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Research paper

Dexamethasone-loaded nanoparticle-coated microparticles: Correlation between *in vitro* drug release and drug transport across Caco-2 cell monolayers

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Abstract

This work reports the preparation of dexamethasone in nanoparticle-coated microparticles and the study of the influence of such microencapsulation on drug absorption across Caco-2 cell monolayers. Nanoparticle-coated microparticles were prepared by spray-drying using nanocapsules (NC) or nanospheres (NS) in aqueous suspensions as coating material. Drug contents ranged from 64 to 134 mg g^{-1}, yields between 49% and 67% and moisture content below 2.0%. SEM and AFM analysis demonstrated that the nanoparticle-coated microparticles (20–53 μm) show nanostructures on their surface with a similar diameter compared to the aqueous suspensions. The type of nanocoating material had a significant influence on the drug release profile and on the drug permeation across Caco-2 cells: NC-coated microparticles led to a prolonged release and slower transport across Caco-2 cell monolayers, while the NS-coated microparticles showed a faster release and Caco-2 transport compared to uncoated microparticles. The correlation between the amount of drug permeated and the drug released (%) suggests that the drug absorption from such a delivery system is controlled mainly by the release rate rather than by epithelial permeability. Caco-2 transport studies appear to be a useful characterization tool for the development of microparticulate oral controlled release systems.

Keywords: Caco-2 cells; Dexamethasone; Microparticles; Nanoparticles; Spray-drying

1. Introduction

Microparticles are a common approach to modify drug release behavior [1–5]. Oral microparticulated delivery systems exhibit several advantages including ready distribution on a large surface area, more constant plasma levels, better dose by dose reproducibility and smaller decrease in bioavailability compared to single-unit systems [6]. It was also demonstrated that microparticles are promising for the treatment of inflammatory bowel diseases (IBD) [7,8]. The main advantage of microparticles is given by their small size. Decreasing the diameter of drug carrier systems increases their residence time in the gut even in the case of diarrhea, a frequent symptom of IBD [7–10].

We recently have reported on a novel strategy to prepare microparticulated controlled release systems by spray-drying, using polymeric nanoparticles (nanospheres or

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nanocapsules) as coating material of microparticles [11,12]. Such systems can be prepared by nanoparticle-coating of an organic–inorganic core, compounded of silicon dioxide and the anti-inflammatory drug diclofenac. The nanoparticle-coating was demonstrated by SEM analysis which showed the presence of nanostructures forming a polymeric coating on the organic–inorganic core surface. This observation was also accompained by a modified drug release from the nanoparticle-coated microparticles compared to the uncoated core. In another work of our group, we have demonstrated that for nanoparticle-coated microparticles the mechanism of drug release is non-Fickian and it depends on the particle desagglomeration [13]. Besides, this method presents significant advantages in comparison to the main methods reported to prepare Eudragit and other polymeric microparticles, such as the avoidance of organic solvents during the coating step, the reduced environmental burden and risk of explosion during the spray-drying process and the possibility to control the micro-powder coating using different nanoparticle suspensions, like polymeric nanocapsules, nanospheres or nanodispersion [11,12]. This surface control could allow the modulation of the drug release profile as previously reported [11] and the modulation of the drug transport across biological barriers.

Caco-2 cells, a human colon adenocarcinoma cell line, undergo spontaneous enterocytic differentiation in culture, forming monolayers of polarized enterocytes that exhibit morphological and functional similarities to the small intestinal epithelium [14]. They have been used to study drug transport by all four different transport routes across the intestinal epithelium: passive transcellular and paracellular routes, carrier mediated route and by transcytosis [15]. Caco-2 cells have been widely accepted as a potent *in vitro* model for the rapid screening of intestinal drug absorption [16–20] and their use has dramatically increased in the pharmaceutical sciences [15,21].

However, relatively few studies have been carried out concerning the influence of microencapsulation of a drug on its absorption across Caco-2 cell monolayers [22–26]. Typically these studies report different results in relation to the drug permeability, depending on the type of drug and microparticle. Insulin-loaded microparticles showed an increase in the permeability of the drug across Caco-2 cell monolayers [22–25], whereas famotidine-loaded microparticles showed a decrease in permeability depending on the polymer type and the particle size [15]. On the other hand, ibuprofen-loaded chitosan microparticles did not show any difference in their permeation across Caco-2 cells when a polymer with fixed positive charges (*N*-trimethyl chitosan chloride) was used in combination with chitosan [26].

Considering all these previous studies, this work reports the preparation and characterization of nanoparticle-coated microparticles and the study of the influence of microencapsulation on the absorption of a drug model (dexamethasone) across Caco-2 monolayers. Dexamethasone was chosen due to its low aqueous and pH-independent solubility, and its pharmacological applications for the treatment of IBD, as its anti-inflammatory and immunosuppressive activities [27–29]. The microparticles were characterized in terms of process yields, drug contents, water contents, particle sizes, X-ray diffraction, *in vitro* drug release, morphological analysis by scanning electronic microscopy and atomic force microscopy and cytotoxicity. Mathematical modeling of drug release profiles and of the permeability of dexamethasone across Caco-2 monolayers was also carried out.

2. Materials and methods

2.1. Materials

Dexamethasone was obtained from Henrifarma (São Paulo, Brazil); Eudragit S100® (EUD) was supplied from Almapal (São Paulo, Brazil). Caprilic/capric triglyceride mixture was delivered from Brasquim (Porto Alegre, Brazil); polyethylene glycol 6000 (PEG 6000), sorbitan monostearate (Span 60®) and polysorbate 80 (Tween 80®) were supplied by Delaware (Porto Alegre, Brazil). Colloidal silicon dioxide (Aerosil 200®) was acquired from Degussa (São Paulo, Brazil). The Caco-2 cell line was obtained from the ATCC (American Type Culture Collection, clone C2BBE1). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin (0.25%)–EDTA (1 mM) were purchased from Gibco Laboratories (New York, USA). Non-essential amino acids (NEAA) were obtained from Sigma (Steinheim, Germany). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from Carl Roth (Karlsruhe, Germany). All others chemicals and solvents were of pharmaceutical or chromatographic grade and were used as received.

2.2. Methods

2.2.1. Preparation and characterization of colloidal suspensions

Nanocapsules (NC) and nanospheres (NS) from Eudragit S100® (EUD) were prepared by the nanoprecipitation method as described by Fessi and co-workers [30]. For NC preparation, the organic solution consisted of a capric/caprilic mixture (3.3 ml), Span 60® (0.1532 g), the polymer (EUD S100®) (1.0 g) and acetone (267.0 ml). This organic phase was added under moderate magnetic stirring to an aqueous solution (533.0 ml) containing Tween 80® (0.1532 g). The magnetic stirring was maintained during 10 min. Thus, the acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure (40 °C); then the final volume was adjusted to 100 ml, corresponding to a polymer concentration of 10 mg ml^{-1}. The NS suspensions were prepared as described above for NC, omitting the capric/caprilic triglyceride mixture. The colloidal suspensions were characterized by pH measurements (Micronal, B-474, São Paulo, Brazil) and particle size and

polydispersity determination using photon correlation spectroscopy (Zetasizer Nano ZS, Malverns Instruments, UK) after dilution of samples (500 times) with water (Milli-Q®).

2.2.2. Preparation of nanoparticle-coated microparticles

To obtain the core of the microparticles (uncoated core), 50 ml of dexamethasone acetone solution (5.00 mg ml^{-1}) was added to Aerosil 200® (1.5 g). The acetone was removed under reduced pressure to obtain a solid product (Fig. 1). This powder (the core) was maintained in a desiccator at room temperature during 48 h. For the coating step, the core material (1.5 g) was carefully milled in a mortar for 10 min, and dispersed into 50 ml of the NS or NC aqueous suspension under magnetic stirring at room temperature. The mixture was fed into a mini-spray-dryer Büchi 190® (Flawil, Switzerland) with a two-component nozzle and co-current flow. The inlet temperature at the drying chamber was maintained around 170 °C ± 4 °C and the spray rate feed was 3 ml min^{-1}. The powders were called MP-NS and MP-NC, for samples obtained with the NS or NC suspensions, respectively.

These formulations were also prepared using polyethylene glycol 6000 (PEG 6000), as a plasticizer. The PEG 6000 [20% (w/w) in relation to the polymer] was added to the nanoparticle suspensions (NC or NS) that were maintained under magnetic stirring for 24 h, followed by spray-drying. The powders were called MP-NS-PEG and MP-NC-PEG, for samples obtained with the NS or NC suspensions, respectively.

All formulations were prepared in triplicate ($n = 3$).

2.2.3. Characterization of microparticles

2.2.3.1. Determination of yield and drug content. The yields of the formulations were calculated by the ratio between the experimental weight of product and the sum of the weights of all components, discounting the content of water in the suspension. To determine the drug content, the powders (cores and nanoparticle-coated microparticles) were dispersed in acetonitrile during 60 min at room temperature, followed by centrifugation of the dispersions. Thus, the supernatants were appropriately diluted with the mobile phase and filtered through an hydrophilic membrane (GVWP, 0.22 μm, Millipore). The samples were analyzed by HPLC [31]. The chromatographic system consisted of a Lichrospher® column RP 18 (250 × 4 mm, Merck, Darmstadt, Germany) and a Perkin–Elmer instrument (Series 200 Pump, Series 200 Autosampler and Series 200 UV/Vis Detector, Shelton, EUA). The mobile phase consisted of acetonitrile/water (55:45 v/v) at a flow rate of 1.2 ml min^{-1}. The volume injected was 20 μl. Dexamethasone was detected at 254 nm. The drug content of each formulation was calculated by the correlation of the theoretical and the experimental dexamethasone concentrations and expressed as percentage (%). Validation of the HPLC assay demonstrated that this methodology was linear ($r^2 = 0.9995$) in the range of 3–15 μg ml^{-1}, precise (RSD: 1.79% for repeatability and <3.5% for intermediate precision) and accurate within this range of concentration (3–15 μg ml^{-1}). The specificity was tested in presence of the microparticles adjuvants and in different pH media (phosphate buffer pH 6.0 and 7.4) and demonstrated that these factors did not alter the dexamethasone assay [32,33].

2.2.3.2. Determination of moisture content. The moisture content was determined by the Karl-Fisher coulometric method (Mettler DL 37, Greifensee, Switzerland).

2.2.3.3. Scanning electron microscopy. The uncoated core and nanoparticle-coated microparticles were examined under scanning electron microscope (SEM) (JEOL Scanning Microscope, JSM-5800, Tokyo, Japan), at different

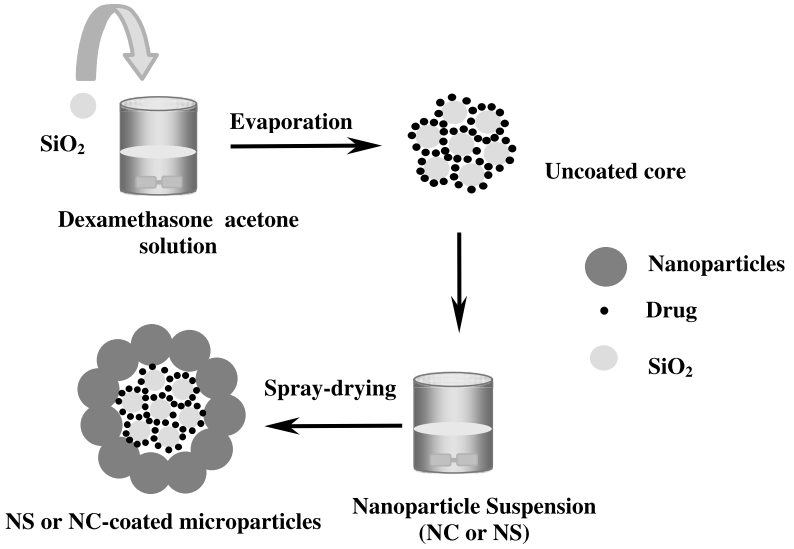

Fig. 1. Schematic process for preparation of nanoparticle-coated microparticles.

magnifications between 1,000× and 90,000×. Samples were analyzed after they had been gold sputtered (JEOL Jee 4B SVG-IN, Tokyo, Japan). Analyses were performed in the Centro de Microscopia Eletrônica (UFRGS).

2.2.3.4. Atomic force microscopy. The surface morphology of the nanoparticle-coated microparticles was analyzed by atomic force microscopy (AFM) Nanoscope IV Bioscope™ (Digital Instruments, Veeco Instruments, Germany) in tapping mode using ultrasharp silicon cantilevers (NSC16/AIBS/15, Mikromasch, Spain) with a spring constant of about 34 N/m and a resonance frequency of about 200 kHz. Scanning was performed at a scan speed of 0.4 Hz with a resolution of 512 × 512 pixels. Image were processed and analyzed using the Nanoscope 5 software (Digital Instruments,Veeco Instruments, Germany).

2.2.3.5. Particles size distribution and average diameter. The particle size distribution was determined by laser diffractometry with a Beckman Coulter – Tornado (Beckman Instruments, USA) by dry dispersion. The mean diameter over the volume distribution $d_{4.3}$ was used as a particle size distribution parameter. $d_{0.1}$ and $d_{0.9}$ are also determined and correspond to the diameters at 10% and 90% cumulative volumes, respectively.

2.2.3.6. X-ray analyses. X-ray analyses were performed for the polymer, the drug (dexamethasone) and the microparticles. Diffraction powder patterns were obtained with a Siemens diffractometer (model D500) using Cu Kα radiation at 35 kV.

2.2.4. In vitro drug release studies

The *in vitro* drug release experiments were carried out using a flow-through cell technique. The apparatus is constituted by recycling flow-through cells (Desaga, Wiesloch, Germany), containing as a fluid inlet a bed of glass beads and connected to a peristaltic pump (Desaga, Wiesloch, Germany). The flow rate was 1 ml min^{-1}. Assays were carried out using phosphate buffer at pH 6.0 and 7.4. The dissolution medium was maintained at 37 °±0.5 C.

An exact amount of each powder (equivalent to 2 mg of dexamethasone) was placed in the cells for the release tests ($n = 3$). The samples were collected at predetermined time intervals and filtered through a hydrophilic membrane (GVWP, 0.22 μm, Millipore). The drug release/dissolved was assessed through HPLC analysis according to the method described above.

Dexamethasone release kinetics were mathematical modeled in order to better understand the influence of the type of the coating material on the release behavior of the microparticles. Table 1 presents the equations tested (MicroMath Scientist® software, Salt Lake City, USA).

2.2.5. Caco-2 cell culture

Caco-2 cells, clone C2BBe1, were purchased at passage 60 from American Type Culture Collection (ATCC; Manassas, VA) and used at passages 70–92. Cells were grown to ~90% confluency in 75 cm^2 T-flasks with DMEM supplemented with 10% FBS and 1% NEAA. Culture medium was changed every second day and cells were grown at a temperature of ~37 °C in an atmosphere of ~85% relative humidity and ~5% CO_2. For the transport assay, cells were seeded on top of Transwell polyester inserts (pore size 0.4 μm, 1.13 cm^2) at a density of ~60,000 cells/cm^2. The culture medium (0.5 ml in the apical compartment, 1.5 ml in the well) was replaced every 48 h. Transepithelial electrical resistance (TEER) was measured and only monolayers with a TEER >350 Ω cm^2, with background subtracted, were used for transport studies.

2.2.6. Transport studies

Caco-2 monolayers were used 21–25 days after seeding. Apical to basolateral permeability of dexamethasone was assessed in the presence (apical pH 6.0, basolateral pH 7.4) and absence (pH 7.4 on both sides) of a pH gradient. After 1 h of preincubation with drug-free transport medium (Hanks' balanced salt solution, HBSS), the medium containing the drug or the microparticle suspension was introduced to the apical side (0.6 ml). To determine the initial concentration (C_0), a sample of 100 μl was taken from the apical side (0.5 ml remaining at the apical side) and dispersed in acetonitrile during 60 min and filtered. Sample aliquots (400 μl) were taken from the basolateral side at given time intervals (0, 20, 40, 60, 90, 120, 150, 180, 240 min). After each sampling, an equal volume of fresh transport buffer (~37 °C) was added to the receiver compartment. All experiments were performed at 37 °C ($n = 6$). Throughout the experiments, monolayers were agitated using an orbital shaker (IKA®-Werke GmbH & CO KG; Staufen, Germany) at 100 ± 20 rpm. Apparent permeability P_{app} (cm/s) was calculated according to the following equation:

$$P_{app} = dQ/dt \times 1/AC_0$$

where dQ/dt is the rate of appearance of the drugs on the basolateral side (nmol s^{-1}), C_0 is the initial concentration on the apical side (mM) and A is the surface area of the monolayer (cm^2). Additionally, a study of dexamethasone transport across filters without cell monolayers was conducted.

2.2.7. Cytotoxicity studies

Lactate dehydrogenase is a cytosolic enzyme that is readily released upon cell membrane damage; hence,

Table 1
Categories of employed methods to compare the dissolution profiles

Method	Equation[a,b,c]
Zero-order	$\%\ \text{diss} = kt$
First-order	$\%\ \text{diss} = 100(1 - e^{-kt})$
Biexponential	$\%\ \text{diss} = 100[1 - (C_1 \cdot e^{-k \cdot t} + C_2 \cdot e^{-k' \cdot t})]$

% diss: percentage dissolved at time t.
k and k': dissolution rate constants.
C_1 and C_2: initial concentration of drug.

LDH may be used as a tool to monitor cellular toxicity. LDH released into the assay medium can be measured via a coupled enzymatic assay: conversion of the yellow tetrazolium salt (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) into a red formazan salt which is colorimetrically detected at 490 nm. Caco-2 cells were grown on 96-well tissue culture plates with a flat bottom (Greiner Bio-One GmbH, Frickenhausen, Germany) for 7 days as previously described. Cells were washed two times with KRB + 1% BSA (pH 7.4) medium and monolayers were incubated with the appropriate test suspensions in KRB + 1% BSA (pH 7.4) at 10 different concentrations (0.025; 0.050; 0.100; 0.200; 0.400; 0.800; 1.600; 3.200; 6.400 and 12.800 mg of microparticles/ml). After 120 min of incubation, LDH release into the supernatant was determined using the cytotoxicity LDH kit (Roche Diagnostics, Mannheim, Germany). In parallel, control experiments with 0.1% Triton X-100 (positive control) and with KRB + 1% BSA pH 7.4 medium (negative control) were carried out.

2.2.8. Analytical methods

The concentration of dexamethasone in the sample solutions from the transport studies was determined using a reversed-phase (Merck Lichrospher RP 18 column, Merck, Germany) high performance liquid chromatography system consisting of a L6220 Intelligent Pump, a L-4259 UV/Vis Detector, an AS 2000A Autosampler and a D-6000 Interface (Merck Hitachi, Germany). The mobile phase consisted of acetonitrile/water (55:45 v/v) with a flow rate of 1.0 ml min^{-1}. The volume injected was 20 µl. Dexamethasone was detected at 240 nm. The HPLC method was validated according to the following characteristics [32,33]: linearity ($y = 16{,}541 \times -636.95$, $n = 3$, $R^2 = 0.9999$), range (0.1–5.0 µg ml^{-1}), precision (repeatability: 0.24–0.53%; intermediate precision: 0.54–1.07%), accuracy (99.07–101.46%) and quantification limit (0.08 µg ml^{-1}).

2.2.9. Statistical analysis

One-way analysis of variance was employed in the comparison of the experimental data. Post hoc multiple comparisons were done by Tukey's test for significance at p–values less than 0.05.

3. Results

3.1. Preparation of nanoparticle-coated microparticles

The polymeric nanoparticle suspensions were prepared by nanoprecipitation of Eudragit S100® using a capric/caprilic triglyceride as oily phase in the case of nanocapsules (NC), or by omitting this triglyceride phase in the case of nanospheres (NS). These colloidal suspensions, NC and NS, presented acidic pH values (3.61 ± 0.05 and 3.60 ± 0.01, respectively), particle sizes of 189 nm and 109 nm and polidispersity indices of 0.23 and 0.15 respectively. After the preparation, the polymeric nanoparticles were used as aqueous coating suspension to prepare the dexamethasone-loaded microparticle powders by spray-drying. Table 2 presents the characteristics of these powders.

Except for the uncoated core, all the MP formulations presented yields between 39% and 67%. The uncoated core presented a drug content of 133.51 ± 3.51 mg g^{-1} and the microparticles presented values between 64 and 104 mg g^{-1}. The microparticles presented different drug contents according to the type of the nanoparticle suspension used as coating material (MP-NS: 104.10 ± 4.73 mg $g^{-1}$and MP-NC: 67.38 ± 1.91 mg g^{-1}).

Regarding the determination of the particle size (Table 2), all formulations presented a mean particle size ranging from 20 to 53 µm. A broader dispersity of microparticle sizes can be observed for the NC-coated microparticles (MP-NC: 3.27–161.30 µm and MP-NC-PEG: 3.02–166.60 µm) in comparison to the NS-coated formulations (MP-NS: 1.73–94.27 µm and MP-NS-PEG: 1.56–59.19 µm) and to the uncoated core (1.16–74.59 µm). These differences could be observed independently of the presence of the plasticizer.

In addition, all formulations presented moisture content below 2.00% and did not demonstrate any influence of the presence of the plasticizer (PEG) on this parameter (Table 2).

3.2. Morphological analysis

The morphological analysis of the uncoated core showed irregular shaped microparticles (Fig. 2a). At a higher magnification rugged surfaces with some cavities were visible (Fig. 2b). MP-NS and MP-NC on the other hand were more spheroidal shaped (Fig. 2c and e).

Table 2
Characteristics of the dexamethasone-loaded NP-coated microparticles ($n = 3$)

Formulation	Yield (%) (mean ± SD)	Drug content (mg g^{-1}) (mean ± SD)	Particle size (µm) $d_{4.3}(d_{0.1}–d_{0.9})$	Moisture (%) (mean ± SD)
Uncoated core	100[c]	133.51 ± 3.51[a]	22.32 (1.16–74.59)	1.49 ± 0.09[b]
MP-NS	49 ± 2[a]	104.10 ± 4.73[a,b]	41.10 (1.73–94.27)	1.90 ± 0.03[c]
MP-NC	39 ± 6[a]	67.38 ± 1.91[c]	52.71 (3.27–161.30)	0.88 ± 0.03[a]
MP-NS-PEG	67 ± 4[b]	96.10 ± 3.11[a]	20.31 (1.56–59.19)	1.01 ± 0.06[c]
MP-NC-PEG	49 ± 7[a]	64.74 ± 1.88[c]	51.54 (3.02–166.60)	1.85 ± 0.08[a]

Means, in column, with the same letter are not significantly different (ANOVA, Tukey test, $p \leqslant 0.05$). $d_{4.3}$ is the mean diameter over the volume distribution; $d_{0.1}$ and $d_{0.9}$ are the diameters at 10% and 90% cumulative volumes, respectively.

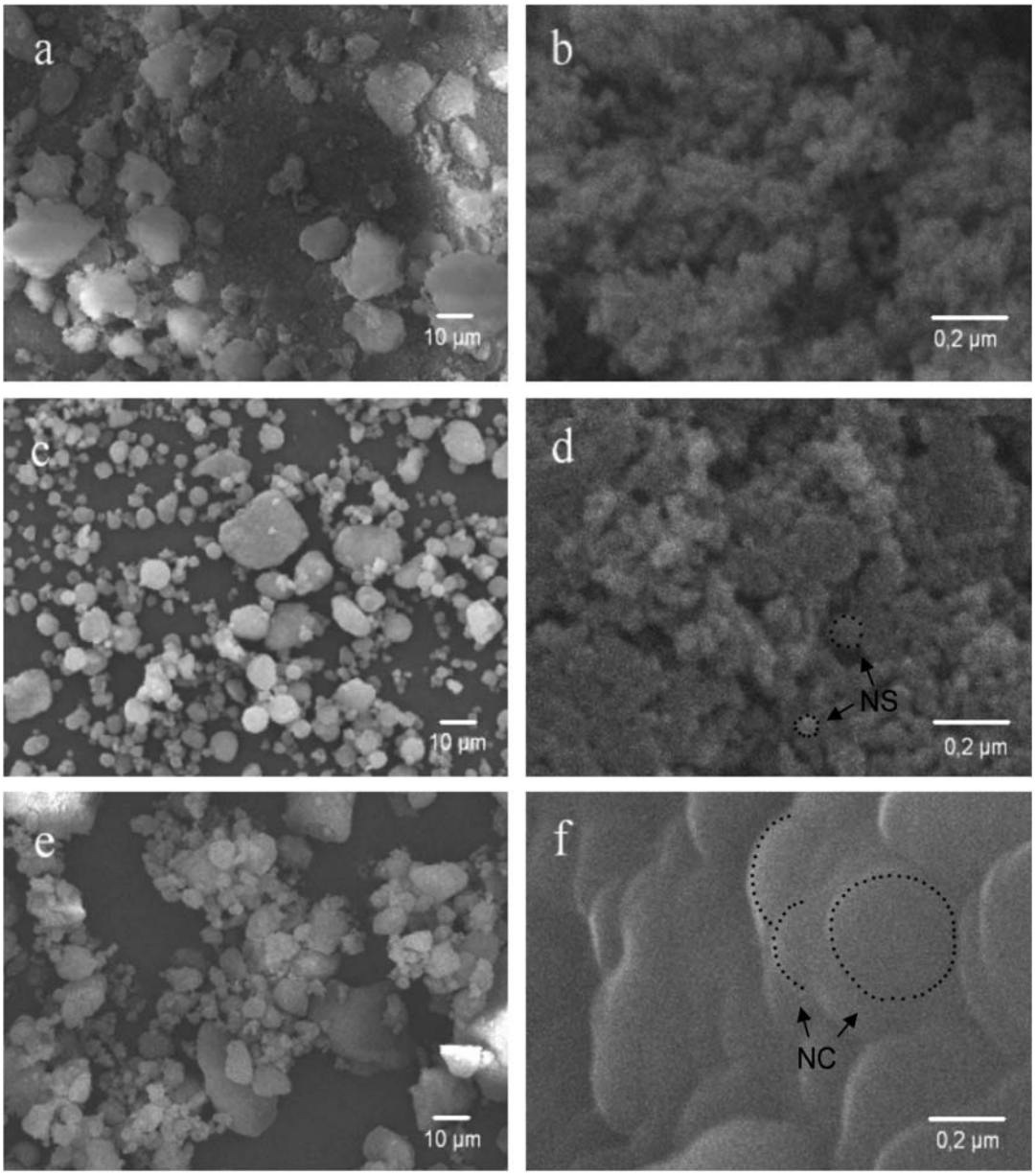

Fig. 2. SEM micrographs of dexamethasone-loaded nanoparticle-coated microparticles: (a) uncoated core (132 μm); (b) uncoated core (1.39 μm); (c) MP-NS (132 μm); (d) MP-NS (1.39 μm); (e) MP-NC (132 μm) and (f) MP-NC (1.39 μm).

At higher magnification these microparticles presented nanostructures with diameters around 60–70 nm and 170–200 nm at their surfaces, for MP-NS (Fig. 2d) and MP-NC (Fig. 2f), respectively. The morphological analyses (photomicrographs not shown) of MP-NS-PEG and MP-NC-PEG showed similar characteristics to the correspondent powders prepared in the absence of the plasticizer. In addition, the presence of nanostructures on the surface of the microparticles could also be observed by atomic force microscopy, as shown in Fig. 3 for MP-NC-PEG.

In order to verify the presence of drug crystals in the formulations, X-ray analyses were performed. Fig. 4 shows the X-ray diffraction patterns for dexamethasone (A) and the polymer Eudragit S100® (B). The diffractograms from C to F present the experimental results for the different microparticle samples (MP-NC, MP-NC-PEG, MP-NS and MP-NS-PEG). Diffraction peaks which correspond to the pattern of the dexamethasone (A) can be seen in all dexamethasone-loaded microparticles (C–F). These peaks are superimposed on the amorphous silica pattern, which has a very broad diffraction line at $2\theta \approx 22°$.

3.3. In vitro drug release

A first evaluation of effect of the different coatings on the drug release from the different microparticles is possible on the basis of the percentage of the drug released after 120 min. Release experiments were also done during 120 min aiming to compare the results to those obtained in Caco-2 transport studies. At pH 6.0, the uncoated core

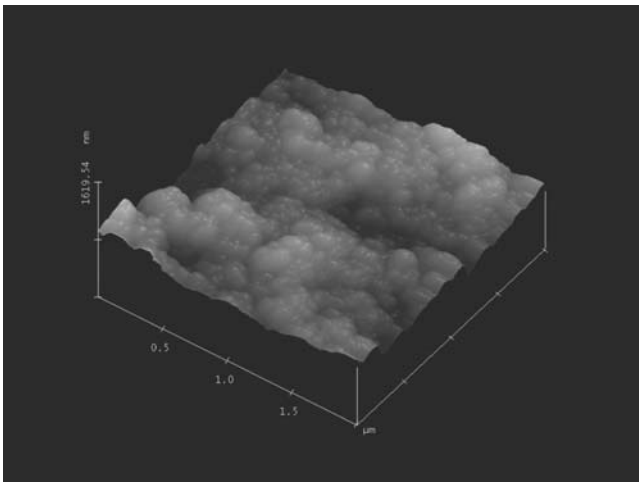

Fig. 3. AFM image showing the surface topography of MP-NC-PEG (2 μm × 2 μm image).

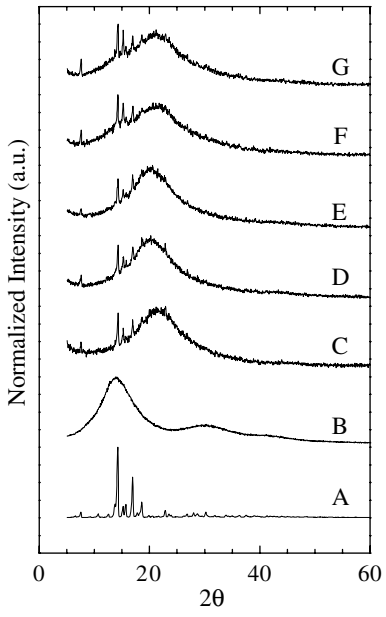

Fig. 4. X-ray diffraction pattern of: (A) dexamethasone; (B) Eudragit S100®; (C) uncoated core; (D) MP-NC; (E) MP-NC-PEG; (F) MP-NS; (G) MP-NS-PEG.

released 69 ± 7% of dexamethasone after 120 min. Nanoparticle-coated microparticles presented a drug release of 76 ± 4% and 79 ± 2%, for MP-NC and MP-NC-PEG, respectively (Fig. 5) while the NS-coated microparticles released 70 ± 3% and 77 ± 3% for MP-NS and MP-NS-PEG, respectively. This means that at pH 6.0, none of the coatings had a significant effect on the drug release rate compared to the uncoated core.

In contrast, at pH 7.4, the release rate was virtually unchanged for the uncoated particles (62 ± 7%), while the amount of drug released from the NS-coated microparticles was significantly faster and higher (82 ± 2% and 80 ± 3%), also in comparison to the release rate from either particles measured at pH 6.0.

Furthermore, at pH 7.4 NC-coated microparticles presented a lower drug release after 120 min (70 ± 3% and 71 ± 3%, for MP-NC and MP-NC-PEG, respectively) compared to the release observed for the same particles at pH 6.0 (76 ± 4% and 79 ± 2%, for MP-NC and MP-NC-PEG,), while the release rate at this pH was still not much different from that of uncoated particles (69 ± 7%).

Mathematical modeling was used to analyze the drug release profiles (Table 1). The selection of the model was based on the best correlation coefficient, the best model selection criteria (MSC) and the best graphic adjustment. At pH 6.0 the best fitting was the monoexponential equation for all formulations (Table 3). On the other hand, at pH 7.4 the best fitting was the monoexponential for the uncoated core, MP-NS and MP-NS-PEG. The other formulations (MP-NC and MP-NC-PEG) presented best fitting according to the biexponential model (Table 4).

3.4. Caco-2 cells transport studies

The best linear fit for the apical to basolateral transport of dexamethasone free solution and microparticle suspensions across Caco-2 cell monolayers was found over the first 120 min of the experiment, either at pH 6.0 or at pH 7.4, and P_{app} values were calculated from this period of time. In a first experiment the transport of dexamethasone solution across filters with and without cell monolayers in HBSS pH 6.0 or 7.4 was investigated (Fig. 6).

Fig. 7 shows the results of the transport studies of dexamethasone from nanoparticle-coated microparticles across the Caco-2 cell monolayers at pH 6.0 and 7.4. At pH 6.0, all formulations (except the MP-NS) presented P_{app} values that were significantly lower compared to the free dexamethasone solution ($p \leqslant 0.05$). Regarding the transport studies at pH 7.4 we also observe significant differences ($p \leqslant 0.05$) between the P_{app} value of dexamethasone free drug solution ($0.87 \pm 0.02 \times 10^{-5}$ cm/s) and each NC-coated formulation ($0.64 \pm 0.04 \times 10^{-5}$ cm/s and $0.62 \pm 0.05 \times 10^{-5}$ cm/s for MP-NC and MP-NC-PEG, respectively). On the other hand, the NS-coated formulations ($0.81 \pm 0.10 \times 10^{-5}$ cm/sand $0.70 \pm 0.05 \times 10^{-5}$ cm/s, for MP-NS and MP-NS-PEG, respectively) did not show any difference in relation to the free drug. The uncoated core also demonstrated a P_{app} value significantly lower ($p \leqslant 0.05$) compared to the P_{app} value of the free dexamethasone solution (uncoated core: $0.56 \pm 0.06 \times 10^{-5}$ cm/s; free dexamethasone solution: $0.87 \pm 0.02 \times 10^{-5}$ cm/s).

3.5. TEER and cytotoxicity studies

The TEER values of the monolayers were routinely checked before and after the experiment. Considering these values above 300 Ω cm^2 as a parameter for intact Caco-2 cell monolayers the results demonstrated that all nanoparticle-coated microparticles did not alter TEER values in Caco-2. The cytotoxicity indices were below 5% for all

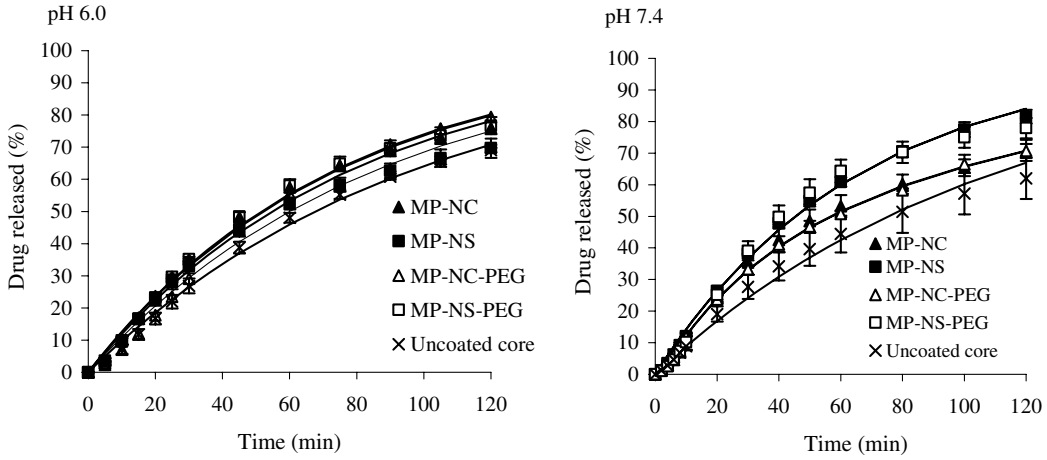

Fig. 5. *In vitro* dexamethasone release profiles at pH 6.0 and 7.4 ($n = 3$).

the microparticle formulations at all tested concentrations (0.025–12.8 mg ml^{-1}).

4. Discussion

Nanoparticle-coated microparticles were prepared by spray-drying using polymeric colloidal suspensions as coating material. The polymeric nanoparticles (NC or NS) presented mean diameter in the nanometer range according to their structural composition (NC formulations presented bigger mean diameter compared to NS formulations).

Considering the process of the preparation of the nanoparticle-coated microparticles by spray-drying the yields were until 70% (Table 2). These values are adequated considering a laboratorial scale of preparation [34] and are in agreement with the diclofenac-loaded nanoparticle-coated microparticles reported in our previous work [11]. These yield values are related to the adhesion of powder to the chamber walls and the loss of the smallest particles through the exhaust of the spray dryer apparatus [34]. The presence of the plasticizer (PEG 6000) increased significantly ($p < 0.05$) the yields of the process for the microparticles prepared with the NS suspension as coating material (MP-NS: $49 \pm 2\%$ and MP-NS-PEG: $67 \pm 4\%$). Even though such a significant influence could not be detected

Table 3
Mathematical parameters calculated from the monoexponential fitting for the *in vitro* dexamethasone release from microparticles at pH 6.0 ($n = 3$)

Formulation	k (mean ± SD)	MSC	r	$t_{1/2}$ (min)
Uncoated core	0.0103 ± 0.0005	4.93	0.9979	67
MP-NS	0.0116 ± 0.0006	3.91	0.9932	60
MP-NC	0.0134 ± 0.0007	4.61	0.9959	52
MP-NS-PEG	0.0135 ± 0.0008	4.59	0.9959	51
MP-NC-PEG	0.0127 ± 0.0004	4.02	0.9989	55

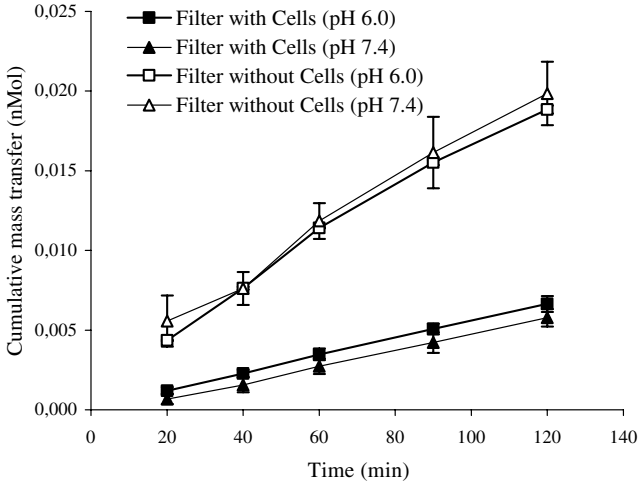

Fig. 6. Dexamethasone transport (0.1 μM) across filters with or without Caco-2 cell monolayers from a dexamethasone solution in HBSS pH 6.0 and 7.4 (mean ± SD, $n = 6$ or 3, for filters with or without cells, respectively).

Table 4
Mathematical parameters calculated from the best fitting model for the *in vitro* dexamethasone release from microparticles at pH 7.4 ($n = 3$)

Formulation	Model	k (mean ± SD)	MSC	r	$t_{1/2}$ (min)
Uncoated core	Monoexponential	0.0094 ± 0.0015	4.19	0.9942	74
MP-NS	Monoexponential	0.0147 ± 0.0012	5.40	0.9986	47
MP-NC	Biexponential	k_1:0.0275 ± 0.0030	6.71	0.9997	$t_{1/2}(1)$:25
		k_2:0.0040 ± 0.0001			$t_{1/2}(2)$:173
MP-NS-PEG	Monoexponential	0.0150 ± 0.0013	3.99	0.9931	46
MP-NC-PEG	Biexponential	k_1:0.0436 ± 0.0186	6.72	0.9997	$t_{1/2}(1)$:16
		k_2:0.0071 ± 0.0038			$t_{1/2}(2)$:98

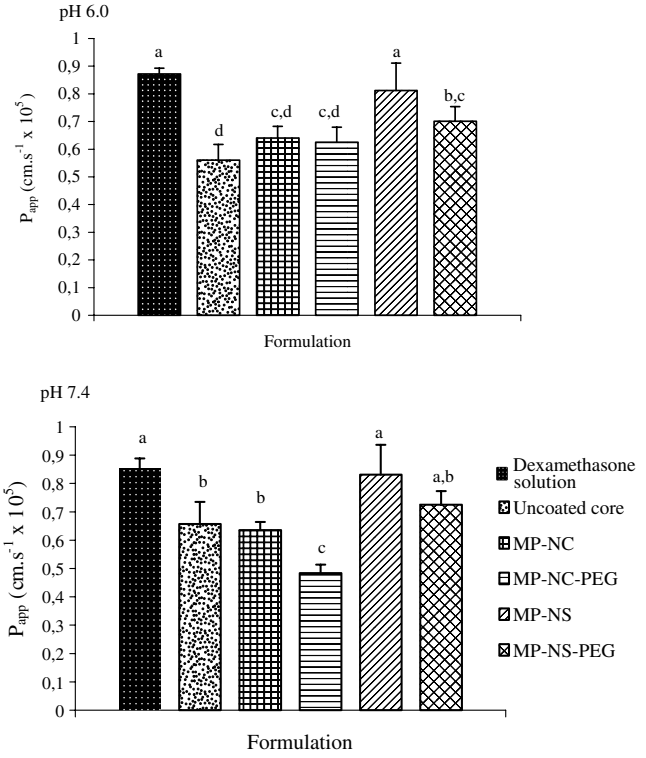

Fig. 7. Permeabiliy (P_{app}) of dexamethasone from a dexamethasone solution (0.1 μM) and from microparticle suspensions across Caco-2 cell monolayers at pH 6.0 and 7.4 (mean ± SD, $n = 6$); Means, in the same pH, with the same letter are not significantly different (ANOVA, Tukey test, $p \leqslant 0.05$).

for the NC-coated microparticles ($p > 0.05$), there is a tendency of increasing the yield in the presence of the plasticizer (MP-NC: 39 ± 6% and MP-NC-PEG: 49 ± 7%).

Regarding the values obtained for the drug content this parameter showed influence according to the type of nanoparticle suspension used as coating material. NC-coated microparticles presented lower drug content compared to the NS-coated microparticles (Table 2). These results can be explained by the presence of the oil in the case of the NC-coated microparticles, which increases the percentage of excipients in the final formulation. In addition, the presence of the plasticizer did not significantly influence any of these characteristics. The particle sizes data showed bigger particle sizes than the uncoated core for all formulations, except the MP-NS-PEG, and the moisture content data demonstrated that the operational conditions of spray-drying were able to give satisfactory dried formulations.

SEM analyses showed more spheroidal shaped nanoparticle-coated microparticles (Fig. 2c and e) compared to the uncoated core because of the influence of the spray-drying process. The nanostructures could be observed at their surfaces as a nanostructure coating layer whose diameters are related to the type of nanostructure employed as coating material (Fig. 2d and f). Similar morphologic characteristics have been reported in our previous work for diclofenac-loaded nanoparticle-coated microparticles [11]. AFM analyses confirmed the presence of these nanostructures on the surface of microparticles (Fig. 3).

X-ray analyses performed to investigate the presence of drug crystals in the formulations demonstrated the presence of dexamethasone in all formulations as its crystalline form. These analyses also served as a quality control. The spray-drying process did not alter the crystalline form of the dexamethasone (Fig. 4).

In vitro drug release experiments were carried out for 120 min in order to compare the drug release profiles to the drug permeation studies across Caco-2 cell monolayers. Phosphate buffer media at pH 6.0 and 7.4 were employed for this evaluation considering the solubility of the polymer at pH above 7.0.

Comparing the amount of drug released from the formulations between the two different pH media (pH 6.0 and 7.4) after 120 min (Fig. 5), the NC-coated formulations (MP-NC and MP-NC-PEG) presented lower amounts of drug released at pH 7.4 while the uncoated core and MP-NS-PEG presented similar amount of drug released, both values being similar to those observed at pH 6.0. On the other hand, the MP-NS showed an increase of release of dexamethasone, which was significantly faster than even for uncoated particles at pH 7.4. This result can be explained by the rearrangement of NS structures during the spray-drying process [35] leading to a more porous nanostructure confirmed by the higher surface area obtained for NS-coated microparticles (131 $m^2 g^{-1}$) compared to the NC-coated microparticles (61 $m^2 g^{-1}$) [36]. This NS rearrangement is also related to the lack of gastrointestinal protection observed in our previous works [36,37]. These differences in the release behavior at both pH media could not be explained as an influence of the particle size of the different formulations. At pH 6.0, the formulations MP-NC and MP-NS-PEG showed the same drug release after 120 min (76 ± 4% and 77 ± 3%, respectively) independently of their great difference in the particle size (52.71 and 20.31 μm, respectively). At pH 7.4, similar results can be observed for the formulations MP-NS and MP-NS-PEG, which presented different particle sizes (41.10 and 20.31 μm, respectively) and similar drug release after 120 min (70 ± 3% and 77 ± 3, respectively). Also, in our previous report we showed that the surface area did not influence the drug release profile of a drug model from these microparticulated systems [36].

At pH 6.0 the best fitting was the monoexponential equation for all formulations as commented before (Table 3). The uncoated core and the MP-NS formulation ($p \leqslant 0.05$) presented the lowest rate constants as can be observed in Fig. 5. Although, only the MP-NS formulation presented a lower fraction of drug release after 120 min compared to the other formulations. The $t_{1/2}$ for the slower formulations were 67 and 60 min for the uncoated core and MP-NS, respectively. On the other hand, the MP-NC, MP-NC-PEG, MP-NS-PEG presented similar $t_{1/2}$ (52, 55 and 51 min, respectively). Regarding the modeling of the drug release profiles at pH 7.4 the best fitting was the

monoexponential for the uncoated core, MP-NS and MP-NS-PEG (Table 4). There was no significant difference ($p \leqslant 0.05$) between the k values for the uncoated core and for the MP-NS-PEG in both pH media. However, the release was faster for the MP-NS formulation at pH 7.4 in relation to the release at pH 6.0. The presence of the plasticizer did not show influence on the drug release from NS-coated formulations as can be seen by the $t_{1/2}$ calculated for these formulations (MP-NS: 47 min and MP-NS-PEG: 46 min). On the other hand, the best fitting for the NC-coated microparticles was achieved with the biexponential model (Table 4). This biexponential fitting cannot be related to the burst release of drug from the smaller microparticles because the NS-coated formulations presented smaller microparticles without presenting the same biexponential behavior. The $t_{1/2}$ for the burst release phase were 25 min and 16 min for MP-NC and MP-NC-PEG, respectively, and 173 min and 98 min for the slow release phase, respectively.

Comparing the values obtained for both formulations a slower release in the burst phase can be observed from the microparticles prepared in the presence of the plasticizer (MP-NC-PEG). These results are in agreement with the percentage of the drug related to this phase (C_1, Table 1) which were 60% and 33% for MP-NC and MP-NC-PEG, respectively. This parameter reflects the percentage of drug more superficially associated on the microparticles and quickly available to the medium. In addition, the parameter C_2 (Table 1) reflects the fraction of the drug more internalized within the particles (MP-NC: 44% and MP-NC-PEG: 70%). From these results it can be suggested that the presence of the plasticizer could lead to a delay in the absorption of dexamethasone due to this more internal localization of the drug in this formulation (MP-NC-PEG) [11].

In order to verify this hypothesis Caco-2 transport studies were carried out at the same pH media. Besides the dissolution of the polymer (Eudragit S100®) above pH 7.0 the best results to differentiate the microparticle formulations were observed at pH 7.4. These findings could be explained by the better resolution of the data when not only diffusion but also other processes were occurring, *e.g.* dissolution and/or erosion of the polymer.

In the first experiment (Fig. 6) we demonstrated that the Caco-2 cell monolayer was a barrier to dexamethasone transport at both pH media (pH 6.0: $P_{app} = 2.37 \pm 0.04$ and $0.87 \pm 0.02 \times 10^{-5}$ cm/s for filters without and with cells, respectively; pH 7.4: $P_{app} = 2.24 \pm 0.24$ and $0.85 \pm 0.04 \times 10^{-5}$ cm/s for filters without and with cells, respectively). No significant difference could be observed between the P_{app} of dexamethasone at different pH media ($p \leqslant 0.05$); P_{app} values were comparable to those described in the literature, although slightly lower ($1.21 \pm 0.05 \times 10^{-5}$ cm/s for dexamethasone in HBSS supplemented with 25 mM glucose solution in pH 7.4 [15] and $1.14 \pm 0.09 \times 10^{-5}$ cm/s in Krebs–Henseleit physiological solution [38]).

Regarding the experiments carried out with nanoparticle-coated microparticles lower P_{app} values were demonstrated for dexamethasone from the NC-coated formulations (MP-NC and MP-NC-PEG) and the uncoated core compared to the free drug solution at pH 7.4 (Fig. 7). These results are in agreement with a previous report for famotidine-loaded microparticles [25]. Additionally, they are also in accordance with the results obtained in the *in vitro* drug release studies in the same pH medium where the same formulations (uncoated core, MP-NC and MP-NC-PEG) presented the lowest percentages of the drug released after 120 min ($62 \pm 7\%$, $70 \pm 3\%$ and $71 \pm 3\%$, respectively). As observed in the *in vitro* drug release studies we could demonstrate the influence of the type of the coating (NS or NC) on the absorption of dexamethasone across Caco-2 cell monolayers. The NC-coated microparticles presented a lower P_{app} value (MP-NC: $0.64 \pm 0.03 \times 10^{-5}$ cm/s) compared to the NS-coated microparticles (MP-NS: $0.83 \pm 0.11 \times 10^{-5}$ cm/s). Except for the MP-NC-PEG formulation, all other formulations did not show any statistical difference ($p > 0.05$) for the P_{app} value between the different media (pH 6.0 and 7.4). However, there was a statistical difference ($p \leqslant 0.05$) between the P_{app} values obtained from the MP-NC-PEG ($0.48 \pm 0.03 \times 10^{-5}$ cm/s) and all other formulations (MP-NC: $0.64 \pm 0.03 \times 10^{-5}$ cm/s; MP-NS: $0.83 \pm 0.11 \times 10^{-5}$ cm/s; MP-NS-PEG: $0.72 \pm 0.05 \times 10^{-5}$ cm/s). These results support our previous hypothesis that the presence of the plasticizer in the preparation of NC-coated formulation (MP-NC-PEG) leads to a more internal localization of the drug in relation to the formulation without plasticizer (MP-NC), followed by a significant delay in the absorption of dexamethasone from these microparticles across Caco-2 cell lines, according to our previous report [11]. It must be emphasized, however, that the *in vitro* transport study does not address a delay of the gastrointestinal transit time of microparticles, as shown in previous works in the literature [6,10]. But, such a delay could further increase the advantages of the encapsulation of dexamethasone in these nanoparticle-coated microparticles to retard its absorption. Among the major problems in the treatment of IBD are the necessity of administration of high doses of anti-inflammatory drugs and the low residence time of these drugs in the gut due to diarrhea, a frequent symptom of IBD [7–10]. Thus, our system could conjugate the control of the drug release and the more time of the microparticles remaining in the site of action, decreasing the frequency of the drug administration. In addition, although the nanoparticle-coated microparticles presented small differences in permeability coefficients, some other advantages or strategies could increase their biological importance, as the protection of gastrointestinal mucosa showed by diclofenac-loaded nanocapsule-coated microparticles [36] and/or the use of polymers with bioadhesive properties.

In addition, the TEER results (above 300 $\Omega\,cm^2$) suggest that the microparticles are non-toxic to the cells. Furthermore, it indicates that the drug absorption by the paracellular pathway cannot be significant under these conditions. In agreement with these results the cytotoxicity studies also

demonstrated that these formulations are not toxic to the membrane of the Caco-2 cells as expected, considering the excipients employed in their preparation.

From the results discussed above, it can be observed that the *in vitro* drug release and the permeation studies lead to similar results. The type of the nanoparticle used in the coating step has a significant influence on the drug release profile and on the permeation across Caco-2 cell monolayers. As commented previously, the results obtained at pH 7.4 allowed a better differentiation among the formulations, probably due to different processes happening in this pH range (diffusion, dissolution and/or erosion of the polymer). At this pH, NS-coated microparticles presented a higher fraction of the drug released after 120 min than the NC-coated microparticles. These results are in agreement with the results of the transport studies. The former presented a higher P_{app} value compared to the latter. In the case of the NC-coated formulations the data allowed their mathematical modeling according to the biexponential equation. From the parameters calculated for both kinetic phases it could be observed that the drug was more internally localized in the formulation MP-NC-PEG in relation to the MP-NC. This hypothesis was confirmed by the lowest transport rate of dexamethasone observed across the Caco-2 cells for the MP-NC-PEG formulation. Additionally, a correlation between *in vitro* drug release and *in vitro* drug permeation can be observed in Fig. 8 which shows the drug released (%) as a function of the cumulative mass transferred of dexamethasone (nMol) across Caco-2 cells. The exponential correlation shows that the permeation of the drug across the cells is related to the gradient of concentration of dexamethasone at the absorption site. Also, the differences observed in the permeation across the Caco-2 cells among the NS or NC-coated formulations can be correlated to the best fitting model of the drug release profiles at pH 7.4 (mono or biexponential models).

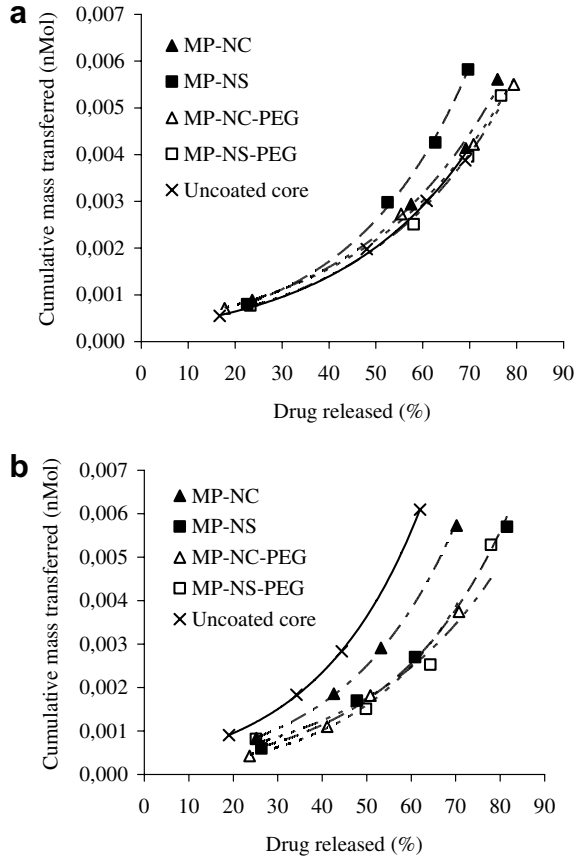

Fig. 8. Correlation between cumulative mass transferred of dexamethasone (nMol) in Caco-2 cells transport experiment as a function of the fraction of dexamethasone *in vitro* released in different times: (a) pH 6.0 and (b) pH 7.4.

5. Conclusion

Dexamethasone-loaded nanoparticle-coated microparticles were sucessfully prepared by spray-drying. They showed satisfactory technological characteristics (process yields, encapsulation efficiency and moisture content) and the particle sizes ranged from 20 to 53 μm. Nanoparticle-coating was demonstrated by the SEM and AFM analyses, that showed nanostructures on the surface of the micropartilces, presenting diameters similar to those shown by the original aqueous suspensions. *In vitro* drug release studies demonstrated the influence of the polymeric coating layer on the drug release behaviour mainly at pH 7.4. The NC-coated microparticles presented the lowest fraction of drug released after 120 min. The mathematical modeling of the drug release data from the NC-coated formulations showed a more internal localization of the drug in the formulation prepared in presence of the plasticizer (MP-NC-PEG). Transport studies across Caco-2 cell monolayers also demonstrated the feasibility of these microparticles to control the absorption of dexamethasone across the Caco-2 cell lines depending on the type of the coating material. NC-coated microparticles presented lower P_{app} values compared to the free dexamethasone solution. In accordance with the *in vitro* drug release data and their mathematical modeling, the MP-NC-PEG presented the more pronounced delay in the absorption of dexamethasone across Caco-2 cells. Finally, the results from the transport and drug release studies can be correlated indicating that the former can become a powerful tool to confirm that it is indeed the drug release rate which controls drug transport across the epithelial barrier, and that epithelial permeability is not the only rate limiting barrier to drug absorption when delivered by such formulations. Therefore *in vitro* studies on Caco-2 monolayers appear as a useful step in the development of microparticulate oral controlled release forms prior to their evaluation in clinical studies. Apart from those nanoparticle-coated microparticles, mainly NC-coated microparticles, can represent a promising platform for the development of oral drug delivery systems.

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