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Sodium risedronate-loaded poly(ε-caprolactone) nanoparticles: Development, physicochemical characterization, and in vitro drug release study

Desenvolvimento de nanopartículas de poli(ε-caprolactona) contendo risedronato de sódio: desenvolvimento, caracterização físico-química e estudo de liberação in vitro.

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ABSTRACT:

Objective: Population in all over the world is affected by bone diseases, such as osteoporosis and loss bone tissue. Sodium risedronate (Na-Ris) is one of the most used drugs to its treatment. However, it has low oral bioavailability and exhibits many side effects. In order to overcome these limitations, the search for new dosage forms is necessary. One of these alternatives is the development of nanoparticles, which are able to transport the drug to its target directly, promoting maximization of the therapeutic efficiency and minimization of the toxicity. Due to its great versatility, these systems can be applied to an assorted administration routes, such as oral, pulmonary, intravenous, among others. Thus, the objective of this study was to develop and characterize Na-Ris-loaded nanoparticles, as well as determine the drug release profile. Methods: Nanoparticles were prepared by solvent emulsification/evaporation method and characterized by mean size, polydispersity index, zeta potential, granulometric distribution, drug content and encapsulation efficiency. Afterwards, in vitro drug release was performed using the dialysis bag technique as well as the release kinetics were also studied. Results: The developed system has shown mean size of 193 ± 14 nm and polydispersity index around 0.2. Zeta potential was -9.76 ± 0,52 mV and slightly acid values for pH. The granulometric distribution demonstrated nanoparticles with a narrow size distribution and the absence of particles in the micrometer range. Regarding the in vitro release, the drug was released completely from the system in 240 minutes and the release kinetics has follow the zero-order equation. Final considerations: Thus, a nanocarrier containing a water-soluble drug was successfully developed, presenting adequate physicochemical characteristics, which can be promising for biological evaluations.

KEYWORDS: Osteoporosis; Sodium risedronate; Nanostructured systems; Solvent emulsification; evaporation method.

RESUMO:

Objetivo: A população mundial tem sido afetada por doenças ósseas, como osteoporose e perda da massa óssea. O risedronato de sódio (Na-Ris) é um dos medicamentos mais utilizados para seu tratamento. No entanto, tem baixa biodisponibilidade oral e exibe muitos efeitos colaterais. Para contornar essas limitações, é necessária a busca por novas formas farmacêuticas. Uma dessas alternativas é o desenvolvimento de nanopartículas, que sejam capazes de transportar o fármaco diretamente até seu alvo, promovendo a maximização da eficiência terapêutica e minimização da toxicidade. Devido à sua grande versatilidade, esses sistemas podem ser aplicados nas mais diversas vias de administração, como oral, pulmonar, intravenosa, entre outras. Assim, o objetivo deste estudo foi desenvolver e caracterizar nanopartículas contendo Na-Ris, bem como determinar o perfil de liberação do fármaco. Métodos: As nanopartículas foram preparadas pelo método de emulsificação / evaporação de solvente e caracterizadas em relação ao tamanho médio, índice de polidispersão, potencial zeta, distribuição granulométrica, teor de fármaco e eficiência de encapsulação. Posteriormente, foi realizada a liberação do fármaco in vitro pela técnica de saco de diálise e também estudada a cinética de liberação. Resultados: O sistema desenvolvido apresentou tamanho médio de 193 ± 14 nm e índice de polidispersão em torno de 0,2. O potencial zeta foi de -9,76 ± 0,52 mV e valores ligeiramente ácidos para pH. A distribuição granulométrica demonstrou nanopartículas com distribuição de tamanho estreita e ausência de partículas na faixa micrométrica. Em relação à liberação in vitro, o fármaco foi liberado completamente do sistema em 240 minutos e a cinética de liberação seguiu a equação de ordem zero. Considerações finais: Assim, um nanocarreador contendo um fármaco solúvel em água foi desenvolvido com sucesso, apresentando características físico-químicas adequadas, o que pode ser promissor para avaliações biológicas.

PALAVRAS-CHAVE: Osteoporose; Risedronato de sódio; Sistemas nanoestruturados; Método de emulsificação; Evaporação de solvente.

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1 INTRODUCTION

Osteoporosis is a chronic disease defined as a loss of bone mass tissue, characterized as an imbalance between the bone formation and reabsorption and causing changes in the tissue microarchitecture, leading to a major risk of fractures due to an extensive bone fragility [1,2]. A crescent number of patients committed by the disease has been registered in the past years, being the female and elderly population more likely to develop it. Estimates indicate there are more than 200 million people throughout the world suffering of this disease, whose discovery generally occurs after a serious event [2–4].

Considering its chronic and epidemic characteristic, osteoporosis is still a reason of worrying, being target of many studies. Amongst the alternatives available for its treatment, bisphosphonates are the most utilized pharmacologic class. One of these molecules is sodium risedronate (Na-Ris), being widely applied in osteoporosis treatment and object of study in scientific community. However, because of the high number of hydroxyl groups in its structure, this drug is highly hydrophilic, promoting low bioavailability of fewer than 1%, being still reduced whether the patient is in fed state [5] Moreover, the drug shows many gastrointestinal adverse effects when administrated by oral route, commonly related as gastric discomfort, abdominal pain, nausea, diarrhea and constipation [6] . In order to extend the bioavailability of the drug and minimize its adverse effects, it is necessary to seek alternative solutions to improve its administration

Nanostructured systems have paid attention as their sizes fewer than 500 nm and a great proportion between their surface and volume, whose characteristics are attractive for drug loading, becoming interesting for effective formulation developing in order to treat many diseases [7,8]. These systems are capable of carrying drugs to their action sites, promoting control release and therapeutic efficiency improvement [7,9]. Moreover, there is an extension on circulating time of the drug in human body, a uniform distribution of the drug on the target site, a huge system stability against chemical and enzymatic degradation and improving drug bioavailability due to its prolonged release [9,12].

A couple of studies of nanoparticles developing containing Na-Ris have been conducted in the latest years, such as Dissette and collaborators (2010), Sahana and collaborators (2013), Vaculikova and collaborators (2014), Fazil and collaborators (2015), Rawat and collaborators (2015), and Khajuria and collaborators (2016) [28,29,30, 31,32,33]. The studies employed different kind of polymers, as poly lactic acid (PLA), poly lactic-glycolic acid (PLGA) and others; however, none of them use PCL or use the method proposed in this study. Therefore, this study aimed to design a poly(**ε**-caprolactone) nanoparticles to Na-Ris incorporation by solvent evaporation/emulsification method. The physicochemical characterization was performed as well as the in vitro drug release by dialysis bag diffusion technique.

2 MATERIALS AND METHODS

2. 1 MATERIALS

Na-Ris was acquired from Pharmanostra (Rio de Janeiro, Brazil). Sorbitan monooleate (Span 80®) and poly-(ε-caprolactone) (PCL) were acquired from Sigma Aldrich (São Paulo, Brazil). Polysorbate 80 (Tween 80®) was acquired from Delaware (Porto Alegre, Brazil). Ethyl acetate (EAc) was acquired from Dinâmica (Diadema, São Paulo). Methanol was acquired from Tedia (Rio de Janeiro, Brazil) and acetonitrile was acquired from Merck (Darmstadt, Germany).

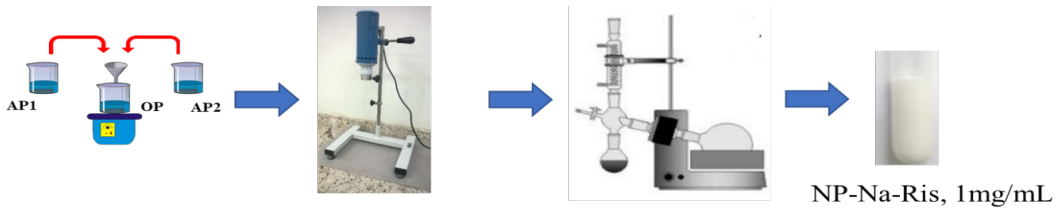
2. 2 ANALYTICAL PROCEDURE

The Na-Ris quantification was assessed by High Performed Liquid Chromatography (HPLC). The chromatographic conditions were adapted from the work carried by Kyriakides and Panderi (2007) [34]. The chromatograph utilized was LC-10A model (Shimadzu, Japan), LC 20-AT pump, CBM 20A controller, manual Rheodyne injection valve, UV/Vis SD M20A detector. The separation was achieved at room temperature (15-25°C) using a Phenomenex C18 4 x 30 mm pre-column and Phenomenex C18 250 x 4.00 mm x 5 µm column. The mobile phase was consisted of water alkalinized to pH 9.0 with triethylamine /acetonitrile (70:30, v/v) at 0.5 mL/min of flow rate, 20 µL injection volume and drug detection at 262 nm wavelength, with retention time of 3.5 minutes. A calibration curve was performed at the concentration range of 5 – 25 µg/mL.

2 . 3 PREPARATION OF NA-RIS-LOADED POLY(ε-CAPROLACTONE) NANOPARTICLES

Nanoparticles were prepared by solvent evaporation/emulsification method, consisting in a multiple emulsion system, according to Bitencourt and collaborators [13], as illustrated on figure 1. Firstly, an aqueous solution (AP1) containing 20 mL distilled water alkalinized to pH 9.0 with triethylamine, 0.01g Na-Ris and 0.077g hydrophilic surfactant polysorbate 80 was prepared. Concomitantly, an organic solution (OP) containing 40 mL EAc, 0.1g PCL and 0.077g of lipophilic surfactant sorbitan monooleate was kept under magnetic stirring at 40 ◦C. After 60 minutes, the AP1 was emulsified with OP, forming the primary emulsion, which was kept under strong magnetic stirring during 20 min. Then, another aqueous solution (AP2) compounded by 65 mL distilled water alkalinized to pH 9.0 with triethylamine and 0.144g polysorbate 80 was incorporated into the primary emulsion to achieve a multiple A/O/A emulsion. The formulation was taken, under ice bath, to a high-performance disperser (Marconi MA-102/Plus) at 7,000 rpm for 10 minutes in order to reduce its size particle and, in sequence, to rotary evaporator to eliminate EAc and part of the water to achieve 10 mL final volume and a Na-Ris final concentration of 1mg/mL. The formulations were prepared in triplicate. A blank formulation was prepared by Bitencourt and collaborators, whose data were used in order to compare to this work.

Figure 1 : Preparation of NP-Na-Ris. Firstly, a multiple emulsion is constituted and then taken to the high-performance disperser and to the rotary evaporator, forming a colloidal suspension.
*AP1: aqueous phase 1; OP: organic phase; AP2: aqueous phase 2



2 . 4 CHARACTERIZATION OF THE POLYMERIC NANOPARTICLES

The formulations were characterized in terms of such physicochemical parameters: pH, mean particle size, polydispersity index (PDI), zeta potential, granulometric distribution, total drug content and encapsulation efficiency. All of the analysis were carried in triplicate at room temperature (15-25°C).

2 . 4 . 1 PH DETERMINATION

The verification of pH was conducted utilizing a potentiometer (pH 21, Hanna, São Paulo) calibrated with 4.0 and 7.0 buffer solutions, by its direct immersion in the nanoparticles suspension.

2 . 4 . 2 MEAN PARTICLE SIZE, POLYDISPERSITY INDEX AND ZETA POTENTIAL

Mean size and PDI were evaluated by photon correlation spectroscopy at 25ºC after the samples were diluted in ultrapure water (1:500) (Zetasizer Nanoseries, ZEN 3600 model, Malvern Instruments, United Kingdom). The values of zeta potential were determined by dilution of the samples in NaCl 10 mM (1:500) utilizing microelectrophoresis technique at the same equipment.

2 . 4 . 3 GRANULOMETRIC DISTRIBUTION

Granulometric distribution was carried by laser diffraction technique (Mastersizer 3000, Malvern Instruments, United Kingdom). Samples were diluted in distilled water until reach a laser obscuration of 15%. A refractive index of 1.59 was used to perform the measurement.

2 . 4 . 4 DRUG CONTENT

The total drug content in the formulations was determine by HPLC method describes in "2.2 Analytical procedure" section. For this, 150 µL of the nanoparticles were transferred to 10 mL mobile phase containing water alkalinized to pH 9.0 with triethylamine/acetonitrile (70:30, v/v), taken to an ultrasonic water bath for 15 minutes and, afterwards, centrifugation for 10 minutes at 3,500 rpm to extract drug from the nanoparticles. Then, the supernatant was filtered in a 0.45 µm

membrane and injected into the HPLC system.

2 . 4 . 5 ENCAPSULATION EFFICIENCY

Encapsulation efficiency (EE) was conducted by ultrafiltration/ultracentrifugation method. For this, 300 µL of the formulation was transferred to a centrifugal filter device (Amicon Ultra 10.000 MV, Millipore) and the free drug was separated from the nanostructures at 7,000 rpm for 10 minutes. The non-associated drug was found at the ultrafiltered which was analyzed by HPLC method describes above (2.2 section). In order to determine EE, according to Bitencourt and collaborators [13], the difference between the free and total drug concentrations was calculated, in percentage (%), by the following equation:

$$EE = \frac{Total\ drug\ load - free\ drug\ load}{Total\ drug\ load} x100 \qquad (1)$$

2 . 5 IN VITRO RELEASE PROFILE

To determine the Na-Ris in vitro release profile, the dialysis bag diffusion technique was applied. For this, 2 mL of the nanoparticle's formulation and the Na-Ris aqueous solution (free drug - 1mg/mL) was transferred to a dialysis bag and subsequently immersed in 150 mL buffer solution. In order to mimic a pulmonary release, phosphate buffer pH 7.4 was used as release medium. At predetermined periods, aliquots of 1mL were withdrawn and replaced by the same volume of fresh medium, in order to keep the sink conditions. The amount of drug released was determined using the HPLC conditions previously mentioned (2.2 section).

2 . 6 DRUG RELEASE KINETICS

The drug release rate kinetics of Na-Ris was determined and the best fit observed indicates the reaction order (Microsoft Excel 2013® software). The kinetic equations used were as described by Blass and collaborators (2018) [35]: zero order (C=C0-kt), first order (lnC=lnC0-kt) and second order equation (1/C=1/C0+kt). After fitting the data to equations, the following parameters were determined: the linear correlation (r), the release rate (k) and half-life (t1/2), using the equations below:

$$k = C_o - C/t \qquad (2)$$

$$t_{1/2} = 0{,}5\ C_o / k \qquad (3)$$

2 . 7 STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation (SD). The GraphPad Prism® version 6 software was used to perform the statistical analysis: Student's t test and analysis of variance (ANOVA) followed by post hoc Tukey's test. Values of $p<0.05$ were considered statistically significant.

3 RESULTS AND DISCUSSION

3.1 NA-RIS-LOADED POLY(ε-CAPROLACTONE) NANOPARTICLES: PREPARATION AND CHARACTERIZATION

After the preparation, the macroscopic analysis of the nanoparticle suspensions indicated they showed a milked and opalescent aspect typical of colloidal formulations. Tyndall effect was observed as well, characterized by a bluish reflection due to the Brownian movement of the particles [8,14,15]. Such formulations did not show visible precipitation. Prior to our study, Bitencourt and co-workers had already utilized the evaporation/emulsification method to prepare a formulation with same composition in order to encapsulate hydrophilic extract from Syzygium cumini seeds. The nanoparticles had adequate physicochemical characteristics, maintained Syzygium cumini extract antioxidant properties and improved the antifungal activity in vitro. Regarding the physicochemical characteristics of nanoparticles without the extract, formulations presented mean size of 191 ± 6 nm, polydispersion index of 0.20 ± 0.02, zeta potential of -9.90 ± 0.69 mV, and pH of 6.5 ± 0.53 [13,16,17]. These parameters are in accordance with results displays in our study (table 1), indicating that the Na-Ris association to nanoparticles did not cause any significant change in the particle structure.

Table 1: Characterization of Na-Ris-loaded poly(**ε**-caprolactone) nanoparticles

	Physicochemical analysis					
	Mean size (nm)	**PDI***	**ZP* (mV)**	**pH**	**DC* (mg/mL)**	**EE* (%)**
NP-Na-Ris	193 ± 14	0.25 ± 0,01	-9,76 ± 0,52	5,6 ± 0,05	0,99 ± 0,03	92,8
	Granulometric distribution					
	D_{10} (nm)	**D_{50} (nm)**	**D_{90} (nm)**		**SPAN**	
	145 ± 0.003	279 ± 0,015	625 ± 0,049		1,71 ± 0,08	

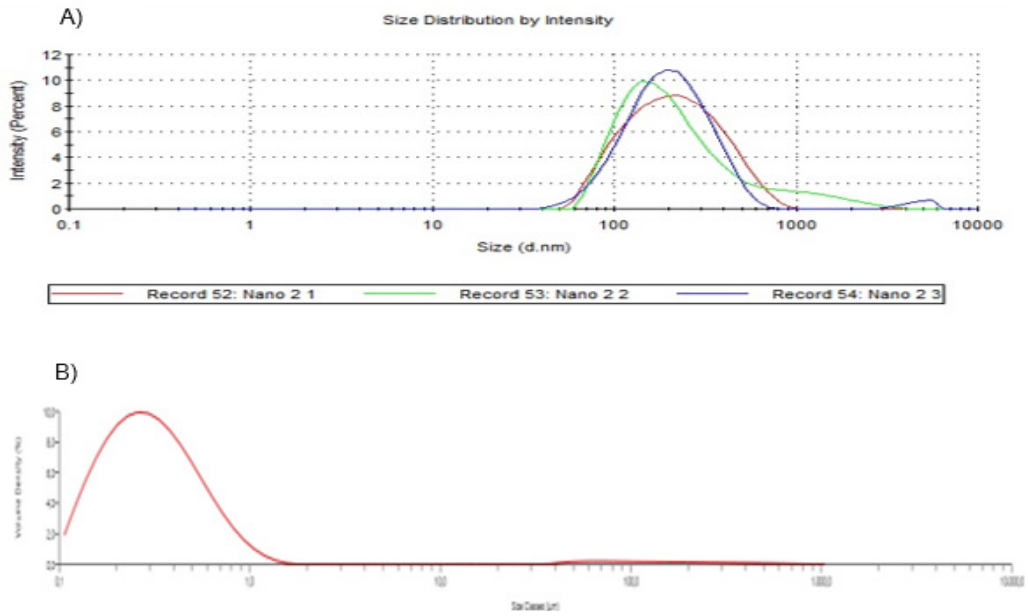
*PDI: polydispersity index; ZP: zeta potential; DC: drug content; EE: encapsulation efficiency

Mean particle size and dispersion of nanoparticles are the most important characteristics of the nanostructured systems because of their influence on in vivo distribution, toxicity and drug release [8,18]. The developed suspensions in this study exhibited submicrometric size, lower than 200 nm (Fig. 2A). To reach this mean size, there are some important components, such as ethyl acetate, due to its partial miscibility in water; the time of agitation longer that usually applied, due to its emulsification in three different phases; and the use of a high-performance disperser, responsible to reduce

the size particle. The PDI indicates the size particle distribution on the current system and, therefore, its homogeneity [8,19]. Usually, PDI values near 0.2 indicate low dispersion of the size. The developed system shows values near 0.2 with unimodal distribution, indicating good homogeneity [15,20].

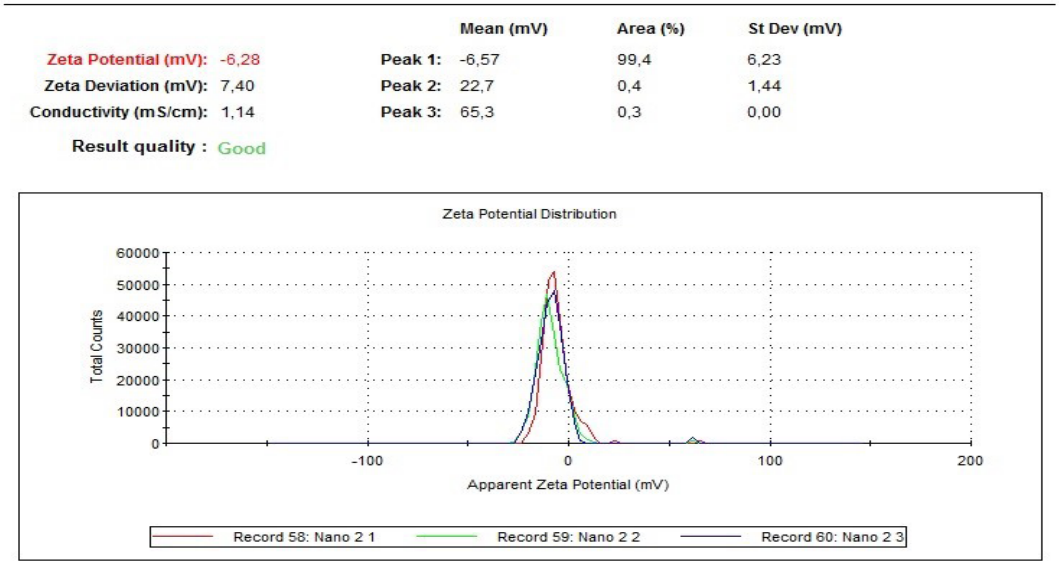
The particle size and granulometric distribution determinations by laser diffraction has been used in the nanostructured systems characterization in order to exclude the particles in the micrometer range size presence [21]. Zetasizer apparat, by photon correlation spectroscopy, analyses the size particles in the range between 200 and 1x104 nm, whilst Mastersizer apparat, by laser diffraction, operates between 10 and 3x106 nm. As it is possible to observe in Table 1, 10 % of the particles had size lower than 145 nm; 50 %, lower than 279 nm; and 90 %, lower than 625 nm. Such values are in accordance to the mean size analyzed by photon correlation spectroscopy, about 193 nm. The granulometric analysis indicates SPAN as well with values lower than 2, which indicated a narrow size distribution (Fig. 2B)

Figure 2 : Particle size analysis of the Na-Ris loading nanostructured system (n =3) by photon correlation spectroscopy (A). Granulometric distribution of the Na-Ris nanoparticles (n = 3) by laser diffraction analysis (B).



Zeta potential indicates the superficial characteristics of the particles, indicating the surface electrical potential. Such value estimates the possibility of aggregates formation, then how great it is the module value higher is the particles repulsion, suggesting the obtention of more stable systems [8,22]. Zeta potential is dependent, mainly, of the polymers and surfactants utilized and of the external phase pH, which alteration indicates the possibility of dissociation of functional groups on the surface of the particle of the absorption of ions provided by the dispersing medium [7]. Zeta potential data exhibited negative values (Fig. 3) which is probably because of the anionic polymer nature. Moreover, it can be also attributed to polysorbate 80, which is responsible for the steric stabilization and presents a negative charge surface density, due to the presence of the oxygen atoms in the molecule [13,20].

Figure 3: Zeta potential of the Na-Ris particles (n = 3).



pH evaluation allows to obtain data about the stability and check it over time. Changes in pH values may indicate the presence of degradation products provided from the polymer and/or the drug, being common for these formulation values between 3.0 and 7.5 [14,22]. pH determination was verified afterwards the suspension preparation, showing acid character, whose mean value was 5.67. The blank formulation developed previously have slight acid pH of 6.7 [13], indicating that Na-Ris may has some influence in this parameter. As it is possible to observe in table 1, formulations have exhibited drug contents around the theoretical concentration of 1 mg/mL, demonstrating there are no losses during the preparation of the formulations. For comparison, Kotak and Devarajan (2020) has encapsulated a Na-Ris-complex to hydroxyapatite nanoparticles, which loading was reached to 85% [23].

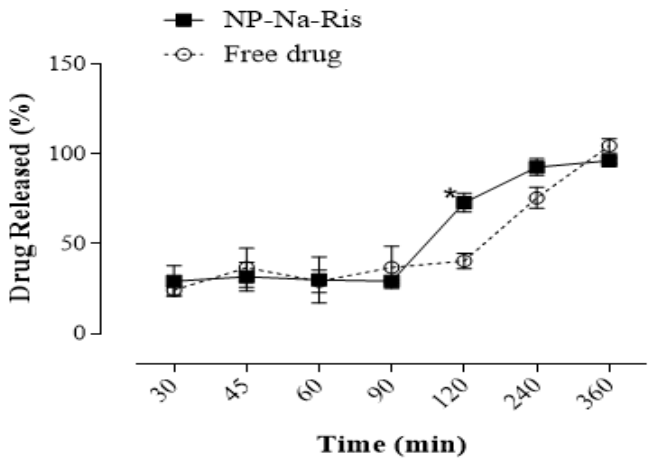
EE determination is important to know how the drug is associated to the nanostructures. Such parameter can be influenced by the pH of the external medium, drug content and lipophilia polymer nature and type of surfactant [8,14]. The value found in our study was 92.8% which is in accordance to Rawat et al. (2016) that achieve 93 % of Na-Ris EE in nanoparticles containing PLGA and hydroxyapatite [24]. Meanwhile, our EE results was superior than described by Elnaggar and co-workers which found about 33.1% of EE [25], Nasr and co-workers, who presented about 34.4% [26] and 58.96% [27] of EE for its Ris nanoparticles. The lower EE is common for hydrophilic drugs due to the high affinity for aqueous medium, then there is a major tendency to be found in this phase [24]; however, we achieve an EE around 100 %, indicating that the nanoparticle developed are adequate as Na-Ris nanocarrier.

3. 2 IN VITRO RELEASE PROFILE

In vitro release profile is a predict analysis that determines the time of drug is released from the nanostructured

system under physiological conditions. As it is observed at figure 4, the drug is released in its totality in 240 minutes after the beginning of the experiment. Excepted for the time of 120 minutes, there are no significant difference on Na-Ris release from the nanoparticles and drug solution ($p>0.05$). In comparison to other studies described with Na-Ris incorporated to nanostructured systems, our system is similar to related by Dissette and co-workers, which released 86% of the drug at the same period [28], and a more controlled release in relation to Khajuria and co-workers, whose release was to 93% in 90 minutes [29].

Figure 4: In vitro release of Na-Ris from the nanoparticles and aqueous solution.



Aiming to elucidate the kinetics of in vitro Na-Ris release from the nanoparticles, experimental data were fitted to zero, first and second order equations. As it was demonstrated in table 2, by comparing the correlation coefficients of the nanoparticles and free drug, the samples showed a better adjustment to the zero-order kinetics giving $r = 0.9063$ and $r = 0.9725$, respectively for Na-Ris nanoparticle and aqueous solution. It indicates that the release rate is proportional over time and does not depend on the drug concentration. Thus, it was possible to calculate the drug release constant rate (k) and the drug half-life (t1/2), both for Na-Ris nanoparticle and aqueous solutions.

Table 2: Correlation coefficient (r), drug release constant rate (k) and half-life (t1/2) of free and Na-Ris incorporated to nanoparticles after 240 minutes.

Sample	Reaction order	*r*	*k* (mg/min)	$t_{1/2}$ (h)
NP-Na-Ris	Zero	0.9063	0.267	1.872
	First	0.8748		
	Second	0.8452		
Free drug	Zero	0.9725	0.261	1.916
	First	0.9190		
	Second	0.8305		

In fact, the release constant and half-life were similar for encapsulated and unencapsulated Na-Ris. We attribute this as a limitation of the in vitro technique. As Na-Ris is a hydrophilic drug, it has a low oral bioavailability; this limitation can be circumvented or minimized with its incorporation in nanostructured systems, since, due to their reduced size, the

particles are more uniformly distributed in the gastrointestinal tract, providing a broader release of the drug, which can favor its absorption. Furthermore, this study was initially designed for an intended pulmonary administration. Unfortunately, biological activity studies still need to be performed, however, it is known that the pulmonary mucosa has great circulation and a high surface area. Therefore, we believe that in a biological system the bioavailability of Na-Ris can be improved by its incorporation into nanoparticles, even though it was not possible to infer this with our in vitro results.

4 CONCLUSIONS

Considering the data exhibited in this study, it can be stated that nanostructured system containing Na-Ris is a promising formulation for drug release. According to the physicochemical proprieties evaluated, the system is suitable to be applied, exhibiting submicrometric mean size and low dispersion of the particles, negative zeta potential values, pH within the acceptable range and high drug content and encapsulation efficiency. Regarding its in vitro release, all of the drug was released from the nanoparticles in 240 minutes. Due to the zero-order release kinetics of the drug, it is possible to observe that its release is constant over time. In such context, the Ris nanoencapsulation can be an advantageous formulation, mainly in terms of enhanced oral bioavailability and therapy adhesion, once gastrointestinal discomfort may be minimized.

Therefore, the developed formulation in this study is able to encapsulate Na-Ris successfully, even though this is a hydrophilic drug, exhibiting suitable characteristics for colloidal systems, being a promising formulation for bone diseases management. As perspectives of studies, the optimization of the aerodynamic design of the nanoparticles, the conduction of the stability study of the developed formulation, and the in vitro and in vivo analysis intending a pulmonary administration.

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