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Development of nitazoxanide-loaded colon-targeted formulation for intestinal parasitic infections: centre composite design-based optimization and characterization

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Abstract

Background: The current investigation is focused on the development and characterization of Eudragit S100 coated nitazoxanide-loaded microbeads as colon-targeted system utilizing central composite design (CCD) and desirability function. The study initiated with the selection of a BCS class II drug nitazoxanide and its preformulation screening with excipients, selection of polymer and identification of concentration for CCD, selection of optimized formulation based on desirability function, and in vitro release studies in simulated gastric and colonic media and stability studies. A two-factor, three-level CCD was employed with two independent variables, i.e. X1 (chitosan % w/v) and X2 (sodium tripolyphosphate % w/v), and three dependent variables, i.e. Y1 (particle size in micrometres), Y2 (percentage yield) and Y3 (percent entrapment efficiency), were chosen. Additionally, surface morphology, mucoadhesion and in vitro drug release studies were also conducted.

Result: Chitosan concentration showing maximum entrapment and optimum particle size was selected to formulate chitosan beads. The polynomial equation and model graphs obtained from the Design-Expert were utilized to examine the effect of independent variables on responses. The effect of formulation composition was found to be significant ($p < 0.05$). Based on the desirability function, the optimized formulation was found to have 910.14 μm ± 1.03 particle size, 91.84% ± 0.64 percentage yield and 84.75% ± 0.38 entrapment efficiency with a desirability of 0.961. Furthermore, the formulations were characterized for in vitro drug release in simulated colonic media (2% rat caecal content) and have shown a sustained release of ~92% up to 24 h as compared to in vitro release in simulated gastric fluid.

Conclusion: The possibility of formulation in enhancing percentage yield and entrapment efficiency of nitazoxanide and the utilization of CCD helps to effectively integrate nitazoxanide microbeads into a potential pharmaceutical dosage form for sustained release.

Keywords: Central composite design, Desirability function, 3D surface plots, Simulated colonic media, Eudragit S100, Multiple regression, Contour plots

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Background

As per World Health Organization (WHO) 2020 fact-sheets, soil-transmitted helminths (STHs) also known as intestinal nematodes or roundworms have triggered high morbidity and obstruction of an individual's well-being by inducing infection in approximately 1.5 billion of population worldwide. Children and pregnant women are the prime host of these infections. As per WHO latest estimates, more than 880 billion children need therapy against these parasites as these infections are common in tropical and sub-tropical areas [1]. The WHO has also released the control interventions centred on the intermittent anthelminthic administration (deworming) to the population at risk, supplemented with improved sanitation [2]. Parasitic infections can be instigated by three kinds of microorganisms: ectoparasites, protozoa and helminths. These are responsible for many ill health conditions, including diarrhoea, gastrointestinal upset, vaginal irritation, joint pain, nervous diseases, immune dysfunction and chronic fatigue. *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus* and *Ancylostoma duodenale* are the major nematode species which cause STH [3, 4]. Since many times, two main categories of active molecules, i.e. benzimidazoles and nicotinic acetylcholine receptor agonists, are being utilized for the treatment of parasitic infections. But with time, resistance which has been developed in intestinal nematodes with these drug categories in humans has been reported. Thus, to overcome these STH infections, there is a requisite to discover highly effective new therapies and measures to the existing therapies. Nitazoxanide is a promising alternative for the treatment of intestinal nematode infections [5]. Nitazoxanide (NTZ) is a new antiparasitic agent and, chemically, a nitrothiazolyl salicylamide compound. It is the first antiparasitic agent which shown positive activity against both protozoa and helminths, especially for the intestinal parasitic infection therapy. It is basically used in diarrhoea caused by *Giardia lamblia*/*intestinalis* or *Cryptosporidium parvum* [6]. The main metabolites of nitazoxanide have been identified as deacetyl-NTZ or tizoxanide (TIZ). NTZ has poor water solubility due to which it has low bioavailability, and hence, high doses and frequent dosing are needed for the treatment. It is a class II drug as per the Biopharmaceutical Classification System (BCS). Around two thirds and one third nitazoxanide oral dose is excreted in faeces and in urine, respectively [7].

The oral route of administration has been used for both conventional and novel formulations. An oral controlled-release formulation should exhibit the following individualities: able to attain optimal therapeutic drug concentration in the plasma with the smallest fluctuation, enhance pharmacotherapy of drugs with short half-life, minimize repeated dosing, reduce the dose-related adverse effects in high dose, and advance therapy, efficacy and safety with improved patient compliance [8]. Furthermore, the colon-targeted formulations should have the capability of drug protection via oral route to the colon, i.e. the dissolution and absorption of drug should occur only in colon; degradation should be prevented [9, 10]. Currently, the physiological features of colon aid in achieving colonic targeting, viz., colon pH (6.5–7.5), microbial flora-rich segment, slow peristaltic movement, etc. Exploiting these features of colon, several colon-targeted formulations had been suggested like pH and time-dependent, pressure-controlled and some passive targeting drug delivery systems like enzyme-triggered, prodrug and enzyme-degradable polymer-coated drug delivery systems and complex colon-targeting drug delivery systems [11].

Chitosan, a naturally occurring polysaccharide, is emerging as a promising polymer in the development of novel drug delivery system. It is biocompatible and non-toxic in nature. Chitosan is a weak cationic polysaccharide composed of (a (1→4) 2-amino-2deoxy-β-D-glucan) which is acquired by chitin alkaline deacetylation [12]. The properties of smart polymers such as biodegradability and bioadhesion are the features of chitosan. Poorly soluble drugs or the drugs readily soluble in acidic medium are the good candidates for being developed as chitosan beads as they have the synergistic characteristics of bioadhesion and floating [13]. The beads function as depot basin which will permit the steady release of minute quantities of active in solution state to the upper part of small intestine resulting in greater and more constant drug blood levels. Microbeads are tiny, solid and free-flowing particulate carriers comprising of dispersed active moieties in solution or crystalline state which allow a sustained release or multiple release profiles of treatment with various active agents without any major side effects [14]. Additionally, the beads maintain functionality under physiological conditions; it can incorporate the drug to deliver locally at high concentration ensuring that therapeutic levels are reached at the target site while reducing the side effects by keeping the systemic concentration low.

In this research, optimization of drug-loaded microbeads was carried out by one of the designs of response surface methodology (RSM), i.e. central composite design (CCD). A two-factor, three-level CCD was chosen as it avoids the usage of complete three-level factorial experiment and provides quadratic and rotatable model for the response variables. CCD also gives an extra benefit with prediction of responses at an additional level 'α', apart from high, medium and low levels [15]. The α is considered as the distance of axial points from the centre. The present investigation was to design, optimize and characterize nitazoxanide-loaded chitosan microbeads using CCD in terms of responses, viz., particle size, percentage yield and entrapment efficiency. Furthermore, formulations were also evaluated for in vitro drug release in simulated gastric fluid (SGF) and release in the presence of rat ceacal content (simulated colonic media) and stability studies.

Methods

Materials

Nitazoxanide was received as a gift sample from Ind-Swift Ltd., Jammu & Kashmir, India. Chitosan and sodium tripolyphosphate (NaTPP) were obtained from CDH, New Delhi, India. Analytical grade chemicals and reagents were used in the investigation.

Methods

Preformulation studies

Prior to the development of microbeads, the physicochemical properties of drug, i.e. nitazoxanide, were studied via several significant parameters, viz., organoleptic evaluation (color, odor and texture), melting point range (digital melting point apparatus), ultraviolet-visible (UV) spectroscopy and drug-excipient compatibility studies (Fourier transform-infrared spectroscopy). A standard solution (1 μg/ml) of nitazoxanide was prepared in methanol and scanned by UV-VIS spectrophotometer (Shimadzu UV-1800) between 200 and 800 nm. Furthermore, calibration curve was also plotted by the method suggested by Kapse et al [16]. Nitazoxanide was dissolved in 20 ml of methanol in a volumetric flask and treated with a solution (10 ml of 5 N HCl). Different concentrations of chitosan, i.e. 1.5% w/v, 2% w/v, 2.5% w/v, 3% w/v to 3.5% w/v, in 1% v/v glacial acetic acid were taken to form beads with 2.5% w/v sodium tripolyphosphate as crosslinking agent and 1 g of zinc granules in portion while shaking, kept at room temperature for 1 h, and then filtered through a cotton wool. The residue was washed with a 10 ml portion of methanol three times, and 0.5 ml of 2% v/v para-dimethyl aminobenzaldehyde (PDAB) solution prepared in methanol was added and the volume was made up to 100 ml. The final concentration of reduced nitazoxanide was made up to 100 μg/ml. From the stock solution, concentrations of 5 to 25 μg/ml were prepared and subjected to measurement via a UV-visible spectrophotometer [16]. FT-IR studies were conducted by storing the drug, excipient and drug-excipient mixture (1:1) for 15 days at room temperature in separate glass vial drug-KBr pellet, excipient KBr pellet and drug-excipient-KBr pellet (1:1) and subjected for scanning from 4000 to 400 cm^{-1} using FT-IR spectrophotometer in a reflectance mode (Perkin Elmer Spectrum Rx, Serial No. – 79225).

Selection of polymer

For the development of nitazoxanide microbeads, two polymers based on their mucoadhesive and release-modifying behaviour were selected from the literatures. Chitosan and alginate are commonly used polymers in modifying the drug release of microbeads. Likewise, different sodium alginate concentrations, i.e. 1.5% w/v, 2% w/v, 2.5% w/v, 3% w/v to 3.5% w/v, in distilled water were taken to manufacture beads with 3% w/v anhydrous calcium chloride as crosslinker. The polymer showing maximum entrapment of drug was selected and further subjected to the selection of formulation technique [17] (Gulati et al., 2014). All the readings were taken in triplicate (n = 3), and standard deviation (S.D.) was calculated from the average value (mean ± S.D.)

Selection of formulation technique

Two techniques were taken, viz., ionotropic gelation and emulsion gelation technique. Maximum entrapment, particle size and the microbead structure (observed by compound microscope) were three the parameters for the selection of technique. Additionally, optimization of concentration of selected polymer and its respective crosslinking agent was carried out based on maximum entrapment of drug and optimum particle size [17]. All the readings were taken in triplicate (n = 3), and standard deviation (S.D.) was calculated from the average value (mean ± S.D.).

Ionotropic gelation technique

Nitazoxanide-loaded colon-targeted microbeads were prepared by dispersing chitosan (3% w/v) in 1% v/v glacial acetic acid with agitation for 24 h. Nitazoxanide was dissolved in acetone and then gradually added to chitosan solution with stirring. Nitazoxanide-loaded chitosan solution was added dropwise using 20-G hypodermic needle fitted with syringe into 2% w/v sodium tripolyphosphate (NaTPP) solution in deionized water at RT with continuous magnetic stirring at 100 rpm. The pH of the system was kept as slightly acidic, i.e. 5 ± 1. After 30 min curing time, the microbeads were filtered and washed with distilled water and dried at room temperature for 24 h in a desiccator [18].

Emulsion gelation technique

Different concentrations of sodium alginate (2.5% w/v, 3% w/v, 3.5% w/v) were prepared separately in distilled water. Nitazoxanide and castor oil were added to the solution. Each mixture (containing nitazoxanide 100 mg and castor oil) was stirred properly to prepare homogenous mixtures. The mixture was extruded, using a 20-G syringe needle into 100 ml calcium chloride solution (1% w/v) using a magnetic stirrer (TANCO) at room temperature and 100 rpm speed. After 30 min curing time, the microbeads were filtered and washed with distilled water and dried at 40 °C for 12 h.

Coating of microbeads

The coating of optimized microbead formulation was conducted with Eudragit S100 employing solvent evaporation technique. The formulated beads were dispersed into an acetone-based solution of Eudragit S100 to obtain 5% of weight gain. By applying vaccum at 300 mg

Hg in a rotary evaporator, solvent was evaporated at 50 rpm and further dried in a desiccator for 12 h with the aid of a vacuum [19].

Optimization of microbeads: Central Composite Design (CCD)

For the nitazoxanide-loaded microbead optimization, an orthogonal block, two-factor, three-level CCD was employed (Design-Expert® software, version 12, Stat-Ease, USA). The preferred independent variables were polymer concentration (% w/v) and crosslinker concentration (% w/v). For each independent variable, the CCD has given two axial points (− α and + α) in addition to low, medium and high as shown in Table 1. The preferred responses were particle size (um), percentage yield (%) and entrapment efficiency (%). With the selected aspects, CCD had advocated total 09 runs (04 factorial runs, 04 axial runs and 01 centre run). Nitazoxanide-loaded microbeads were formulated, and matching values of responses or variables were entered in the design. Ultimately, to obtain the formulation of maximum desirability, constraints with their relevant importance were applied [20].

Evaluation of nitazoxanide-loaded chitosan microbeads

Particle size, percentage yield and entrapment efficiency

For the analysis of microbead particle size, randomly 100 microbeads were selected, and their diameter was analysed using the Medical Pro software with the help of optical microscope. The smear formed by the dispersion of microbeads in liquid paraffin was viewed under compound microscope, and then, the diameter of the particles was noted down [21].

The percentage yield of various formulated microbead formulation was computed using the dried final microbead weight with respect to the primary complete weight of the drug and polymer utilized for microbead formulation [22]. Percentage yield was computed with the help of the following formula:

$$\text{Percentage yield} = (\text{practical yield}/\text{theoretical yield}) \times 100$$

Accurately weighed microbeads (50 mg) were crushed and dissolved in 10 ml methanol, vortexed for 5 min and filtered through a 0.45-μm Whatman filter no. 4. The filtered samples were added with 0.5 ml of 2% v/v para-dimethylaminobenzaldehyde (PDAB) solution prepared in methanol [23]. Then, the solution was heated for 10 minutes at 60–70 °C temperature on water bath. After cooling, the volume of sample solution was made up to 10 ml by methanol and analysed spectrophotometrically using UV-VIS spectrophotometer (Shimadzu, Japan) at 559 nm. The drug entrapment efficiency (DEE) was computed with the given formula:

$$\%\text{Drug entrapment efficiency} = \text{Actual drug content}/\text{Theoretical drug content} \times 100$$

All the readings were taken in triplicate (n = 3), and standard deviation (S.D.) was calculated from the average value (mean ± S.D.).

Surface morphology

The structural descriptions of the bead's surface such as shape were investigated with the help of scanning electron microscopy (Model-ZEISS EVO50, special edition, Japan) using gold sputter technique. The dried microbead particles were coated to 200 A° thickness with gold palladium. The operating parameters were 20 nm working distance, zero-degree tilt and 15 kV accelerating voltage [22]. Photomicrographs were clicked within a range of 50–5000 magnifications.

Mucoadhesive testing

A small intestinal tissue freshly cut was acquired from local slaughterhouse within 1 h of goat slaughter which was cleansed with washing by isotonic saline solution.

Table 1 Independent variables, dependent variables and constraints for central composite design

	Levels				
Independent variables	**Low (− 1)**		**Medium (0)**	**High (+ 1)**	
X_1 = chitosan (% w/v)	2.75		3	3.25	
X_2 = NaTPP (% w/v)	1.75		2	2.25	
Constraints applied on dependent variables					
	Independent variables		**Dependent variables**		
	X_1: chitosan (% w/v)	**X_2: NaTPP (% w/v)**	**Y1: particle size (um)**	**Y2: percentage yield (%)**	**Y3: entrapment efficiency (%)**
Constraints	In range	In range	In range	Maximize	Maximize
Importance	+++	+++	++++	++++	++++

The jejunum was separated and soaked in receptor medium (phosphate buffer, pH 6.4). Therefore, for experimentation, a piece of the jejunum mucosa (2 × 3 cm) was mounted onto glass slide (2 × 1 cm) using thread. An exact weight of microbeads (50 mg) was kept on the mucosal surface [22]. The glass slides were put in the grooves of the USP tablet disintegrating test apparatus, and a regular up and down movement was performed in a beaker holding phosphate-buffered saline pH 7.4. The beads which stayed on the mucosal surface were totalled at a regular interval for up to 10 h. All the readings were taken in triplicate (n = 3), and standard deviation (S.D.) was calculated from the average value (mean ± S.D.).

$$\text{Percent Mucoadhesion} = \text{Number of beads adhered} / \text{Total number of beads applied} \times 100$$

In vitro drug release studies in simulated gastric fluid

In vitro drug release studies of coated nitazoxanide microbeads (equivalent to 100 mg of nitazoxanide) were performed using USP dissolution test apparatus II (DS 8000 Labindia). The dissolution studies were performed in 900 ml of simulated gastric fluid as dissolution medium, which was stirred at 100 rpm at 37 ± 0.1 °C following a pH progression method, i.e. pH 1.2 for the first 2 h and pH 7.2 phosphate-buffered solution for the rest of 24 h studies. Aliquots were withdrawn periodically and replaced with fresh medium to maintain sink condition throughout the period. Samples were withdrawn at predetermined time intervals using a pipette; the tip of which was covered with filter paper to avoid drug particles [24]. The withdrawal samples were treated with 0.5 ml 2% w/v methanolic solution of para-dimethylaminobenzaldehyde (PDAB) solution. All samples were kept for 10 min at 60–70 °C temperature on water bath. Nitazoxanide content in the aliquots after that adding of PDAB solution was assayed spectrophotometrically (UV-VIS spectrophotometer) at 559 nm for all samples. The experiments were done in triplicate (n = 3), and standard deviation (SD) was calculated from the mean value.

Desirability function approach for optimization

Derringer and Suich had given a numerical optimization technique which aids to optimize all responses at one time. In this research, a desirability function approach was utilized to optimize all three responses altogether with the help of Design-Expert 12. To each independent variable and response, an aim/constraint is assigned, i.e. minimize, maximize, in range, target and equal to. With individual responses (dependent variables), a desirability function is related which has the value between 0 and 1. The value 0 is for unanticipated responses, and between 0 and 1 (minimum to most preferred) is for desired response. Hence, to establish the anticipated responses in the design space, desirability function approach is most helpful [25].

Evaluation of optimized formulation

Based on the desirability function, the optimized formulation (FF1) was selected and prepared. It shows a polymeric concentration of 2.912% w/v and a crosslinking agent concentration of 1.947% w/v. It was further evaluated for the abovementioned parameters, viz., particle size, percentage yield and entrapment efficiency. Additionally, it was evaluated for in vitro drug release in SGF and simulated colonic media.

Preparation of rat caecal content (simulated colonic media)

Conventional dissolution tests have some limitations, viz., unable to investigate the effect of bacterial flora specifically present in colon on drug formulations. To surmount this drawback, rat caecal contents are utilized as a substitute to dissolution media. This media is also known as simulated colonic media. The investigation was conducted in accordance with the Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by the Institutional Animal Ethical Committee (IAEC). Rat caecal content was created by the technique as described by Van Den Mooter et al. [26]. Two rats of constant body weight (150–200 g) with no previous drug therapy were kept on normal diet and were given 1.0 ml of 1% w/v chitosan solution (in 1% v/v glacial acetic acid) via oral route for 7 days. This triggers the induction of specific enzymes in rat colon to digest the chitosan. Thirty minutes before the investigation starts, euthanasia was performed on each rat with their abdomen cut and open. The caecum was located, and both ends were ligated and dissected. Simulated colonic fluid pH 6.8 (bubbled with CO_2) was used to keep the caecal content [27, 28]. The caecal contents were separately weighed, merged and appended in buffer to produce 4% w/v final caecal concentration. The media was then sonicated using probe sonicator for 5 min at 4 °C to break the bacterial cells. The complete procedure was done in CO_2 environment to maintain the anaerobic conditions.

In vitro drug release study in the presence of rat caecal content

Eudragit S100 coated chitosan microbeads were evaluated for in vitro drug release in rat caecal content in a USP Dissolution Test Apparatus II (DS 8000, Labindia) using 900 ml dissolution medium, at 100 rpm and 37 ± 0.5 °C, maintaining sink condition throughout the study. Drug release rate studies were performed initially in GI fluid for 2 h. From the third hour onwards, the release

study was performed in simulated colonic fluid containing rat caecal content. The experiment was performed with a continuous supply of carbon dioxide into the dissolution media to simulate the colonic environment [29]. All the readings were taken in triplicate (n = 3), and standard deviation (S.D.) was calculated from the average value (mean ± S.D.).

Stability studies as per ICH guidelines

The stability study of optimized nitazoxanide beads was carried out at different storage conditions, i.e. refrigerator, room temperature and accelerated temperature and humidity conditions 40 ± 2 °C/75 ± 5% RH for 60 days [30].

Results

Preformulation studies

Nitazoxanide was found to be yellow crystalline powder which revealed melting with decomposition in the range of 199 ± 1 °C to 201 ± 1 °C as reported, thus indicating purity of sample. The UV spectrum of nitazoxanide showed the absorption maxima at 559 nm in methanol with further derivatization using *p*-dimethylaminobenzaldehyde (PDAB). Hence, all further UV estimation was done at maximum a wavelength of 559 nm. Calibration curve was plotted with a series of dilutions as shown in Fig. 1. The regressed equation is $y = 0.0066x - 0.0068$. The value of r^2 is close to 1, i.e. 0.9924. The FT-IR spectra of pure nitazoxanide, chitosan and nitazoxanide-chitosan physical mixture (1:1) confirmed the absence of any chemical interaction between them after 15 days of sample storage. The values of functional group reported in samples are compiled in Table 2. For the selection of polymers, two mucoadhesive polymers, viz., chitosan and sodium alginate, were chosen at different concentrations (1.5, 2, 2.5, 3, and 3.5% w/v), and their effect on DEE was recorded as shown in Fig. 2. From the results, it was found that sodium alginate beads possess DEE in the range of 23.56% ± 0.75 to 58.99% ± 0.48, while chitosan microbeads have shown greater DEE in the limits of 50.44% ± 1.23 to 75.87% ± 0.42. After the selection of polymer, the best formulation technique was chosen based on DEE and PS. The results demonstrated that emulsion gelation technique produced microbeads larger in size, i.e. 960.74 μm ± 1.02 and less drug entrapment, i.e. 58.07 ± 1.75 as compared to the beads prepared by ionotropic gelation which are possessing high entrapment, i.e. 71.46 ± 1.02, and less particle size, i.e. 719.56 ± 0.79 as shown in Fig. 3. Apart from this, emulsion gelation method also results in tailing of microbeads, while ionotropic gelation technique results in discretely round free-flowing microbeads as shown in Fig. 4.

Central Composite Design (CCD) for the optimization of polymeric microbeads

In this study, CCD was selected to investigate the effect of two independent variables on responses. The constraints and importance of independent variables and responses are mentioned in Table 1. As per CCD, 09 formulations were developed and analysed for their responses, i.e. particle size (Y1), percentage yield (Y2) and entrapment efficiency (Y3). All the values are acquired utilizing Design-Expert 12. A full quadratic equation was applied to all the dependent variables which were significantly tested by ANOVA and multiple correlation coefficient (R^2) test. The model p value was also checked for significance. The value of R^2 should be close to 1 and helps to determine the extent of variation from the average.

The observed highest and lowest particle size of all formulations varied from 845.45 ± 0.34 μm to 942.06 ± 0.67 μm. The investigated higher and lower range for percentage yield and entrapment efficiency were obtained to be 78.00% ± 1.04 to 92.82% ± 1.78 and 65.23 ± 0.034 to 91.87 ± 0.011%, respectively, as shown in Table 3. The best fit polynomial model for

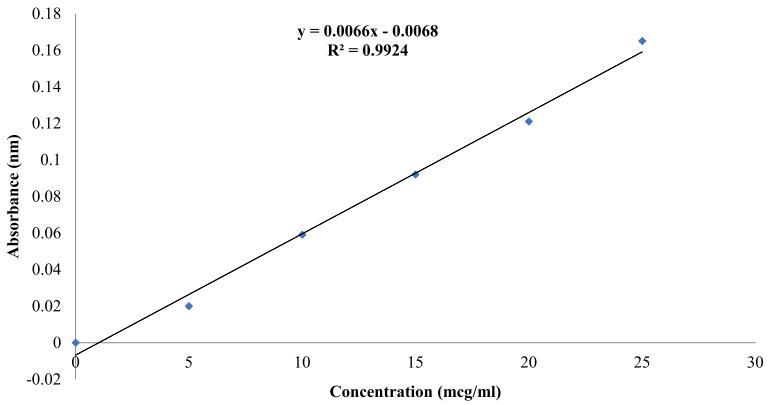

Fig. 1 Calibration curve of nitazoxanide at 559 nm absorption maxima

Table 2 Observed wave numbers and the corresponding frequency assignments

S. No.	Frequency assignment	Wave number (cm^{-1})
Observed wavenumbers (cm^{-1}) of nitazoxanide		
1	–C=O stretching (amide linkage)	1662.11
2	–C=C– ring benzene	1471.73
4	–CH aromatic ring	3087.92
5	–C=O stretching (ester linkage)	1772.27
6	–N–H stretching	3356.15
Observed wavenumbers (cm^{-1}) of chitosan		
7	–OH stretching	3460.81
8	Symmetric (CH_3) stretching and asymmetric (CH_2) stretching	2927.94
9	Amide II band and amide I band	1600 and 1650
10	C=O stretching in $NHCOCH_3$ group	1662.39
Observed wavenumbers (cm^{-1}) of drug: polymer (1:1) physical mixture after 15 days		
11	–C=O–N stretching	1662.75
12	–C=C– ring benzene	1473.77
13	–C–H aromatic ring	3088.32
14	C=O stretching	1773.37
15	–N–H stretching	3358.30

all three responses, viz., particle size (Y1), percentage yield (Y2) and entrapment efficiency (Y3), was found to be quadratic (p value < 0.005) as mentioned in Table 4. The variance between "Adjusted R^{2}" and "Predicted R^{2}" values for particle size, percentage yield and entrapment efficiency were less than 0.2.

Effect of independent variables on particle size

The results of observed responses and predicted responses for Y_1 are mentioned in Table 3. Based on CCD, the combinations of independent factors, i.e. chitosan (X_1) and sodium tripolyphosphate (X_2), for response (Y_1) resulted in a mathematical relationship in the form of polynomial equation as mentioned below:

$$\text{Particle size}(Y_1) = 892.98 + 57.91X_1 + -28.93\,X_2 + -17.47X_1X_2 + -143.83{X_1}^2 + -5.96{X_2}^2 \ldots\ldots\ldots\ldots\text{Coded equation} \quad (1)$$

The measurable effect of independent variables (X_1 and X_2) and their interactions (X_1X_2) on the response Y_1

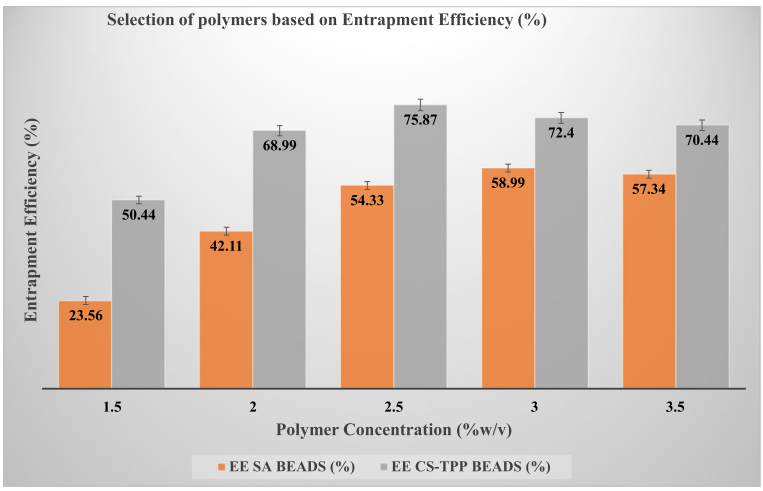

Fig. 2 Selection of polymer based on percent entrapment efficiency

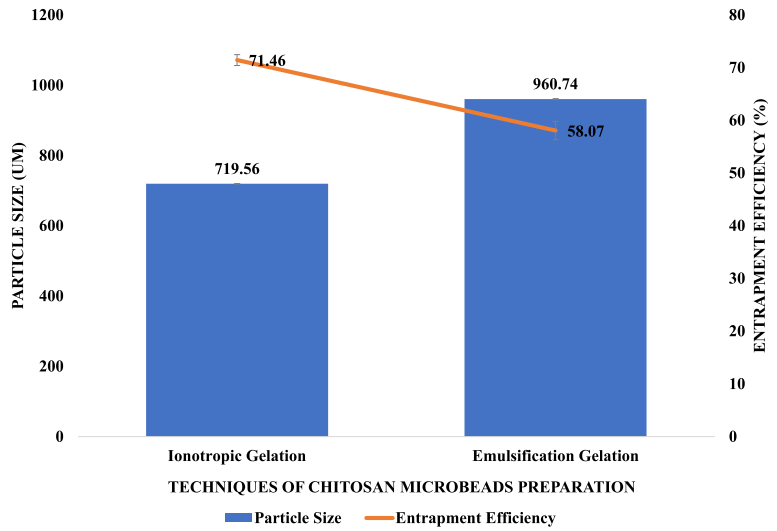

Fig. 3 Comparison of chitosan microbeads preparation based on particle size and entrapment efficiency

can be noted from the above equation. The value of p (< 0.05) of coefficients specifies the significance of response as shown in Table 5. The sign and value of coefficients give a direct idea about the nature and extent of effect on responses. If the coefficients of independent variables and their interactions has a positive sign, the effect will be collegial, and if it has a negative sign, the effect will be contrasting. The greater value of factor signifies the considerable influence on the response. Quadratic model was applied to the design. The model effectiveness was confirmed by multiple correlation test (R^2) and ANOVA. The R^2 value was found to be 0.9985, and the p value is 0.0001 which confirms the significance of independent variables in response prediction as shown in Table 4. Furthermore, variance inflation factor (VIF) was utilized to interpret multi-collinearity. Here, the value of VIF is 1 which indicates absence of multi-collinearity. The results of particle size are compiled in the form of response plot, contour plot and predicted vs actual plot in Figs. 5, 6 and 7a, respectively. The difference between adjusted R^2 (0.9960) and predicted R^2 (0.9987) is less than 0.2 which shows a good fit of the model. The model F value of this model was found to be 398.22.

Effect of independent variables on percentage yield

The results of observed responses and predicted responses for Y_2 are mentioned in Table 3. The results of percentage yield are expressed in the form of full quadratic polynomial equation given as:

$$\begin{aligned}\text{Percentage Yield } (Y_2) = {} & 83.42 + 6.88X_1 + 1.98X_2 \\ & + -0.0350X_1X_2 + -1.75{X_1}^2 \\ & + -0.4250{X_2}^2 \ldots\ldots \text{Coded equation}\end{aligned} \quad (2)$$

This equation exhibited a positive value of both variable coefficients, i.e. X_1 (chitosan % w/v) and X_2 (sodium tripolyphosphate % w/v), but the quantitative effect of X_1 is large as compared to X_2 as shown in Table 5. This clearly implies that as the concentration of chitosan increases, the percentage also increases. Adjusted R^2 and predicted R^2 show a difference less than 0.2 as shown in Table 4. The analysis of this model via ANOVA revealed the p value of less than 0.05 which suggests the

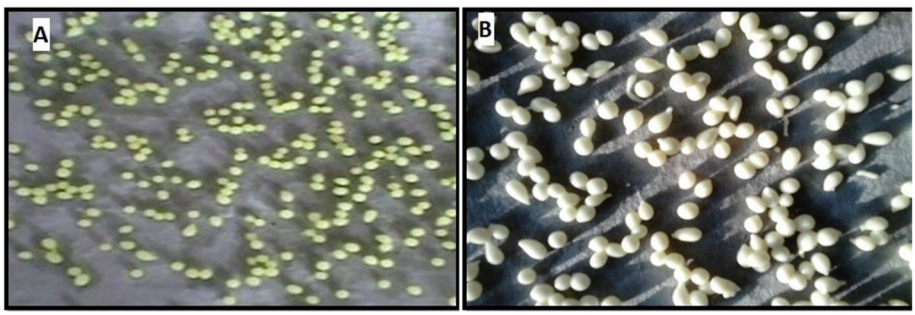

Fig. 4 Lab photographs of nitazoxanide loaded microbeads by **a** ionotropic gelation and **b** emulsion gelation

Table 3 Experimental and predicted responses of formulations with their coded factors

Formulation code	Independent variables		Dependent variables					
	Coded factors		Observed responses			Predicted responses		
	X_1	X_2	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Factorial points								
F1	− 1	− 1	700.72	72.13	79.13	696.75	72.36	78.85
F3	− 1	+ 1	672.87	76.13	83.12	673.83	76.38	83.48
F7	+ 1	− 1	848.36	86.31	82.17	847.50	86.18	81.52
F9	+ 1	+ 1	750.62	90.17	90.54	754.69	90.07	90.52
Centre points								
F5	0	0	892.78	83.17	88.67	892.98	83.42	88.09
Axial points								
F2	− 1	0	688.23	75.28	81.22	691.25	74.80	81.14
F4	0	− 1	911.12	81.12	83.78	915.95	81.02	84.71
F6	0	+ 1	863.12	85.13	91.87	858.09	84.98	91.53
F8	+ 1	0	810.27	88.32	85.32	807.06	88.55	85.99
Optimized nitazoxanide-loaded chitosan microbeads								
Optimized nitazoxanide microbeads (FF1)	3.184	2.250	829.14 ± 1.03	90.84 ± 0.64	89.75 ± 0.38	809.649	89.071	91.662

significance of the model applied. F5 exhibited the higher percentage yield (92.82%). The graphical presentation of effect of independent variables on percentage yield is shown in the form of response plot, contour plot and predicted vs actual plot (Figs. 5, 6 and 7b, respectively.). Model *F* value of this model was found to be 356.37.

Effect of independent variables on entrapment efficiency

The results of observed responses and predicted responses are mentioned in Table 3. The full polynomial quadratic equation is shown as:

$$\begin{aligned}\text{Entrapment Efficiency } (Y_3) = {} & 88.09 + 2.43X1 \\ & +3.41X2 + 1.09X1X2 \\ & + -4.52{X_1}^2 + 0.0317{X_2}^2 \\ & \ldots\ldots\ldots\ldots\text{Coded equation}\end{aligned} \tag{3}$$

In the above equation, positive values of coefficients of X_1, X_2 and their interaction factors symbolize the mutual effect of these variables on response (Y_3) with significant *p* values as shown in Table 5. This reveals that as the polymer and crosslinking agent concentration increases, entrapment efficiency also increases. High concentration of polymer shall entrap the more quantity drug, while the larger concentration of cross-linking agent will prevent drug leakage during formulation. The *p* value (˂ 0.05) of this model displays significance of the model. The model *F* value was found to be 37.53 with less than 0.2 difference between adjusted R^2 and predicted R^2 value as shown in Table 4. The results are compiled in the form of contour plot, response plot and predicted vs actual response in Figs. 5, 6 and 7c, respectively.

Surface morphology and mucoadhesion

The SEM photomicrographs of the drug-loaded microbeads and their surface morphology are shown in Fig. 8. Morphology of the drug-loaded chitosan microbeads was discrete and spherical in shape with a rough outer surface and visible large wrinkles.

The mucoadhesion of all prepared formulations was found to be in the range of 79.09 ± 0.21 to 91.23 ± 0.56% as shown in Fig. 9.

In vitro drug release in simulated gastric fluid and simulated colonic media

For testing the statistical significance of in vitro drug release, an ordinary one-way ANOVA was utilized for the comparison of the mean of one column to the mean of

Table 4 Summary of model fitting statistics and analysis of variance (ANOVA) for the responses

Response	Model fitting	R^2	Adjusted R^2	Predicted R^2	SD	Model *F* value	Model *p* value	%CV
Y_1	Quadratic	0.9985	0.9960	0.9817	5.84	398.22	0.0001	0.735
Y_2	Quadratic	0.9983	0.9955	0.9817	0.419	356.37	0.0207	0.511
Y_3	Quadratic	0.9843	0.9580	0.8320	0.894	37.53	0.0131	1.05

Table 5 Compiled data of coefficients of independent variables and their associated probability (*p*) value

Response	Intercept	A	B	AB	A^2	B^2
Particle size	892.979	57.905	− 28.9317	− 17.4725	− 143.828	− 5.95833
***p* values**		0.0002	0.0012	0.0093	< 0.0001	0.2445
Percentage yield	83.4233	6.87667	1.97833	− 0.035	− 1.75	− 0.425
***p* values**		< 0.0001	0.0014	0.8781	0.0097	0.2474
Entrapment efficiency	88.0856	2.42667	3.40833	1.095	− 4.52333	0.0316667
***p* values**		0.0070	0.0026	0.0922	0.0057	0.9633

another column. All formulations revealed an initial burst release as shown in Fig. 10. This may be attributed to the drug leaching from the outer layer of the bead with subsequent entry of dissolution fluid inside the bead matrix resulting in the dissolution and diffusion of the drug. Up to 2 h, 22.89% ± 0.14 to 30.03 ± 0.18 release has been observed for all formulations. As the pH is altered from acidic to alkaline, the release of drug gradually increases. The in vitro release of all formulations was found to be 73.05 ± 1.09 to 89.60 ± 1.10%, respectively. For multiple comparisons, Tukey's test was recommended which was observed to be accurate. ANOVA displays the significant results, but the application of Tukey's test after applying ANOVA reveals where the actual differences lie. Based on the desirability approach, FF1 was formulated and tested for in vitro drug release which shows initial burst release with subsequent sustained release at alkaline pH up to 86.27% ± 1.02.

The release of optimized Eudragit S100 coated nitazoxanide microbeads (FF1) was found to be 92.05% ± 1.13 in the presence of rat caecal content at 37 °C ± 0.015 in pH 7.4 phosphate buffer as compared to without caecal content, i.e. 86.27% ± 1.05. The comparative drug release is shown in Fig. 11.

Stability studies

The stability study of optimized formulation (FF1) was carried out at different storage conditions, i.e. refrigerator, room temperature and 40 ± 2 °C/75 ± 5% RH for 60 days, as shown in Fig. 12. The study displays that the microbeads were stable at refrigeration and room temperature while slight degradation was observed at accelerated conditions, i.e. 40 ± 2 °C/75 ± 5% RH.

Discussion

Nitazoxanide is a nitrothiazolyl-salicylamide derivative, chiefly comprising of two moieties, i.e. nitrothiazole connected to salicylic acid via peptide bond. When administered orally, its main active metabolite is tizoxanide with a broad spectrum of antihelminthic and antiprotozoal activity. Precisely, it inhibits pyruvate ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reactions which are involved in anaerobic organism metabolism. In the research literatures available, nitazoxanide had been investigated as non-mutagenic, i.e. resistance against this drug can be substantially deferred. It has been established that nitazoxanide is more effective in vitro against both metronidazole-susceptible and metronidazole-resistant strains of *Helicobacter pylori* and *Trichomonas vaginalis* [6].

Preformulation studies

The results of preformulation studies clearly established the quality and the standard of drug, i.e. nitazoxanide. The value of r^2 is close to 1, i.e. 0.9924, which demonstrates the accuracy of dilutions as shown in Fig. 1. The

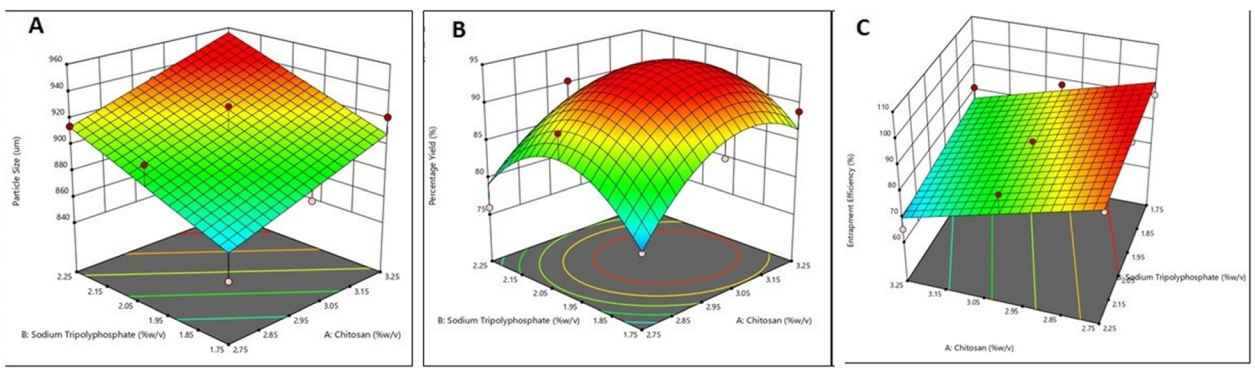

Fig. 5 3D response surface plots illustrating the effect of independent variables for three responses. **a** For response Y1. **b** For response Y2. **c** For response Y3

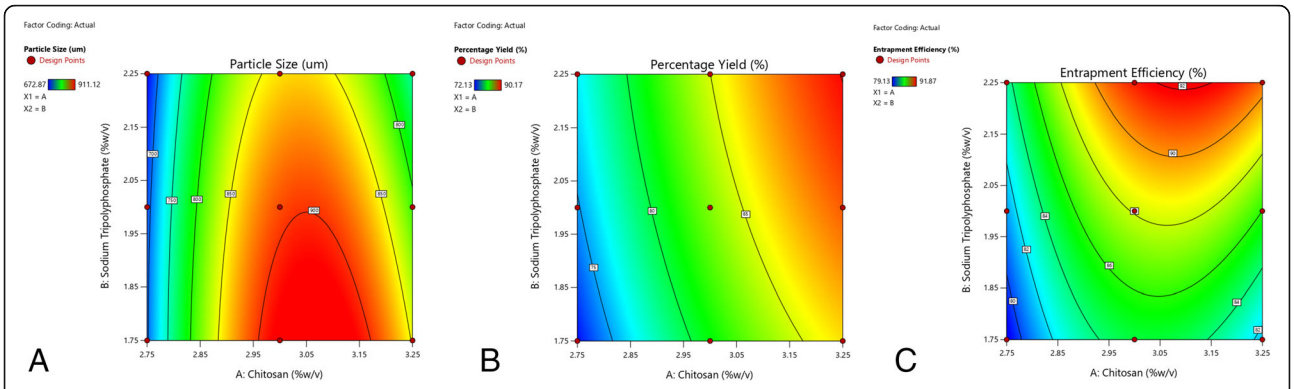

Fig. 6 Contour plots illustrating the effect of independent variables for three responses. **a** For response Y1. **b** For response Y2. **c** For response Y3

FT-IR spectra of pure nitazoxanide, chitosan and nitazoxanide-chitosan physical mixture (1:1) confirmed the absence of any chemical interaction between them after 15 days of sample storage. The values of functional group reported in samples are compiled in Table 2. There is no extra peak observed in the physical mixture of drug and excipient which clears the path for the development of formulation and its characterization.

The selection of polymer for the development of beads is considerable as it forms the matrix for active moiety and hold the drug till its release. Polymer selection was based maximally upon the extent of drug entrapment efficiency (DEE). Two mucoadhesive polymers, viz., chitosan and sodium alginate, were chosen at different concentrations (1.5, 2, 2.5, 3, 3.5% w/v), and their effect on DEE was recorded as shown in Fig. 2. From the results, it was found that sodium alginate beads possess smaller DEE as compared to chitosan beads which has greater DEE. The reason attributed for this difference is the basic structure of both linear polysaccharides. In the case of alginate beads, the gel beads are fabricated via sol-to-gel conversion of alginate with the help of divalent cations. Primely, guluronic acid present in alginate forms gel with the cations in the solution which leads to the formation of matrix that has open lattice and porous structure [31]. Thus, alginate beads have low entrapment as compared to chitosan which is a N-deacetylated derivative of chitin. Based on results, chitosan showed higher drug entrapment than sodium alginate microbeads as shown in Fig. 2. After the selection of polymer, the best formulation technique was chosen based on DEE and PS. The results demonstrated that emulsion gelation technique produced microbeads larger in size and less drug entrapment as compared to the beads prepared by ionotropic gelation which are possessing high entrapment and less particle size as shown in Fig. 3. Apart from this, emulsion gelation method results in tailing of microbeads, while ionotropic gelation technique results in discretely round free-flowing microbeads as shown in Fig. 4. Additionally, ionotropic gelation is very eco-friendly and a green process which avoids the usage of surfactants and organic solvents. The higher entrapment of nitazoxanide by ionotropic gelation technique may be due to slightly acidic nature of the system during formulation. It can be hypothesized

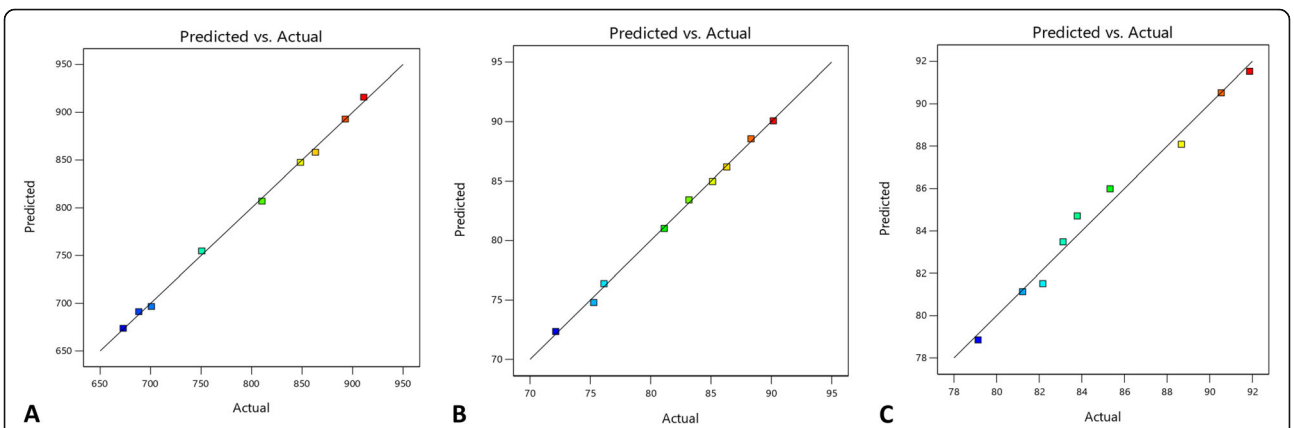

Fig. 7 Predicted vs. actual plots illustrating the effect of independent variables for three responses. **a** For response Y1. **b** For response Y2. **c** For response Y3

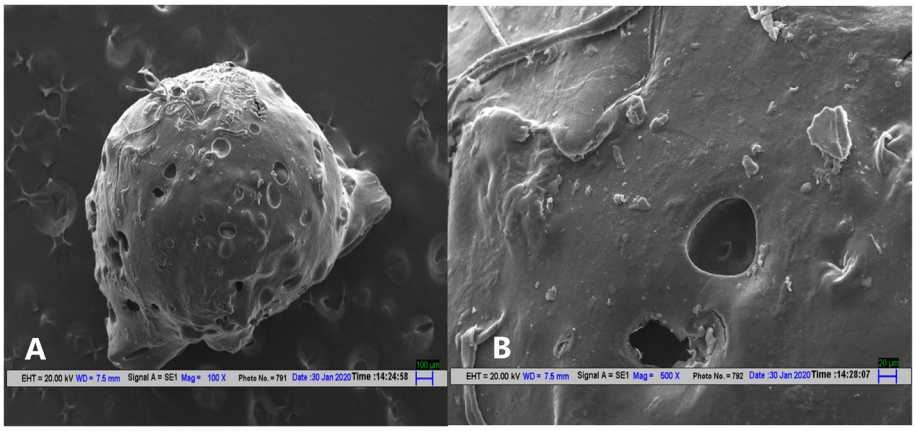

Fig. 8 SEM photomicrograph of nitazoxanide-loaded chitosan microbeads

that at a basic pH (more OH^- ions), there will be a competition for cross-linking between OH^- ions and TPP $P_3O_{10}^{5-}$ ions with the NH_3^+ group of chitosan, while at an acidic pH (more H^+ ions), there will be a strong interaction between the $P_3O_{10}^{5-}$ and NH_3^+ groups. Hence, more spherical, smaller, stable and homogeneous beads are formed [32, 33]. Consequently, ionotropic gelation technique was selected for the development of nitazoxanide-loaded chitosan microbeads.

Central Composite Design (CCD) for the optimization of polymeric microbeads

The central composite design (CCD) is a two-factor, three-level design which is desirable since it coerces fewer runs and contains extreme point combination along with points within the design space. Thus, maximum chances are there to obtain relevant and preferred results. Therefore, CCD was selected and employed here to estimate the effect of independent variables, i.e. polymer and cross-linking agent concentration, on three dependent variables, i.e. particle size, percentage yield and entrapment efficiency. As per CCD, 09 formulations were prepared and characterized for responses. Every formulation composition effect was also investigated. The regression calculations generated from the software assist to interpret the type of polynomial model fitting to the preferred responses. The best fit polynomial model for all three responses, viz., particle size (Y1), percentage yield (Y2) and entrapment efficiency (Y3), was found to be quadratic (p value < 0.005). The variance between "Adjusted R^2" and "Predicted R^2" values for particle size, percentage yield and entrapment efficiency were less than 0.2 which reveals the proper fitting of this polynomial model as indicated in Table 4. Three responses are given in Table 4.

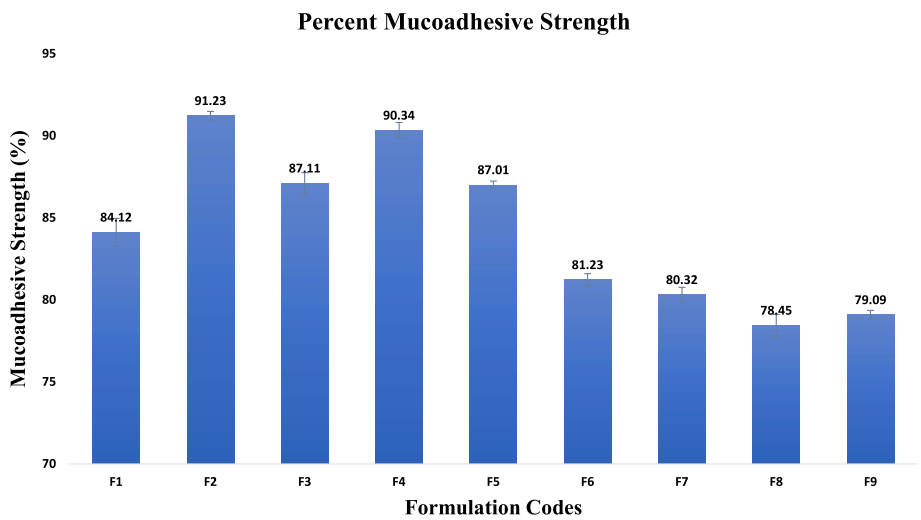

Fig. 9 Mucoadhesive strength of nitazoxanide-loaded chitosan microbead formulations

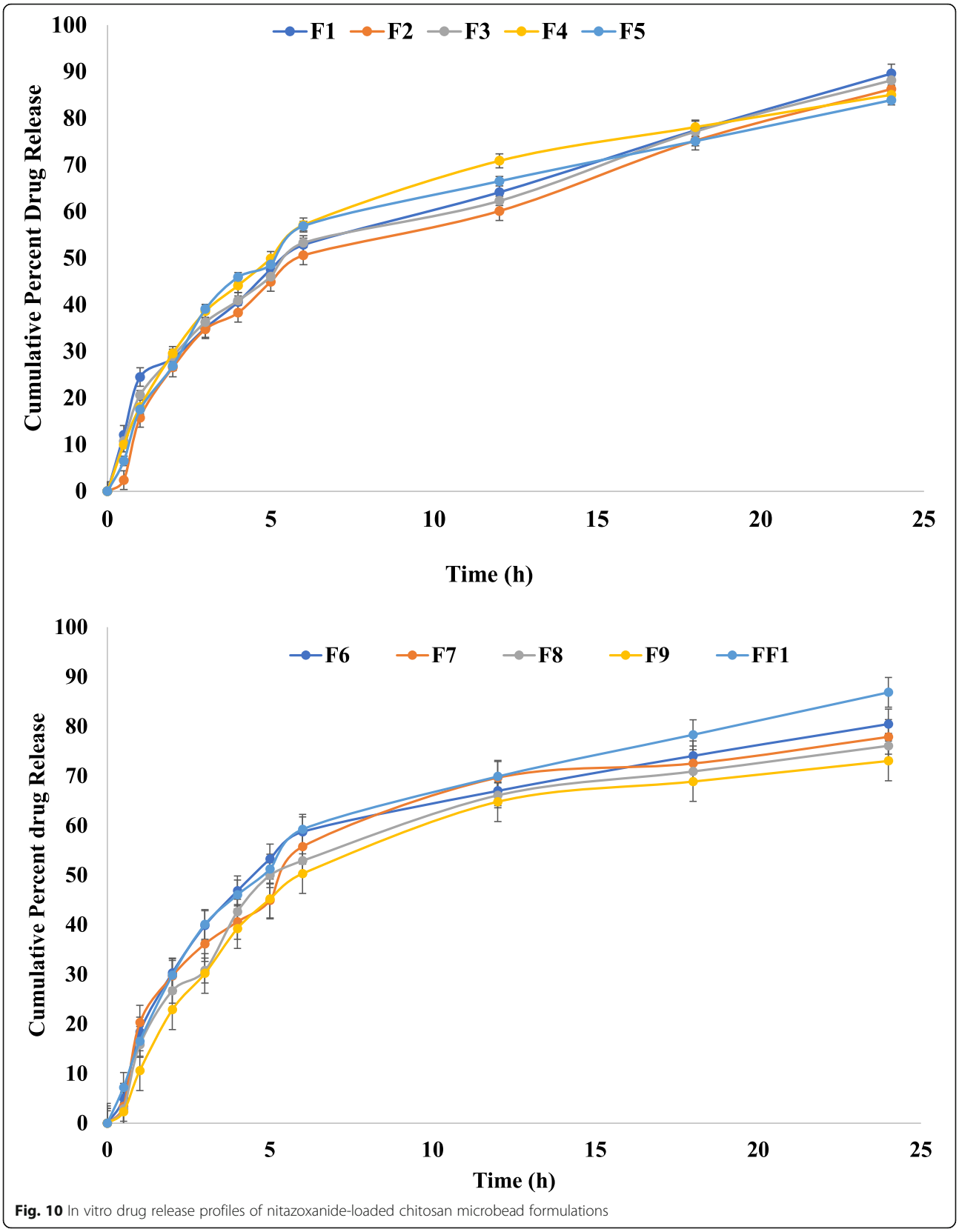

Fig. 10 In vitro drug release profiles of nitazoxanide-loaded chitosan microbead formulations

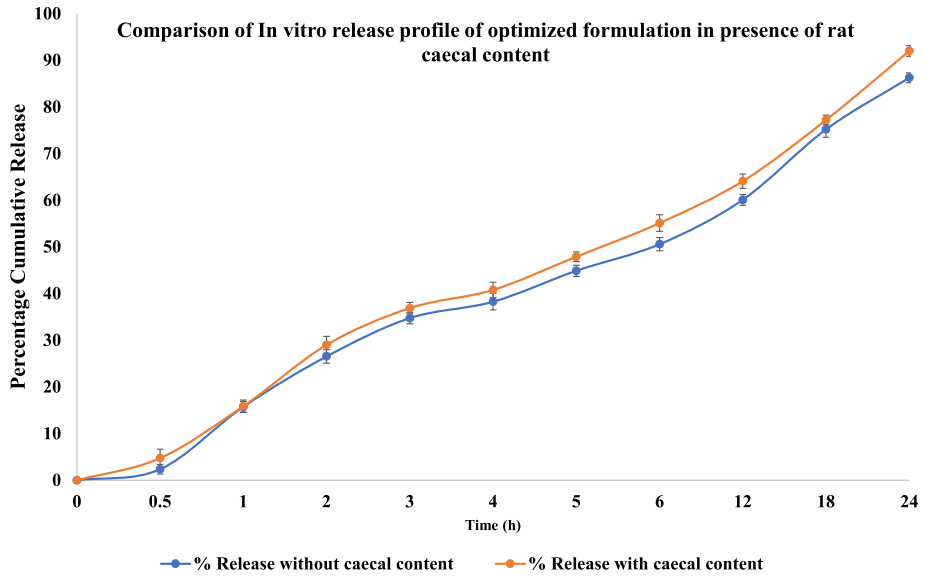

Fig. 11 In vitro release profile of optimized formulation in the presence and absence of rat caecal content

Alteration in the concentration of selected variables effects the particle size of developed formulation as shown in Coded equation (1). The lowest particle size observed was 845.45 ± 0.34 μm for the formulation F1 (2.75% w/v X_1 and 1.75% w/v X_2), while the highest particle size was observed as 942.06 ± 0.67 μm for the formulation F9 (3.25% w/v X_1 and 2.25% w/v X_2). From the coded equation 1 and Figs. 5 and 6a, a remarkable increase in

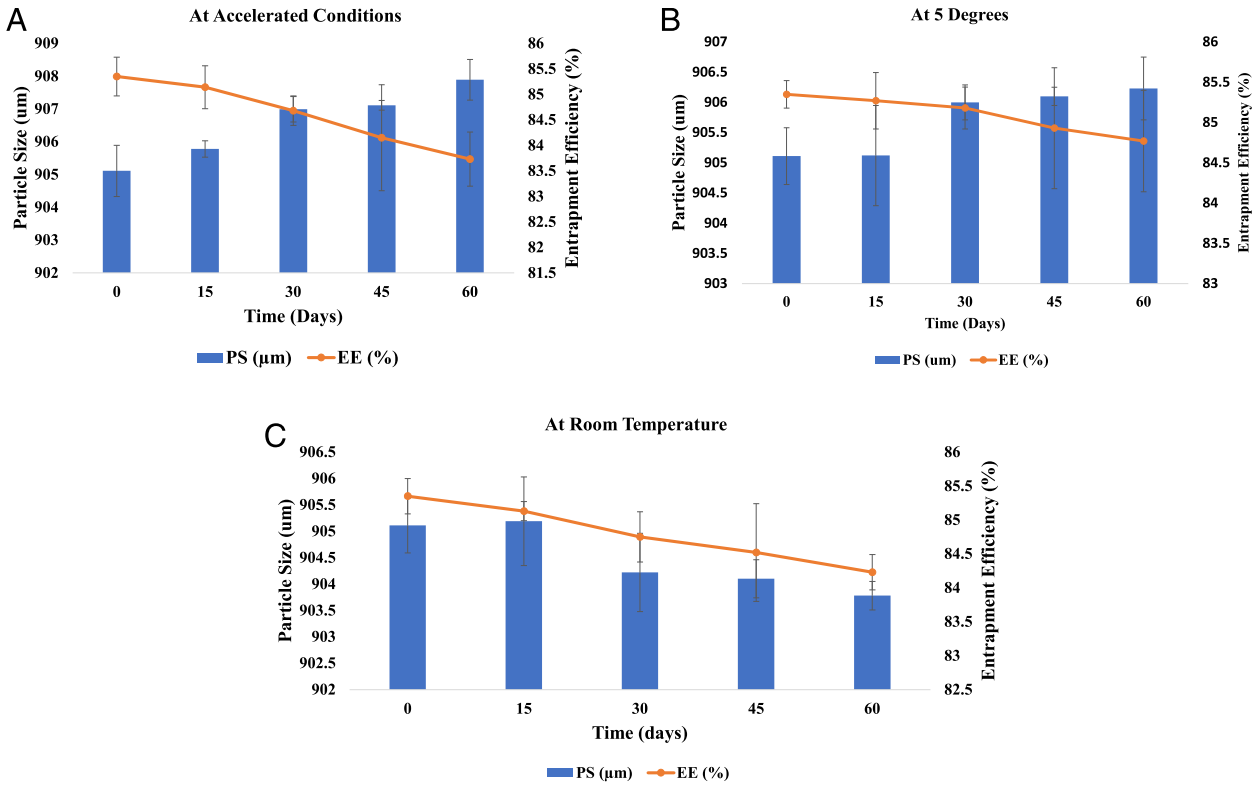

Fig. 12 Stability studies of optimized nitazoxanide-loaded chitosan microbeads at different temperatures

particle size was observed on increasing the polymer concentration. This is a quadratic equation which involves the quantitative effect of independent variables (X_1 and X_2) and their interactions (coefficient with more than one-factor term; X_2^2, X_1X_2) on the response Y_1. Herein, X_1 has a positive coefficient which signifies that as the chitosan concentration increases, the particle size correspondingly upsurges, whereas interaction factors have a negative effect on particle size which can be correlated with the fact that insufficient amount of Na-TPP would be available for cross-linking with increase in chitosan concentration. High polymeric concentration will yield high viscosity solutions due to which cross-linker would be incapable to diffuse into the particles [34, 35]. It can also be observed from Fig. 5a that an increase in polymer concentration leads to increase in particle size up to a specific concentration. Furthermore, the model was verified by ANOVA and multiple correlation test (R^2), and their results are mentioned in Table 4. The p value and R^2 value for the Y1 response was found to be < 0.05 and 0.9985, respectively, which clearly demonstrates the independent variables had a considerable effect in response estimation. Moreover, the value of variance inflation factor (VIF) is 1 which is used to interpret multi-collinearity of independent factors. The result implies no multi-collinearity amongst the independent variables in this quadratic model (VIFs > 1 indicate multi-collinearity and VIFs less than 10 are tolerable. Likewise, from the coded equation 1 and Table 5, the value of coefficient of X_1 has a positive value, indicating its large impact on Y_1, while a negative coefficient value of X_2 indicates its inverse effect on Y_1. As per the CCD, the formulation (F5: 3% w/v X_1 and 2% w/v X_2) was very near to optimized formulation.

Percentage yield or product yields are studied to interpret the quantity of excipients utilized to obtain the product. It helps the manufacturer or researcher to closely observe the formulation economically. This dimensionless figure can be used to analyse the exact efficiency of excipients for the development of formulation in terms of improvement of yield, using minimum excipients, reduction of cost and waste [36]. The investigated lower range of percentage yield was found to be 78.00% ± 1.04 for the formulation F1 (2.75% w/v X_1 and 1.75% w/v X_2), while higher range for percentage yield was 92.82% ± 1.78 for the formulation F5 (3% w/v X_1 and 2% w/v X_2). From the coded equation 2 and Figs. 5 and 6b, it is established that both X_1 and X_2 have positive impact on percentage yield of beads. The result implies that as the concentration of chitosan and tripolyphosphate increases, the product yield also increases. Coded equation 2 showed a perfect fit to the dependent variable/response (Y_2) as the R^2 (0.9983) is in the accordance with the adjusted R^2 (0.9955). It can also be observed from Fig. 5b that an increase in polymer concentration leads to increase in percentage yield. The values of regression analysis of Y2 illustrated a positive sign for X_1 and X_2 while a negative sign for interaction factors, i.e. X_1X_2. This suggested that enhancement in chitosan and sodium tripolyphosphate concentration will considerably improve the product percentage yield. The result of $p < 0.05$ for independent variables after ANOVA (Table 4) analysis reveals significant effect on Y_2. F9 possessed a higher percentage yield which supports the above fact.

Entrapment efficiency of microbeads is reliant upon the extent and nature of ionic interaction between chitosan and TPP in terms of charge density and solution pH [37]. The calculated values of entrapment efficiency were lower (65.23% ± 0.034) for formulation F9 (3.25% w/v X_1 and 2.25% w/v X_2) while higher (91.87 ± 0.011%) for formulation F2 (2.75% w/v X_1 and 2% w/v X_2). Here, interaction factors are also dominant on Y3 along with X_1 and X_2. Coded polynomial equation 3 is a quadratic equation which is suitable for the response variable Y_3 as the difference between R^2 (0.9843) and adjusted R^2 (0.9580) is less than 0.2 as compiled in Table 4. It can also be observed from Fig. 5c that an increase in polymer concentration leads to an increase in entrapment efficiency. The values of regression analysis for Y_3 expressed a positive sign for chitosan, sodium tripolyphosphate and their interaction factors. This suggested that the high concentration of chitosan and NaTPP collectively has a synergistic effect on entrapment efficiency. High concentration of polymer and cross-linker will help to accommodate maximum quantity of drug within the polymeric matrix and cross-link tightly to avoid leakage of drug, respectively, in order to give maximum entrapment. Additionally, high TPP concentration increases the cross-linking junction points between TPP polyanion and chitosan.

The SEM photomicrographs of the drug-loaded microbeads and their surface morphology are shown in Fig. 8. Morphology of the drug-loaded chitosan microbeads was discrete and spherical in shape with a rough outer surface and visible large wrinkles.

The mucoadhesion of all prepared formulations was found to be in the range of 79.09 ± 0.21 to 91.23 ± 0.56% as shown in Fig. 9. The in vitro wash-off test showed that as the concentration of chitosan is increased in the formulation, its mucoadhesiveness also increases. But on further increasing the chitosan concentration, the mucoadhesiveness decreases. This may be attributed to the higher concentration of chitosan at which coiling of the polymer molecules may occur, which reduces the flexibility of the polymeric chain, thereby reducing the mucoadhesive strength [22].

The in vitro release of all formulations was found to be in the range of 73.05 ± 1.09 to 89.60 ± 1.10%, respectively, up to 24 h. The dissolution profiles of microbeads are

shown in Fig. 10. An initial burst release of drug was observed from all the microbead formulations which may be influenced by two reasons: the leaching of drug on the bead outer layer and faster entry of dissolution media inside the bead matrix and subsequent outer diffusion of drug. However, on changing the pH from acidic to alkaline level, the drug release was slowed down. The pH responsive release can best be explained based on charge density on the beads, which is an important factor in electrostatic interaction and depends on the pH of solution [27]. In SGF, protonation of phosphate ions causes hydrogen ions to break, leading to weaker electrostatic interaction. This caused higher swelling and release in pH 7.4 phosphate buffer than acid environment. The optimized formulation was selected and then coated with Eudragit S100 till 5% of weight gain.

The release of Eudragit S100 coated nitazoxanide microbeads was found to be 92.05% in the presence of rat caecal content at 37 °C ± 0.015 in pH 7.4 phosphate buffer as compared to without caecal content. After 2 h, a negligible amount of drug release in acidic environment was observed. The initial release of drug in phosphate buffer was found to be low. The release of drug in pH 7.4 phosphate buffer was very high due to the presence of enzymes. The Eudragit polymer contains carboxyl group; hence, ionization takes place at pH 7.4 due to which membrane coating gets dissolved and beads were exposed to dissolution media following which the polymer matrix swells and erodes releasing entrapped drug [29]. Additionally, eroding of coating membrane may also result due to bacterial presence in the dissolution media. The in vitro cumulative release of microbeads is shown in Fig. 11.

The stability study of optimized batch was carried out at different storage conditions, i.e. refrigerator, room temperature and 40 ± 2 °C/75 ± 5% RH for 60 days. The study displays that the microbeads were stable at refrigeration and room temperature, while slight degradation was observed at 40 ± 2 °C/75 ± 5% RH. This can be attributed to the fact that at accelerated conditions, chitosan gets degraded as it has the tendency to get swell at high humidity conditions. These stability study data for optimized formulation are given in Fig. 12.

Conclusion

The developed nitazoxanide-loaded chitosan microbeads were characterized considering every aspect of formulation, and from the results, it is concluded that the coating of chitosan microbeads with Eudragit S100 was efficacious in attaining a sustained release of drug with due course of time in simulated colonic media. Furthermore, CCD is being an efficient tool in the optimization of chitosan beads with minimum number of formulations. This model has adequately predicted the significant responses in terms of particle size, percentage yield and entrapment efficiency. The model graphs (3D surface plot, contour plots and predicted vs actual plots) and polynomial equations were utilized to study the effect of independent variables (polymer and cross-linking agent) on the dependent variables (particle size, percentage yield, entrapment efficiency). From the results, it is evident that independent variables revealed a significant effect on the measured responses ($p < 0.05$). Increase in chitosan concentration increases the particle size and yield but up to a certain level; after that a decrement was observed. The stability data revealed the lowest degradation was observed at 4° as per ICH guidelines. Thus, nitazoxanide-loaded microbeads can prove to be a potential pharmaceutical dosage form for sustained effect and targeted release.

Abbreviations

CCD: Central composite design; WHO: World Health Organization; STHs: Soil-transmitted helminths; NTZ: Nitazoxanide; TIZ: Tizoxanide; BCS: Biopharmaceutical Classification System; FT-IR: Fourier transform-infrared spectroscopy; PDAB: Para-dimethylaminobenzaldehyde; S.D.: Standard deviation; SGF: Simulated gastric fluid; NaTPP: Sodium tripolyphosphate; TPP: Tripolyphosphate; SEM: Scanning electron microscopy; RH: Relative humidity; DEE: Drug entrapment efficiency; PS: Particle size; ANOVA: Analysis of variance; VIF: Variance inflation factor

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Authors' contributions

The authors read and approved the final manuscript. CB: design of work and writing of the original draft. UN: interpretation of data. JN: interpretation of data. SJ: revision of the draft. NJ: conception and design of the work and review and editing of the draft

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Availability of data and materials

All data generated or analysed during this study are included in this published article. Any additional data could be available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors report no conflict of interest.

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