Send Orders for Reprints to reprints@benthamscience.ae

Current Drug Delivery, 2016, *13*, 1021-1033

RESEARCH ARTICLE

Preparation and Optimization of Moxifloxacin Microspheres for Colon Targeted Delivery Using Quality by Design Approach: *In Vitro* and *In Vivo* Study

Bhumika Prajapati[1], Prasant Kumar Jena[2], Tejal Mehta[3] and Sriram Seshadri[*]

[1]*Institute of Science, Nirma University, Ahmedabad, Gujarat, India 382481;* [2]*Department of Medical Pathology and Laboratory Medicine, Davis Medical Centre, University of California, Sacramento, California 95817;* [3]*Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India 382481*


S. Seshadri

ARTICLEHISTORY

Received: January 07, 2016
Revised: March 07, 2016
Accepted: April 25, 2016

DOI: 10.2174/1567201813666160512145625

Abstract: ***Background***: Gut microbiota has a significant role in the pathogenesis of diabetes. Colonic microflora modulation using an antibiotic might have an emerging role to treat the metabolic disorders. The present study was aimed to optimize the Moxifloxacin loaded chitosan microspheres (MCMs) by emulsion cross linking method for colon targeted delivery to alter the microflora.

Methods: Preliminary optimization of MCMs was carried out using Placket-Burman design (PBD) following by final optimization with Box-Behnken design (BBD). Optimized MCMs were evaluated for yield, particle size, entrapment efficiency and *in vitro*/ *in vivo* antimicrobial activities.

Results: FTIR spectroscopy of MCMs confirms the absence of chemical interactions during the formulation. MCMs were found to be smooth, spherical with particle size around 20µm. An enteric coating of MCMs prevented the drug release in the acidic environment of the stomach and ileum with complete release at the colon. MCMs had followed the korsmeyer - peppas model of drug release, indicating the drug release by non-fickian diffusion pattern. MCMs showed significant *in vitro* antimicrobial activity against *Lactobacillus casei* and *Escherichia coli*. *In vivo* results of MCMs exhibited prolonged antimicrobial effect of drug in the cecal content of rats. Significant protective activity observed in the ileum and colon histology in rats treated with MCMs compared to the pure drug.

Conclusion: MCMs were formulated by emulsion cross linking method using QBD approach. An enteric coating around the microspheres prevented the premature drug release at upper gastrointestinal tract, while chitosan cross linking has provided the sustain release of the drug in the colonic region over the time.

Keywords: Box-behnken, chitosan, microspheres, moxifloxacin, placket-burman, quality by design.

1. INTRODUCTION

Trillions of the microbes, which reside in the mammalian gut, have an important role in the normal human physiology and diseases progression. Colonic microflora plays a significant role in the production of various microbial metabolites i.e SCFAs (Short chain fatty acids) through fermentation, which have a key role in the pathogenesis of the diseases. Dysbiosis of the commensal colonic microflora has shown to progress the inflammation mediated insulin resistance [1, 2]. Recent reports suggest that, colonic microflora modulation through application of various antibiotics, probiotics and prebiotics can play a pivotal role in the management of certain metabolic disorders such as diabetes and obesity [3, 4].

Moxifloxacin hydrochloride (MFX) (Drug Bank ID: DB00218) is a synthetic antimicrobial drug of fluroquinolones derivatives. It blocks the bacterial DNA replication by binding to an enzyme: DNA gyrase, thus inhibits the DNA replication. The drug demonstrates 100 times higher affinity for bacterial DNA gyrase than mammalian. It was selected for its excellent broad-spectrum antibacterial activity against both gram-positive and gram-negative bacteria. It is absorbed by the gastrointestinal tract and has complete oral bioavaibility [5, 6].

*Address correspondence to this author at the Institute of Science, Nirma University, Sarkhej-Gandhinagar Highway, Chharodi, Ahmedabad-382481, Gujarat, India; Tel: +91 2717 241901-04 ext752; Fax: +91 2717 241916; E-mail: sriram.seshadri@nirmauni.ac.in, and sriramsjpr@gmail.com

1567-2018/16 $58.00+.00

Various mucoadhesive and biodegradable polymers such as, Chitosan, sodium alginate, pectin, xanthan are used for the targeted drug delivery [7]. Chitosan is a naturally occurring, linear polyaminosaccarides *i.e.* obtained from the chitin through the alkaline deacetylation. Chitosan is composed of β-(1, 4)-2-acetamido-2-deoxyglucopyranose and 2-amino-2-deoxyglucopyranose units presenting mucoadhesive and biodegradable properties. Chitosan microspheres (CMs) and nanoparticles are widely used as drug carriers, antimicrobial agents, immuno-enhancers and immobilization agents [8, 9]. The chitosan was chosen to encapsulate the MFX for achieving sustained release properties by a process of slow erosion from a hydrated compressed matrix. Moreover, chitosan is completely degraded by the colonic microflora ensuring the complete drug release at the colonic site.

Eudragit S100 (ES) is composed of methacrylic acid and methyl methacrylate; which is pH sensitive polymer due to its unique dissolution at pH 7.0 and widely used for enteric coating and drug delivery [10]. Eudragit can also be used in the combination with several other polymers such as hydroxypropyl methyl cellulose (HPMC) and talc for controlled drug delivery system [11].

It was well documented about CMs formulation methods, such as emulsion cross-linking, solvent evaporation, multiple emulsions, ionic gelation and spray drying. The cross linking of CMs is carried out by various agents, *i.e.* citric acid, glutaraldehyde, formaldehyde and sodium tri-polyphosphate [12]. Amongst these, glutaraldehyde is widely used cross linker due to its higher efficiency. Different properties of CMs, such as yield, entrapment efficiency (EE) and drug release are mainly affected by the polymer and cross linker concentration (Conc.) [13]A well designed controlled drug delivery system can overcome several limitations of conventional systems and enhance the therapeutic efficacy. To enhance the maximum therapeutic efficacy, it becomes necessary to deliver the drug at a target site with the appropriate dose showing least toxicity [14]. Various formulations, such as nanoparticles, liposomes etc. have been developed for delivering a drug at target site in a controlled fashion. One of the advanced approaches is using microspheres as a drug carrier. The microspheres are free flowing powder, consisting of proteins or synthetic polymers; which are biodegradable in the nature and ideally have a particle size less than 200 μm [15, 16].

Quality by design (QBD) refers to the achievement of certain predictable quality with desired and predetermined specifications. A very useful component of QBD is the understanding of factors and their interaction effect with a desired set of experiments. To understand the variables and their interactions, many statistical experimental designs have been recognized as useful techniques [17]. Box Behnken design (BBD) is a popular form of response surface methodology (RSM), which is more effective and acknowledged as one of the best statistical and analytical models than other response surface designs [18].

The objective of the present investigation was to design and formulate the multiparticulate system by QBD approach for the colon targeted delivery of MFX using chitosan and enteric coating polymer. Combination of chitosan and ES100 was selected to release the MFX only at the colonic milieu. Enteric polymer coating of microspheres will control the drug release at stomach and small intestine region, due to its pH sensitive release behavior, while cross linking of microspheres with natural bioadhesive polymer i.e chitosan will selectively provide the adhesion to the colonic mucosa for increasing the drug retention time to give the sustain release. This system is anticipated to prevent the drug loss in the upper gastrointestinal tract and deliver it efficiently to the colon with the desired dose [19]. The system is hypothesized to significantly modulate the colonic microbial population, without demonstrating toxic effect on the intestinal tissue architecture.

2. MATERIALS AND METHODS

2.1. Materials

The active pharmaceutical ingredient (API) of MFX was kindly gifted by Unimark Pharma Ltd, Ahmedabad. Chitosan having medium molecular weight, 85-90% deacetylation degree was obtained from Sigma Aldrich (Bangalore, India). ES was kindly gifted from Evonik Pvt. Ltd. (USA). Light liquid paraffin; span-80, glutaraldehyde, petroleum ether, acetone, ethanol, acetic acid and n-hexane were purchased from Himedia (India). All the reagents and solvents used were of analytical grade and used without purification.

2.2. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR study of physical mixtures of polymers and drug as well as enteric coated MCMs was carried out to find the possible interaction amongst them during the formulation process. FTIR spectra were obtained in KBr pellets using a Perkin Elmer model spectrum BX-FTIR spectrophotometer in the ranges, 4000- 400 cm^{-1} [20].

2.3. Chitosan Microspheres Preparation

The MFX loaded CMs (MCMs) were formulated by water in oil emulsion (w/o) cross linking method using glutaraldehyde [21]. In brief, chitosan solutions of various conc. (1%, 2% and 3%) were prepared by dissolving the respective quantities of chitosan in dilute acetic acid. MFX dissolved in the chitosan solution would act as an aqueous phase. Light liquid paraffin containing different conc. of span 80; as surfactant; was used as organic phase. The aqueous phase was added drop by drop to the organic phase solution using a gauze syringe to give stable w/o emulsion and was allowed to cross link for a definite period of the time. Thereafter, cross-linked microspheres were centrifuged, collected and washed three to four times with petroleum ether, air dried and stored in air tight vials [21].

Formation and properties of the MCMs such as yield, particle size, EE and drug release were affected by seven different factors: polymer conc. (X_1), cross linker conc. (X_2), surfactant conc. (X_3), drug to polymer ratio (X_4), phase volume ratio (X_5), stirring speed (X_6) and cross linking time (X_7). To clearly investigate the influence of the seven variable factors and maximize the responses; placket burman design (PBD) followed by BBD had been applied using Design Expert 7 (DX_7) software.

2.4. Experimental Design

2.4.1. Placket Burman Design (PBD)

The placket burman factorial design was applied as the primary step in the study to screen the significant factors, which have a considerable effect on the formation and properties of MCMs [22]. The applied PBD was on the basis of seven factors-two level factorial design. PBD cannot give any significant information regarding the interaction effects amongst all the variable factors. However, it is mainly applied to screen out the important independent variables (X), which have the major impact on responses (Y). On the basis of the design, each variable was examined at two different levels: (-1) for low levels and (+1) for high levels and the (-) and (+) values of the all seven variables have mentioned (Table **1A**). Total 8 runs were carried out in this model and 6 different responses such as yield, particle size, EE and drug release at 60 and 480 minutes (T_{60} and T_{480}) were analyzed and shown (Table **1B**). The regression analysis result of (p<0.05) significance at the 95% level was considered as significant and were further taken for the optimization of the BBD model.

2.4.2. Final Optimization by Box-Behnken Design (BBD)

A three factors-three levels BBD was applied for the optimization of the significant independent variables, obtained from the PBD. Three factors such as, polymer conc. (X_1), surfactant conc. (X_2) and cross-linker conc. (X_3) were found to have the most significant effect on the MCMs formation and properties. These factors have been analyzed for three different levels: (-1) for low levels, (0) for medium level and (+1) for high levels. Total 15 formulation batches were prepared (Table **3**). High, medium and lowest levels of the factors were selected from the results obtained by previous experimentations. After generation of the polynomial equation, relating the dependent and independent variable, the process was optimized [23].

2.5. Characterization of Microspheres

2.5.1. Particle Size Analysis

The average particle size distribution of the optimized MCMs was estimated through the optical microscopy method using both calibrated stage micrometer and eyepiece

Table 1A. Placket-burman design for various formulation batches.

Formulation	X1 Polymer Conc. (%)	X2 Cross Linker Conc. (%)	X3 Surfactant Conc. (%)	X4 Drug Polymer Ratio	X5 Phase Volume Ratio	X6 Stirring Speed (RPM)	X7 Cross Linking Time (hr)
F1	-1 (1)	-1 (1)	-1 (1)	1 (1:6)	-1 (1:5)	1 (1200)	1 (4)
F2	1 (3)	-1 (1)	-1 (1)	-1 (1:2)	1(1:10)	-1 (800)	1 (4)
F3	1 (3)	-1 (1)	1 (3)	-1 (1:2)	-1(1:5)	1 (1200)	-1 (3)
F4	-1 (1)	1 (3)	1 (3)	-1 (1:2)	-1(1:5)	-1 (800)	1 (4)
F5	1 (3)	1 (3)	-1 (1)	1 (1:6)	-1(1:5)	-1 (800)	-1 (3)
F6	-1 (1)	-1 (1)	1 (3)	1 (1:6)	1(1:10)	-1 (800)	-1 (3)
F7	-1 (1)	1 (3)	-1 (1)	-1 (1:2)	1(1:10)	1 (1200)	-1 (3)
F8	1 (3)	1 (3)	1 (3)	1 (1:6)	1(1:10)	1(1200)	+1 (5)

Table 1B. Responses of placket-burman design.

Formulation	Y1 Yield (%)	Y2 Size (µm)	Y3 Entrapment Efficiency (%)	Y4 T_{60}	Y5 T_{480}
F1	36.111	9.88	37.65	55.76	94.87
F2	53.456	10.66	44.12	58.83	97.44
F3	31.76	9.42	39.93	37.91	82.29
F4	36.754	15.87	56.67	59.76	97.11
F5	59.54	20.5	63.43	43.41	89.5
F6	39.86	17.76	57.92	41.46	88.92
F7	64.34	19.92	61.62	64.73	98.98
F8	48.76	11.97	46.12	36.56	85.02

[24]. The particle size of around 100 microspheres was observed and D50 value was calculated from it.

2.5.2. Percentage Entrapment Efficiency

Accurately weighed amount of MCMs was taken and added into pH 7.4 phosphate buffer. The mixture was continuously agitated on a magnetic stirrer for 5 to 6 hours (hrs). Further, the solution was filtered and drug content of the filtrate was determined by UV spectrophotometer at 290 nm. The percentage drug EE was calculated using below formula [25].

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Practical Drug Content}}{\text{Theoretical Drug Content}} \times 10$$

2.5.3. Scanning Electron Microscopy (SEM)

A scanning electron microscope (ESEM TMP with EDAX, Philips, Holland) was used to characterize the surface morphology of formulated MCMs. The surface was scanned and images were taken at 30 KV accelerating voltage and two different magnifications, *i.e.* 200x and 1500x for the drug-loaded microspheres [26].

2.6. Enteric Coating of Chitosan Microspheres

MCMs were coated with pH sensitive polymer i.e ES by solvent evaporation method [27]. Three different ratios of uncoated microspheres with ES (core to coat) such as 1:1, 1:3 and 1:5 were taken to efficiently coat the MCMs. ES was dissolved in an equal mixture of acetone and alcohol, in which the MCMs were further dispersed. This mixture was then added drop wise in a continuous stirred oil phase containing light liquid paraffin and span 80. The resultant mixture was continuously stirred for 3 to 4 hrs until complete evaporation of the solvent. The encapsulated MCMs were recovered, centrifuged, filtered and washed with n-hexane and dried overnight at 50ºC.

2.7. *In vitro* Drug Release Study

2.7.1. Core Microspheres

Accurately weighed core microspheres (uncoated) containing 300 mg of equivalent drug were dispersed in 500 ml of 7.4 pH phosphate buffer. Microspheres were filled into dialysis bag and loaded into the basket of the USP dissolution apparatus. 5ml sample withdrawn from the dissolution media at suitable time intervals and the same amount of buffer was replaced with fresh buffer to maintain the sink condition. The withdrawn samples were filtered and analyzed for the drug content by measuring the absorbance at 290nm by UV spectrophotometer.

2.7.2. Coated Microspheres

Accurately weighed coated microspheres containing 300 mg of equivalent drug were filled in the dialysis bag and dispersed in 500 ml of 0.1 N HCl for initial 2 hrs. The dissolution media in the USP apparatus were maintained at 37ºC and 100 RPM. After 2 hrs, the microspheres were transferred to the pH 5.5 phosphate buffer for 2 hrs. Consequently, the pH of the buffer was increased by further addition of Na_2HPO_4 to pH 7.0 and maintained till the completion of study. 5 ml of the sample was withdrawn hourly replacing each withdrawn sample with the fresh release medium. The samples were filtered and analyzed for drug content at spectrophotometrically. The drug content in the withdrawn samples from 0.1N HCl, pH 5.5 and pH 7.0 phosphate buffer was measured by spectrophotometer at 294nm, 288 nm, and 290 nm respectively.

2.8. Release Kinetic Modeling

The cumulative drug release vs. time data were entered into various kinetic models to find out the mechanism of the drug release from the MCMs. The data were calculated for the various kinetic models, such as zero order, first order, higuchi model and korsmeyer-peppas model. The model coefficient and regression co-efficient values for all the kinetic models were deliberated using DDsolver tool. The coefficient and regression values were observed for all the models and appropriate values were further considered [28].

2.9. *In vitro* Antimicrobial Study

As described earlier, *in vitro* antimicrobial study of MCMs was performed with modification [29]. The MFX and MCMs were dissolved separately in distilled water to obtain the drug conc. 5 µg/ml. 1 ml of overnight grown culture of *Escherichia coli* MTCC443 and *Lactobacillus casei* MTCC1423 were inoculated in freshly prepared Luria-Bertani and MRS broths respectively. After 16 hrs of incubation at 37°C with shaking at 150 RPM, drug and MCMs were added to both the cultures individually and again incubated at 37°C with shaking at 150 rpm. Changes in the optical density (600 nm) were recorded at every hr up to 10 hrs. Antimicrobial activities of MCMs and MFX were compared with control microbial cells without any treatment.

2.10. Animal Experimentation

Around 8 to 10 weeks old healthy male wistar rats, weighing about 150–200 g were procured from the animal research facility (Cadila pharmaceutical limited, Ahmedabad (India) under the approval of Institutional animal ethics committee with protocol no. IS/BT/PhD11-12/1004, and were maintained in the animal house of Institute of pharmacy (Nirma university, India). The animals were acclimatized at temperature 25±2°C with relative humidity of 50–60 % under 12/12 h light/dark conditions for 1 week before the initiation of the experiment. Animals were assigned to control group without any treatment (control; n=6), uncoated MCMs treated group (uMCMs; n=6) and coated MCMs treated group (MCMs; n=6). The MCMs were orally administrated into the rat models on a daily basis for 7 days.

2.11. The *Ex vivo* Antimicrobial Study

The *Ex vivo* antimicrobial activity of enteric coated and uncoated MCMs was estimated from the colon fecal samples; isolated from test animals at the end of the experiment. The antimicrobial activity was determined using culturable method through selective media. For the quantitative determination of microflora, 1g colon fecal sample was suspended in 10 ml of 0.85% NaCl solution for homogenization and 10 fold serial dilutions were prepared. The appropriate dilutions were spread plated onto two selective agar medium such as;

MRS medium for *Lactobacillus* and MacConkey agar for *Escherichia.* The plates were incubated aerobically for 48 hrs at 37°C and the resultant colonies were counted in cfu/mg of feces.

2.12. Histopathological Analysis

The test animals were sacrificed using the euthanasia method at the end. Small portions of the ileum and proximal colon were excised from animals of each group, fixed with 10% v/v formalin saline, and processed for standard histopathological procedures. Paraffin embedded specimens were cut into 5 µM sections (Yorco sales Pvt. Ltd., New delhi) and stained with hematoxylin as well as eosin (H&E). The histopathological tissue sections were viewed and digitally photographed using a Cat-Cam 3.0MP trinocular microscope with an attached digital 3XM picture camera (Catalyst Biotech, Mumbai, India) [30, 31].

3. RESULTS

3.1. Drug Excipient Compatibility Studies

It is advisable to check the drug excipient compatibility amongst drug and polymers used for the formulation using FTIR. The FTIR spectra of the chitosan showed the prominent peak at 3154 cm^{-1} for N-H bond, 2811 cm^{-1} for C-H bond, 1592cm^{-1} for the protonated amine group. The IR spectra of the MFX pure drug had shown the characteristic peak at 1708 cm^{-1} due to carboxylic acid C=O stretching, C−N stretching at 1340 cm^{-1}, aromatic C=C stretching at 1612 cm^{-1}, 1518 cm^{-1} and 1448 cm^{-1}, and C−H bending for the substituted benzene at 2308 cm^{-1}(Fig. **1**). FTIR spectra for the eudragit polymer coated MCMs have shown characteristic peaks at 3045cm^{-1}, 2815 cm^{-1}, 2348 cm^{-1} and 1058 cm^{-1} similar to the physical mixture of all components, which indicates that drug and polymers are not going in any chemical interactions during the process of the formulation [32].

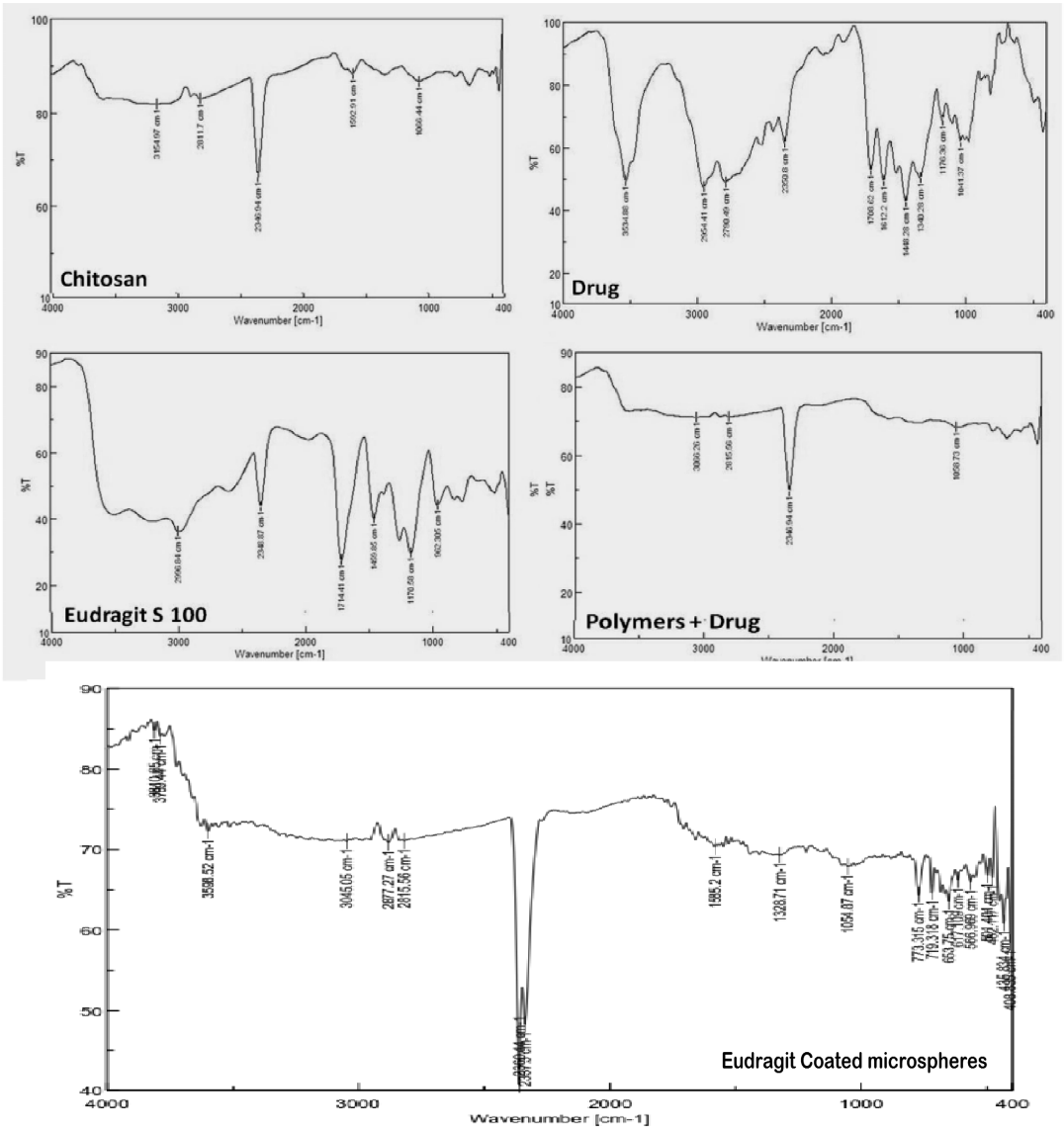

Fig. (1). Fourier-transform infrared spectra of components i.e chitosan, ES, pure drug (MFX), physical mixture of all polymers and drug as well as enteric coated drug loaded microspheres. FTIR spectra of the physical mixture indicate that there are not any chemical interactions amongst all the components.

Table 2. Box-behnken design.

Formulation	Variable Factors			Responses				
	X1 Polymer Conc. (%)	X2 Surfactant Conc. (%)	X3 Cross Linker Conc. (%)	Y1 Yield (%)	Y2 Size (µm)	Y3 Entrapment Efficiency (%)	Y4 T_{60} (%)	Y5 T_{480} (%)
F**1**	1(3)	0(2)	-1 (1)	60.23	15.12	59.29	37.12	84.88
F**2**	-1 (1)	0(2)	-1 (1)	38.89	12.89	47.67	41.78	88.08
F**3**	1 (3)	-1(1)	0 (2)	57.98	11.9	44.12	43.87	94.14
F**4**	0 (2)	-1 (1)	-1 (1)	49.11	8.89	41.2	42.12	89.45
F**5**	0 (2)	0 (2)	0 (2)	57.25	13.87	56.89	45.05	97.56
F**6**	0(2)	0 (2)	0 (2)	59.35	12.89	58.91	49.43	98.88
F**7**	-1 (1)	-1 (1)	0 (2)	35.24	7.14	38.98	50.14	95.75
F**8**	1(3)	1 (3)	0 (2)	64.11	17.81	63.11	41.66	92.86
F**9**	0 (2)	1 (3)	-1 (1)	62.67	16.09	62.09	36.14	85.44
F**10**	0 (2)	-1 (1)	1 (3)	55.09	9.76	42.34	56.78	98.87
F**11**	1 (3)	0 (2)	1 (3)	62.32	14.56	61.67	52.34	96.54
F**12**	-1 (1)	1 (3)	0 (2)	42.56	15.89	59.88	48.78	91.27
F**13**	0 (2)	1 (3)	1 (3)	64.98	16.33	65.54	51.78	95.22
F**14**	-1(1)	0(2)	1 (3)	41.13	13.78	53.07	53.89	99.06
F**15**	0 (2)	0 (2)	0 (2)	61.09	14.09	55.45	51.09	96.44

Table 3. Statistical analysis of placket burman design.

	Yield (%)		Size (µm)		Entrapment Efficiency (%)		T_{60} (%)		T_{480} (%)	
Factors	**Co-efficient**	**p-value**	**Co-efficient**	**p-value**	**Co-efficient**	**p-value**	**Co-efficient**	**p-value**	**Co-efficient**	**p-value**
Polymer Conc. (%) (X1)	+8.83	0.0002	+1.43	0.0125	+3.10	0.0001	+1.29	0.0156	+0.78	0.0310
Cross-linker Conc. (%) (X2)	+3.07	0.0053	+4.07	0.0012	+8.71	0.0001	+2.68	0.0001	+1.88	NS
Surfactant Conc. (%) (X3)	+1.54	NS	+0.44	NS	-0.83	0.0035	+9.41	NS	+5.27	0.0004
Drug to polymer ratio (X4)	-0.43	0.0469	+0.035	NS	-0.21	NS	+1.21	NS	+0.11	NS
Phase to volume ratio (X5)	0.61	NS	+0.33	NS	-0.21	NS	-0.53	NS	+0.056	NS
Stirring speed (RPM) (X6)	-0.068	NS	+0.42	0.0034	-0.29	0.0856	+0.60	NS	+0.39	NS
Stirring time (hrs) (X7)	-0.62	NS	-0.12	NS	-0.092	NS	+0.493	NS	-0.84	NS

3.2. Statistical Analysis of Placket-Burman Design

PBD is useful and efficient design to select the most efficient parameters which affect the formation and properties of MCMs. MCMs yield was significantly varied from 36.11% to 64.34% under different levels of factors (Table **2**). From the regression analysis of PBD, the confidence level of the factors above 95% (P<0.05) was selected as most significant variables (Table **4**). From the significance analysis of the seven variables, first three variables (X_1, X_2, and X_3) had shown a more significant effect on the responses, while other four variables (X_4, X_5, X_6 and X_7) had not shown any prominent effect on the responses. Moreover, the factors showing the confidence level below 95% (P>0.05) were not included

Table 4. Statistical analysis of box-behnken design.

Source	Yield (%)		Size (μM)		Entrapment Efficiency (%)		T_{60} (%)		T_{480} (%)	
	Sum of Squares	p>F	Sum of Squares	p>F	Sum of Squares	p>F	Sum of Squares	p>F	Sum of Squares	p>F
(A) Model Analysis	43883.16	-	2527.73	-	42987.27	-	32201.67	-465.25	130000.12	-
Mean vs. Total	1128.37	0.0003	111.11	<0.0001	1012.50	<0.0001	<0.0001	0.9400	247.75	0.0007
Linear Vs. Mean	2.61	0.9940	1.72	0.6831	6.00	0.8988	2.50	0.5625	1.50	0.9810
2FI Vs Mean	249.52	0.0034	5.15	0.1955	62.07	0.0601	16.17	0.6748	60.23	0.0156
Quadratic Vs. 2FI	13.95	0.4717	1.46	0.7561	16.50	0.3117	16.75	-	8.25	0.2779
Cubic Vs Quadratic	7.39	-	2.28	-	4.67	-	18.67	-	2.00	-
Residual	45285.60	-	2649.44	-	44089.00	-	32721.00		13040	-
Total										
(B) Lack of Fit	266.08	0.1160	8.33	0.6637	84.57	0.2147	35.42	0.8512	69.98	0.1191
Linear	263.47	0.0797	6.61	0.5896	78.57	0.1589	32.92	0.7402	68.48	0.0827
2FI	13.97	0.4717	1.46	0.7561	16.50	0.3117	16.75	0.6748	8.25	0.2779
Quadratic	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
Cubic	7.39	-	2.28	-	4.67	-	18.67	-	2.00	-
Pure error										
(C) R-square Analysis	Adjusted R^2	PRESS	Adjusted R^2	PRESS	Adjusted R^2	PRESS	Adjusted R^2	PRESS	Adjusted R^2	PRESS
Linear	0.7517	499.53	0.8891	20.29	0.8969	163.09	0.8675	85.35	0.7135	107.60
2FI	0.6619	1080.31	0.8722	37.12	0.8678	325.51	0.8262	146.59	0.6142	179.63
Quadratic	0.9574	239.80	0.9139	28.51	0.9462	274.50	0.8091	310.00	0.9102	136.50
Cubic	0.9631	-	0.8688	-	0.9703	-	0.7484	-	0.9562	-

in the next optimization experiment. The effect of all three variables *i.e.*, X_1, X_2 and X_3 was positive for most of the responses. On the basis of PBD, these three factors were set at their maximum values *i.e.* polymer conc. (X_1), cross-linker conc. (X_2), surfactant conc. (X_3) from 1% to 3% in the final optimization process. To investigate the optimum levels of significant factors and their interaction effects on the MCMs production, the BBD was performed to achieve the optimum domain of the maximum response.

3.3. Analysis of BBD

The BBD was performed to study the interactions among the three significant factors and also to determine their optimal levels for CMs formation. The design matrix of the variables and experimental results has been described (Table **2**). The BBD comprised of 15 experimental runs, including 3 runs under the similar conditions were performed. By applying multiple regression analysis on the experimental data; second-order polynomial equation was obtained. A suitable polynomial equation was selected based on several statistical parameters [33]. The resulting equations for all the responses are mentioned as follows:

$$\textbf{Yield } (\mathbf{Y_1}) = 50.42 + 22.64X_1 + 1.78X_2 - 1.44X_3 - 0.30X_1X_2 - 0.037X_1X_3 - 0.75X_2X_3 - 8.20X_1^2 - 1.05X_2^2 - 0.38X_3^2 \quad (1)$$

$$\textbf{Size } (\mathbf{Y_2}) = 13.22 + 1.19X_1 + 3.53X_2 - 1.07X_3 - 0.55X_1X_2 - 0.33X_1X_3 - 0.11X_2X_3 + 0.39X_1^2 - 1.05X_2^2 + 0.22X_3^2 \quad (2)$$

$$\textbf{EE } (\mathbf{Y_3}) = 62.83 + 4.92X_1 + 5.17X_2 - 0.83X_3 - 0.50X_1X_2 - 1.00X_1X_3 + 0.50X_2X_3 - 1.42X_1^2 - 3.92X_2^2 + 0.082X_3^2 \quad (3)$$

$$\mathbf{T_{60}}\ (\mathbf{Y_4}) = 42.83 - 7.54X_1 - 6.04X_2 + 5.33X_3 - 0.006X_1X_2 + 0.75X_1X_3 + 0.25X_2X_3 - 1.67X_1^2 - 1.17X_2^2 - 0.92X_3^2 \quad (4)$$

$$\mathbf{T_{480}}\ (\mathbf{Y_5}) = 97.00 - 0.87X_1 - 1.62X_2 + 5.25X_3 + 0.50X_1X_2 + 0.25X_1X_3 + 0.25X_2X_3 + 2.00X_1^2 + 2.00X_2^2 - 3.25X_3^2 \quad (5)$$

All the above equations were found to be statistically significant (p<0.05) as determined by ANOVA, under the provision of design expert software. Formulation batches prepared by the BBD yielded smooth and spherical microspheres with size in the range of **7.14 - 17.81** μm (Table **2**). Polymer conc. at medium level (X_1, 0), while cross linker and surfactant conc. at high levels (X_2-X_3, +1), yielded the MCMs with the highest yield and drug EE *i.e.* 64.98% and 65.54% respectively (Table **2**). Factor effects of the Box-

Behnken model associated *p*-values and coefficients were presented for all the responses (Table **3**). A positive sign indicates a synergistic effect while a negative sign indicates an antagonistic effect of the factor on the selected response.

Press value is a measure of the fit the model to the points in the design. The smaller the press statistic value, the better the model fits to the data points. From the p-values (Table **4**), it was concluded that cross product contribution (2FI) was not significant, indicating the absence of the interaction effect between various factors. Product yield, size, EE and T480of MCMs showed R2 values of above equations to be 0.9317 & 0.9892 respectively; demonstrating a very good fit (Table **4**). It is concluded that the second order model adequately approximated the true surface. For estimation of significance of the model, the ANOVA was applied using 5% significance level [34].

The differential effect of the three variables on the five responses in BBD is mentioned (Table **2**). After analyzing the effect of the variables on the responses, four batches of formulation (B8, B9, B11 and B13) were selected as optimized batches for further analysis. Optimized formulation batches have higher yield (≈60%), smaller particle size (≈17µm), optimum EE (≈60%) and almost 90% drug release in 10 hrs of the gastric transient time (Table **2**).

3.4. Scanning Electron Microscopy

The shape and surface morphology of the MCMs were investigated by SEM. SEM analysis of optimized MCMs had confirmed the smooth and spherical properties of the microspheres without any clump formation (Fig. **2**).

3.5. *In vitro* Drug Release

In vitro drug release pattern from the optimized batches (B8, B9, B11 and B13) of the uncoated MCMs were investigated in pH 7.4 phosphate buffer for 10 hrs. An initial burst release of around 50% was observed in the first hr, due to the surface bound drug in the microspheres. From the cumulative drug release profile, it can be seen that MCMs follows the birelease drug release pattern with the first burst release within 2 hrs and then continues sustained drug release period for 10 hrs. More than 90% of the drug release was observed at around 9 hrs, which is in accordance with the gastrointestinal transient time (Fig. **3A**).

In vitro drug release from the ES coated MCMs was studied pH progressive media. The drug release from the coated MCMs was studied in 0.1N HCl (pH 1.2) for initial 2 hrs, then transferred it to pH 5.5 and pH 7.0 phosphate buffer up to 10 hrs to check the efficiency of the enteric coating. Coated MCMs have shown very negligible amount of drug release in 0.1N HCl and pH 5.5 phosphate buffer, which indicates that eudragit coating protects the drug from being released completely in the physiological environment of the stomach and small intestine. All formulation batches showed no significant release in 0.1 N HCl and pH 5.5 buffer and then it showed a constant drug release pH 7.0 phosphate buffer up to 10 hrs (Fig. **3B**).

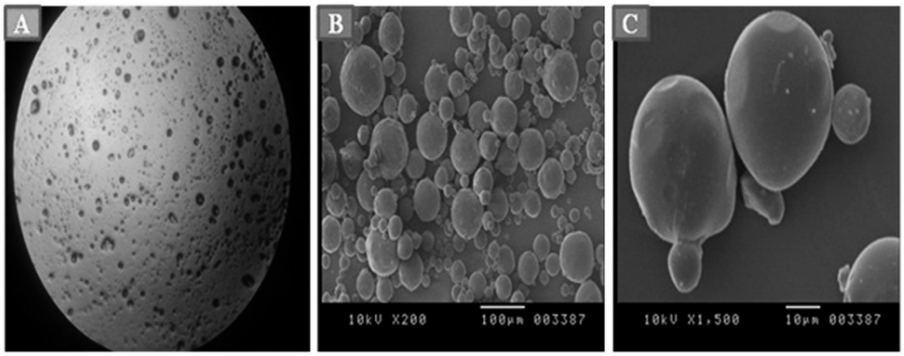

Fig. (2). Scanning electron microscopy (SEM) images of crystalline MCMs. (**A**) Microscopic view of formulated microspheres (**B**) SEM image of formulated microspheres at 200x magnification (**C**) SEM image of formulated microspheres at 1500x magnification.

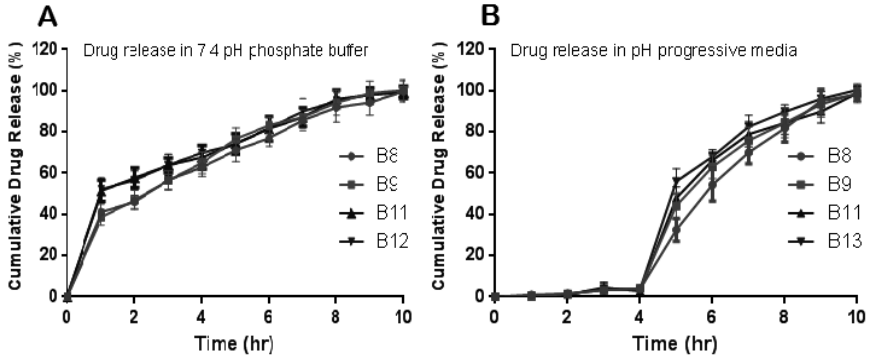

Fig. (3). Cumulative drug release (%) profile from the microspheres for 10 hrs (**A**) Drug release profile from uncoated MCMs in pH 7.4 phosphate buffer (**B**) Drug release profile from enteric coated MCMs was performed in pH progressive media i.e pH 1.2 (2 hrs), pH 5.5 (2 hrs) and pH 7.0 (6 hrs) with the help of 0.1N HCl and phosphate buffer.

3.6. Drug Release Kinetics

In order to determine the mode of the drug release from the dried uncoated MCMs, the cumulative drug release data were analyzed with the following models: zero order kinetics (1), first order kinetics (2), higuchi model kinetics (3) and korsmeyer-peppas model (4). The equations for the models are mentioned below [35]

$$Q=k_0t \quad (1)$$

$$\ln (100-Q) = \ln Q_0 - k_1t \quad (2)$$

$$Q= k_Ht^{1/2} \quad (3)$$

$$M_t/M\alpha=kt^n \quad (4)$$

In the equations, Q is the percent of drug released at time t, and k_0, k_1, and k_H are the coefficients (intercept) of the equations of zero order, first order and higuchi model respectively; k_P is a coefficient constant incorporating structural and geometric characteristics of korsmeyer peppas model; $M_t=M_1$ is the fraction of drug release at time t; k is the release rate constant; and n is the release exponent, which indicates the mechanism of release [36].

The cumulative drug release data were treated with above mentioned kinetic model equations. The kinetic parameters (obtained using Eqs. 2 to 4) of cross-linked MCMs are given (Table **5**).

The correlation coefficient with zero order release model was not satisfactory (0.5678 to 0.9662). The results of the first order release and higuchi kinetic study model showed slightly better fit with value of (0.9162 to 0.9704) and (0.9165 to 0.9952) respectively. The correlation coefficient and n values with korsmeyer-peppas release kinetic model were found to be satisfactory with value of (0.9675 to 0.9987) and (0.311 to 0.542) respectively [37].

3.7. *In vitro* Antimicrobial Activity

The antimicrobial activity of MCMs and MFX (pure drug) against gram positive (*L. casei* MTCC1423) and gram-negative (*E. coli* MTCC443) strains were checked and the results are summarized (Fig. **4**). This study indicated that, MCMs have almost an equal antimicrobial activity as MFX alone against *L. casei* maximum grown culture during 10 hrs of the drug release, which indicates the significant entrapment of the drug in the chitosan matrix. The MCMs and pure MFX alone had identical inhibition properties against *E. coli* culture also. However, *L. casei* had shown quite slower growth cycle compared to *E.coli* due to its higher generation time.

3.8. *Ex vivo* Antimicrobial Activity

The antimicrobial activity of the uncoated and coated MCMs on the ileum and colon residents *Lactobacillus* and *Escherichia* was checked from the fecal samples isolated from the all the treated groups as well as control animals (Fig. **5**). In the ileum, uncoated MCMs have shown significant (p<0.01) decrease in the *Lactobacillus* species as compared with control and coated MCMs treated group, due to their nonspecific drug release in the ileum region, while, enteric coated MCMs had avoided the drug release at the ileum. The same effect was also observed for the *Es-*

Table 5. Release Kinetic model analysis.

Formulation	Zero Order		First Order		Higuchi		Korsmeyer Peppas		
	R^2 Value	k_0 Value	R^2 Value	k_1 Value	R^2 Value	k_H Value	R^2 Value	k_{KP} value	n value
F1	0.9662	10.996	0.9194	0.226	0.9655	29.50	0.9675	27.29	0.542
F2	0.7041	11.72	0.9368	0.282	0.9830	31.78	0.9894	35.845	0.435
F3	0.6126	12.295	0.9594	0.330	0.9820	33.45	0.9962	39.63	0.408
F4	0.6730	11.67	0.9293	0.277	0.9801	31.62	0.9855	35.32	0.440
F5	0.6299	12.35	0.9413	0.327	0.9782	33.56	0.9883	38.86	0.421
F6	0.6123	12.65	0.9322	0.360	0.9572	34.25	0.9874	43.51	0.370
F7	0.6345	13.18	0.9315	0.363	0.9508	34.08	0.9925	44.83	0.351
F8	0.6692	11.96	0.9555	0.297	0.9880	32.44	0.9935	36.38	0.440
F9	0.7060	12.355	0.9704	0.318	0.9952	34.45	0.9987	35.88	0.462
F10	0.7156	13.12	0.9161	0.422	0.9165	35.33	0.9895	49.98	0.311
F11	0.5678	10.56	0.9162	0.372	0.9281	34.14	0.9866	46.92	0.327
F12	0.7987	15.65	0.9227	0.328	0.9561	33.06	0.9911	42.59	0.362
F13	0.7245	14.24	0.9237	0.369	0.9441	34.22	0.9896	45.53	0.345
F14	0.7088	13.99	0.9172	0.381	0.9375	34.66	0.9818	46.01	0.346
F15	0.6245	13.12	0.9301	0.382	0.9381	34.41	0.9925	46.73	0.333

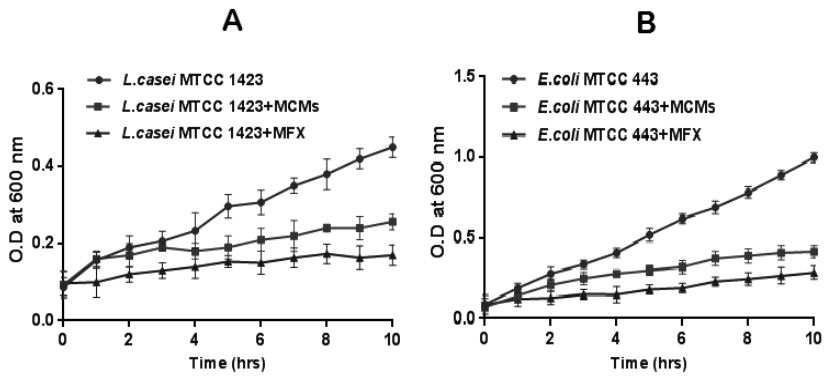

Fig. (4). *In vitro* antimicrobial activities of MFX and MCMs against fully grown pure bacterial culture for 10 hrs. (**A**) Antimicrobial activity against *Lactobacillus casei* standard strain procured from MTCC 1423 (**B**) antimicrobial activity against *Escherichia coli* standard strain procured from MTCC 443. Antimicrobial activities of MFX and MCMs were compared with each other as well as with control bacterial cells having not any treatment. The experiment performed in triplicate and result presented as Mean±SD.

cherichia, in which the uncoated MCMs treated group have shown significant (p<0.05) decrease in the species count as compared to control and coated MCMs treated group. In the colonic region, coated MCMs treated animals had shown significant (p<0.01) decrease in *Lactobacillus* and *Escherichia* species count as compared to control and uncoated MCMs treated group, due to the pH specific release of the MCMs with the desired dose at colonic milieu only.

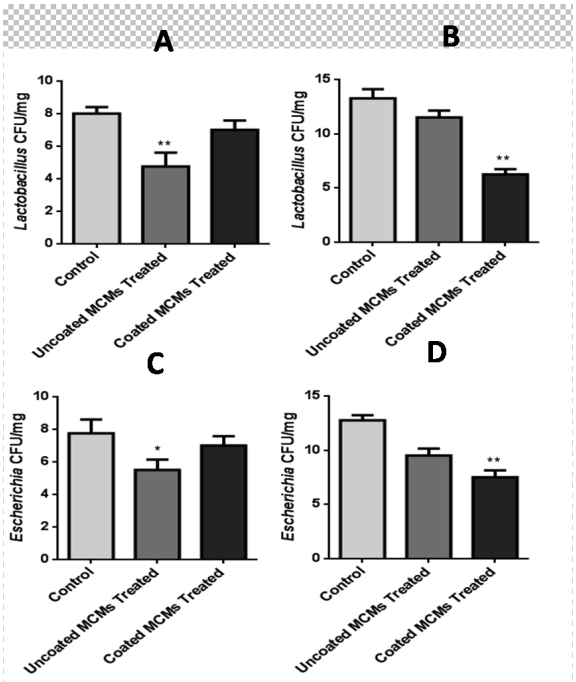

Fig. (5). *Ex vivo* antimicrobial activities of uncoated and enteric coated MCMs against bacterial colonies grown from the fecal samples of the test animals after oral administration. (**A**) Antimicrobial activities against (**A**) *Lactobacillus* isolated from ileum fecal (**B**) *Lactobacillus* isolated from colonic fecal (**C**) *Escherichia* isolated from ileum fecal (**D**) *Escherichia* isolated from colonic fecal. Antimicrobial activities of uncoated and enteric coated MCMs were compared with each other as well as with control bacterial cells having not any treatment. The experiment performed on 3 animals and result presented as Mean±SD.

3.9. Histopathological Analysis

It is very important to study the effect of drug and microspheres on the architecture of the mucosal tissues. Histopathological analysis of the ileum from the control group has shown the intact structure of epithelium layer and enterocytes, while colon had shown intact goblet cell, crypts and absence of the intraepithelial space between two colonic cells. Ileum and colon from MFX and uncoated MCMs treated group had shown the destruction of villus structure, enlarged goblet cells and increased intraepithelial space between two colonic cells compared to control group. On the other side, enteric coated MCMs treated group had shown significantly lesser damage on the colonic cells compared to MFX and MCMs uncoated treated group, which indicates the pH specific release of the coated MCMs as well as bio protective effect of the chitosan. Results also indicate that, the crude drug application has destructive effects on the mucosal barrier structure, while chitosan drug cross linked microspheres have significant protective effects on the colonic mucosal tissue architecture (Fig. **6**).

4. DISCUSSION

To specifically deliver drug to the colon, its loss during the gastrointestinal transit through upper GIT should be kept minimum, so that maximum dose of the drug reaches to the colon. The MCMs were successfully prepared by emulsion cross linking method and further coated with eudragit by the solvent evaporation method. It is worth to note that design of experiment is an essential element of the QbD. The study indicates the significance of PBD for the identification of the important process variables, which have significant impact on the responses. BBD was used to statistically optimize the formulation parameters and evaluate the main effects and interaction effects of the independent variables on the yield, size, EE and *in vitro* drug release from microspheres.

PBD has shown that polymer, cross linker and surfactant conc. have more significant effects on the most of the responses, i.e yield, particle size, EE and drug release as compared to other process variables (Table **1**). Polymer and cross-linker conc. have positive co-efficient value, but polymer has more significant impact on the yield. An increase in

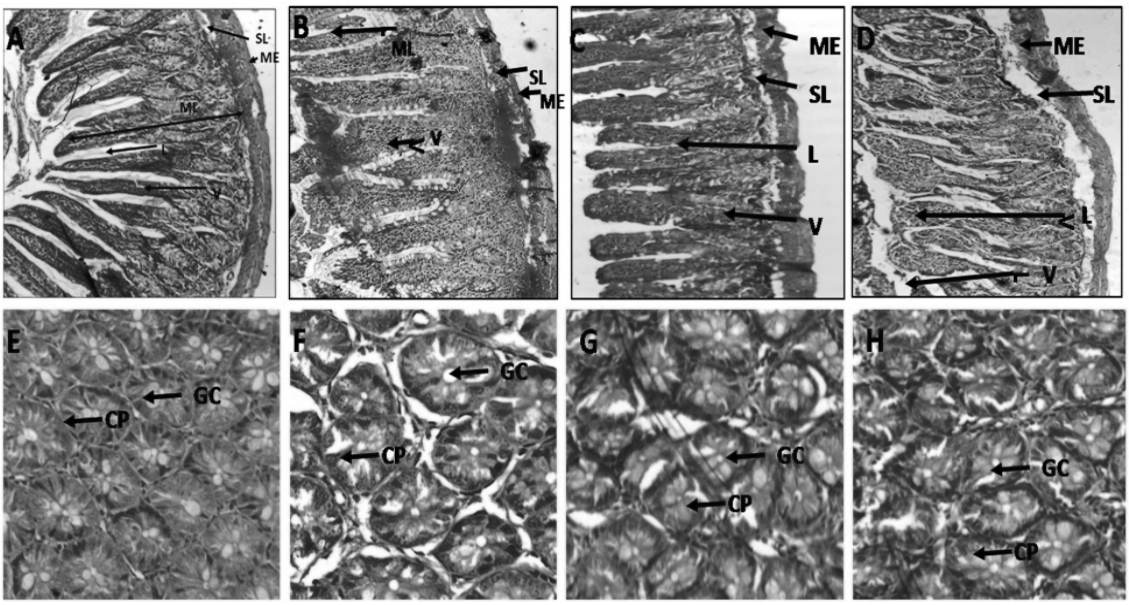

Fig. (6). Histopathological analysis of ileum and colon from all experimental animals (**A**) Ileum of control group (Untreated) (**B**) Ileum of MFX treated group (**C**) Ileum of uncoated MCMs treated group (**D**) Ileum of ES coated MCMs (**E**) colon of control group (Untreated) (**F**) colon of MFX treated group (**G**) colon of uncoated MCMs treated group (H) colon of ES coated MCMs treated group (ME, Muscularis Epithelium; L, lumen; V, villi; CP, crypts; GC, goblet cells).

the polymer conc. increases the viscosity of the polymer solution, which enhances the yield of the microspheres. Particle size is positively affected by cross linker conc., while negatively affected by surfactant amount. Increased cross-linker conc. had resulted in the formation of larger particles, which might be attributed to increase in viscosity of the primary emulsion. Therefore, large emulsion droplets were formed which would not undergo size reduction at the shear force energy supplied to the system and eventually get precipitated leading to an increase in the mean particle size [38]. Surfactant conc. has a negative impact on the size of MCMs. It can be justified that, interfacial tension between the aqueous droplets and the organic phase decreases with increasing the conc. of emulsifier, which further decrease the size of microspheres. The drug EE is also an important parameter for determining the drug-loading capacity of the microspheres. Our results have shown that, polymer and cross linker conc. have more significant positive impact on EE. Number of reports has shown that, EE increases with an increase in the polymer conc, which prevents the drug crystals from leaving the droplet [39, 40]. Increased conc. of cross-linker increases the drug EE due to formation of mesh like structure of polymer.

In the cumulative drug release analysis, initial burst release within 2 hrs was observed due to the surface associated drug crystals. A significant reduction in the drug release rate was found with an increase in the amount of cross linker [41]. It can be explained by the increased cross linking density of chitosan with the increased amount of cross-linker agent. During the microsphere formation, reaction takes place between -NH_2 (amino) group of chitosan and –COO group of glutaraldehyde that forms new bonds and matrix structures. Drug release from the chitosan matrix initiates after its swelling. An increase in the cross linking density increases the hydrophobicity of the chitosan matrix, which increases the hydration time and thus decreases the drug release. Initial burst release can also be explained by the particle size reduction, which increases the effective surface area in contact with dissolution medium to result in the enhanced surface bound drug release. Problem of the drug burst release from MCMs due to solubilisation of chitosan at acidic pH can be overcome by enteric coating of MCMs with ES, which exhibits pH-dependent solubility with a threshold of pH7.0 only [36]. Enteric coating of the MCMs had prevented the premature drug release at acidic environment of the stomach as well as in small intestinal fluid, which ensures the complete drug release at the colonic region only.

Drug release kinetic model analysis had shown that drug release from the uncoated MCMs follows the korsmeyer-peppas model. In the peppas model, the value of n characterizes the release mechanism of the drug. The value, $0.45 \leq n$ corresponds to a fickian diffusion mechanism, $0.45 < n < 0.89$ to non-fickian transport. To study the release kinetics, data obtained from *in vitro* drug release studies were plotted as log cumulative percentage drug release *versus* log time. In our formulation data, the n value range varies from 0.311 to 0.542, but almost all the formulation batches n values are $0.45 \leq$ n, which means they follow the drug release by the diffusion mechanism [42].

Formulation batches having optimum particle size, yield, drug loading and drug release were further investigated in the animal experimentation. The purpose of the *in vitro* antimicrobial study was to find out the drug release from the formulation and its efficacy to inhibit the growth of the microorganism. MFX showed broad spectrum antibacterial activity against both *Lactobacillus* (Gram positive) and *E. coli* (Gram negative) bacteria. The antimicrobial potency of the drug needs to be tested as the desired minimum inhibi-

tory concentration (MIC) has to be achieved. Thus optimized batches of the microsphere formulations along with pure drug were tested for the antibacterial activity. *In vitro* antimicrobial activity of MCMs at pH specific media against *Lactobacillus* and *Escherichia* has confirmed the sustain drug release from MCMs with the desired dose at desired time over 10 hrs.

To check the efficacy of MCMs, it is required to analyze the effect of MCMs on animal models after oral administration. ES coated MCMs had not released the drug in the stomach or small intestine, which was confirmed by analyzing the microbial content in the fecal samples of small intestine after oral administration from the animal models. ES coated MCMs had significantly decreased microbial counts of *Lactobacillus* and *Escherichia* in the fecal samples isolated from the animal model after oral administration of it, which indicates the significant drug efficacy and its antimicrobial effects on the colonic microflora only. Chitosan is a biodegradable and biocompatible natural polymer. In the oral drug delivery system, it protects the mucous membrane of the upper gastrointestinal tract from the irritation of the drug. In this study, colon targeted drug loaded chitosan microspheres have shown a significant mucoprotective effect on the intestinal and colonic tissue architecture as compared with the crude drug. These results prove the bioprotective and the biocompatible nature of the chitosan polymer in the colonic drug delivery system.

5. CONCLUSION

Moxifloxacin loaded chitosan microspheres (MCMs) were successfully formulated by emulsion cross linking method using two QBD designs i.e PBD and BBD. Optimized MCMs were found to be nearly spherical and having a good surface morphology with average particle size of around 20 µm. An enteric coating of MCMs with eudragit polymer have prevented the premature drug release in stomach and ileum for the sustained drug release at colon over 10 hours. Optimized MCMs have shown significant *in vitro* antimicrobial activities against both gram positive and gram negative microorganisms proving entrapped drug efficacy. *In vivo* histopathological analysis of the ileum and colon has revealed that MCMs administration in animals has shown significant protective effects on mucosal tissue architecture compared to the crude drug. Thus, designed and optimized formulation was found suitable for the small intestine and colon targeted drug delivery for the selective manipulation of the microflora.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors are thankful to Nirma Education and Research Foundation (NERF), Ahmedabad for financial support.

PATIENT CONSENT

Declared none.

REFERENCES

[1] Carvalho, B.M.; Guadagnini, D.; Tsukumo, D.M.; Tsukumo, M.L.; Schenka, A.A.; Latuf-Filho, P.; Vassallo, J.J.; Dias, C.; Kubota, L.T.; Carvalheira, J.B.; Saad, M.J. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. *Diabetologia,* **2012,** *55*, 2823-2834.

[2] Cani, P.D.; Bibiloni, R.; Knauf, C.; Waget, A.; Neyrinck, A.M.; Delzenne, N.M.; Burcelin, R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. *Diabetes,* **2008,** *57(6)*, 1470-1481.

[3] Jena, P.K.; Singh, S.; Prajapati, B.; NareshKumar, G.; Mehta, T.; Seshadri, S. Impact of targeted specific antibiotic delivery for gut microbiota modulation on high-fructose-fed rats. *Appl. Biochem. Biotechnol.,***2014**, *172(8),* 3810-3826

[4] Murphy, E.F.; Cotter, P.D.; Hogan, A.; O'Sullivan, O.; Joyce, A.; Fouhy, F.; Clarke, S.F.; Marques, T.M.; O'Toole, P.W.; Stanton, C.; Quigley, E.M.; Daly, C.; Ross, P.R.; O'Doherty, R.M.; Shanahan, F. Divergent metabolic outcomes arising from targeted manipulation of the gut microbiota in diet-induced obesity. *Gut*, **2013**, *62*, 220-226.

[5] Askal, H.F.; Refaat, I.H.; Wani, T.A.; Darwish I.A. Novel high-throughput microwell spectrophotometric assay for determination of non-fluorinated quinolone antibiotic rosoxacin in its bulk and capsules. *Dig. J. Nanomater. Biostruct.*, **2014,** *9(1),* 205-212.

[6] Moxifloxacin: www.igenericdrugs.com

[7] Sonia, T.A.; Sharma, C.P. Chitosan and its derivatives for drug delivery perspective. *Adv. Polym. Sci.*, **2011**, *243*, 23-54.

[8] Nagda, C.; Chotai, N.; Patel, S. *et al.* Chitosan microspheres of aceclofenac: *in vitro* and *in vivo* evaluation. *Pharm. Dev. Technol.*, **2010**, *15(5),* 442-451.

[9] Tao, Y.; Zhang, H.L.; Hu, Y.M.; Wan, S.; Su, Z.Q. Preparation of chitosan and water-soluble chitosan microspheres *via* spray-drying method to lower blood lipids in rats fed with high-fat diets. *Int. J. Mol. Sci.*, **2013**, *14(2),* 4174-4184.

[10] Yoo, J.W.; Giri, N.; Lee, C.H. pH-sensitive Eudragit nanoparticles for mucosal drug delivery. *Int. J. Pharm*., **2011,** *403,* 262-267.

[11] Del Curto, M.D.; Palugan, L.; Foppoli, A.; Zema, L.; Gazzaniga, A.; Maroni, A. Erodible time-dependent colon delivery systems with improved efficiency in delaying the onset of drug release. *J. Pharm. Sci.*, **2014**, *103(11)*, 3585-3593.

[12] Dhawan, S.; Singla, A.K.; Sinha, V.R. Evaluation of mucoadhesive properties of chitosan microspheres prepared by different methods. *AAPS Pharm. Sci. Tech.*, **2004**, *5(4)*, 122-128.

[13] Roy, S.; Panpalia, S.G.; Nandy, B.C.; Rai, V.K., Tyagi, L.K., Dey, S., Meena, K.C. Effect of method of preparation on chitosan microspheres of mefenamic acid. *Int. J. Pharm. Sci. Drug. Res.*, **2009**, *1 (1)*, 36-42

[14] Singh, P.; Prakash, D.; Ramesh, B.; Singh, N.; Mani. T.T. Biodegradable polymeric microspheres as drug carriers; A review. *IJNDD*, **2011,** *3(2)*, 70-82.

[15] Saxena, P.; Vats, S.; Easwari, T.S.; Shukla, V.K. Floating microspheres: A novel approach used to develop gastroretentive drug delivery system. *IJPRS*, **2014**, *3(2)*, 699-716.

[16] Nayak, U.Y.; Gopal, S.; Mutalik, S.; Ranjith, A.K.; Reddy, M.S.; Gupta, P.; Udupa, N. Glutaraldehyde cross-linked chitosan microspheres for controlled delivery of zidovudine. *J. Microencapsul.*, **2009,** *6(3)*, 214-22.

[17] Zidan, A.S.; Sammour, O.A.; Hammad, M.A.; Megrab, N.A.; Habib, M.J.; Khan, M.A. Quality by design: understanding the formulation variables of a cyclosporine A self-nanoemulsified drug delivery systems by Box-Behnken design and desirability function. *Int. J. Pharm.*, **2007**, *332(1-2)*, 55-63.

[18] Chandrakala, V.; Saral, A.M. Quality by design approach in formulation of bioadhesive levodopa microspheres. *Int. J. Pharm. Pharm. Sci*., **2013**, *5(4)*, 172-178.

[19] LinCai, P.; YuanCai, C. Conversion of paper sludge to ethanol by separate hydrolysis and fermentation (SHF) using *Saccharomyces cerevisiae*. *Biomass Bioenerg.*, **2011**, *35(4)*, 1600-1606.

[20] Chaus, W.N.; Shrivastava, B.; Rao, K.P.; Judge, D.R.; Satpute, K.L. Characterization and *in vitro* evaluation of drug implants. *IJCPS,* **2014**, *3*(3)*,* 5005.

[21] Ramachandran, S.; Nandhakumar, S.; Dhanaraju, M.D. Formulation and characterization of glutaraldehyde cross-linked chitosan

biodegradable microspheres loaded with Famotidine. *Trop. J. Pharm. Res.*, **2011,** *10(3)*, 309-316.

[22] Kenari, H.S.; Alinejad, Z.; Imani, M.; Nodehi, A. Effective parameters in determining cross-linked dextran microsphere characteristics: screening by Plackett-burman design-of-experiments. *J. Microencapsul.,* **2013**, *30 (6)*, 599-611.

[23] Jain, A.K.; Swarnakar, N.K.; Godugu, C.; Singh, R.P.; Jain, S. The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen. *Biomaterials*, **2011, 32***(2)*, 503-515.

[24] Chawla, A.; Sharma, P.; Pawar, P. Eudragit S-100 coated sodium alginate microspheres of naproxen sodium: formulation, optimization and *in vitro* evaluation. *Acta. Pharma.*, **2012**, *62 (4)*, 529-545.

[25] Kuchekar, A.B.; Pawar, A.P. Screening of factors using Plackett Burman design in the preparation of Capecitabine-loaded nano polymeric micelles. *Int. J. Pharm. Pharm. Sci.*, **2014**, *6(5)*, 489-496.

[26] Swathi Krishna, K.V.; Chandra Shekhar, K.B.; Lakshman Murthy, G.; Sandhya Rani, N. Formulation and evaluation of abacavir microspheres. *Int. J. Res. Pharm. Sci.*, **2015**, *6(2)*, 172-177.

[27] Shivani, N.; Hetal, P.; Rajesh, K.; Murthy, RR. Colon delivery of 5-fluoro uracil using cross-linked chitosan microspheres coated with eudragit S 100. *Int. J. Drug. Deliv.*, **2011**, *3*, 260-268.

[28] Modi, S.; Anderson, B.D. Determination of drug release kinetics from nanoparticles: Overcoming pitfalls of the dynamic dialysis method. *Mol. Pharma.*, **2013**, *10(8)*, 3076-3089.

[29] Aleem, O.; Kuchekar, B.; Pore, Y.; Late, S. Effect of β-cyclodextrin and hydroxypropyl β-cyclodextrin complexation on physicochemical properties and antimicrobial activity of Cefdinir. *J. Pharm. Biomed. Anal.*, **2008**, *47*, 535-540.

[30] Kevadiya, B.D.; Patel, T.A.; Jhala, D.D.; Thumbar, R.P.; Brahmbhatt, H.; Pandya, M.P.; Rajkumar, S.; Jena, P.K.; Joshi, G.V.; Gadhia, P.K.; Tripathi, C.B.; Bajaj, H.C. Layered inorganic nanocomposites: a promising carrier for 5-fluorouracil (5-FU). *Eur. J. Pharm. Biopharm.*, **2012**, *81(1)*, 91-101.

[31] Chaudhary, H.; Jena, P.K.; Seshadri, S. *In vivo* evaluation of Eclipta alba extract as anticancer and multidrug resistance reversal agent. *Nutr. Cancer*, **2014**, *66(*5), 904-913.

[32] Mudgil, M.; Pawar, P.K. Preparation and *in vitro*/*ex vivo* evaluation of moxifloxacin-loaded PLGA nanosuspensions for ophthalmic application. *Sci. Pharm.*, **2013**, *81(2)*, 591-606.

[33] Zhou, X.; Cheng, X.J.; Liu, W.F.; Li, J.; Ren, L.H.; Dang, Q.F.; Feng, C.; Chen, X.G. Optimization and characteristics of preparing chitosan microspheres using response surface methodology. *J. Appl. Polym. Sci.*, **2012**, *127*(6), 4433-4439.

[34] Mothilal, M.; Nagalakshmi, M.; Swati P.S., Damodharan, N.; Manimaran, V.; Lakshmi, K.S. Optimization and characterization of chitosan microspheres of aceclofenac. *Int. J. Pharm. Sci. Res.*, **2012**, *3(2)*, 305-315.

[35] Akbari, J.; Nokhodchi, A.; Farid, D.; Adrangui, M.; Siahi-Shadbad, M.R.; Saeedi, M. Development and evaluation of buccoadhesive propranolol hydrochloride tablet formulations: effect of fillers. *Farmaco.*, **2004**, *59(2)*, 155-161.

[36] Desai, K.H. Preparation and characteristics of high-amylose corn starch/pectin blend microparticles: A technical note. *AAPS Pharm. Sci. Tech.*, **2005,** *6(2)*, E202-E208.

[37] Liu, C.; Desai, K.H.; Tang, X.; Chen, X. Drug release kinetics of spray-dried chitosan microspheres. *Dry. Technol.*, **2006**, *24(6)*, 769-776.

[38] Essays, UK. (November 2013). Solvent evaporation *in vitro* histopathology biology essay. Retrieved from http://www.ukessays.com/essays/biology/solvent-evaporation-in-vitro-histopathology-biology-essay.php?cref=1

[39] Arora, N.; Khattar, H.; Parashar, D.; Arora, N.; Garg, T. Evaluation of entrapment efficiency of Glipizide microsphere. *IOSR J. Pharm.*, **2012**, *2(2)*, 180-181.

[40] Maravajhala, V.; Dasari, N.; Sepuri, A.; Joginapalli, S. Design and evaluation of Niacin microspheres. *Indian. J. Pharm. Sci.*, **2009**, *71(6)*, 663-669.

[41] Bulut, E. In-vitro evaluation of Ibuprofen-loaded microspheres prepared from novel chitosan/poly (vinyl alcohol) interpenetrating polymer network. *Polym-Plast. Technol.*, **2014**, *53(4)*, 371-378.

[42] Fu, Y.; Kao, W.J. Drug release kinetics and transport mechanisms of non-degradable and degradable polymeric delivery systems. *Expert. Opin. Drug. Deliv.*, **2010**, *7(4)*, 371-378.