76.02 ± 0.61
VMS 10	90.05 ± 0.42	15.09 ± 0.51	40.24 ± 0.61	72.32 ± 0.42
VMS 11	86.66 ± 0.61	8.26 ± 0.45	60.06 ± 0.51	78.31 ± 0.32
VMS 12	83.40 ± 0.51	7.89 ± 0.52	64.02 ± 0.21	81.3 ± 0.22

Data are represented as mean ± SD ($n = 3$)

VMS Vancomycin HCl microspheres

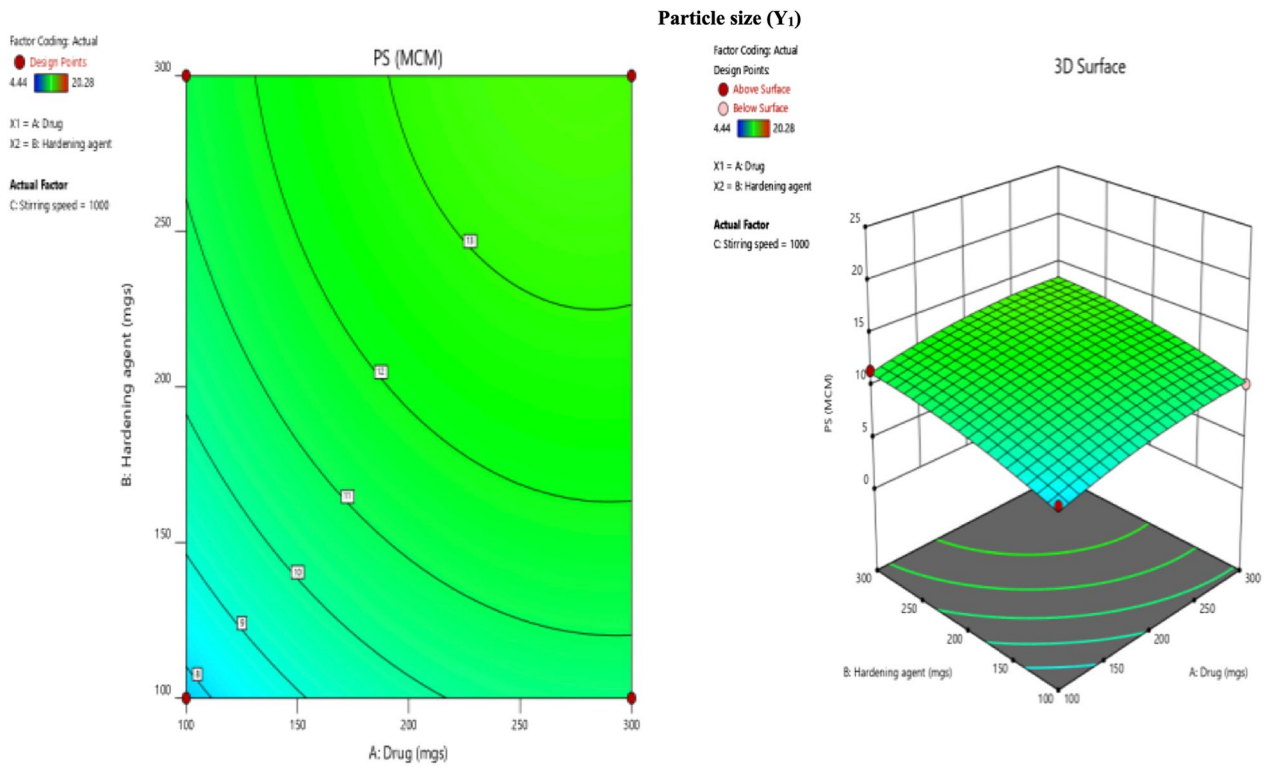

Fig. 1 Contour plot and response surface plot for particle size (Y_1)

contour, response surface plots (Fig. 1) and the coefficient of the quadratic model are given in Eq. (2), respectively.

$$Y_1 = 12.12 + 1.24X_1 + 1.66X_2 - 5.53X_3 - 0.1375X_1X_2 + 0.0525X_1X_3 - 1.20X_2X_3 - 0.7200X_1^2 - 0.6750X_2^2 \quad (2)$$

The effect of X_1, X_2 and X_3 on Y_1 was analyzed by its polynomial Eq. (2). The coefficient of X_1 and X_2 was positive indicated that with an increase in drug and hardening agent the proportional increase in particle size was achieved. However, coefficient of X_3 was negative indicated that an increase in the stirring speed decreased the particle size. The combined effect of X_1 and X_2 was negative that indicated the decreased particle size. The coefficient of X_3 was 5.53, which was highest among all coefficients, i.e., X_1 (1.24) and X_2 (1.66). The results showed that the effect of X_1, X_2 and X_3 was significant on Y_1. Thus, it was observed that the stirring speed (X_3) was the major contributing variable for particle size than drug concentration (X_1) and hardening agent (X_2). The model proposed the quadratic equation and the same results were found from respective 3D response surface plots and contour plots. The particle size of 1–10 µm was needed for targeting of the drug in the knee joint to retain in synovial fluid for prolonging release without leakage to surrounding fluids. Figure 1 shows the contour plot and 3D response surface plot for particle size (Y_1).

Entrapment efficiency (Y_2)

The entrapment efficiency (EE) of all drug-loaded microspheres is shown in Table 2. It was between 27.12 and 80.42%. The respective surface plot, contour plot (Fig. 2) and coefficient of the linear model are shown in Eq. (3).

$$Y_2 = 48.11 - 1.67X_1 + 8.28X_2 + 12.09X_3. \quad (3)$$

The effect of X_1, X_2 and X_3 were analyzed by a polynomial equation. The value of coefficient of X_1 was negative and indicated the negative effect (− 1.67) on Y_2. However, the coefficient variable X_2 in Eq. (3) indicated the positive effect, i.e., (+8.28); thus, it was concluded that X_2 had positive effect on the Y_2 variable. The results exhibited that increase in the concentration of hardening agent (X_2) significantly increased EE. It may be due to an increase in the density of matrix and porous surface around the polymer that allowed the drug to get entrapped more. The coefficient of X_3 in Eq. (3) indicated the positive effect and thus, X_3 had a positive effect on Y_2. It was found that increase in the stirring speed (X_3), significantly increased EE. It may be due to the decrease in particle size on increasing the revolution per minute (rpm). So,

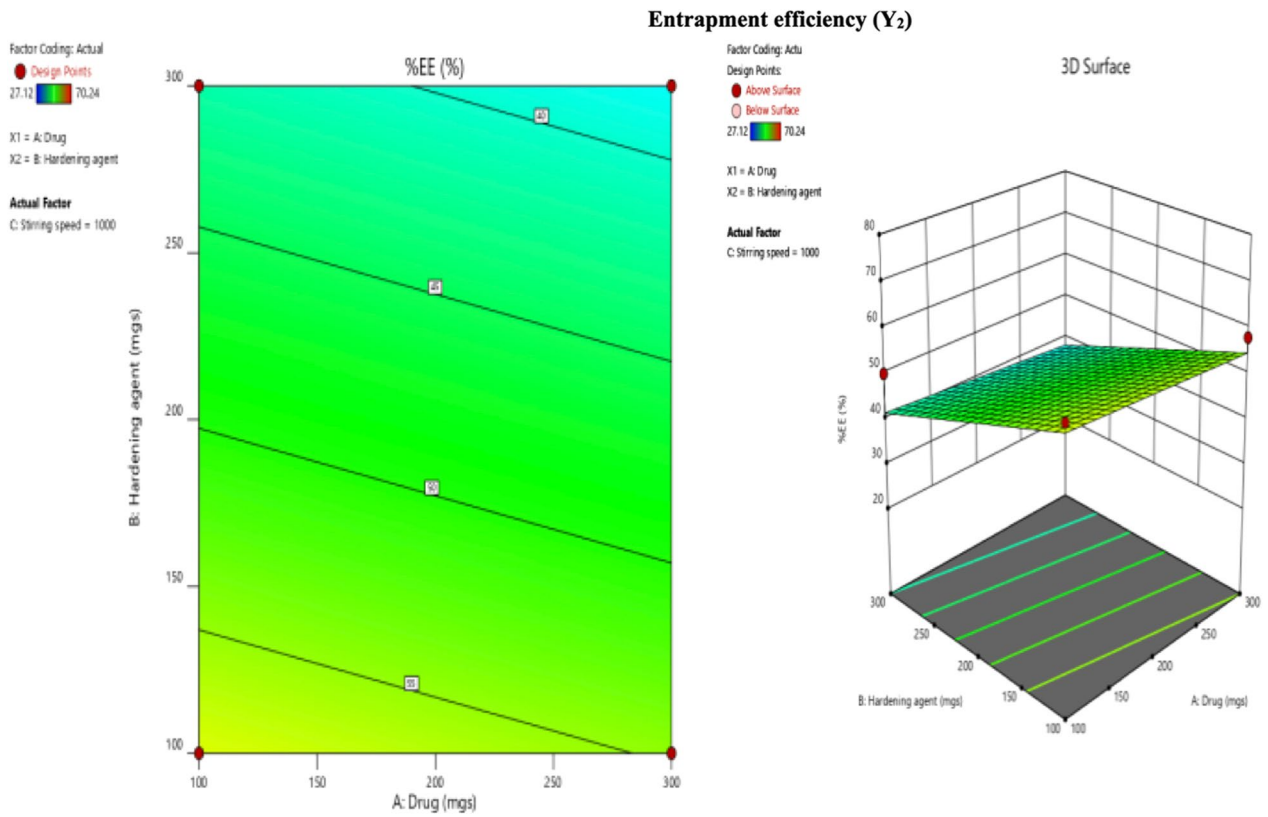

Fig. 2 Contour plot and response surface plot for entrapment efficiency (Y_2) optimized formulation

the smaller particles had a large surface area and shown more entrapment efficiency. It was concluded that the X_2 and X_3 had a significant effect on Y_2 as compared to X_1. The effect of concentration of drug was not significant on the entrapment efficiency. The model proposed for entrapment efficiency is a linear equation. Figure 2 shows the contour plot and 3D response surface plot for entrapment efficiency (Y_2).

Drug release (Y_3)

Table 2 shows the drug release profile of vancomycin HCl microspheres (VMS). Its contour, response surface plots (Fig. 3) and the coefficient of the quadratic model are shown in Eq. (4).

$$Y_3 = 77.35 - 1.37X_1 - 1.91X_2 + 6.40X_3 - 0.9750X_1X_2 - 0.6100X_1X_3 + 0.6975X_2X_3 \quad (4)$$

The effect of variables X_1, X_2 and X_3 on drug release (Y_3) revealed that the increase in the drug concentration caused no significant change in the drug release. The coefficient of X_1 and X_2 in Eq. (4) indicated a negative effect on Y_3. Thus, increase in the concentration of the drug (X_1) and hardening agent $CaCl_2$ (X_2) a significant decrease in drug release was achieved. It may be due to the dense matrix formed on the microspheres with increased hardening agent which reduced the drug release. The coefficient of X_3 in Eq. (4) indicated a positive effect (+6.40) in drug release. The significant increase in drug release (Y_3) was seen with an increase in stirring speed. It may be due to the decrease in the particle size with an increase in surface area that allowed the drug to release fast. The results of BBD revealed that the major contributing factor was X_3 when compared with X_1 and X_2 on Y_3 in the preparation of the microspheres. Figure 3 shows the contour plot and 3D response surface plot for drug release (Y_3).

Statistical analysis

The significance of the model for all responses (Y_1–Y_3) is generated using software and summary is shown in Table 3. The ANOVA results exhibited that models were significant ($P < 0.05$) for investigated responses (Y_1–Y_3). The values of P and $F > 0.0500$ indicated that the terms were significant and value greater than 0.100

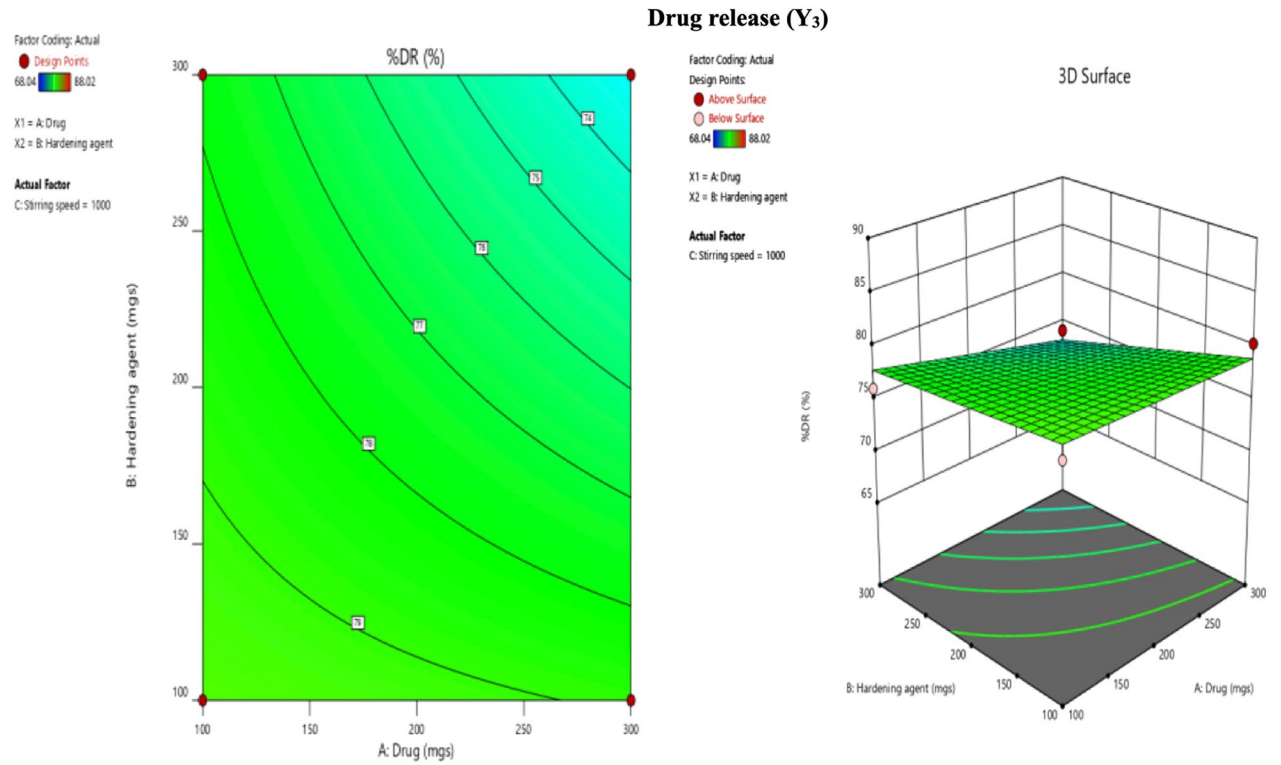

Fig. 3 Contour plot and 3D response surface plot for drug release (Y_3)

indicated that the terms were not significant. The model *F*-value of 52.63 for Y_1 implied that the model was significant. In the case of particle size (Y_1) X_1, X_2 and X_3 were significant model terms and X_1X_2, X_2, X_3 and X_1^2 were not significant terms.

The model *F*-value of 13.84 for Y_2 implied that the model was significant. In the case of Y_2X_2, X_3 were significant model terms and X_1 was not significant. The model *F*-value of 14.73 for Y_3 implied that the model was significant. In the case of drug release (Y_3) X_2, X_3 were significant model terms and X_1, X_1X_2 and X_2X_3 were not significant terms. The results exhibited that the models were significant for all the parameters (Y_1–Y_3).

The composition of the optimized formulation (OVMS) (Table 4) was obtained by desirability plot and over layout plot (Fig. 4). The predicted and experimental values of all the parameters (Y_1–Y_3) tested with OVMS were compared and the respective percent relative error calculated (Table 5) was found to be less than 5%. Thus, the model predicted was valid for manufacturing of optimized Vancomycin HCl targeted release microspheres.

In vitro drug release profile of VMS

In vitro drug release study of VMS was performed to confirm the controlled release of the drug (Fig. 5), and the mechanism of drug release was determined from the in vitro release profile of OVMS by subjecting its profile to release kinetic studies. The R^2 values for zero-order kinetics were (0.580) lesser than the first order (0.904). The regression value was closer to unity in case of first-order kinetics; thus, it indicated that the release was the first-order release from the optimized formulation of microspheres. The correlation coefficient obtained for Korsmeyer Peppas was found to be more superior in comparison with an R^2 value of Higuchi plot, it indicated the best fit model. The "*n*" value in Korsmeyer Peppas indicated that the formulation followed the Fickian release.

Characterization of the optimized formulation

Scanning electron microscopy (SEM) The surface morphology and surface texture of the OVMS and blank microspheres was determined by using a scanning electron microscope. SEM images of OVMS revealed that morphology of microspheres was spherical and has a porous surface. Figure 6 shows the SEM images of blank microspheres and OVMS.

Table 3 Statistical ANOVA results for contour and surface responses (Y_1–Y_3)

Source	Sum of squares	*Df*	Mean squares	*F*-value	*P* value	Remark
Particle size (Y_1)						
Model	286.44	8	35.80	52.63	0.0039	Significant
A: Drug	12.35	1	12.35	18.15	0.0237	Significant
B: Hardening agent	22.04	1	22.04	32.40	0.0107	Significant
C: Stirring speed	244.87	1	244.87	359.92	0.0003	Significant
AB	0.0756	1	0.0756	0.1112	0.7608	Not significant
AC	0.0110	1	0.0110	0.0162	0.9068	Not significant
BC	5.78	1	5.78	8.50	0.0517	Significant
A^2	1.04	1	1.04	1.52	0.3049	Not significant
B^2	0.9112	1	0.9112	1.34	0.3309	Not significant
Entrapment efficiency (Y_2)						
Model	1739.36	3	579.79	13.84	0.0016	Significant
A: Drug	22.28	1	22.28	0.5319	0.4866	Not significant
B: Hardening agent	548.47	1	548.57	13.09	0.0068	Significant
C: Stirring speed	1168.62	1	1168.62	27.90	0.0007	Significant
Drug release (Y_3)						
Model	379.23	6	6	14.73	0.0048	Significant
A: Drug	14.96	1	1	3.49	0.1208	Not significant
B: Hardening agent	29.22	1	1	6.81	0.0477	Significant
C: Stirring speed	327.81	1	1	76.40	0.003	Significant
AB	3.80	1	1	0.8863	0.3897	Not significant
AC	1.49	1	1	0.3469	0.5815	Not significant
BC	1.95	1	1	0.4536	0.5305	Not significant

$A = X_1$(Drug), $B = X_2$(Hardening agent), $C = X_3$(Stirring speed), $AB = X_1X_2$, $BC = X_2X_3$, $AC = X_1X_3$, $A^2 = X_1^2$, $B^2 = X_2^2$

Table 4 Composition of optimized vancomycin HCl microspheres (OVMS)

S.No.	Name of ingredient	Quantity
1	Drug	100 mg
2	CaCl2	200 mg
3	Stirring speed	1350 rpm
4	Acetic acid (0.2%)	40 ml
5	Glutaraldehyde (25%)	2 ml
6	Distilled water	Q.S.

Differential scanning calorimetry (DSC) Thermal analysis using a DSC method was carried out on pure drug and optimized formulation of microspheres (OVMS). The DSC thermograms of pure drug and OVMS showed the exothermic peak at 80.69 °C and 82.41 °C, respectively, which was nearly the same thermal behavior as the individual components (Fig. 7). It indicated that there was no interaction or incompatibilities between the drug and the polymer.

FTIR spectroscopy FTIR study of pure drug vancomycin and optimized Vancomycin HCl microspheres (OVMS) revealed the presence of different functional groups as shown in Table 6.

Micromeritic properties The results of micromeritic properties for OVMS revealed angle of repose 25.05°, bulk density 0.48 g/cm^3, tapped density 0.54 g/cm^3, Carr's index 11.11 and Hausner's ratio 1.12.

In vitro antibacterial activity of OVMS

In vitro antibacterial activity of OVMS exhibited zone of inhibition of 1.5 cm, which was near to reference marketed formulation (IV powder), i.e., 1.9 cm as shown in Table 7. The results showed no significant difference in the zone of inhibition between test and reference formulations. It indicated that the antibiotic possessed the same activity with both test and reference formulations.

In vivo efficacy of OVMS

Gross examination of septic arthritis The left knees of the all the animals were examined for swelling, erythema and redness at the site of infection after treatment with OVMS. The results exhibited that OVMS significantly

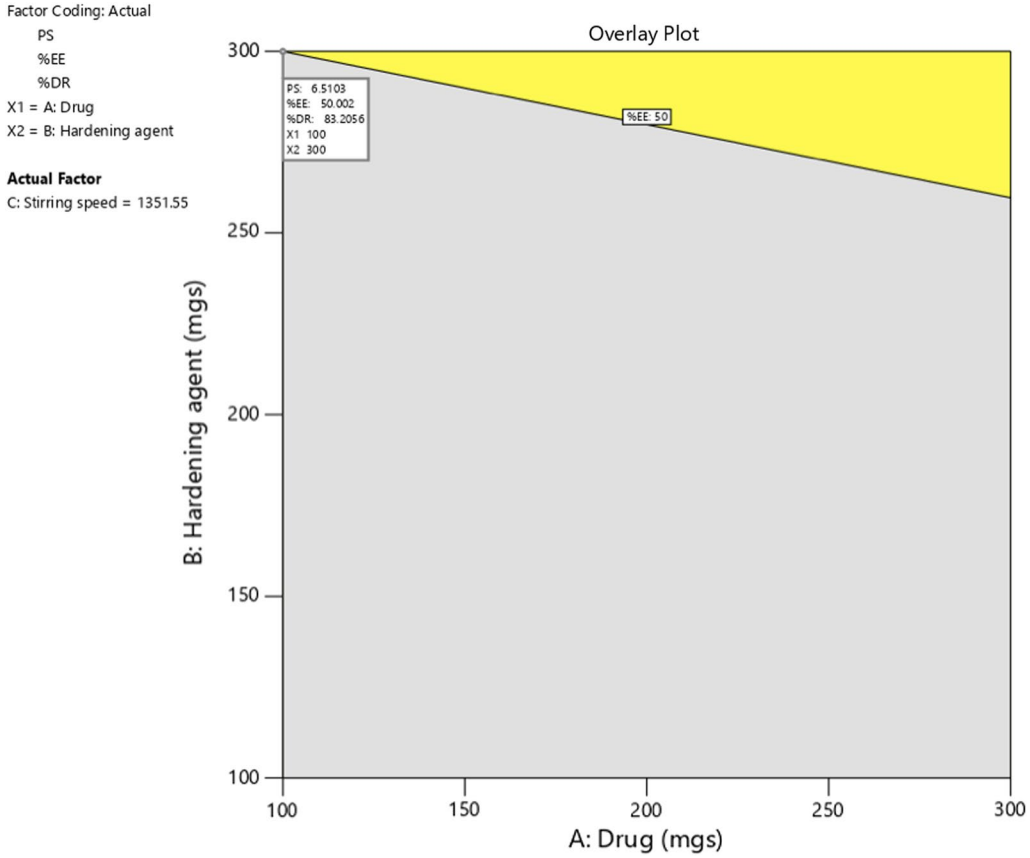

Fig. 4 Overlay plot for responses (Y_1–Y_3) of optimized formulation

restored the normal physiology of knees (Table 8). The arthritic index revealed that OVMS reduced the intensity of sepsis. The diameter of the knee joint of animals was restored to normal of 8.0 mm on day 14th in comparison with diseases control group, i.e., 19.2 mm as shown in Table 8. The reference group was also effective in normalizing septic arthritis 8.5 mm on day 14th. The clinical score for the reference group and test group on day 14th was 2 and 1, respectively. Figure 8 shows the gross examination of rat knees after treatment with the test drug.

Table 5 Comparison between predicted and experimental values of OVMS

Parameters	Predicted values	Experimental value	Percent relative error
Particle size	6.5102	6.29 ± 0.63	3.39
%EE	50	49.09 ± 1.70	1.85
%DR	83.20	81.25 ± 2.51	2.4

Determination of bacterial load The bacterial load in synovial fluid from the infected joint was determined by counting the number of colonies formed after infection. The results exhibited that OVMS significantly reduced the bacterial colonies at the knee joint. The number of bacterial colonies on day 14th was 110.1 ± 6.29 CFU/ml in the test group, 148 ± 5.29 CFU/ml in reference group in comparison with control group, i.e., 850 CFU/ml as shown in Fig. 9.

Histopathological studies

Figure 10 shows the histopathological status of the knee joint after treatment with OVMS. The knee joint was infected with *S. aureus* suspension (1×10^3 CFU/ml) in physiological saline by intraarticular injection. In the diseased control group, the joint of rats were swelled and also observed erythema and redness on the knee joint. On 13th day, histopathology of the knee joint was performed and in that bacteria was proliferated more in the synovial fluid and also the synovial membrane and synovial lining were enlarged by causing inflammation. Sterile OVMS was administered by intraarticular injection at knee joints of rats on alternative days up to 13 days. The knee joint was

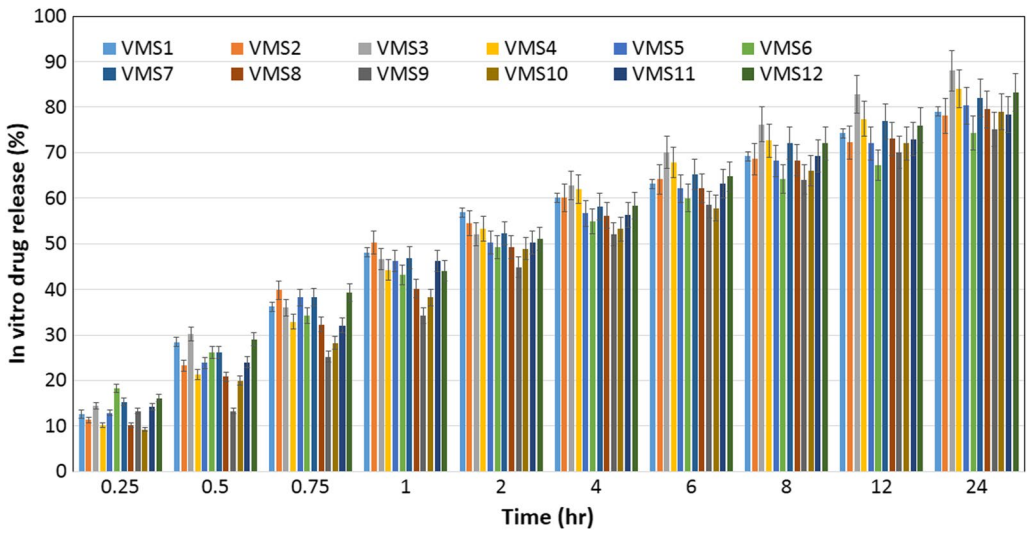

Fig. 5 In vitro drug release profile of VMS

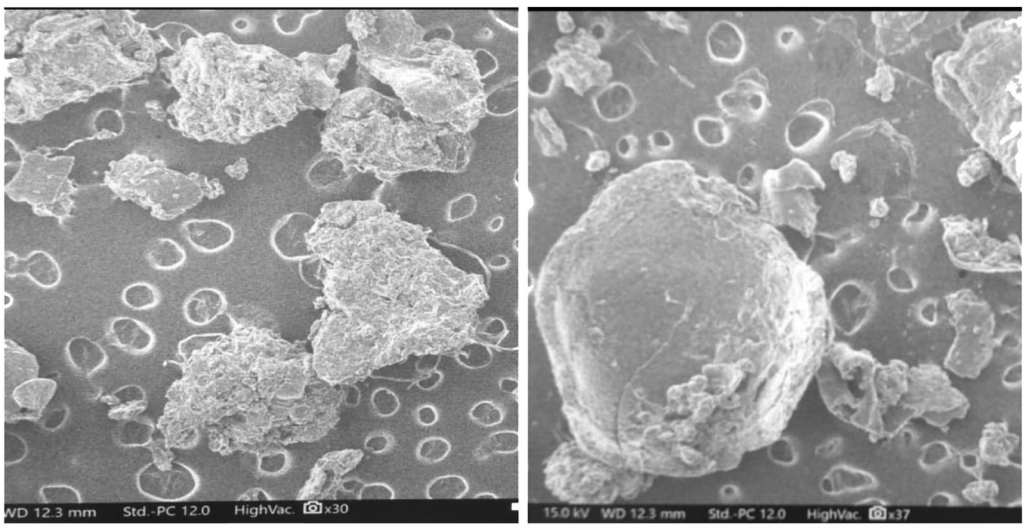

Fig. 6 Scanning electron microscopic images of microspheres. **A** Blank microspheres, **B** OVMS

examined on 13th day under a fluorescence microscope and observed the decreased bacterial infection and inflammation in the synovial membrane and synovial lining. However, in the reference group, there is also decreased infection and inflammation in the synovial fluid and membrane with reduced infiltration in the synovial fluid.

Discussion

In the present study, the optimized Vancomycin HCl microspheres (OVMS) were prepared for the treatment of septic arthritis. VMS was developed by applying a factorial design of Box–Behnken design (BBD). Vancomycin HCl was formulated in the form of microspheres to increase the therapeutic effect of the drug at the target site, to decrease the frequency of dosing or administration, to reduce toxic effects and to improve patient compliance. Twelve formulations (VMS1–VMS12) were prepared by chemical denaturation method using the varying the quantities of the drug (X_1), hardening agent $CaCl_2$ (X_2) and stirring speed (X_3) (Table 1) and evaluated for different in vitro parameters such as percentage yield, particle size (Y_1), entrapment efficiency

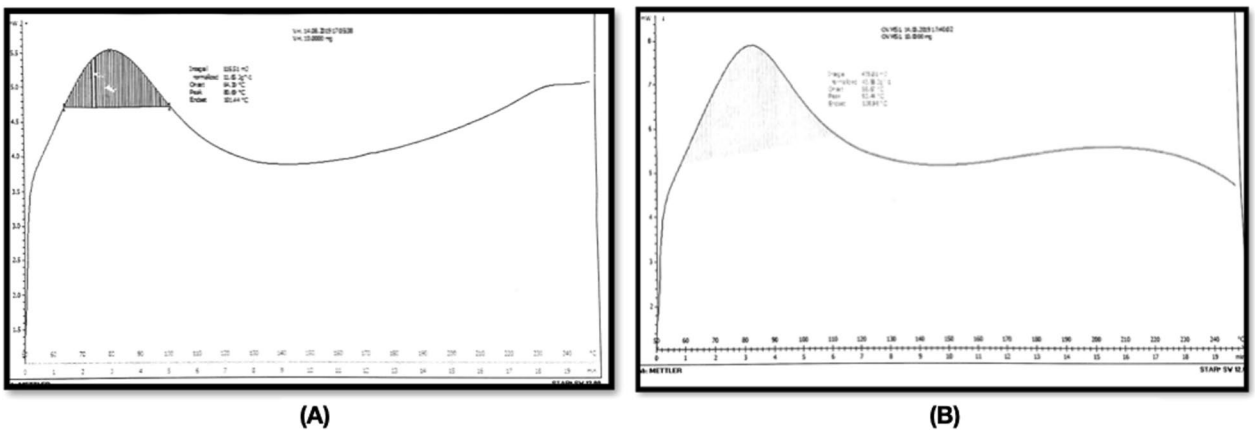

Fig. 7 Differential scanning calorimetry thermograms of microspheres. **A** Pure drug, **B** OVMS

Table 6 Identification of functional groups present in VMS based on FTIR ranges

Functional groups	Absorption peaks at wave number	
	Pure drug	OVMS
O–H Stretch	3205.26	3260.68
C=C Stretch	2344.83	2323.64
C–F Stretch	1226.24	1233.74
C=N Stretch	1890	1905.35
NO_2 Stretch	1306.64	1313.66

Table 7 Inhibitory effect of OVMS against *S. aureus*

Formulation	Zone of inhibition (cm)
Marketed formulation (IV powder)	1.9 ± 0.1
Test formulation (VMS)	1.5 ± 0.1

Data are represented as mean ± SD ($n = 3$)

(Y_2) and drug release (Y_3) followed by in vivo antibacterial effect on animals.

The results of Y_1, Y_2 and Y_3 of all the twelve formulations are shown in Table 2. The increase in drug and hardening agent led to an increase in the particle size, but increase in the stirring speed decreased the particle size. Increase in the concentration of hardening agent (X_2) and stirring (X_3) speed, significant rise in %DEE was achieved. It may be due to increase in the density of matrix and porous surface of polymer that allowed the drug to get entrapped more. The decrease in particle size may be due to large surface area that produced by increasing the rpm and thus showed more entrapment efficiency. Increase in the concentration of hardening agent caused significant decrease in percentage drug release (DR). It may be due to denser matrix that allowed slow drug release but there was a significant increase in %DR (Y_3) with increase in stirring speed as shown in Table 2. It may be due to the decreased particle size that allowed the drug to release fast (Fig. 5).

Further effect of X_1, X_2 and X_3 on Y_1, Y_2 and Y_3 was analyzed by BBD of design expert software version

Table 8 Effect of VMS against septic arthritis in different groups of animals

Days	Diseased control group		Reference group		Test group	
	Clinical score	Knee size (mm)	Clinical score	Knee size (mm)	Clinical score	Knee size (mm)
0	0	4.2 ± 0.01	0	3.8 ± 0.02	0	4.0 ± 0.03
3	3	15.5 ± 0.02	3	14.8 ± 0.02	3	14.6 ± 0.03
6	3	16.2 ± 0.03	2	13.3 ± 0.04	2	12.4 ± 0.04
9	3	17.3 ± 0.02	2	11.9 ± 0.03	2	10.5 ± 0.02
12	3	18.5 ± 0.01	2	10.0 ± 0.02	2	9.5 ± 0.01
14	3	19.2 ± 0.04	2	8.5 ± 0.03	1	8.0 ± 0.05

Data are represented as mean ± SD ($n = 6$), significantly different at $P < 0.05$ in comparison with diseased control group

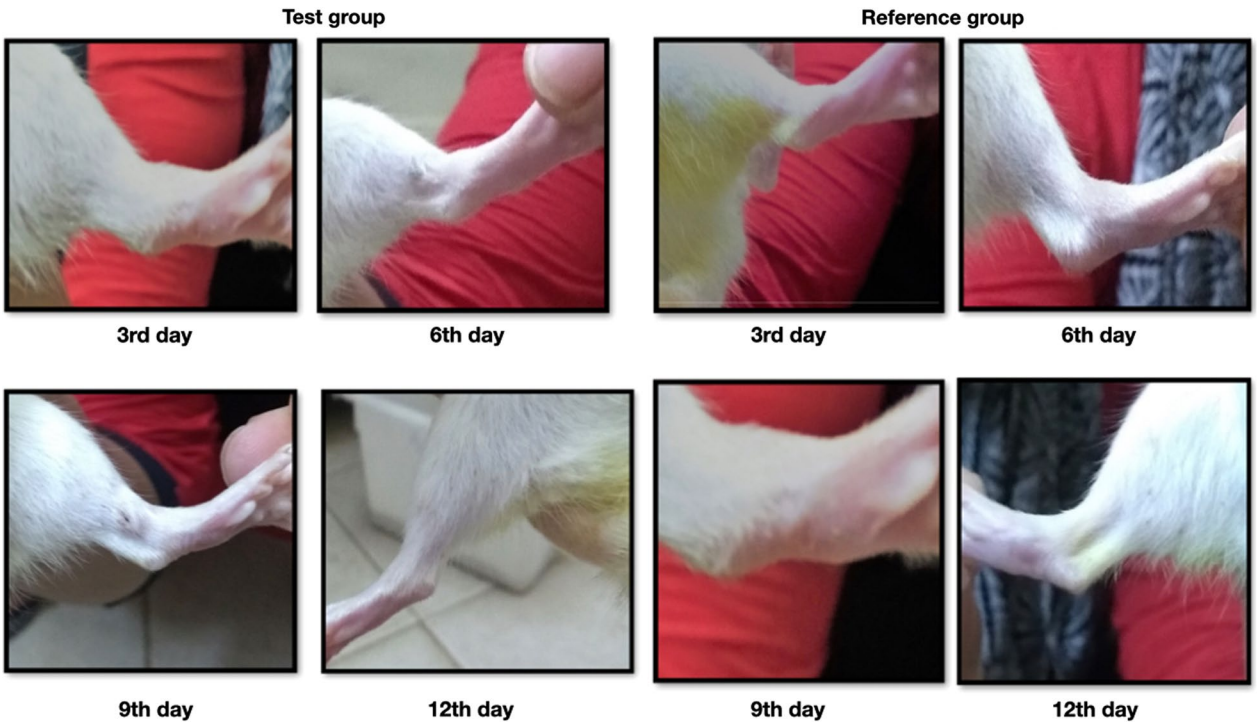

Fig. 8 Gross examination of knee joints in two different groups on various days

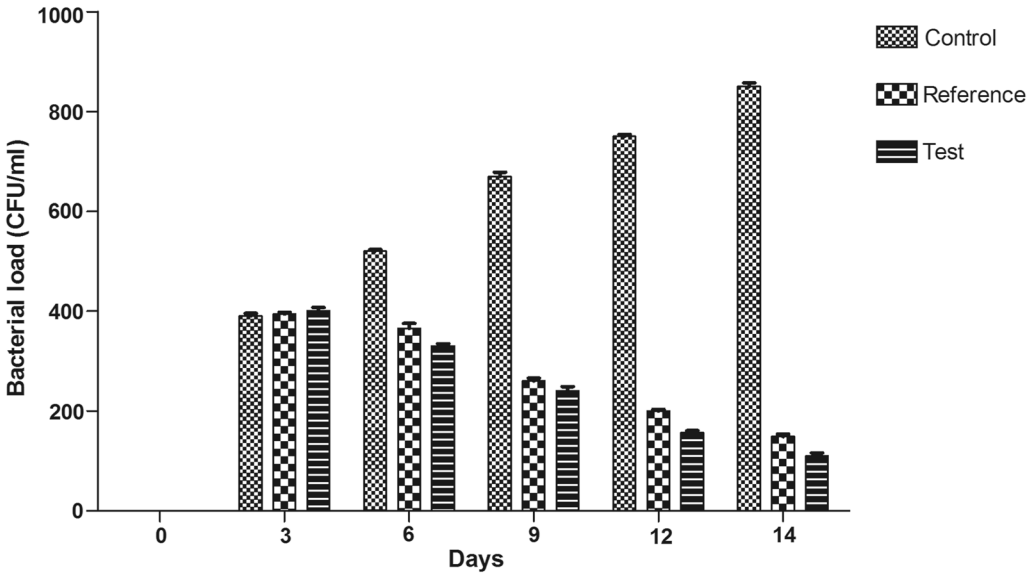

Fig. 9 Effect of OVMS on bacterial load in synovial fluid

12 using polynomial equations, contour plots and 3D response surface plots. As per statistical analysis (Table 3) all X_1, X_2 and X_3 have shown a significant effect on Y_1. The model proposed the quadratic equation for particle size (Y_1) but X_2 and X_3 only have shown a significant effect on Y_2 and Y_3, which were also confirmed from respective 3D response surface plots and contour plots (Figs. 1, 2, 3) Then, the formula for optimized formulation (Table 4) was collected by desirability and overlay plots. BBD used for generating a surface

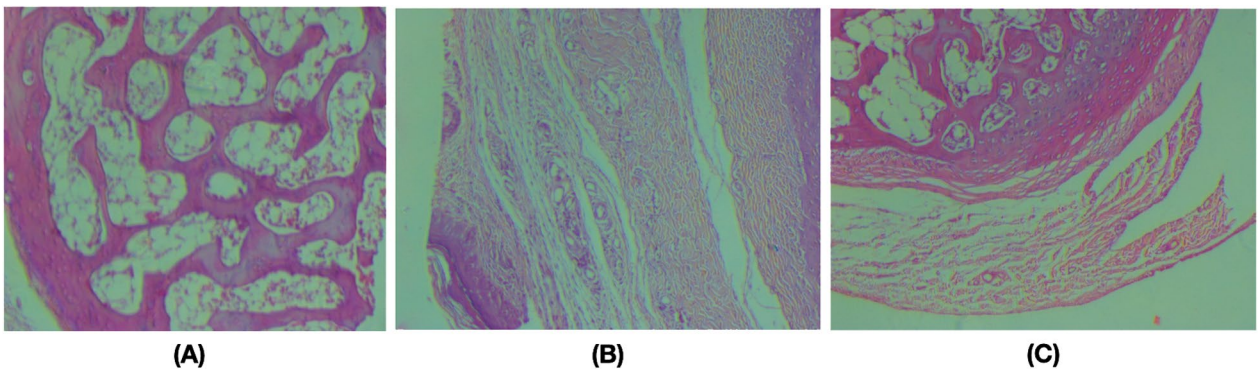

Fig. 10 Histopathological slides of knee joint after treatment with VMS **A** Disease control **B** Test group **C** Reference group

response with fewer runs as compared to factorial technique [21]. Response surface plots are important in designing, formulation and development of new products [22].

The compatibility between polymer and the drug in optimized Vancomycin HCl microspheres (OVMS) was determined by DSC and FTIR. The DSC thermograms of Vancomycin hydrochloride pure drug and optimized vancomycin HCl microspheres (OVMS) (Fig. 8) showed exothermic peaks at 80.69 and 82.41 °C, respectively, indicated nearly the same thermal behavior. FTIR spectra of OVMS and pure drug demonstrated the almost similar characteristic absorption peaks. DSC thermograms and FTIR absorption peaks confirmed that there were no interactions between the drug and polymer. The drug did not lose any of its thermal behavior and spectral characteristics after entrapment into the microspheres. These results were in agreement with the study of Cevher et al. 2006 for the preparation of vancomycin microspheres [23]. The surface morphology of the hardening agent $CaCl_2$ (X_2) and stirring speed (X_2) (Table 1) and evaluated for different in vitro such as percentage yield, particle size (Y_1), entrapment efficiency (Y_2) and drug release (Y_3) followed by in vivo antibacterial effect on animals. The morphology of chitosan microspheres loaded with Vancomycin HCl was porous and spherical in shape. The characterization profile of OVMS including particle size, surface morphology and drug entrapment was according to the study of Chakraborty et al. 2019 [24]. OVMS exhibited an angle of repose of 25.05° which indicated the good flow property. Carr's index and Hauser's ratios were 11.11 and 1.12, respectively, that confirmed good flow property of the optimized microspheres. The micromeritics study of OVMS was similar to the results reported by Sahoo et al. 2005 [25].

Vancomycin is an effective antibacterial drug against *S. aureus* and other microorganisms [26]. So, in vitro antibacterial activity of OVMS against *S. aureus* was compared with the marketed Vancomycin hydrochloride IV powder. The results showed that no significant difference in the zone of inhibition between test and reference formulations. It indicated that the antibiotic possessed the same activity with both test and reference formulations and thus confirmed that activity of drug against bacteria was not lost by formulating into microspheres. These in vitro antibacterial results of OVMS were in agreement with the finding of Claudius et al. 1999 [27].

The mechanism of drug release from OVMS was first-order release as the regression value was closer to unity for first order compared to zero-order kinetics. The correlation coefficient obtained for Korsmeyer Peppas was found to be more superior on comparison with R^2 value of Higuchi plot indicated the best fit model. The "n" value in Korsmeyer Peppas model was 0.296 indicated that the formulation followed the Fickian release, as it is below 0.4 and above 0.4 suggests non-Fickian release.

The results of in vivo studies exhibited that there was a significant decrease in the diameter of the knee joint of animals treated with intraarticular injection of OVMS when compared with the control untreated animals. Additionally, a significant decrease in the bacterial load in synovial fluid of the animals treated with OVMS was seen when compared with the control and reference group animals. The results showed that intraarticular injection of OVMS may be effective in treating septic arthritis. The above in vivo results against *S. aureus* induced septic arthritis are similar to the reports of the study of Darley and MacGowan (2004) [28]. Thus, the present study showed that the formulation of VMS designed by BBD may be an effective drug therapy against septic arthritis with prolonged release of drug and increased therapeutic effect at the site of infection with a decrease in the frequency of dosing.

Conclusions

In this study, the prepared optimized VMS showed a similar effect as that of the marketed vancomycin formulation by reducing infection in the knee joint at a reduced frequency of dose administration. However, the significant effect was achieved with OVMS for treating the septic arthritis joints. The intraarticular administration of OVMS can reduce dose size, dosing frequency and systemic exposure with local targeted delivery. It can also reduce systemic toxicity. Additionally, OVMS may retain a knee joint for more time to give sustained action due to the biodegradable polymer matrix. The intraarticular administration of OVMS may also increase the therapeutic efficacy of the drug by limiting the rapid clearance of the drug.

Abbreviations

BBD: Box–Behnken design; VMS: Vancomycin hydrochloride-loaded microspheres; OVMS: Optimized Vancomycin hydrochloride-loaded microspheres; RA: Rheumatoid arthritis; DSC: Differential scanning calorimetry.

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Authors' contributions

PSL contributed to design and writing of the work. MV have drafted the work or substantively revised it. SKRV contributed to guidance and mentoring. All authors read and approved the final manuscript.

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Availability of data and materials

All data and material are available upon request.

Declarations

Ethics approval and consent to participate

The animal study was approved by Institutional Animal Ethical Committee (Approval No. CPCSEA//1677/PO/Re/S/2019/IAEC/23).

Consent for publication

Not applicable.

Competing interests

There is no competing of interest.

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