School of Chinese Materia Medica[1], Beijing University of Chinese Medicine; State Key Laboratory of Natural and Biomimetic Drugs and Department of Natural Medicines[2], School of Pharmaceutical Sciences, Peking University, Beijing, China

Dulbecco's modified eagle's medium and minimum essential medium – which one is more preferred for establishment of Caco-2 cell monolayer model used in evaluation of drug absorption?

Wu Xiu-Wen[1], Wang Ru-Feng[1]*, Yuan Ming[1], Xu Wei[2], Yang Xiu-Wei[2]

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Wang Ru-feng, School of Chinese Materia Medica, Beijing University of Chinese Medicine, No.6, Wangjing Zhonghuan Nanlu, Chaoyang District, Beijing, 100102, China
wangrufeng@tsinghua.org.cn

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The Caco-2 cell monolayer model is widely used in drug absorption studies. Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM) have been used alternatively in the development of this model, although they are different in composition which may affect the differentiation and junction formation of the Caco-2 cell monolayer. Two Caco-2 cell monolayers cultured in both media were compared herein in order to underlay the standardization of this model. These two monolayers were comparatively evaluated regarding reliability and stability by morphology, transepithelial electrical resistance (TEER), alkaline phosphatase (AKPase) activity and transport experiments. Although the results showed that characteristic microvilli were present at the apical side of both monolayers, the dynamic change of TEER of the monolayer cultured in DMEM was more stable than that cultured in MEM, and AKPase activity of the former was stronger than that of the latter. Furthermore, the quantity of atenolol, a key indicator usually used for assessment of this model, across the monolayer cultured in MEM was significantly more than that cultured in DMEM. Therefore, the Caco-2 monolayer cultured in DMEM was more reliable and stable than that cultured in MEM, and thus the former was preferred for drug absorption investigation *in vitro*.

1. Introduction

It has been widely accepted that bioavailability has a significant effect on the efficacy of a drug and absorption is one of the key factors in determining bioavailability of orally administered drugs. Appropriate and correct evaluation of absorption not only underlies the development of new drugs, but also benefits the identification of effective components of complex drugs, e.g., herbal drugs which are usually composed of multi-components and used in oral dosage forms. It has been well recognized that only the compounds being absorbed into the circulation can be candidates for effective constituents of herbal drugs. For this reason, various models for absorption investigation have been used to elucidate the effective substances and to screen bioactive compounds. Permeability across intestinal mucosa is one of the most important parameters in defining oral drug absorption (Turco et al. 2011), thus, a number of *in vivo*, *in situ* and *in vitro* experimental procedures have been developed to determine intestinal permeability (Yamashita et al. 1997). As reviewed in some papers, *in vitro* models for intestinal absorption are the most popular ones because of their high efficiency, standard operation, and easy availability (Turco et al. 2011). In recent decades, in cell-based evaluation methods, for example, the Caco-2 cell monolayer model has been extensively employed in assessment of drug absorption.

Caco-2 cells, derived from a human colon adenocarcinoma, are able to differentiate in long-term culture and polarize when being seeded on microporous membrane. They form a continuous monolayer with tight junctions on this membrane, resembling the intestinal barrier and exhibiting morphological and functional properties of mature enterocytes, whereby they have become a preferred surrogate for human intestinal epithelium in drug absorption studies. In addition, Caco-2 cells have been used to evaluate the positive or negative effects of numerous substances such as additives and contaminants in food and environmental pollutants (Pinton et al. 2009; De Walle et al. 2010). Although it has been shown that Caco-2 cells behave as mature enterocytes after differentiation, the Caco-2 cell monolayer is heterogeneous and highly dependent on culture conditions. There are several culture-related factors that lead to variable transport properties and permeability. One is the selection of sub-populations of cells. Others include the passage number, the addition of growth-promoting factors such as paracrine and atocrine, the seeding density, the culture time, and, most importantly, the culture medium. The culture medium is able to influence the biology of Caco-2 cells either by its physicchemical properties or by its composition, thus modulating the cell morphology and functions of proliferation, differentiation and permeability (Sambuy et al. 2005). These variations of cell morphology and functions in turn make the monolayer unstable, resulting in poor repeatability, accuracy, and reliability of the experimental results. In recent years, although many efforts, such as studies on the influences of composition and porosity of filter in transport unit, use of extracellular matrix coating on

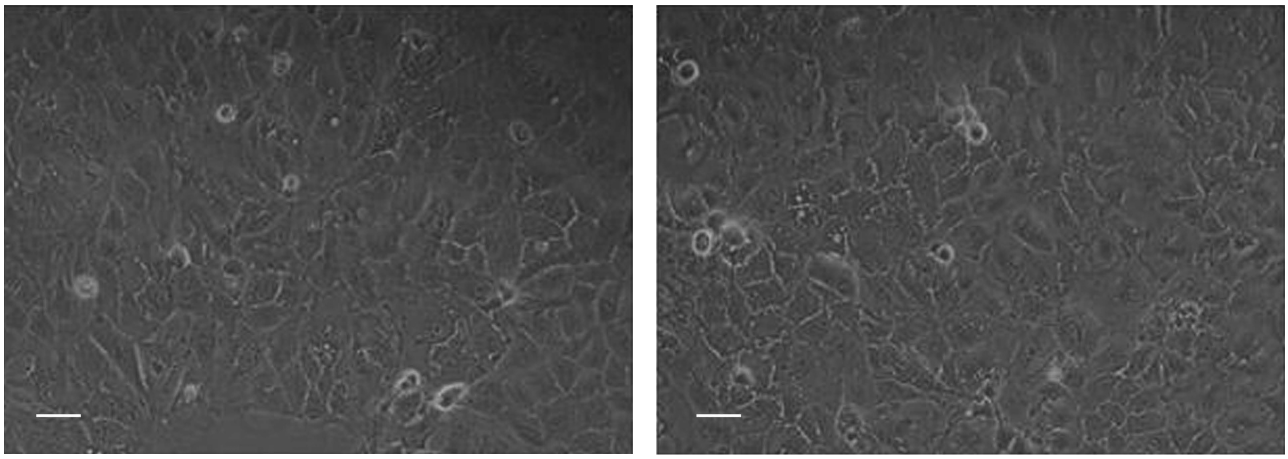

Fig. 1: Cell morphology in culture flasks (optical micrograph) **Notes:** The bars in A and B are equal to 50 μm, respectively.

filter, cell sources, time span of cell growth on filters, composition and pH of transport buffer, have been made to improve or optimize Caco-2 cell cultures in order to overcome the variability and heterogeneity of this model, almost none of the reports was about the screen of culture media except the influence of single components like glucose, serum and glutamine on Caco-2 cells (Sambuy et al. 2005; Bailey et al. 1996; Markowska et al. 2001; Ferruzza et al. 2012b; DeMarco et al. 2003). Both DMEM and MEM were used in the cell monolayer developing process (Yang et al. 2007; Ma et al. 2006; Ji et al. 2007); however, the question which culture medium is more suitable for establishing a reliable and stable research model for pharmacological *in vitro* studies has not been answered yet. As a part of standardization program, we compared these two media in the development of Caco-2 cell monolayer model.

2. Investigations, results and discussion

2.1. Morphological observation

The optical micrographs of the cells in flasks are shown in Fig. 1 and the transmission electron micrographs of characteristic microvilli on the apical side of the differentiated Caco-2 monolayers are shown in Fig. 2.

Based on these micrographs of both light and electron microscope, the cells cultured in either DMEM or MEM developed characteristic microvilli on the apical side on the membrane, after 23 days of culture indicating that these two Caco-2 monolayers were morphologically similar to intestinal epithelial cells. However, almost no significant morphological difference was observed between the cells cultured in DMEM and those cultured in MEM. This might be one of the superficial reasons that both media were used alternatively in the establishment of Caco-2 cell monolayer model.

2.2. Determination of TEER

TEER profile of the Caco-2 cell monolayers cultured in both DMEM and MEM in 23 days is illustrated in Fig. 3 as a function of time. In general, the TEER values of the monolayers cultured in both DMEM and MEM increased with the extension of culture time and reached a maximum on day 14. However, the trend line of the values for DMEM seemed more stable than that for MEM. Although the TEER values for both DMEM and MEM appeared to increase in the first half of the culture period, the former displayed essentially a plateau without larger fluctuation in the second half whereas the latter exhibited lager fluctuation after day 16 and decreased abruptly in the last 3 days.

TEER is one of the key parameters to estimate the integrity of monolayers and to qualify the model to be used in the transportation experiment. TEER values reported by different research groups were different, ranging from 130 to 900 $\Omega \cdot cm^2$ (Narai et al. 1997; Aiba et al. 2005). In this study, the values of the monolayers cultured in both DMEM and MEM were within the reported range, but the fluctuation after day 16 and the abrupt decrease in the last 3 days in MEM group demonstrates that MEM is inferior to DMEM as the medium for Caco-2 model.

2.3. Determination of AKPase activity

The test results of AKPase activity of the Caco-2 cell monolayers are illustrated in Fig. 4. At the sampling time points, days 8, 15 and 23, the AKPase activity of the Caco-2 cell monolayers cultured in both DMEM and MEM was enhanced with time. However, the values determined at all time points for the DMEM group were significantly greater than those for the MEM group ($P < 0.001$ on day 8; $P < 0.001$ on day 15; $P < 0.05$ on day 23).

In the small intestine, the differentiation of enterocytes results in increased expression and activity of several enzymes involved in digestion (Ferruzza et al. 2012a). Similar increase of these brush border enzymes upon *in vitro* differentiation has also been observed in Caco-2 cells. The activity of these enzymes gradually increases after cell confluence and can be used as a reliable marker to evaluate differentiation of Caco-2 cells (Ferruzza et al. 2012a). AKPase is one of the brush border enzyme markers indicating the polarization of the cell membrane of Caco-2 monolayers. On the basis of our test results, the increasing value of AKPase activity demonstrated that the monolayers cultured in both DMEM and MEM were polarized, yet the polarization rates were different. The cells cultured in DMEM (from 391.9 *U/g protein* on day 8 to 516.3 *U/g protein* on day 15 to 649.9 *U/g protein* on day 23) polarized faster than those cultured in MEM (from 222.0 *U/g protein* on day 8 to 341.6 *U/g protein* on day 15 to 581.1 *U/g protein* on day 23).

2.4. Monolayer permeability

In this study, the calculated average P_{app} values of propranolol were 2.53×10^{-5} cm/s (DMEM group) *vs* 2.85×10^{-5} (MEM group), and of atenolol were 5.92×10^{-6} cm/s (DMEM group)

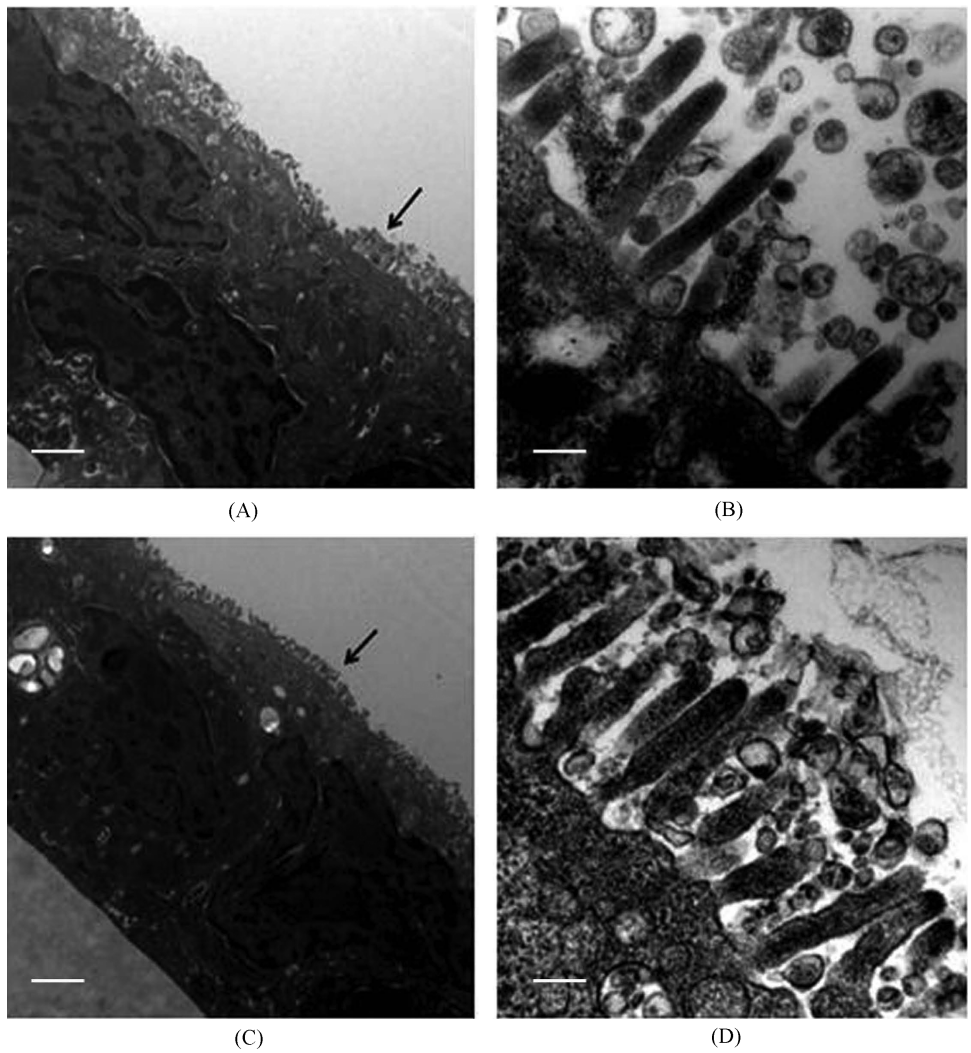

Fig. 2: Characteristic microvilli of apical side of the differentiated Caco-2 monolayer (transmission electron micrograph) **Notes:** A and C are Caco-2 monolayers cultured in DMEM and MEM, respectively. B and D are the microvilli of the differentiated Caco-2 monolayers cultured in DMEM and MEM, respectively. The bars in A and C are equal to 4 μm, and those in B and D are equal to 400 nm, respectively.

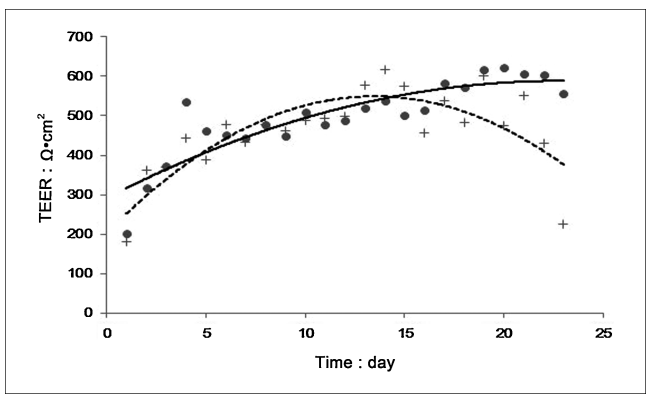

Fig. 3: TEER values of the developing Caco-2 monolayer as the function of time **Notes:** "•" and "+" indicate the TEER values of the developing Caco-2 monolayers cultured in DMEM and MEM, respectively. The full line and the dotted line denote the tendency of TEER change of the developing Caco-2 monolayers cultured in DMEM and MEM, respectively.

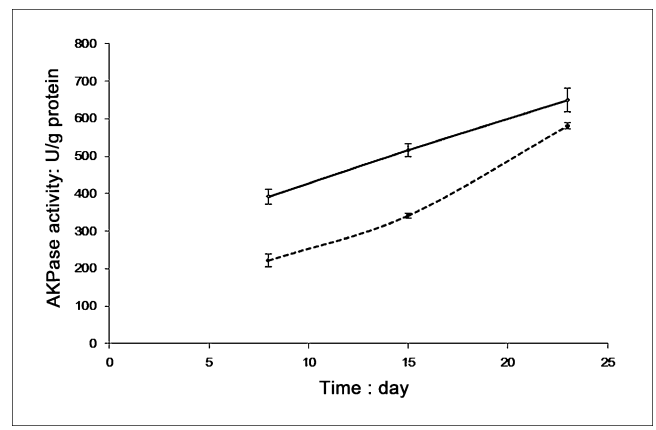

Fig. 4: AKPase of the developing Caco-2 monolayers at different time points **Notes:** The full line and the dotted line denote the tendency of activity changes of alkaline phosphatase of the developing Caco-2 monolayers cultured in DMEM and MEM, respectively. The bars indicate the SD of measurement (n = 3).

vs 1.13×10^{-5} (MEM group). The transport was monitored for a period of 90 min (Table).

Propranolol is usually used as the marker to indicate high permeability and transcellular transport, whereas atenolol is the marker for poor permeability and paracellular transport (Yang et al. 2007). Although the P_{app} values of propranolol in the MEM group were only slightly highes than those in the DMEM group, those of atenolol in MEM group were significantly more than in DMEM group (Fig. 5) ($P < 0.01$), demonstrating that more compounds permeate paracellularly across the monolayer in the MEM group than in the DMEM group. This suggested that the monolayer cultured in DMEM was tighter than that cultured in

Table: P_{app} Values of propranolol and atenolol in the cell monolayer model

Compounds	$P_{app}1$ (DMEM)	$P_{app}2$
Propranolol/ $\times 10^{-5}$ cm/s	2.53 ± 0.02 (U/g prot)	2.85 ± 0.04
Atenolol/ $\times 10^{-6}$ cm/s	5.92 ± 0.24	11.34 ± 1.91

$P_{app}1$ and $P_{app}2$ were the P_{app} values of the Caco-2 cell monolayers cultured in DMEM and MEM, respectively. All data were expressed as mean ± S.D ($n = 3$) and the incubation time was up to 90 min.

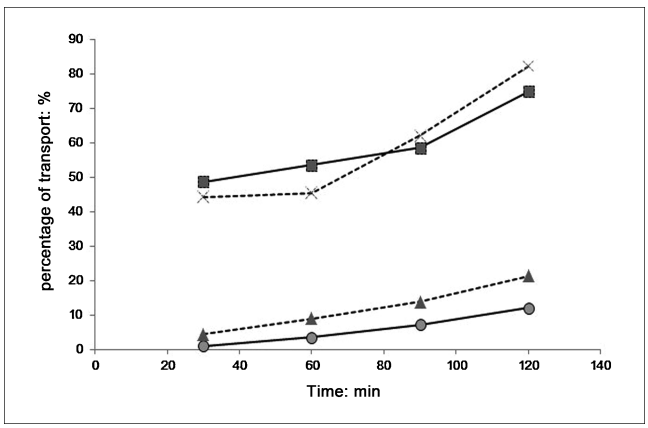

Fig. 5: Percentage of propranolol and atenolol transported across Caco-2 cell monolayers as a function of time **Notes:** "—■—", "- -✕- -" indicate the percentage of propranolol transported from apical to basolateral across monolayers cultured in DMEM and MEM, respectively. "—●—", "-▲-" indicate the percentage of atenolol transported from apical to basolateral across monolayers cultured in DMEM and MEM, respectively.

MEM, which was coherent to the results of determination of TEER and AKPase activity.

These two media differ in composition, i.e., DMEM contains more nutrient substances such as amino acids and carbohydrates than MEM, though the concentration of L-Arginine hydrochloride in DMEM is lower than that in MEM. The most obvious difference between DMEM and MEM is the glucose level (25 mM glucose in DMEM *vs* 0.56 mM glucose in MEM). Glucose is the simplest and most essential energy source for cell cultures, including Caco-2 cells. Previous research showed that Caco-2 cells grow properly in media containing glucose ranging from 1 mM to 25 mM (Rodolosse et al. 1996). In the present study, the two media were replaced every other day at most and morphologic observation showed that cells were in good condition, thus, these two media were able to meet the growth requirements of cells. Nevertheless, the qualified cell monolayer requires not only cell proliferation but also cell differentiation to form intercellular tight junctions. According to the literature, cells maintain their viability when they were fed with media containing glucose levels higher than 18.75 mM and high glucose concentration altered cell permeability through altering the tight junction and membrane fluidity (D'Souza et al. 2003). As a result, it could be inferred that a high concentration of glucose profits the formation of tight junctions and maintenance of the stability of the model.

In summary, the Caco-2 monolayers cultured in both DMEM and MEM had no significant morphological difference; however, these two monolayers were different in cell differentiation and tightness of cell junction. The monolayer cultured in DMEM was more stable, more polarized and tighter than that cultured in MEM. Based on the results of this study, the former is more reliable and suitable than the latter for absorption study of drugs *in vitro*.

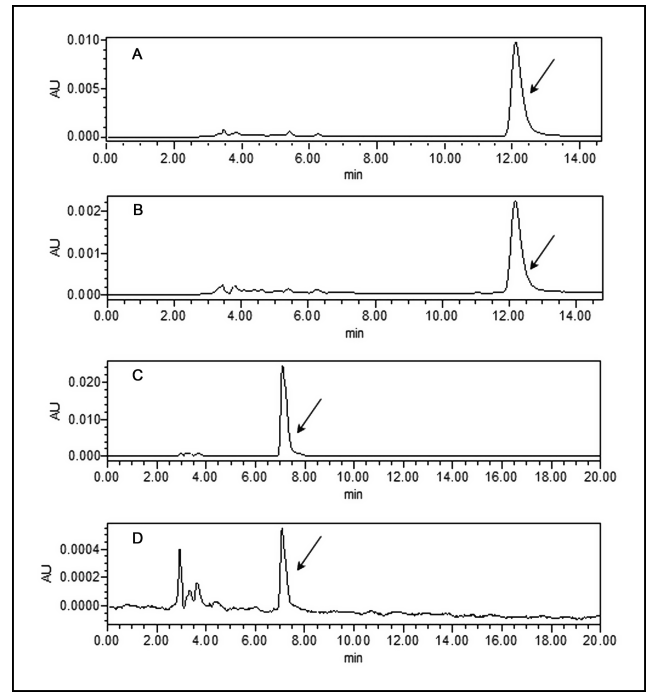

Fig. 6: HPLC chromatograms of reference solution (A) and sample solution (B) of propranolol, and reference solution (C) and sample solution (D) of atenolol **Notes:** The target chromatographic peaks are marked with arrows.

3. Experimental

3.1. Materials

3.1.1. Cell line

The human colon adenocarcinoma cell line Caco-2 (No. 3111C0001CCC000100) was purchased from the Cell Resource Center, Peking Union Medical College (CRC/PUMC, China). Caco-2 cells used in this study were between passages 40 and 48.

3.1.2. Reagents and chemicals

DMEM (No. CCCM005) containing 4500 mg/L D-glucose, 584 mg/L L-glutamine and 3700 mg/L $NaHCO_3$, MEM Eagles with Earle's Balanced Salts (MEM-EBSS, CCCM015) containing 100 mg/L glucose, 292 mg/L L-glutamine, 2200 mg/L $NaHCO_3$, and penicillin-streptomycin solution were obtained from the Cell Resource Center, Peking Union Medical College (CRC/PUMC, China). Fetal bovine serum (FBS) and trypsin were supplied by GiBco (California, USA). Nonessential amino acids (NEAA) were purchased from Hyclone (Utah, USA). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), ethylene diaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), and propranolol and atenolol with purity of minimum 98% were products of Sigma Chemical Co. (Deisenhofen, Germany). AKPase kit (JC-A0059) was provided by Nanjing Jiancheng Bioengineering Research Institute (Nanjing, Jiangsu, China). Other chemicals were of analytical grade and solvents used in HPLC were of HPLC grade. Transwell™ plates of 12-well (insert diameter 12 mm, pore size 3.0 μm, insert membrane growth area 1.12 cm^2) were purchased from Corning Costar (Cambridge, MA, USA).

3.1.3. Apparatus

Morphologic observation of Caco-2 cells was conducted under a Nikon TE2000S Inverted Phase Contrast Microscope (Nikon Corp., Tokyo, Japan) and a Hitachi 7100 Transmission Electron Microscope (Hitachi Ltd., Tokyo, Japan). The TEER values of Caco-2 cell monolayer were measured by a Millicell®-ERS system (Millipore Corp., Bedford, USA). The SpectraMax 190 Spectrophotometer (MDS Inc., California, USA) was used for reading the absorbance of microplates in determination of the activity of AKPase. HPLC analysis was performed on a Waters 1500 series equipped with a 1525 Binary HPLC pump, an on-line degasser, a manual injector holding a 20 μl loop and a 2489 UV/Visible detector. The signals were acquired and processed using Windows XP-based Waters Breeze 2 software. An Agilent TC-C_{18} Polar-RP 80A column (250 mm × 4.60 mm i.d., 5 μm particle size) with a guard column was applied to the analysis of the compounds. Other equipment such as JB-CJ-1500 super clean bench (Beijing Changping Great Wall Air Purification Company, Beijing, China), MCO-18AIC CO_2

incubator (SANYO Inc., Osaka, Japan), HYG-B thermostatic oscillator (Beijing Jiayuan Xingye Technology Ltd., Beijing, China) and TGL-16C high speed tabletop centrifuge (Shanghai Anting Scientific Instruments Plant, Shanghai, China) was also used in this study.

3.1.4. Preparation of solutions

3.1.4.1. DMEM

DMEM was supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The culture medium was stored at 4 °C.

3.1.4.2. MEM

MEM was supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The culture medium was stored at 4 °C.

3.1.4.3. Phosphate Buffer Solution (PBS)

8 g of NaCl, 0.2 g of KCl, 3.48 g of $Na_2HPO_4 \cdot 12H_2O$ and 0.2 g of KH_2PO_4 were added to 1000 ml of tri-distilled water and dissolved by magnetic stirring. The final pH value was adjusted to 7.2, and the solution was stored at 4 °C after high-pressure steam sterilization.

3.1.4.4. Hank's Balanced Salt Solution (HBSS)

8 g of NaCl, 0.4 g of KCl, 47.5 mg of Na_2HPO_4, 60 mg of KH_2PO_4 and 1 g of D-glucose were dissolved in 1000 ml of tri-distilled water and the final pH value was adjusted to 7.4. The solution was stored at 4 °C after membrane filter sterilization.

3.1.4.5. 0.25%Trypsin solution

2.5 g of grinded trypsin was dissolved in 1000 ml of PBS and the final pH value was adjusted to 8.0. The solution was stored at 4 °C after membrane filter sterilization.

3.1.4.6. Reference solutions

Stock solutions of propranolol and atenolol were prepared in DMSO and diluted with HBSS to the appropriate concentration (the percentage of DMSO was maintained at 0.5% in the transport experiment, which did not have any detectable effect on the cell monolayer) and their concentrations were 100 μM and 1 mM, respectively. Further dilution was carried out on the stock solution to afford a series of solutions for calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for each compound. Diluted concentrations were 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 (only for atenolol) μM. All stock and working solutions were stored at 4 °C until used.

3.2. Methods

3.2.1. Cell maintenance and differentiation

Caco-2 cells were respectively cultured in DMEM and MEM and were grown in a humidified atmosphere of 5% CO_2 in air at 37 °C. Stock cultures were grown in 25 cm^2 flasks and were split 1:2 or 1:3 at 70 to 80% confluence. The culture medium was replaced every other day. When the cells were in good condition and the flasks reached 80% confluence once again, they were washed three times with PBS and were removed from the flasks by incubating the monolayers with trypsin for 5–10 min at 37 °C. The cells were collected into centrifuge tubes, spun at 800 rpm for 5 min, and the obtained pellet was resuspended in culture medium. For differentiation, Caco-2 cells were seeded at a cell density of 1.25×10^5 cells/cm^2 on a 12-well Transwell insert filter to grow for 23 days. Apical and basal compartments of the inserts were full of 0.5 ml of cell suspension and 1.5 ml of culture medium, respectively. The inserts were fed with culture medium at 2 days intervals in the first week, and then at daily intervals until they were used for the transport experiment 23 days after seeding. The integrity of the cell monolayers was evaluated by measuring TEER values in culture medium at 37 °C using a Millicell®-ERS system. The cell inserts were used after the resistance reached not less than 400 Ω·cm^2.

3.2.2. Transport experiment

On day 23, a transport study was initiated by careful removal of the culture medium from apical and basal compartments. Caco-2 cells were rinsed twice with pre-warmed HBSS and were incubated by pre-warmed HBSS 0.5 ml in the apical compartment and 1.5 ml in the basal compartment at 37 °C for 30 min. Apical and basal solutions were carefully removed, and 0.5 ml of stock solution of test compounds (propranolol and atenolol) and 1.5 ml of HBSS were added to the apical and basal compartment, respectively. Each 0.5 ml of solution was sampled from the basal compartment after shaken (50 rpm, 37 °C) in a thermostatic oscillator for 30, 60, 90 and 120 min, then was frozen immediately and preserved below -20 °C before analysis, and the volume was made up with HBSS. Upon completion of all permeation experiments, TEER values were measured to ensure that cell monolayer integrity and viability had not been adversely affected by experimental conditions.

3.2.3. Determination of AKPase activity

In order to study the functional polarization of the Caco-2 cells, the presence of AKPase, a brush border enzyme marker, was followed (Hilgers et al. 1990). Monolayers grown on the Transwell filters were rinsed twice with PBS on days 8, 15 and 23 after seeding. Cells were detached from the membrane of the inserts by a cell scraper and put into 1.5 ml Eppendorf tubes with 1 ml of tri-distilled water. The optimum ultrasonic and repeated freezing and thawing were adopted simultaneously in the cell disruption. And then cell debris was removed by centrifugation and samples were obtained. The total intracellular protein was determined by the Lowry method, and an AKPase kit was used to determine AKPase activity (Yang et al. 2007). In accordance with the package insert of the kit, 30 μl of sample, each 50 μl of buffer solution and substrate solution were transferred to 96-well microplates in sequence and incubated for 15 min at 37 °C after thoroughly mixed. Finally, the reaction product was colored by development reagent and the absorbance of microplates was read at 520 nm by the SpectraMax 190 Spectrophotometer. Meanwhile, the same quantity of tri-distilled water and 0.024 mg/ml standard working solution were added to microplates, respectively, and other experimental steps were the same as above. The absorbance was converted to concentration after blank subtraction with reference to the standard working solution. Then, the concentration was corrected for the reaction volume and presented as a function of time. AKPase activity was calculated using the formula in the package insert and expressed as *U/g protein*. *U* was defined as that 1 mg of phenol was generated when 1 g of cellular protein reacted chemically with substrate for 15 min at 37 °C.

3.2.4. HPLC analysis

3.2.4.1. Chromatographic condition optimization

Different mobile phase compositions, volume ratios of solvents and column temperatures were investigated to optimize the chromatographic condition in order to establish a good resolution of adjacent peaks within a reasonable analysis time. At last, an Agilent TC-C_{18} Polar-RP 80A column (250 mm × 4.60 mm i.d., 5 μm particle size) with a guard column was applied to the experiments under the following conditions. Samples were filtered through a 0.45 μm filter and 10 μl of aliquots were used for assay by a reversed-phase HPLC system. The mobile phase consisted of methanol-0.02 M KH_2PO_4 in H_2O (50:50, v/v) at a flow rate of 0.90 ml/min for propranolol, and acetonitrile-0.05 M KH_2PO_4 in H_2O (10:90, v/v) at a flow rate of 0.80 ml/min for atenolol. UV detector was set at 290 nm for propranolol and 277 nm for atenolol and temperature was 30 °C for both analytes. Quantification was carried out by peak area measurements in comparison with standard curves for each analyte.

3.2.4.2. Validation of the HPLC method

3.2.4.2.1. Linearity, limits of detection and quantification and system suitability

The calibration equations of propranolol and atenolol were obtained by plotting HPLC peak areas (Y) *versus* the concentrations (X, in μM). The results were as follows: for propranolol, $Y = 1756.5\ X - 2721.7$ ($R^2 = 0.9972$), with a good linearity over the range from 2 μM to 50 μM; for atenolol, $Y = 474.64\ X - 277.9$ ($R^2 = 0.9996$), with a good linearity over the range from 2 μM to 100 μM. On the basis of signal-to-noise ratio (S/N), the limits of detection (LOD) and quantification (LOQ) for propranolol and atenolol were determined as 1.73 ng and 1.78 ng (S/N = 3), and 5.20 ng and 5.33 ng (S/N = 10), respectively. The system suitability test was conducted using the reference solution. The tailing factors of propranolol and atenolol were determined as 1.326 and 1.289, and the theoretical plate numbers were determined as 16321 and 24071, respectively.

3.2.4.2.2. Precision and stability

Precisions were determined by investigating the two analytes in sextuplicates during a single day (injected volume 10 μl) as intra-day precision and duplicating the intra-day experiment on three consecutive days as inter-day precision. The RSD values for intra-day precision of propranolol and atenolol were 1.16 % and 0.75 % and those for inter-day precision were 3.80% and 1.76%, respectively. One sample solution was kept at room temperature for 24 h, and then the stability was determined by injecting it into the apparatus at 0, 1, 2, 4, 6, 12 and 24 h. The RSD values for stability of

propranolol and atenolol were 3.52% and 1.52%, respectively. This showed that the samples were stable within 24 h, and the methods used were suitable.

3.2.5. Data analysis

The *TEER* values were calculated according to Eq. (1), wherein *TEER* (Ω) is the electrical resistance across Caco-2 monolayers and $TEER_{\text{background}}$ (Ω) is the electrical resistance across the insert only (without cells). The *area* (cm^2) is the area of the insert, 1.12 cm^2. All experiments were conducted in decuplicate and the data were expressed as mean ± S.D.

$$TEER\,(\Omega\,\text{cm}^2) = (TEER\,(\Omega) - Teer_{\text{background}}(\Omega)) \times area\,(\text{cm}^2) \quad (1)$$

The calculation of *AKPase activity* is described by Eq. (2), wherein A is the absorbance of tested samples, A_0 is the absorbance of blank samples, A_s is the absorbance of standard working solution, C_s is the concentration of standard working solution (0.024 mg/ml), $C_{protein}$ is the total protein concentration of tested samples (*g protein/ml*). All experiments were conducted in triplicate and the data were expressed as mean ± S.D.

$$AKPase\,activity\,(U/g\,protein) = (A - A_0)/(A_S - A_0) \times C_S/C_{protein} \quad (2)$$

P_{app} values were determined in this study by HPLC quantification of the concentration of the compound in the receiver compartment after transport across the Caco-2 monolayer. The calculation is described by the Eq. (3), wherein P_{app} is the permeability coefficient (cm/s); *dQ/dt* is the rate of appearance of the test compound on the receiver compartment (μmol/s); C_0 is the initial test compound concentration on the donor compartment (μmol/ml); and A is the surface area of Caco-2 monolayer (cm^2). All experiments were conducted in triplicate and the data were expressed as mean ± S.D.

$$P_{\text{app}} = (dQ/dt) \times (1/A) \times (1/C_0) \quad (3)$$

The percentage transported was calculated by Eq. (4), wherein the amount of compounds (in μmol) in the donor compartment is denoted by the product of C_S and V_{D}. Q_B is the amount of compounds in the receiver compartment.

$$\%\text{Transported} = Q_B/(C_S \times V_D) \times 100 \quad (4)$$

Data are given as means ± S.D. The statistical difference between these two Caco-2 monolayer models cultured in DMEM and MEM was investigated by analysis of variance, followed by an independent-samples T test to compare different evaluation parameters using the SPSS16.0 software. Statistical significance was set at the 0.05 level.

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References

Aiba T, Ishida K, Yoshinaga M, Okuno M, Hashimoto Y (2005) Pharmacokinetic characterization of transcellular transport and drug interaction of digoxin in Caco-2 cell monolayers. Biol Pharm Bull 28: 114–119.

Bailey CA, Bryla P, Malick AW (1996) The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. Adv Drug Deliv Rev 22: 85–103.

DeMarco VG, Li N, Thomas J, West CM, Neu J (2003) Glutamine and barrier function in cultured Caco-2 epithelial cell monolayers. J Nutr 133: 2176–2179.

De Walle JV, Sergent T, Piront N, Toussaint O, Schneider YJ, Larondelle Y (2010) Deoxynivalenol affects *in vitro* intestinal epithelial cell barrier integrity through inhibition of protein synthesis. Toxicol Appl Pharmacol 245: 291–298.

D'Souza VM, Shertzer HG, Menon AG, Pauletti GM (2003) High glucose concentration in isotonic media alters Caco-2 cell permeability. AAPS J 5: 17–25.

Ferruzza S, Rossi C, Scarino ML, Sambuy Y (2012a) A protocol for in situ enzyme assays to assess the differentiation of human intestinal Caco-2 cells. Toxicol *In Vitro* 26: 1247–1251.

Ferruzza S, Rossi C, Scarino ML, Sambuy Y (2012b) A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. Toxicol *In Vitro* 26: 1252–1255.

Hilgers AR, Conradi RA, Burton PS (1990) Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm Res 7: 902–910.

Ji HY, Shim HJ, Yoo M, Park ES, Lee HS (2007) Transport of a new erectogenic undenafil in Caco-2 cells. Arch Pharm Res 30: 1168–1173.

Markowska M, Oberle R, Juzwin S, Hsu CP, Gryszkiewicz M, Streeter AJ (2001) Optimizing Caco-2 cell monolayers to increase throughput in drug intestinal absorption analysis. J Pharmacol Toxicol Methods 46: 51–55.

Ma Y, Li PB, Su WW, Chen DW (2006) Evaluation indexes of Caco-2 cell model in pharmacology experiment. Zhong Yao Cai 29: 946–948.

Narai A, Arai S, Shimizu M (1997) Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayers models by cytotoxic membrane perturbents. Toxicol *In Vitro* 11: 347–354.

Pinton P, Nougayrède JP, Del Rio JC, Moreno C, Marin DE, Ferrier L, Bracarense AP, Kolf-Clauw M, P.Oswald I (2009) The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. Toxicol Appl Pharmacol 237: 41–48.

Rodolosse A, Chantret I, Lacasa M, Chevalier G, Zweibaum A, Swallow D and Rousset M (1996) A limited upstream region of the human sucrase-isomaltase gene confers glucose-regulated expression on a heterologous gene. Biochem J 315: 301–306.

Sambuy Y, Angelis ID, Ranaldi G, Scarino ML, Stammati A, Zucco F (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 21: 1–26.

Turco L, Catone T, Caloni F, Consiglio ED, Testai E, Stammati A (2011) Caco-2/TC7 cell line characterization for intestinal absorption: How reliable is this *in vitro* model for the prediction of the oral dose fraction absorbed in human? Toxicol *In Vitro* 25: 13–20.

Yamashita S, Tanaka Y, Endoh Y, Taki Y, Sakane T, Nadai T, Sezaki H (1997) Analysis of drug permeation across Caco-2 monolayer: Implication for predicting *in vivo* drug absorption. Pharm Res 14: 486–491.

Yang XW, Yang XD, Wang Y, Ma L, Zhang Y, Yang XG, Wang K (2007) Establishment of Caco-2 cell monolayer model and standard operation procedure for assessing intestinal absorption of chemical components of traditional Chinese medicine. Chin J Integr Med 5: 634–641.