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Transport evaluation of alendronate across Caco-2 cell monolayers

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The transport of alendronate through Caco-2 monolayers in the absence and presence of absorption enhancers (sodium taurocholate-STC and dimethyl-β-cyclodextrin-DM-β-CD) was studied. The viability of Caco-2 cells was determined by MTT assay. The effects of the experiment period and serum existence in Dubelco's Modified Eagle's Medium (DMEM) on cell viability were examined. The least toxic concentrations of alendronate, STC and DM-β-CD were found as 0.2% (w/v), 5 mM and 0.3% (w/v), respectively. Transport experiments were performed with these concentrations in DMEM supplemented with serum for an 8 h period. DM-β-CD increased the transport of alendronate through Caco-2 monolayers significantly. No significance was observed with STC. Cell integrity was determined by measuring the electrical resistance values at the end of the transport experiments and found to be decreased to a greater extent with DM-β-CD. These results indicate that DM-β-CD is a promising agent for improving the transport of alendronate.

1. Introduction

Bisphosphonates are pyrophosphate analogues in which the oxygen bridge has been replaced by a carbon with various side chains (Francis 1997). These compounds have been known by chemists since the 19th century (Menschutkin 1865).

Alendronate, like other bisphosphonates, is a bone resorption inhibitor and is used in the treatment of postmenopausal osteoporosis, osteoporosis in men, glucocorticoid-induced osteoporosis and treatment of Paget's disease (Bone et al. 2000). Alendronate is bound to bone because of its high affinity for calcium ions (Sharpe et al. 2001) and is bound where bone turnover takes place (Sato et al. 1991).

The bioavailability of alendronate is very low, less than 1% (Gertz et al. 1995). Oral bioavailability is 0.64% in women when 5–70 mg is taken after all night fast and two hours before breakfast. Bioavailability of 10 mg alendronate in men under the same conditions is 0.59% (Physicians' Desk Reference 2005). Its bioavailability is diminished when the drug is taken with food, divalent cations like calcium, beverages other than water, and with breakfast or within 2 h after breakfast. Its bioavailability is increased with increasing gastric pH (Gertz et al. 1995). The fasted oral bioavailability of alendronate has been reported as 0.9%, 1.8% and 1.7% in rats, dogs and monkeys, respectively (Lin et al. 1991).

Alendronate is absorbed paracellularly in the gastrointestinal tract. It is absorbed better in the gastrointestinal sites with wide surface areas (jejunum > duodenum > ileum) (Lin et al. 1994).

Cell culture models have many advantages when compared with classical drug absorption models (Audus et al. 1990); rapid estimation of the permeation or metabolism of drugs, opportunity for studying a drug absorption mechanism under controlled conditions, rapid estimation of increasing drug absorption methods by using prodrug, absorption enhancer or other pharmaceutical materials, opportunity to explain the drugs which partition into the lymphatic system, opportunity to study in human cells, opportunity to decrease time limitation, cost and sometimes animal studies, and rapid estimation of drug-targeting strategies.

The effects of different absorption enhancers on Caco-2 cells (Sakai et al. 1998) and their effects on drug transport (Meaney and O'Driscoll 2000; Udata et al. 2003) have been investigated in different studies. Cyclodextrins increase the absorption of drugs that are poorly soluble in water by affecting the membrane structure and by increasing the solubility rate (Gerloczy et al. 1994). The most commons are dimethyl and hydroxypropyl-β-cyclodextrin (Rajewski and Stella 1996). Bile salts can increase the absorption by changing the barrier properties of the cell membrane (O'Reilly et al. 1994) or mucus layer (Poelma et al. 1990) or paracellular route (Lane et al. 1996).

This study was carried out in order to evaluate the transport of alendronate in the absence and presence of absorption enhancers by using Caco-2 monolayers. For this purpose, two different absorption enhancers (sodium taurocholate – STC and dimethyl-β-cyclodextrin – DM-β-CD) were used. The effects of alendronate and absorption enhancers on the viability and integrity of Caco-2 mono-

layers were investigated by using MTT test and by measuring the electrical resistance values, respectively.

2. Investigations and results

According to MTT tests performed for different concentrations of alendronate, viability of Caco-2 cells decreased with increasing concentrations up to 1.2% for an 8 h period and for DMEM supplemented with and without serum. Over a 24 h period, cell viability decreased up to the concentration of 0.8%. Above these concentrations, viability increases were observed (Fig. 1a).

DM-β-CD caused a decrease in cell viability up to the concentration of 0.5% for 8 and 24 h periods and also with DMEM supplemented with and without serum. Above this concentration, viability was approximately constant for all groups (Fig. 1b).

When we investigated the effects of different concentrations of STC, we observed that during the 8 h experimental period for both serum-free and serum-supplemented DMEM, cell viability decreased up to 2.5 mM after which a slight increase was observed until 5 mM. For the 24 h period with serum-free and serum-supplemented DMEM, a decrease up to 1.25 mM and later an increase up to 5 mM were observed. Percentage viability decreased rapidly between the concentrations of 5–10 mM in all groups, then a slight decrease was observed in an 8 h period in DMEM supplemented with and without serum and in a 24 h period including serum. Percentage viability was observed to be increased between the concentrations of 10–20 mM over a 24 h period with DMEM without serum (Fig. 1c).

Based on the results obtained from MTT assay tests, it was decided to use the concentrations of 0.2% (w/v), 0.3% (w/v) and 5 mM for alendronate, DM-β-CD and STC, respectively, in the transport experiments.

The cumulative amounts of alendronate transported through the membrane without Caco-2 cells in the absence and presence of absorption enhancers during the 8 h experiment period were quite similar ($p > 0.05$) and found as 2.3%, 2.2%, 2.4% with alendronate, alendronate and DM-β-CD, and alendronate and STC, respectively (Fig. 2).

The cumulative amount of alendronate transported through Caco-2 monolayers after 8 h was (0.6 ± 0.3) % and was significantly different from the ones obtained with blank membranes ($p < 0.01$) (Fig. 2).

The cumulative amounts of alendronate transported through Caco-2 monolayers at the end of 8 h were (2.0 ± 0.2) % and (1.7 ± 0.6) % with alendronate – DM-β-CD and alendronate – STC, respectively. A significant increase in alendronate transport through Caco-2 cells was obtained in the presence of DM-β-CD ($p < 0.01$), but there was not a significant increase with STC ($p > 0.05$) (Fig. 2).

As a marker of the integrity of Caco-2 cells, electrical resistance of the monolayers was measured at the end of the experiment periods. Low electrical resistance indicated the destroyed monolayer. When compared with the results obtained with alendronate, STC caused a slight decrease while DM-β-CD had a strong effect on the electrical resistance values.

We calculated the flux and permeability coefficients (log k) of alendronate in the absence and presence of absorption enhancers. Flux of alendronate increased 1.5- and 3.3-fold and permeability coefficients (log k) increased

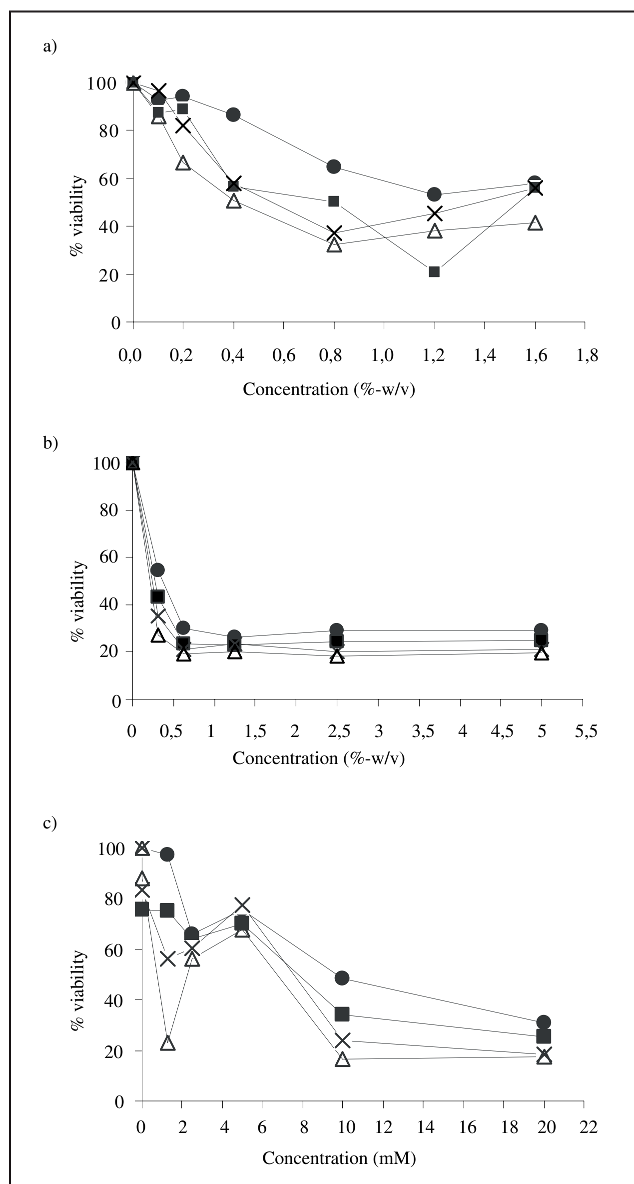

Fig. 1: The effects of different concentrations of (a) alendronate, (b) DM-β-CD, (c) STC on the viability of Caco-2 cells (●; DMEM supplemented with serum and 8 h period, ■; DMEM without serum and 8 h period, ×; DMEM supplemented with serum and 24 h period, □; DMEM without serum and 24 h period)

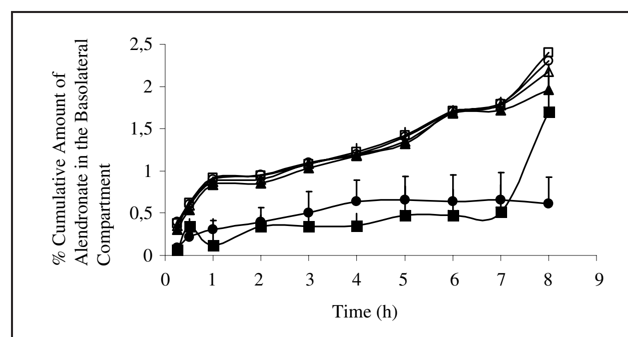

Fig. 2: % Cumulative amount of alendronate in the basolateral compartment resulting from its transport through membrane with and without Caco-2 cells in the absence or presence of absorption enhancers [○; Blank membrane (alendronate), ●; Caco-2 (alendronate), △; Blank membrane (alendronate and DM-β-CD), ▲; Caco-2 (alendronate and DM-β-CD), □; Blank membrane (alendronate and STC), ■; Caco-2 (alendronate and STC)] (Experiments were performed triplicate, n = 3)

1.1- and 1.2-fold with STC and DM-β-CD, respectively. It is obvious that DM-β-CD increased to a greater extent both the flux and permeability coefficient of alendronate when compared with STC.

3. Discussion

Performing cytotoxicity tests with cell culture models before starting the transport experiments guides cell culture studies. The least toxic concentrations of drugs or absorption enhancers to be used and the transport period can be decided utilizing these tests. There are many ways to determine cytotoxicity (Abid-Essefi et al. 2003; Boulenc et al. 1995 a, b; Foss and Peppas 2004; Meaney and O'Driscoll 2000; Palamakula and Khan 2004; Raiman et al. 2003; Silano et al. 2004; Zhou et al. 2005). The MTT test is the most commonly used method.
MTT tests for different bisphosphonates have been performed (Boulenc et al. 1995 b; Raiman et al. 2001) but there has been no study with alendronate. Different time periods have been used for the transport of different bisphosphonates. In the studies carried out by Raiman et al. (2001, 2003) and Boulenc et al. (1995 a, b) 3 h transport period was constructed after performing 3 h MTT test and 2 h transport period was constructed after performing 1 h MTT test, respectively. There have also been studies constructed with longer transport periods (Degim et al. 2004; Greenwood and Al-Achi 1997). To understand the effects of exposure time of alendronate and absorption enhancers on the viability of cells, we performed MTT tests for 8 and 24 h separately. According to the results, we decided to construct our transport experiments over an 8 h period.
We used DMEM supplemented with and without serum in all groups in an effort to understand if serum had any effect on cell viability. The effects of serum in cell culture studies are listed in the literature (Keenan et al. 1998). The viability results obtained with DMEM supplemented with serum were better when compared with those obtained with DMEM alone. As can be ascertained from the results, serum presence in DMEM decreased the toxicity of alendronate, DM-β-CD and STC on Caco-2 cells, thus all transport experiments were performed in serum-supplemented DMEM.
According to MTT results constructed for alendronate, we observed that the concentrations lower than 0.2% were less toxic for Caco-2 cells. As it was thought that 0.1% alendronate would not be sufficient for determining transport, we decided to use a concentration of 0.2%, which maintained the cell viability as high as 0.1%. The viability of Caco-2 cells in the presence of 0.2% alendronate was 94.2%.
One of the strategies for improving the absorption of drugs is use of absorption enhancers (Raiman et al. 2003). The absorption enhancement effects of ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) on the transport of tiludronate (bisphosphonate) (Boulenc et al. 1995 b) and the effects of palmitoyl carnitine chloride, *N*-trimethyl chitosan chloride, sodium caprate and EGTA on transport of clodronate (bisphosphonate) (Raiman et al. 2003) through Caco-2 cell monolayers have been investigated. However, there has been no study on the effects of DM-β-CD and STC on transport of bisphosphonates through Caco-2 cells. For this reason, the concentrations of DM-β-CD and STC as used in different studies were established (Anderberg et al. 1992; Udata et al. 2003). Based on these data, concentrations of DM-β-CD and STC similar to those used in different studies were prepared and their effects on cell viability were investigated. According to MTT results obtained for DM-β-CD, we decided to use the concentration of 0.3%, which affects cell viability the least. Cell viability was found to be 54.6% with this concentration.
Electrone microscope studies indicated that exposure of Caco-2 cells to 20 mM or more STC for 1 h resulted in only a slight widening of tight junctions. MTT results obtained from this study had shown that a STC concentration of 10 mM caused a 50% decrease in enzyme activity on Caco-2 cells with a 10 min incubation time (Meaney and O'Driscoll 2000). Caco-2 cell viability decreased 50% with incubation of 10 mM STC for 10 min and of 9.7 mM for 24 h (Anderberg et al. 1992). In light of these data, we decided to use 5 mM STC in order to observe the transport increase. Cell viability was 75.6 % with 5 mM STC which was considered high.
We sometimes observed increases in viability during the experiments which could be attributed to the increase in tetrazolium salt transported inside the cells (Boulenc et al. 1995 a; Raiman et al. 2003).
Transepithelial electrical resistance (TEER) results can be used as an indication of cell integrity (Boulenc et al. 1995 a, b). A monolayer with low TEER was assumed to exhibit extensive leakage through imperfect occluding junctions or holes in the monolayer (Boulenc et al. 1995 b). Johansson et al. (2002) observed that TEER of Caco-2 cells decreased and insulin permeability increased with 20–25 mM STC. They also concluded that concentrations above 30 mM caused a decrease in cell viability and an increase in the permeability.
Udata et al. (2003) studied the effects of absorption enhancers on the permeability of cosalene through Caco-2 cells and 5% DM-β-CD was determined to cause an increase in its transcellular absorption. In this study, they also used STC and concluded that concentrations of more than 20 mM affected the cellular integrity of Caco-2 cells.
We used STC as an absorption enhancer and found no significant effect on alendronate transport through Caco-2 cells. However, this effect could be attributed to the concentration selected based on the results of the MTT test, since in the literature STC concentrations are generally more than 10 mM (Johansson et al. 2002; Udata et al. 2003). According to our MTT results, concentrations of 10 mM and above STC decreased cell viability, so 5 mM concentration was considered more suitable for use in the transport studies. After the seventh hour of the transport experiment, a considerable increase in alendronate transport was observed. If the experiment was extended, there may have been a significant effect on the transport of alendronate. However, we thought that this sharp increase could be the result of cell death and thus decided not to continue the experiment.
The properties of Caco-2 cells can be affected by the passage number and culture medium in and between laboratories. This may account in part for the differences observed between our results and those obtained by other investigators.
We determined that DM-β-CD enhanced the transport of alendronate through Caco-2 cells. The electrical resistance results and permeability coefficient values were also in accordance with the transport experiments. It was observed that the effects of DM-β-CD on Caco-2 cell integrity were more effectual. log k of alendronate increased more with DM-β-CD.
Caco-2 cells cover the membrane as a monolayer without any holes and serve as a barrier for alendronate. The re-

sults obtained with the blank membrane were thus higher compared with those obtained with Caco-2 cells.
In conclusion, transport experiments performed through Caco-2 cells allowed us to demonstrate the effects of two different absorption enhancers on the transport of alendronate. Using DM-β-CD as an absorption enhancer can be a strategy to increase the transport of alendronate. STC can be a potential absorption enhancer for many drugs but we could not find a significant effect on alendronate. For enhancing absorption with STC, high concentrations are needed, but cell viability and integrity should be taken into account in deciding the optimum concentration.
Current results indicate that cell culture systems can be an alternative model for *in vivo* studies. Nevertheless, these results should be supported by animal experiments.

4. Experimental

4.1. Materials

Alendronate sodium trihydrate was kindly provided from Sanovel Pharmaceutical Company (Turkey). DM-β-CD and STC were obtained from Cyclo Laboratory (Hungary) and Sigma (Germany), respectively. Dubelco's Modified Eagle's Medium (DMEM), medium components and other reagents for cell culture were obtained from Gibco Life Technologies (England). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma (Germany).
All other reagents and solvents were of analytical grade except for those used in the procedure which were of HPLC grade.

4.2. Cell culture

Caco-2 cells orginating from a human colorectal carcinoma (Fogh et al. 1977) were obtained from American Type Culture Collection (ATCC) and grown by the Food and Mouth Diseases Institute (Turkey). Cells which were kept at −196 °C were dissolved in water bath at 37 °C then centrifuged at 800 rpm for 5 min. Precipitant was homogenized with DMEM supplemented with 10% heat-inactivated fetal calf serum, 1% sodiumbicarbonate, and 1% gentamicin and then grown in 25 cm^2 cell culture flasks (Costar-Germany). Flasks were kept at 37 °C and the medium was changed every 48 h until the flasks reached 100% confluence. Cells were passaged and the volume was enlarged to 75 cm^2. Cells were detached from the flasks by incubating the monolayers with trypsin EDTA (Fig. 3). The cells were collected, counted with a hemocytometer and seeded at a density of 80 000 cells/mL on the transwell polycarbonate cell culture inserts (12 mm diameter, 0.4 μm pore size, Costar-Germany) for transport experiments. A model of the cell culture insert which was used in this study is illustrated in Fig. 4. The medium was changed every 48 h for 21 days. The monolayers for the transport studies were used 21 days after seeding on the membranes (Karamustafa et al. 2006a, b) The integrity of the cells were controlled with an inverted microscope.

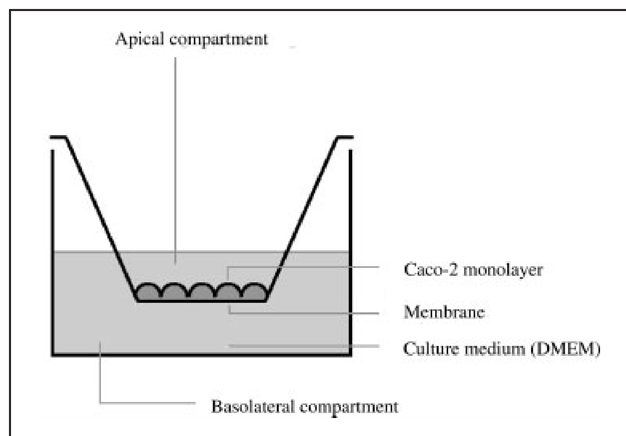

Fig. 4: A schematic representation of Caco-2 cells which were grown on on the transwell polycarbonate cell culture inserts as a monolayer

4.3. Cell viability

MTT assay is a colorimetric method for the determination of cell viability based upon reduction of the yellow tetrazolium salt MTT to a purple formazan dye by mitochondrial succinate dehydrogenase in viable cells (Mosmann 1983).
The effects of alendronate, DM-β-CD and STC on Caco-2 cell viability were studied by MTT method before starting the transport experiments. MTT tests were conducted by applying DMEM supplemented with serum and DMEM without serum, for 8 and 24 h periods in all groups. Caco-2 cells in DMEM (100 μL) were placed in the microwells of 96 well tissue culture plates. Plates were kept at 37 °C for 24 h then emptied. Concentrations ranging between 0.1% to 1.6% alendronate (w/v), 0.3% to 5% DM-β-CD (w/v) and 0.00625 to 20 mM STC in DMEM were placed and kept at 37 °C for 8 or 24 h. The incubation medium containing the drug or the absorption enhancers was removed. Monolayers were incubated for 3 h at 37 °C with 100 μL fresh medium and 13 μL MTT. Plates were emptied and 100 μL isopropanol was added to dissolve the formazan precipitate. The developed color was measured at a wavelength of 570 nm with spectrophotometer (Versamax Molecular Devices).

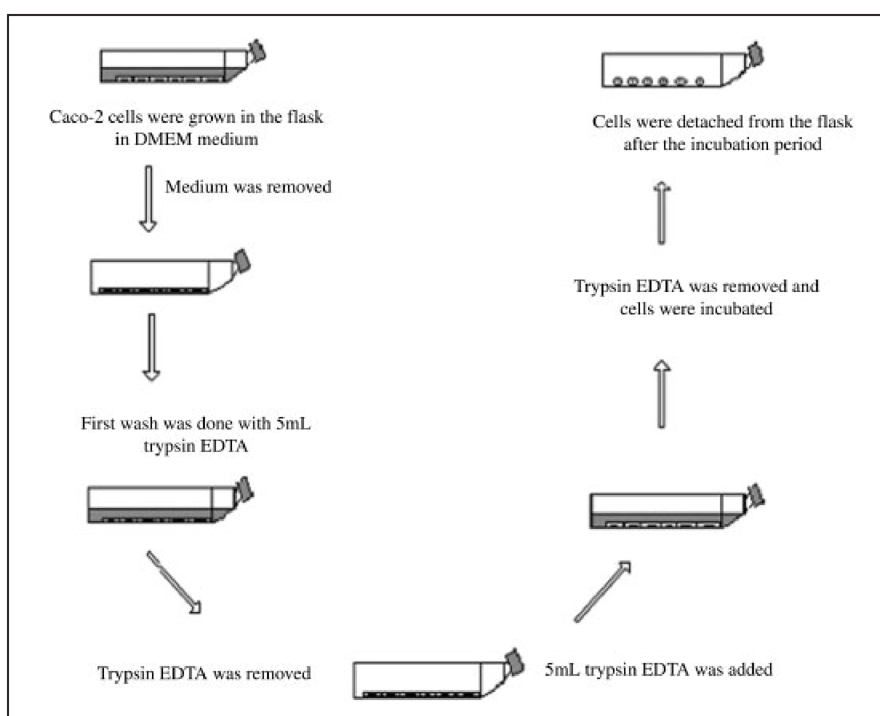

Fig. 3:
The schematic representation of detaching Caco-2 cells from the flasks

According to MTT results, the least toxic concentrations of alendronate and absorption enhancers on Caco-2 cells were determined and these were used for the transport experiments.

4.4. Measurement of drug transport

Based on MTT results, we observed that serum-free DMEM and 24 h transport period decreased cell viability, so it was decided to perform the transport experiments using DMEM supplemented with serum for an 8 h period. Alendronate, DM-β-CD and STC concentrations for performing transport experiments were decided as 0.2%, 0.3%, and 5 mM, respectively. Caco-2 cells were prepared by cultivating on polycarbonate membrane filters for 21 days (Fig. 4). The membrane with Caco-2 cells monolayer was placed between the donor and receptor compartments of the radial diffusion cells (Harvard Apparatus Company-USA). Transport experiments were performed from apical to basolateral direction at 37 °C. 95% O_2/5% CO_2 was provided continuously to both compartments for retaining the viability of Caco-2 cells. On the day of the experiment, the medium on the apical compartment was replaced with 4 mL DMEM containing 0.2% alendronate with and without absorption enhancers. The medium on the basolateral compartment was replaced with 4 mL prewarmed fresh medium. The samples (2 mL) were withdrawn over time over an 8 h period from the basolateral compartment. The volume removed from the basolateral compartment was replaced with fresh prewarmed DMEM. The same procedure was repeated using blank membranes (i.e., membranes with no layer of cells). The samples were analyzed for alendronate content by HPLC method.

HPLC analysis was performed on a Surveyor High Performance Liquid Chromatograph with autosampler and LC pump. The direct analysis of alendronate is complicated due to the lack of a suitable UV chromophore for conventional HPLC analysis. Derivatization with 9-fluorenylmethyl chloroformate (FMOC) ensures UV properties which enables assay by conventional HPLC (De Marco et al. 1989). We thus used FMOC for the analysis of alendronate. Alendronate was separated on a column (ODS-3, particle size 5 μm, 4.6 × 250 mm) from GL sciences. UV detection was monitored at 262 nm using PDA detector. Elution was carried out isocratically at 1 mL/min using the mixture of acetonitrile (25%), methanol (10%) and 0.05 M sodium citrate – 0.05 M potassium phosphate buffer (65%). The volume of injection was 50 μL and the temperature was set at 25 °C. HPLC method was validated with the constituents of linearity, accuracy, precision, selectivity-specificity, stability, limit of detection and limit of quantification (Karamustafa 2005).

4.5. Electrical resistance (cell integrity)

The integrity of the monolayers was determined by measuring the electrical resistance values at the end of each experiment. For this purpose, Bostek type instrument with two electrodes (Ag^+ and Ag^+Cl^-) was used and the results were expressed as Ω/cm^2.

4.6. Calculation of the flux and permeability coefficient values (log k)

Flux values were calculated by graphing time versus the values obtained by dividing the cumulative transported alendronate to the membrane area.

Permeability coefficients of alendronate (log k-cm/h) in the absence and presence of absorption enhancers were calculated according to the following equation (Boulenc et al. 1995a).

$$\log k = \frac{dQ}{dt} \times \left(\frac{1}{A \times C_0}\right) \qquad (1)$$

where dQ/dt is the permeability rate, A (cm^2) is the diffusion area of the membrane and C_0 (μg/mL, μg/cm^3) denotes the initial concentration in the donor compartment.

4.7. Statistical analysis

The one-way ANOVA test was used for analysis of the data.

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