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Lipid-core nanocapsules restrained the indomethacin ethyl ester hydrolysis in the gastrointestinal lumen and wall acting as mucoadhesive reservoirs

Vitória Berg Cattani[a,1], Luana Almeida Fiel[a,1], Alessandro Jäger[b], Eliézer Jäger[a], Letícia Marques Colomé[a], Flavia Uchoa[a], Valter Stefani[b], Teresa Dalla Costa[a], Sivia Stanisçuaski Guterres[a], Adriana Raffin Pohlmann[a,b,*]

[a] *Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Avenida Ipiranga, 2752, Porto Alegre, RS, 90610-000, Brazil*
[b] *Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, P Box 15003, Porto Alegre, RS, 91501-970, Brazil*

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ABSTRACT

The aim of this work was to investigate if the indomethacin ethyl ester (IndOEt) released from lipid-core nanocapsules (NC) is converted into indomethacin (IndOH) in the intestine lumen, intestine wall or after the particles reach the blood stream. NC–IndOEt had monomodal size distribution (242 nm; PDI 0.2) and zeta potential of −11 mV. The everted rat gut sac model showed IndOEt passage of 0.16 μmol m^{-2} through the serosal fluid (30 min). From 15 to 120 min, the IndOEt concentrations in the tissue increased from 6.13 to 27.47 μmol m^{-2}. No IndOH was formed *ex vivo*. A fluorescent-NC formulation was used to determine the copolymer bioadhesion (0.012 μmol m^{-2}). After NC–IndOEt oral administration to rats, IndOEt and IndOH were detected in the gastrointestinal tract (contents and tissues). In the tissues, the IndOEt concentrations decreased from 459 to 5 μg g^{-1} after scrapping, demonstrating the NC mucoadhesion. In plasma (peripheric and portal vein), in spleen and liver, exclusively IndOH was detected. In conclusion, after oral dosing of NC–IndOEt, IndOEt is converted into IndOH in the intestinal lumen and wall before reaching the blood stream. The complexity of a living system was not predicted by the *ex vivo* gut sac model.

1. Introduction

Polymeric nanoparticles have been proposed to increase the apparent solubility of lipophilic drugs, protect them from enzymatic attack, control drug release, and improve activity or even to reduce their side effects (Couvreur et al., 2002; Schaffazick et al., 2003). The term nanoparticles refers to nanospheres or nanocapsules that are, respectively, matricial (Pohlmann et al., 2007) and vesicular structures (Jäger et al., 2007) varying in size from 10 to 1000 nm (Soppimath et al., 2001; Torchilin, 2006). The polymeric nanoparticles promote drug selective passage through biological barriers when their distribution sizes range from 100 to 600 nm.

The encapsulation of diclofenac or indomethacin, non-steroidal anti-inflammatory drugs, in polymeric nanospheres and nanocapsules has been reported as a strategy to reduce their gastrointestinal toxicity (Ammoury et al., 1993; Chasteigner et al., 1995; Guterres et al., 1995; Müller et al., 2001). Indomethacin has also been used as a drug model in several studies concerning nanoparticulated systems (Calvo et al., 1996; Guterres et al., 2000; Kim et al., 2001; Vinogradov et al., 2002; Pohlmann et al., 2002). More recently, comparative studies have been performed in order to propose the nanoencapsulation mechanisms for indomethacin and its ethyl ester derivative (Pohlmann et al., 2004, 2007, 2008; Cruz et al., 2006a; Poletto et al., 2008), as well as to determine the different drug release behavior from nanospheres, nanoemulsion and nanocapsules (Cruz et al., 2006b). The nanoencapsulation of indomethacin ethyl ester within semi-crystalline polyester nanocapsules (NC) has been used to simulate a perfect sink condition by releasing the drug after its interfacial alkaline hydrolysis creating a concentration gradient in the system (Pohlmann et al., 2004). Regarding the biological activity, the indomethacin-loaded lipid-core nanocapsules (NC–IndOH) and the co-encapsulation of indomethacin and indomethacin ethyl ester in nanocapsules showed selective cytotoxicity after treating glioma cell lines (U138-MG and C6) (Bernardi et al., 2008). Furthermore, after intraperitoneal administration, the NC–IndOH were

* Corresponding author at: Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, P Box 15003, Porto Alegre, RS, 91501-970, Brazil. Tel.: +55 51 3308 7237; fax: +55 51 3308 7304.
E-mail address: pohlmann@iq.ufrgs.br (A.R. Pohlmann).
[1] Both authors have contributed equally to this paper.

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able to increase the intratumoral penetration of the drug reducing the growth of the glioma (C6) implanted in rats (Bernardi et al., 2009a). In addition, the effects of systemic treatment with NC–IndOH were evaluated in rat models of acute and chronic edema. The drug nanoencapsulation increased the anti-inflammatory effect in long-term models of inflammation, allied to an improved gastrointestinal safety (Bernardi et al., 2009b). Previously, the *in vitro* release experiment using gastric or intestinal simulated fluids containing enzymes (pepsin and pancreatin) did not show any indomethacin ethyl ester release or degradation (Cruz et al., 2006a; Pohlmann et al., 2007). The experiment showed that polymeric nanocapsules and nanospheres containing indomethacin ethyl ester are stable *in vitro* even in the presence of pepsin and pancreatin. However, indomethacin ethyl ester-loaded lipid-core nanocapsules (NC–IndOEt) showed antiedematogenic activity in acute treatment in rats, indicating that the drug was released *in vivo* from the nanocapsules (Cruz et al., 2006a). That *in vivo* experimental protocol was not designed to show which substance, indomethacin or indomethacin ethyl ester, was responsible for the biological effect. In this way, we performed a pharmacokinetic study using the same formulations to evaluate the plasma levels of each substance after i.v. and oral dosing (Cattani et al., 2008). In the study, we demonstrated that the indomethacin ethyl ester acts as a pro-drug. However, if indomethacin ethyl ester is converted into indomethacin after oral dosing of nanocapsules, either at the intestine lumen, intestine wall or after the nanocapsules reach the blood stream, remains to be elucidated.

After oral administration of nano- and microparticles the main uptake route has been considered the follicle-associated epithelium (FAE) in small intestine due to the presence of gut-associated lymphoid tissue (Peyer's patches). There are three possible transport routes for macromolecules, microorganisms and particles in FAE: (1) paracellular transport; (2) transcellular uptake across enterocytes by endocytic process; and (3) endocytosis by M cells (Carreno-Gómez et al., 1999). The literature described several experimental models to investigate the interaction between particles and gastrointestinal tract (Hussain et al., 2001; Ponchel and Irache, 1998). Among the *ex vivo* models, the everted intestinal sac model (Wilson and Wiseman, 1954) has been used in the past 50 years to study bioadhesive properties and substances uptake and transport in the intestine (Chen et al., 2003; Carreno-Gómez et al., 1999; Mainardes et al., 2006). Furthermore, bioadhesion and uptake of particles were previously studied *in vivo* (Jani et al., 1990; Limpanussourn et al., 1998; Jani et al., 1996; Damge et al., 1996; Florence and Hussain, 2001) and *in vitro* (Hussain et al., 1997; Santos et al., 1999; Santos et al., 2003; Mainardes et al., 2006).

Recently, we prepared lipid-core nanocapsules varying the concentrations of sorbitan monostearate and oil (capric/caprylic triglyceride), both constituents of the core, to investigate if the sustained release from those carriers could be a consequence of the variation of the core viscosity and the particle surface area (Jäger et al., 2009). The augmentation in the sorbitan monostearate concentration enhanced the resistance to the pro-drug diffusion due to the core viscosity increase. On the other hand, after increasing the oil concentration, despite the possibility of diminishing the core viscosity, the pro-drug release was delayed due to the decrease in the surface area of the colloidal system as a consequence of the increase in the particle mean size.

Taking those considerations into account, the aim of this work was to investigate if indomethacin ethyl ester released from the lipid-core nanocapsules is converted into indomethacin in the intestine lumen, intestine wall or after the particles reach the blood stream. First, the everted rat gut sac model was used to verify the transport of the pro-drug and the bioadhesion of the nanocapsules. Secondly, after oral administration of the pro-drug-loaded lipid-core nanocapsules to rats, the concentrations of indomethacin ethyl ester and indomethacin were determined in stomach, small intestine, liver, spleen, as well as in plasma from peripheric and portal vein blood.

2. Materials and methods

2.1. Materials

Poly(ε-caprolactone) (PCL) (M_n = 42,500 and M_w = 65,000 g mol^{-1}) was supplied by Aldrich® (Strasbourg, France). Dicyclohexylcarbodiimide, 4-(*N*,*N*-dimethyl)aminopyridine and indomethacin were obtained from Sigma (St. Louis, USA). Caprylic/capric triglyceride (density 0.94 g ml^{-1}), sorbitan monostearate and polysorbate 80 were acquired from Delaware® (Porto Alegre, Brazil). TC 199 Earle® intestinal simulated medium was obtained from Sigma–Aldrich (Strasbourg, France).

o-Aminophenol, 1,2-phenylenediamine, 5-aminosalicylic acid were acquired from Aldrich® (reagent grade) and used without purification. Diethyl β-ethoxymethylenmalonate and polyphosphoric acid were purchased from Acros Chemicals®. Methyl methacrylate, acquired from Aldrich®, was purified before polymerization by passing it through activated alumina (Merck®). 2,2′-Azo-*bis*-isobutyrylnitrile (AIBN) was obtained from Merck® and purified before use by recrystallization from methanol and maintained under vacuum. Silica gel 60 (Merck®) was used for preparative chromatographic column separations. All solvents were used as received or purified using classical standard procedures. Spectroscopic grade solvents (Merck®) were used for fluorescence and UV–vis measurements and chromatographic grade solvents (Merck®) were used for liquid chromatography (HPLC) measurements.

^{1}H NMR analyses were performed at room temperature on a Varian® (model VXR-200, 200 MHz) using tetramethylsilane as internal standard and DMSO-d_6 (Aldrich) or $CDCl_3$ (Merck) as solvents. The UV–vis and fluorescence analyses were carried out using a Shimadzu® UV-1601 PC spectrometer and a Hitachi spectrofluorometer (model F-4500), respectively. Fluorescence spectrum correction was performed to enable measuring a true spectrum by eliminating instrumental response such as wavelength characteristics of the monochromator or detector using Rhodamine B as standard (quantum counter).

2.2. Synthesis and nanocapsule preparation

2.2.1. Synthesis of indomethacin ethyl ester

The synthesis of indomethacin ethyl ester (IndOEt) was carried out under argon by preparing a solution of indomethacin (5 mmol) in ethanol (20 ml) that was added of 4-(*N*,*N*-dimethyl)aminopyridine (0.2 mmol) (Pohlmann et al., 2004). The solution was stirred for 10 min at 0 °C following the addition of dicyclohexylcarbodiimide (5 mmol). After 30 min at 0 °C and 16 h at room temperature, the solvent was evaporated under reduced pressure. The residue was added of dichloromethane (30 ml) and the suspension was filtered. The filtrate was extracted with saturated $NaHCO_3$ aqueous solution (3 × 10 ml) to remove the unreacted indomethacin. Then, the organic phase was dried with anhydrous $MgSO_4$, filtered and evaporated. The product was purified by column chromatography (Silica gel 60, 70–230 mesh) using ethyl acetate and cyclohexane (1:1, v/v) as eluent. The isolated product was obtained as a solid (80% of yield) presenting a melting point (uncorrected) of 82–83 °C.

^{1}H NMR 200 MHz (δ, ppm) $CDCl_3$: 7.66 and 7.46 (AB system, 2H and 2H, ArH *p*-chlorobenzoyl), 6.97 (d, 1H *J* = 2.5 Hz, H-4), 6.87 (d,

1H J = 9.0 Hz, H-7), 6.67 (dd, 1H J = 9.0 and 2.5 Hz, H-6), 4.16 (q, 2H J = 7.1 Hz, OCH_2), 3.84 (s, 3H, OCH_3), 3.65 (s, 2H, CH_2), 2.38 (s, 3H, CH_3), 1.27 (t, 3H J = 7.1 Hz, CH_3CH_2O).

^{13}C NMR 75 MHz (APT, δ, ppm) $CDCl_3$: 170.9 (CO-ester), 168.3 (CO-amide), 156.0, 139.2, 135.9, 134.0, 130.8, 130.7 and 112.7 (7 × Cq), 131.1 and 129.1 (4 × CH *p*-chlorobenzoyl), 114.9, 111.6 and 101.3 (3 × CH indol), 61.0 (OCH_2), 55.7 (OCH_3), 30.4 (CH_2), 14.2 and 13.3 (CH_3 and CH_3CH_2).

2.2.2. Lipid-core nanocapsules containing indomethacin or indomethacin ethyl ester

Nanocapsules were prepared by interfacial deposition of preformed polymer as previously described (Jäger et al., 2009). Briefly, at 40 °C, poly(ε-caprolactone) (0.500 g), capric/caprylic triglyceride (1.65 ml), sorbitan monostearate (0.385 g) and indomethacin or indomethacin ethyl ester (0.050 g) were dissolved in acetone (135 ml). Then, at room temperature, the solution was injected into an aqueous solution (265 ml) of polysorbate 80 (0.385 g). After 10 min, acetone was eliminated and the suspension concentrated under reduced pressure to a final volume of 50 ml (drug or prodrug final concentration at 1 mg ml^{-1}). Formulations were made in triplicate.

2.2.3. Synthesis of the fluorescent-labeled copolymer

The syntheses of the fluorescent-labeled copolymer was performed as previously described (Jäger et al., 2007). The reaction was based on the radical copolymerization of methyl methacrylate and the fluorescent benzazole derivative. Briefly, the 5-aminosalicylic acid was condensed with the 1,2-phenylenediamine. The 2-(4′-amino-2′-hydroxyphenyl)benzimidazole was isolated and purified by column chromatography. Diethyl β-ethoxymethylenmalonate was added to a solution of 2-(4′-amino-2′-hydroxyphenyl)benzimidazole in ethanol. The vinylene derivative was obtained with satisfactory yield and its chemical identity was verified by NMR. The copolymer (PMMA-HBN) was prepared by polymerization of vinylene derivative with methyl methacrylate in the presence of AIBN. PMMA-HBN was purified by dissolution in chloroform (1 ml) and precipitation into petroleum ether (20 ml), as much as any dye was not detected in the filtrate by UV spectroscopy. Then, PMMA-HBN was characterized by UV spectroscopy and by size exclusion chromatography (LDC Analytical Model Constametric 3200).

2.2.4. Fluorescent-labeled nanocapsules

Nanocapsules were prepared using PMMA-HBN synthesized as described above. A solution of PMMA-HBN (0.075 g), capric/caprylic triglyceride (0.825 ml) and sorbitan monostearate (0.190 g) in acetone (33.8 ml) was injected into an aqueous solution (66.2 ml) of polysorbate 80 (0.190 g). Acetone was eliminated and the suspension concentrated under reduced pressure to a final volume of 25 ml (copolymer final concentration at 3 mg ml^{-1}). Formulations were made in triplicate.

2.3. Physicochemical characterization of nanocapsules

The pH values were determined at 25 °C using a potentiometer DPMH-2 (Digimed). Size, polydispersity index and zeta potential were determined using a Zetasizer Nano ZS (Malvern) after diluting the samples with water (MilliQ®) for the size measurements or 10 mM NaCl aqueous solution for zeta potential analysis. Measurements were made in triplicate.

Transmission electron microscopy (TEM) was carried out using nanocapsule suspensions diluted in water. The sample was deposited on specimen grid (Formvar-Carbon support film, Electron Microscopy Sciences) and negatively stained with uranyl acetate solution (2%, w/v). The analyses were performed using a microscope JEM 1200 Exll, operating at 80 kV (*Centro de Microscopia Eletrônica*- UFRGS).

Indomethacin or indomethacin ethyl ester contents in nanocapsules were determined by HPLC (PerkinElmer S-200 with injector S-200, detector UV–vis), a guard-column (C_{18}) and a column (LiChrospher® 100 C_{18}, 250 mm, 4 mm, 5 μm, Merck) using a mobile phase (1.2 ml min^{-1}) consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0 with 10% (v/v) acetic acid. The methodology was previously validated (Jäger et al., 2009) showing linearity between 1.00 and 50.00 μg ml^{-1} (r > 0.9999), inter- and intraday variability lower than 2.0%, accuracy between 99.42% and 104.78% and limit of quantification of 1.00 μg ml^{-1}.

2.4. Ex vivo drug absorption using rat everted gut sac

Male Wistar rats (250–300 g, CREAL/UFRGS, Porto Alegre, Brazil) were handled in accordance with the provisions of the Guide to care and use of experimental animals in all experimental procedures (UFRGS Ethics in Research Committee, # 2005403). Animals were sacrificed and the entire small intestine quickly excised and flushed through several times with a NaCl solution (0.9%, w/v) at room temperature. The intestine was immediately placed in warm (37 °C) TC 199 (Earle's salts), pH 7.4, and, then, gently everted over a glass rod (2.5 mm diameter). The intestine was filled with fresh oxygenated medium and clamped at both ends. The resulting large gut sac was divided into eight small sacs of approximately 2.5 cm in length using braided silk suture, starting from the end of the duodenum to ensure maximal metabolic activity from the upper/mid jejunum. Each replicate was analyzed using the sacs of the intestine from one rat.

NC–IndOH and NC–IndOEt suspensions (2 ml for each flask) were added in flasks containing TC 199 medium (18 ml each) at 37 ± 1 °C. At the appropriate time intervals (2, 5, 10, 15, 30, 60, 90 and 120 min) each sac was removed and maintained over the recipient for 5 s to drip the liquid. Each sac was cut open and the serosal fluid drained into a tube. The serosal fluid and the sac tissue were analyzed. The serosal fluid aliquot was added of acetonitrile, centrifuged (5 min at 5290 × g) and the supernatant was collected, filtered (0.45 μm) and analyzed by HPLC. The sac tissue was macerated with acetonitrile (1 min) and centrifuged (5290 × g) to collect supernatants, which were filtered (0.45 μm) and analyzed by HPLC. The transport of indomethacin and indomethacin ethyl ester from the mucosal to the serosal side was determined, considering an average area of 4.61 ± 0.45 cm^2 for the sacs. The integrity of the sacs was verified by glucose measurement in both the external medium and the serosal fluids using a glucose meter (Accu-Check, Roche, Switzerland). As glucose is actively transported by the small intestine, healthy metabolically active sacs that are not leaking accumulate glucose in the serosal fluid (Barthe et al., 1999).

2.5. Ex vivo fluorescent nanocapsules uptake using rat everted gut sac

Fluorescent nanocapsules uptake was evaluated using the same procedure described for the indomethacin and indomethacin ethyl ester *ex vivo* absorption with some modifications. Briefly, each sac was placed in flasks containing 19 ml of TC 199 medium at 37 ± 1 °C and added of 1 ml of NC–PMMA-HBN suspension (3 mg ml^{-1} of copolymer). At the appropriate time intervals (0, 15, 30, 60, 90, 120 and 150 min), each sac was removed and maintained over the recipient for 5 s to drip the liquid. Each sac was cut open and the serosal fluid drained into a tube. The drained serosal fluid was extracted with chloroform (vortex, 5 min), centrifuged (5 min at 5290 × g) and the supernatant was collected for analysis.

The sac tissue was macerated with chloroform (1 min) and centrifuged (5 min at 5290 × *g*). The organic phase was collected for analysis.

2.6. Validation of the PMMA-HBN quantification

The quantification of PMMA-HBN extracted from the fluorescent nanocapsules was performed by fluorescence (Hitachi Spectrofluorometer, model F-4500). To validate the methodology, different proportions of fluorescent nanocapsules were added into TC 199 Earle® intestinal simulated medium under magnetic stirring at 37 ± 1 °C, providing concentrations of 7.5, 12.5, 25, 50, 75, 100, 125 and 150 μg ml^{-1} of fluorescent copolymer. One milliliter of each mixture was extracted with 1 ml of chloroform in vortex (5 min). Each organic phase was gently transferred to a quartz cuvette for analysis. Linear calibration curves (9 curves) showed correlation coefficients higher than 0.99. Inter- and intraday variability did not exceed 10.6% and 7.3%, the limit of detection was 3.5 μg ml^{-1} and the accuracy was 97.9%. Considering that a blank of tissue (intestinal tissue extracted with chloroform) showed fluorescent intensity between 40.2 and 44.7 arbitrary units, at each time interval (0–150 min) a blank of tissue was carried out in parallel with the tested sample containing NC–PMMA-HBN. The fluorescence of each blank was subtracted from the fluorescence of the sample at each time interval.

2.7. In vivo intestinal drug absorption

2.7.1. Animals

Animals in this study were handled in accordance with the provisions of the Guide to care and use of experimental animals and all experimental procedures were approved by UFRGS Ethics in Research Committee (# 2005478). The experiments were performed on male Wistar rats weighing 290–320 g, which were purchased from *Fundação Estadual de Produção e Pesquisa em Saúde* (Porto Alegre, Brazil) and were kept at constant room temperature (22 ± 1 °C) and 60% relative humidity, under controlled 12 h light–dark cycle (light on from 7 a.m. to 7 p.m.) during the acclimation period with free access to water and food. For the experiments, the animals were kept fasten overnight (14–18 h) with free access to water.

2.7.2. In vivo intestinal absorption after oral administration of NC–IndOEt

The intestinal absorption was investigated in male Wistar rats ($n = 4$) after oral administration of nanocapsules corresponding to a 10 mg kg^{-1} indomethacin ethyl ester dose. After pre-determined time points (0.25, 1 and 2 h), the rats were sacrificed and liver, spleen, blood samples (from peripheric and from portal vein), stomach and small intestine and their contents were harvested, weighted and quantified. Stomach, duodenum, jejunum and ileum were isolated and the tissue and the luminal contents were separated and homogenized with acetonitrile (0.5–2.0 ml) using a PowerGen125 apparatus for approximately 3 min in an ice bath. The homogenized samples were transferred to Falcon tubes and centrifuged at 5292 × *g* for 20 min. A 100 μl aliquot of the supernatants were treated with acetonitrile (200 μl) and analyzed by a validated HPLC/UV method. When necessary, samples were properly diluted with the mobile phase considering the range of the calibration curve. The indomethacin and indomethacin ethyl ester tissue and luminal content concentrations were calculated by Eq. (1):

$$C_T = \frac{C_S \cdot V_S}{P} \quad (1)$$

where C_T is the analite concentration (μg g^{-1}) in tissue, C_S is the analite concentration in supernatant, V_S is the supernatant volume and P is the tissue weight.

To evaluate the possibility of nanocapsule bioadhesion to the intestinal tissue an additional group was studied. One hour after the oral administration of NC–IndOEt in a dose of 10 mg kg^{-1} of drug, the rats were sacrificed and blood samples, liver, spleen, stomach and small intestine and their contents were harvested and weighed. After the content removal, stomach and intestinal tissues were cut along the longitudinal axis and the intestinal mucosa of the segments was scrapped with a wet-swab slide. Then, the tissues were processed as described above.

Blood samples were collected into heparinized reaction tubes and centrifuged (14,811 × *g* for 15 min at 21 °C) to separate the plasma. Before the analysis, plasma samples were deproteinized by adding 200 μl of acetonitrile, vortexed for 10 s and centrifuged at 20,200 × *g*, 21 °C for 10 min. The supernatant was separated to inject into the HPLC system.

Indomethacin and indomethacin ethyl ester were quantified in plasma and tissues, using a previously validated HPLC method with UV detection (Cattani et al., 2008). The HPLC system consisted of a Waters 600 solvent delivery system, a Waters 717 plus auto-injector and UV 2487 (Dual λ Absorbance, Waters) detector. Chromatographic separation was achieved on a 4 μm Nova-Pak® 150 × 3.9 mm C18 column (Waters®, Dublin, Ireland), at 45 °C, using a mixture of acetonitrile:0.02 M ammonium dihydrogen phosphate (70:30, v/v), apparent pH 5, as mobile phase. The analyses were carried out using a flow rate gradient, starting with a 0.8 ml/min until 3.5 min, swifting to 1.3 ml/min over 2.5 min and returning to the initial conditions over 0.5 min. The autosampler was set to inject 50 μl sample aliquots and indomethacin and indomethacin ethyl ester were detected at 267 nm. The assay for both plasma and tissue samples was linear over a concentration range of 0.5–40 μg ml^{-1}, had a low limit of quantification (0.5 μg ml^{-1}) and the determination coefficients were higher than 0.98. The accuracy of the method was higher than 85%. Intra- and interday relative standard deviations were less than 11.8% and 9.2%, respectively.

3. Results and discussion

3.1. Preparation and characterization of the nanocapsules

The indomethacin ethyl ester was prepared and isolated with yields of 80%. In parallel, the fluorescent copolymer (PMMA-HBN) was successfully obtained presenting M_n of 276,000 g mol^{-1} and M_w of 1,603,000 g mol^{-1}. The fluorescent dye was chemically bound at 558 μg/g of copolymer (2.5 mol mol^{-1}).

All nanocapsule formulations showed macroscopic homogeneous appearance, like a milky white bluish opalescent liquid. NC–IndOH, NC–IndOEt and NC–PMMA-HBN were directly obtained by interfacial deposition of pre-formed polymers without any subsequent step of filtration or centrifugation. The diameters (*z*-average) and the polydispersity indexes were 262, 242 and 215 nm and 0.18, 0.20 and 0.09, respectively. The suspensions were formed by nanoparticles distributed in one narrow population in each case. Fig. 1a shows a schematic representation of the nanocapsule, composed by a lipid core and a polymeric wall, containing the drug retained in the particle. Fig. 1b shows the size distributions of three different batches of NC–IndOEt. The lipid-core nanocapsules are spherical shape as depicted in Fig. 1c. The size distributions are in accordance with other nanocapsule systems described in the literature (Chasteigner et al., 1995; Calvo et al., 1996). The pH values of suspensions were 6.0, 6.0 and 6.1 for

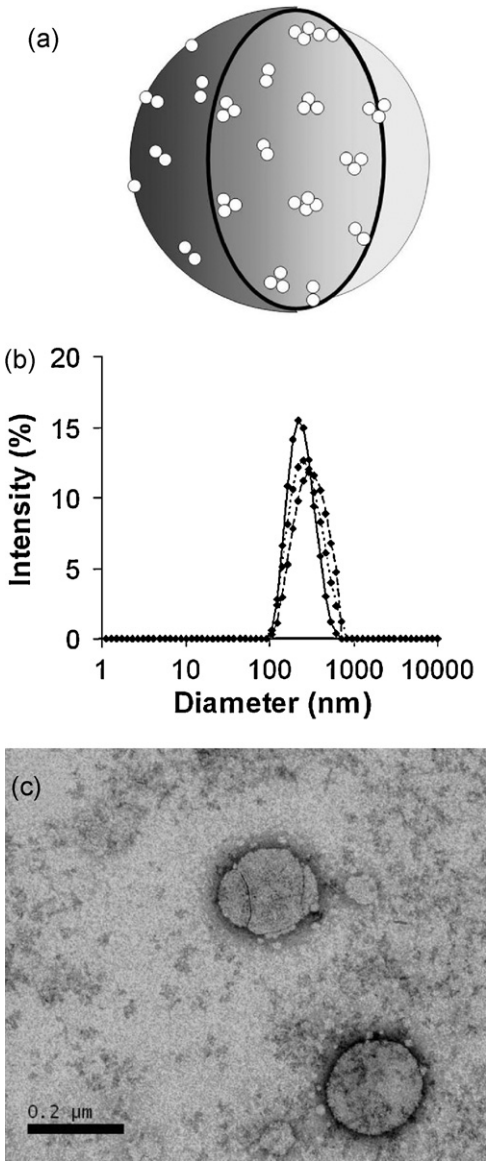

Fig. 1. Schematic illustration of the lipid-core nanocapsules containing indomethacin ethyl ester (longitudinal cut) (a); size distribution profiles of three different batches of NC–IndOEt (b); and TEM photomicrograph of a NC–IndOEt formulation.

NC–IndOH, NC–IndOEt and NC–PMMA-HBN, respectively. The zeta potential values were -10.6 ± 0.4, -11.3 ± 1.2, and -10.7 ± 1.2 mV for NC–IndOH, NC–IndOEt and NC–PMMA-HBN, respectively, as a consequence of a polymeric wall coated with polysorbate 80. The negative density of charge is related to the chemical structures of the polymers (PCL and PMMA-HBN), and the low modulus values of zeta potential are due to the polysorbate 80 coating, which stabilizes the nanocapsules by a steric mechanism.

3.2. Ex vivo drug absorption using rat everted gut sac

Indomethacin ethyl ester was quantified as a function of time in the serosal fluid and intestinal tissue. The total time of experiment was 120 min, *i.e.* 7200 s. The intestinal tissue showed an increase of indomethacin ethyl ester from 0.04% to 0.24% of the initial dose within 120 min. Indomethacin ethyl ester ranged from 6.13 to 27.47 μmol m^{-2} within 7200 s (120 min) (Fig. 2). After 15, 60 and 120 min, the concentrations of indomethacin ethyl ester were

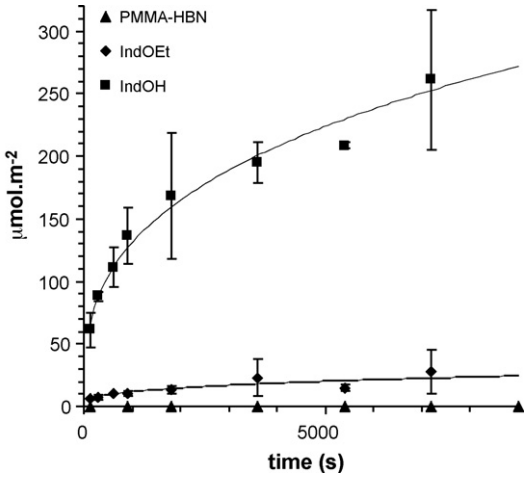

Fig. 2. Amounts of indomethacin, indomethacin ethyl ester and fluorescent copolymer per square meter of intestine as a function of time in the *ex vivo* rat everted gut sac experiment after adding the NC formulations: NC–IndOH, NC–IndOEt and NC–PMMA-HBN (mean + S.D.).

11.93, 27.31 and 32.88 μg/g of tissue, respectively. In the serosal fluid, the indomethacin ethyl ester concentration was below the pro-drug limit of quantification between 2 and 30 min of experiment. After 30 min, 0.03% of the initial dose was determined in the serosal fluid corresponding to a passage of 0.16 ± 0.02 μmol m^{-2}. This value remained constant within 30 and 120 min.

It is noteworthy to mention that no indomethacin (free acid) was formed within the period of experiment, indicating that no hydrolysis of indomethacin ethyl ester took place. In a previous work, the pro-drug release was assayed adding indomethacin ethyl ester-loaded nanocapsules in simulated gastric fluid containing pepsin, as well as in simulated intestinal fluid containing pancreatin, and no hydrolysis was observed independent of the medium (Cruz et al., 2006a).

Fluorescent-labeled nanocapsules (NC–PMMA-HBN) were assayed to determine the bioadhesion and if the nanocapsules were able to cross the gut wall. The physicochemical stability of the fluorescent dye chemically bound to the polymer wall has been already demonstrated (Jäger et al., 2007). The encapsulation of indomethacin ethyl ester in NC–PMMA-HBN was not possible due to a lack of specificity in the copolymer quantification by fluorescence spectroscopy. So, placebo NC–PMMA-HBN was added into the external medium. Regarding the *ex vivo* uptake of NC–PMMA-HBN, the serosal fluid showed low fluorescence intensity within 150 min of experiment. The copolymer concentrations were below the validated limit of quantification (7.5 μg ml^{-1}; 4.7×10^{-6} μmol ml^{-1}) for the whole experiment. The intestinal tissue showed a copolymer bioadhesion of 0.012 μmol m^{-2} (Fig. 2). After 15, 60 and 120 min, the concentrations of PMMA-HBN in the tissue were practically constant (43.77, 42.28 and 49.33 μg g^{-1}, respectively). The main factors which affect the particle bioadhesion and uptake are the average particle size, zeta potential and hydrophilicity–hydrophobicity balance (Florence, 1997; Ponchel and Irache, 1998; Hussain et al., 2001; Shakweh et al., 2004). So, the bioadhesion observed for the lipid-core nanocapsules (NC–PMMA-HBN) is due to the hydrophobic and nanoscopic nature of the particles. On the other hand, the formulation showed a neglected and not quantifiable uptake *ex vivo*.

In parallel, indomethacin-loaded nanocapsules (NC–IndOH) were also evaluated in the *ex vivo* experiment to verify the molecular diffusion through the gut wall. Indomethacin solubilizes at pH 7.4 by interfacial ionization and desorption from the nanocap-

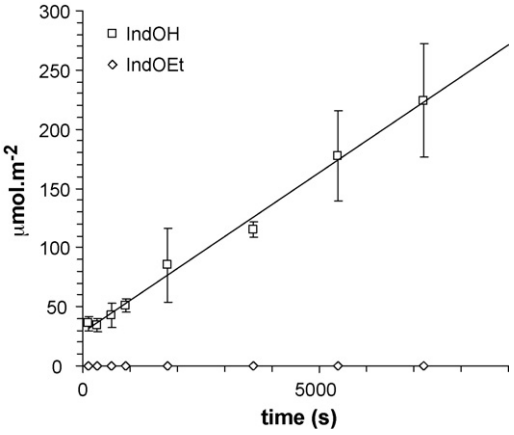

Fig. 3. Transport across *ex vivo* gut sac (mean + S.D.) after adding NC–IndOH or NC–IndOEt in the external medium.

sules (Pohlmann et al., 2004). Indomethacin ranged from 61.30 to 261.04 μmol m^{-2} within 7200 s (120 min) (Fig. 2), corresponding to 0.46–1.93% of the drug initial dose. After 15, 60 and 120 min, the concentrations of indomethacin were 151.90, 216.65 and 289.74 μg/g of tissue, respectively.

The profiles of indomethacin and indomethacin ethyl ester determined in the gut sac as a function of time fit to a power law model, $y = ax^n$ ($r = 0.9929$ and 0.9360, respectively). The n coefficients were 0.33 for both substances, indicating that both substances diffuse in the tissue (Fickian diffusion). After the addition of NC–IndOH in the external medium, the permeation of indomethacin through the gut sac wall was 0.27–1.69% of the initial dose (2–120 min of experiment). The cumulative mass transfer as a function of time showed an indomethacin flux of 0.027 ± 0.004 μmol m^{-2} s^{-1} ($r = 0.9941$) (Fig. 3).

Since indomethacin is adsorbed at the polymeric wall of the nanocapsules, as previously demonstrated (Pohlmann et al., 2004), and no drug crystal was simultaneously formed with the nanocapsules (Pohlmann et al., 2008), the indomethacin transport occurred by interfacial ionization (nanocapsules/aqueous phase interface), dissolution (in the aqueous phase) and drug permeation through the gut wall by molecular diffusion. In the case of indomethacin ethyl ester, considering its low solubility in the aqueous medium (less than 1 μg ml^{-1}) allied to the lack of interfacial hydrolysis *ex vivo*, its release occurred exclusively at the gut sac after the nanocapsule bioadhesion. Indomethacin was mostly released from the nanocapsules in the external medium, while indomethacin ethyl ester, a non-ionizable substance, is exclusively released in the *ex vivo* tissue. The different solubility of those substances in aqueous media can explain the results.

3.3. *In vivo intestinal absorption after oral administration of NC–IndOEt*

After NC–IndOEt oral administration (10 mg kg^{-1}), the indomethacin ethyl ester and indomethacin concentrations in the gastrointestinal tissues and theirs contents were evaluated. The indomethacin ethyl ester concentration was practically constant in the content of the stomach ranging between 990 and 1480 μg ml^{-1} within 120 min after oral dosing (Fig. 4). Regarding the content of duodenum, the indomethacin ethyl ester concentrations showed a tendency to increase from 1715 to 2620 μg ml^{-1} after 15 and 60 min of dosing, respectively. After 120 min, it was observed a tendency to decrease to 940 μg ml^{-1}. After the same period, it showed a tendency to decrease from 2450 (15 min) to 335 μg ml^{-1} (120 min) in the content of jejunum. Finally, the

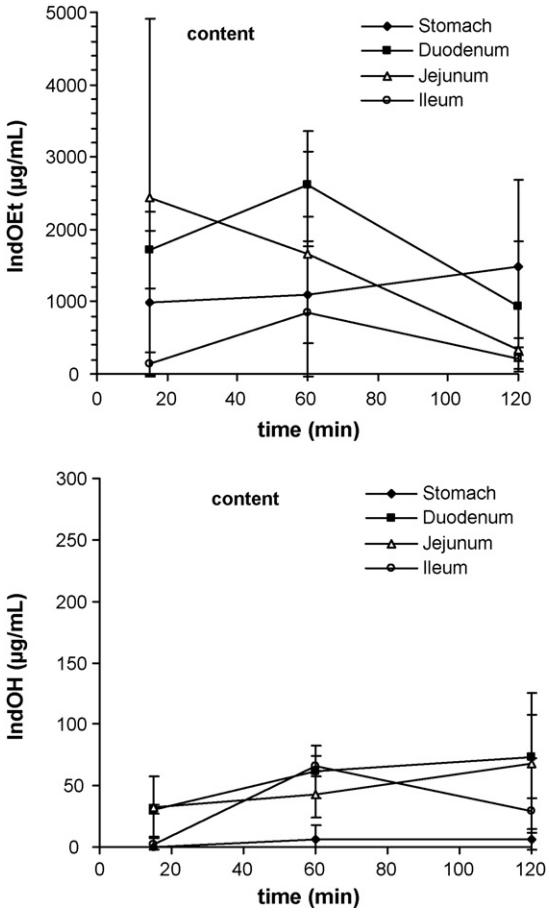

Fig. 4. Indomethacin ethyl ester (top) and indomethacin concentrations (bottom) in the luminal contents of stomach and small intestine (mean + S.D.) after oral administration of NC–IndOEt (pro-drug at 10 mg kg^{-1}) ($n = 4$).

indomethacin ethyl ester concentration reached a maximum (854 μg ml^{-1}) in the content of ileum after 60 min.

After NC–IndOEt oral administration (10 mg kg^{-1}), indomethacin was also detected in the contents of the stomach and the small intestine. The indomethacin concentration in the content of the stomach was lower than 6 μg ml^{-1}, whereas in the contents of the duodenum and jejunum it ranged between 30 and 74 μg ml^{-1} after 15 and 120 min (Fig. 4). In the content of ileum, the indomethacin concentrations showed a tendency to increase from 2 to 65 μg ml^{-1} after 15 and 60 min, respectively, and then a tendency to decrease to 29 μg ml^{-1} after 120 min. The results demonstrated that indomethacin ethyl ester was hydrolyzed at least in some extension in the lumen of the small intestine.

The gastrointestinal tissues were analyzed after the NC–IndOEt oral administration. The indomethacin ethyl ester concentrations in the stomach were practically constant between 15 and 120 min ranging from 603 to 895 μg g^{-1} (Fig. 5). In the duodenum, the indomethacin ethyl ester concentration rose from 770 to 3020 μg g^{-1} after 15 and 60 min, respectively, and decreased to 440 μg g^{-1} after 120 min. In the jejunum, the indomethacin ethyl ester concentration showed an augmentation from 950 to 1530 μg g^{-1} after 15 and 60 min, respectively, followed by a decrease to 107 μg g^{-1} after 120 min. In the ileum, the indomethacin ethyl ester concentrations were very low, showing a tendency to decrease from 93 to 16 μg g^{-1} from 15 to 120 min.

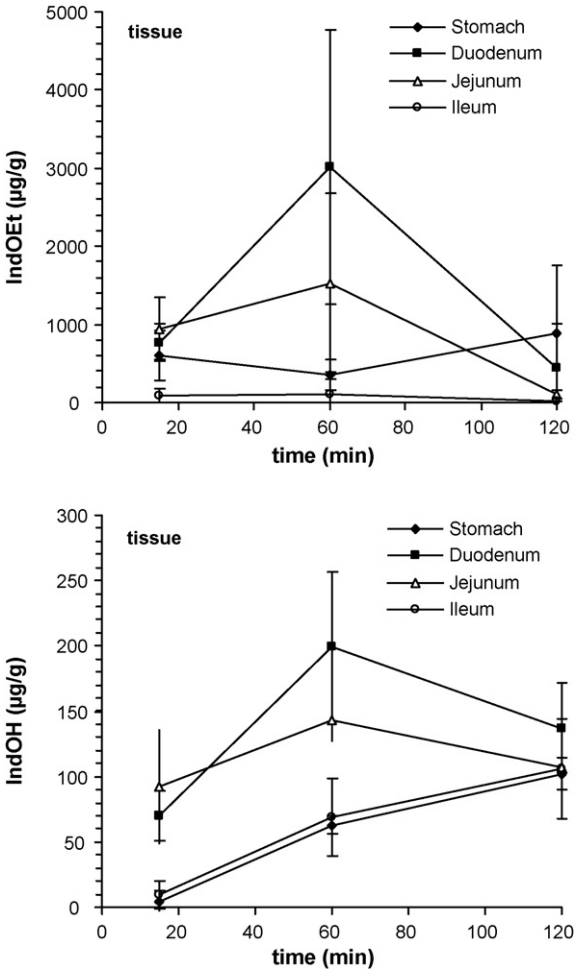

Fig. 5. Indomethacin ethyl ester (top) and indomethacin concentrations (bottom) in stomach and small intestine tissues (mean + S.D.) after oral administration of NC–IndOEt (pro-drug at 10 mg kg^{-1}) (n = 4).

After NC–IndOEt oral administration, the indomethacin concentrations in stomach rose from 4 to 102 µg g^{-1} after 15 and 120 min, respectively (Fig. 5). In the duodenum and jejunum, the concentrations were similar rising from 70 and 92 (15 min) to 137 and 107 µg g^{-1} (120 min), respectively. In the ileum, the indomethacin concentration increased from 9 to 106 µg g^{-1} after 15 and 120 min, respectively. The hydrolysis of the indomethacin ethyl ester was likely occurring in the tissue wall.

A parallel experiment was carried out to evaluate the nanocapsule adhesion to the gastrointestinal luminal mucus. After 60 min of NC–IndOEt oral administration, the stomach and small intestine were scrapped for mucus removal before homogenization. The indomethacin ethyl ester concentrations in the tissues were much lower than before scrapping. Its concentrations decreased from 459 to 5 µg g^{-1} from the stomach to the ileum (Fig. 6). On the other hand, for indomethacin formed by ester hydrolysis, a slight difference was barely observed before and after scrapping. Its concentrations in stomach, duodenum, jejunum and ileum were 58, 126, 88 and 56 µg g^{-1}, respectively, after 60 min (Fig. 6). The mucoadhesion probably delayed the ester hydrolysis in the lumen as previously described for poly(alkyl cyanoacrylate) nanoparticles (Dembri et al., 2001).

The results showed that the indomethacin ethyl ester is mainly accumulated in the mucus. The indomethacin ethyl ester uptake was more intense at the stomach and proximal small intestine,

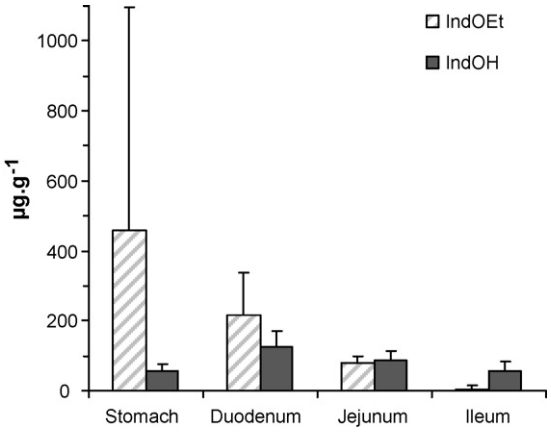

Fig. 6. Indomethacin ethyl ester and indomethacin concentrations in the scrapped tissues for mucus removal, after oral administration of NC–IndOEt (pro-drug at 10 mg kg^{-1}) (n = 4).

where higher concentrations were observed. The indomethacin ethyl ester tissue concentrations without mucus revels a strong nanoparticles interaction with the polysaccharide chains of mucus. The nanoparticles interaction with the mucus gel layer and mucus membrane is a consequence of the physicochemical interactions between mucus and non-swellable and hydrophobic polymers, such as poly(ε-caprolactone). Considering the probable mechanism of indomethacin absorption after NC–IndOEt administration *in vivo*, part of the indomethacin ethyl ester released in the mucus is hydrolyzed by the lumen esterases. Besides, nanocapsules adhered to the epithelium can likely release the indomethacin ethyl ester, which penetrates the gastrointestinal tissues to be hydrolyzed to indomethacin.

After NC–IndOEt oral administration (10 mg kg^{-1}), exclusively indomethacin was detected in plasma (peripheric and portal vein), spleen and liver. After 15, 60 and 120 min, indomethacin concentrations in plasma and portal vein were similar ($p > 0.05$) ranging from 6 to 35 µg ml^{-1} (Fig. 7). The indomethacin concentrations in spleen were lower ($p < 0.05$) than in liver at all time points investigated and increase with time up to 1 h, reaching a plateau, between 1 and 2 h, of 70 and 120 µg g^{-1}, for spleen and liver, respectively (Fig. 7). The results showed that indomethacin ethyl ester was totally hydrolyzed in gastrointestinal lumen, and probably tissue, before reaching the blood stream.

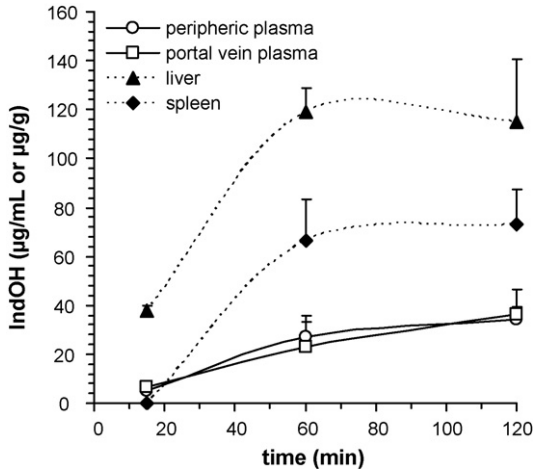

Fig. 7. Indomethacin concentration–time profiles in peripheric plasma, portal vein plasma, liver and spleen after oral administration of NC–IndOEt (pro-drug at 10 mg kg^{-1}) (n = 4/time) (mean + S.D.).

Theoretically, ester pro-drugs could be absorbed and, by first pass metabolism, be converted in free acid drugs, detected in plasma. However, in the present work this hypothesis was refused considering the absence of indomethacin ethyl ester in portal vein plasma and in liver. Additionally, the liver, despite its high esterase activity, can be marginally involved in the conversion of ester pro-drugs after their uptake by the gastrointestinal tract (Braggio et al., 2002).

4. Conclusion

The *ex vivo* experiment showed a limited passage of indomethacin ethyl ester from the luminal side through the serosal fluid. In addition, the lipid-core nanocapsules were stable and no indomethacin ethyl ester was released in the luminal side. So, indomethacin was not formed by hydrolysis of the ester in the external medium. The lipid-core nanocapsules released indomethacin ethyl ester at the tissue after bioadhesion, but no enzymatic hydrolysis took place. In this way, indomethacin was not formed in the tissue. The comparison between the different formulations (NC–IndOEt, NC–IndOH and NC–PMMA-HBN) showed that indomethacin ethyl ester is absorbed in the tissue by a diffusion mechanism.

The *in vivo* experiment showed that the lipid-core nanocapsules were able to restrain the indomethacin ethyl ester hydrolysis in the gastrointestinal lumen and wall acting as mucoadhesive reservoir systems for the pro-drug. Furthermore, after oral dosing of lipid-core nanocapsules, our study showed that indomethacin ethyl ester is converted into indomethacin before the pro-drug reach the blood stream. Lipid-core nanocapsules are stable in the gastrointestinal tract and can be used for oral delivery of lipophilic drugs.

Finally, distinct results were observed comparing the *ex vivo* and *in vivo* models. The complexity of a living system was not mimic by the *ex vivo* experiment, and the gut sac model was not able to predict the *in vivo* behavior of indomethacin ethyl ester-loaded lipid-core nanocapsules.

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