

DEVELOPMENT AND EVALUATION OF A LYOPHILIZED PRODUCT CONTAINING INSULIN¹

DESENVOLVIMENTO E AVALIAÇÃO DE PRODUTO LIOFILIZADO CONTENDO INSULINA

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ABSTRACT

This study aimed at developing and evaluating lyophilized products from F127 (F127) Pluronic solutions containing insulin (INS) and oleic acid (OA) as absorption enhancer. The release of INS from gelatin capsules containing the lyophilized products in different media and at the average size of micelles/vesicles generated in the release media were the parameters evaluated to characterize the formulations. Results indicated that the release media played an important role in the liberation of INS from capsules containing the lyophilized products. Additionally, small micelles/vesicles were formed in the release media during the INS release, which may be decisive for in vivo INS permeation through the intestine membrane. Taking into consideration the smaller size of micelles/vesicle formed in media containing bile salts, and a slower release of INS in these media, F127 formulations containing unsaturated fatty acids are possible candidates for developing oral formulations of insulin.

Keywords: In vitro release, Micelles/vesicle size, Hydrogel.

RESUMO

Este trabalho teve como objetivo desenvolver e avaliar produtos liofilizados a partir de soluções de Pluronic F127 (F127) contendo insulina (INS) e ácido oleico (OA) como promotor de absorção. A liberação de INS em diferentes meios a partir de cápsulas de gelatina contendo o produto liofilizado e o tamanho médio das micelas/vesículas geradas no meio de liberação foram os parâmetros avaliados para caracterizar as formulações. Os resultados demonstraram que o meio de dissolução influenciou a liberação de INS a partir de cápsulas contendo o produto liofilizado. Adicionalmente, pequenas micelas/vesículas foram formadas nos meios de dissolução contendo sais biliares durante a liberação de INS, fato que pode ser decisivo in vivo para a permeação de INS através da membrana do intestino. Levando em consideração as micelas/vesículas de menor tamanho formadas em meio contendo sais biliares e uma liberação mais lenta de INS nestes meios, formulações de F127 contendo ácidos graxos insaturados são possíveis candidatos ao desenvolvimento de formulações orais de INS.

Palavras-chaves: Liberação in vitro, Tamanho de micelas/vesículas, Hidrogel

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INTRODUCTION

Noninvasive methods for peptides administration, such as insulin (INS), to the systemic circulation and, thus, to a target cell or organ have received considerable attention from the pharmaceutical industry and the medicine (SILVA *et al.*, 2003). Alternative routes for INS administration have been investigated and includes: pulmonary, oral, transdermal, nasal, ocular and rectal routes (OBACH *et al.*, 1999). However, the subcutaneous administration of INS is still remained as the main route used for treating diabetes type 1. To date, only the pulmonary route was clinically approved to replace it (SIEKMEIER *et al.*, 2008).

Oral administration of INS would be the most convenient for diabetic patients since the passage of this hormone by the liver could prevent or mitigate systemic hyperinsulinemia and its complications, common in subcutaneous administration (FONTE *et al.*, 2013). The high molecular weight (~ 6 kDa) and the low lipophilicity are limiting factors that carry INS to a very low absorption by the mucosal membrane. The proteolytic activity of enzymes in the stomach and small intestine also account for the rapid degradation of INS orally administered (FONTE *et al.*, 2013). Additionally, the INS mucosal administration without the use of absorption promoters produces no appreciable change in INS plasma concentrations. Therefore, besides the protection of the molecule, the use of absorption promoters, such as surfactants, bile acids or unsaturated fatty acids, are required to increase INS bioavailability after oral administration (CHOONARA *et al.*, 2014).

Fatty acids are endogenous compounds present in human skin (LAMPE *et al.*, 1983) and biomembranes (FISCHER, 1989). They are very often used as absorption promoters in drug delivery systems for lungs and intestine (SHARMA *et al.*, 2005). The use of 2% oleic acid (OA) in a W/O/W emulsion as an absorption enhancer of INS was demonstrated to cause an important hypoglycemic effect with little damage to the rectal mucosa, and no tissue damage to the colon when the formulation was administered rectally (MORISHITA *et al.*, 2000).

Pluronic® F-127 (F127) is a non-ionic surfactant whose aqueous solutions at 20% or more are micellar liquids at low temperatures, turning into a semi rigid gel by increasing the temperature above 25°C (DUMORTIER *et al.*, 2006). We have previously demonstrated that F127 formulations containing unsaturated fatty acids resulted in an important hypoglycemic effect of INS after rectal (BARICHELLO *et al.*, 1999a) and buccal (MORISHITA *et al.*, 2001) administration to normal rats.

In this work, we designed a lyophilized product from F127 solutions containing OA and INS with the perspective to use it in the future development of colon-specific formulations for oral administration of INS. The Lyophilized product was evaluated *in vitro* in regard to INS release profile in different media and the mean size of the micelles aroused in the different release media tested.

MATERIAL AND METHODS

Material

Pluronic F-127 (F127), D,L- α -tocopherol, Oleic acid (OA), bile salts (BS) and trifluoroacetic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Potassium dihydrogenphosphate, di-sodium hydrogen phosphate anhydrous, acetonitrile (HPLC grade) and sodium chloride (NaCl) were purchased from Merck (Darmstad, Germany). Hydrochloric acid (HCl) titrant solution was purchased from Synth (Diadema, SP, Brazil) and sodium hydroxide (NaOH) titrant solution was purchased from Cromoline (Diadema, SP, Brazil). Erythrosine dye was purchased from Vetec (Duque de Caxias, RJ, Brazil). The human insulin (INS) was a kind gift from Novonordisk (Montes Claros, MG, Brazil) and the hard-gelatin capsules Catalent (Sorocaba, SP, Brazil), size 00, were a kind gift from Farmácia Acurácia (Mariana, MG, Brazil).

Preparation of F127 solutions

The F127 solutions were prepared as previously described (BARICHELLO *et al.*, 1999b). A weighed amount of F127 (12 and 16%) was slowly added to a phosphate buffer saline solution pH 7.4 (PBS 7.4) in an ice-water bath under gentle mixing by using a magnetic stirrer. The F127 solution was stored in a refrigerator overnight to allow complete dissolution of the polymer. The F127 solution was placed in an ice-water bath and D,L- α -tocopherol (0.06%) was dispersed under gentle mixing. Then, OA (2,0%) was added under vigorous agitation until a milky solution was obtained. INS (1 mg/g of solution) was dissolved in 0.1M HCl solution and directly added to the F127 solution. The acid was neutralized with a 0.1M NaOH solution.

Freeze-drying process

The Freeze-drying process was realized as previously described (REY *et al.*, 1999). The F127 solutions were poured into capsules body (00 size) and the capsules body were rapidly frozen for 20 minutes in a -80°C freezer (Forma-86C freezer ULT, Thermo Scientific, Brazil). The weights of capsules body were registered before and after filling. The frozen capsules body containing the formulations were lyophilized at a condition of -40°C, under vacuum (pump speed of 10 m³/h), for approximately 20 hours, in a lyophilizer L101 (Liobrás, Brazil). The capsules body containing the lyophilized products were weighted, closed with the capsule cup and stored in a -20°C freezer until use.

***In vitro* insulin release study**

The *in vitro* INS release from capsules containing the lyophilized products was done as follows: one capsule (about 0.8 g) was submersed in a flat-bottomed falcon tube containing 5 mL of a release medium. The release media evaluated were: 0.1M hydrochloric acid, pH 0.8 (0.1M HCl); phosphate buffer saline solution, pH 7.4 (PBS 7.4); 0.1% bile salt aqueous solution (0.1% BS) and; 1.0% bile salt aqueous solution (1.0% BS). The tubes were fixed to a horizontal shaking tray of a water bath. The temperature of the water bath was set to 37°C and the horizontal movement set to 75 rpm. After two hours, the shaking was stopped and 1 mL of the release media was collected and filter through a 0,45 µm PTFE filter, 13 mm Ø (Allcrom, São Paulo, Brazil) to quantified the INS released. Each formulation was evaluated in triplicate.

Quantification of insulin

The quantification of INS was proceeded as previously described (BARICHELLO *et al.*, 1999c) The INS amount in each sample was quantified in an auto sampler Waters HPLC System Alliance 2695 with a Waters 2996 PDA detector. A Phenomenex Luna C18 column (150x4.60 mm) (Allcrom, São Paulo, Brazil), with a 5µm particle diameter and a pore size of 100Å was used. The mobile phase composed of acetonitrile, 0.1% trifluoroacetic acid and sodium chloride (31:69:0.58, v/v/w) was eluted at a flow rate of 1.0 ml/min. The injection volume was 100µL and the detection of INS was realized in the wavelength of 214 nm.

Mean micelle/vesicles size in the release media

Aliquots of the release media were collected at the end of the INS release study to determine the mean micelle/vesicle size in the release media. Those aliquots were properly diluted with ultrapure water and transferred to a quartz cuvette for analysis by dynamic light scattering in a Nanosizer N5 Submicron Particle Size Analyser (Beckman Coulter, USA). The incident scattering angle was set to 90°. The analyses were realized in quadruplicate and the lower diffusion coefficient obtained was discharged. The mean micelle/particle sizes were compared among the dissolution media and between the formulations.

Statistical analysis

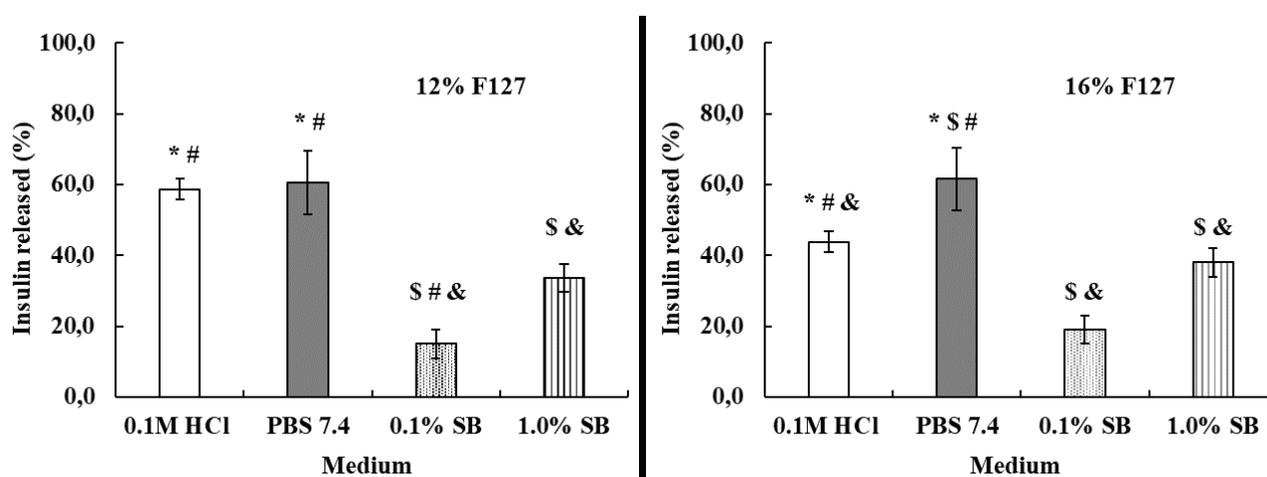
The unpaired Student t test or one-way ANOVA was used. Thereafter, post hoc multiple comparisons of data were analyzed with Tukey-Kramer test. The existence of difference between the amounts of INS released was considered significant when $P < 0.025$, adopting the confidence interval

of 95%. The release data were expressed as the mean \pm standard deviation. The BioEstat software (Belém, PA, Brazil) was used for statistical analysis (AYRES *et al.*, 2007).

RESULTS AND DISCUSSION

The *in vitro* release study permits to evaluate the effect of different formulation compositions on drug release, as well as the effect that the release media exert on formulation performance. The release of INS from capsules containing the lyophilized products was evaluated in 0.1M HCl, PBS 7.4, 0.1% BS and 1.0% BS media and the results were compared among them.

Figure 1- Percentage of insulin released from capsules containing 12% and 16% F127 lyophilized product after two hours in different dissolution media. Legend: significant difference between the INS released amount in the medium compared to: (*) BS 0.1%; (#) BS 1.0%; (\$) 0.1M HCl and (&) PBS 7.4.



The INS release profile from capsules containing 12% F127 lyophilized product in different media is shown in Fig. 1. It is observed in Fig. 1 that the release profile of INS from capsules containing 12% F127 was affected by the dissolution media used. In 0.1% BS media, the percentage of INS released was the lowest (15.02 \pm 4.04%), followed by 1.0% BS (33.69 \pm 3.94%), 0.1M HCl (58.62 \pm 2.92%) and PBS 7.4 (60.61 \pm 8.95%). For capsules containing 12% F127, the higher percentage of INS released were in 0.1M HCl and PBS 7.4, where INS was freely soluble.

The INS release profile from capsules containing 16% F127 lyophilized product in different media is also shown in Fig. 1. It is observed in Fig. 1 that the release profile of INS from capsules containing 16% F127 was also affected by the dissolution media used. The crescent order of INS release from capsules containing 16% F127 lyophilized product was 0.1% BS (19.05 \pm 1.64%), 1.0% BS (37.98 \pm 1.99%), 0.1M HCl (43.75 \pm 8.81 %) and PBS 7.4 (61.53 \pm 2.31%) media, following the same order observed for capsules containing 12% F127. The percentage of INS released from capsules containing 16% F127 lyophilized product was also negatively affected by the presence of BS in the dissolution media.

Collecting together, these results showed that the dissolution media significantly affected the release behavior of INS from capsules containing 12% and 16% F127 lyophilized products, and that the presence of BS in the media has promoted the slowest release of INS.

In a lyophilized product, it was expected that the 16% F127 formulation could dissolve slower than the 12% F127 formulation because the higher concentration of the polymer would generate smaller ice crystals during the freezing step, reducing the pores size and the diameter of network connections formed by the dry layer after water sublimation (ANDERSON *et al.*, 2001; BOSS *et al.*, 2004; MUDASSIR *et al.*, 2019, YOU *et al.*, 2019). When comparing the effect of F127 concentration on the percentage of INS released in the same medium, statistically significant difference was only observed in 0.1M HCl medium ($P = 0.0022$). These result might indicate that the higher surface area created during the lyophilization process, when in contact with the release medium, favored the rapid rehydration of the capsules and the lyophilized product inside of them, overshadowing the effect of the concentration of the polymer, leading to a similar release profile of INS, except in acidic medium wherein INS is readily soluble, and the higher concentration of F127 has promoted the slowest release of INS (BOSS *et al.*, 2004).

Table 1 - Mean micelle/vesicles size formed in different media after insulin release from gelatin capsules containing 12% and 16% F127 lyophilized products.

Lyophilized product	Dissolution media	Mean micelle size (nm)	Polydispersity index ^b
12% F127	0.1M HCl	1151.77 ± 146.76	1,60 ± 0.22
	PBS 7.4	98.23 ± 26.09	1.16 ± 0.36
	0.1% BS	63.93 ± 3.20	0,98 ± 0.04
	1.0% BS	514.45 ± 53.81	1,09 ± 0.10
16% F127	0.1M HCl	713.55 ± 2.89	1.38 ± 0.11
	PBS 7.4	38.90 ± 1.57	0.96 ± 0.22
	0.1% BS	41.15 ± 3.86	0.96 ± 0.07
	1.0% BS	252.00 ± 94.46	1.75 ± 0.36

Data are expressed as mean ± SD, n = 3.

Fonte: elaborada pelos autores.

In Table 1 is presented the mean size of micelle/vesicle formed in the media after INS release from capsule containing 12% and 16% F127 lyophilized products. According to Table 1, the smallest mean micelle/vesicle size was observed in 0.1% BS medium. Additionally, it was observed that the mean micelle/vesicle size formed from capsules containing 12% F127 lyophilized product in all media were bigger than capsules containing 16% F127 lyophilized products, but both formulations followed the same ascending order of mean micelle/vesicle size of 0.1% BS, PBS 7.4, 1.0% BS and 0.1M HCl medium. Interestingly, the data in table 1 also indicated that the increase in BS concentration in the dissolution medium increased the mean micelles/vesicle size over eight times from 12% F127 formulation and six times from 16% F127 formulations.

BS are organic surfactants present in the intestinal lumen responsible for the *in vivo* emulsification of fat material to assist in the absorption of fatty acids, monoglycerides, cholesterol and other lipids (DAVIES *et al.*, 2002). This ability makes BS capable of improving the bioavailability of hydrophilic drugs by increase its rate of solubilization/dissolution on the intestinal tract, at the same time it promotes the emulsification of fat material (AHMAD *et al.*, 2017).

As amphiphilic molecules with hydrophobic and hydrophilic regions, bile salts accommodate their selves at the lipid/water interface (DAVIES *et al.*, 2002). It is suggested that the amphiphilic behavior of BS could facilitate their incorporation into the dried micelles of F127 during rehydration of capsules containing the lyophilized products. Its incorporation into the micellar structure of F127 could increase the micelles size, reducing the number and the dimension of the water channels and, consequently, obstructing the INS release.

Furthermore, Torcello-Gómez *et al.* (2013) have demonstrated that BS are able to form mixed micelles in presence of other amphiphilic molecules like F127. The formation of mixed micelles in presence of BS was shown to affect the micellization ability of F127. Therefore, in 1.0% BS medium, the concentration of BS could be higher enough to form micelles and to solubilize lipids and hydrophobic substances (SHARMA *et al.*, 2005), affecting the erosion and dissolution of the F127 hydrogel matrix in comparison to 0.1% BS medium and, consequently, accelerating the release of INS from the formulations (see Fig. 1 and 2).

The mean micelle/vesicles size is an inherent factor for permeation in biological membranes (MÄLKIÄ *et al.*, 2004). The results demonstrated that the presence of BS in the release media leded to the formation of small micelle/vesicles. The formation of small mixed micelles on the intestinal tract can be a key factor to improve the absorption of INS administered by oral route, since BS can induce changes that increases membrane permeability to various substances (MIKOV *et al.*, 2006), as well as to affect the transfer of hydrophilic drugs dissolved in the media through the absorption barrier due to interactions among BS and the mucous layer covering the cells membrane (SILVA *et al.*, 2003; MIKOV *et al.*, 2006; CHUN *et al.*, 2012).

Morishita *et al.* (2004) observed that smaller was the INS microparticles, better was its adhesion to the intestine mucosa and its absorption into the small intestine, increasing the INS bioavailability and its hypoglycemic effect. The authors suggested that this phenomenon was due to the increased contact area and protection against enzymatic degradation promoted by the smaller microparticles (MORISHITA *et al.*, 2004).

CONCLUSION

The results described here demonstrated that capsules containing the 12% and 16% F127 lyophilized products could be useful formulations for oral administration of INS, if considering that

in vivo, the BS present on the intestine lumen could help on the emulsification of INS and OA, leading to the formation of small mixed vesicles/micelles, assisting on INS permeation/absorption through the intestinal mucosa.

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