



Evaluation of Caco-2 cell permeability of ritonavir nanosuspensions

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ABSTRACT

Background and Aims: Poor aqueous solubility limits drug absorption through intestinal mucosa. Nanosuspensions with nanometer range particle size provides enhanced aqueous solubility and hence permeability. The objective of this study was to investigate the cytotoxicity and *in vitro* cell permeability through human adenocarcinoma (Caco-2) cells of ritonavir (RTV) nanosuspensions.

Methods: The Microfluidization method was used to prepare nanosuspensions. Particle size (PS), polydispersity index (PI) and zeta potential (ZP) values were measured as characterization. MTT test was applied to evaluate the cytotoxic effect. Caco-2 cell lines were used for transport studies with RTV coarse powder, physical mixtures and nanosuspension.

Results: Approximately 600 nm PS, 0.4 PDI and 22 mV ZP values were observed for nanosuspensions. The sample groups showed no cytotoxicity on the cell lines in any RTV concentration. However, significant cytotoxic effect was determined in groups with high amounts of sodium dodecyl sulfate. The transported RTV in nanosuspension formulation enhanced by 5.3-fold and 1.5-fold in comparison with RTV coarse powder and physical mixture, respectively. Rate of the transportation and also the amount of the transported RTV were improved with nanosuspension formulation.

Conclusion: Particle size reduction of RTV into nanometer size and preparing nanosuspension system was found effective to obtain enhanced cell permeability.

Keywords: Ritonavir, nanosuspension, Caco-2, permeability

INTRODUCTION

The solubility in intestinal fluids and permeability from intestinal membrane effect the drug absorption efficiency. Poor drug solubility limits dissolution and hence lower concentration gradient occurs across the intestinal mucosa (Lenhardt, Vergnault, Grenier, Scherer, & Langguth, 2008). Nanosuspensions are colloidal systems with a particle size below 1 µm. The increased surface area of particles by reducing their particle size under nano meter range improves the saturation solubility and dissolution velocity (Xie, Luo, Liu, Ma, Yue, & Yang, 2019). Hence, the poorly soluble drug may have sufficient therapeutic effects with reduced side effects and better transportation to cells of the drug molecules Karakucuk, Alptug, Teksin, Ero-

glu, & Celebi, 2019). The conventional precipitation method or top-down methods are used to prepare nanosuspensions. More preferred approaches are the top-down methods such as media milling or high pressure homogenization, which downsize the particles by high energy creating cavitation and shear forces (Tashan, Karakucuk, & Celebi, 2019). Nanosuspensions are thermodynamically unstable system and stabilization process by using surfactant or polymer is mandatory. Steric or electrostatic stabilization can be achieved in the system with surfactant and/or polymers.

Cell and tissue culture studies are used to investigate the transport of drug from delivery system into specific cells and across specific barriers. Caco-2 cell lines demonstrate the morpho-

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logical and biochemical features of adult differentiated enterocytes and goblet cells, which bring benefit to study intestinal epithelial differentiation and function (Wilson, 1990). The well-established Caco-2 cell monolayer system allows direct measurement of the drug fluxes across an epithelium (Lenhardt et al., 2008). They also, indicate P-gp and metabolizing enzymes to predict the absorption of orally administrated compounds (Matsumoto, Kaifuchi, Mizuhara, Warabi, & Watanabe, 2018).

The *in vitro* (Karakucuk, Celebi, & Teksin, 2016) and *in vivo* studies (Karakucuk et al., 2019) were evaluated in previous studies. Therefore, this study mainly focused on the investigation of cytotoxicity and Caco-2 cell permeability of RTV nanosuspensions. Possible P-gp effect of the formulation was also examined.

MATERIALS AND METHODS

Materials

Ritonavir (RTV) was kindly gifted by Mylan Pharmaceuticals (India) and hydroxypropyl methyl cellulose (HPMC) (3 cps) was donated by Colorcon Limited (Istanbul, Turkey). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). Human epithelial colorectal adenocarcinoma cell (Caco-2, ATCC HTB 37) was obtained from ATCC. Methyl-thiazolyl tetrazolium (MTT), Hank's balanced salt solution (HBSS), and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Other chemicals were HPLC or analytical grade.

Preparation of ritonavir nanosuspensions

RTV nanosuspensions were prepared according to previous study (Karakucuk et al., 2016). Briefly, the stabilizer solution, which was consisted of 4% HPMC 3 cps and 0.8% SDS was prepared. 2% w/w of RTV was dispersed this solution and pre-homogenized for 10 minutes at 10 000 rpm using ultraturrax. The pre-treatment step allowed to reduce particle size below 84 μm in regard to d_{99} . It is because 84 μm Z-type chamber was used to perform interaction of particles on microfluidization. Then, the coarse suspension was processed by jet stream homogenizer (Microfluidizer® Processors, Westwood, Massachusetts, USA) under high pressure (30 000 psi). After 20 cycles of homogenization nanosuspensions were obtained. The process continued with lyophilization, and nanosuspensions were freeze-dried to fill in the capsules as dosage form and also improve physical stability. For the lyophilization process approximately 2 mL of the nanosuspensions were frozen at $-80\text{ }^{\circ}\text{C}$ for 2 h. Freeze-dry process was performed at $-50\text{ }^{\circ}\text{C}$, 0.021 mbar for 40 h using Christ Alpha 1-2 LD Freeze Dryer.

Cell culture studies

The Caco-2 cells were used to investigate permeability of coarse RTV and compare permeability values with nanosuspensions. The Caco-2 cells were cultured using DMEM with L-glutamine, supplemented with fetal bovine serum (10%, v/v) and penicillin/ streptomycin (50 IU/mL and 50 $\mu\text{g}/\text{mL}$, respectively) in a humidified incubator at 37°C in air containing 5% CO_2 .

In vitro cytotoxicity

Before performing permeability studies, the effect of the formulations on cell viability of Caco-2 cells was investigated by

MTT assay. Cells were seeded at a density of 5×10^3 cells per well to 96-well plate and incubated overnight and then the drug solution and formulations (RTV coarse powder, physical mixtures, stabilizer mixtures and nanosuspension) were added to the wells at different concentrations and incubated for 4 hours. DMSO was used to solubilize the drug; therefore, DMSO solution (0.25-4 %) was also applied as control. After incubation period, 25 μL MTT solution was added to wells and incubated for 4 hours. Then the medium was removed and 200 μL of DMSO was added to wells to solubilize the formazan crystals and optical density of wells was measured at 570 nm.

In vitro permeability studies

For permeability studies, cell monolayers were prepared by seeding harvested cells onto inserts (12 mm Snapwell Insert with 0.4 μm pore) at a density of 7×10^4 cells per well. After 21 days of culturing (changing the medium every other day), TEER values were measured to determine monolayer integrity. Apical to basolateral transport studies were conducted after the application of formulations in Hank's Balanced Salt Solution (HBSS pH 7.4) to the donor compartment (0.5 mL). RTV bulk powder, nanosuspension, and physical mixtures of stabilizers with or without RTV were used as samples (RTV concentration was 50 mg/mL in all RTV containing samples, n=6). At predetermined time intervals (30, 60, 90, 120 and 240 mins), 0.5 mL sample was withdrawn from basolateral compartment and then fresh medium (HBSS) was added to the wells. Samples were isolated from the receiver compartment (1.5 mL) 4 hours after the incubation. The apparent permeability coefficients (P_{app} , cm/sn) were calculated according to equation below (Eq. 1):

$$P_{app} = \frac{dQ/dt}{A \cdot 60 \cdot C_0} \quad (\text{Eq. 1})$$

Where dQ/dt ($\mu\text{mol}/\text{L}\cdot\text{min}$) is the cumulative amount of RTV which has been transported over the membrane, A (1.12 cm^2) is the surface area of the inserts and C_0 ($\mu\text{mol}/\text{L}$) is the initial concentration of the RTV on the donor site.

The HPLC method was used to analyze of the samples at 239 nm, which was validated according to accuracy, precision, repeatability, specificity, detection limit, quantitation limit, linearity, and range. The HPLC instrument was HP 1050 series and the C18 RP column (250 mm x 4.6 mm, 5 μm) was equipped as an analytical column. The mobile phase was consisted of acetonitrile: 0.05 M phosphoric acid (55:45 v/v). Injection volume was 25 μL and flow rate was 1 mL/min. Retention time of RTV was 11-12 minutes.

Statistical analysis

Statistical evaluation was performed with one-way ANOVA followed Tukey HSD Post hoc test at the significance level of 0.05. Mean \pm standard deviation was provided for all data.

RESULTS AND DISCUSSION

Preparation of ritonavir nanosuspensions

The main process factors for the microfluidization method that effect quality of final nanosuspensions are the chamber type and size, temperature, homogenization cycle, and pressure

(Salazar, Heinzerling, Müller, & Möschwitzer, 2011). Additionally, formulation parameters such as physicochemical properties of the drug, amount of the drug, type and amount of the stabilizers should be considered and optimized (Van Eerdenbrugh, Van den Mooter, & Augustijns, 2008). In this study, 84 μm Z-type chamber was used to obtain nanosuspensions, which is the appropriate size and type for microfluidization. Temperature was controlled during the study. 20 homogenization cycles were applied. The well-known stabilizers, which were HPMC and SDS were used to stabilize the nanosuspensions. 2% of RTV was added in the formulations considering the final oral dose for capsule dosage form. The nanosuspensions were obtained approximately 600 nm PS, 0.4 PDI and -22 mV ZP values. The final RTV nanosuspensions were found physically stable for 30 days of storage time at room temperature. Also, physicochemical properties of RTV were preserved according to differential calorimetry and X-ray diffraction results which is shown in previous study (Karakucuk et al., 2016).

Caco-2 cell viability and permeability

Monolayer integrity of Caco-2 cell was shown with TEER values of 500-1000 $\text{ohm}\cdot\text{cm}^2$. MTT assay was conducted to understand the in vitro toxicity of the RTV in different formulations. Cell viability (%) was observed after the interaction between Caco-2 cells and RTV formulations for 4 hours. Cytotoxicity was not observed by using RTV coarse powder even in the highest dose which was 400 μg . The cytotoxicity was resulted from the formulations with SDS either in the existence of RTV or not. In the findings from different concentrations, the increased use of SDS caused toxicity on Caco-2 cells. Nanosuspension and the other formulations had no cytotoxic effect at the 50 μg dose used in the permeability study (Figure 1). Cell viability results were above 100% for some treatment groups, especially for coarse RTV powder. MTT is converted to formazan by viable cells with active metabolism so MTT reduction indicates viable cell metabolism rather than cell proliferation (Riss et al., 2016). Thus our results suggest that increasing RTV concentration, increases cellular metabolic activity of Caco-2 cells.

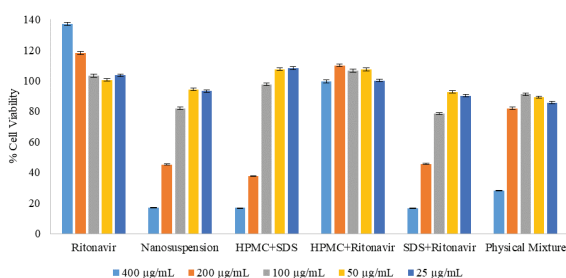


Figure 1. Cell viabilities (%) for the sample groups (n=6).

The Permeability value of coarse RTV and physical mixture were determined 1.57×10^{-5} cm/s and 5.75×10^{-5} cm/s , respectively (Figure 2). The permeability value of coarse RTV was found similar to the literature which is at about 2×10^{-5} cm/s (Alsenz, Steffen, & Alex, 1998; Holmstock, Annaert, & Augustijns, 2012). As the permeability value of RTV was found above 1×10^{-5} cm/s , it was concluded that the RTV has high permeability property which means RTV belongs to BCS Class II drug with this per-

meability value. The literature data have also proven this result (Chowdary, Annamma Devi, & Dhanalakshmi, 2012; Sinha, Ali, Baboota, Ahuja, Kumar, & Ali, 2010).

The permeability value of nanosuspension was increased and was found 8.38×10^{-5} cm/s (Figure 2). The permeability value of nanosuspension was higher than the other physical mixtures ($p < 0.05$). The increment of the permeability value of nanosuspension can be resulted from decreasing particle size under micrometer scale. Beside the nanometer particle size values, nanosuspension was prepared by SDS and it is known that SDS can inhibit P-glycoprotein (P-gp) which can result in permeability enhancement (Koga, Ohyashiki, Murakami, & Kawashima, 2000; Miller, Batrakova, & Kabanov, 1999; Rege, Yu Lawrence, Hussain, & Polli, 2001). It is also known that RTV is p-gp substrate by itself (Schmitt, Kaeser, Riek, Bech, & Kreuzer, 2010) and studies showed that RTV induced protein expression of p-gp and increased cellular drug exclusion (Perloff, Von Moltke, Marchand, & Greenblatt, 2001). Using RTV as drug efflux transporter is a strategy to improve intracellular concentration of drug molecules (Janneh, Jones, Chandler, Owen, & Khoo, 2007).

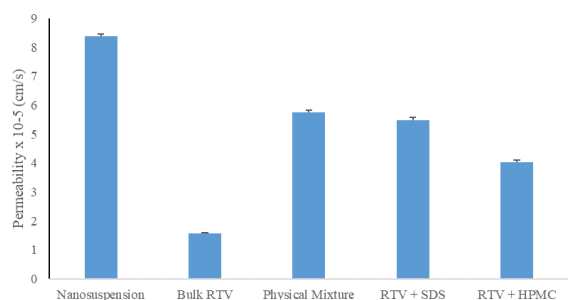


Figure 2. Permeability values of different groups (n=6).

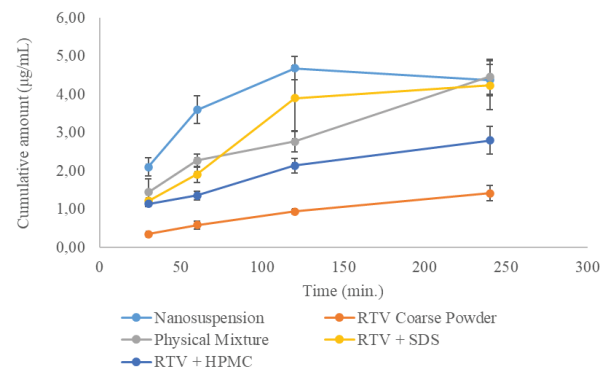


Figure 3. Cumulative amount of RTV in basolateral membrane (n=6).

Cumulative transported RTV amount to the basolateral membrane is given in Figure 3. No further drug transportation was observed after 120 minutes by nanosuspension exposure.

Even the nanosuspension showed faster drug transport, the cumulative drug transported at the end of the assay was similar among nanosuspension, physical mixture, and RTV with SDS formulations. SDS was provided P-gp inhibition which was resulted in an increase in RTV amount in basolateral

membrane. However, RTV coarse powder showed slower and lower drug transport to the basolateral membrane even in comparison with RTV and HPMC mixture. As a polymer, HPMC possessing p-gp inhibiting activity (Rehman et al., 2017), this could also explain that HPMC might be caused P-gp inhibition. It is known that inhibition or induction of functionality of P-gp can affect the pharmacokinetics, efficacy, safety, or tissue levels (Matsumoto et al., 2018).

CONCLUSION

The microfluidization method was found an effective method to develop nanosuspension formulation regarding to fewer process parameters and easy for scale-up. Neither nanometer range RSV nor using stabilizer combination did not show cytotoxic effect on Caco-2 cells at desired concentration. The nanosuspensions with particle size below 1000 nm showed enhanced permeability through Caco-2 cells due to increased saturation solubility. It could also be said that stabilizers showed P-gp effect, which resulted in improved cumulative drug transport. It is concluded that beside nanosuspensions being beneficial to improve saturation solubility, dissolution and bioavailability, they also increase the cell permeability of poorly soluble drugs.

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Informed Consent: Written consent was obtained from the participants.

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Conflict of Interest: The authors have no conflict of interest to declare.

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