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Research paper

Reduced food interaction and enhanced gastrointestinal tolerability of a new system based on risedronate complexed with Eudragit E100: Mechanistic approaches from in vitro and in vivo studies

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ABSTRACT

Novel complexes consisting of Eudragit E100-risedronate are presented. The oral bioavailability of risedronate in rats was determined through its percentage excreted in urine after administration of complexed or free risedronate in fed and fasted conditions. The evaluation of the risedronate gastroduodenal irritation potential was carried out by macroscopic and histological analyses in an experimental rat model. The degree of counterionic condensation between Eudragit E100 and risedronate was assessed by dialysis with, mechanistic information about the interaction with calcium and the release of risedronate from the complexes being obtained using physiological solution and simulated gastric fluid without pepsin. Non-significant differences were observed in the urinary excretion of risedronate when the complex or free risedronate was administered to fasted rats. However, the urinary excretion of risedronate in the complex group was 4-times higher than in the free risedronate group when animals were concomitantly administered with food. This behavior was related to the high degree of counterionic condensation in the complex (86.5%), which led to a reduction in the calcium induced rate and magnitude of risedronate precipitation and resulted in a decrease in the gastroduodenal damage from the complex, as evidenced by a lower frequency of gastric mucosae hemorrhage. A sustained release of risedronate from the complex was observed toward water, simulated gastric fluid or physiological solution, through an ionic-exchange mechanism. In conclusion, complexation with Eudragit E100 could be a useful strategy to overcome the unfavorable properties of risedronate.

1. Introduction

As osteoporosis is a chronic disease, a long term treatment is required to reduce the risk of fracture and maintain the quality of life, with aminobisphosphonates being currently the first line drugs most widely and frequently used [1,2].

Aminobisphosphonates are zwitterionic molecules with a high polarity (e.g. Alendronate octanol/buffer partition coefficient at pH 7 = 0.0017) [3] and are almost completely ionized at physiological pH, which prevents transcellular transport across the epithelial barriers. As a consequence, their absorption after oral administration is very low with a bioavailability below 1% and significant inter- and intra-individual variability [1–4]. Furthermore, it is well known that the strong interaction between aminobisphosphonates and food significantly decreases their absorption [4,5], with this behavior being related to their ability to form insoluble complexes with calcium and other divalent cations in the intestinal lumen, which tend to precipitate at the absorption site [6].

The absorption of risedronate (Ris, a third generation aminobisphosphonate) has been shown to be drastically reduced by the effects of food. Ris concentrations as well as its cumulative urinary excretion decreased to about one tenth of that in fasted subjects, when administered 30 min after a meal to healthy volunteers. Moreover, such wide differences were reduced as the time between an oral administration of Ris and food intake was extended from 0.5 to 2 or 3 h [7]. Similarly, another investigation showed that the oral absorption of sodium alendronate (AlNa) in

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fasted rats was 4–5 times higher than after concomitant administration with food [3].

The most common reasons for patients to discontinue oral treatment with aminobisphosphonates are gastrointestinal side effects such as erosions or bleeding ulcers, which are more frequent in the stomach than in the intestine [8–10]. To ensure an effective treatment that can minimize both the risk of gastrointestinal damage and interactions with food, rigorous guidelines have been proposed for the oral administration of aminobisphosphonates, such as taking the drug with a full glass of water (180–250 mL) 30 min before the first food or drink of the day, with the patient in a standing position [9]. It should be emphasized that non-adherence to treatment can lead to even more severe ulcerative gastrointestinal effects; thus, compliance with these assumptions is essential to ensure clinical success. These side effects, however, can imply an obstacle for the adherence to treatment, and therefore be a cause for treatment interruption, particularly in the elderly for whom aminobisphosphonates are mostly prescribed. According to Kothawala et al. [11], one-third to one-half of patients do not take their medication as directed, with this non-adherence often occurring shortly after treatment initiation. Treatment response is related to dosage and its correct administration. In the case of aminobiphosphonates, the need to fast has been identified among the top three issues reported by patients as the reason for poor adherence [12]. Although an oral bioavailability of 1% is clinically effective for osteoporosis treatment, higher doses of aminobisphosphonates or parenteral administration are required for osteolysis and tumor treatment [13]. Therefore, the development of aminobisphosphonates, whose performance leads to an improvement in their bioavailability or tolerability is urgently required, which represents a challenging task.

Eudragit E100 (EuE100) is a cationic polymer based on dimethylaminoethyl methacrylate (DMAEMA) and other neutral methacrylic acid esters, which is widely used in oral and topical formulations and generally regarded as being non-toxic, non-irritant and essentially safe for humans. It is currently used as a coating in solid pharmaceutical dosage forms for taste masking and for protection [14], and its utilization as a dissolution modifier and as a granulating agent has also been explored [15,16], with several transdermal drug delivery systems having been reported [17]. Modified drug release has been achieved as a result of the interaction between reactive groups of copolymer pairs of EuE100 and polyanionic polymers containing carboxylic acids such as EuL100 or EuS100 [18]. Previous studies have also shown interesting properties related to the high degree of counterionic condensation established between the phosphate ester groups of drugs such as dexamethasone phosphate and the DMAEMA groups of EuE100 [19]. These properties could be further exploited to improve unfavorable characteristics of aminobisphosphonates. Related to this, our hypothesis is that the affinity between aminobisphosphonates and DMAEMA groups of EuE100 could help in reducing the interaction of calcium with food, as well as improving oral absorption or reducing gastrointestinal damage effects.

The goal of this work was to obtain a new material by complexing EuE100 and Ris (Fig. 1) and to investigate some biopharmaceutical properties and the effects of complexation on the oral bioavailability of Ris when concomitantly administrated with food or in fasting conditions. In addition, the gastroduodenal irritation potential of the complexes was evaluated.

2. Materials and methods

2.1. Reagents

Poly (butyl methacrylate, 2-dimethyl aminoethyl methacrylate, methyl methacrylate) (DMAEMA) 1:2:1 (Eudragit® E100, granules, pharmaceutical grade, Rohm, Germany) was donated by Etilfarma SA (Buenos Aires, Argentina). The equivalents of amine groups per gram of EuE100 (3.10×10^{-3}) were assayed by acid-base titration, which represents 23.8% of the ionogenic group in the DMAEMA polymer (theoretical value 20.8–25.5) [14].

Risedronate monosodium monohydrate (RisNa) was kindly provided by IVAX Argentina S. A., and risedronic acid (Ris) was obtained through RisNa neutralization, by dissolving RisNa in water and adding 1 M HCl to a pH value of <1. The solid precipitated was washed with distilled water, filtered and dried at 45 °C under vacuum to constant weight, and the melting point of the solid obtained was determined by DSC (onset 245 °C, maximum rate of melting 251 °C, maximum rate of decomposition 261 °C). The solid resulting was free of chlorides, as determined by the limit test described by the Argentinian Pharmacopeia [20].

Physiological solution (sodium chloride 0.9% w/v), simulated gastric fluid without pepsin (SGF, to represent gastric pH in the fasted state), and phosphate buffer pH 6.8 (to represent intestinal pH), were prepared according to USP specifications, using analytical grade reagents [21]. $CaCl_2$ solutions were obtained by dissolving anhydrous $CaCl_2$ (Ciccarelli®, analytical grade) in water. The sodium hydroxide 30% w/v aqueous solution was prepared in an ice bath, by dissolving granules of NaOH (Ciccarelli®) in previously boiled water to remove the CO_2 content. The standard 1 M HCl solution was purchased from Anedra®.

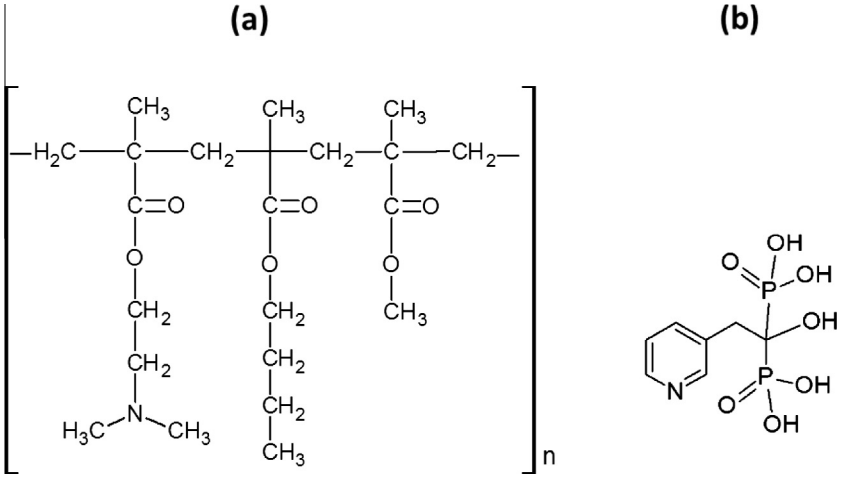

Fig. 1. Structural formula of EuE100 monomer (a) and Ris (b).

Disodium EDTA ($EDTANa_2$) solutions were obtained by dissolving dihydrate (Anhedra®, analytical grade) in water. These solutions were used at concentrations of 100 mg/mL for processing the urine samples, and at 0.18 mg/mL for preparing the mobile phase for HPLC analysis.

A solution of tetrabutylammonium bromide (Sigma®) was prepared in water at a concentration of 1.4 mg/mL and methanol (HPLC grade) was purchased from Anedra®.

Distilled water was used for all assays except for HPLC, in which Milli Q water was used.

2.2. Preparation of EuE100-Ris complexes

EuE100-Ris_x complexes were prepared as aqueous dispersions by adding 10 mL of water to a mixture of Ris and EuE100 (as solids), as shown in Table 1. The subscript "x" represents the percentage of DMAEMA groups of EuE100 neutralized with Ris, considering a 1:1 M proportion between Ris and each DMAEMA group in EuE100. Clear and low viscosity dispersions were observed after approximately 10 min of sonication, suggesting a spontaneous formation of the complexes in the presence of water. These dispersions were diluted with water in order to obtain the required concentration for each experiment.

Complexes were prepared before each experiment, with the dispersion of the complex EuE100-Ris_{50} being arbitrarily selected for all the studies. The complexes EuE100-Ris_{25} and EuE100-Ris_{75} were also used for compatibility studies, as explained in Section 2.5.

2.3. Ris release kinetic

Preliminary experiments revealed that when a tableted EuE100-Ris solid complexes were assayed by the dissolution apparatus, it rapidly dissolved in acidic media as well as in water, and as a consequence, the released free Ris could not be easily differentiated from complexed Ris, so bicompartimental Franz cells were used to study the release behavior of the aqueous complex dispersions.

Aqueous dispersions of EuE100-Ris_{50} (1 mL of a dispersion containing 5.1 mg of Ris, pH = 4.7) were subjected to drug release analysis. In order to prepare a Ris reference solution with the same concentration as its complex, RisNa, which is more soluble, was used (pH = 4.5). This use of the sodium salt of the drug as a reference has been reported previously by other authors [19,22–24].

All experiments were performed at 37 ± 1 °C. A semipermeable acetate cellulose membrane (12,000 Da, Sigma) was placed between the donor and the receptor compartments, and complex dispersions were placed in the donor compartment. The receptor compartment was filled with 16 mL of water, physiological solution or SGF, and stirred with a Teflon-coated magnetic stirring bar. At selected times, 1 mL aliquots were withdrawn from the receptor medium and replaced by the same volume of medium pre-warmed to 37 °C, with the data obtained being corrected for dilution. Ris concentrations were determined by UV spectroscopy at λ = 262 nm. The pH values of the receptor compartments recorded at the end of the experiments were 4.8 ± 0.3, 5.5 ± 0.2 (when water and physiological solution, respectively, were used as the media for EuE100-Ris_{50} complex release) and 5.1 ± 0.2 (after RisNa release toward water).

To assess sink conditions, the volume of the receptor compartment, sampling frequency and concentration of drugs in the donor compartment were considered [21]. The profiles obtained were compared statistically using the difference factor (f_1, Eq. (1)), with two profiles being considered to be different when the f_1 value calculated between them (see Eq. (1)) was equal to or >15.

$$f_1 : \left[\sum(R_t - T_t) \Big/ \sum T_t\right] \times 100 \quad (1)$$

2.4. Ionic complexation of EuE100 with Ris

The proportion of Ris associated with EuE100 was determined by dialysis using a cellulose membrane tube (12,000 Da, Sigma). Then, 10 mL of EuE100-Ris_{50} aqueous dispersion containing 47.6 mg of Ris was placed in the dialysis tube (donor compartment), which was introduced into a flask containing 100 mL of water (receptor compartment). All assays were performed in triplicate. The system reached equilibrium after 24 h of dialysis at 25 °C and the pHs of the donor and receptor phases were recorded. The Ris concentration in the receptor compartment was spectrophotometrically assayed at λ = 262 nm, and the assay was repeated using EuE100-Ris_{50} with NaCl added in the donor compartment at a molar proportion of Ris: NaCl 1:2. The percentage of counterionic condensation of Ris with EuE100 was calculated according to Eq. (2):

$$\%\ Ris_{cc} = 100 - (Q_R/Q_D \times 100) \quad (2)$$

where Q_R and Q_D are the quantities of Ris in the receptor and donor compartments, respectively.

2.5. Aqueous compatibility of EuE100-Ris_x in the presence or absence of calcium

Aqueous dispersions of the complexes were freeze dried. To carry this out, 10 mL of samples was frozen at −18 °C before being lyophilized at −50 °C and 150 mBar with a Labconco® Freeze Dry System/Freeze Zone 6 (Kansas City, MO, USA) for 48 h, and the resulting solid samples were stored in tight containers at room temperature. An interaction between Ris and EuE100 in the solid state was confirmed using powder X-ray diffraction by the absence of reflections of crystalline Ris. The profiles of Ris, EuE100-Ris_x complexes and the physical mixtures (PM) are included as supporting information.

In order to assay complex aqueous compatibility, increasing amounts of obtained solids were added to eppendorf tubes containing 1 mL of distilled water and thermostated at 25 °C. This methodology was selected because high amounts of complexes led to high viscosity dispersions. The samples were periodically vortexed and equilibrated for 24 h, and the maximum quantities of the complexes dispersible without precipitation of a solid phase were recorded.

For comparison, Ris and RisNa solubility in distilled water were also determined. To carry this out, an excess of the drugs was placed into suitable stoppered containers and distilled water was added. In addition, RisNa solubility was also determined by adjusting the pH to 6 using a NaOH solution.

The samples (in triplicate) were immersed in a thermostatized water bath at 25 °C and periodically shaken. Once equilibrium was reached, the pH of the supernatant was recorded.

Table 1
Composition of aqueous dispersions of the EuE100-Ris_x complexes.

EuE100 (g)[a]	Ris (g)	Denomination of the complex obtained[b,c]
1	0.24	EuE100-Ris_{25}
1	0.47	EuE100-Ris_{50}
1	0.71	EuE100-Ris_{75}

[a] Containing 3.10×10^{-3} equivalents of amine groups per gram of EuE100.
[b] Considering 1 mol of Ris per equivalent of EuE100.
[c] The dispersions were clear and non-viscous.

Aliquots of the filtrate supernatant, adequately diluted with distilled water, were analyzed by UV spectrophotometry (Thermo Electronic Corporation, Evolution 300 BB, England) at λ = 262 nm.

The precipitation of Ris was also evaluated in the presence of calcium in a phosphate buffer pH 6.8, for both EuE100-Ris_{50} and a RisNa solution of an equivalent concentration. For this purpose, a concentration of Ris equivalent to a dose of 70 mg in 250 mL (0.3 mg/mL) was selected. Briefly, aliquots of EuE100-Ris_{50} or RisNa solutions were added with $CaCl_2$ 0.68 mg/mL to obtain calcium/Ris molar at ratios of 0.5, 1, 2 and 5. After 24 h, an aliquot was centrifuged and suitably diluted with buffer pH 6.8. The concentration of Ris was quantitated by UV spectroscopy at λ = 262 nm and statistically assayed using a one-way ANOVA. Samples taken at 45 min and 2 h after the addition of $CaCl_2$ were analyzed in order to estimate the precipitation rate. All these experiments were performed in triplicate.

2.6. Oral absorption of Ris in fast and fed conditions

The oral bioavailability of Ris in rats was determined through an analysis of its percentage excreted in urine. The experimental design was completely randomized with a 50% loaded complex (EuE100-Ris_{50}) being arbitrarily selected for the bioavailability studies. Forty male rats were used, which were housed in metabolic cages at standardized temperature with free access to water and standard rodent chow.

Approximately three (3) mL of an aqueous dispersion of EuE100-Ris_{50} (containing 35.7 mg of Ris) of an aqueous solution of RisNa (with both solutions being equivalent to a dose of 116 mg/kg of Ris) was administered intragastrically via a silicone gavage tube, as follows:

- *RisNa fasted group* (n = 10): animals fasted for 18 h prior to and 2 h after administration of RisNa solution.
- *EuE100-Ris_{50} fasted group* (n = 10): animals fasted for 18 h prior to and 2 h after administration of the EuE100-Ris_{50} dispersion.
- *RisNa fed group* (n = 10): animals administered with RisNa solution with free access to food.
- *EuE100-Ris_{50} fed group* (n = 10): animals administered with EuE100-Ris_{50} dispersion with free access to food.

The dose of Ris was selected according to Hui-Juan et al. [25] Urine samples were collected over 48 h during 6 h periods after which the fractions were combined and the total volume was measured and samples were frozen at −20 °C until HPLC analysis.

After administration, the elimination of aminobisphosphonates is biphasic with an initial half-life of a few hours and a terminal one of 10 years, with this long terminal half-life being due to their slow release from the bone [26]. Based on this, the harvesting period was the time for the elimination of 50% of the absorbed dose.

2.6.1. Quantification of Ris from rat urine

For the extraction and quantification of Ris from rat urine, a methodology described by Hui-Juan et al. [25] was adapted and validated. For the calibration curve, a stock solution of RisNa (64 μg/mL) in water was prepared. Then, 2 mL of blank urine was centrifuged at 9000 rpm for 10 min at room temperature. Aliquots of 0.025, 0.05, 0.1, 0.15, 0.20 and 0.35 mL of the stock solution were added to 1 mL of the supernatant to obtain concentrations of Ris in urine between 1.4 and 19.6 μg/mL. In order to cause selective precipitation of Ris, 50 μL of $CaCl_2$ solution and 80 μL of the sodium hydroxide 30% w/v aqueous solution were added to each sample. After stirring using a vortex, these samples were centrifuged at 9000 rpm for 15 min, and the supernatant was discarded using a Pasteur pipette and the precipitate redissolved in 150 μL of 1 M HCl and 1 mL of distilled water. These precipitation and redissolution steps were repeated twice, and finally, the precipitate was redissolved with 200 μL of $EDTANa_2$ + 500 μL of water.

The addition of sodium hydroxide 30% w/v aqueous solution in successive steps ensured a more efficient extraction of Ris, and the final addition of $EDTANa_2$ removed the excess of calcium and dissolved the precipitate. The obtained solutions were filtered through 0.45 μm cellulose filters (Millipore) and then quantified by HPLC. All solutions were prepared in triplicate.

Linearity was found over the concentration range of 1.4–19.6 μg/mL (R^2 = 0.998). The specificity of the method was verified and the lower quantification and detection limits (LQL and LDL) were 1.4 ± 0.01 μg/mL and 0.56 μg/mL, respectively. The percentage of recovery at low, medium and high concentrations was 93.5 ± 4.2%, with no significant variations (1.8%) being found after 2 cycles of freezing/thawing [27].

2.6.2. HPLC analytical procedures

Chromatography was performed using a Waters® HPLC system equipped with a 1500 HPLC pump, a 717 auto sampler and a Waters 2996 PDA detector, with data acquisition and processing being performed using Empower® system software. The temperature was maintained at 25 °C with a Waters 1500 series column heater. Chromatographic separations were carried out using a Phenomenex® C18 reverse phase column (250 × 4.6 mm, 5 μm particle size) and a Phenomenex® guard column (C18 4 × 3 mm ID). The mobile phase for the separation of Ris consisted of 88/12, v/v buffer (0.11% tetrabutylammonium bromide, 0.15% monobasic sodium phosphate and 0.055% $EDTANa_2$)/methanol, adjusted to pH 6.75 with 1 M solution of NaOH. The flow rate was 1.0 mL/min, the injection volume was 100 μL and the detection wavelength was 262 nm, with peak areas being used for quantitative analysis.

2.6.3. Sample analysis

For quantification of Ris in the urine samples, 2 mL was taken from the combined fractions and processed as described above. The percentage of Ris excreted in urine (Eq. (3)) was calculated as follows:

$$\% \, Ris_{excreted} = C_{urine} \times V \times 100/D_0 \quad (3)$$

where C_{urine} is the concentration (mg/mL) of Ris in the sample of the processed urine, V is the total volume of urine excreted in 48 h (mL) and D_0 is the dose of Ris administered (mg).

The averaged excreted percentage of each group was statistically compared using a one-way ANOVA.

2.7. Evaluation of gastric/duodenal effects

A fasted non-clinical model of indomethacin-treated rats described by Blank et al. was used [28], which generates gastric lesions in an acute single dose treatment for rapid assessment of the gastric effects of aminobisphosphonates. Eighteen hours prior to dosing, rats were food fasted and individually housed with free access to tap water. Rats were injected subcutaneously with 40 mg/kg of indomethacin, while being intragastrically administered with 3 mL of RisNa solution or EuE100-Ris_{50} dispersion (30 mg Ris and 63.5 mg of EuE100) equivalent to a dose of 300 mg/kg [29] (N = 6 per experimental group). The control group (N = 6) received an EuE100 dispersion at the same concentration as EuE100-Ris_{50}. Before administration, the pHs of all the treatments were adjusted to 5.66 ± 0.05 (pH value obtained for the EuE100-Ris_{50} dispersion) by adding 1 M NaOH or 1 M HCl. Animals were sacrificed 4 h later by CO_2 inhalation, and the stomach and the first 2-cm portion of the duodenum were removed. The organs were opened at the greater curvature and rinsed with saline for further analysis. For damage assessment, the rats were classified according

to the different pathological criteria of mucosal damage. Internal organ surfaces were macroscopically observed during autopsy in order to identify indomethacin-induced hemorrhagic lesions in different quadrants, and samples were also photographed. The less severe injuries were petechiae with or without exudates, whereas the advanced stage included clear-cut hemorrhages. Mucous membranes were evaluated microscopically by histopathology using hematoxylin-eosin staining in organ samples fixed with 10% neutral buffered formalin. Then, dehydrated paraffin-embedded 4-μm thick sections were mounted on glass slides and stained prior to being examined with an Olimpus-BX41 light microscope connected to a digital Lumenera-Infinity camera. Rats were classified according to the severity of the predominant lesions found in the microphotographs, as follows: exulceration (foci of lost epithelial), inflammation (mucosal infiltration with exudates), hemorrhage (bleeding lesion with lost mucosal).

The distribution groups (18 rats) according to treatments (EuE100, EuE100-Ris_{50}, or RisNa) and categories of mucosal damage (macro: petechiae/hemorrhage; micro: exulceration/inflammation/hemorrhage) were analyzed using the chi-square test with a significant level of $p < 0.05$, by observing at least four fields per animal and using the Infostat v.2012 software.

2.8 Animals

Adult male Wistar rats (average weight 200–250 g) were used in all studies. The trials were approved by the Evaluation Committee for Experimental Protocols on the Use of Animals for Scientific Projects of the Faculty of Chemical Sciences, National University of Córdoba (Exp n° 55668/2012).

3. Results and discussion

In the present study, clear and no viscous dispersions were observed after approximately 10 min of sonication, thus suggesting the spontaneous formation of complexes in the presence of water. Such behavior has been previously reported by other authors and ascribed to electrostatic interaction between the drug and the polyelectrolyte [19,22–24].

3.1. Ionic complexation and Ris release kinetics

The acid-base properties of Ris in an aqueous solution were investigated by Meloun et al. [30] in a pH range from 2 to 12, and described in terms of four dissociation steps: pKa2, pKa4 and pKa5 (related to the dissociation of POH groups) and pKa3 (related to the dissociation of the protonated amino group R_2NH^+) (Fig. 2). The important dissociation constants in the intestinal pH range are pKa3 and pKa4, which are associated with the loss of the third and fourth protons from the pentaprotic acid [30].

In the current investigation, the pH of the donor compartment at the end of the dialysis experiment in water was 5.51 ± 0.1. According to the equilibrium depicted in Fig. 2, the most abundant species in such a solution would be $RisH^{3-}$ and $RisH_2^{2-}$, whose negative charges would be attracted by the positively charged amine groups of EuE100. Consequently, the EuE100-Ris_{50} system revealed a high degree of counterionic condensation, with 86.2 ± 1.0% of Ris being ionically condensed with EuE100. When NaCl was added to the donor compartment, the proportion condensed decreased to 54 ± 4%, with a final pH of 5.7 ± 0.1 in the donor compartment. Notice that the species present at this pH would be almost the same as in the experiment using water without NaCl. Hence, the reduction in the counterionic condensation may be assigned to the ionic exchange with Na^+ and Cl^- that produced Ris salts, which were able to diffuse through the dialysis membrane.

Ris release toward physiological solution and SGF in bicompartimental Franz cells from EuE100-Ris_{50} aqueous dispersions was also evaluated. The same approach has been previously used by other authors to obtain mechanistic and quantitative information regarding the rate and the kinetics of release [19,22–24,31]. The plot of Ris released over time is shown in Fig. 3. When the receptor compartment was filled with water, a fast diffusion of Ris from the RisNa reference solution was observed. However, in the same medium, the release rate of Ris from the aqueous dispersion of its complex was substantially slower. Moreover, as depicted in Fig. 3, when water in the receptor compartment was replaced by a solution of NaCl, the release rate from the Ris complex was significantly increased as a consequence of the ionic exchange produced by the diffusion of Na^+ and Cl^- to the donor compartment.

When the receptor compartment was filled with SGF, an additional increase in Ris release from the complex was observed, which might be assigned to the higher concentration of the $RisH_4^+$ zwitterionic species at the expense of the anionic $RisH_3^-$ and $RisH_2^{2-}$ ones (Fig. 2). The weaker strength of attraction between the positively charged macro-ion and the zwitterion resulted in a faster release rate at acidic pH. In addition, the ionic exchange produced by the diffusion of H^+ to the donor compartment (whose pH was reduced from 5.1 to 1.2), may have also increased the release of Ris. It should be noted that, at the concentrations used in the experiment, the complexes presented a negligible viscosity, suggesting that ionic exchange played a major role in the release mechanism of Ris from the complexes.

These results show that EuE100 behaved as a Ris carrier, and was able to function as a "smart" release system designed to release the drug slowly in aqueous dispersions, with the delivery rate being maintained for at least 5 h in all the conditions evaluated. Previous studies have shown that the high binding affinity of the complexes formed by dexamethasone phosphate (a drug containing a phosphate ester group) and EuE100 is a consequence of the intervention of the second acidic proton of the drug and hydrophobic interactions [19]. Similarly, Ris has more than one possible acidic site of interaction with EuE100 with the faster release rate observed possibly being due to its higher hydrophilic nature (logP dexamethasone phosphate: 1.9; logP Ris −2.5) [32,33]. Therefore, a lower association with EuE100 (a greater affinity for the aqueous medium compared to dexamethasone phosphate) would be expected.

3.2. Aqueous compatibility of EuE100-Ris_x and interaction with calcium

Table 2 displays the estimated proportion of Ris in dispersion from the respective complexes, and also the aqueous solubility of Ris and RisNa in distilled water; the pHs of their supernatants are also shown. In contact with water, the complexes EuE100-Ris_x generated a clear environment in which viscosity increased with the concentration, thereby preventing the saturation of the system by not allowing addition of the solid.

The solubility of Ris and RisNa in distilled water was 0.89 mg/mL and 61.80 mg/mL respectively, and the pHs of their supernatant were 2.60 and 4.54. The solubility of RisNa at pH 6 was 100.72 mg/mL.

It has been previously reported that EuE100 precipitates at pH values above 5 [14]. As can be observed in Table 2, a clear and viscous dispersion of EuE100-Ris_{25} presented a pH value of 6.97, suggesting an increase in EuE100 aqueous compatibility due to complexation with Ris. Notice that, Ris was completely dissolved at all pHs used, and consequently available for dissolving and complexing the EuE100.

The addition of $CaCl_2$ to a pH 6.8 solution containing Ris at a concentration equivalent to a dose of 70 mg in 250 mL caused

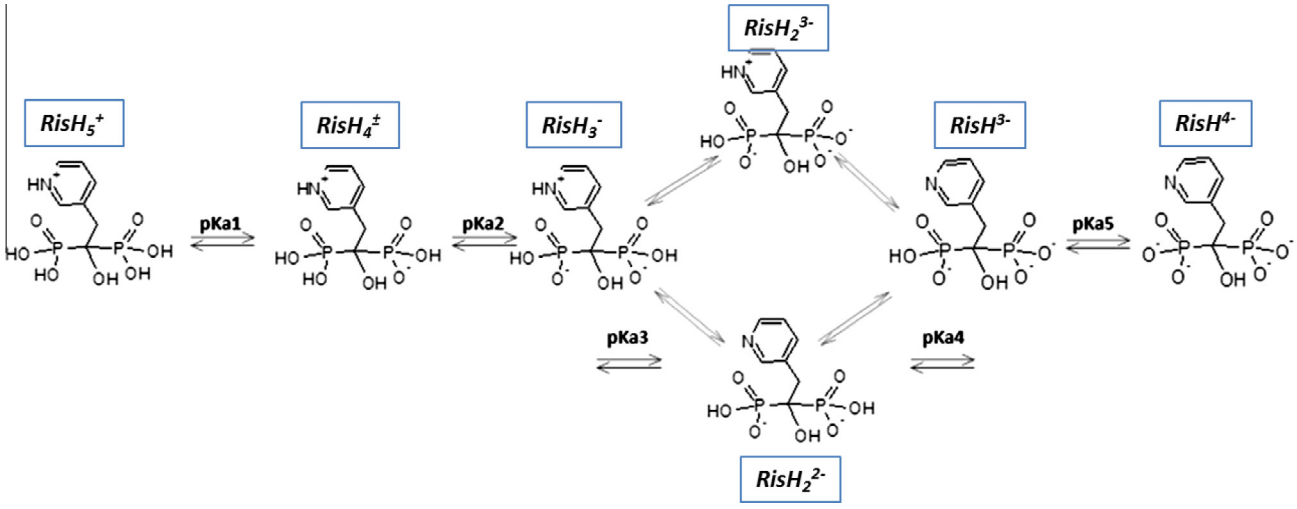

Fig. 2. Schema of the dissociation of pentaprotic acid Ris. pKa1 = 0.32, pKa2 = 2.00, pKa3 = 5.99, pKa4 = 7.07 and pKa5 = 9.86.

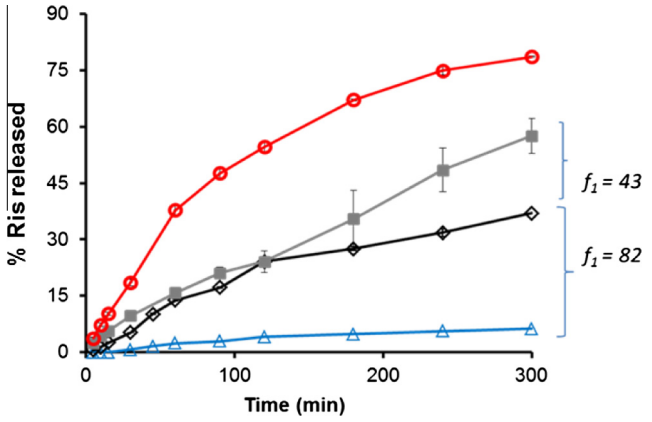

Fig. 3. Ris released from EuE100-Ris$_{50}$ to Water (△), Physiological solution (NaCl 0.9%) (◇) and SGF (■); and reference solution of RisNa to water (○).

Table 2
Solubility evaluation of Ris and its complexes with EuE100 in water at 25 °C.

Compound	mg/mL of Ris	pH
EuE100 –Ris$_{25}$	>90[a]	6.97
EuE100 –Ris$_{50}$	>120[a]	5.02
EuE100 –Ris$_{75}$	>150[a]	4.19
Ris	0.89	2.60
RisNa	57.40[b]	4.54
RisNa	93.55[c]	6.05[d]

[a] When in contact with water the complexes generated a viscous environment that prevented saturation of the system by not allowing addition of the solid.
[b] Equivalent to 61.80 mg/mL of RisNa.
[c] Equivalent to 100.72 mg/mL of RisNa.
[d] This value was adjusted with a NaOH solution.

the precipitation of a solid from both the RisNa solution and EuE100-Ris$_{50}$ dispersions. The amount of Ris remaining in the supernatant at the equilibrium (24 h) was significantly higher ($p \leqslant 0.05$) for the systems containing EuE100-Ris$_{50}$ at all the molar Ris: calcium ratios studied (Fig. 4), suggesting an increase in the aqueous compatibility of Ris in the presence of calcium due to complexation with EuE100. In fact, the higher the proportion of calcium, the lower was the amount of Ris that remained in the supernatant with the same trend being observed at 45 min and 2 h indicating that the rate of precipitation of the solids was also slower for the complex than that for the RisNa solution. More

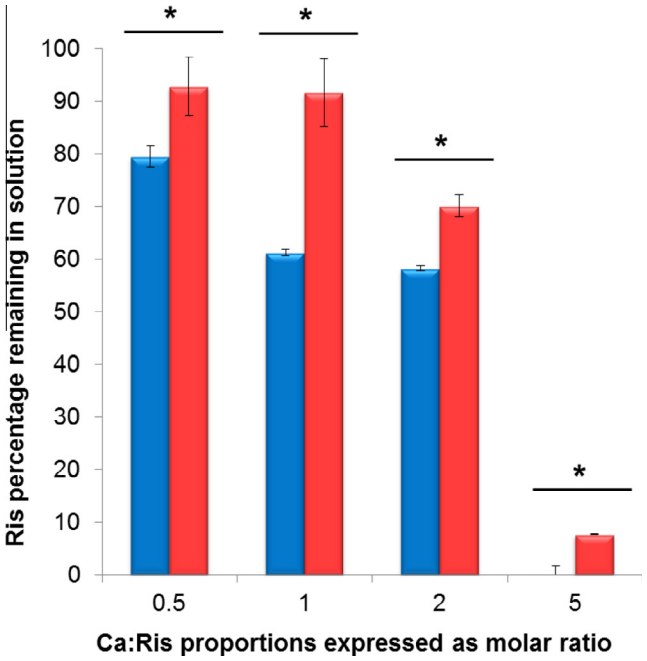

Fig. 4. Ris percentage remaining at equilibrium (24 h) in the supernatant of a RisNa solution ■ (red) and an EuE100-Ris$_{50}$ dispersion ■ (blue) after the addition of $CaCl_2$. Initial concentration of Ris was equivalent to a dose of 70 mg in 250 mL. Ca: Ris proportions are expressed as molar ratios. * P value < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detailed studies are now required to assess whether the magnitude of the interaction with calcium is influenced by the proportions of EuE100 and Ris.

3.3. Oral absorption of Ris in fast and fed conditions

Since systemic aminobisphosphonates are widely sequestered in bone, the plasma concentrations are usually too low for analytical quantification. For this reason, and because renal excretion is the only elimination route, urinary collection of aminobisphosphonates is commonly used to estimate their absorption [7,34]. Fig. 5 presents the percentage of Ris excreted in urine (after 48 h), found in our study, where it can be observed that when free RisNa was

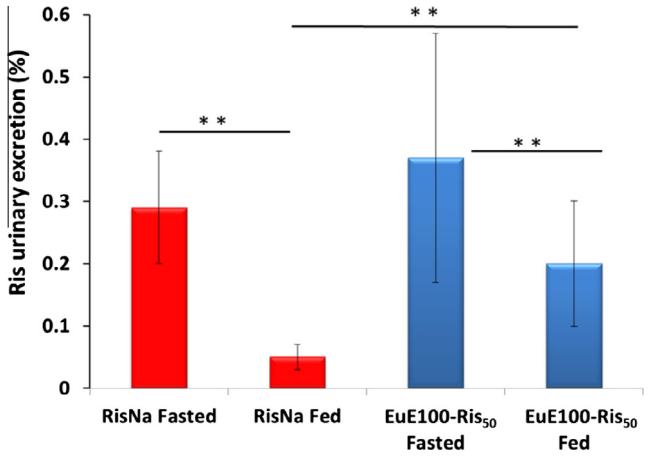

Fig. 5. Ris percentage excreted in urine (48 h) after administration of EuE100-Ris_{50} and RisNa (116 mg Ris/kg) to rats in fasted and fed conditions. **P value < 0.01.

administered, the presence of food led to a strong reduction (5.8 times) in the urinary excretion of Ris. In contrast, this reduction was only 1.8 times when the complex EuE100-Ris_{50} was administered. Consequently, Ris urinary excretion in the EuE100-Ris_{50} fed group was around 4-times higher than that of the RisNa fed group (Fig. 5). Moreover, Ris urinary excretion in the EuE100-Ris_{50} fasted group was 1.3-times higher than that of the RisNa fasted group, although this difference was not statistically significant.

The reduction in the urinary excretion of Ris in the presence of food has been previously described by other authors [5,7] and ascribed to the ability of aminobisphosphonates to form insoluble complexes with calcium or other divalent cations in the intestinal lumen [4,6]. Therefore, the increase in the aqueous compatibility of Ris in the presence of calcium due to its complexation with EuE100 could be the reason for a reduced interaction in the presence of food, with potential safety benefits.

Pazianas et al. [35,36] developed a formulation of Ris (35 mg) which eliminates the need for fasting using an enteric-coating and association with EDTA (100 mg). This ensured that calcium and other divalent or trivalent cations present in the food would be preferentially bound by EDTA instead of by Ris. In a similar fashion, in the present study, EuE100 competed with calcium for binding Ris, thereby increasing Ris aqueous compatibility in the presence of calcium, and also reducing food interaction. Interestingly, this resulted without using a protective coating, and therefore could probably be optimized by applying one.

A number of strategies have been reported to improve aminobisphosphonate oral bioavailability, although without assessing the interaction with food. Among these microemulsions [37], polymeric microparticles [38], and complexes with EDTA [39] or TiO_2 [40] can be mentioned.

3.4. Evaluation of gastric/duodenal effects

3.4.1. Macroscopic evaluation of damage

Rat stomachs showed statistically significant differences among treatments ($p = 0.0052$). Animals treated with RisNa presented a higher frequency of gastric hemorrhage, whereas those treated with EuE100 or EuE100-Ris_{50} mainly exhibited indomethacin-related petechiae (Fig. 6). Thus, these latter treatments were safer, with the polymer preventing deleterious effects of Ris.

3.4.2. Microscopic evaluation of damage

The highest frequency of animals with gastric hemorrhage was found after RisNa treatment, in agreement with macroscopic observations. The EuE100 experimental group mainly showed epithelial exulcerations, with the EuE100-Ris_{50} complex being associated with inflammation and hemorrhage, although to a

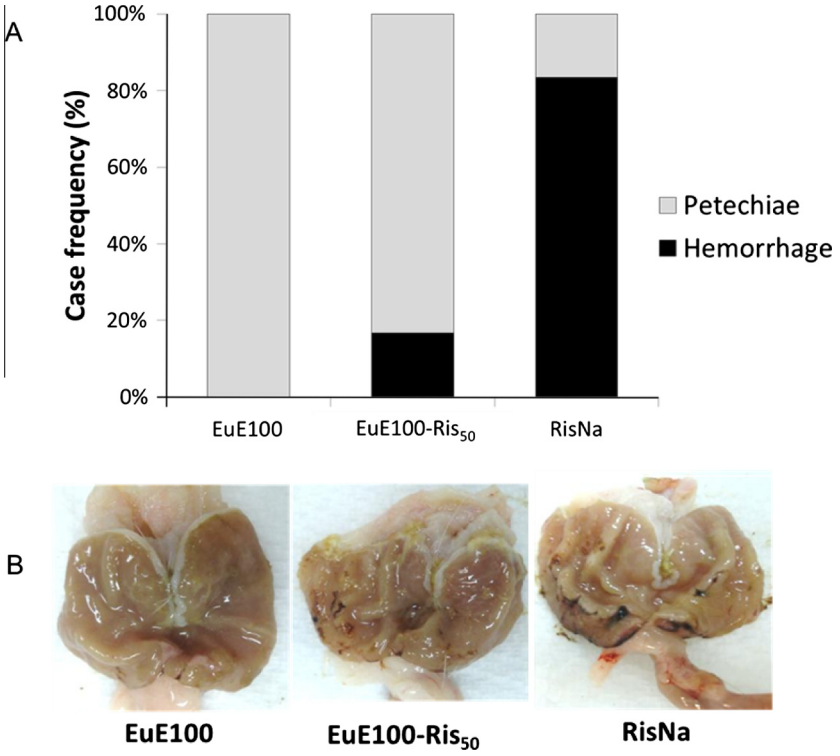

Fig. 6. Macroscopic evaluation of the damage after administration of EuE100, EuE100-Ris_{50} or RisNa to fasted rats. A: Absolute frequency of macroscopic damage: petechiae or hemorrhage. B: Representative photographs of the stomach after the treatments.

lower extent than RisNa (Fig. 7). All differences observed among the treatments were statistically significant ($p = 0.0026$).

In the case of rat duodena, the EuE100 treatment was associated principally with exulcerations, whereas all rats treated with EuE100-Ris_{50} presented mucosal inflammation. Only RisNa-treated animals developed hemorrhagic lesions with lost villi (Fig. 8). Thus, the EuE100-Ris_{50} complex prevented Ris-related damage in this intestinal section in a more efficient way than in the stomach. Again, all differences were statistically significant ($p < 0.0001$).

It is known that gastric damage caused by aminobisphosphonates is a result of their topical irritant effects on the mucosa

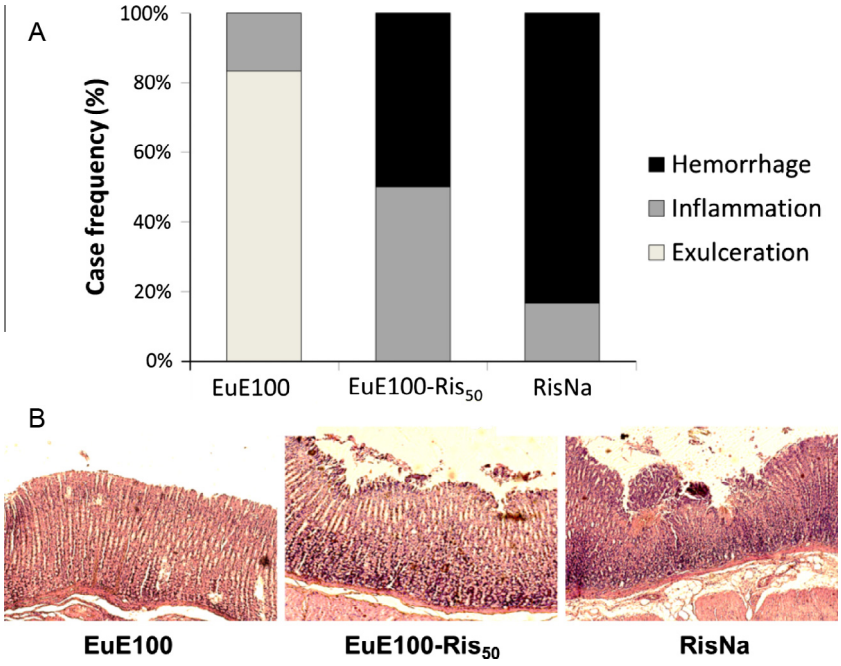

Fig. 7. Microscopic evaluation of the gastric damage after administration of EuE100, EuE100-Ris_{50} or RisNa to fasted rats. A: Absolute frequency of microscopic damage: hemorrhage, inflammation or exulceration. B: Representative photomicrographs of the glandular stomach mucosa after the treatments.

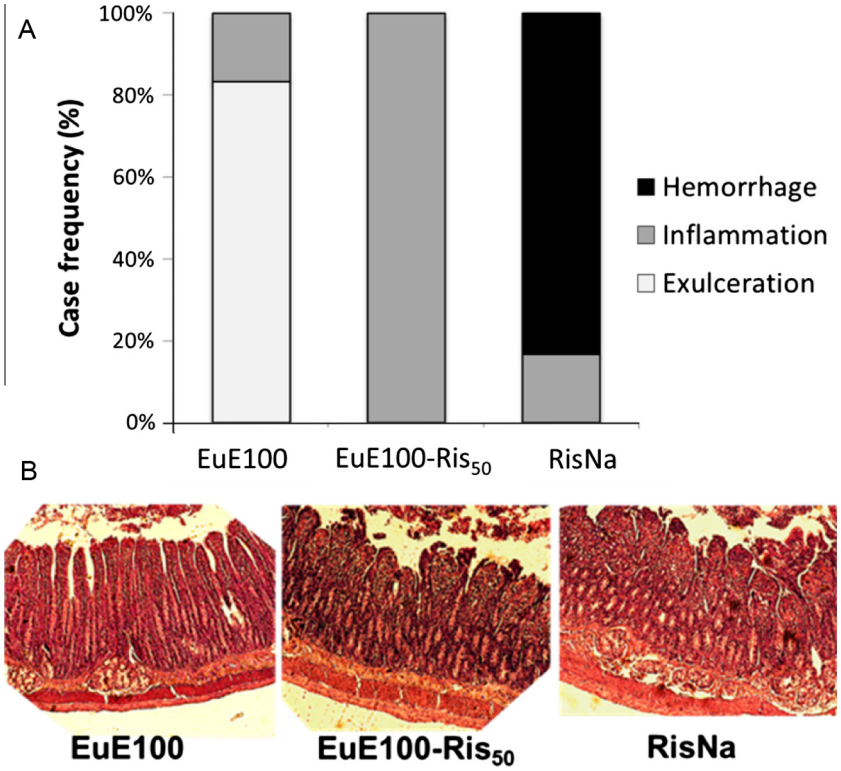

Fig. 8. Microscopic evaluation of the duodenal damage after administration of EuE100, EuE100-Ris_{50} or RisNa to fasted rats. A: Absolute frequency of microscopic damage: hemorrhage, inflammation or exulceration. B: Representative photomicrographs of the glandular stomach mucosa after the treatments.

[29,41,42]. Thus, the reduction in the gastric irritation potential observed with the EuE100-Ris$_{50}$ treatment could have been related to the reduction in the concentration of free Ris observed in this work. Related to this, it has been reported that insoluble aminobisphosphonate - calcium complexes formed in the intestinal lumen are more detrimental than soluble forms. Twiss et al. [43] demonstrated that AlNa and pamidronate insoluble complexes with calcium are more toxic to the gastric mucosa than their soluble uncomplexed forms, thus establishing a connection between the solubility of the aminobisphosphonate and gastrotoxicity. In the present study, the experimental model used to evaluate the potential for gastric irritation allowed the type and severity of damage that the EuE100-Ris$_{50}$ system generated compared to the RisNa reference solution to be classified.

Summing up, in this model indomethacin facilitated the observation of damage through the exacerbation of injuries produced by aminobisphosphonates. However, indomethacin can also produce a small foci of mucosal necrosis [28]. Another aspect to be considered is that in this model a dose of Ris was used, which was more than 100 times the dose normally used to treat osteoporosis. Consequently, although the damage observed did not reflect a condition that can be directly extrapolated to treatment, it permitted the observation of differences in the damage produced by EuE100-Ris$_{50}$ relative to the other groups.

4. Conclusions

New complexes between EuE100 and Ris were obtained, with the EuE100-Ris$_{50}$ complex revealing a high degree of counterion condensation that enabled a controlled release of Ris in water as well as in other media such as SGF and physiological solution. This material also permitted a reduction in the magnitude and rate of precipitation of Ris in the presence of calcium.

The lower proportion of free Ris in the EuE100-Ris$_x$ complexes and in the interaction with calcium was related to the decrease in the gastro-duodenal irritation potential. When EuE100-Ris$_{50}$ was administered orally, a reduced influence of the coadministered food on urinary excretion of Ris was observed, with the reduction in the magnitude and rate of precipitation of Ris in the presence of calcium possibly accounting for this behavior. Interestingly, these results were obtained without using a protective coating. Thus, the simplicity of the methodology to produce EuE100-Ris$_{50}$ makes this material a worthy and versatile candidate for improved Ris formulations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2016.07.012.

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