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Novel nelfinavir mesylate loaded D-α-tocopheryl polyethylene glycol 1000 succinate micelles for enhanced pediatric anti HIV therapy: *In vitro* characterization and *in vivo* evaluation

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ABSTRACT

Worldwide more than 35 million people are living with Human Immunodeficiency Virus (HIV) where 3.3 million are children. This translates in approximately 700 new daily infections in children only in 2012. Prolonged High Activity Antiretroviral Therapy (HAART) regimes could present low-patient compliance, especially in children, affecting therapeutic success. Nelfinavir mesylate (NFV) is a non-peptidic HIV-1 protease inhibitor (IP) which was the first IP recommended for pediatric use (>2 years-old). It exhibits pH-dependant aqueous solubility which results highly restricted at physiological pH values. The former represents a main clinical limitation due to the reduction on drug absorption along the small intestine after an oral administration, leading to unpredictable drug bioavailability. Moreover a liquid formulation of NFV is not available worldwide, preventing appropriate dose adjustment and more convenient administration. In this framework, the present investigation reports the development of a NFV highly concentrated aqueous formulation for a more appropriate management of pediatric anti-HIV therapy. The aim was to encapsulate NFV within D-α-tocopheryl polyethylene glycol 1000 succinate micelles to improve its aqueous solubility and its oral pharmacokinetic parameters. Results show that NFV aqueous solubility was increased up to 80.3 mg/mL. NFV-loaded micelles exhibited a hydrodynamic diameter of 5.6 nm and a spherical morphology as determined by dynamic light scattering and transmission electronic microscopy, respectively. *In vitro* NFV release profile demonstrated a cumulative drug release of 56% at 6 h. Finally, *in vivo* data showed a significant ($p < 0.01$) increase of Area-Under-the-Curve between 0 and 24 h for NFV encapsulated in micelles in comparison with a NFV suspension prepared with glycerin 20% v/v and carboxymethylcellulose sodium 0.5% w/v, representing an increment on drug oral relative bioavailability of 1.71-fold. Thereby, this formulation represents an innovative nanotechnological platform to improve pediatric HIV pharmacotherapy.

1. Introduction

Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) remains as a major public health issue worldwide [1]. In 2012, according to the last World Health Organization (WHO) statistics, more than 35.3 million people are infected with HIV of which 3.3 million are children. Also, around 2.3 million people became infected with HIV, which 260,000 are children [2].

Actually, patients with HIV/AIDS receive a treatment called High Activity Antiretroviral Therapy (HAART), which comprises the chronic administration of at least three combined antiretroviral drugs which can temporarily suppress viral replication [3]. Nevertheless, prolonged HAART regimes could present low-patient compliance, especially in children, affecting therapeutic success [4] The number of antiretroviral drugs, approved for pediatric use by the regulatory agencies, and liquid formulations, commercially available, is actually limited [5]. Pediatric HAART remains as a health challenge where antiretroviral drugs are still "therapeutic

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orphans" [6]. In this context, the World Health Assembly has promoted the worldwide campaign entitled "*Make medicines children size*" due to the need of pharmaceutical formulations development tailored for pediatric patients [7]. Actually, children therapy consist in adults fixed-dose-combination tablets that are fractionated in order to adjust dose per child weight. Although this is a common clinical practice, it could involve prescription mistakes. Therefore, the development of liquid dosage forms such flavored syrups, solutions or suspensions could improve oral drug administration and minimize dosing errors [8].

Nelfinavir mesylate (NFV) is a non-peptidic HIV-1 protease inhibitor (IP) which was the first IP recommended for pediatric use [9]. It has been indicated in HAART for children of 2 years and older [10]. According to the Biopharmaceutic Classification System, NFV is classified as a class IV drug (low aqueous solubility and low permeability). However its aqueous solubility results pH-dependant (pKa_1: 6.0 and pKa_2: 11.1) being ~4.5 mg/mL at pH < 3 and it dramatically decreases at pH > 4. At physiological pH values (7.4), NFV solubility is highly restricted [11,12]. This represents a main clinical limitation since the drug could precipitate in the small intestine media affecting the amount of NFV oral absorbed [13]. Also, IP drugs are usually P-glycoprotein (P-gp) substrates leading to a decrease on drug intestinal absorption and oral bioavailability [14]. Clinical studies have demonstrated a clear inter-individual variability on NFV pediatric pharmacokinetics profiles related with HAART failure [15]. The drug is commercialized as tablet or powder for oral drug suspension, being the powder the only pediatric formulation available (Viracept® Oral Powder). Prepare this last is a very difficult task for hospital pharmacists or caregivers because a great amount of powder should be dispersed in a small volume of water. Therefore, achieve the content uniformity could be extremely difficult. Moreover, this pediatric formulation was poorly tolerated in infants because its oral administration resulted extremely difficult [16].

Nanotechnology has provided a feasible platform to overcome different bio(pharmaceutical) limitations of a wide vary of poor-water soluble and/or instable drugs [17]. In this framework, drug encapsulation within polymeric micelles is one of the most promising and well-investigated approaches [18,19]. These nano-sized carriers are conformed by self-aggregation of an amphiphilic polymer in water due to non-polar interactions between the hydrophobic portions of the polymer chains. Polymeric micelles structure comprises a hydrophilic corona and a hydrophobic core which provides a clear enhancement on drug water solubility and chemical stability since drugs can be hosted into the hydrophobic core. Also the outsider hydrophilic corona provides steric stabilization to the micellar aggregates [20]. Particularly, amphiphilic block copolymers have demonstrated its potential application for nano-sized carrier design [21].

D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) is a water-soluble derivative of natural vitamin E, appearing as an excellent biomaterial and a versatile pharmaceutical excipient. Moreover, it has been used in commercially available soft capsules of the anti-HIV amprenavir. TPGS contains 260 mg/g of vitamin E and has an average molecular weight of 1513 g/mol [22]. More recently, it has been approved by the Food and Drug Administration (FDA) as a "pharmaceutically safe adjuvant" [23]. Due to its amphiphilic nature, it has been used as an absorption enhancer of poorly-absorbed drugs [24–26] related with its micelle-forming properties. Also *in vitro* and *in vivo* studies have demonstrated the inhibition of P-gp activity by TPGS resulting in an enhanced oral bioavailability of paclitaxel, cyclosporine and talinolol [25,27].

Based on the previous discussion, the present work investigated the *in vitro* parameters that govern the encapsulation of poorly-water soluble NFV within TPGS micelles. Thereafter, we established a comparative study of the oral pharmacokinetic profiles between the NFV-loaded micelles and a drug control suspension. The investigation was focused on the TPGS micelles potential as a nanotechnological platform for the development of a liquid highly-concentrated NFV formulation to improve patient adherence and HAART effectiveness, especially among pediatric population.

2. Experimental

2.1. Materials

Nelfinavir mesylate (NFV) was provided by Richmond Laboratories (Argentina), D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) was supplied by Eastman Chemical Company (USA), Na_2HPO_4, NaOH, HCl, acetic acid, $NaC_2H_3O_2{\cdot}3H_2O$, polysorbate 80 (Tween 80), solvents of analytical or HPLC grade were used as received.

2.2. TPGS micelle preparation

TPGS micelles (1–10% w/v) were prepared by dissolving the appropriate amount of polymer in water or phosphate buffer pH 7.4 under magnetic stirring at 25 °C. Then samples were equilibrated for 24 h before use.

2.3. Determination of the critical micellar concentration (CMC)

The critical micellar concentration (CMC) of TPGS in aqueous and buffer solution was determined by the hydrophobic probe solubilization method employing insoluble-water 1-(2-pyridylazo)-2-naphthol (PAN) as a dye. A TPGS micellar dispersion was prepared (final concentration: 0.3% w/v) and diluted with an appropriate media to get a polymer concentration range of 0.001–0.3% w/v. Samples were equilibrated 24 h at 25 °C before used. Then, PAN was dissolved in pentane (1.6×10^{-3} M) and an aliquot (0.5 mL) was added to 3 mL of the micellar dispersion into a 5 mL-glass vial. Samples were gently magnetic stirred at room temperature until pentane evaporation (1 h) and the absorbance solution was determined by UV-Visible spectrophotometry (λ: 470 nm, UV-260, UV-Visible Recorder Spectrophotometer, Shimadzu, Japan). Thereafter, CMC value was graphically determined by plotting absorbance *versus* the logarithm of TPGS concentration.

2.4. Preparation and characterization of NFV-loaded TPGS micelles

To evaluate the capacity of TPGS micelles to encapsulate NFV, an excess of drug was added to 10 mL of micellar dispersion (1%, 3%, 5%, 7% and 10% w/v) in an amber 15-mL glass vial. Then, samples were filtered (0.45 μm cellulose nitrate membranes, Sartorius Stedim Biotech GmbH, Goettingen, Germany) to remove insoluble NFV. Different aliquots (10–70 μL) were collected and diluted to 10 mL with ethanol in a volumetric flask. NFV concentration was determined by UV-Visible spectrophotometry (λ: 253 nm, UV-260, UV-Visible Recorder Spectrophotometer, Shimadzu, Japan) at 25 °C. The linearity range was established between 18 and 92 μg/mL (R^2: 0.9999) and NFV-free TPGS micellar dispersions were used as controls. Assays were done by triplicate and the results were expressed as the average ± S.D. NFV solubility factors (f_s) were calculated according to the equation:

$$f_s = \frac{S_a}{S}$$

where, S_a and S are the apparent solubility of NFV in the micellar systems and the intrinsic drug solubility in water or buffer solution, respectively. Statistical analysis was performed by one-way

analysis of variance (ANOVA) and Tukey's *post hoc* ($p < 0.05$, GraphPad Prism version 5.02 for Windows, GraphPad Software, USA).

Additionally, an aliquot (5 mL) of NFV (50 mg/mL)-loaded micellar dispersion in water (10% w/v) was frozen (−20 °C) and lyophilized using a freeze-dryer (FIC-L05, shelf temperature −14 °C and condenser temperature −39 °C, Scientific Instrumental Manufacturing, Argentina) for 48 h. Then samples were re-disperse in: (i) deuterated water (D_2O, Sigma-Aldrich, Argentina) and (ii) deuterated dimethylsulfoxide (DMSO-d_6, Sigma-Aldrich, Argentina) and analyzed by proton nuclear magnetic resonance (^{1}H NMR, 300 MHz, Bruker MSL300 spectrometer, Germany) at room temperature. The drug encapsulation within polymeric micelles was evaluated by ^{1}H NMR spectra comparison.

2.5. Measurement of the micellar size

The average hydrodynamic diameter (D_h) and micellar size distribution of free- and NFV-loaded (50 mg/mL) TPGS (10% w/v) micelles were measured by dynamic light scattering (DLS, scattering angle of $\theta = 173°$ to the incident beam, Zetasizer Nano-Zs, Malvern Instruments, United Kingdom) at 25 °C. Samples were filtered (0.45 μm acetate cellulose filters, Microclar, Argentina) and equilibrated for 5 min at 25 °C before the measurements. The results were expressed as the average of five measurements.

2.6. Morphological characterization

Free- and NFV-loaded (50 mg/mL) TPGS (10% w/v) micelles were visualized by means of transmission electron microscopy (TEM, Philips CM-12 TEM apparatus, FEI Company, The Netherlands). An aliquot (5 μL) was placed onto a clean grid and covered with a Fomvar film. Then, sample was negatively stained with 5 μL of phosphotungstic acid solution (1% w/v), washed with distilled water (5 μL) and dried into a silicagel container before the analysis.

2.7. Thermal analysis

The thermal behavior of lyophilized drug-loaded (50 mg/mL) TPGS (10% w/v) micelles in water was characterized by differential scanning calorimetry (DSC, Mettler Toledo, TA-400 differential scanning calorimeter, USA). Samples (7 mg) were sealed into 40 μL Al-cruicible pans and heated from 25 to 200 °C at 10 °C/min. Then, the melting temperature (T_m) of each sample was determined. NFV, TPGS and a physical mixture of drug/polymer at a weight ratio of 1/2 were analyzed for comparison.

2.8. In vitro NFV-loaded micelles stability

TPGS (5% and 10% w/v) micellar dispersions containing NFV (25 and 50 mg/mL, respectively) prepared in water and in buffer pH 7.4 were stored at 25 °C over 28 days. At different time points (0, 1, 4, 7, 14, 21 and 28 days), aliquots (10 and 20 μL) were diluted with ethanol (10 mL) and the percentage of NFV in solution (NFV%) was monitored by UV-Visible spectrophotometry at 253 nm (UV-260, UV-Visible Recorder Spectrophotometer, Shimadzu, Japan). Free-loaded micellar systems were used as blank.

Additionally, in order to mimic micellar dilution and pH gradient values after an oral administration, NFV-loaded (50 mg/mL) TPGS (10% w/v) micelles prepared in water were diluted (1/10 and 1/50) with (i) HCl 0.1 N, (ii) acetate buffer pH 4.2 and (iii) acetate buffer pH 5.6, to get a final volume of 50 mL. Then samples were stored at 37 °C for 4 h and aliquots of 100 and 500 μL were taken from samples diluted 1/10 and 1/50, respectively. Samples were diluted with ethanol to 10 mL and NFV% was determined by UV-Visible spectrophotometry (λ: 253 nm, UV-260, UV-Visible Recorder Spectrophotometer, Shimadzu, Japan). Free-loaded micellar dispersions were used as controls. Assays were done by triplicate and results were expressed as an average ± S.D.

2.9. In vitro NFV release

The release of NFV from TPGS micelles was assessed by triplicate employing the membrane dialysis method. Briefly, micelles (TPGS 10% w/v, NFV 50 mg/mL) were diluted with distilled water (1/10) in order to mimic the sample dilution after an oral administration [28]. Then, samples (10 mL) were placed inside dialysis membranes (MWCO 1000 Da, Spectra/Por®6 Dialysis Membrane, nominal flat width 38 mm, diameter 24 mm, USA) and immersed into the release medium (250 mL) for 6 h at 37 ± 0.5 °C under gentle magnetic stirring (50 RPM). External media was composed of (i) HCl 0.01 N (pH 2.0) during the first 2 h and (ii) acetate buffer (pH 4.2) with Tween 80 1% v/v for the last 4 h. At different time points (0.5, 1, 2, 3, 4, 5 and 6 h) the total release medium was removed and NFV concentration was determined spectrophotometrically at 249 and 258 nm for HCl 0.01 N and acetate buffer, respectively. The linearity range was established between 0.013 and 0.1 mg/mL (R^2: 0.9991–0.9990) in both external medias. Then, the NFV release profiles were obtain by plotting the mean values (±S.D.) *versus* time.

Finally, NFV release profiles were fitted to zero order, first order and Higuchi diffusion model (Microsoft® Excel 2007 software). In this case, the model with the highest correlation coefficient (R^2) was considered the best fitting.

2.10. In vivo NFV oral pharmacokinetics

Plasma drug concentration–time profiles after NFV oral administration were assessed in fed male Winstar rats (300–350 g). Animal experiments were performed in accordance with the published Guide for the Care and Use of Laboratory Animals (NIH, eighth edition, 2011). Animals were divided into two groups ($n = 6$) and the performance of a NFV (50 mg/mL)-loaded TPGS (10% w/v) micellar dispersion in water was compared to those of an oral NFV (50 mg/mL) aqueous extemporaneous suspension prepared with glycerin 20% v/v and carboxymethylcellulose sodium 0.5% w/v. This suspension was prepared due to the impossibility of using the commercial formulation. In this way, to prepare a NFV suspension of 50 mg/mL, the great amount of oral powder (1 g) could not be homogeneously dispersed in the required amount of water (1 mL). Thereafter an appropriate extemporaneous NFV suspension was developed as described above. Animals were maintained on a 12 h light/dark routine at 22 ± 2 °C receiving a standard rodent diet (Asociación Cooperativas Argentinas, Argentina) with the following composition (w/w): 3% fat, 2% fiber, 20% proteins, 69% starch, 6% minerals, and vitamin supplements. Formulations were orally administered by gavage at a single dose of 200 mg/kg. Sample volumes were adjusted according to the drug concentration in the formulation (50 mg/mL) and the required dose per individual weight. Then blood aliquots (70 μL) were collected from the tail vein at 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8 and 24 h and plasma samples were obtained by centrifuging blood samples at 10,000 RPM for 10 min at 4 °C. Supernatants were collected and aliquots (40 μL) were deproteinized with acetonitrile (55 μL), centrifuged (13,000 RPM, 2 min, 4 °C) and NFV concentration was determined by reversed-phase HPLC-UV. Briefly, the analytical method consisted in a Fluophase™ PFP Thermo column (250 mm × 4.6 mm, i.d. 5 μm, Thermo Fisher Scientific Inc., USA) with a mobile phase composed of methanol: KH_2PO_4 25 mM (75:25, pH: 3.5 adjusted with phosphoric acid). The flow rate was maintained at 1 mL/min, the injection volume was 50 μL, the column temperature was 45 °C and UV detection was performed at 210 nm (Spectra System UV2000, Thermo Scientific Inc., USA).

Linearity range was established between 0.2 and 25 μg/mL (R^2: 0.9998–0.9999).

2.11. Evaluation on in vivo data and statics

Oral pharmacokinetic parameters denoted as: (i) the maximum plasma concentration (C_{max}), (ii) the time to the maximum plasma concentration (t_{max}), (iii) the elimination rate constant (k_e) and (iv) the area-under-the-curve between the administration time and 24 h (AUC_{0-24}) were estimated by a non-compartmental analysis of NFV plasma concentrations profiles using the TOPFIT program (version 2.0, Dr. Karl Thomae Gmbh, Schering AG, GödeckeAG, Germany).

The relative bioavailability (F_r%) of NFV after oral administration of drug-loaded TPGS micelles and NFV suspension was calculated with the following equation:

$$F_r(\%) = \frac{AUC_{mic}}{AUC_{susp}} \times 100$$

where, AUC_{mic} and AUC_{susp} are the AUC_{0-24} of micellar dispersion and suspension, respectively.

Pharmacokinetic parameters were log transformed for statistical analysis in order to reduce heterogeneity of the variance, and further compared by one-way analysis of variance and the Bonferroni *post hoc* test using GraphPad Prism version 5.02 for Windows® (GraphPad® Software, San Diego, CA, CA). Statistical significance was defined as $p < 0.01$.

3. Results and discussion

3.1. Self-aggregation of TPGS

Polymeric micelles have been extensively investigated as nanoscopic carriers to enhance aqueous solubility and chemical stability of hydrophobic/instable drugs [18]. A minimal amphiphilic polymer concentration is needed to establish a dynamic equilibrium between individual polymer chains and polymeric micelles. This concentration is denominated CMC. Below CMC, TPGS unimers are disperse in the aqueous media. At a certain concentration, hydrophobic domains interact with each other generating a state of minimum energy which promotes the formation of colloidal micellar structures. Above CMC, a sharp increment of apparent solubility is associated with the encapsulation of drug molecules into the micellar inner domain (core). As micelles are dynamic systems, it has been proposed that the CMC value could be used as an excellent parameter to characterize their stability [29]. In the present study, CMC values were determined by the hydrophobic probe solubilization method in order to establish the TPGS concentrations for NFV encapsulation assays. At low TPGS concentrations (below CMC), no PAN partition was observed due to the absence of micelles. However, above the CMC, an increase on absorbance was observed as the polymer concentration was increased after pentane evaporation. Micellar systems in distilled water showed a CMC value of 0.02% w/v at 25 °C being in good concordance with previously reports [23].

Since the thermodynamic stability of micellar dispersions could vary with different micro-environmental factors such as surfactant nature, pH and ionic strength, a more extended assay was performed in order to evaluate possible changes for micellar dispersions formulated in pH-regulated solution. Results demonstrated that TPGS prepared in phosphate buffer pH 7.4 decreased the CMC value obtained in water from 0.02% to 0.013% w/v. This result could be explained in terms of the number of surfactant molecules present in the micelles upon the CMC denoted as the aggregation number. After salt incorporation into the external media, there is an increase on the aggregation number for surfactants with a hydrophilic/lyphophilic balance (HLB) between 13 and 15 [30]. Therefore, the decrease on CMC value in buffer solution was expected since TPGS exhibits an HLB value of approximately 13.2 [22]. A similar behavior, upon pH and ionic strength modification, was observed with other non-ionic poly(ethylene oxyde)-based copolymer as Pluronic® F127 [31,32].

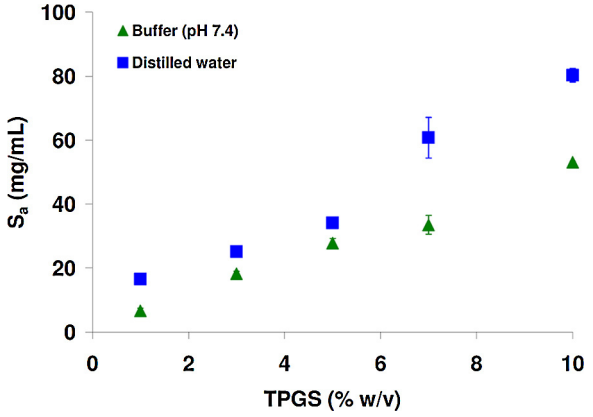

Fig. 1. NFV apparent solubility (S_a) *versus* TPGS concentration in distilled water and phosphate buffer pH 7.4 at 25 °C. Data represents mean ± standard deviation (S.D.), $n = 3$.

3.2. Characterization of NFV-loaded TPGS micelles

3.2.1. Drug encapsulation

One of the main objectives of the present study was to investigate the NFV encapsulation within TPGS micelles to enhance drug aqueous solubility and optimize its oral bioavailability. Particularly, the aim was focused on reaching a clinically relevant NFV concentration for an oral pediatric administration which could enhance child adherence to HIV therapy. In this way, TPGS results an excellent micelle-forming biomaterial since it is biocompatible and it can spontaneously self-assemble in aqueous solutions without employing organic solvents. Table 1 summarizes the S_a and f_s values in distilled water and phosphate buffer (pH 7.4) at 25 °C.

Initially, the present study involved the experimental determination of drug intrinsic aqueous solubility in each media assayed. NFV solubility in distilled water was found to be 4.08 mg/mL (pH 2.3) being in good concordance with previous investigations [33]. Interestingly, a sharp decrease on drug solubility was observed in phosphate buffer solution where solubility value was 0.003 mg/mL. It has been reported that NFV aqueous solubility is highly pH-dependant and it dramatically decreases at physiological pH, [11] as we have also observed.

Secondly, NFV was solubilized in TPGS dispersions between 1% and 10% w/v (values above CMC), which were formulated in water and buffer solutions. In this case, clear dispersions were obtained for every polymer concentration assayed. As it was expected, an increase on the polymer concentration led to a higher NFV solubility due to the inclusion of poorly water-soluble drug molecules into the micelles (Fig. 1). These results are in good concordance with previously investigations where an increase on the polymer concentration produces an increase in the solubilization of hydrophobic drugs [20,28]. In this case, NFV solubility was increased up to 80.28 mg/mL ($f_s = 19.7$) in distilled water. Its counterparts in phosphate buffer solution demonstrated S_a value of 52.10 mg/mL ($f_s = 17{,}698.4$) at a TPGS concentration of 10% w/v. In this case S_a values in distilled water resulted significantly ($p < 0.05$) higher than those observed in phosphate buffer (Table 1).

Table 1
NFV apparent solubility (S_a) and solubility factors (f_s) for TPGS micellar dispersions in distilled water and phosphate buffer pH 7.4 at 25 °C. Data represents mean ± standard deviation (S.D.), $n = 3$.

TPGS concentration (% w/v)	Distilled water		Phosphate buffer pH 7.4	
	S_a (mg/mL) (±S.D.)	f_s	S_a (mg/mL) (±S.D.)	f_s
0	4.08 (0.09)	1.0	0.003 (0.009)*	1.0
1	16.55 (0.62)	4.1	6.59 (0.86)*	2197.7
3	25.10 (1.27)	6.2	18.18 (0.79)*	6059.7
5	34.15 (0.17)	8.4	27.82 (1.42)	9273.7
7	60.79 (6.34)	14.9	33.51 (2.95)*	11,168.7
10	80.28 (2.11)	19.7	53.10 (0.31)*	17,698.4

* $p < 0.05$ *versus* distilled water.

However, f_s values demonstrated an opposite behavior as is shown in Table 1. For example, the f_s value for micellar dispersions (10% w/v) in phosphate buffer was 900-fold higher that the f_s value for the micelles prepared in distilled water. Moreover, a similar pattern was observed at every TPGS concentration assayed. An explanation of these results could be related with the low drug intrinsic solubility at pH 7.4 respect to the aqueous media (Table 1). This behavior is consistent with the formation of ionizated species at low pH and neutral species at physiological pH. In this case, NFV resulted 1300 times more soluble in water than in phosphate buffer, thereafter higher f_s values for micellar systems at pH 7.4 were observed.

Certainly, the NFV aqueous solubility increments obtained with TPGS micelles demonstrate clinical relevance since pediatric NFV doses are usually between 45 and 55 mg/kg/12 h or 25–35 mg/kg/8 h [34]. Therefore, only a small volume of clear drug-loaded micellar dispersion could be easily administered by the oral route promoting child acceptance and compliance to HAART.

Moreover, to confirm NFV encapsulation within TPGS micelles a ^{1}H NMR spectroscopic assay was performed. Typical spectra of TPGS and NFV in DMSO-d_6 are shown in Fig. S1a and b. Initially the characteristic peak (4H, multiplet, 3.50 ppm) of methylene protons of the PEG portion was observed in the ^{1}H NMR spectrum of TPGS. Also resonant peaks corresponding to aromatic protons could be individualized on NFV spectra (8H, 6.85–7.35 ppm) (Fig. S1b). After NFV-loaded TPGS micelles lyophilization and re-suspension in D_2O, an intense peak at 3.50 ppm is observed probably related with PEG blocks solvated in D_2O conforming the micellar corona. However, the absence of resonant peaks corresponding to the aromatic protons of NFV suggested the inclusion of poorly-water soluble drug molecules into the micellar core (Fig. S1c). Moreover, the spectrum of freeze-dried drug-loaded TPGS micelles in DMSO-d_6 showed both, methylene protons peak (PEG, 3.50 ppm) and the resonant peaks (6.85 and 7.35 ppm) of drug aromatic protons. In this case, due to the disruption of the micellar carrier in DMSO-d_6, the overlapped ^{1}H NMR spectra of polymer and drug could be observed (Fig. S1d).

Overall, NFV was successfully encapsulated within TPGS polymeric micelles leading to a sharp increment on drug aqueous solubility in both, distilled water and buffer solution at pH 7.4. This enhancement on drug solubility could overcome NFV dissolution rate limitation, especially in the small intestine, improving its oral absorption and bio-performance.

3.2.2. Micellar size distribution and morphology

Micellar size and size distribution of TPGS micelles in absence and presence of NFV was determined by DLS. Initially, drug-free dispersions exhibited a narrow and unimodal size distribution along with a D_h of 10.5 ± 0.1 nm and a polydispersity index (PDI) value of 0.16 ± 0.01 (Fig. 2a). Then, after NFV encapsulation, TPGS micelles presented also a unimodal size distribution with a D_h of 5.6 ± 0.3 nm along with a PDI value of 0.29 ± 0.01 (Fig. 2c). It is worth stressing that there was a slight decrease on micellar size (1.9-fold) for NFV-loaded micelles in comparison with their drug-free counterparts. It is well known that polymeric micelles are dynamic core–shell structures and drug incorporation into the inner core could impact on the micellar aggregates stability [29]. In this case, findings suggested that after drug encapsulation within the micellar core, NFV altered the aggregation pattern of TPGS leading to a slight shift to lower average hydrodynamic diameters as is shown in Fig. 2. Similar results were assessed for the first-line antitubercular drug rifampicin and poly(epsilon-caprolactona)-*b*-PEG-*b*-poly(epsilon-caprolactona) "flower-like" polymeric micelles [35].

Table 2
DSC analysis of NFV-loaded TPGS micelles in distilled water. Pristine NFV, TPGS and a TPGS/NFV physical mixture were used as controls.

Sample	T_m (°C)
NFV	148.2
TPGS	39.4
Micelles[a]	37.1
Physical mixture[b]	41.8/141.2

T_m: melting temperature.
[a] NFV-loaded (50 mg/mL) TPGS micelles in distilled water (10% w/v).
[b] TPGS/NFV weight ratio: 2/1.

For an oral administration, it has been proposed that nano-sized carriers (<300 nm) could be able to rapidly overcome gastrointestinal mucilliary clearance [36]. Therefore NFV-loaded TPGS micelles could be able to diffuse through the mucus barrier and reach the gastrointestinal mucosa surface promoting not only drug absorption but also NFV oral bioavailability.

To access the morphology of TPGS micelles, they were visualized by TEM. Initially, TPGS micelles in absence of NFV showed a spherical morphology with an average diameter of ~10 nm and only one size population (Fig. 2b). The core–corona spherical structure was expected since the length of the TPGS hydrophilic domain (PEG) results longer than its hydrophobic alkyl tail (vitamin E succinate), promoting this type of morphology [37]. Moreover the micellar size calculated by TEM was in good concordance with the average hydrodynamic diameter obtained by DLS. After NFV encapsulation, TPGS micelles remained as spheres with a unimodal size distribution (Fig. 2d) and no difference on average micellar size was observed in comparison with the drug-free dispersion.

3.2.3. Thermal analysis

To gain further insight on the physical status of NFV inside the polymeric micelles, a DSC analysis was performed to elucidate whether the drug is in crystalline or amorphous state.

As is shown in Table 2, pristine NFV denoted the presence of only one melting endothermic peak at 148.2 °C. Then, pure TPGS exhibited a single endotherm (39.4 °C) which corresponds to the melting of crystalline polymer blocks. Thereafter, the same thermal transitions were observed when a physical mixture of TPGS/NFV was analyzed (Table 2). Interestingly, NFV-loaded TPGS micelles denoted only a single endothermic peak at 37.1 °C which probably

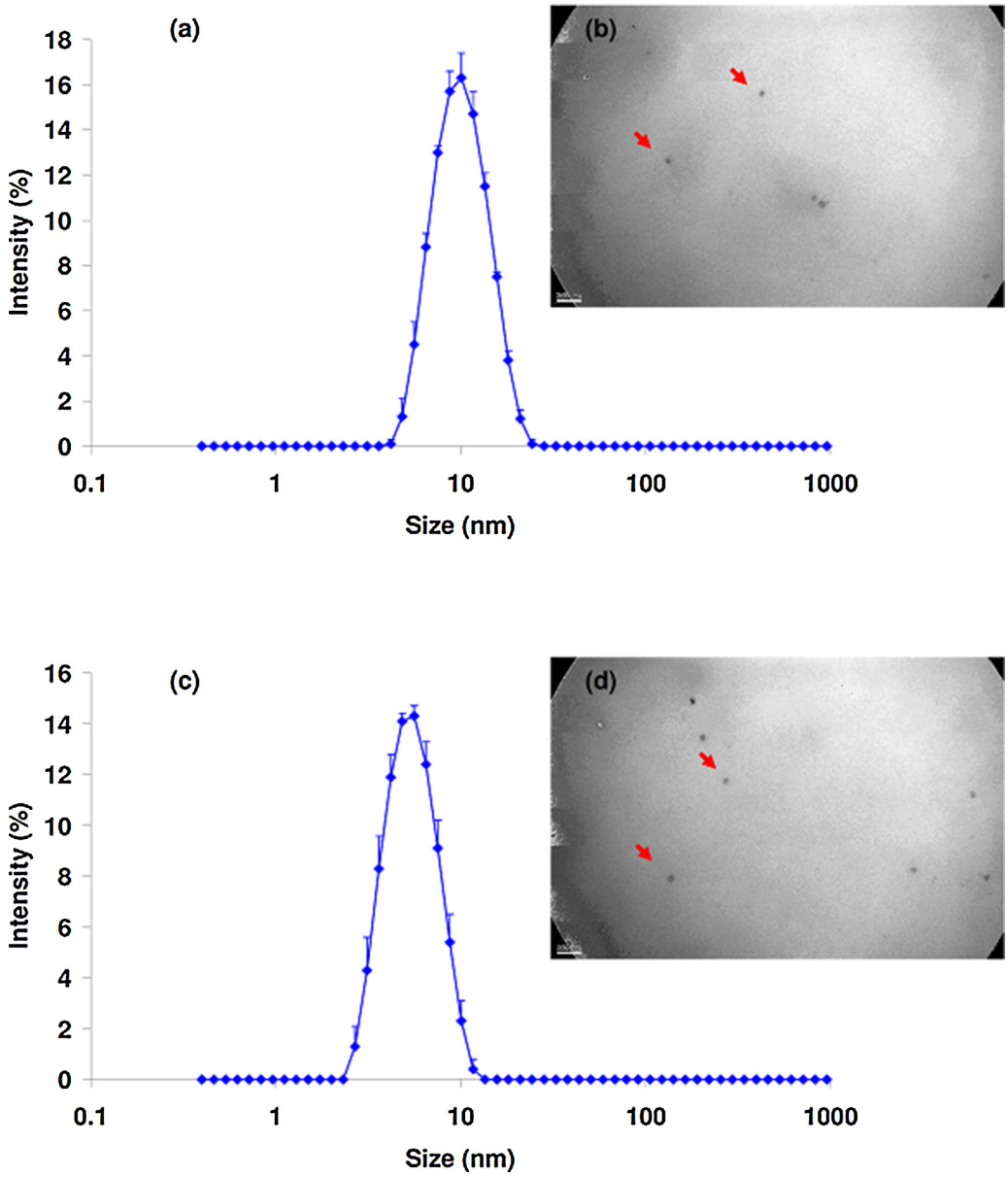

Fig. 2. Size distribution of TPGS micelles (10% w/v) in (a) absence and (b) presence of NFV (50 mg/mL). TEM micrographs of (c) drug-free and (d) NFV-loaded TPGS micelles (10% w/v). Scale bar: 200 nm.

corresponded to the polymer melting. These results suggested that the thermal properties of the pure drug were altered after its encapsulation within micelles. The absence of the melting endotherm suggests NFV conversion into an essentially amorphous form.

3.3. *In vitro NFV-loaded micelles stability*

Initially, NFV-loaded micellar dispersions were stored at 25 °C and the remaining drug content was determined at 1, 4, 7, 14 and 28 days to assess micelle stability in aqueous media. Fig. S2 shows the results obtained for TPGS (5% and 10% w/v) micellar dispersions formulated in water and buffer phosphate pH 7.4, at different time points. Results demonstrated that dispersions prepared in water remained stable up to 28 days, regardless the polymer concentration. In this case, NFV content was 97% and 93% at a TPGS concentration of 5% and 10% w/v, respectively (Fig. S2a). In contrast, in buffer phosphate pH 7.4, only TPGS 10% w/v dispersions remain stable up to 28 days (NFV content: 91%). In this case, we observed that a lower TPGS concentration in the dispersions produced a decrease on drug remaining content. For these systems, only 76% of NFV remained solubilized after 28 days (Fig. S2b). This behavior indicated that the samples with lower concentration of TPGS (5%) rendered relatively unstable systems that unassembled, releasing the drug to the medium. This was probably because the presence of salts in buffer solution of pH 7.4 could induce a dehydration process of the PEG chains of the micellar corona decreasing the polymer solubility in water [38] and therefore, the number of available micelles. In contrast, more concentrated systems displayed much higher stability after 28 days of study. Based on the previously results, TPGS micelles formulated in distilled water demonstrated the highest stability during storage at 25 °C and they were assayed for further investigations. Another important aspect was to assay the ability to re-disperse NFV-loaded TPGS micelles that were previously freeze-dried under controlled conditions. Findings showed that all the samples could be easily re-dispersed in aqueous media after simple hand-shaking for 1 min (Fig. S3).

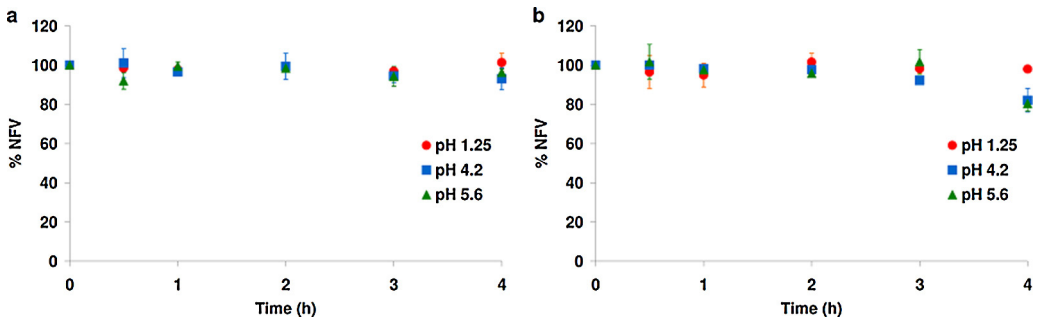

Fig. 3. NFV remaining concentration (%) after micellar dilution (a) 1/10 and (b) 1/50 with HCl 0.1 N, acetate buffer pH 4.2 and 5.6 at 37 °C for 4 h. Data represents mean ± standard deviation (S.D.), $n = 3$.

For oral drug delivery, micelles should remain stable upon dilution in the gastrointestinal tract. Furthermore, after an oral administration, nanocarriers will be exposed to the variation of pH levels and the presence of bile salts and digestive enzymes which could strongly influence their kinetic stability. In order to asses drug-loaded TPGS micellar dispersion stability, samples formulated in distilled water (TPGS 10% w/v, NFV 50 mg/mL) were diluted 1/10 and 1/50 (above the CMC) with different media to establish a variation of pH values between 1.25 and 5.6. Thereafter, each sample diluted was stored at 37 °C for 4 h and the remaining NFV content was determined by UV–vis (253 nm).

NFV-loaded micelles, diluted 1/10, were highly stable, regardless of the pH used. The NFV content was ranged between 93.0% and 101.3% for every media assayed after 4 h (Fig. 3a). Moreover, micellar dispersions diluted 1/50 presented the follow behavior, for example at pH 1.25, the NFV content was 97.9% at 4 h, then as the pH value was increased, a gradually decrease on NFV content was observed after 4 h; 82.1% and 80.3% at pH 4.2 and 5.6, respectively (Fig. 3b). As expected, a higher dilution (1/50) could favor the disassembly of the nanocarrier in a greater extend. Moreover, if micelles resulted disassembled, a greater amount of drug could be release into the external media. Also, in terms of micelle stability, the presence of salts in buffer solutions of pH 4.2 and 5.6 used in this study could produce the same effect mentioned above, favoring micelle disassembly. According to these results, and despite the dilution and acidification in the gastric environment, NFV-loaded micelles are not expected to dissociate (and drug precipitate) substantially in their transit through the stomach.

3.4. *In vitro* NFV release

Based on the previously *in vitro* stability results, the release of NFV from the micellar dispersions (TPGS 10% w/v, NFV 50 mg/mL) was investigated.

Since the main objective of the present study is the development of an oral liquid NFV micellar formulation, the release profile of NFV from the micellar nanocarrier was assessed in two external media (pH 2.0 and 4.2) at 37 °C over 6 h. Because NFV exhibits a pH-dependant aqueous solubility and it has been reported that it decreases upon pH 4.0, [11,12] we added a non-ionic surfactant (Tween 80) to the acetate buffer pH 4.2 in order to increase NFV solubility in the external medium and maintain sink conditions.

Fig. 4 shows the NFV release profile from the micellar nanocarrier. Results demonstrated that 19% and 41% of NFV diffused through the dialysis membrane at 2 and 4 h, respectively. Moreover an almost linear NFV release profile was seen over 6 h where the total drug cumulative release was 56%.

The release from polymeric micelles is a complex process that involves different mechanisms such as diffusion of drug molecules, polymer chain relaxation and erosion [39]. In the present study,

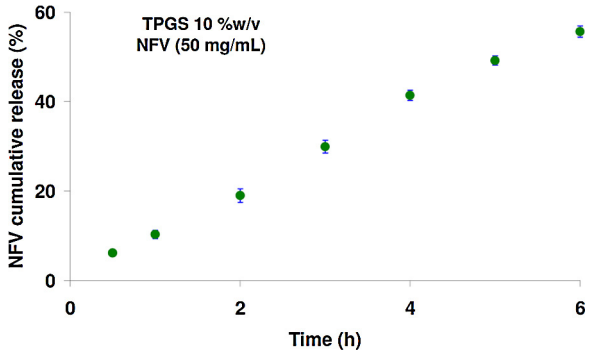

Fig. 4. *In vitro* NFV release profile from TPGS micelles at 37 °C over 6 h. Release medium was compound of: HCl 0.01 N (pH 2.0) for the initial 2 h and acetate buffer (pH 4.2) with Tween 80 1% v/v for 4 h. Data represents mean ± standard deviation (S.D.), $n = 3$.

the mathematical analysis comprised the fitting to zero order, first-order and Higuchi model. R^2 values obtained for each model were: (i) R^2: 0.9943 (zero-order), (ii) R^2: 0.9937 (first order) and (iii) R^2: 0.9790 (Higuchi model). Based on the best goodness-of-fit (R^2), the release profile could be best fitted to zero-order which correlates with the linear release profile observed in Fig. 4. Thereafter, the NFV release rate remains constant being independent of the amount of drug remaining inside the micelles [40].

3.5. *NFV oral pharmacokinetics*

The main goal of the present investigation was the development of a nanosized micellar carrier which could enhance the oral bioavailability of NFV. Particularly this investigation was focused on the development of a liquid highly concentrated drug formulation which could be easily prepared from a biomaterial as TPGS. Based on the *in vitro* performance, we use NFV-loaded (50 mg/mL) TPGS micelles (10% w/v) to investigate the following pharmacokinetic parameters: maximum plasma concentration (C_{max}), time to maximum plasma concentration (t_{max}), area under the curve between 0 and 24 h (AUC_{0-24}) and the elimination rate constant (k_e) using Wistar rats at a dose of 200 mg NFV/kg. These parameters of NFV were obtained by non compartmental analysis of plasma concentrations at selected time points. The pharmacokinetic curves of the NFV plasma concentration *versus* time after oral administration are presented in Fig. 5. *In vivo* data showed that C_{max} values increased from 3.1 μg/mL (extemporaneous suspension) to 4.4 μg/mL for the TPGS micellar dispersion (Table 3). These results represented an increase of 1.4-fold respect to the extemporaneous suspension. Moreover, the t_{max} values remained almost unchanged (Table 3). The oral NFV bioavailability encapsulated within TPGS micelles exhibited a significant ($p < 0.01$) increase in comparison with the drug extemporaneous suspension. As is shown in Table 3,

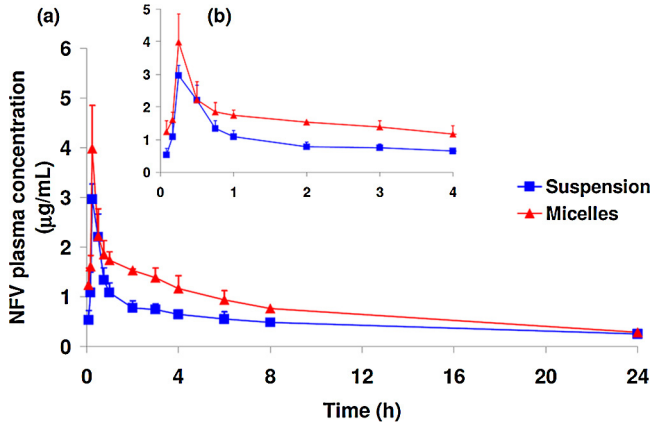

Fig. 5. (a) NFV plasma concentrations upon oral administration of drug-loaded (50 mg/mL) TPGS micelles (polymer concentration 10% w/v) and an extemporaneous NFV suspension (50 mg/mL). (b) Magnification of (a) between 0.083 and 4 h. Results are expressed as mean ± standard error (S.E.), $n = 6$.

Table 3
Pharmacokinetic parameters of NFV formulations (50 mg/mL) administered orally. Results are expressed as mean ± standard error (S.E.), $n = 6$.

Pharmacokinetic parameter	Formulation	
	Extemporaneous suspension	Micellar dispersion[a]
$C_{max.}$ (μg/mL)	3.1 ± 0.3	4.4 ± 0.9
$t_{max.}$ (h)	0.28 ± 0.03	0.29 ± 0.04
AUC_{0-24} (μg/mL/h)	14.1 ± 0.9	24.2 ± 2.8*
k_e (h^{-1})	0.057 ± 0.005	0.078 ± 0.007
F_r (%)	100	171.6

$C_{max.}$: maximum plasma concentration. $t_{max.}$: time to maximum plasma concentration. AUC_{0-24}: area under the curve between 0 and 24 h. k_e: elimination rate constant. F_r: relative oral bioavailability.

[a] TPGS concentration: 10% w/v.

* $p < 0.01$ *versus* extemporaneous suspension.

the AUC_{0-24} values for the micellar systems (24.2 μg/mL/h) were significantly ($p < 0.01$) higher than those observed for the drug suspension (14.1 μg/mL/h), representing an increment on NFV oral relative bioavailability of 1.71-fold (F_r: 171.6%).

The improvement observed in the drug pharmacokinetic parameters could be explained in terms of (i) drug encapsulation within TPGS micelles and (ii) size of the micellar nanocarrier. The former effect has previously demonstrated to increase NFV apparent aqueous solubility as it was observed in the *in vitro* characterization assays. In this way, a greater drug aqueous solubility could facilitate intestinal drug absorption after an oral administration [41]. Another interesting point is the hydrodynamic diameter of the TPGS micelles employed to encapsulate NFV. Nano-sized carriers with a $D_h < 300$ nm could overcome gastrointestinal mucilliary clearance, [39] promoting drug absorption and bioavailability. Thereafter the improvement on AUC_{0-24} values for the micellar systems could be related with the ability of the NFV-loaded nanocarrier (D_h: 5.6 nm) to diffuse though the mucus barrier, reaching the intestinal mucosa and promoting NFV absorption. These results are in good concordance with previously investigations where an enhanced oral bioavailability of a poorly-water soluble drug was observed after its encapsulation within nano-sized micelles [39,41–43]. Interestingly, TPGS has been reported as a potent inhibitor of P-gp [22] and NFV results a P-gp substrate as other IP drugs [11,14]. Thereafter, a synergist performance for TPGS could be suggested where this biomaterial could act not only as NFV nanocarrier but also a P-gp inhibitor improving NFV intestinal absorption and its bioavailability.

Finally, the k_e value of the drug was similar comparing micellar dispersions and control suspension (Table 3). This result suggested that the drug elimination was not modified by the micellar systems.

Overall *in vivo* pharmacokinetic parameters analysis confirmed that TPGS micelles could effectively improve NFV bioavailability in comparison with a drug control suspension after an oral administration.

4. Conclusions

In the present investigation the water-poorly soluble NFV was successfully solubilized by its encapsulation within TPGS micelles for the first time in this kind of nanocarrier. NFV aqueous solubility was increased promoting the development of a highly-concentrated drug aqueous formulation (50 mg/mL) which could fit pediatric patient needs in terms of (i) dose adjustment and (ii) easy swallowing. Finally, the comparative preclinical evaluation confirmed that the micellar nanocarriers significantly ($p < 0.01$) enhanced NFV oral bioavailability in comparison with a drug aqueous suspension. In this framework, this novel nano-sized micellar formulation represents an excellent and simple nano-technological strategy for the development of anti-HIV/AIDS liquid pediatric formulations, especially in low, middle-income regions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.09.031.

References

[1] http://www.who.int/mediacentre/factsheets/fs360/en/index.html (accessed July 2014).
[2] http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf (accessed July 2014).
[3] http://www.who.int/hiv/pub/guidelines/bhutan_art.pdf (accessed July 2014).
[4] B. Nacro, E. Zoure, H. Hien, H. Tamboura, F. Rouet, A. Ouiminga, A. Drabo, S. Yameogo, A. Hien, H. Peyriere, O. Mathieu, D. Hirt, J.-M. Treluyer, J. Nicolas, V. Foulongne, M. Segondy, P. van de Perre, S. Diagbouga, P. Msellati, Bull. World Health Org. 89 (2011) 451.
[5] A. Sosnik, K.P. Seremeta, J.C. Imperiale, D.A. Chiappetta, Expert Opin. Drug Deliv. 9 (2012) 303.
[6] A. Bowen, P. Palasanthiran, A.H. Sohn, Drug Discov. Today 13 (2008) 530.
[7] http://www.who.int/childmedicines/en/ (accessed July 2014).
[8] M.C. Nahata, L.V. Allen Jr., Clin. Ther. 30 (2008) 2112.
[9] S.J. Schuval, Pharmacotherapy of pediatric and adolescent HIV infection, Ther. Clin. Risk Manag. 5 (2009) 469.
[10] http://apps.who.int/prequal/whopar/whoparproducts/HA409Part1v1.pdf (accessed July 2014).
[11] G.C. Williams, P.J. Sinko, Adv. Drug Deliv. Rev. 39 (1999) 211.
[12] M. Longer, B. Shetty, I. Zamansky, P. Tyle, J. Pharm. Sci. 84 (1995) 1090.
[13] Y. Shono, E. Jantratid, J.B. Dressman, Eur. J. Pharm. Biopharm. 79 (2011) 349.
[14] R.B. Kim, F.M. Fromm, C. Wandel, B. Leake, A.J.J. Wood, D.M. Roden, G.R. Wilkinson, J. Clin. Invest. 101 (1998) 289.
[15] A.S. Bergshoeff, P.L.A. Fraaij, A.M.C. van Rossum, T.F.W. Wolfs, S.P.M. Geelen, R. de Groot, D.M. Burger, Antivir. Ther. 8 (2003) 215.
[16] http://www.aidsinfo.nih.gov/contentFiles/GlChunk/GLChunk_133.pdf (accessed July 2014).
[17] A. Sosnik, D.A. Chiappetta, A. Carcaboso, J. Control. Release 138 (2009) 2.
[18] D.A. Chiappetta, A. Sosnik, Eur. J. Pharm. Biopharm. 66 (2007) 303.
[19] Y. Lu, K. Park, Int. J. Pharm. 453 (2013) 198.
[20] D.A. Chiappetta, J. Degrossi, S. Teves, M. DíAquino, C. Bregni, A. Sosnik, Eur. J. Pharm. Biopharm. 69 (2008) 535.
[21] X-B. Xiong, Z. Binkhathlan, O. Molavi, A. Lavasanifar, Acta Biomater. 8 (2012) 2017.

[22] E. Bernabeu, D.A. Chiappetta, J. Biomater. Tissue Eng. 3 (2013) 122.
[23] Y. Guo, J. Luo, S. Tan, B.O. Otieno, Z. Zhang, Eur. J. Pharm. Sci. 49 (2013) 175.
[24] L. Yu, A. Bridgers, J. Polli, A. Vickers, S. Long, A. Roy, R. Winnike, M. Coffin, Pharm. Res. 16 (1999) 1812.
[25] T. Chang, L.Z. Benet, M.F. Hebert, Clin. Pharmacol. Ther. 59 (1996) 297.
[26] E.A. Argao, J.E. Heubi, B.W. Hollis, R.C. Tsang, Pediatr. Res. 31 (1992) 146.
[27] K. Bogman, Y. Zysset, L. Degen, G. Hopfgartner, H. Gutmann, J. Alsenz, J. Drewe, Clin. Pharmacol. Ther. 77 (2005) 24.
[28] D.A. Chiappetta, C. Höcht, C. Taira, A. Sosnik, Nanomedicine (London) 5 (2010) 11.
[29] S.C. Owen, D.P.Y. Chana, M.S. Shoichet, Nano Today 7 (2012) 53.
[30] P. Chandrasekharan, D. Maity, C.X. Yong, K.-H. Chuang, J. Ding, S.-S. Feng, Biomaterials 32 (2011) 5663.
[31] I. Pepić, J. Filipović-Grčić, I. Jalšenjak, Colloid. Surf. A: Physicochem. Eng. Asp. 336 (2009) 135.
[32] X. Zhai, G. Xu, Y. Chen, T. Liu, J. Zhang, J. Yuan, Y. Tan, J. Zhang, Colloid. Polym. Sci. 291 (2013) 2825.
[33] N.A. Kasim, M. Whitehouse, C. Ramachandran, M. Bermejo, H. Lennerna, A.S. Hussain, H.E. Junginger, S.A. Stavchansky, K.K. Midha, V.P. Shah, G.L. Amidon, Mol. Pharm. 1 (2004) 85.
[34] C.K. Taketomo, J. Hurlburt Hodding, D.M. Kraus (Eds.), Pediatric Dosage Handbook, 17th ed., Lexi-Comp. and American Pharmacist Association, Ohio, Washington, 2010.
[35] M.A. Moretton, R.J. Glisoni, D.A. Chiappetta, A. Sosnik, Colloid. Surf. B: Biointerfaces 79 (2010) 467.
[36] C. Primard, N. Rochereau, E. Luciani, C. Genin, T. Delair, S. Paul, B. Verrier, Biomaterials 31 (2010) 6060.
[37] H.K. Cho, I.W. Cheong, J.M. Lee, J.H. Kim, Korean J. Chem. Eng. 27 (2010) 731.
[38] Y. Kadam, U. Yerramilli, A. Bahadur, P. Bahadur, Colloid. Surf. B: Biointerfaces 83 (2011) 49.
[39] M.A. Moretton, C. Höcht, C. Taira, A. Sosnik, Nanomedicine (London) (2014), http://dx.doi.org/10.2217/nnm.13.154.
[40] P. Costa, J.M. Sousa Lobo, Eur. J. Pharm. Sci. 13 (2001) 123.
[41] D.A. Chiappetta, C. Höcht, C. Taira, A. Sosnik, Biomaterials 32 (2011) 2379.
[42] J. Dou, H. Zhang, X. Liu, M. Zhang, G. Zhai, Colloid. Surf. B: Biointerfaces 114 (2014) 20.
[43] M.A. Moretton, L. Cohen, L. Lepera, E. Bernabeu, C. Taira, C. Höcht, D.A. Chiappetta, Colloid. Surf. B: Biointerfaces 122 (2014) 56.