Box-Behnken design: Optimization of insulin-loaded nanoparticles as reservoirs for oral delivery

C. Woitiski[1], F. Veiga[1] A. Ribeiro[1] and R. Neufeld[2] *

[1] Laboratory of Pharmaceutical Technology, University of Coimbra, Coimbra, Portugal (cwoitiski@ci.uc.pt);
[2] Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada.

Introduction

The design of strategies that improve the absorption of insulin through the gastrointestinal tract is a considerable challenge in the pharmaceutical sciences and would significantly enhance the treatment of diabetes mellitus (C. Woitiski et al. (2008)). Several strategies have been devised to overcome physiological and morphological barriers to insulin absorption, including formulation of carrier systems. Nanoparticles are potential candidates in terms of engineering novel carrier systems by providing a stable and biocompatible environment to ensure that insulin remains biologically active during particle formulation and delivery to the target site.

The design of a functional delivery system involves distinct, yet interconnected processes of identification of the objectives and the parameters of physiological and pharmacological properties, formulation and characterization of the delivery system, followed by the development of experimental and possibly computational models for optimization of structure-function relationships, and performance of clinical studies for verification of the optimal delivery system (C. Woitiski et al. (2008)).

Chemometric tools have been frequently applied to the design and development of drug delivery systems, considering the advantages such as reduction in the number of experiments that need to be performed (S. Ferreira et al. (2007)). Systematic optimization procedures are carried out by selecting an objective function, screening the contributing factors and investigating the relationship between responses and factors by the response surface methodology (S. Motwani et al. (2008)). The optimum strategies are attained by using experimental designs such as Box-Behnken design. Box-Behnken is a useful design for response surface methodology because it permits estimation of the parameters of the quadratic model; building of sequential designs; detection of lack of model fit; and use of blocks (S. Ferreira et al. (2007)).

The purpose of this study was to develop and optimize reservoir systems for insulin delivery combining mucoadhesive, biodegradable, biocompatible and acid-protective biomaterials. The current study was performed to evaluate the effect of three factors (calcium chloride, chitosan and bovine serum albumin concentrations) on particle size, loading efficiency and insulin *in vitro* release from nanoparticles, and to optimize the levels of the factors using response surface methodology combined with Box-Behnken experimental design.

Materials and Methods

Low viscosity sodium alginate, chitosan (50 kDa), bovine serum albumin (BSA) (Sigma-Aldrich Chemie, France), dextran sulfate (5 kDa) (Fluka Biochemika, Switzerland), poloxamer 188 (BASF, Germany) calcium chloride (Riedel-Haen, Germany) and actrapid INN- insulin human (rDNA) (Novo nordisk, Denmark).

Preparation of insulin-loaded nanoparticles - The current study reports a slightly modified method to prepare insulin-loaded nanoparticles based on ionotropic pre-gelation followed by polyelectrolyte complexation of polymers carrying opposite charges under controlled pH and stoichiometric conditions. (B. Sarmento et al, 2007). Nanoparticles were prepared under 800 rpm at room temperature by dropwise extrusion of 7.5 mL of calcium choride solution into 117.5 mL of a solution at pH 4.9 containing 0.06% (w/v) of sodium alginate, 0.04% (w/v) of dextran sulfate, 0.04 (w/v) of poloxamer 188 and insulin equivalent to 200 UI. Into the pre-gel, 25 ml of chitosan solution at pH 4.6 was added dropwise, and 25 mL of BSA solution at pH 5.1 was added to stabilize the pre-gel nuclei into nanoparticles. Nanoparticles were concentrated by dialysis using regenerated cellulose membranes with tubing nominal dry thickness of 10K MWCO**.**

Experimental Design - A three factor, three-level Box-Behnken design was used for the optimization of formulation of insulin-loaded nanoparticles with calcium chloride (X1), chitosan (X2) and BSA (X3) concentrations as the independent variables (Table 1). The factors and levels of these three parameters were determined from preliminary studies. The particle size, loading efficiency and insulin release in simulated digestive fluids after 120 and 300 minutes were used as dependent variables (Table 1). Design-Expert software (v.7.0 Stat-Ease Inc., Minneapolis, USA) was used for the generation and evaluation of the statistical experimental design.

Factor	Levels used		
Independent variables	-1	0	+1
X1=Calcium chloride (% w/v)	0.20	0.22	0.24
X2=Chitosan (% w/v)	0.04	0.07	0.11
X3=BSA(% w/v)	0.25	0.50	0.75
Dependent variables	Constraints		
Y1= Particle size (nm)	Minimize		
Y2= Loading Efficiency (%)	Maximize		
Y3= Release simulated gastric fluid (120 minutes) (%)	Minimize		
Y4= Release simulated intestinal fluid (300 minutes) (%)	Maximize		

Table 1: Variables in Box-Behnken design

Particle size analysis - Particle size was determined by photon correlation spectroscopy at 20ºC with a detection angle of 90º and water as diluent ($n \geq 6$). (N5 Particle Analyzer - Beckman Coulter Inc., USA).

Loading efficiency analysis - Loading efficiency was determined by a validated HPLC technique (LC-2010 HT HPLC system - Shimadzu Co., Japan) and calculated by the difference between the total amount of insulin used to prepare the particles and the amount of insulin analyzed in the supernatant after centrifugation (20,000xg/45 min).

Insulin *in vitro* release from nanoparticles – 10 mg of nanoparticles were incubated (37ºC/2h) in 10 mL of simulated gastric fluid without pepsin (USP XXX), and 200 µL of supernatant was collected at predetermined times and replaced by the same volume of fresh incubation medium. Samples were centrifuged (20,000xg/15 min) and the supernatant was used for insulin determination by HPLC technique. Nanoparticles were recovered by centrifugation and incubated (37ºC/3 h) in 10 mL of simulated intestinal fluid without pancreatin (USP XXX), and the same procedure was performed for sampling and insulin determination.

Results and Discussion

The preparation of insulin-loaded nanoparticles, based on an ionotropic gelation process, involves gelification of alginate by calcium ions and the formation of nanoparticles by complexation of biomaterials. Because of the effect of calcium ions on formation of the pregel state, and the effect of

chitosan and BSA concentrations on formation of the nanoparticles, experiments were performed in order to screen the optimum conditions correlating the dependent variables and response variables. The observed responses of particles size, loading efficiency and insulin *in vitro* release from nanoparticles in simulated digestive fluids are presented in Table 2.

Batch	Dependent variables			Independent variables			
	X1 (%)	X2 (%)	X3 (%)	Y1 (nm) (mean ± SD)	Y2 (%)	Y3 (%)	Y4 (%)
1	0.20	0.04	0.50	394 ± 193	100	0	92
2	0.24	0.04	0.50	540 ± 259	100	0	81
3	0.20	0.11	0.50	472 ± 225	89	14	93
4	0.24	0.11	0.50	543 ± 264	93	12	100
5	0.20	0.07	0.25	488 ± 247	94	0	93
6	0.24	0.07	0.25	588 ± 285	100	0	91
7	0.20	0.07	0.75	406 ± 188	85	33	98
8	0.24	0.07	0.75	507 ± 247	88	28	98
9	0.22	0.04	0.25	516 ± 286	100	0	89
10	0.22	0.11	0.25	546 ± 264	100	8	86
11	0.22	0.04	0.75	444 ± 214	90	19	95
12	0.22	0.11	0.75	495 ± 240	90	24	93
13a	0.22	0.07	0.50	551 ± 264	94	0	94
14a	0.22	0.07	0.50	552 ± 270	94	0	96
15a	0.22	0.07	0.50	561 ± 271	95	0	94

a Indicates the center point of the design

Table 2: Composition and observed responses in Box-Behnken design

It is observed that the nanoparticles size, as previously reported (S. Motwani et al. (2008)), is dependent upon the concentration of chitosan, and also of calcium chloride. The minimum size i.e. 394 nm corresponds to the lowest chitosan and calcium chloride concentrations. It confirms that smaller nanoparticles results when the availability of the function groups on the alginate and chitosan for interactions is in stoichiometric proportion, and when low concentrations of calcium ions are available, being higher concentrations leading to intermolecular crosslinking of alginates resulting in aggregates (B. Sarmento et al. (2007). The loading efficiency of insulin was primarily affected by BSA concentration. It was observed that the entrapment of insulin was highest when BSA concentration was lowest. The hypothesis is based on a competitive interaction between insulin and BSA with the polyelectrolytes. It also explains the influence of BSA concentration on insulin release from nanoparticles observed in the simulated gastric fluids. It is also dependent of chitosan concentrations. Chitosan dissolves easily at low pH and the mechanism of pH sensitive swelling involves the protonation of amine groups that leads to chain repulsion, diffusion of proton and counter ions with water inside the particles and dissociation of secondary interaction (K. Yao et al. (1994)). This mechanism has probably more influence when higher concentrations of chitosan are presented. The results of insulin release from nanoparticles in simulated intestinal fluid can demonstrate the feasibility of this reservoir in releasing insulin at the desirable site for its absorption with is facilitated by the multifunctional biomaterials like bioadhesiveness to the intestinal mucosa.

Three-dimensional response surface plots drawn for the graphical optimization of insulin-loaded nanoparticles are presented in Figure 1-4, which are very useful to study the interaction effects of two independent variables on the responses, when the third is kept at constant level. All the relationships among the three variables were non-linear.

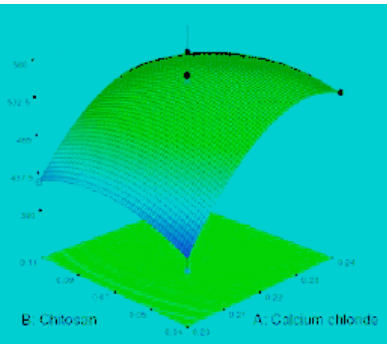

Figure 1: Response surface plot representing the effect of calcium chloride (X1) and chitosan (X2) on particle size (Y1).

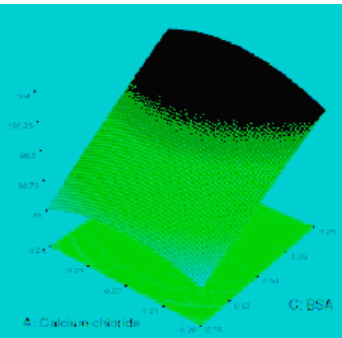

Figure 2: Response surface plot representing the effect of calcium chloride (X1) and BSA (X3) on loading efficiency (Y2).

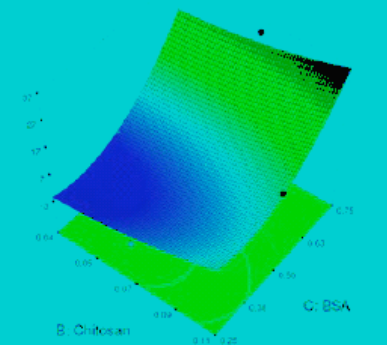

Figure 3: Response surface plot representing the effect of chitosan (X2) and BSA (X3) on insulin release from nanoparticles in simulated gastric fluid (Y3).

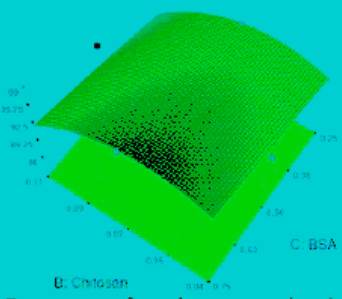

Figure 4: Response surface plot representing the effect of chitosan (X2) and BSA (X3) on insulin release from nanoparticles in simulated intestinal fluid (Y4).

Conclusion

Box-Behnken design was used to statistically optimize the formulation parameters and evaluate the main effects and interaction effects of the independent variables on the particle size, loading efficiency and insulin *in vitro* release from nanoparticles. A 3-factor, 3-level design was used to explore the quadratic response surfaces and for constructing a second order polynomial model (data not shown). Insulin-loaded nanoparticles as reservoirs for oral delivery were successfully formulated and optimized by using experimental design. Upon trading of various response variables and comprehensive evaluation of the feasibility search, the formulation composition with 0.20 % of calcium chloride, 0.04% of chitosan and 0.47% of BSA was determined to fulfill requisites of an optimum formulation.

Bibliography

C. Woitiski et al. (2008) *Strategies toward the improved oral delivery o insulin nanoparticles via gastrointestinal uptake and translocation.* Biodrugs 22(4): 223-237.

S. Ferreira et al. (2007) *Box-Behnken design: An alternative for the optimization of analytical methods.* Anal Chim Acta 597: 179-186.

S. Motwani et al. (2008) *Chitosan-sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: Formulation, optimization and in vitro characterization.* Eur J Pharm Biopharm 68: 513-525.

B. Sarmento et al. (2007) *Insulin-loaded nanoparticles are prepared by alginate ionotropic pre-gelation followed by chitosan polyelectrolyte complexation.* J Nanosci Nanotechnol 7: 1-9.

K. Yao et al. (1994) Swelling kinetics and release characteristics of crosslinked chitosan-polyether polymer network (semi-IPN) hydrogels. J Polym Sci, A, Polym Chem 32: 1213-1223.