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Comparison of Gastrointestinal Permeability of Caffeine, Propranolol, Atenolol, Ofloxacin, and Quinidine Measured Using Ussing Chamber System and Caco-2 Cell Monolayer

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Abstract : The purpose of this study was to develop a cocktail approach for the measurement of the permeability of marker compounds, caffeine and propranolol (high permeability), ofloxacin (intermediate), atenolol (low), and quinidine (P-glycoprotein substrate), simultaneously. Then we compared the permeability in Caco-2 cells with that in rat intestinal segments. The difference between individual measurement and cocktail approach was less than 20 %, and the permeabilities of these compounds were similar to those previously reported, suggesting that the cocktail transport study and simultaneous drug analysis were successfully developed and validated in this study. Additionally, in the application of this cocktail method, the permeability of five drugs in rat jejunum was similar to that in ileum but different from that in colon, which was measured using the Ussing chamber system. Moreover, permeability in jejunum and ileum was similar to that in Caco-2 cells. In conclusion, the permeability in Caco-2 cells was equivalent to the permeability in rat jejunum and ileum determined with the Ussing system. Therefore, this newly developed cocktail assay and its application to the Ussing system can be a useful tool for robust and rapid screening for site-specific permeability in rat intestine, thus accelerating the prediction of absorption of new chemical entities.

Keywords : Cocktail method, Caco-2 cells, Ussing system, intestinal permeability

Introduction

Measurement of intestinal permeability of drugs or new chemical entities is very important in predicting the bioavailability of orally administered drugs. Caco-2 cells are reported to be the gold standard for measuring intestinal permeability since the morphology of Caco-2 cells reflects the shape of the human intestinal membrane including microvilli and crypts.[1] Moreover, the approach to expect intestinal absorption from this permeability data is a key stage in the drug development process.[1,2]

However, for the permeability test using Caco-2 cell monolayer, cell cultures require a period of 3 weeks after seeding onto the insert membrane of a transwell plate to differentiate into the required intestinal morphology.[3,4] The Caco-2 cell monolayer system is also limited by being unable to assess site-specific absorption from the gastrointestinal tract; for example, from the jejunum, ileum, and colon, since these intestinal segments vary in morphology and cell integrity, which could affect drug absorption properties.[5,6]

For these reasons, measurement of drug permeability in live intestinal segments using a Ussing chamber system is a good substituent.[7] This system could be applied, without a cell culture period, after dissecting rat intestinal segments. It also could assess the permeability of drugs in different intestinal segments such as jejunum, ileum, and colon.

Therefore, the purpose of this study was to develop a cocktail approach to simultaneously measure the permeability of marker compounds in Caco-2 cells and rat intestinal segments, and to compare these permeability data. For this study, caffeine and propranolol were selected as highly permeable compounds.[8,9] Ofloxacin was selected as an intermediately permeable compound and atenolol was chosen as a low permeability drug.[9] Quinidine was selected as a low permeability drug with efflux function.[10]

Experimental Methods

Chemicals and reagents

Caffeine, propranolol, ofloxacin, atenolol, quinidine, and

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Hank's balanced salt solution (HBSS) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal bovine serum and Dulbecco's Modified Eagle's medium (DMEM), Non-essential amino acid (NEAA), penicillin-streptomycin, and Trypsin-EDTA were purchased from Hyclone Laboratories (Logan, UT, USA). All other chemicals used were of analytical grade.

Cells

Caco-2 cells (passage no 42-48) purchased from ATCC (Rockville, MD, USA) were grown in tissue culture flasks in DMEM supplemented with 10% fetal bovine serum, 1% NEAA and 1% penicillin-streptomycin.

Animals

Male Sprague−Dawley rats (aged 8−9 weeks, weighing 250-300 g) were obtained from the Samtako Korea, Inc. (Osan, Korea). Animals were acclimatized for 1 week in a temperature-controlled room (23 ± 2^{o}C) under a 12 h illumination cycle. Food and water were given ad libitum. All animal procedures were approved by the Animal Care and Use Committee of the Kyungpook National University (No. 2014-0157).

Permeability study using Caco-2 cell monolayer

Caco-2 cells were seeded on collagen-coated 12 transwell membranes at a density of 5×10^{5} cells/mL and were maintained at 37°C in a humidified atmosphere with 5% CO_2/95% air for 21 days. The culture medium was replaced every 2 days. The integrity of cell monolayers was evaluated prior to transport experiments by measuring the transepithelial electrical resistance (TEER) values. TEER values of plated cells ranged from 400-600 $\Omega\cdot cm^2$. On the day of transport experiments, the growth media were discarded and the attached cells were washed with HBSS and preincubated for 20 min in HBSS at 37°C.

To measure apical to basal (A to B) transport of marker compounds, 0.5 mL of HBSS media containing marker compounds was added to the apical side and 1.5 mL of HBSS media without marker compounds was added to the basal side of the insert. The insert was transferred to a well containing fresh transport medium at 15, 30, 45, and 60 min. Aliquots (0.4 mL) from the basal side were transferred to clean tubes and samples were stored at -80°C until analysis. To measure basal to apical (B to A) transport of marker compounds, 1.5 mL of transport media containing marker compounds was added to the basal side and 0.5 mL of fresh transport media was added to the apical side of the insert. Aliquots (0.4 mL) from the apical side were transferred to clean tubes and the transport medium in the apical side was replaced with 0.4 mL of fresh transport media at 15, 30, 45, and 60 min. Samples were stored at -80°C until analysis.

Concentrations of caffeine, propranolol, ofloxacin, atenolol, and quinidine in the samples were measured simultaneously. For the analysis of these compounds, the thawed 200 μL samples were extracted using 200 μL of acetonitrile containing 20 ng/mL metformin (IS) and vigorous mixing for 10 min followed by sonication for 5 min and centrifugation at 14,000 g for 5 min at 4°C. After centrifugation, an aliquot (5 μL) was injected into an LC-MS/MS system.

Permeability analysis using the Ussing chamber system

Rat intestinal segments were excised at three sites along the small intestine (i.e., the jejunum, ileum, and colon). Jejunum was excised approximately 10 cm aboral to the duodenal section. The ileum and colon (approximately 20 cm of each) were excised following jejunal dissection. Prior to the experiment, the rat jejunal, ileal, and colonic segments on the chamber inserts were submerged in fresh, preheated HBSS for 15 min for acclimatization. The chambers were continuously bubbled with carbogen gas (5% CO_2/95% O_2) during the experiment. The experiments began by changing the HBSS media on both sides of the intestinal segments with 1 mL of preheated HBSS with and without marker compounds to the apical (A) or basal (B) compartments, respectively. Aliquots (400 μL) of media in the receiver side were withdrawn at 0, 30, 60, 90, and 120 min from the receiver compartment and replaced with equal volume of fresh preheated HBSS. The concentrations of marker compounds used were identical for the Caco-2 cells.

Aliquots (200 μL) of the samples were mixed for 10 min with 200 μL acetonitrile containing 20 ng/mL metformin (internal standard, IS) and centrifuged at 14,000 g for 5 min. Following this process, 5 μL of supernatant was injected directly into an LC-MS/MS system.

LC-MS/MS Analysis

Concentrations of caffeine, propranolol, ofloxacin, atenolol, and quinidine in the samples were analyzed using an Agilent 6430 Triple Quadrupole LC-MS/MS system (Agilent, Wilmington, DE, USA) equipped with an Agilent 1260 HPLC system. Separation was performed on a Luna CN column (2.0 mm × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA) using a mobile phase consisting of water and acetonitrile (50:50 v/v) with 0.1% formic acid at a flow rate of 0.2 mL/min. The operating parameters of the mass spectrometer detector were as follows: ion spray, 4000 V in negative mode; capillary temperature, 350°C; vaporizer temperature, 300°C; sheath gas pressure, 35 arbitrary units; auxiliary gas, 10 arbitrary units; and nitrogen gas flow rate, 15 L/min. Quantification was carried out using multiple reaction monitoring (MRM) mode at m/z 195 → 138 for caffeine, m/z 260 → 116 for propranolol, m/z 267 → 145 for atenolol, m/z 362 → 318 for ofloxacin, m/z 325 → 184 for quinidine, and m/z 130 → 71 for metformin (IS) in the positive ionization mode, and the CE was 10-25 eV.

Data Analysis

The apparent permeability (P_{app}) of the drug was calculated by dividing the initial drug transport rate (V, pmol/cm^2·min) by the initial drug concentration in the donor compartment of the insert (C) multiplied by the surface area of the insert (A):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_o} \text{ . [10]}$$

Where dQ/dt represents the rate of drug permeation, and C_0 and A are initial drug concentration and a gross surface area, respectively.

All data are expressed as means ± standard deviation (SD) of three independent experiments. Differences between treatments were evaluated using the unpaired *t*-test. In addition, linear regression analysis of the permeability of drugs in Caco-2 cells and rat intestines was also performed using SPSS software. A p value < 0.05 was considered to indicate statistical significance.

Results and Discussion

Development of a simultaneous analysis of marker compounds

In an approach for the assessment of intestinal absorption of drugs or new chemical entities, the use of a validated experimental system with high efficiency is vital. In this study, we explored the optimal transport condition to avoid potential interaction between marker compounds (e.g., caffeine, propranolol, ofloxacin, atenolol, and quinidine) of high, intermediate, and low permeability. The concentration of each marker compounds was optimized as follows: caffeine, 2 μM; propranolol, 5 μM; ofloxacin 10 μM; atenolol, 50 μM; and quinidine 10 μM. Furthermore, we developed an experimental and analytical method for the simultaneous determination of these five compounds. As shown in Fig. 1, the marker compounds were selectively detected in the positive mode and separated based on differential retention time. Atenolol was eluted in 2.4 min (Figure 1B-c), caffeine in 2.4 min (Figure 1B-e), ofloxacin in 2.5 min (Figure 1B-a), quinidine in 2.6 min (Figure 1B-b), propranolol in 3.0 min (Figure 1B-d), and metformin in 2.3 min (IS; Figure 1B-f). Intra- and inter-day precision and accuracy for each marker compounds had coefficients of variance of less than 15%.

Development of a cocktail approach in Caco-2 cells

The interactions of the marker compounds were evaluated by comparing the transport rate of each compound from the individual transport study with the transport rate of the same compound from the 5 drug cocktail set. The difference between the individual experiment (Figure 2B) and cocktail transport study (Figure 2A) was less than 20% in Caco-2 cells. The relative standard deviations ranged from 2.98 to 11.3% (n = 3) for the results of the cocktail incubation procedure. Apparent permeability (P_{app}) values of 5 marker compounds ranged from 0.33 to 35.8 × 10^{-6} cm/s, which is also consistent with other research findings.[8,9,10]

Taken together, the cocktail transport study and simultaneous analysis method were successfully developed and validated in this study.

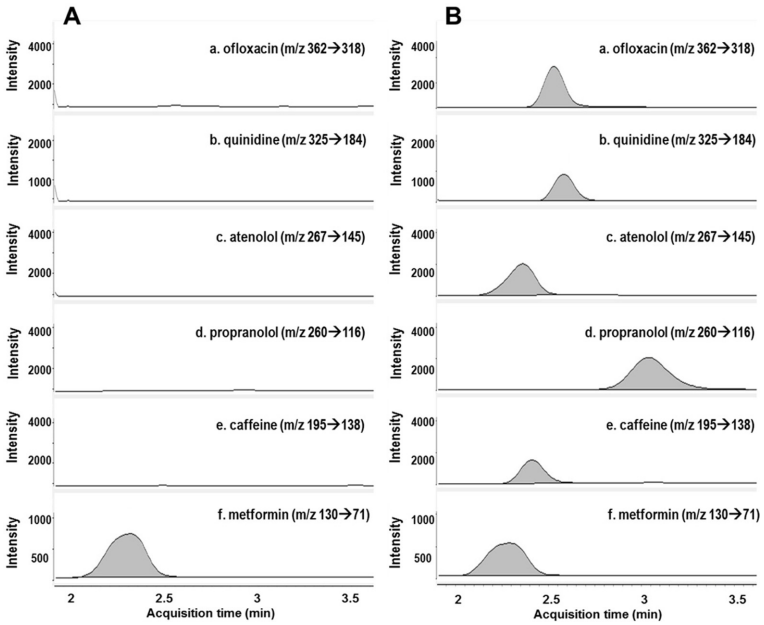

Figure 1. Representative LC-MS/MS chromatogram of ofloxacin (a), quinidine (b), atenolol (c), propranolol (d), caffeine (e), and metformin (IS; f) in zero blank (A) and samples (B) from transport study in Caco-2 cell monolayer.

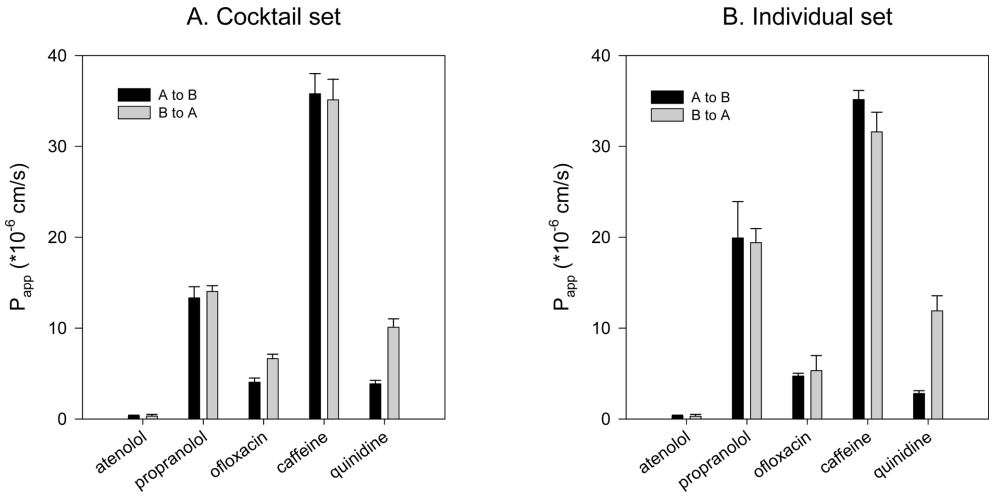

Figure 2. The A to B and B to A permeability of atenolol, propranolol, ofloxacin, caffeine, and quinidine was measured from the cocktail set (A) and individual set (B). Bar represents the mean ± S.D. of three independent experiments.

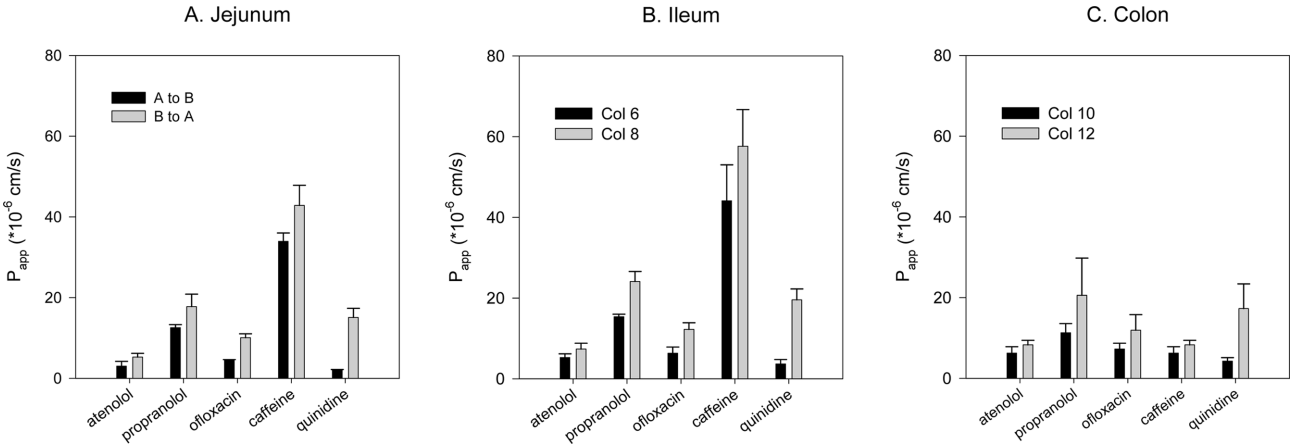

Figure 3. The A to B and B to A permeability of atenolol, propranolol, ofloxacin, caffeine, and quinidine was measured in rat jejunum (A), ileum (B), and colon (C) using a Ussing system. Bar represents the mean ± S.D. of three independent experiments.

Assessment of intestinal permeability in rat intestinal segments using the Ussing system

Since we developed an efficient cocktail approach for the measurement of intestinal permeability, we applied this method to the measurement of intestinal permeability in the different intestinal segments using the Ussing chamber system.

We dissected three different intestinal segments (i.e., jejunum, ileum, and colon) and measured the A to B and B to A permeability of 5 marker compounds. As shown in Fig. 3A, the A to B permeability of atenolol, propranolol, ofloxacin, and caffeine was lower than the B to A permeability of each compound but the efflux ratio, calculated by dividing B to A permeability by A to B permeability was less than 2.0. This suggested minimal involvement of an efflux system in the absorption of these compounds. However, the efflux ratio of quinidine was 7.4, suggesting the involvement of an efflux system. This is consistent with a previous report that quinidine is a substrate for P-glycoprotein, a representative efflux system in the gut.[10]

The permeability of the five drugs in the jejunum was comparable to those in the ileum but different from those in the colon (Figure 3B and 3C). These results showed the different permeability of drugs or new chemical entities varies between the intestinal segments, which is important when predicting the absorption windows or long absorption sites.

Comparison of gastrointestinal permeability of caffeine, propranolol, atenolol, ofloxacin, and quinidine measured by Ussing Chamber system and Caco-2 Cells

To investigate the correlation between the permeability of 5 drugs in Caco-2 cells and those in different intestinal segments in a Ussing chamber system, linear regression analysis was performed using SPSS software. As shown in Figure 4A, the correlation between Caco-2 permeability

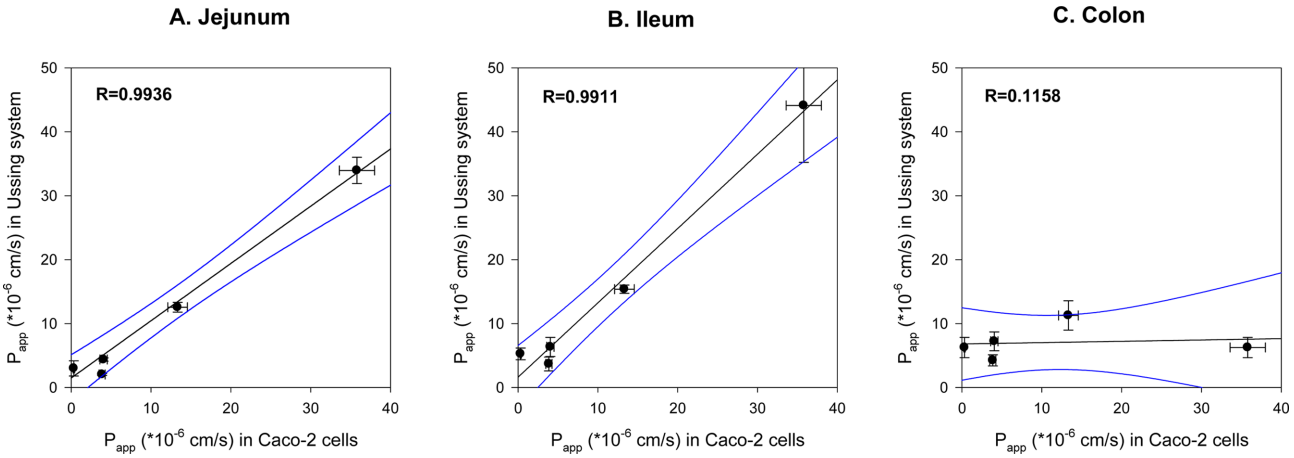

Figure 4. Correlation between the A to B permeability of atenolol, propranolol, ofloxacin, caffeine, and quinidine in Caco-2 cells and the A to B permeability of same drugs in the rat jejunum (A), ileum (B), and colon (C) using a Ussing system. Each data point represents the mean ± SD of three independent experiments. Lines were generated from the linear regression analysis and dotted lines represent the 90% confidence interval from the geometric mean value.

and the permeability of 5 drugs in the jejunum was statistically significant and the correlation coefficient was 0.9936 (p<0.05). Similar results were also found for Caco-2 permeability and the permeability of 5 drugs in the ileum with a correlation coefficient of 0.9911 (Figure 4B). The slopes of the Caco-2 permeability and the permeability of 5 drugs in jejunum and ileum were 0.92 and 1.16, respectively. Therefore, the permeability in Caco-2 cells was substitutable with that in the jejunum and ileum. However, the correlation between Caco-2 permeability and the permeability of 5 drugs in the colon was not statistically significant and the correlation coefficient was 0.1158 (p>0.05) (Figure 4C).

Conclusion

In this study, we developed a LC-MS/MS method for the simultaneous determination of five marker compounds with different permeability characteristics. Apparent permeability calculated from individual transport studies was comparable to the permeability from the optimized cocktail incubation. This process allowed us to simultaneously evaluate the permeability of 5 marker compounds as well as new chemical entities. Moreover, to assess the site-specific permeability of rat intestines, we developed a method for a Ussing chamber system and compared the permeability characteristics with that in Caco-2 cells. As our results show, permeabilities of 5 drugs in the jejunum were comparable to those in the ileum and were very similar to those in Caco-2 cells. Therefore, these results suggest that this newly developed assay may be a useful tool for robust and rapid screening for site-specific permeability in rat intestine, thus accelerating the prediction of absorption of new chemical entities.

Acknowledgments

The authors have no conflict of interest

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