Carbohydrate Polymers 101 (2014) 220–230

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Development of calcium pectinate-tamarind seed polysaccharide mucoadhesive beads containing metformin HCl

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ARTICLE INFO

Article history:
Received 20 July 2013
Received in revised form 23 August 2013
Accepted 10 September 2013
Available online 19 September 2013

Keywords:
Tamarind seed polysaccharide
Pectin
Ionotropic-gelation
Mucoadhesion
Controlled drug release
Metformin HCl

ABSTRACT

Novel mucoadhesive beads containing metformin HCl made of low methoxy (LM) pectin-tamarind seed polysaccharide (TSP) polymer-blend was developed through ionotropic-gelation technique and optimized using 3^2 factorial design. Effects of LM pectin and TSP amounts on drug encapsulation efficiency (DEE), and cumulative drug release at 10 h (R_{10h}) were analyzed using response surface methodology. The optimized calcium pectinate-TSP beads containing metformin HCl showed DEE of 95.12 ± 4.26%, R_{10h} of 46.53 ± 3.28%, and mean diameter of 1.93 ± 0.26 mm. The *in vitro* drug release from these beads was followed controlled-release (zero-order) pattern with super case-II transport mechanism. These beads were also characterized by SEM and FTIR. The optimized beads also exhibited pH-dependent swelling, good mucoadhesivity with goat intestinal mucosa and significant hypoglycemic effect in alloxan-induced diabetic rats over prolonged period after oral administration.

1. Introduction

Polysaccharides are composed of many monosaccharidic residues that are joined one to the other by O-glycosidic linkages and have a large number of reactive groups varying in chemical composition with broad range of molecular weights; thus, contributing to their diversity to structures and properties (Kaur, Ahuja, Kumar, & Dilbaghi, 2012). Among them, natural polysaccharides exhibits some excellent properties, which make them the biopolymer group with the longest and widest biomedical applications, experience non-toxicity (monomer residues are not hazardous to health), high water solubility or swelling ability, stability to pH variations and a broad variety of chemical structures (Barbosa, Granja, & Barrias, 2005; Kaur, Yadav, Ahuja, & Dilbaghi, 2012; Manjana, Kumar, & Shivakumar, 2010). Recently, the development of natural polysaccharide-based multiple-unit drug delivery systems like nanoparticles, microspheres, microparticles, beads, *etc.*, for controlled drug release applications has been the focus of drug delivery research (Gaba, Singh, Gaba, & Gupta, 2011; Guru et al., 2013; Jana, Das, Nayak, Sen, & Basu, 2013; Jana, Saha, Nayak, Sen, & Basu, 2013; Kulkarni, Mangod, Mutalik, & Sa, 2011; Liu, Jiao, Wang, Zhou, & Zhang, 2008; Maiti et al., 2010; Maiti, Ranjit, Mondal, Ray, & Sa, 2011; Nayak & Pal, 2013b; Nayak, Pal, & Das, 2013; Nayak, Pal, Pradhan, & Hasnain, 2013).

Tamarind seed polysaccharide (TSP) is a water soluble natural polysaccharide, obtained from the endosperm of *Tamarindus indica* L. seeds (Kaur, Ahuja, et al., 2012; Kaur, Yadav, et al., 2012). TSP is composed of (1 → 4)-β-D-glucan backbone substituted with side chains of α-D-xylopyranose and β-D-galactopyranosyl (1→2)-α-D-xylopyranose linked (1→6) to glucose residues (Jana, Saha, et al., 2013). The glucose, xylose and galactose units are present in TSP in the ratio of 2.8:2.25:1 (Kaur, Yadav, et al., 2012). TSP is noncarcinogenic, biocompatible and stable enough even in acidic pH range (Pal & Nayak, 2012). It is used as binder, gelling agent, thickener, emulsifier, suspending agent and release modifier in different pharmaceutical formulations (Avachat, Dash, & Shrotriya, 2011; Deveswaran et al., 2009; Khanna, Dwivedi, & Singh, 1997; Kulkarni, Dwivedi, Sarin, & Singh, 1997; Kulkarni, Gowthmara, & Dhobe, 2002; Mishra & Khandare, 2011; Prajapati, Jani, Moradiya, & Randeria, 2013). Moreover, it finds its use in the development of various types of mucoadhesive drug delivery systems due to its hydrophilic and mucoadhesive properties (Dutta & Bandyopadhyay, 2006; Pal & Nayak, 2012; Nayak & Pal, 2013a). In previous literature, several investigations were reported by our research group on the use of TSP as polymer-blend with other natural polysaccharides to produce multiple-unit controlled drug release carriers (Jana, Saha, et al., 2013; Nayak & Pal, 2011, 2013a; Pal & Nayak, 2012). In most of the cases, sodium alginate was used as polymer-blend with TSP to produce TSP-based microspheres/beads

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http://dx.doi.org/10.1016/j.carbpol.2013.09.024

Table 1
Experimental plan of 32 full factorial design (coded values in bracket) with observed response values with bead size for different formulations of calcium pectinate-TSP beads containing metformin HCl (F—O).

Codes	Factors with normalized levels		Responses[a]		Bead diameter (mm)[b]
	LM pectin (mg, X_1)	TSP (mg, X_2)	DEE (%)	R_{10h} (%)	
F-1	650.00 (−1)	175.00 (−1)	68.80 ± 3.27	90.45 ± 4.26	1.52 ± 0.12
F-2	650.00 (−1)	200.00 (0)	70.75 ± 3.13	82.44 ± 4.18	1.58 ± 0.14
F-3	650.00 (−1)	225.00 (+1)	74.92 ± 3.44	73.32 ± 3.02	1.65 ± 0.20
F-4	700.00 (0)	175.00 (−1)	70.08 ± 2.93	88.86 ± 5.37	1.54 ± 0.22
F-5	700.00 (0)	200.00 (0)	72.72 ± 3.15	81.04 ± 3.44	1.70 ± 0.23
F-6	700.00 (0)	225.00 (+1)	77.53 ± 3.68	71.18 ± 3.07	1.75 ± 0.18
F-7	750.00 (+1)	175.00 (−1)	73.89 ± 2.97	81.02 ± 4.68	1.71 ± 0.12
F-8	750.00 (+1)	200.00 (0)	76.98 ± 4.01	70.90 ± 3.72	1.82 ± 0.20
F-9	750.00 (+1)	225.00 (+1)	82.84 ± 3.98	60.62 ± 3.02	1.88 ± 0.23
F—O	710.61	277.49	Observed values		1.93 ± 0.26
			95.12 ± 4.26	46.53 ± 3.28	
			Predicted values		
			96.95	45.00	
	% Error[c]		−1.89	3.40	

[a] Mean ± S.D., $n = 3$.
[b] Mean ± S.D., $n = 20$.
[c] Error (%) = (observed value − predicted value)/predicted value × 100.

through ionotropic-gelation technique (Nayak & Pal, 2011, 2013a; Pal & Nayak, 2012). To the best of our knowledge, there is still no report is available in the literature on the development of multiple-unit controlled release systems composed of TSP and pectin.

Pectin is an inexpensive, nontoxic, water soluble, natural polysaccharide extracted industrially from citrus peels, sugar beet roots, apple pomaces, *etc.*, and has been used as food additive, thickener and gelling agent (Munarin, Tanzi, & Petrini, 2012; Sriamornsak, Sungthongjeen, & Puttipipatkhachorn, 2007). Pectin consists mainly of linearly connected (1-4)-linked α-D-galacturonic acid residues interrupted by some rhamnogalacturonic acid residue and α-L-rhamnopyranose by α-1-2 linkage (Sharma & Ahuja, 2011). The galacturonic acid residue of the backbone is partially esterified. Usually, low methoxy (LM, <50% degree of esterification) pectins have more carboxylic groups, which are available to be ionotropically gelled cross-linked with divalent cations (*e.g.*, calcium, zinc, *etc.*) to produce rigid pectinate gels from the use as effective vehicles in drug delivery applications (Das, Ng, & Ho, 2010; Sriamornsak, Nunthanid, Cheewatanakornkool, & Manchun, 2010). During last few decades', various ionotropically gelled calcium pectinate gel beads as oral drug delivery matrices have been investigated (Assifoui, Chambin, & Cayot, 2011; Lee, Chung, & Lee, 2008; Lee, Kim, Chung, & Lee, 2009; Sriamornsak et al., 2010). However, these pectinate gel beads experienced with low drug encapsulation and premature release of encapsulated drugs (Lee et al., 2008, 2009). To limit these disadvantages, several modifications of calcium pectinate beads have been investigated by various research groups (Atyabi, Majzoob, Iman, Salehi, & Dorkoosh, 2005; Chakraborty et al., 2010; Desai, 2005; Sharma & Ahuja, 2011; Soares, de Castro, Cury, & Evangelista, 2013). In the current work, the efficiency of TSP, as mucoadhesive polymer-blend with LM pectin to develop ionotropically gelled calcium pectinate-TSP mucoadhesive beads for controlled metformin HCl release was investigated.

Metformin HCl is an oral antihyperglycemic drug, which is widely used in the treatment of non-insulin dependent diabetes mellitus (Type II) (Maji, Ray, Das, & Nayak, 2012). Its biological half-life is 1.5–1.6 h (Nayak, Pal, & Das, 2013; Nayak, Pal, Pradhan, & Hasnain, 2013). It shows dose-dependent and saturable transport with absorption window in the upper part of the intestine (Kumar & Ahuja, 2012). Its bioavailability is reported to be improved by gastric-retention (Kumar & Ahuja, 2012; Sharma & Bhattacharya, 2008). Therefore, the proposed mucoadhesive beads might facilitate an intimate contact with the absorbing surfaces of mucous membranes (*i.e.*, mucoadhesion) and thus, the gastric residence could be prolonged to deliver entrapped drug at a controlled rate at the absorption site to get optimal therapeutic effect. A 3^2 factorial optimization technique based on response surface methodology was employed to investigate the composition of polymer-blend (here, LM pectin and TSP) on the drug entrapment and drug release of the inotropically gelled pectinate-TSP mucoadhesive beads containing metformin HCl.

2. Experimental

2.1. Materials

Metformin HCl (Abhilash Chemicals Pvt. Ltd., India), LM pectin (MW ~30,000–100,000, Loba-chemie, India), calcium chloride (Merck Ltd., India) were used. TSP was isolated from the seeds of *T. indica* L. The procedure of TSP isolation has been described by Nayak and Pal (2011). All other chemicals and reagents were commercially available and of analytical grade.

2.2. Preparation of calcium pectinate-TSP beads containing metformin HCl

Calcium pectinate-TSP beads containing metformin HCl were prepared using calcium chloride ($CaCl_2$) as cross-linking agent by ionotropic-gelation technique. Briefly, required amounts of LM pectin and isolated TSP were dissolved in deionized water (20 ml) using magnetic stirring for 30 min. Afterwards, metformin HCl was added to the mixture solutions of LM pectin-TSP for each formulation maintaining polymer to drug ration 2:1, and mixed thoroughly using a homogenizer (Remi Motors, India). The final LM pectin-TSP mixture solutions containing metformin HCl were ultra-sonicated for 5 min for debubbling. The resulting dispersion was then added *via* a 21-gauge needle drop wise into 100 ml of 5% (w/v) $CaCl_2$ solution. Added droplets were retained in the $CaCl_2$ solution for 15 min to complete the curing reaction. The wet beads were collected by decantation. These wet beads were washed two times with distilled water and dried at 37 °C for overnight. The dried beads were stored in a desiccator until used.

2.3. Experimental design for optimization

To reduce the number of trials necessary to attain maximum numbers of information on product properties, the screening was performed applying a 3^2 factorial design. The amount of LM pectin

(X_1, 650–750 mg) and TSP (X_2, 100–200 mg) as polymeric blend were defined as the selected independent variables, which were varied at three levels, low level (−1), medium level (0), and high level (+1). The drug encapsulation efficiency (DEE, %), and cumulative drug release after 10 h (R_{10h}, %) were used as dependent variables (responses). Design-Expert® Version 8.0.6.1 software (Stat-Ease Inc., USA) was used for the generation and evaluation of the statistical experimental design. The matrix of the design including investigated responses *i.e.*, DEE (%), and R_{10h} (%) are shown in Table 1. For optimization, the effects of independent variables upon the responses were modeled using quadratic mathematical model generated by 3^2 factorial design (Malakar, Nayak, & Pal, 2012):

$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2$; where, Y is the response; b_0 is the intercept, and b_1, b_2, b_3, b_4, b_5 are regression coefficients. X_1 and X_2 are individual effects; X_1^2 and X_2^2 are quadratic effects; X_1X_2 is the interaction effect. One-way ANOVA was applied to estimate the significance of the model ($p < 0.05$).

2.4. Determination of DEE

100 mg of beads was taken and were crushed using pestle and mortar. The crushed powders of drug containing beads were placed in a 250 ml volumetric flask and the volume was made up to 250 ml by phosphate buffer, pH 7.4, and kept for 24 h with occasionally shaking at 37 ± 0.5 °C. After the stipulated time, the mixture was stirred at 500 rpm for 20 min using a magnetic stirrer (Remi Motors, India). The polymer debris formed after disintegration of bead was removed filtering through Whatman® filter paper (No. 40). The drug content in the filtrate was determined using a UV–vis spectrophotometer (Shimadzu, Japan) at 233 nm against appropriate blank. The DEE (%) of these prepared beads was calculated by the following formula (Nayak & Pal, 2013a):

$$\text{DEE}\ (\%) = \frac{\text{Actual drug content in beads}}{\text{Theoretical drug content in beads}} \times 100$$

2.5. Bead size measurement

Particle size of 100 dried beads from each batch was measured by optical microscopic method for average particle size using an optical microscope (Olympus). The ocular micrometer was previously calibrated by stage micrometer.

2.6. Scanning electron microscopy (SEM) analyses

Beads containing metformin HCl were gold coated by mounted on a brass stub using double-sided adhesive tape and under vacuum in an ion sputter with a thin layer of gold (3–5 nm) for 75 s and at 20 kV to make them electrically conductive and their morphology was examined by scanning electron microscope (ZEOL, JSM-5800, Japan).

2.7. Fourier transform-infrared (FTIR) spectroscopy analyses

Samples were reduced to powder and analyzed as KBr pellets by using a Fourier transform-infrared (FTIR) spectroscope (Perkin Elmer Spectrum RX I, USA). The pellet was placed in the sample holder. Spectral scanning was taken in the wavelength region between 3600, and 500 cm^{-1} at a resolution of 4 cm^{-1} with scan speed of 1 cm/s.

2.8. In vitro drug release studies

The release of metformin HCl from various calcium pectinate-TSP beads was tested using dissolution apparatus USP (Campbell Electronics, India). The baskets were covered with 100-mesh nylon cloth to prevent the escape of the beads. The dissolution rates were measured at 37 ± 1 °C under 50 rpm speed. Accurately weighed quantities of beads containing metformin HCl equivalent to 100 mg were added to 900 ml of 0.1 N HCl (pH 1.2). The test was carried out for 2 h and then continued in phosphate buffer (pH 7.4) for next 8 h. 5 ml of aliquots was collected at regular time intervals, and the same amount of fresh dissolution medium was replaced into dissolution vessel to maintain the sink condition throughout the experiment. The collected aliquots were filtered, and suitably diluted to determine the absorbance using a UV–vis spectrophotometer (Shimadzu, Japan) at 233 nm against appropriate blank.

2.9. Analysis of in vitro drug release kinetics and mechanism

In order to predict and correlate the *in vitro* drug release behavior from formulated various calcium pectinate-TSP beads containing metformin HCl it is necessary to fit into a suitable mathematical model. The *in vitro* drug release data were evaluated kinetically using various important mathematical models like zero-order, first-order, Higuchi, and Korsmeyer–Peppas models (Nayak, Das, & Maji, 2012).

Zero-order model: $Q = kt + Q_0$; where Q represents the drug released amount in time t, and Q_0 is the start value of Q; k is the rate constant.
First-order model: $Q = Q_0e^{kt}$; where Q represents the drug released amount in time t, and Q_0 is the start value of Q; k is the rate constant.
Higuchi model: $Q = kt^{0.5}$; where Q represents the drug released amount in time t, and k is the rate constant.
Korsmeyer–Peppas model: $Q = kt^n$; where Q represents the drug released amount in time t, k is the rate constant and n is the diffusional exponent, indicative of drug release mechanism. Again, The Korsmeyer–Peppas model was employed in the *in vitro* drug release behavior analysis of these formulations to distinguish between competing release mechanisms: Fickian release (diffusion-controlled release), non-Fickian release (anomalous transport), and case-II transport (relaxation-controlled release). When n is ≤0.43, it is Fickian release. The n value between 0.43 and 0.85 is defined as non-Fickian release. When, n is ≥0.85, it is case-II transport (Nayak & Pal, 2011).

2.10. Evaluation of swelling behavior

Swelling behavior evaluation of optimized calcium pectinate-TSP beads containing metformin HCl were carried out in two different aqueous media: 0.1 N HCl (pH 1.2), and phosphate buffer (pH 7.4). 100 mg beads were placed in vessels of dissolution apparatus (Campbell Electronics, India) containing 500 ml respective media. The experiment was carried out at 37 ± 1 °C under 50 rpm paddle speed. The swelled beads were removed at predetermined time interval and weighed after drying the surface by using tissue paper. Swelling index was determined using the following formula:

$$\text{Swelling index}\ (\%) = \frac{\text{Weight of beads after swelling} - \text{Dry weight of beads}}{\text{Dry weight of beads}} \times 100$$

2.11. Ex vivo mucoadhesion testing

The mucoadhesive property of optimized calcium pectinate-TSP beads containing metformin HCl was evaluated by *ex vivo* wash-off method (Pal & Nayak, 2011). Freshly excised pieces of goat intestinal mucosa (2 cm × 2 cm) (collected from slaughterhouse) were

mounted on glass slide (7.5 cm × 2.5 cm) using thread. About 50 beads were spread onto the wet tissue specimen, and the prepared slide was hung onto a groove of disintegration test apparatus. The tissue specimen was given a regular up and down movement in a vessel containing 900 ml of 0.1 N HCl (pH 1.2) and phosphate buffer (pH 7.4), separately, at 37 ± 0.5 °C. After regular time intervals, the machine was stopped and the number of beads still adhering to the tissue was counted.

2.12. Pharmacodynamic evaluation

In vivo pharmacodynamic studies were performed in alloxan-induced diabetic male albino rats of either sex (weighing 315–340 g). The acclimatized rats were kept fasting for 24 h with water *ad libitum*. All experiments were performed between 8 AM and 12 PM to minimize circadian influences. The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethical Committee and was cleared before starting. The experimental animals were handled as per guidelines of committee for the purpose of control and supervision on experimental animals (CPCSEA). All efforts were made to minimize both the suffering and number of animals used.

The male albino rats were made diabetic by intraperitoneal administration of freshly prepared alloxan solution at a dose of 150 mg/kg dissolved in 2 mM citrate buffer (pH 3.0). After one week of alloxan administration, alloxanized rats with fasting blood glucose of 300 mg/dl or more were considered diabetic and were employed in the study for 10 h. After initial collection of blood samples from the alloxan-induced diabetic rats, they were divided randomly into 2 groups of 6 rats each and treated as follow: Group A was administered with pure metformin HCl (100 mg/kg body weight) in suspension form and Group B were administered with optimized calcium pectinate-TSP beads containing metformin HCl, both at a dose equivalent to 100 mg metformin HCl/kg body weight using oral feeding needle. Blood samples were withdrawn (0.1 ml) from tail tip of each rat at regular time intervals under mild ether anesthesia, and were analyzed for blood glucose by oxidase–peroxidase method using commercial glucose kit. Comparative *in vivo* blood glucose level in alloxan-induced diabetic rats after oral administration of pure metformin HCl and optimized calcium pectinate-TSP beads containing metformin HCl were evaluated.

2.13. Statistical analysis

Statistical optimization was performed using Design-Expert® Version 8.0.6.1 software (Stat-Ease Inc., USA). All measured data are expressed as mean ± standard deviation (S.D.). Each measurement was done in triplicate ($n = 3$). The *in vivo* data was tested for significant differences ($p < 0.05$) by paired samples *t*-test. All other data was analyzed with simple statistics. The simple statistical analysis and paired samples *t*-test were conducted using MedCalc software, version 11.6.1.0.

3. Results and discussion

3.1. Preparation of calcium pectinate-TSP beads containing metformin HCl

Currently, polymer-blending is a familiar practice to achieve desired functional properties like gel strength, swelling, mucoadhesivity and sustained drug release in both biomedical and pharmaceutical applications (Chakraborty et al., 2010; Desai, 2005; Mishra, Datt, & Banthia, 2008; Pal & Nayak, 2012; Soares et al., 2013). Blending of ionic polysaccharides with other polymers improves functional properties such as gel strength, stability, swelling, drug encapsulation and desired sustained drug release (Ahuja, Yadav, & Kumar, 2010; Hua, Ma, Li, Yang, & Wang, 2010; Pal & Nayak, 2011). Blending of mucoadhesive polymers with other polymers can also improve mucoadhesivity of various systems for the use in the development of mucoadhesive drug delivery (Nayak & Pal, 2013a, 2013b; Nayak, Pal, & Das, 2013; Nayak, Pal, Pradhan, et al., 2013; Pal & Nayak, 2011, 2012). The blends of pectin and other biocompatible second polymers have also been employed in some investigations for the development of ionotropically gelled calcium pectinate-based beads to prevent premature release of incorporated small molecular drugs (Atyabi et al., 2005; Chakraborty et al., 2010; Desai, 2005; Nayak, Pal, & Das, 2013; Soares et al., 2013). In the present investigation, calcium pectinate-TSP beads containing metformin HCl were prepared by ionotropic-gelation using $CaCl_2$ as cross-linker.

The interactions between the negative charged carboxylic groups of LM pectin-backbone and the positive charged divalent cross-linking cations (here Ca^{2+}) induces the formation of so-called "Egg-Box" model structure, even though it slightly differs from the "Egg-Box" model originally defined for alginates (Fang et al., 2008; Munarin et al., 2012). According to the "Egg-Box" model originally hypothesized for LM pectin, there is an initial dimerization step of two homogeneous galacturonic chains by cooperative bridging of parallel facing chains through Ca^{2+} ions. Cooperatively, it is possible because homogalacturonic chains are relatively rigid and binding of a first Ca^{2+} ion by two pectin chain facilitates their alignment with respect to each other, which in turn allows easier binding in a next chain ion, and so on along the sequence (Axelos & Thibault, 1991; Renard & Jarvis, 1999). The anti-parallel orientation of the two chains seems to be the most favorable arrangement, and in addition to electrostatic interactions, this initial dimer association is strongly stabilized by hydrogen bonding. Subsequent Ca^{2+}-induced aggregation of the "Egg-Box" dimmers in tetramers, hexamers, *etc.* can occur, but these subsequent associations of dimmers have no particular specificity and seem to be merely governed by electrostatic interactions (Munarin et al., 2012). The multimers are therefore easily disrupted by competing monovalent ions, which is initially formed chain dimmers are not. Thus, various rigid and spherical calcium pectinate-TSP beads containing metformin HCl were formulated due to electrostatic interaction between negatively charged pectin and positively charged Ca^{2+} ion, when the mixtures of LM pectin-TSP blends containing metformin HCl was dropped into the $CaCl_2$ solutions.

3.2. Optimization

The conventional method of pharmaceutical optimization based on changing one variable at a time while keeping others fixed is laborious and time consuming (Nayak et al., 2012). In contrast, design of experiment is the methodology of systemically determining the relationship between variables affecting the formulation and/or process and output of that formulation and/or process (Li et al., 2011). Among various statistical experimental design for optimization, factorial design is a design by which the factors all factors involved in a process are studied in all possible combinations by analyzing the influence of individual variables and their interactions using minimum experiments (Malakar & Nayak, 2012; Malakar, Nayak, & Pal, 2012). Based on the factorial design experiment, the optimization technique encompasses factorial design experiment that will reliably measure responses, generating mathematical model equations using experimental data, conducting appropriate statistical tests to select best possible model and determining the values of independent variables to produce optimum responses (Guru, Nayak, & Sahu, 2013).

For the 3^2 factorial design, 9 trial formulations were suggested by Design-Expert® Version 8.0.6.1 software (Stat-Ease Inc., USA) for

Table 2
Summary of ANOVA for response parameters.

Source	Sum of square	d.f.[a]	Mean square	*F*-Value	*p*-Value prob > *F*
For DEE (%)[b]					
Model	154.21	5	30.84	2351.24	<0.0001 (S)
X_1	61.70	1	61.70	4703.32	<0.0001 (S)
X_2	84.53	1	84.53	6443.40	<0.0001 (S)
X_1X_2	2.00	1	2.00	152.64	0.0011 (S)
X_1^2	3.14	1	3.14	239.50	0.0006 (S)
X_2^2	2.85	1	2.85	217.12	0.0007 (S)
For R_{10h} (%)					
Model	730.70	5	146.14	638.21	<0.0001 (S)
X_1	188.94	1	188.94	825.14	<0.0001 (S)
X_2	508.02	1	508.02	2218.60	<0.0001 (S)
X_1X_2	2.67	1	2.67	11.67	0.0419 (S)
X_1^2	30.45	1	30.45	132.96	0.0014 (S)
X_2^2	0.61	1	0.61	2.66	0.2015 (NS)

X_1 and X_2 represent the main effects (factors) – the amounts of LM pectin and TSP (in mg), respectively.
X_1^2 and X_2^2 are the quadratic effect; X_1X_2 is the interaction effect.
S and NS indicate significant and non significant, respectively.
[a] d.f. indicates degree of freedom.

two independent variables: amount of LM pectin (X_1, 650–750 mg) and TSP (X_2, 175–225 mg) as polymeric blend, which were varied at three levels: low level (−1), medium level (0), and high level (+1). The DEE (%), and R_{10h} (%) were evaluated as dependent variables (responses). Design-Expert® Version 8.0.6.1 software (Stat-Ease Inc., USA) was used for the generation and evaluation of the statistical experimental design. According to this trial plan, calcium pectinate-TSP beads containing metformin HCl were prepared by ionotropic-gelation technique. Overview of matrix of the design including investigated responses (*i.e.*, DEE and R_{10h}) is presented in Table 1. The results of the ANOVA indicated that the investigated models were significant for all response parameters (Table 2). The values of investigated responses measured for all trial formulations were fitted in the 3^2 factorial design to get model equations for responses analyzed in this investigation.

The model equation relating DEE (%) became:

$$\text{DEE}\ (\%) = 398.99 - 0.75X_1 - 1.01X_2 + 5.66 \times 10^{-4}X_1X_2 + 5.01 \times 10^{-4}X_1^2 + 1.91 \times 10^{-3}X_2^2$$

$$[R^2 = 0.9997;\quad F = 2351.24;\quad p < 0.05]$$

The model equation relating R_{10h} (%) became:

$$R_{10h}\ (\%) = -658.69 + 2.20X_1 + 0.44X_2 - 6.54 \times 10^{-4}X_1X_2 - 1.56 \times 10^{-3}X_1^2 - 8.83 \times 10^{-5}X_2^2$$

$$[R^2 = 0.9991;\quad F = 638.21;\quad p < 0.05]$$

Model simplification was carried out by eliminating non-significant terms ($p > 0.05$) in model equations resulting from the multiple regression analysis (Nayak & Pal, 2011), giving:

$$\text{DEE}\ (\%) = 398.99 - 0.75X_1 - 1.01X_2 + 5.66 \times 10^{-4}X_1X_2 + 5.01 \times 10^{-4}X_1^2 + 1.91 \times 10^{-3}X_2^2$$

$$R_{10h}\ (\%) = -658.69 + 2.20X_1 + 0.44X_2 - 6.54 \times 10^{-4}X_1X_2 - 1.56 \times 10^{-3}X_1^2$$

The influences of main effects (factors) on responses (here, DEE, and R_{10h}) were further elucidated by response surface methodology (RSM). RSM is a widely proficient approach in the development and optimization of drug delivery devices (Lee et al., 2008; Nayak & Pal, 2011). The purpose of RSM is to understand a model as fully as possible the effect of factors and their levels, over the whole of the experimental domain, and to predict the response inside the domain. Moreover, it can be used for optimizing a formula (*i.e.* maximizing one or more of the responses, keeping the formulation variable setting within a satisfactory range), carrying out simulations with the model equations and plotting the responses. The three-dimensional response surface graph is very useful in learning about the main and interaction effects of the independent variables (factors), whereas two-dimensional contour plot gives a visual representation of values of the response (Malakar, Nayak, & Goswami, 2012; Malakar, Nayak, & Pal, 2012). The three-dimensional response surface plots (Fig. 1a and b) and corresponding contour plots (Fig. 1c and d) were presented to estimate the effects of the independent variables (factors) on each response investigated. The three-dimensional response surface graph relating DEE (Fig. 1a) indicates the increment of DEE with the increasing of LM pectin amount (X_1), and TSP amount (X_2) in the formulated calcium pectinate-TSP beads containing metformin HCl prepared by ionotropic gelation technique. However, a decrease in R_{10h} values with the increasing LM pectin amount (X_1), and TSP amount (X_2) is indicated by the three-dimensional response surface graph relating R_{10h} (Fig. 1b). All of the contour plots relating measured responses (Fig. 2a and b) indicate nonlinear relationships between two independable variables (here, LM pectin amount, X_1 and TSP amount, X_2) on all measured responses, investigated in this study.

A numerical optimization technique using the desirability approach was employed to develop new formulations with desired response (optimum quality). The desirable ranges of the undependable variables (factors) were restricted to $650 \le X_1 \le 700$ mg, and $250 \le X_2 \le 400$ mg; whereas the desirable ranges of responses were restricted to $95 \le \text{DEE} \le 100\%$, and $45 \le R_{10h} \le 50\%$. The optimal values of responses were obtained by numerical analysis using the Design-Expert® 8.0.6.1 software based on the criterion of desirability. The desirability plot indicating desirable regression ranges for optimal process variable settings and overlay plot indicating the region of optimal process variable settings was presented in Fig. 1e and f, respectively. In order to evaluate the optimization capability of the mathematical models generated according to the results of 3^2 factorial design, optimized calcium pectinate-TSP beads containing metformin HCl were prepared using one of the optimal process variable settings proposed by the design ($R^2 = 1$). The selected optimal process variable setting used for the formulation of optimized formulation was $X_1 = 710.61$ mg and $X_2 = 277.49$ mg. Optimized calcium pectinate-TSP beads containing metformin HCl (F—O) were

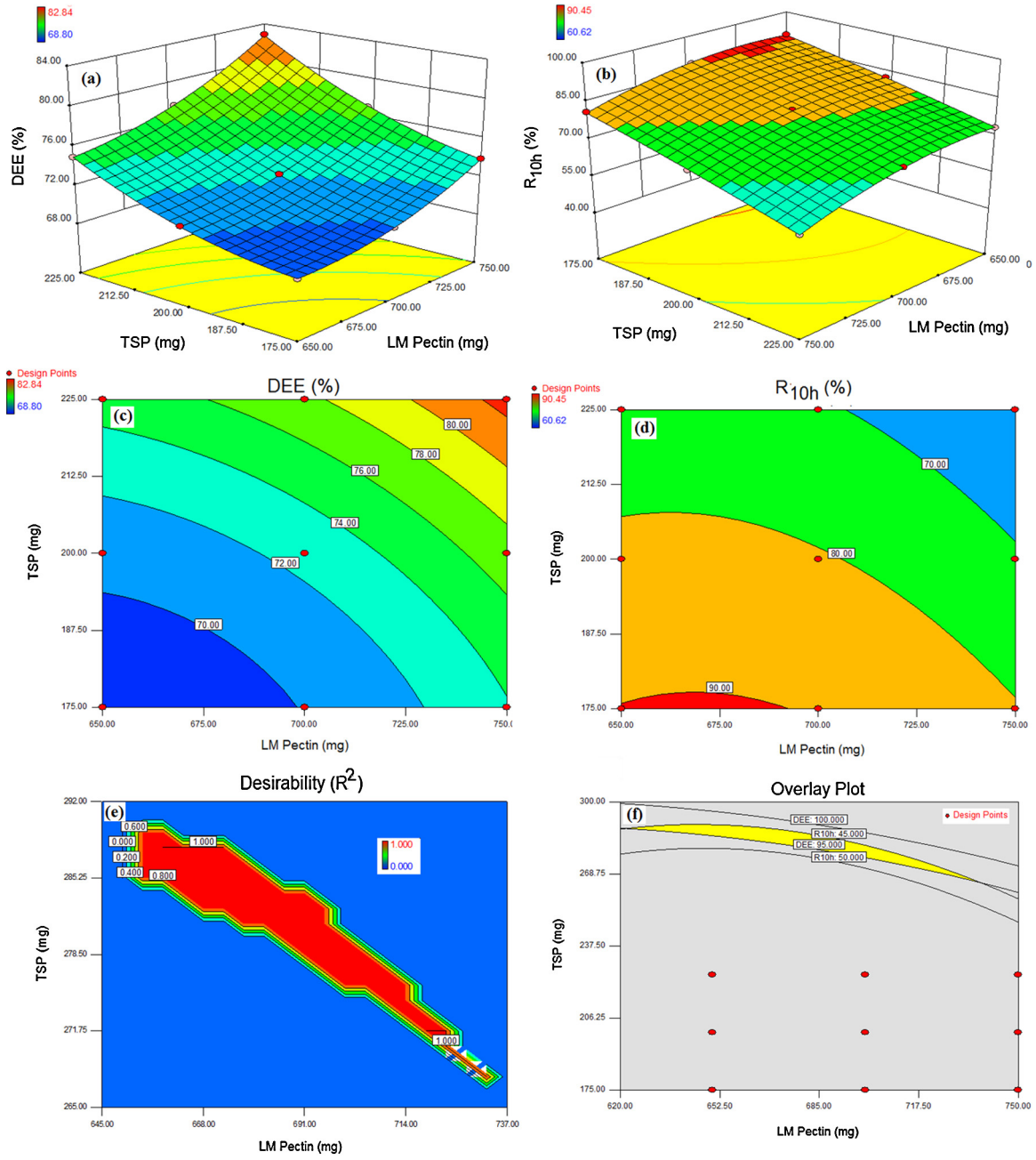

Fig. 1. Three-dimensional response surface plots showing the effects of amount of LM pectin (mg) and TSP (mg) on (a) DEE (%) and (b) R_{10h} (%); two-dimensional corresponding contour plots showing the effects of amount of pectin (mg) and TSP (mg) (c) DEE (%) and (d) R_{6h} (%); the desirability (R^2) plot (c) indicating desirable regression ranges and the overlay plot (d) indicating the region of optimal process variable settings.

evaluated for DEE (%) and R_{10h} (%). The optimized beads containing metformin HCl (F—O) showed DEE of 95.12 ± 4.26% and R_{10h} of 46.53 ± 3.28% with very small percentage error-values (−1.89 and 3.40, respectively) (Table 1). The very small percentage error values indicate that mathematical models obtained from the full 3^2 factorial design were well fitted.

3.3. DEE

The DEE (%) of all these calcium pectinate-FSM beads containing metformin HCl ranged 68.80 ± 3.27 to 95.12 ± 4.26% (w/w) (Table 1). Both pectin and TSP amount used as polymer-blend significantly influenced DEE (%) ($p < 0.05$). It was also observed that the drug encapsulation in these beads containing metformin HCl was increased with the increasing of both LM pectin and TSP amount used as polymer-blend. The increased DEE with the increasing amount of both LM pectin and TSP in these newly developed beads could be due to the increase in viscosity of the polymer-blend solutions with the increasing amount of polymer addition. This might have been prevented drug leaching from the prepared beads to the cross-linking solution. In addition, the increasing amount of pectin in polymer-blend solution might have been elevated the cross-linking by $CaCl_2$ through availing more numbers anionic of sites of pectin molecules for ionic cross-linking by Ca^{2+} ions. This phenomenon might prevent leakage of the drug into the cross-linking solution during the preparation process.

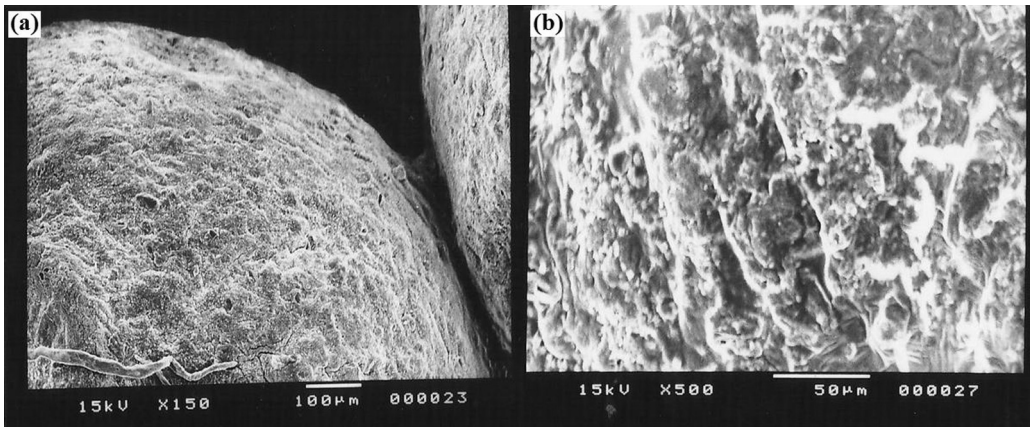

Fig. 2. SEM photograph of the optimized calcium pectinate-TSP beads containing metformin HCl prepared through ionotropic gelation (F—O).

3.4. Bead size

The average size of these formulated dried beads containing metformin HCl ranged from 1.52 ± 0.12 to 1.93 ± 0.26 mm (Table 1). Increasing the bead size was found with the increasing amount of the polymers, LM pectin and TSP into bead formulations containing metformin HCl. This might be explained by an increase in the viscosity of the polymer-blend (LM pectin and TSP) solutions with incorporation of both the polymers in increasing ratio, which therefore flowed more slowly out of the needle. Consequently, the drops and then the beads had a larger diameter. In addition, the increment average bead size was found proportional with the increasing of DEE.

3.5. SEM analyses

The morphological analysis of the optimized calcium pectinate-TSP beads containing metformin HCl (F—O) was visualized by SEM and is presented in Fig. 2. The SEM photograph of the bead morphology at lower magnification (150×) showed that these beads were spherical in shape with a rough surface, which was also noticed even at higher magnification (500×). Detailed examination of the bead surface topography revealed cracks and wrinkles, which might be caused by partly collapsing the polymeric gel network during drying (Malakar, Nayak, Pal, & Jana, 2013). However, polymeric derbies were seen on the bead surface, which could be due to the method of preparation (*i.e.*, simultaneous gel bead preparation and formation of the polymer-blend matrix).

3.6. FTIR spectroscopy analyses

FTIR spectroscopic analysis was performed to confirm the compatibility of metformin HCl with LM pectin and TSP used to prepare ionotropically gelled calcium pectinate-TSP beads containing metformin HCl. The FTIR spectra of LM pectin, TSP, calcium pectinate-TSP blank beads, calcium pectinate-TSP beads containing metformin HCl, and metformin HCl are shown in Fig. 3. The FTIR spectra of LM pectin showed principal absorption peaks at 3400.50 cm^{-1} and 2933.00 cm^{-1} that were due to —OH and —CH stretching vibration peaks. The peaks at 1459.27 cm^{-1} and 1356.45 cm^{-1} could be assigned to CH_2 and —OH bending vibration peaks respectively. In the FTIR spectrum of TSP, the characteristic peaks showed principal peaks at 1043.06 cm^{-1}, 1650.37 cm^{-1}, 2925.55 cm^{-1}, and 3403.48 cm^{-1} for —HC=O stretching vibration, —CH—OH stretching vibration, aliphatic C—H stretching, and for the OH groups, respectively. The FTIR spectrum of calcium pectinate-TSP blank beads showed all characteristic peaks of both LM pectin and TSP without any significant interaction. In the FTIR spectrum of metformin HCl, the principal absorption peaks appeared at 3169 cm^{-1} due to the N—H stretching of the primary amine group (—NH_2) and at 1063 cm^{-1} due to C—N stretching, and a peak at 1584 cm^{-1} occurs due to N—H bending vibrations of the primary amine group. In the FTIR spectrum of calcium pectinate-TSP beads containing metformin HCl, various characteristic peaks of LM pectin, TSP, and metformin HCl were appeared without any significant shifting. In short, the calcium pectinate-TSP beads containing metformin HCl had significant characters of metformin HCl in the FTIR spectrum, suggesting absence of any interaction between the drug, metformin HCl and the polymers used (LM pectin and TSP).

3.7. In vitro drug release

The *in vitro* drug release behavior of these newly developed ionotropically gelled calcium pectinate-TSP beads containing metformin HCl showed prolonged metformin HCl release over 10 h (Fig. 4). Metformin HCl release from these beads in the acidic medium was slow (less than 15% after 2 h) due to compactness

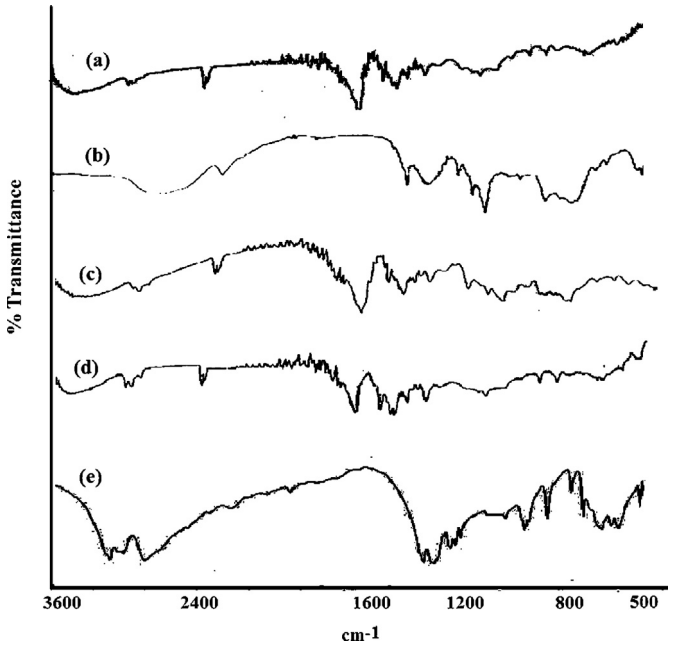

Fig. 3. FTIR spectra of (a) pectin, (b) isolated TSP, (c) calcium pectinate-TSP blank bead, (d) optimized calcium pectinate-TSP beads containing metformin HCl (F—O), and (e) pure metformin HCl.

Table 3
Results of curve fitting of the *in vitro* metformin HCl release data from various calcium pectinate-TSP beads containing metformin HCl.

Formulation code	Correlation coefficient (R^2)				Release exponent (n)
	Zero order	First order	Higuchi	Korsmeyer–Peppas	
F-1	0.9958	0.9015	0.6072	0.9906	1.02
F-2	0.9962	0.9126	0.5273	0.9923	1.11
F-3	0.9950	0.9152	0.6334	0.9944	1.10
F-4	0.9955	0.8937	0.5554	0.9893	1.11
F-5	0.9966	0.8998	0.5519	0.9918	1.09
F-6	0.9927	0.9010	0.5751	0.9929	1.10
F-7	0.9975	0.9035	0.5694	0.9930	1.05
F-8	0.9930	0.9146	0.5322	0.9949	1.10
F-9	0.9964	0.9122	0.5862	0.9923	1.12
F—O	0.9980	0.9117	0.5320	0.9944	1.12

of the gel-network through the shrinkage of pectinate gel at acidic pH. The trace amount of drug release could probably be due to the presence of drug crystals onto bead surface. After that, faster drug release was observed in phosphate buffer (pH 7.4) comparatively, probably due to the higher swelling rate of these beads in phosphate buffer. However, there was an increase in the amount of drug release with time. The cumulative percentage drug released from calcium pectinate-TSP beads containing metformin HCl after 10 h (R_{10h}, %) was within the range of 46.53 ± 3.28–90.45 ± 4.26%. The results of the drug release study indicated a decrease in drug release with the increasing of polymer amounts. In case of beads containing higher polymer contents, the more hydrophilic property of the polymers could probably binds better with water to form viscous gel structure, which might blockade the pores on the surface of beads and could delayed the drug release from these formulated beads (Nayak, Pal, & Das, 2013). The another reasonable explanation of the delayed drug release can be attributed to the increase in densities of the polymer matrix resulting in larger beads size and this in turn increase the diffusional path-length (Mishra et al., 2003).

The kinetics of drug release from the drug delivery devices is important because they influence the drug bioavailability, dosage intervals and the occurrence of toxic or untoward side effects (Odeku, Okunlola, & Lamprecht, 2013). The *in vitro* drug release data from various calcium pectinate-TSP beads containing metformin HCl were evaluated kinetically using various mathematical models like zero order, first order, Higuchi, and Korsmeyer–Peppas model. The results of the curve-fitting into these above-mentioned mathematical models are given in Table 3. When respective correlation coefficients of these beads were compared, the metformin HCl release from these beads was found to follow zero order model (R^2 = 0.9927–0.9980) over a period of 10 h. In addition, Korsmeyer–Peppas model (R^2 = 0.9893–0.9944) was found to be closer to the best-fit zero order model. The best-fit of zero order model indicated that the drug release from these beads followed controlled-release pattern. The values of release exponent (n) determined from *in vitro* drug release data of various calcium pectinate-TSP beads containing metformin HCl ranged from 1.02 to 1.12. This result indicates that the drug release from these beads followed the super case-II transport mechanism controlled by swelling and relaxation of polymeric-blend (calcium pectinate-TSP) matrix. Thus, a pronounced acceleration in drug release by the polymer-blend matrix was evidenced toward the latter stages of drug release, resulting in a more rapid relaxation-controlled transport (Odeku et al., 2013). This could be attributed due to polymer dissolution and enlargement or relaxation of polymeric chain. However, the release kinetic result is consistent with previous reports on release kinetics of various ionotropically gelled natural polymeric-blend beads containing metformin HCl (Nayak & Pal, 2013a, 2013b; Nayak, Pal, & Das, 2013; Nayak, Pal, Pradhan, et al., 2013).

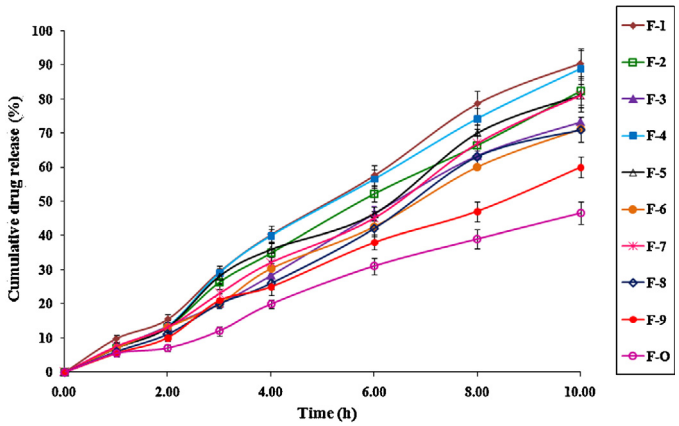

Fig. 4. *In vitro* drug release from various calcium pectinate-TSP beads containing metformin HCl prepared through ionotropic-gelation [mean ± S.D., n = 3].

3.8. Swelling behavior

The swelling index of optimized calcium pectinate-TSP beads containing metformin HCl was found lower in 0.1 N HCl, pH 1.2 in comparison with that of in phosphate buffer, pH 7.4, initially (Fig. 5a). This was occurred due to shrinkage of pectinate at acidic pH. Maximum swelling of these beads was noticed at 2–3 h in phosphate buffer, pH 7.4 and after which, erosion and dissolution took place. The swelling behavior of these beads containing metformin HCl in phosphate buffer, pH 7.4 could be explained by the ion exchanging between Ca^{2+} ions of the ionically cross-liked beads and the Na^+ ions present in phosphate buffer with the influence of Ca^{2+}-sequestrant phosphate ions. This could result in disaggregation in the calcium pectinate-TSP matrix structure leading to matrix erosion and dissolution of the swollen beads. The slow erosion of these beads could occur through slight degradation of polymeric-backbone into smaller molecular weight component. The pH-sensitive swelling properties of these beads might be suitable for intestinal delivery of encapsulated metformin HCl as these beads were found to get rapidly dissolved in the higher pH prevailing in the intestine.

3.9. Mucoadhesivity

The results of *ex vivo* wash-off test of optimized calcium pectinate-TSP beads containing metformin HCl using goat intestinal mucosa in gastric pH (0.1 N HCl, pH 1.2) and intestinal pH (phosphate buffer, pH 7.4) are presented in Fig. 5b. The *ex vivo* wash off of these newly developed beads showed that the percentage of beads attached to the intestinal mucosa at gastric pH was more than at intestinal pH. The less percentage of beads attached

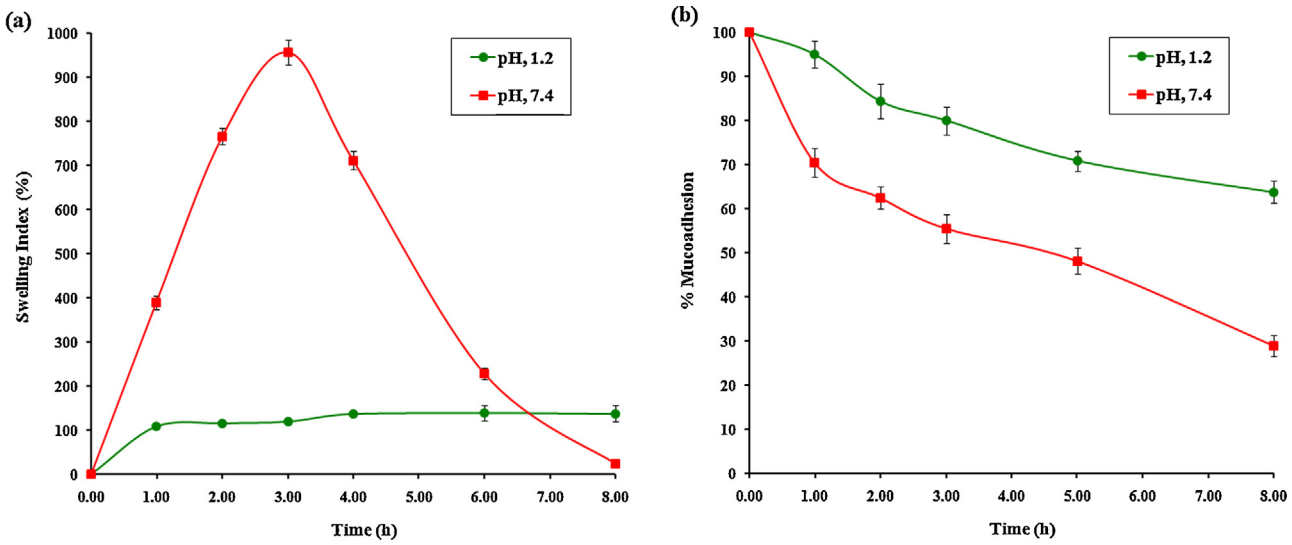

Fig. 5. (a) Swelling behavior of optimized calcium pectinate-TSP beads containing metformin HCl in 0.1 N HCl (pH 1.2), and phosphate buffer (pH 7.4) [mean ± S.D., $n = 3$]; (b) mucoadhesive behavior of optimized calcium pectinate-TSP beads containing metformin HCl in 0.1 N HCl (pH 1.2), and phosphate buffer (pH 7.4) [mean ± S.D., $n = 3$].

to the intestinal mucosa at intestinal pH could be due to ionization of carboxyl and other functional groups of polymers, which increased their solubility with reduced adhesive strength. Therefore, the results of the wash off test confirmed that the developed optimized calcium pectinate-TSP beads containing metformin HCl had good mucoadhesivity. The mucoadhesive property of these beads could be attributed to the presence of —OH groups of both the polymers used as polymer-blend, which form hydrogen bonds with mucus molecules. However, hydrophilic polymers like LM pectin and TSP also have the ability to form non-covalent bonds like van der Waal's forces or ionic interactions, resulting in mucoadhesion.

3.10. Pharmacodynamic evaluation

The comparative *in vivo* blood glucose level and the mean percentage reduction in blood glucose level in alloxan-induced diabetic rats after oral administration of pure metformin HCl and optimized calcium pectinate-TSP mucoadhesive beads containing metformin HCl is presented in Fig. 6 (a and b, respectively). In case of the group treated with pure metformin HCl (Group A), a rapid reduction in blood glucose level was observed within 3 h of administration, and after that, the blood glucose level recovered rapidly toward the normal level. In case of the group (Group B) treated with optimized mucoadhesive beads containing metformin HCl, the reduction in blood glucose level was found slower than that of the group treated with pure metformin HCl (Group A) up to 3 h. Significant differences ($p < 0.05$) were found between the blood glucose level after administration of pure metformin HCl and optimized calcium pectinate-TSP mucoadhesive beads containing metformin HCl (F—O) at each time-point measured. However, the reductions in glucose level were increased gradually with the increment of time in case of Group B (treated with optimized calcium pectinate-TSP mucoadhesive beads containing metformin HCl), and were sustained over 10 h. A 25% reduction in glucose level is considered a significant hypoglycemic effect (Pal & Nayak, 2012). Therefore, the significant hypoglycemic effect by the calcium pectinate-TSP mucoadhesive beads containing metformin HCl (F—O) was observed over 10 h. The results clearly demonstrated that the ability of the developed calcium pectinate-TSP mucoadhesive beads containing metformin HCl to maintain tight blood glucose level and improved patient compliance by enhancing, controlling and prolonging systemic absorption of metformin HCl.

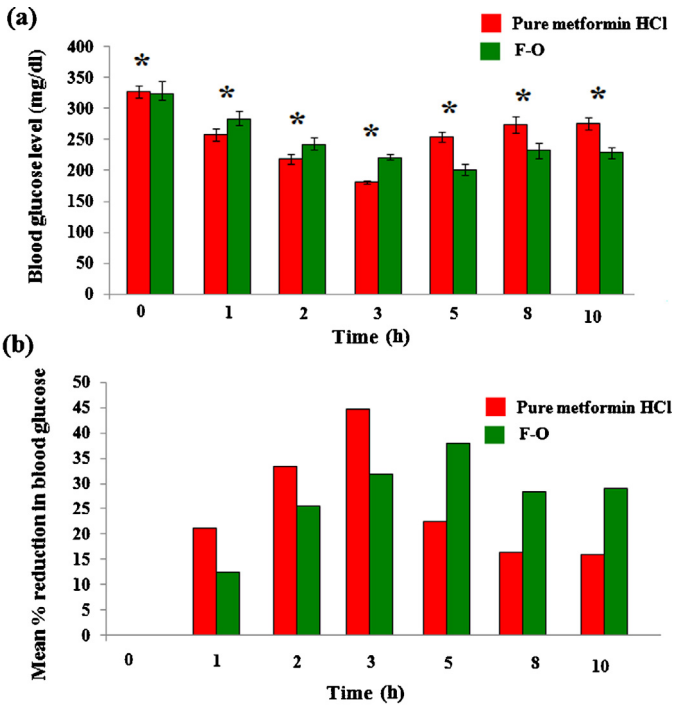

Fig. 6. (a) Comparative *in vivo* blood glucose level in alloxan-induced diabetic rats after oral administration of pure metformin HCl (standard) and optimized calcium pectinate-TSP beads containing metformin HCl (F—O) [mean ± S.D., $n = 6$]. The data were analyzed for significant differences (*$p < 0.05$) by paired samples t-test. The statistical analysis was conducted using MedCalc software version 11.6.1.0; (b) comparative *in vivo* mean percentage reduction in blood glucose level in alloxan-induced diabetic rats after oral administration of pure metformin HCl (standard) and optimized calcium pectinate-TSP beads containing metformin HC (F—O).

4. Conclusion

In this investigation, novel mucoadhesive beads containing metformin HCl made of LM pectin-TSP polymer-blend was developed through ionotropic-gelation technique and optimized. The optimized calcium pectinate-TSP mucoadhesive beads containing metformin HCl displayed high drug encapsulation, good mucoadhesivity with the biological membrane, suitable controlled *in vitro* drug release pattern and also significant hypoglycemic activity

in alloxan-induced diabetic rats over prolonged period after oral administration. The results of this investigation suggested that the optimized calcium pectinate-TSP mucoadhesive beads containing metformin HCl swelled slowly in the stomach and accordingly adhered to the stomach mucosa allowing more drug to be absorbed minimizing the diffusion barriers to increase the absorption period by prolonging the gastric residence time. Then, these beads were subsequently moved to the upper part of the intestine, where they swelled more and released drug through the polymeric gel layer, formed at the matrix-periphery. Thus, these newly developed calcium pectinate-TSP mucoadhesive beads containing metformin HCl could possibly be lucrative in terms of prolonged systemic absorption of metformin HCl maintaining tight blood glucose level and advanced patient compliance.

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