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Research paper

Quality by design approach for developing chitosan-Ca-alginate microspheres for colon delivery of celecoxib-hydroxypropyl-β-cyclodextrin-PVP complex

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ABSTRACT

The aim of the present work was to develop a new multiparticulate system, designed for colon-specific delivery of celecoxib for both systemic (in chronotherapic treatment of arthritis) and local (in prophylaxis of colon carcinogenesis) therapy. The system simultaneously benefits from ternary complexation with hydroxypropyl-β-cyclodextrin and PVP (polyvinylpyrrolidone), to increase drug solubility, and vectorization in chitosan-Ca-alginate microspheres, to exploit the colon-specific carrier properties of these polymers. Statistical experimental design was employed to investigate the combined effect of four formulation variables, i.e., % of alginate, $CaCl_2$, and chitosan and time of cross-linking on microsphere entrapment efficiency (EE%) and drug amount released after 4 h in colonic medium, considered as the responses to be optimized. Design of experiment was used in the context of Quality by Design, which requires a multivariate approach for understanding the multifactorial relationships among formulation parameters. Doehlert design allowed for defining a design space, which revealed that variations of the considered factors had in most cases an opposite influence on the responses. Desirability function was used to attain simultaneous optimization of both responses. The desired goals were achieved for both systemic and local use of celecoxib. Experimental values obtained from the optimized formulations were in both cases very close to the predicted values, thus confirming the validity of the generated mathematical model. These results demonstrated the effectiveness of the proposed jointed use of drug cyclodextrin complexation and chitosan-Ca-alginate microsphere vectorization, as well as the usefulness of the multivariate approach for the preparation of colon-targeted celecoxib microspheres with optimized properties.

1. Introduction

Celecoxib (CXB), 4-[5-(4-methylphenyl)-3-trifluoromethyl-1H-pyrazol-1-yl] benzenesulfonamide, is a specific inhibitor of cycloxygenase-2 (COX-2) widely used as analgesic and in the treatment of osteoarthritis and rheumatoid arthritis, familial adenomatous polyposis, and primary dysmenorrhea [1,2]. It also showed a chemopreventive activity against colon carcinogenesis [3]. Moreover, it exhibits a similar therapeutic efficacy and a lower incidence of gastrointestinal complications in comparison with conventional NSAIDs [1,4]. However, CXB is a class II drug according to the biopharmaceutical classification system, and its very poor water solubility gives rise not only to formulation problems but also to low and highly variable bioavailability [5]. It has been shown that CXB solubility can be significantly improved by complexation with native β-cyclodextrin [6], and even more with its hydroxypropyl-derivative [7], particularly in the presence of hydrophilic polymers [8]. Other attempts of improving the CXB therapeutic effectiveness include incorporation in chitosan microspheres aimed for intra-articular administration [9], encapsulation in alginate microspheres as dispersion in a self-microemulsifying system [10], solid dispersion in a polymethacrylate carrier prepared by hot-melt extrusion [11], or formation of spherical agglomerates with hydrophilic polymers [12].

In recent years, colon-specific drug delivery systems gained increasing importance, not only for a more effective treatment of local pathologies, such as Chron's diseases, ulcerative colitis, and colorectal cancer, but also for the systemic therapy of both conventional and labile molecules [13,14]. In fact, the colonic region exhibits some peculiar advantages in comparison with stomach and small intestine, such as a less hostile environment, a longer residence time, and a better responsiveness to drug absorption enhancers [14]. Thus, colon-specific delivery could allow improvement in drug oral bioavailability, reduction in the total administered dose, and a decrease in side effects [15]. Moreover, it has been shown that colon-targeted drug delivery can be also exploited as a means of achieving chronotherapy for diseases sensitive to circadian rhythms, such as asthma,

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angina, and arthritis [16–19]. Different strategies have been proposed to target orally administered drugs to the colon, including prodrugs, pH-sensitive polymer, time-dependent systems, and microflora-activated systems [20,21]. Several natural polysaccharides have been investigated for their potential as colon drug carrier systems, and, among them, alginate and chitosan are considered particularly attractive due to their nontoxicity, biocompatibility, mucoadhesion properties, and biodegradability by colonic microflora [22,23]. Alginate is a water-soluble linear polysaccharide, formed of alternating blocks of 1–4 linked α-L-guluronic and β-D-mannuronic acid residues, while chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine. Alginates gel in aqueous solutions in the presence of divalent ions, such as Ca^{2+}, due to the formation of intermolecular cross-links with the carboxyl groups of guluronate moieties, giving rise to the well-known "egg-box" structure. However, the effectiveness of Ca^{2+} alginate microspheres as drug carriers is mainly limited by the hydrogel high porosity, responsible for possible burst effect or too fast release of the entrapped drug, as well for low drug loading ability due to drug leakage through the pores during their preparation [24]. Combination of Ca^{2+} alginate with chitosan, by interaction between the free carboxyl groups of the first and the aminic groups of the second one, has proven to be a successful strategy not only to overcome these drawbacks, increasing the gel mechanical properties and reducing its permeability, but also to combine the favorable properties of both polymers, improving their ability as carriers for achieving colon-specific drug delivery [24–27].

Considering on all these premises, we thought it worthy of interest to develop a colon delivery system of CXB, aimed at improving its therapeutic effectiveness, by reducing its gastrotoxicity and obtaining a release suitable to the circadian rhythm of rheumatoid arthritis. In fact, the selective delivery to colon is considered as a chronopharmaceutical approach for a better treatment of diseases such as asthma, angina, or rheumatoid arthritis, whose symptoms are characteristically most severe in the first hours of the morning [18,19,28]. Moreover, a colon-targeted delivery system of CXB could be also useful in the prophylaxis of colorectal cancer [3,29]. With this purpose, we investigated the possibility of creating a new biodegradable carrier by incorporating CXB into chitosan-Ca-alginate microspheres as ternary complex with hydroxypropyl-β-cyclodextrin and polyvinylpyrrolidone obtained by co-grinding, which were previously selected, respectively, as the most effective polymer-cyclodextrin combination and preparation method to increase the drug dissolution properties [30]. This novel delivery system should allow for both improving CXB dissolution and bioavailability, by cyclodextrin complexation, and providing an effective colon delivery, by complex encapsulation in chitosan-Ca-alginate microspheres, exploiting the pH-sensitive properties and the specific colon biodegradability of these polymers.

The US Food and Drug Administration Quality by Design (QbD) initiative [31] encourages the use of statistical tools for improving the development of pharmaceuticals with high quality. The International Conference on Harmonization describes in different guidelines a series of principles and tools for implementing QbD [32,33]. One of these tools is the experimental design, or design of experiments (DoE), that allows for understanding how formulation variables can influence the product quality by defining a "design space". The "design space" is the region of the experimental space where a multidimensional combination and interaction of input variables and process parameters have been demonstrated to provide assurance of quality [33] and it can be described in terms of mathematical relationships. By means of DoE, the relationship among different independent variables and the system performance can be found [34]. The "design space" can be then defined, allowing an in-depth understanding of the problem and, in turn, the maintaining of the product final quality. DoE was previously successfully used in preformulation and formulation studies to facilitate the screening process of excipients, to study the effects of different formulations and/or process variables or to determine the best levels of excipients that provide optimal drug release or dissolution properties [35–40].

In the present study, the planned CXB microsphere formulations were optimized in terms of encapsulation efficiency and drug amount released in the colon after a given time using the QbD approach. DoE was used in the development of such a multiparticulate system in the context of QbD, since it enables simultaneous evaluation of the effects of different variables, as well as their actual significance on the considered responses and possible interrelationships among them [37,38,40]. The considered variables were the relative amounts of alginate, calcium chloride, and chitosan and the time of cross-linking used for microsphere preparation. The selected QbD strategy allowed an efficient selection of the best formulation composition and of the most suitable experimental conditions in the shortest time and with the minimum number of experiments.

2. Materials and methods

2.1. Materials

Celecoxib (CXB) was extracted from capsules of Celebrex® (Pfizer, Italy). Identity and purity of CXB was checked by means of spectroscopic methods (IR, ^{1}HNMR) combined with powder X-ray diffractometry and DSC analysis. Chitosan at low molecular weight (CSL, average M_W 150 kDa, deacetylation degree 75–85%) and sodium alginate (40–50% guluronic acid subunits and 50–60% mannuronic acid subunits; viscosity 20–40 MPa s for 1% (w/v) aqueous solution) were provided by Sigma–Aldrich (Italy). Polyvinylpyrrolidone K30 (povidone, PVP, average M_W 40 kDa), sodium lauryl sulfate, and calcium chloride (solubility 74% w/v) were purchased from Fluka-Sigma–Aldrich (Italy). Hydroxypropyl-β-cyclodextrin (Hydroxypropylbetadex, HPβCD) DS 0.6 (average M_W 1400) was a kind gift from Roquette (Lestrem, France).

2.2. Preparation and characterization of CXB-HPβCD-PVP ternary system

Previous phase solubility studies indicated the formation of soluble complexes of 1:1 stoichiometry between CXB and HPβCD, both in the absence and presence of PVP, and pointed out the favorable effect of the polymer on the solubilizing and complexing ability of HPβCD toward the drug [8]. Accordingly, equimolar CXB-HPβCD solid systems added with a 10% w/w of PVP were prepared. However, the systems were prepared by the co-grinding method [30], proved by us as more effective than the kneading method used by Chowdary et al. [8]. Briefly, the physical mixture of the components (accurately weighed in the above indicated relative amounts) was co-ground in a high-energy vibration micromill (Retsch, GmbH, Germany) for 30 min at 24 Hz. The 210–250 μm sieve granulometric fraction was then collected and stored in desiccator until use. The product was characterized by X-ray powder diffractometry (Bruker D8, 5–30° 2θ range, scan rate 0.05° s^{-1}) (Bruker, Wisconsin, USA) and DSC analysis (Mettler TA4000 Star^e System, 10 °C min^{-1} in the 30–300 °C temperature range under static air) (Mettler Toledo, Switzerland) and evaluated for dissolution properties (USP apparatus 2 (paddle method); 10 mg of drug (or drug equivalent) added to 900 mL pH 7.4 phosphate buffer containing 0.17% w/v sodium lauryl sulfate to increase CXB solubility in the medium, thus allowing for maintaining sink conditions during dissolution test [8,41]; paddle rotation 100 rpm, 37 ± 0.5 °C).

2.3. Preparation and characterization of microspheres

Weighed amounts (3–5% w/v) of alginate were dispersed in distilled water (10 mL), and then, the drug (20 mg) was added as co-ground system with HPβCD and PVP. CSL was dissolved at different concentrations (0–4% w/v) in 5% (w/v) glacial acetic acid, and 5 mL of this solution added to an aqueous solution containing different concentrations (7–12% w/v) of $CaCl_2$. Alginate solutions were then dropped with a nozzle (inner diameter 0.9 mm) into the CSL–Ca^{2+} solution, under stirring, at room temperature. The microspheres thus obtained were allowed to stand in the cross-linking solution for a fixed time (5–20 min), then washed with distilled water, filtered, and dried at 40 °C for 24 h, and finally stored in a dryer. The microspheres obtained at the end of preparation of each batch were weighed, and the production yield was determined as percentage with respect to the initial amounts of excipients and drug used for their preparation. The mean diameter was calculated on 20 microspheres for each batch and measured with a stainless steel digital caliber endowed with a liquid crystal display and a fine adjustment roller (precision reading 0.01 mm). Shape and surface morphology of microspheres were examined using a FEI Quanta200™ environmental scanning electron microscope (ESEM), with a spatial resolution at 30 kV of 3 nm (FEI Company, Oregon, USA). A microanalysis was performed by an Energy Dispersive Spectrometer to check the homogeneity of the microsphere composition.

2.4. Entrapment efficacy

Weighed amount of microspheres of each batch was finely powdered, added with methanol, and maintained under stirring 24 h at room temperature, to ensure complete drug dissolution. Samples were then filtered (0.45 μm pore size) and assayed for drug content by HPLC, as described below. The drug entrapment efficiency (EE%) was then calculated according to Eq. (1):

$$EE\% = Qr/Qt * 100 \quad (1)$$

where Qt is the total drug amount initially added during the batch preparation, and Qr is the drug amount recovered in the microspheres.

2.5. HPLC assay of CXB

The drug HPLC assay was performed using a Merck Hitachi Elite Lachrom apparatus equipped with a L2130 model isocratic pump, a L-2400 UV–Vis spectrophotometric detector, a Rheodyne injector fitted with a 20 μL loop, and a reversed phase Supercodil™ C_{18} column (5 μm particle size; 25 cm × 4 mm). A methanol–water 70:30 v/v mixture was used as the mobile phase; the flow rate was 0.9 mL/min, the temperature was 25 °C, and the detection wavelength was set at 250 nm. The retention time of CXB under these experimental conditions was 6.9 min. The assay was linear in the concentration range 1.5–10 μg/mL; the regression coefficient was 0.9992, and the linear regression equation was $y = 266{,}472x + 11{,}436$. The detection (LOD) and quantification (LOQ) limits were 0.06 and 0.21 μg/mL, respectively.

2.6. Release studies

Release studies were performed using the basket apparatus (USP Apparatus 1) (Sotax AT7, dissolution testing, Sotax, Switzerland). Microspheres equivalent to 50 mg of drug (as ternary system with CD and PVP) were added to 900 mL of dissolution medium thermostated at 37 ± 0.5 °C and stirred at 50 rpm, which was varied according to the following sequence, in order to mimic the gastrointestinal transit [42]: 2 h artificial gastric juice (pH 1.1, 0.1 N HCl solution); 2 h artificial small intestinal fluid (pH 6.8 phosphate buffer); 4 h artificial colonic fluid (pH 7.4 phosphate buffer). According to other authors [8,41], sodium lauryl sulfate (0.17% w/v) was added in the medium to increase drug solubility and achieve sink conditions. At suitable time intervals, aliquots were withdrawn from the dissolution medium and assayed for CXB content by HPLC analysis as described above.

2.7. Software for experimental design

The software NEMRODW was used for the generation and evaluation of the statistical experimental design. [43]. Multivariate linear regression was used to generate the models. Response surface and desirability function were used to define the design space and find the optimum conditions. Analysis of variance (ANOVA) was applied for testing the significance and validity of the models.

3. Results and discussion

3.1. Characterization of CXB-HPβCD-PVP ternary system

The solid CXB-HPβCD-PVP ternary system obtained by co-grinding was characterized by DSC and X-ray powder diffractometry, to confirm the formation of the solid complex (Fig. 1). The thermal curve of CXB was typical of a pure anhydrous crystalline substance, exhibiting a sharp fusion peak at 162.2 °C (98.7%

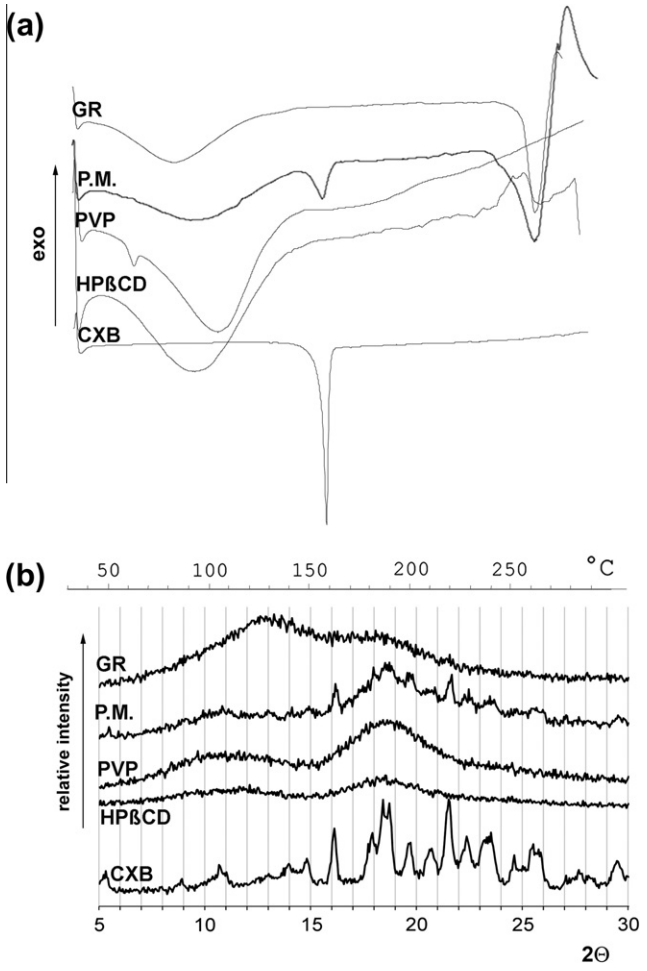

Fig. 1. DSC curves (A) and powder X-ray diffraction patterns of pure celecoxib (CXB), HPβCD, and PVP and of their ternary physical mixture (PM) and co-ground product (GR).

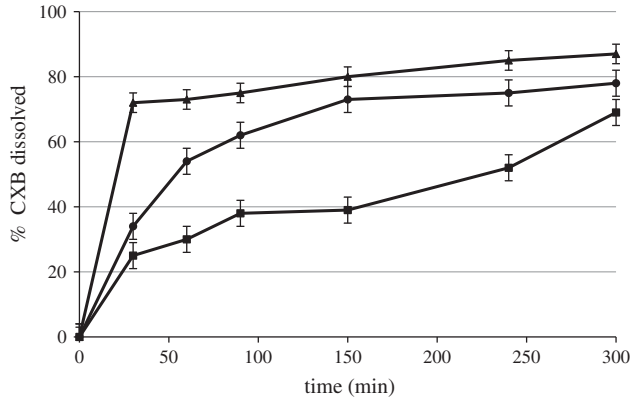

Fig. 2. Dissolution profiles of celecoxib (CXB) alone (■) or as co-ground system with HPβCD (●) or as ternary co-ground system with HPβCD and PVP (▲) in colonic artificial medium (phosphate buffer pH 7.4) at 37 °C.

purity), while HPβCD and PVP showed a broad endothermal band, consistent with their hydrated amorphous nature (Fig. 1A). The drug melting endotherm was still well detectable in the ternary physical mixture, while it completely disappeared in the co-ground product, as a consequence of interactions between the components. The X-ray diffraction pattern of the drug was characterized by the presence of several intense and sharp peaks, confirming its crystalline nature, while both HPβCD and PVP (Fig. 1B) exhibited a flat pattern, typical of amorphous substances. Some of the most intense diffraction peaks of CXB, emerging from the amorphous profile of HPβCD and PVP, can be seen in the ternary physical mixture, while they totally disappeared in the corresponding co-ground product, indicating its complete amorphization, in agreement with DSC results. Dissolution rate studies demonstrated the very better dissolution properties of the ternary co-ground product, whose percent dissolved at 30 min was 2 and 3 times higher than the binary drug-HPβCD co-ground system and the drug alone, respectively (Fig. 2), and also higher than the % of drug alone dissolved after 300 min, thus further supporting the need of its use in the microsphere formulation development. In fact, the higher dissolution rate of CXB-HPβCD-PVP co-ground system allowed its rapid dissolution in the alginate aqueous dispersion and, consequently, its homogeneous distribution within the microspheres during their formation, thus assuring high content uniformity.

3.2. Optimization of microsphere formulation

According to QbD principles, DoE [34] was used for microsphere formulation optimization, so that to ensure a predefined quality of the product [44–46]. In order to define the "design space," the critical process variables (independent variables) and the responses able to measure the product quality were defined. The components used for the microsphere production (alginate, chitosan, and $CaCl_2$) and the time of cross-linking (TCL) were chosen as the independent variables since they were considered critical in determining the performance of the final product; the drug encapsulation efficiency (EE%) and the percent of CXB released after 4 h in the colonic medium (DR%) were instead selected as the most important responses to be maximized to improve the product quality.

3.2.1. Determination of the experimental domain of the independent variables

The experimental domain of each of the selected independent variables was set on the basis of a preliminary screening, also considering some technological factors such as spherical shape regularity, size homogeneity, yield, and consistency of the obtained microspheres. In particular, the alginate concentration range was established between 3 and 5% w/v because polymer amounts lower than 3% gave rise to sticky microparticles of irregular shape, while solutions containing alginate amounts greater than 5% were excessively viscous and difficult to be regularly dropped with the syringe. In case of chitosan, 0% w/v was chosen as the lowest value of this polymer, to evaluate the effect of its absence in the formulation, and 4% w/v as the highest value, since larger quantities, as for alginate, gave rise to too much viscous solutions. As for the $CaCl_2$ concentration range, 12% w/v was fixed as the upper value, in order to avoid possible negative effects due to an excess of free Ca^{2+} ions [47], while 7% w/v was chosen as the lowest value, since lesser amounts did not allow obtainment of microspheres with an adequate consistency. Finally, 5 min of TCL was found to be the minimum time necessary to allow complete microsphere formation, and 20 min was chosen as the highest value, since longer times gave rise to a reduction in encapsulation efficiency, probably due to the drug elution from the beads during the microsphere formation. Other process variables such as temperature, stirring speed, and volume of dispersion medium were kept constant and fixed, respectively, at 25 °C, 300 rpm, and 50 mL, according to preliminary evaluations. Thus, the chosen independent variables ranged as follows:

U_1 = % alginate: 3–5% w/v; U_2 = % $CaCl_2$: 7–12% w/v; U_3 = % chitosan: 0–4% w/v; U_4 = TCL: 5–20 min.

3.2.2. Postulation of the mathematical model and definition of the experimental plan

In order to found the relationship able to describe the response variation inside the design space, a second order degree polynomial model was postulated among responses and factors:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1 x_1 + \beta_{22} x_2 x_2 + \beta_{33} x_3 x_3 + \beta_{44} x_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + +\beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{123} x_1 x_2 x_3 + \beta_{124} x_1 x_2 x_4 + \beta_{134} x_1 x_3 x_4 + \beta_{234} x_2 x_3 x_4 + \beta_{1234} x_1 x_2 x_3 x_4 + \varepsilon$$

Table 1
Experimental plan and obtained responses (entrapment efficiency (EE%) and % drug released after 4 h in colonic medium (DR%)).

Batch	Alginate% U_1	$CaCl_2$% U_2	CSL%[a] U_3	TCL[b] (min) U_4	EE% Y_1	DR% Y_2
1	5.0	9.5	2.0	12.5	94.6	6.50
2	3.0	9.5	2.0	12.5	92.1	12.0
3	4.5	12.0	2.0	5.0	91.4	58.7
4	3.5	7.0	2.0	12.5	98.7	55.3
5	4.5	7.0	2.0	12.5	96.6	27.3
6	3.5	12.0	2.0	12.5	100.0	60.3
7	4.5	10.3	4.0	12.5	91.2	19.1
8	3.5	8.7	0.0	12.5	87.0	62.3
9	4.5	8.7	0.0	12.5	76.5	14.4
10	4.0	11.2	0.0	12.5	85.4	21.0
11	3.5	10.3	4.0	12.5	91.1	31.9
12	4.0	7.8	4.0	12.5	92.0	8.5
13	4.5	10.3	2.5	20.0	96.2	1.9
14	3.5	8.7	1.5	5.0	88.8	25.8
15	4.5	8.7	1.5	5.0	88.6	18.8
16	4.0	11.2	1.5	5.0	89.4	22.7
17	4.0	9.5	3.5	5.0	89.0	51.7
18	3.5	10.3	2.5	20.0	97.5	65.3
19	4.0	7.8	2.5	20.0	91.1	6.8
20	4.0	9.5	0.5	20.0	91.9	1.0
21	4.0	9.5	2.0	12.5	95.9	5.0
22	4.0	9.5	2.0	12.5	96.7	10.3
23	4.0	9.5	2.0	12.5	96.1	9.6

[a] CSL = chitosan at low molecular weight.
[b] TCL = time of cross-linking.

Doehlert design was chosen for an accurate estimation of the model coefficients and in turn for a careful and complete description of the problems under study through the use of the relative response surfaces. This design has the advantage, with respect to other designs useful for response surface study, of studying the factors at a different number of levels, thus giving the possibility to investigate some factors in greater [34,35,39,40]. The number of experiments required by a Doehlert design is $k^2 + k + n$, where k is the number of factors and n the number of replicates at the center of the experimental domain. Therefore, the experiments needed in the present study were 23, including three replicates (Table 1).

3.2.3. Preparation of microsphere batches according to the experimental plan and their evaluation

The different batches were then prepared in compliance with the experimental plan of the matrix**.** All the formulations prepared within the experimental design layout yielded microspheres with a suitable consistency, uniform spherical shape, and homogeneous size. There was no statistically significant variation ($p > 0.05$) in particle dimensions among the different bead types, whose mean diameter varied from 1.23 ± 0.04 mm to 1.32 ± 0.05 mm ($n = 20$). The production yields were around 85–90%.

ESEM analysis (Fig. 3), being a non-invasive technique not requiring any pre-treatment of the sample before analysis and keeping unchanged its structural properties, allowed a careful examination of the actual morphological characteristics of the microspheres. All microspheres looked as regularly shaped, almost spherical particles. The surface of microsphere formulations lacking in CSL appeared perfectly smooth and uniform; typical formations of rectangular shape, homogeneously distributed on the microsphere surface, were instead observed in all other formulations, indicative of the presence of CSL. A section of such microspheres evidenced the absence of these rectangular structures in their internal core, confirming the dominant localization of CSL in the particle wall. Finally, microanalysis, carried out by the Energy Dispersive Spectrometer, allowed the demonstration of the homogeneous chemical composition of the microspheres throughout their whole mass.

All the batches were then evaluated in a randomized order for both encapsulation efficiency and drug release profile under pH gradient. Table 1 shows the experimental plan and the obtained responses, i.e., EE% and % of drug released after 4 h (DR%) in colonic medium.

Analysis of variance (ANOVA) was applied for testing the significance and validity of the postulated model, using a 1% significance level. ANOVA (Tables 2 and 3) indicated that the assumed regression model was significant and valid for both the examined responses [34]. This means that the found relationship was able to describe the response variation in function of factor variations and thus to carefully describe the design space.

As shown in Table 1, all the batches showed high EE% values, never less than 88%, except for microspheres without CSL, where the EE% decreased until 76.5%. The high EE% values are attributable to the very limited aqueous solubility of CXB, even as ternary co-ground system with HPβCD and PVP, which prevents drug diffusion from the gel network to the aqueous medium during the ionotropic gelation process. On the contrary, the DR% in colonic medium from the different batches showed a wide variation ranging from a minimum of 5% to a maximum of 65.3%, indicating that this response was very strongly dependent on the selected

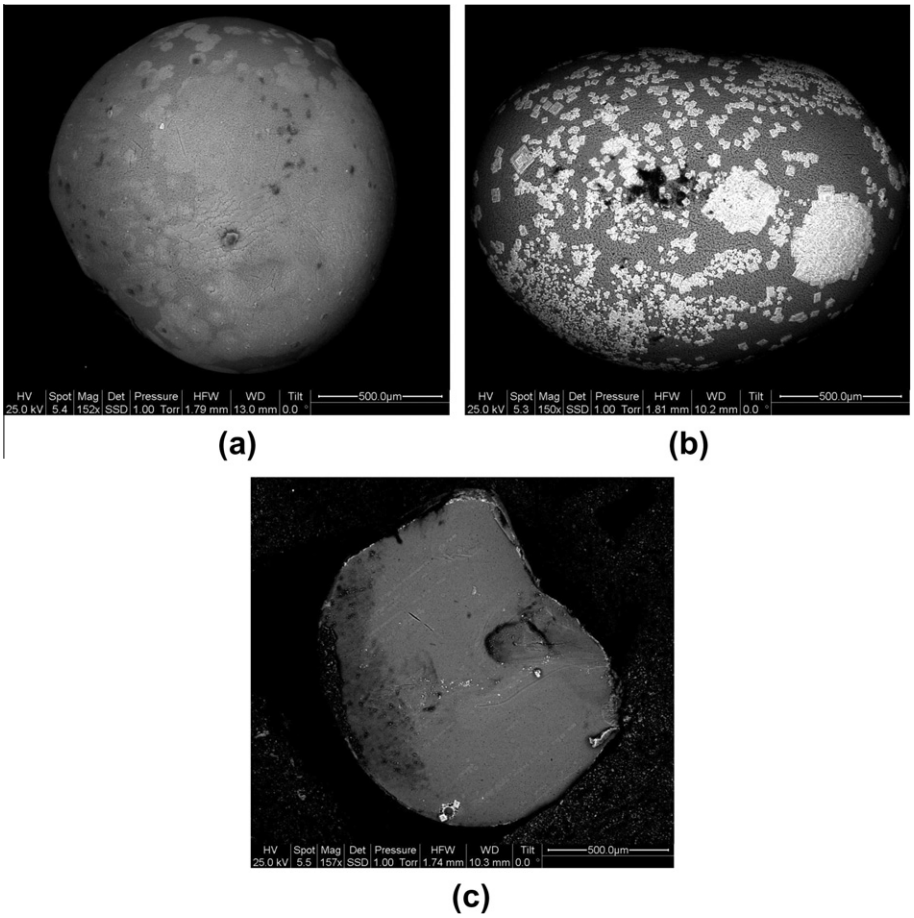

Fig. 3. ESEM photographs of microsphere batches without chitosan (a) or with chitosan (b) and a section of this latter (c).

Table 2
Summary of ANOVA for the response entrapment efficiency (EE%).

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-ratio
Regression	547.2532	14	39.0895	225.5164[a]
Residuals	40.4259	8	5.0532	
Validity	40.0792	6	6.6799	38.5377[b]
Error	0.3467	2	0.1733	
Total	587.6791	22		

[a] $225.51 > F^{crit.} = 5.559$ (with 14 and 8 degrees of freedom and $\alpha = 0.01$).
[b] $38.54 < F^{crit.} = 99.33$ (with 6 and 2 degrees of freedom and $\alpha = 0.01$).

Table 3
Summary of ANOVA for the response % of drug released at 4 h in colonic medium (DR%).

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-ratio
Regression	7.99078	14	5.70770	68.850[a]
Residuals	2.35547	8	2.94434	
Validity	2.33889	6	3.89815	47.022[b]
Error	1.65800	2	8.29000	
Total	1.03462	22		

[a] $68.85 > F^{crit.} = 5.559$ (with 14 and 8 degrees of freedom and $\alpha = 0.01$).
[b] $47.02 < F^{crit.} = 99.33$ (with 6 and 2 degrees of freedom and $\alpha = 0.01$).

variables in the experimental domain under study. On the other hand, it is important to point out that all chitosan-Ca^{2+} alginate microsphere batches proved to be able to effectively carry the entrapped drug up to the colonic medium, preventing its premature delivery in the first gastrointestinal tract. In fact, the percent of CXB released after 2 h in acidic medium (pH 1.1) was in all cases less than 0.5% and after the following 2 h in the small intestinal medium (pH 6.8) less than 1%.

3.2.4. Response surface study

Three-dimensional response surface plots were then generated, using the statistical model obtained from multiple regression analysis, in order to investigate more in depth the effects of changing the independent variables on the considered responses.

By keeping constant U_2, (i.e., $CaCl_2$%) and U_4 (i.e., CTL) variables at their intermediate values (9.5% w/v and 12.5 min, respectively), EE% increased with increasing CSL content in the microspheres, irrespective of the content of alginate (Fig. 4a). On the contrary, in the same conditions, the highest DR% value was observed for the lowest levels of both polymers, and a dramatic reduction in drug release was instead observed for high levels of alginate (Fig. 4b). Both these results can be explained by the electrostatic interaction between the carboxyl groups of alginate and the positively charged amino groups of CSL, leading to the formation of a poly-electrolytic complex between the polymers. As a consequence

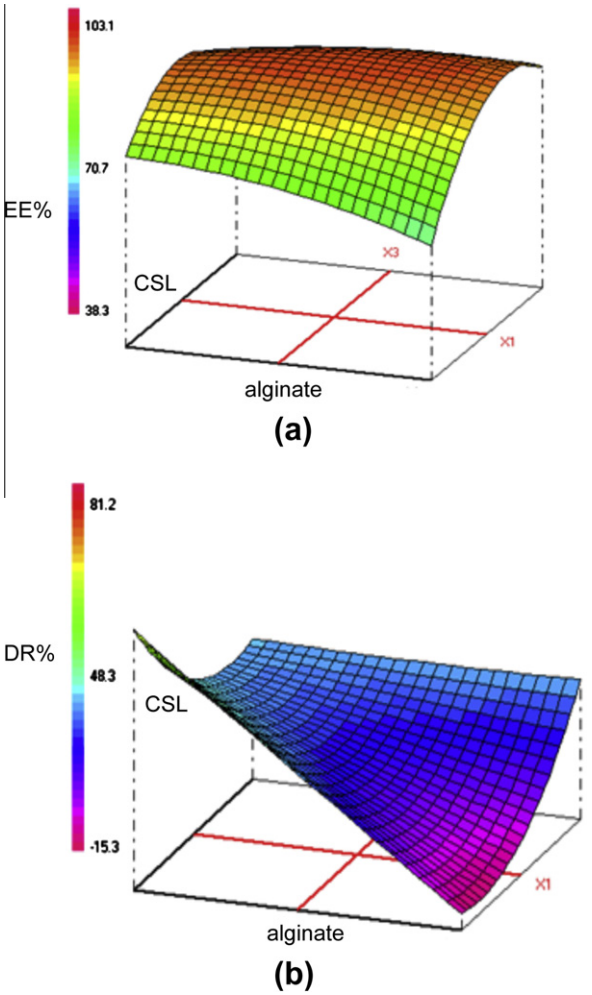

Fig. 4. Response surface plot of the effect of variations of % alginate (U_1) and CSL (U_3) on EE% (a) and % drug released at 4 h (DR%) (b). ($CaCl_2$ (U_2) and TCL (U_4) kept constant at 9.5% and 12.5 min, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

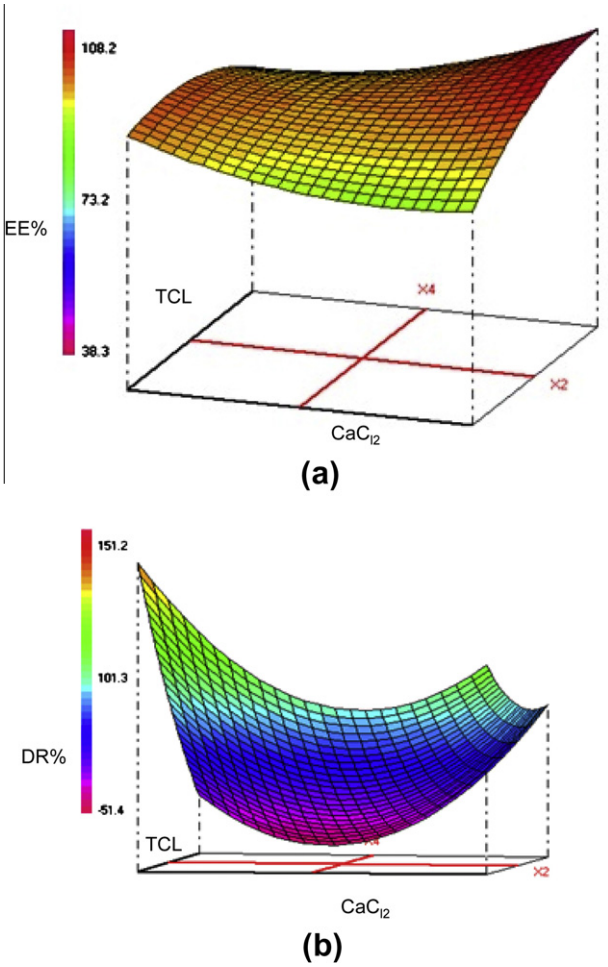

Fig. 5. Response surface plot of the effect of variations of % $CaCl_2$ (U_2) and TCL (U_4) on EE% (a) and % drug released at 4 h (DR%) (b). (% alginate (U_1) and CSL (U_3) kept constant at 4% and 2%, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of this, a more compact structure of the microspheres is formed, which, on the one hand, contributes to a more effective entrapment of the drug, but, at the same time, hampers its diffusion through the matrix. However, when the CSL concentration becomes too high, its positive effect on EE% is reduced, since the increased viscosity of the solution slows down the diffusion of CSL chains, hindering their interaction with the alginate chains. For the DR% response, the consequence of this last effect prevailed in the presence of high levels of alginate, revealing a positive interaction between the factors.

The effect of variations of cross-linking agent concentration and cross-linking time is shown in Fig. 5, where the levels of the other variables, i.e., alginate and CSL, were held constant at their middle values, i.e., 4% w/v and 2% w/v, respectively. EE% increased with increasing both $CaCl_2$% and TCL, because the cross-linking reaction is favored (Fig 5a), while an almost opposite behavior was instead observed for drug release (Fig 5b). The lower rate of CXB release could be a result of the increased rigidity of the polymer chains due to the formation of thicker junction zones [27].

Moreover, while EE% was scarcely influenced by variations of TCL and alginate content (Fig 6a), a negative interaction was found between these variables as for their influence on drug release (Fig. 6b): In fact, the best drug release values were observed for the highest values of the former and the lowest values of the latter and vice versa.

Finally, as expected, the entrapment efficacy was maximized when alginate percentage and $CaCl_2$ percentage were at their highest levels (Fig 7a), while high drug release values were obtained for the lowest levels of these same variables (Fig 7b). However, unexpectedly, high drug release values were also found for the highest values of $CaCl_2$ percentage. This result can be explained by the marked hygroscopicity of microspheres with high content of $CaCl_2$ which, when immersed in colon medium, exhibited the maximum swelling, thus favoring drug release.

3.2.5. Application of the desirability function

The careful analysis of the surface response plots revealed that the considered factors had in most cases an opposite influence on the considered responses. Therefore, it was necessary to apply a multicriteria decision approach, like desirability function, to find the best compromise between the values of the variables, in order to maximize at the same time both entrapment efficiency and drug release. Each response was associated with its own partial desirability function d_i, which varied from 0 to 1, according to the closeness of the response to its target value [34]. The individual

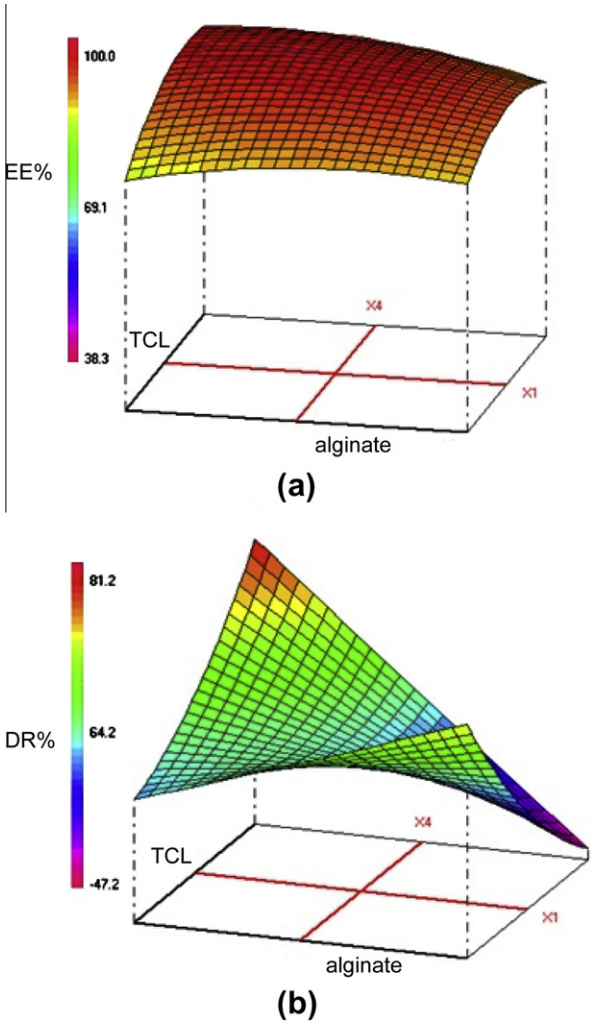

Fig. 6. Response surface plot of the effect of variations of % alginate (U_1) and TCL (U_4) on EE% (a) and % drug released at 4 h (DR%) (b). ($CaCl_2$ (U_2) and CSL (U_3) kept constant at 9.5% and 2%, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

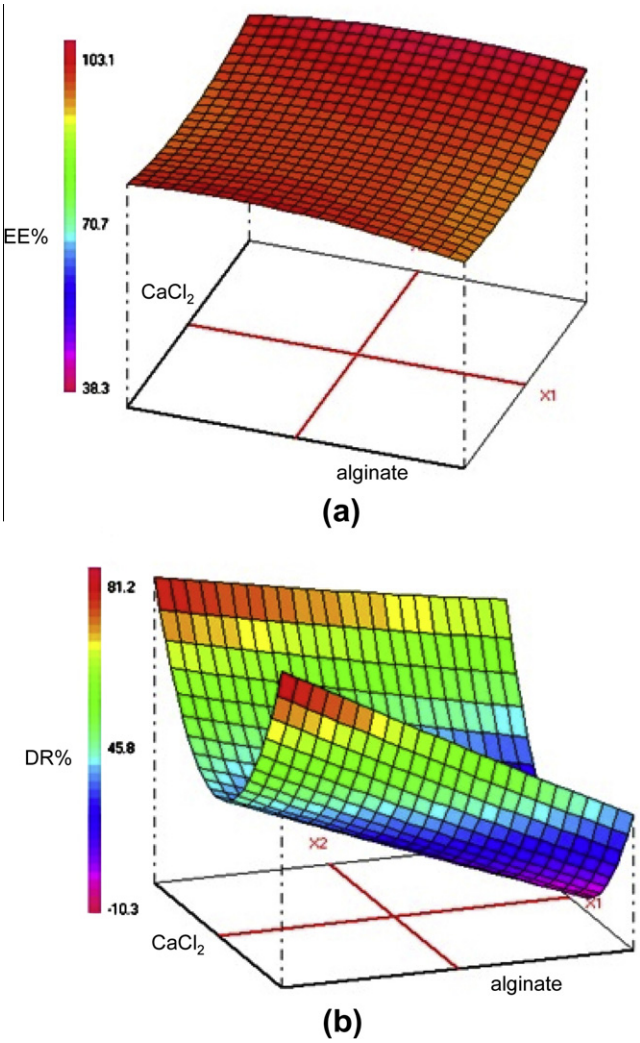

Fig. 7. Response surface plot of the effect of variations of % alginate (U_1) and $CaCl_2$ (U_2) on EE% (a) and % drug released at 4 h (DR%) (b). (CSL (U_3) and TCL (U_4) kept constant at 2% and 12.5 min, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

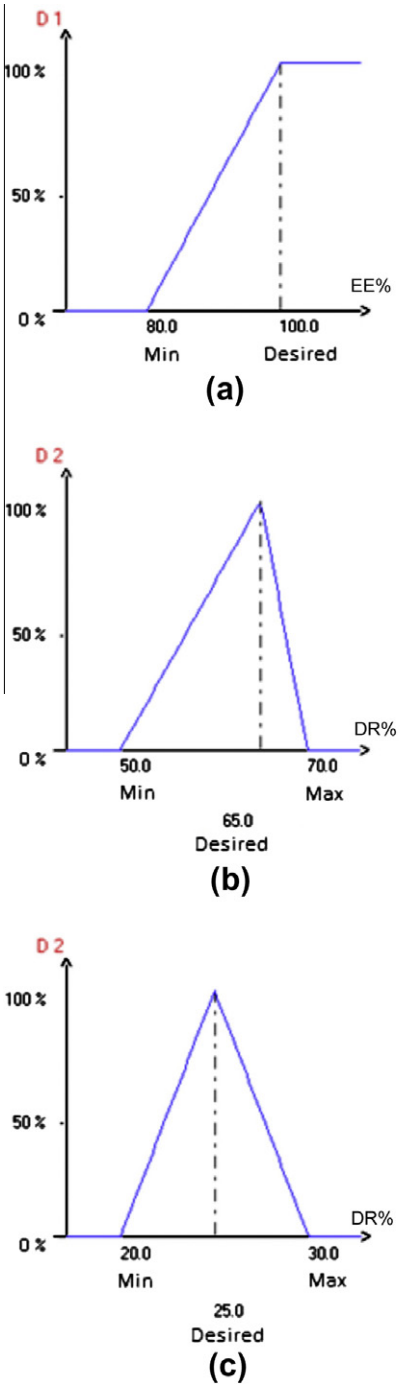

Fig. 8. Graphic representation of partial desirability for EE% (a) and % drug released at 4 h (DR%) in case of systemic use (b) or local use (c) of celecoxib. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

desirability functions were then combined together, as the geometric mean, to obtain the overall desirability function (D) for the system whose maximum value could then be looked for within the domain [37]. In this case, the partial desirability functions (d_1, d_2) for the two responses Y_1 and Y_2, respectively, are presented in Fig. 8. In particular, as for the EE% response, 80% was selected as the lowest value of desirability ($d_i = 0$) and 100 as the highest value ($d_i = 1$). As for the DR%, two different desirability functions have been considered, one related to the obtainment of a colon release profile for a systemic use of CXB in the treatment of rheumatoids arthritis, suitable to the circadian rhythm of this pathology, and the other to the obtainment of a colon release pofile for a local use of CXB in the prophylaxis of colorectal cancer. In the first case, the partial desirability function was set with the lowest value at 50% ($d_i = 0$) and the highest value at 70% ($d_i = 1$) of DR%; in the second case, where the objective is to assure the highest permanence time of the drug at colon level, the lowest acceptable value was fixed at 20% ($d_i = 0$) and the highest one at 30% ($d_i = 1$).

Table 4
Values of the variables for the optimized colon-targeted formulation of celecoxib (CXB) aimed at systemic or local use and predicted responses (entrapment efficiency (EE%) and % drug released after 4 h in colonic medium (DR%)).

CXB use	Alginate% U_1	$CaCl_2$% U_2	CSL%[a] U_3	TCL[b] (min) U_4	EE% Y_1	DR% Y_2
Systemic	3.9	7.2	0.5	12.0	90.0 ± 2.9	65.0 ± 2.9
Local	4.5	11.0	2.6	18.5	99.6 ± 2.1	25.0 ± 2.5

[a] CSL = chitosan at low molecular weight.
[b] TCL = time of cross-linking.

The predicted variable values that simultaneously optimize both EE% and DR% (in case of both systemic and local use of the drug), calculated by the software on the basis of the above defined partial desirability functions, are reported in Table 4. The confidence interval for each predicted response at a probability level of 95% was calculated using the mean and the standard deviation obtained from replicates ($n = 4$) carried out with the predicted optimum conditions.

3.2.6. Evaluation of the optimized formulation and of the model predictive ability

In order to validate the predictive ability of the hypothesized model for each response around the optimized conditions, the agreement between predicted and measured responses was verified. Therefore, CXB microspheres were prepared according to the optimized conditions for both systemic and local use of the drug and characterized for entrapment efficiency and % drug released at 4 h in colonic artificial medium.

The experimental values were Y_1 91.1 ± 0.8% and Y_2 63.8 ± 1.8% for the formulation of systemic use and Y_1 98.7 ± 0.9% and Y_2 23.9 ± 1.3% for the formulation of local use. In both cases, the predicted values (see Table 4) were inside the confidence interval for each response, thus indicating statistical equivalence between experimental data and the predicted ones and demonstrating the validity of the applied model.

4. Conclusion

It was shown that QbD approach can be successfully used in the development of colon-targeted microsphere formulation of CXB with predictable entrapment efficiency and drug release properties.

In particular, DoE allowed the simultaneous evaluation, by a response surface study, of the effects of the selected variables, i.e., % of alginate, calcium chloride, and CSL and time of cross-linking, on the considered responses to be optimized, i.e., microsphere EE% and % of drug released after 4 h (%DR) in colonic artificial medium.

Since the changes in the considered factors exhibited a general opposite influence on the two evaluated responses, the use of desirability function was necessary, to find the best compromise which allowed simultaneous optimization of both the considered responses.

The experimental values of the responses obtained from the optimized microsphere formulations were very close to the predicted values, both in case of those aimed for systemic or for local use of the drug, demonstrating the actual reliability and usefulness of the assumed model in the preparation of colon-targeted CXB microspheres with optimized and predictable properties, suitably

adaptable to obtain the desired drug release profile. Design space was defined according to ICH requirements.

It can be expected that this application of the DoE tools in QbD approach could be useful for further formulation studies, where microspheres with different drug release profiles could be required.

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