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ORIGINAL ARTICLE

In Vitro Caco-2 Cell Permeability Studies of Ziprasidone Hydrochloride Monohydrate Nanocrystals

Ziprasidon Hidroklorür Monohidrat Nanokristallerinin Caco-2 Hücre *İn Vitro* Geçirgenlik Çalışmaları

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ABSTRACT

Objectives: The current study focused on the evaluation of the cytotoxic effect and permeability of ziprasidone hydrochloride monohydrate (ZHM) nanocrystals on Caco-2 cells.

Materials and Methods: ZHM nanocrystals were prepared by the microfluidization method in the presence of polyvinylpyrrolidone as a stabilizer. Particle size (PS), particle size distribution (PDI), and zeta potential (ZP) values were measured in characterization studies. *In vitro* cytotoxic effects of ZHM nanocrystals were investigated using the 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Caco-2 transport studies were conducted with formulations of ZHM coarse powder and nanocrystals.

Results: Nanocrystals were obtained with 400-600 nm PS, 0.1-0.4 PDI, and >20 mV ZP values. The cell viability remained 100% for all sample groups. The permeability value of ZHM nanocrystals through Caco-2 cells increased 2.3-fold in comparison with ZHM coarse powder. Cumulative drug transport also increased at the end of the sampling period.

Conclusion: Nanocrystal technology helps to increase the permeability of drug particles by increasing the saturation solubility.

Key words: Caco-2 cells, permeability, ziprasidone, nanocrystal

ÖZ

Amaç: Bu çalışma, ziprasidone hidroklorür monohidrat (ZHM) nanokristallerinin Caco-2 hücreleri üzerindeki sitotoksik etkisini ve geçirgenliğini değerlendirmeye odaklanmıştır.

Gereç ve Yöntemler: ZHM nanokristalleri, stabilizan olan polivinilpirolidon varlığında mikrofluidizasyon yöntemi ile hazırlanmıştır. Partikül büyüklüğü (PB), partikül büyüklüğü dağılımı (PBD) ve zeta potansiyel (ZP) değerleri karakterizasyon çalışmalarıyla ölçülmüştür. ZHM nanokristallerinin *in vitro* sitotoksik etkisi 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide testi ile belirlenmiştir. Caco-2 hücreleri taşınma çalışmaları, ZHM kaba tozu ve nanokristal formülasyonları ile gerçekleştirilmiştir.

Bulgular: ZHM nanokristalleri 400-600 nm PB, 0,1-0,4 PBD ve >20 mV ZP değerleri ile elde edilmiştir. Tüm çalışma gruplarında hücre canlılığı %100 kalmıştır. Caco-2 hücrelerinden geçiş çalışmalarında, ZHM nanokristalleri, permeabilite değerini ZHM kaba tozuna kıyasla 2,3 kat artırmıştır. Aynı zamanda kümülatif ilaç taşınımı örnek alma süresinin sonunda yükselmiştir.

Sonuç: Nanokristal teknolojisi doygunluk çözünürlüğünü artırmasına bağlı olarak permeabiliteyi artırmada yarar sağlayabilir.

Anahtar kelimeler: Caco-2 hücreleri, geçirgenlik, ziprasidon, nanokristal

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INTRODUCTION

New drug candidates have poor water solubility, which limits absorption and results in low bioavailability.[1] Saturation solubility depends on the radius of the particles according to the Ostwald-Freundlich equation (equation 1).[2,3]

$$\mathrm{Ln}\left(\frac{S}{So}\right) = 2\,M\,\gamma\,/\,\rho\,r\,R\,T \qquad \text{equation 1}$$

Where S is the solubility, r is the radius of the particles, S_o is the normal solubility value (plane surface), M is the molecular weight, γ is the interfacial tension, ρ is the density, R is the gas constant, and T is the temperature (Kelvin).

Reducing particle size (PS) to the nanometer range can increase the surface area, which leads to an increase in kinetic saturation solubility and dissolution velocity according to the Kelvin and Noyes-Whitney equations. Membrane penetration and, finally, enhanced bioavailability can be achieved by PS reduction.[4,5]

Nanocrystals are 100% drug molecules with a PS <1000 nm (typically 200-600 nm).[6] They consist of a minimum amount of stabilizer, such as a polymer and/or surfactants.[7] Because of the advantage with regard to PS, they increase the saturation solubility and hence the permeability and dissolution rate of the drug component, which increases the bioavailability.

Ziprasidone hydrochloride monohydrate (ZHM) is a Biopharmaceutical Classification system (BCS) class II antipsychotic drug with low water solubility and high permeability.[8] The absorption of ZHM is affected by the presence of food.[9] The dissolution-rate-limited performance causes a highly variable bioavailability and absorption profile, which is affected by the fed/fasted state of the patient.[10] Preparing nanocrystals of ZHM can increase the saturation solubility and hence the dissolution rate, which results in elimination of the food effect due to drug absorption and enhances the permeability and bioavailability of ZHM.[11-13]

Intestinal drug absorption is affected by the permeability of drugs, and there are several methods for investigating permeability during the drug development process.[14] One of the methods uses cultured monolayers of suitable cells, which is recommended by the food and drug administration for determining drug substance permeability. The Caco-2 human colon epithelial cancer cell line is used as an *in vitro* model to predict drug permeability. Caco-2 cells are differentiated to mimic the small intestinal epithelium when cultured as monolayers under conventional culture conditions.[15] An excellent correlation has been shown between *in vivo* absorption and *in vitro* apparent permeability obtained from a Caco-2 cell model.[15,16] Besides compound screening in high throughput format during the discovery phase, a Caco-2 model can be used to investigate the formulation effect on permeability. Some excipients can compromise tight junction integrity or cause changes in the efflux system and increase permeability;[17] therefore, it is important to investigate the permeability of formulations during drug development.

Previous studies showed that saturation solubility was dramatically enhanced with nanocrystal formulations of ZHM,[18] and an orally disintegrating tablet form was developed successfully.[19] This study focused on the *in vitro* cytotoxicity as well as the Caco-2 cell permeability of ZHM nanocrystals, and the effect of PS on permeability was investigated.

MATERIALS AND METHODS

Materials

ZHM was a kind gift from Abdi İbrahim Pharmaceuticals (İstanbul, Turkey). Polyvinylpyrrolidone K30 (PVP K30) was purchased from Sigma Aldrich (USA). Human epithelial colorectal adenocarcinoma cells (Caco-2, ATCC HTB 37) were obtained from the ATCC. Hank's balanced salt solution (HBSS), 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Dulbecco's modified eagle medium (DMEM) were purchased from Sigma Aldrich (USA). Other chemicals for high-performance liquid chromatography (HPLC) were of analytical grade.

HPLC analysis of ZHM

The ZHM concentration was determined using HPLC. An Agilent 1200 HPLC (Agilent Technologies, California, USA) system equipped with an autosampler and a ultraviolet (UV)-visible detector was used for this purpose. The concentration range was 12-20 µg/mL. The analytical column used for sample separation was an ODS C18 (RP) column (150 mmx4.6 mm, 5 µm) (TSKgel). The mobile phase consisted of potassium dihydrogen phosphate (6.8 g/L and with pH 3.0 o-phosphoric acid) 10:90 (v/v). The flow rate was 1 mL/min, the injection volume was 20 µL, and the column temperature was 25°C. The detection wavelength was 229 nm, and the retention time was 2.4 minutes.

Preparation of ZHM nanocrystals

ZHM nanocrystals were prepared according to a previous study.[18] The microfluidization technique (Microfluidics LV1, Microfluidizer® Processors, USA) was used to obtain nanocrystals. For this purpose, 0.5% (w/w) ZHM was dispensed into the 0.5% (w/w) PVP K30 solution. Macro suspensions were stirred with a homogenizer (Ultraturrax, Heidolph, Germany) at 15,000 rpm for 10 minutes. The microfluidization method was performed at 30 000 psi pressure for 20 cycles. PS, PS distribution, and zeta potential (ZP) values were measured using Malvern ZetaSizer (Malvern Instruments, UK).

The ZHM amount in ZHM nanocrystals was analyzed with a validated UV spectrophotometric method. Lyophilized nanocrystals were dissolved completely in methanol, filtered through 0.45 µm membrane filter, and then measured at wavelength of 314 nm.

Cell culture studies

Cell culture studies were conducted on Caco-2 cells to determine the permeability values of the ZHM coarse powder and ZHM nanocrystals. The effect of the sample groups on Caco-2 cell viability was investigated with an MTT test.

Preparation of Caco-2 cells

Firstly, Caco-2 cells were removed from a -180°C nitrogen tank and thawed in a water bath at 37°C. The Caco-2 cells in the vial were transferred into 15 mL of culture media under laminar flow. The media was centrifuged for 3 minutes at 2,000 rpm, and the supernatant was removed. The precipitated cells were re-suspended and transferred to 25 cm^2 flasks. The cells were incubated at 37°C, in air containing 5% CO_2, in DMEM with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 IU/mL penicillin and 50 μg/mL streptomycin. The media was changed every other day. After 6-7 days, when the cell frequency reached 80%-90%, the cells were removed by treatment with trypsin-EDTA (0.25%) and transferred to new flasks. Cells were used for permeability studies at passage 24.

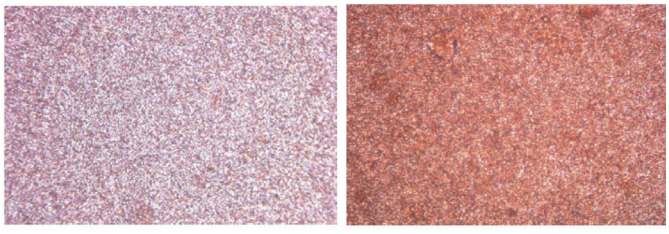
Transfer of Caco-2 cells into inserts

After 24 passage cycles, cells were counted using microscopy with a hemocytometer using trypan blue. The suspended cells were seeded into 6-well plate inserts (Snapswell™), 6 well, 0.4 μm pore diameter) at a density of 70,000 cells per well. Media (1.5 mL and 0.5 mL) were placed into the basolateral and apical sides, respectively. The culture medium was changed every other day for 21 days. Images of the cells in the culture media were acquired with an inverted microscope (Figure 1).

In vitro cytotoxicity

The MTT cell viability test was executed to investigate the effect of the coarse powder of ZHM and nanocrystals on Caco-2 cells and to determine the concentrations to be applied in the permeability study.[20] Caco-2 cells were seeded into 96-well plates at a density of 5,000 cell/well. The ZHM coarse powder and ZHM nanocrystals were dissolved in dimethyl sulfoxide (DMSO) or dispersed in culture media (DMEM) and added into the 96-well plates. DMSO (0.4%) was added as a control group. The plates were incubated at 37°C, in air containing 5% CO_2 for 4 hours, which was the duration for permeability studies. After the incubation period, 25 μL of MTT (5 mg/mL) solution was added to the wells, and the plates were incubated for a further 4 hours. Then, the media in the wells was removed, 200 μL of DMSO was added to each well, and the absorbance was read using a microplate reader at 570 nm to measure optical density. Cell viability was calculated according to equation 2.

$$\text{Cell viability (\%)} = (Abs_{test}/Abs_{control}) \times 100 \qquad \text{equation 2}$$



(a) (b)

Figure 1. Photo of the inserts taken with an inverted microscope. (a) Empty inserts; (b) Caco-2 cells

Evaluation of the monolayer integrity of the cells

The transepithelial electric resistance (TEER) of the Caco-2 cell monolayer was measured with a Millicell-ERS voltohmmeter. Monolayers with TEER >300 Ω.cm^2 were used for permeability studies.[21]

TEER values were calculated according to equation 3, where R_{sample} is the resistance of inserts, which contain cells; R_{empty} is the resistance of inserts, which are empty; a is the surface area of the cell culture inserts (cm^2).

$$TEER_{cell\ layer} = (R_{sample} - R_{empty}) \times A \qquad \text{equation 3}$$

In vitro permeability

HBSS buffer with 10 mM Hepes (pH 7.4) was used for in vitro permeability studies. The samples were prepared in 0.4% DMSO containing HBSS. Stock ZHM concentration of the formulations was 100 μg/mL. The other concentrations were diluted from stock solution. The stock concentration was selected according to the oral ZHM dose.

Firstly, the culture media in the basolateral and apical sides was removed, and these compartments were treated with HBSS. After 30 minutes of incubation, all of the medium was removed. A 0.5 mL aliquot of the sample was placed into the apical compartment, and 1.5 mL of the HBSS buffer was placed into the basolateral compartment (n=6). The plates were incubated at 37°C, at 60 rpm, for 4 hours, and samples were taken at intervals of 30, 60, 90, 120, and 240 minutes. At each sampling time point, 0.5 mL of sample was withdrawn, and fresh HBSS buffer was added to the basolateral side. The samples were analyzed using a validated HPLC method. The apparent permeability coefficient (P_{app}, cm/s) was calculated according to equation 4.

$$P_{app} = dC/dt \times 1 / (A \times C_0) \qquad \text{equation 4}$$

Where dC/dt is the drug permeation rate (μg/s); a is the surface area of the inserts (cell monolayer) (cm^2); and C_0 is the initial concentration at the apical side (μg/mL).

Statistical analysis

Statistical analysis of the data was performed by one-way analysis of variance, followed by Tukey's post-hoc test using SPSS statistics, Version 20 (Armonk, NY: IBM Corp.). The significance level was selected as $p<0.05$. The results were express as means ± standard deviation.

RESULTS AND DISCUSSION

Preparation of nanosuspensions

Microfluidization is one of the top-down methods of producing nanocrystals. The main advantages of this method are the repeatability of experiments, ease of scale up, and relatively fewer process validation parameters.[22]

In this study, ZHM nanocrystals characterized by a 532.4±13.7 nm PS, 0.304±0.01 PS distribution, and 20.5±0.3 mV ZP values were used for cell culture studies. The ZHM amount was found to be 95% ± 5% for the nanocrystals according to the validated UV spectrophotometric method.[18]

Cell culture studies

Caco-2 cell viability

An MTT assay was conducted to understand the *in vitro* toxicity of the coarse ZHM and ZHM nanocrystals. Cell viability (%) was determined after the interaction between Caco-2 cells and ZHM formulations for 4 hours. ZHM coarse powder or ZHM nanocrystals were not cytotoxic, even at the highest dose, which was 200 µg/mL. DMSO 0.4% was used to dissolve ZHM particles and applied to the cells as a control group. No cytotoxic effect was observed for DMSO at this concentration (Figure 2). As the ZHM treatment dose is 20 mg and considering that the drug is taken with 200 mL of water, permeability studies were continued with a concentration of 100 µg/mL. In the literature, it was reported previously that the PVP K30 polymer was not cytotoxic at the concentration we used.[23]

Caco-2 cell permeability

Saturation solubility, dissolution rate, and drug permeability are crucial factors for improving oral bioavailability. It is well known that the dissolution rate can be increased by reducing the PS of a drug substance to the sub-micron level, due to increased surface area. Nanocrystal technology, by taking advantage of this phenomenon, is a useful method for improving the bioavailability of poorly soluble compounds.[24] Besides *in vitro* solubility and dissolution studies, nanocrystal formulations should be tested regarding their permeability to investigate the influence of PS on drug permeability and to complement solubility and dissolution studies.[25] In this regard, the permeability of the nanocrystal formulation was investigated across the Caco-2 cell monolayer.

The monolayer integrity of Caco-2 cells was shown with TEER values of 300-1000 ohm.cm^2, which is in accordance with the literature.[26] The permeability value of coarse ZHM was found to be 8.887x10^{-6} cm/s at the end of 4 hours. The permeability value increased 1.34-fold with the nanocrystal formulation, to a value of 11.931x10^{-6} cm/s (Figure 3). In a previous study, it was shown that ZHM nanocrystals increased the saturation solubility 2.3-fold in comparison with the coarse powder of ZHM.[18] The increase in the permeability value can be explained by the increase in saturation solubility caused by the nanocrystal formulation.[27] The reduced PS of the nanocrystal formulation increases the saturation solubility of ZHM; therefore, the amount of dissolved ZHM on the apical side is greater for the nanocrystal formulation compared with coarse powder.[25] Hence, this results in an increased permeation rate for ZHM nanocrystals.

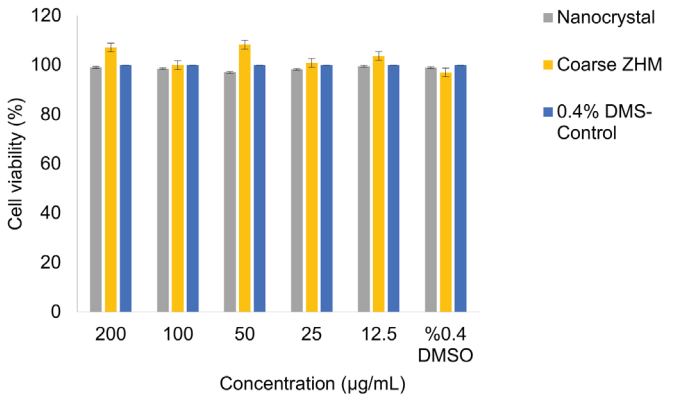


Figure 2. Cell viabilities (%) of the sample groups (n=6)
ZHM: Ziprasidone hydrochloride monohydrate, DMSO: Dimethyl sulfoxide

ZHM is known as a BCS class II drug, which has low water solubility as well as high permeability.[8,28] The log P value of ZHM is 3.6, and the drugs that have a log P value between 2.9 and 5.2 have an expected permeability value of 10^{-5}-10^{-4}.[29] The drugs, which are completely absorbed at Caco-2 monolayers, have a high permeation coefficient. Permeability values can be classified as Papp <1x10^{-6} cm/s, >10x10^{-6} cm/s and between 1 and 10x10^{-6} cm/s for the drugs which have low, high, and moderate permeability properties, respectively.[16] Considering this information, nanocrystal formulation increased the permeability value of ZHM from moderate to high.

The cumulative drug transport was also found to be higher with nanocrystal formulation in comparison with coarse powder, which is considered to be related to the improved solubility of ZHM in nanocrystal form (Figure 4).

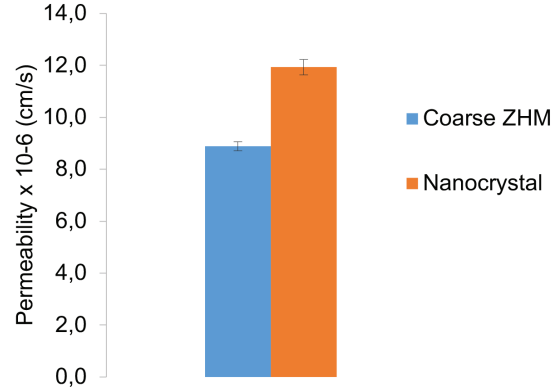


Figure 3. Permeability values of coarse ziprasidone hydrochloride monohydrate and nanocrystals
ZHM: Ziprasidone hydrochloride monohydrate

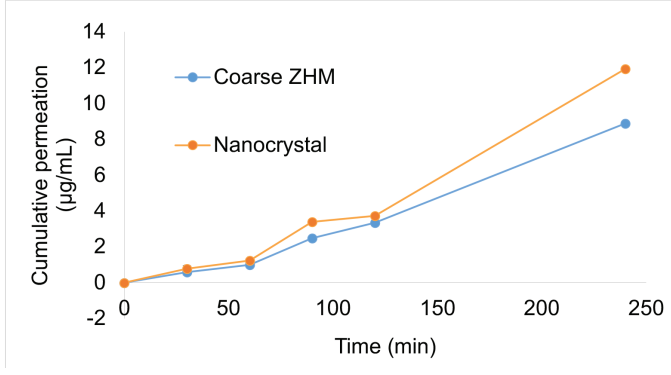


Figure 4. Cumulative amount of ziprasidone hydrochloride monohydrate that penetrated the basolateral membrane
ZHM: Ziprasidone hydrochloride monohydrate

CONCLUSION

The microfluidization method was found to be an effective and easy technique to prepare nanocrystal formulations. ZHM

nanocrystals were successfully obtained, lyophilized, and applied to Caco-2 cells. Coarse powder of ZHM or nanocrystals had no toxic effects on Caco-2 cells regarding the applied dose. ZHM nanocrystals showed an enhanced permeability value in comparison with coarse powder. In addition, the cumulative penetrated drug amount reached a higher concentration by using nanocrystals. In light of these results, it can be concluded that nanocrystal formulations can enhance the permeability of drug substances.

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