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Research Paper

Development of solid nanoparticles based on hydroxypropyl-β-cyclodextrin aimed for the colonic transmucosal delivery of diclofenac sodium

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Abstract

Objectives Nanoparticles were designed for the oral administration and transmucosal colon delivery of drugs.

Methods Preparation parameters were studied in order to develop solid pH-dependent drug-release nanoparticles, constituted by hydroxypropyl-β-cyclodextrin and/or Eudragit® L100 loaded with diclofenac sodium. Nanoemulsions were prepared by the emulsion-evaporation method using various homogenizers. Different preparative conditions were tested. The emulsions obtained were analysed in terms of size and then dried to obtain solid nanoparticles which were characterized *in vitro* (particle size, morphology, dissolution, solid state characterization). The effect of nanoparticles on drug permeation through synthetic membranes, colonic pig mucosa and Caco2 cell line were performed. Toxicity studies were carried out to assess the safety of the raw materials used and the nanosystems produced.

Key findings Appropriate parameters to obtain nanoemulsions stable enough to be desiccated were determined: Panda NS100L was the most suitable homogenizer for the preparation; particle size ranged between 100 and 600 nm depending on the production method. Solid nanoparticles were obtained by an exsiccation process, which does not modify the mean size. pH-dependent drug-release nanoparticles were obtained. The nanoencapsulation process decreased the crystallinity of the drug. Materials and nanoparticles were highly biocompatible. Transmucosal delivery of drug is dependent on the polymer and the test employed: cyclodextrin improved drug permeation across colonic pig mucosa.

Conclusions Formulations containing hydroxypropyl-β-cyclodextrin represent new colon-targeted nanoparticles for transmucosal delivery of drugs.

Keywords colon delivery; Eudragit L 100; hydroxypropyl-β-cyclodextrin; solid nanoparticles; transmucosal administration

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity. The chemical conformation favors the complexation of lipophilic drugs, but even the formation of a stable equilibrium with polar and water-soluble drugs by dissolution of the drug into a solution containing CD is possible.[1–7] Although CDs are not predisposed to include hydrophilic drugs, inclusion of water-soluble molecules has already been described, essentially with β-cyclodextrin and its derivatives.[6] The natural β-cyclodextrin can be found in a number of pharmaceutical formulations in numerous countries throughout the world. Loftsson and Duchêne listed more than 30 different drugs marketed as CD complexes.[7]

CDs are generally used as carriers for oral, parenteral, ocular and nasal administration, but can be used also to prepare new formulations containing peptides and proteins for gastrointestinal delivery.[8,9] They can promote absorption by increasing the permeability of the drug because they are able to act directly on the mucosal membrane.[2,10–13] The application of CDs through the oral route increases drug solubility, bioavailability[14] and stability by avoiding hydrolysis and absorption into the stomach and into the first tract of the gut. On

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the other hand, CDs can be degraded to small polysaccharides in the colon by microflora, consisting of around 500 kinds of bacteria,[3,8,15–17] and they can thus be considered as suitable carriers for drug targeting to the colon.

Oral drug delivery is the chosen route for drug and vaccine administration as its non-invasive nature avoids the use of injections.[15,18] In particular, colon-specific delivery is a good way to administer many drugs because it can be used for topical administration, as in the therapy of Crohn's disease and ulcerative colitis, and also for systemic delivery against chronic diseases of the osteo-articolar apparatus. The absence of endopeptidase enzymes and the residence time of drug in the gut – about 33 and 47 h for men and women, respectively – can be exploited to obtain absorption into the colon of drugs with poor bioavailability, such as proteins and peptides.[19–25]

The particle uptake of drugs by the intestinal cells is size-dependent. The nanometer size range is, indeed, important in allowing access to tissues from which larger carriers are excluded.[18,25,26] Nanoparticles (NPs) have been studied as polymeric or lipidic carriers for pharmaceutical and cosmetic use;[27–29] NPs can be administered through several routes and generally they favor good bioavailability, biocompatibility and efficiency of the drug. Moreover, many studies show that the dimensions of NPs influence diffusion and absorption of the drug through physiological barriers: the smaller dimensions of an NP guarantee a more efficient transport of drugs through the mucosa.[18,26,30,31]

The aim of this research was to project, study and prepare NPs based on hydroxypropyl-β-cyclodextrin (HP) and/or Eudragit® L100 (L100) for colon targeting and transmucosal absorption. HP was chosen as it is listed in both The European Pharmacopeia and The United States Pharmacopeia (USP)/National Formulary. Moreover, 2-hydroxypropyl-β-cyclodextrin is cited in the FDA's list of inactive pharmaceutical ingredients.[7]

A new kind of HP-based NP was produced by the W/O emulsion evaporation method. The inverse-phase emulsion process appears to be an easy method to produce stable emulsions, but the use of oils as the external phase inhibits the possibility of obtaining dried NPs. Since there is little literature on preparation methods for dried NPs using the W/O emulsion process, many technological parameters were studied in order to obtain NPs containing diclofenac sodium (D), as a model drug. Different apparatus was used to prepare nanoemulsions containing various HP concentrations; the effect of the amount of surfactant was also investigated.

To achieve successful colonic delivery, the drug needs to be protected from dissolution and absorption in the upper gastrointestinal tract. For this reason, NPs based on CDs should be resistant to the gastric environment, so they should arrive intact in the colon, where CDs are degraded by the intestinal flora.[15]

Particles can be retained in the colon mucosa depending on their size.[32,33] In an attempt to increase the gastric resistance of HP-based NPs, L100 was added to the nanoemulsion. In fact, the combination of molecular encapsulation with other carrier materials can extend the function of pharmaceutical additives, and it becomes an effective and valuable tool for the improvement of drug formulations. Eudragit is a polymer with methacrylic acid as a functional group. The ratio of the free carboxylic groups to the ester groups is approximately 1 : 1. It dissolves at a pH above 6.0, ensuring a pH-dependent release, and it will protect active compounds that are sensitive to gastric fluid, as well as protecting the gastric mucosa from aggressive substances.[34] The formulation composed of only L100 loaded with D was studied as a comparison.

Considering the modification of the physical structure of D due to the interaction between L100 and D,[35,36] solid-state characterization of NPs by means of DSC, XRPD and FT-IR was performed in order to evaluate the effect of the preparative method on the drug's physicochemical properties. The effect of NPs based on HP and/or L100 on drug permeation, through both synthetic membrane and biological tissue, was evaluated in order to assess the properties of the new delivery system as transmucosal colon-targeted formulations. Permeation studies using Caco2 cells were also performed. Since the safety of the raw materials and formulations should also be assessed,[37–39]the viability of the cells after contact with excipients or drug as well as with the NPs was tested.

Materials and Methods

Materials

Hydroxypropyl-β-cyclodextrin (HP), Cavasol (Mw: 1400 g/mol, average degree of substitution per anhydro glucose unit: 0.65), was purchased from Wacker Chemie AG (Munich, Germany). Diclofenac sodium (D) BP: 99.96%, was bought from Crual srl (Rome, Italy). Eudragit® L100 (L100) was kindly given by Rofarma Italia S.r.l (Gaggiano, Italy). 1-butanol was obtained from Merck (Darmstadt, Germany). Tween80 and dichloromethane were purchased from Acros Organics (Gees, Belgium). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin solution and non-essential aminoacids solution were purchased from Invitrogen srl (Milan, Italy). Fetal bovine serum and trypsin-EDTA 0.25% solution were obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Polycarbonate filters (0.05 μm pore size) were purchased from Whatman (Maidstone, UK). Acetonitrile, Chromasolv® and acetic acid 99.8% were obtained from Riedel-de Haen (Milan, Italy), Nylon and cellulose regenerated membrane (diameter 45 mm, 0.45 μm pore size) and PTFE membrane(diameter 13 mm, 0.20 μm pore size) were purchased from Alltech (Milan, Italy). All other solvents and chemicals were of analytical grade.

Preparation of emulsion

Four different water/oil emulsions coded HP (1–4) were prepared by Silverson mixer SL2 apparatus (Silverson Machines, Inc., Waterside Chesham, UK) using different amounts of HP and surfactant, Tween80. The compositions were as in Table 1. The organic phase, kind of surfactant and the amount of components used were selected on the basis of preliminary experimental trials.

HP was dissolved in the water phase by magnetic stirring; Tween80 was dissolved in 1-butanol, which was chosen as the organic phase. The water phase was slowly added to the organic phase under magnetic stirring and then homogenized by Silverson SL2 at 5000 rpm for 5 min.

Starting from HP4, a second series of emulsions named HPO1–HPO6 was prepared using the high-pressure homogenizer Panda NS100L-Panda-S.N.6890 (Niro Soavi S.p.A

Table 1 Composition of formulations based on HP prepared by using a mixer apparatus

Formulation	Tween80	HP
HP1	2.1	4.9
HP2	2.1	7
HP3	4.2	7
HP4	4.9	7

Amounts of components are expressed in grams (77 g of water and 161 g of butanol were used for all formulations).

Table 2 Composition of formulations based on HP and/or L100 prepared by using high-pressure homogenizer Panda NS100L

Formulation	Tween80	HP	L100	D	Pressure (bar)[a]
HPO1	4.9	7	–	–	1500
HPO2	0	7	–	–	1500
HPO3	4.9	7	–	–	150/1500
HPO4	2.33	7	–	–	100/1000
HPO5	3.03	7	–	–	100/1000
HPO6	3.03	7	–	–	50/800
HPE	3.03	4.66	2.33	–	50/800
HPD	3.03	4.66	–	0.23	50/800
HPED	3.03	4.66	2.33	0.23	50/800
DE	3.03	–	2.33	0.23	50/800

Amounts of components are expressed in grams (77 g of water and 161 g of butanol were used for all formulations). [a]Different pressures are used during the two stages of the emulsification process.

Parma, Italy). The composition of the new emulsions as well as the homogenizing pressures were changed in order to obtain stable and nanosized emulsions (Table 2).

A new formulation named HPE was prepared using HP and L100 (2 : 1 w/w), keeping the total amount of polymer in emulsion (HP plus L100) to 7 g (Table 2). These parameters were chosen in order to maintain unaltered emulsion characteristics and, at the same time, to strengthen the gastroresistant effect of the formulation due to the presence of L100. The emulsion was prepared by dissolution of L100 in 1-butanol following the method described above.

Based on the results obtained from HPO6 and HPE, the loaded formulations, named HPD and HPED (Table 2), containing D as a model drug were produced. Emulsion DE was prepared from L100 and D in order to evaluate the effect of the absence of HP. Drug was dissolved in the aqueous phase and the parameters and process of production were unaltered.

Drying process

Dried NPs were obtained from HP4, HPO6, HPE, HPD, HPED and DE by exsiccation under vacuum using a rotary evaporation apparatus at 80°C (Büchi 011; Büchi, Switzerland) and were named HP4s, HPO6s, HPEs, HPDs, HPEDs and DEs, respectively. Solid particles were stored in an oven at 40°C for 2 days in order to completely eliminate the residual butanol.

Dimensional analysis of emulsions and dried particles

All emulsions prepared, as well as the corresponding solid formulations, were dimensionally analysed by laser diffraction spectroscopy, using a Coulter nanosizer N5 (Beckman-Coulter Inc. Miami, FL, USA), in order to evaluate the mean diameter of the drops or solid particles formed and their dimensional homogeneity, expressed as a polydispersivity index (PI). The effect of the drying process on the dimensional characteristics of particles was investigated.

A few milligrams of the dried particles were resuspended in 1-butanol medium (for HP4s, HPO6s, HPEs, HPDs, HPEDs) or in acidic water, pH 3.65 (for DEs), vortexed for 1 min and sonicated for 2 min. The suspension media were filtered with a 0.2 μm filter before use. The mean diameter of the particles was calculated by choosing the unimodal analysis as method of work of the apparatus and setting the following conditions: fluid refractive index 1.373, temperature 20°C, viscosity 2.826 cP, angle of measurement 90.0°, sample time 3.0 μs and sample run time 200 s.

Morphological analysis

The shape and surface characteristics of the dried NPs were studied by scanning electron microscopy (SEM) (Zeiss DSM962, Zeiss, Germany). They were placed on an aluminium holder and covered with a thin layer of gold. After the gold sputtering process, samples were analysed at a 20 kV acceleration voltage to obtain the image of the NPs.

Characterization of drug-loaded nanoparticles

Drug content

Samples of drug-loaded (19.4 mg) HPDs, HPEDs or DEs were transferred into a 100 ml volumetric flask and dissolved in phosphate buffer at pH 6.8 after stirring for 10 min. The concentration of D in the buffer solution was determined using a UV-spectrophotometer (Hitachi spectrophotometer U-2001, Hitachi Instruments, Tokyo, Japan) at a wavelength of 275 nm, using a calibration curve previously obtained ($R^2 = 0.9997$).

Loading efficiency (LE%) was calculated from the ratio between the real drug content and the theoretical amount of drug in the microspheres and was expressed in percentage terms.

In-vitro drug release test

In-vitro drug-release tests were performed using a USP dissolution apparatus equipped with a basket (Erweka, Heusenstamm, Germany). The rotational speed was set at 50 rpm and the bath temperature at 37°C. An amount of NPs corresponding to 2.8 mg of D was tested by using 500 ml of buffer at pH 1.2, simulating the gastric juice, for 2 h. Then 123 ml of monobasic sodium phosphate solution was added in order to reach pH 6.8[40] and the test was continued for a further 2 h. The drug dissolution rate was determined as a comparison.

Samples (1 ml) were withdrawn at selected time intervals and measured spectrophotometrically at 275 nm to determine the amount of drug released. This amount was calculated by referring to the calibration curve prepared in buffer at pH 6.8 (standard solutions in the range of 2.5 to 250 mg/l, $R^2 = 0.999$). An equal volume of fresh medium was added after each sampling to guarantee sink conditions.

Solid state characterization of nanoparticles

Drug, polymers and NP systems were characterized by X-ray powder diffraction (XRPD), differential scanning calorimetry

(DSC) and Fourier transform infrared spectroscopy (FT-IR) in order to evaluate the crystalline or amorphous nature of drug and polymers and to establish possible solid-state interactions and/or drug encapsulation by polymeric NPs.

X-ray powder diffraction

XRPD analyses were performed with a Siemens D5000 diffractometer equipped with a copper tube and a graphite monochromator on the diffracted beam. The acquisition parameters were 40 kV, 30 mA, 2θ range 2–70°, step size 0.020° (2θ), time per step 2 s; all samples were analysed at room temperature. Results were compared with XRPD patterns from the PDF-2 database using the Bruker Diffracplus package.

Differential scanning calorimetry

Temperature and enthalpy values were measured with a Mettler Stare system (Mettler Toledo, Milan, Italy) equipped with a DSC 821e module and an Itracooler device (Julabo mod FT900) for subambient analysis on 2–5 mg (Mettler M3 Microbalance) samples in sealed aluminium pans with pierced lids (40 μl). Scans were performed between −10 and 300°C ($\beta = 10$ K/min) under a flux of nitrogen (50 ml/min).

Fourier transform infrared spectroscopy

Mid-IR (650–4000/cm) spectra were recorded on powder samples using a Spectrum One Perkin-Elmer FTIR spectrophotometer (resolution /4 cm) equipped with a MIRacleTM ATR device (Pike Technologies).

Cell viability assay

Caco2 cells were cultivated in 75 cm^2 flasks (Nunc, Denmark) using DMEM supplemented with 9.7% foetal bovine serum, 1.4% penicillin-streptomycin solution and 1.4% non-essential amino acids. Cells were maintained in a controlled atmosphere at 37°C, with 95% relative humidity and 5% CO_2, until the cells reach approximately 80–90% of confluence. After that cells were placed in 24-well plates with a density of $2 \times 10^5/cm^2$ until a cell monolayer was obtained.

MTT test

Cell viability was determined using the MTT test, which determines the intracellular dehydrogenase activity conducted by the mitochondria of those cells still alive, by formation of purple Formazan crystals from yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). A volume of 710 μl of solution 5 mg/ml of MTT in medium was added to the monolayer, which was previously deprived of culture medium and washed three times with sterile PBS. Cells were incubated for 15 min, and the Formazan dispersion formed was removed and analysed by UV spectrophotometry at 570 nm wavelength.

Effect of raw material

The effect of D, HP, L100, Tween80 and 1-butanol on cell viability was tested. When the monolayer was obtained, the culture medium was replaced by a solution of HP in a concentration between 15 and 2.5 mg/ml in the appropriate volume of medium. Solutions of 10 μl of Tween80 in medium and 10 μl of 1-butanol in medium were also tested. Three-well plates with only the culture medium were used as positive control. D, HP, excipients and controls were put in contact with the monolayer for 24 h at 37°C and 5% of CO_2 and then the solutions were removed and submitted to the MTT test as previously described.

Effect of formulations

The effect of formulations, both dissolved and suspended in the culture medium, on Caco2 cell viability was evaluated. Loaded NPs, HPDs, HPEDs or DEs, and their correspondent unloaded NPs, HPO6s and HPEs, were washed with 1 ml of 1-butanol, centrifuged for 10 min at 13 000 rpm (Hettich centrifuge) deprived of supernatant, and dried for 3 days at 40°C to eliminate residual Tween80.

As DEs NPs were damaged during the treatment with 1-butanol as well as with water, cytotoxicity tests and permeation trials with Caco2 cells were performed using unwashed NPs.

Solutions with a concentration of 5 mg/ml of HPO6s, HPEs, HPDs, HPEDs or DEs were dispersed on the cellular monolayer which had been previously rinsed with fresh PBS, incubated for 24 h and then submitted to the MTT test.

An exactly weighed amount (5 mg) of loaded (HPDs, HPEDs or DEs) and unloaded (HPO6s and HPEs) NPs, which had been previously washed (except for DEs), were uniformly dispersed on the monolayer, covered with 0.5 ml of medium and incubated for 24 h in order to carry out the MTT test.

Permeation studies

Tests for the evaluation of transmucosal permeation across synthetic or biological membranes were performed using a new modified Franz diffusion system incorporating three in-line flow-through diffusion cells.[41] Each cell consisted of a donor compartment and a receptor compartment. The diffusion membrane was placed between the cell compartments; the diffusional area was 3.14 cm^2. The temperature in the different chambers, flow rate and volume of liquid were previously measured. The receptor solution was continuously stirred by means of a spinning bar magnet. Receptor solution samples were withdrawn through a sampling port in the receptor compartment at various time intervals. The receptor medium was refilled.

Experiments for testing the permeation capability of the drug were carried out by in-vitro penetration studies through a synthetic membrane and by ex-vivo permeation tests through colonic pig mucosa. Moreover, a static in-vitro permeation test using a monolayer of Caco2 cells was performed using cell culture inserts. The use of different experimental models permits in depth analysis of the NP delivery process and thus an assessment of the applicability of nanosystems.[42]

The drug quantification in the acceptor medium was evaluated by a rapid and sensitive HPLC method, using a Varian Prostar 210 liquid chromatography system equipped with a Varian 330 diode array detector (Varian, Inc. Cary, NC, USA).

According to the method of El-Sayed *et al.*,[43] the chromatographic separation and quantification were performed on a 200 × 4.6 mm (i.d.) MOS Hypersil column (5 μm particle size), preceded by a MOS Hypersil, 5 μm, guard column (Hewlett Packard, Waldbronn, Germany). An amount of 20 μl of standard solution (1.0, 5.0, 25.0 and 50 μg/ml) ($y = 161\,661\,x - 36\,875$, $R^2 = 0.9994$) or sample was directly injected onto the column and eluted with a mixture of

acetonitrile and 0.5 M acetic acid solution 50/50 (v/v), at a flow rate of 1.2 ml/min over 12 min. All determinations were performed at room temperature. Detection was set at a wavelength of 280 nm. The peak areas were determined by Varian Star Chromatography Workstation, system control, version 6.20 (Varian, Inc. Cary, NC, USA).

All samples from the permeation test, which was carried out at pH 1.2, were added to the correct volume of monobasic sodium phosphate in order to modify the pH value to 6.8 for better dissolution of D.[40] After that, all samples were centrifuged and filtered using cellulose filters (0.2 μm pores) before injection.

In-vitro permeation test using synthetic membrane

An exactly weighed amount of NP (HPDs, HPEDs or DEs), corresponding to 1 mg of D, was dispersed on a polycarbonate membrane (0.05 μm pore size). Membrane was placed above the acceptor chamber, which was filled with 100 ml of simulated gastric juice at 37°C; the flux of the medium was 6.8 ml/min. After 2 h, 23 ml of monobasic sodium phosphate were added in order to reach pH 6.8. The test was continued for two more hours and samples were taken every 30 min. The acceptor medium withdrawn was refilled.

In-vitro permeation test using Caco2 cells

HPDs, HPEDs or DEs were submitted to an in-vitro permeation test through a monolayer of Caco2 cells. Before testing, HPDs and HPEDs were previously washed with 1-butanol in order to eliminate residues of Tween80 as previously described. DEs, which are not washable with any solvent, was tested as obtained. The experiment was carried out by using cell culture inserts placed into six-well plates. Cells were grown on the surface of the insert and were covered by 3 ml of growth medium composed of DMEM supplemented with 9.7% of fetal bovine serum, 1.4% of penicillin-streptomycin solution and 1.4% of non-essential amino acids. The same amount of medium was also injected into each well in order to guarantee the right environment for growing. Before starting the test, the donor medium contained in the insert, and the acceptor medium in the well, were substituted with the same amount of Hank's balanced salt solution at pH 6.8 (HBSS). An amount of 3.6 mg of NPs were homogeneously dispersed in the donor chamber and, at selected time points (0–4 h), 100 μl of HBSS were taken from the acceptor chamber and analysed by HPLC in order to evaluate the amount of D permeated through the monolayer. Viability of cells, before and after the permeation test, was evaluated by TEER and, at the end of the experiment, also by MTT test, in order to confirm the TEER results.

Ex-vivo permeation studies using colonic pig mucosa

Tissue preparation

A fragment of colon was excised from intestine of pigs obtained from a local slaughterhouse, gently washed with PBS, stored in ice-cold PBS for transport to the laboratory, and finally deprived of the serosal mucosa before use.

Ex-vivo permeation test

The mucosa was positioned to ensure that the mucosal portion was in contact with the NPs (HPDs, HPEDs or DEs) and the muscular mucosa was in contact with the pH 6.8 buffer of the acceptor chamber. An exactly weighed amount of NPs, corresponding to 1 mg of D, was dispersed on the superior portion of the mucosa. The muscular portion was constantly hydrated by the pH 6.8 buffer, thermostated at 37°C. The flux of liquid was set at 6.8 ml/min and saturated with 5% of CO_2. The amount of buffer employed as acceptor medium was 100 ml. At selected time points (0–4 h), the amount of drug permeated was determined by HPLC. The acceptor medium withdrawn was refilled.

Statistical analysis

Statistical analysis of data was performed using Student's *t*-test, one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test and Tukey's test in the case of the cytotoxicity studies and permeation studies, respectively (GraphPad Prism, version 2.01; GraphPad software Incorporated, La Jolla, CA, USA). The differences were considered to be statistically significant when $P < 0.05$.

Results

Preparation of emulsion

The kind of apparatus and manufacturing parameters influenced emulsion characteristics and stability: HP4, produced by Silverson SL2, needed almost 5 g of surfactant in order to guarantee the stability of the emulsion. HPO6, obtained by Panda homogenizer, appeared more stable and homogeneous than HP4, using less Tween80 (about 3 g).

Thus, all the formulations were produced using the high-pressure homogenizer Panda NS100L. The results obtained show that the pressure values have to be set at 50/800 bar in order to obtain the most stable emulsion of unidimensional nanodrops. These preparative conditions also suited the preparation of HPE emulsion and the loaded formulations HPD, HPED and DE; the addiction of D does not influence the stability of the emulsions.

Drying process

The water in oil (W/O) nanoemulsion forms an azeotropic mix and the system has a boiling point lower than 100°C, which allows the complete evaporation of water and the organic phase together by evaporation under vacuum, and the formation of dried NPs.

Dimensional analysis of emulsions and dried particles

As described in Table 3, all the stable emulsions analysed have mean diameters between 277.5 and 497.5 nm and PI between 1.6 and 0.6. HP4 is the smallest homogeneous formulation because its PI is the highest among the formulations analysed. The results obtained prove that the Panda homogenizer ensures better results than Silverson SL2 as the homogenizer of an inverse-phase emulsion ($P = 0.0109$). Loading of drug does not significantly affect drop size in the case of the emulsion made only with HP (HPO6 vs HPD: $P = 0.2982$).

Table 3 Dimensional analysis of emulsions and corresponding dried particles

Emulsion	**HP4**	**HPO6**	**HPD**	**HPE**	**HPED**	**DE**
Mean diameter (nm)	277.5 ± 29	360.1 ± 13.1	425.7 ± 94.2	314.0 ± 58.6	497.6 ± 16.1	262.5 ± 9.8
PI	1.6 ± 0.9	1.1 ± 0.5	0.7 ± 0.7	1.3 ± 0.2	0.6 ± 0.3	0.1 ± 0.14
Dried nanoparticles	**HP4s**	**HPO6s**	**HPDs**	**HPEs**	**HPEDs**	**DEs**
Mean diameter (nm)	516.5 ± 168	662.6 ± 157.7	516.2 ± 59.2	373.6 ± 62.8	529.8 ± 10.85	384.6 ± 11.6
PI	1.9 ± 0.4	1.1 ± 0.5	1.1 ± 0.2	0.9 ± 0.6	1.1 ± 0.4	0.4 ± 0.023

Polydispersivity index (PI) measures the width of dimensional distribution of NP suspended in the medium of analysis.

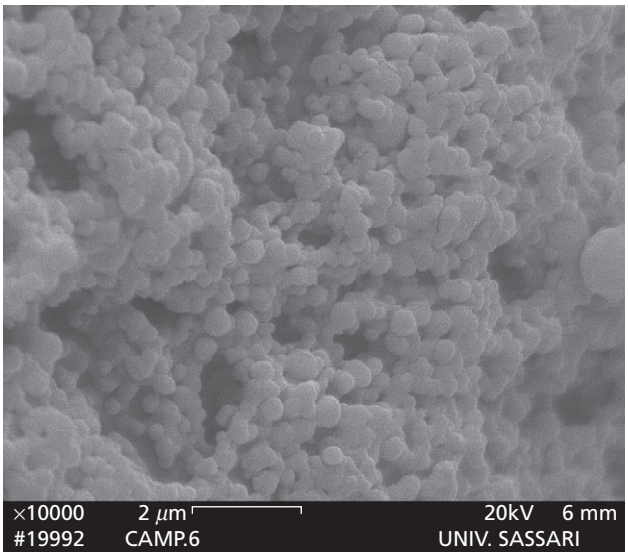

Figure 1 Scanning electron microscope picture of formulation based on HP and L100. Picture is of HPEDs (magnification ×10000).

In contrast, when drug is loaded into HPE to give HPED the drop size significantly increases ($P = 0.0064$). The dimensional difference between HPE and HPED is due to the presence of D in the aqueous phase. The drug, dissolved in water, forms a stable complex with HP[44] before the emulsion process, and this event varies the results of the emulsification process with respect to HPE. In addition, the diameter of HPD is bigger than HPE, confirming the effect of the drug on the drop size of emulsions compared to the polymer L100.

Dried NP size does not show significant differences compared to the correspondent nanoemulsions; only HPO6s has a mean diameter bigger than HPO6 ($P < 0.05$). On the other hand, the PI values are generally between 0.4 and 1.9 (Table 3).

Morphological analysis

Dried particles of HPO6s and the corresponding loaded NP HPDs, morphologically analysed by SEM, have smooth surfaces but were not completely shaped (data not reported).

In contrast, as shown in Figure 1, HPEDs appears very well formed and is characterized by a small dimensional range and a smooth surface. No crystals of drug are visible outside from the NPs. HPEs is not morphologically different from HPEDs (data not reported).

Characterization of drug-loaded nanoparticles

Drug content

HPDs, HPEDs and DEs were analysed in order to evaluate the quantity of D loaded in the NPs. Drug content analysis and loading efficiency results demonstrate that almost all the drug employed during the NP preparation phase is entrapped in the final formulations. Indeed, more than 90% of D is recovered after dissolution of dried NPs from the HPEDs and DEs batches (LE%: 102.5 ± 1.8 and 92.33 ± 6.74, respectively). Only about 87% of drug is recovered from HPDs (LE%: 87.4 ± 1.6).

In-vitro drug release test

Dissolution of more than 80% of D occurs in 5 min. All NPs tested are able to control the drug-release in an acidic medium. However, an in-vitro drug release test shows different kinetics of release between HPDs and HPEDs: about 60% of D is released from HPDs in pH 1.2 medium after 5 min. During the remaining time of analysis, the amount of drug in solution does not vary further. It is known that CDs are scarcely hydrolysed during their passage in the stomach and small intestine, but they are fermented into small saccharides by colonic microflora.[17] This gastroprotective effect is further strengthened by the addition of L100 (HPEDs) to the formulation composition: HPEDs, indeed, is able to control the release of D at pH 1.2 better than HPD, with only 25% of drug detected in the medium. This is mainly due to the presence of L100 in the formulation, as confirmed by the release profile of DEs, which is almost superimposed on HPEDs (Figure 2). At pH 6.8, HPDs and HPEDs release up to about 94% of drug during the first 15 and 30 min, respectively. In contrast, in the formulation without HP, DEs, 80% of D is released immediately after changing the pH of the medium.

Solid-state characterization of nanoparticles

X-ray powder diffraction

The two polymers used for the preparation of NPs, HP and L100, show X-ray patterns compatible with an almost complete amorphous state: only a few broad peaks, with a low ratio of intensity/background, are detectable. The crystalline nature of drug D is confirmed by an XRPD pattern that is in good agreement with the theoretical one drawn from single-crystal X-ray data (file 00-039-1684 in the database).

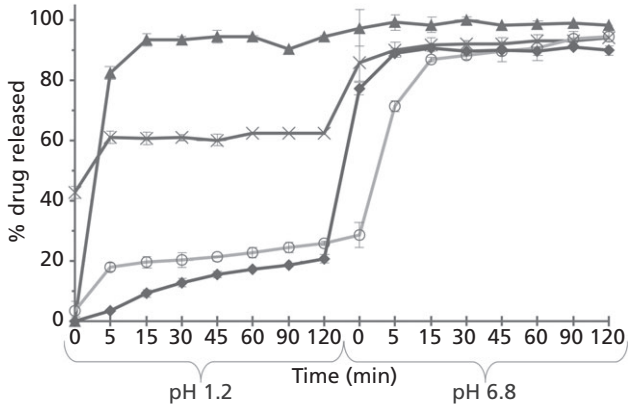

Figure 2 In-vitro drug release kinetics in gastrointestinal simulated fluid. Diclofenac sodium dissolution profiles from NPs HPDs (×), HPEDs (○), DEs (◆) at 1.2 pH and 6.8 pH ($n = 3 \pm$ SD). Dissolution rate of drug (▲) was performed in the same medium ($n = 3 \pm$ SD).

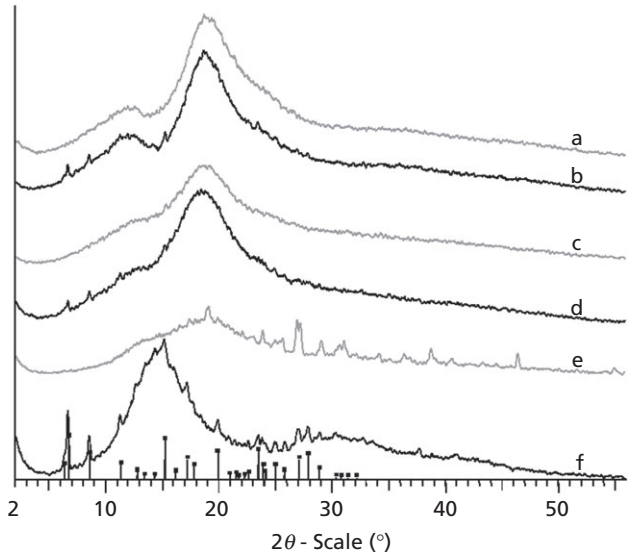

Figure 3 X-ray patterns of loaded formulations compared with the corresponding physical mixtures. (a) HPDs; (b) physical mixture of HPO6s + D; (c) HPEDs; (d) HPEs + D; (e) DEs; (f) L100 + D. Black bars represent the pattern of the drug from the database. All patterns are shifted along the Y scale to evidence their shapes.

The XRPD patterns of all systems prepared, compared to their respective physical mixtures, are reported in Figure 3. HPDs and HPEDs (a and c, respectively) reveal XRPD patterns corresponding to an amorphous nature of the products, so it can be deduced that there is an amorphization of the crystalline drug due to the preparation process. XRPD patterns of the physical mixtures of the corresponding systems (b and d, respectively) still show evident peaks of very small intensity due to the crystalline counterpart of the drug mixed with amorphous polymers. In contrast, Figure 3 shows the XRPD pattern of DEs product (e), with peaks different both in intensity and position with respect to the corresponding physical mixture (f). This is probably due to a new solid phase

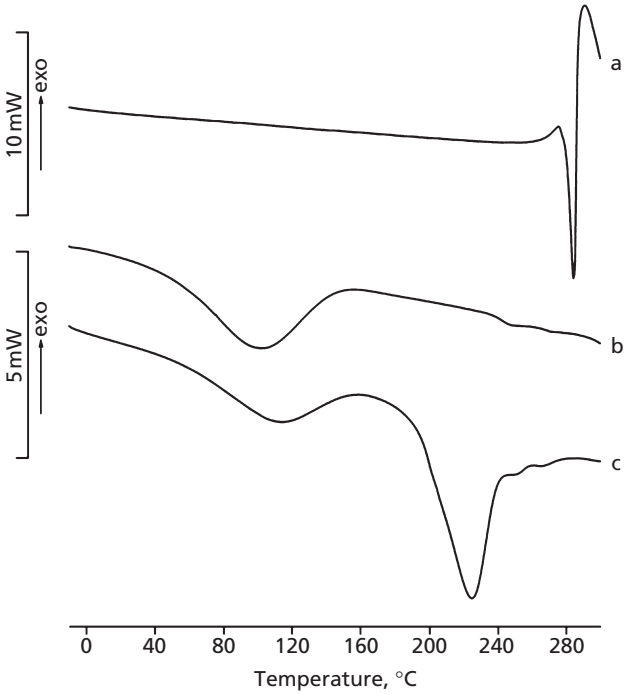

Figure 4 Differential scanning calorimetry profiles of raw materials. DSC profiles of (a) D; (b) HP; (c) L100 ($n = 3$).

formed during DEs preparation as a consequence of an interaction between drug and polymer.

Differential scanning calorimetry

The thermal behaviour of commercial D is typical of an anhydrous crystalline drug, with an endothermic effect due to melting at 283.4 ± 0.1°C (with an associated fusion enthalpy of 69 ± 3/Jg), and a concomitant exothermal decomposition. The melting enthalpy cannot therefore be accurately evaluated (Figure 4, curve a).

HP (Figure 4, curve b) and L100 (Figure 4, curve c) show a typical DSC profile of an amorphous polymer, with a broad endothermal effect between 0 and160°C and 30 and 160°C for HP and for L100, respectively, due to loosely bound water (~4.5% and ~3% for HP and L100, respectively, as mass fraction by thermogravimetric analysis, not shown) followed by sample decomposition at around 220°C.

In Figure 5 DSC profiles of HPO6s (curve a), the physical mixture between HPO6s and D (curve b) and HPDs NPs (curve c) are reported. All the DSC profiles are typical of amorphous products. In the DSC curve of the physical mixtures (curve b), the endothermic effect due to drug melting is not evident because it is probably superimposed on the decomposition effect of the polymer, as confirmed by mass-loss recorded in the corresponding thermogravimetric analysis (data not reported). Similar behaviour is also recorded for HPEDs.

In the DSC curve of the L100 + D physical mixture (Figure 5, curve h) endothermic effects are evident, which can be attributed to polymer decomposition (Figure 5, curve g). A very small endothermic effect at 285°C is probably due to drug melting. The presence of crystalline drug in the physical

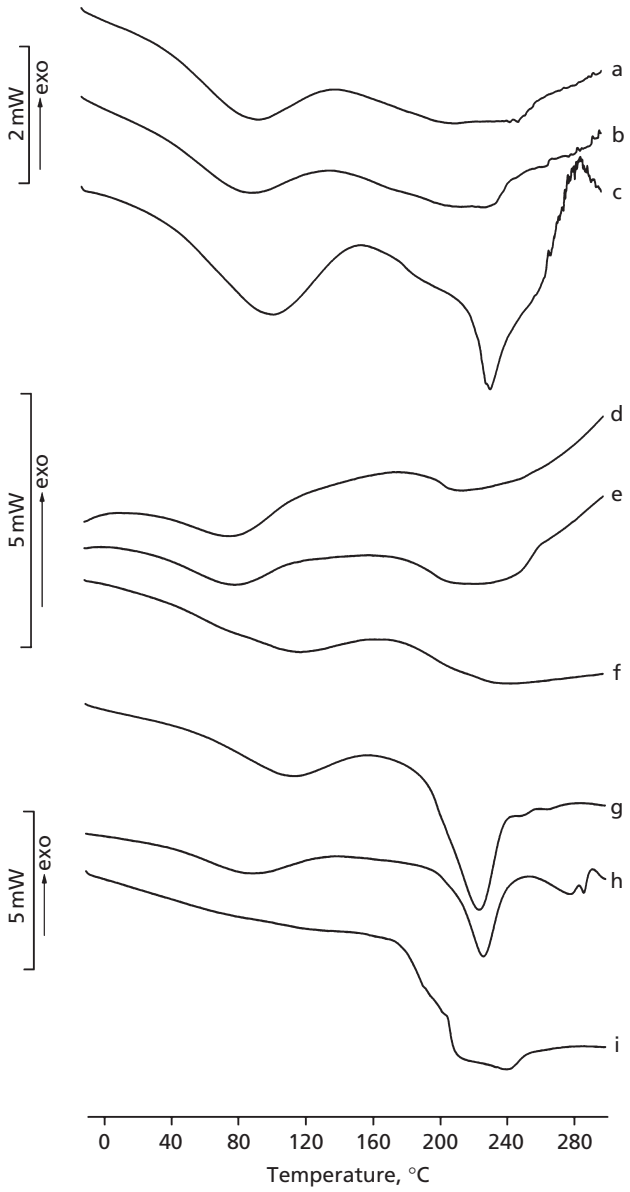

Figure 5 Differential scanning calorimetry profiles of unloaded and loaded NPs compared with physical mixtures. DSC curves of (a) HPO6s; (b) HPO6s + D physical mixture; (c) HPDs; (d) HPEs, (e) HPEs + D; (f) HPEDs; (g) L100; (h) L100 + D; (i) DEs ($n = 3$).

mixture is confirmed by the FT-IR spectrum where absorption bands characteristic of D are evident (data not reported). The thermal behaviour of the DEs formulation (Figure 5, curve i) is typical of an amorphous product, with a broad effect around 200°C due to system decomposition.

Fourier transform infrared spectroscopy

The FT-IR spectra of the crystalline drug and the amorphous polymers were recorded. The broadening band in wave number range 3400–2800/cm, typical of OH and CH stretching vibrations, is present in all the spectra and, in particular, it is better resolved for crystalline D. Peaks at around 1730/cm are due to the carboxylic acid groups of D and L100. Peaks characteristic of D at around 1570/cm are due to C=C vibration.

The FT-IR spectra of NPs DEs, HPEDs and HPDs were collected. All the bands present in HPEDs and HPDs systems are related to characteristic polymer or drug bands. The disappearance of characteristic D bands at around 1570/cm in the DEs spectrum is probably attributable to a new solid phase due to an interaction between D and L100.

Cell viability assay

Effect of raw material

Cell viability in the presence of HP, tested at different concentrations, is 100% regardless of the CD concentration used. The amount of excipients tested were chosen to take into account the corresponding quantities used for formulation preparation. 1-butanol, used as the organic phase during the emulsion process, does not have any toxic activity on Caco2 cells after 24 h of incubation. On the other hand, Tween80 appears to be highly toxic for cells, with cell viability less than 30%. The data obtained are due to the nature of the surfactant, which has solubilization activity on the cellular membrane.[45–47]

Effect of formulations

Consequently to preliminary results obtained, all the formulations prepared were washed before testing in order to avoid residues of the surfactant. Results obtained after incubation of NP solutions on the cell monolayer show that HPDs and HPEDs batches guaranteed viability higher than 80% ($P > 0.05$). The presence of drug increases by about 15–20% the cellular toxicity of formulations compared with empty NPs. Formulation DEs, not washed, has a high toxicity (70% of cell viability) due to the presence of Tween80 on its surface ($P < 0.01$).

Cell viability experiments carried out on NPs suspended in test medium show that direct contact between 5 mg of HPO6s or HPEs and cells after 24 h led to no variation of cell viability with respect to the positive control ($P > 0.05$). HPDs- and HPEDs-loaded NPs have toxic action higher than 25%, with respect to their correspondent unloaded formulations ($P < 0.01$). Unwashed DEs formulation decreased cellular viability by more than 50% ($P < 0.01$), because of the presence of surfactant (Figure 6).

Permeation studies

In-vitro permeation test using synthetic membrane

From Figure 7 it is possible to see that all formulations are able to protect the drug in acidic medium: only 8% of D permeates from HPDs and 4% from HPEDs. The DEs formulation avoids any permeation of drug at pH 1.2. However, at basic pH, NPs composed of HP enhance the permeation of D through membrane, compared with DE ($P < 0.001$). The combination HP-L100 appears more suitable for preparation of colon-specific NPs because HPEDs is the only formulation that favours almost complete D permeation through the nanosized pores of the membrane (HPEDs vs HPDs: $P < 0.001$).

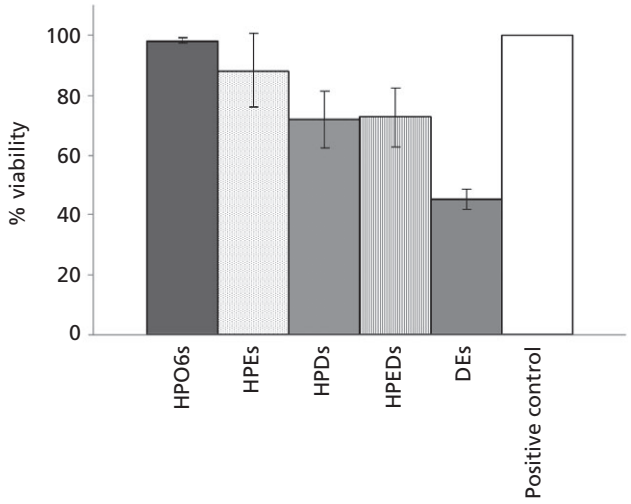

Figure 6 Cell viability test on loaded and unloaded NPs suspended in test medium. Viability test conducted on HPO6s, HPEs, HPDs, HPEDs and DEs suspensions ($n = 3 \pm$ SD).

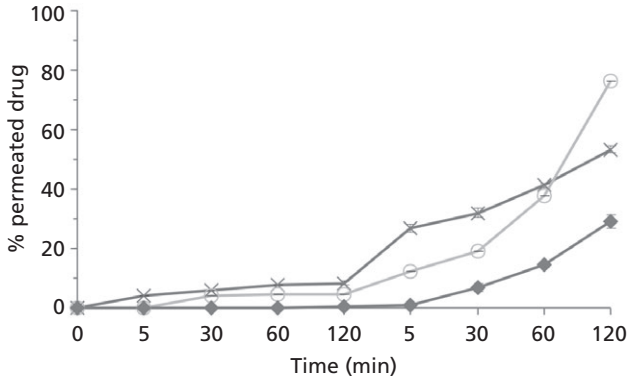

Figure 7 In-vitro permeation test at different pH values using synthetic membrane. Permeation profiles from NPs HPDs (×), HPEDs (○), DEs (◆) at pH 1.2 (first 0–120 min) and 6.8 pH (next 0–120 min) ($n = 3 \pm$ SD).

In-vitro permeation test using Caco2 cells

Tests conducted on the cellular monolayer, simulating the intestinal barrier, show a negative influence of HP on the permeation of D. In fact, as it is possible to observe from Figure 8, HPDs retains almost 90% of drug after 2 h. On the other hand, the presence of L100 in HPEDs and DEs formulations significantly increases the amount of drug permeated, to 50% and 60%, respectively (HPDs vs HPEDs and HPDs vs DEs: $P < 0.001$; HPEDs vs DEs: $P < 0.001$). The results are strongly conditioned by the very high solubility of L100 in the medium and by the static system used for the test, as confirmed by the permeation profile of DEs: after 15 min, due to the polymer dissolution, the percentage of D permeated rises remarkably. HPEDs, as it contains HP, modules the L100 dissolution rate and consequently the amount of drug permeated. Negative performance of HPD could be caused by the composition of the medium – the presence of high amounts of salts. In fact, salts, charges or even pH variation in the medium can modify the equilibrium of the HP:D complex,[8] which can consequently alter the permeation profile of the drug through cells.

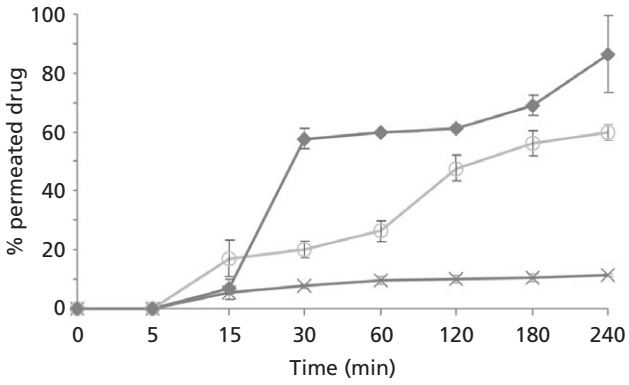

Figure 8 In-vitro permeation experiments on Caco2 cells. Diclofenac sodium permeation profiles from NPs HPDs (×), HPEDs (○), DEs (◆) at pH 6.8 ($n = 3 \pm$ SD).

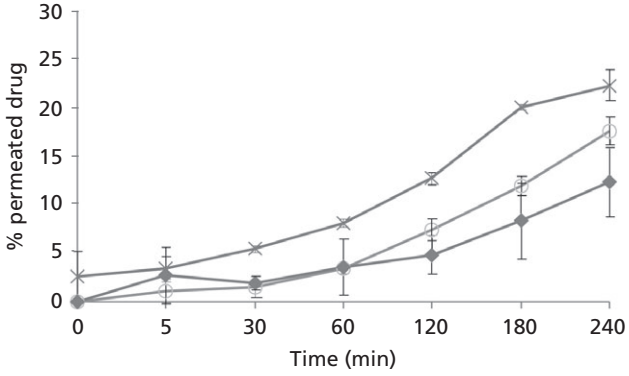

Figure 9 Ex-vivo permeation test using porcine colonic mucosa. Diclofenac sodium permeation profiles from NPs HPDs (×), HPEDs (○), DEs (◆) through biological membrane using intestinal simulated fluid at pH 6.8 ($n = 3 \pm$ SD).

Moreover, viability data obtained by TEER and MTT tests indicate that about 77 and 90% of cell populations are still alive after a 4-h long test with HPDs and HPEDs, respectively. As expected, in the case of DEs, more than 32% of cells die because of the presence of surfactant residues.

Ex-vivo permeation studies using colonic pig mucosa

Results from these experiments (Figure 9) confirm the penetration-enhancement effect of HP, already observed in the in-vitro permeation test. In the case of HPDs, after 2 h about 13% of drug is recovered from the acceptor fluid. The presence of L100 in HPEDs and DEs slows down the permeation rate of the drug with respect to the HPDs formulation (HPDs vs HPEDs and HPDs vs DEs: $P < 0.001$).

Discussion

Dimensionally homogeneous and stable nanoemulsions containing HP and/or L100 can be obtained using the Panda NS100L homogenizer by setting specific preparative

parameters. 1-butanol is a suitable solvent for the organic phase. In fact, the organic solvents usually employed in W/O emulsion preparation have boiling points lower than water. Thus, the evaporation process in order to obtain dried particles leads to the loss of the organic phase, the dissolution of water-soluble polymers in the aqueous phase and consequently emulsion disruption. 1-butanol and water give an eutectic mixture, which evaporates at 80°C under vacuum. The stability of the emulsions obtained is guaranteed by the water-1-butanol ratio of 1 : 2.1 (w/w) and by exact concentration of surfactant (Tween 80). However, by using the homogenizer Panda NS100L, the amount of Tween 80 can be decreased and stable nanodimensioned emulsions can be obtained because the high pressure homogenizer is more efficient than the high shear homogenizer. As already pointed out by Müller and collaborators,[48] during homogenization, different forces are involved, such as cavitation, collision and shear forces: these determine the breaking down of the drug particles to the nanometer range. Process conditions lead to an average particle size that remains constant, as a result of continuous fragmentation and reaggregation processes.[48]

Emulsions can be also dried, generally without any size modification, in order to obtain solid NPs, even if the PI value range is quite wide. This can be explained by the exsiccation method applied: it may be hypothesized that the emulsions start to coalesce during the exsiccation time, varying slightly the dimension and PI of the final product. Drug-loaded formulations show high LE%. Studies show that the real quantity of D encapsulated into the NPs could be higher than the actual amount found but the formation of stable complexes between CD and D does not allow the determination of the real quantity of D.[25,26,30–32] This may be confirmed from results obtained from formulations DEs and HPEDs, which show that almost total recovery of D is achieved; for the HPEDs, the presence of L100 would interfere with CD-D complex formation and thus a high LE% value is obtained.

Solid-state studies demonstrate that the production process of dried NPs leads to the amorphization of crystalline drug, when HP is included in the formulation. High energetic forces involved in the production procedure can also induce a change of crystal structure and/or partial or total amorphization of the sample.[48] On the other hand, analysis of DEs reveals a new solid-phase formation, which may be worth further investigation. Reports in the literature describe the formation of a complex between L100 and drug, the X-ray pattern of which is different to the single components.[36]

The presence of two polymers, HP and L100 is important in order to guarantee gastric protection of formulations from drug release in acidic medium, with formulations containing L100 more effective. On the other hand, there are no differences of release kinetics among NPs in simulated intestinal fluid: almost complete drug release from formulations occurs at pH 6.8.

The raw materials used do not show cytotoxity at the concentrations tested; this agrees with the literature, which states that HP shows only limited toxicity, particularly when dosed orally, in animal species such as rats, mice and dogs; it is also well tolerated in humans.[49]

However, the solid NP risk assessment was also tested *in vitro*. In fact, a number of manufactured NPs have recently been shown to cause adverse effects *in vitro* and *in vivo*.[50,51] Cell viability is the most commonly investigated parameter in cytotoxicity testing: conventional MTT assay was chosen for cytotoxicity screening, as it is known that NP properties could influence in-vitro toxicity assays.[52] Following exposure of Caco-2 cells to polymeric NPs, no negative effect is observed on cell viability.

Permeation studies performed using synthetic or biological membranes prove the prevalent effect of HP on the permeation profile and the rate of D release from formulations. However, experiments on the permeation of formulation DEs, containing only L100, through Caco2 cell monolayer show the more rapid permeation profile because of the high solubility of the polymer at the experimental conditions used. This indicates that the kind of polymer, as well as the sort of test chosen, could influence the permeation behaviour of the drug from formulation.

Conclusions

Stable and nanosized emulsions based on HP and/or L100 can be obtained by a simple and rapid preparation process based on high-pressure homogenization and setting up precise manufacturing and preparative parameters. Emulsions can be simply dried to produce solid polymeric NPs. They show pH-dependent drug-release properties regardless of the polymer used, even if L100 seems to be more effective in giving a gastroresistant formulation. However, in-vitro and ex-vivo permeation studies show that HP is necessary to control drug permeation across synthetic or biological membranes, by enhancing drug transportation. Furthermore, these formulations containing HP do not decrease cell viability. Thus, they could be proposed as new, colon-targeted delivery systems for the transmucosal delivery of drugs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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