ijmps
Vol 04 issue 02
Section: Healthcare
Category: Research
Received on: 02/01/13
Revised on: 04/02/13
Accepted on: 28/02/13

IN VITRO PENETRATION OF THE ANTIBIOTIC FOSFOMYCININ SWINE INTESTINAL CELLS (IPEC J2)

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ABSTRACT

Fosfomycin is an antibiotic widely used for the treatment and prevention of swine infections caused by resistant bacteria. The aim of this research was to study the penetration of fosfomycin in an *in vitro* model of intestinal cells (IPEC-J2 cells). Cells cultures were subjected to 580 µg/mL of calcium fosfomycin. Intracellular concentrations of the antibiotic were analyzed by HPLC MS/MS and they ranged from 23.48 to 45.81 µg/mL (T_{max}: 4 h). Fosfomycin concentrations exceeded the MIC_{90} for the most important pathogens in swine intestinal infections (*Escherichia coli*: 0.50 µg/mL, *Salmonella enterica subsp. Enterica:* 4 µg/mL). Therefore, it is apparent that fosfomycin is an alternative for the treatment of intestinal infections in pigs.

Keywords: Fosfomycin, *in vitro*, penetration, IPEC -2 cells, intestinal infections, pigs

INTRODUCTION

Swine industry provides one of the most important sources of animal protein, being pork industry the largest in production and consumption, followed by poultry meat and beef. In pig production, weaning is considered as a critical period for piglets. It is characterized by a decrease in food intake that leads to a status of under nutrition, affecting other aspects of animal physiology and metabolism (Dirkzwagera et al., 2005). During this period animals are more susceptible to infectious diseases (Nabuurs et al., 1993). The main sites of infection (biophase) of the pathogens responsible for these disorders are the interstitial and intracellular fluid. In this regard, it should be noted that most of enteric diseases are caused by intracellular organisms, such as *Salmonella sp.* (facultative intracellular) and *Lawsonia intracellularis* (obligate intracellular) (Pluske et al., 1996; Pedersen et al, 2008), although other microorganisms that are located at the extracellular level are also important, such as E. coli.

Different antibiotics have been used for decades to reduce pathogen infection in pigs (irrational use). For this reason, many bacteria have become resistant to the most frequently used antimicrobials (Dirkzwagera et al., 2005; Mathew et al., 1998; Rood et al., 1985). Among the most recently used antibiotics is fosfomycin (cis-1,2-epoxyphosphonic acid), an intensive production widely used antibiotic (Serrano, 2002) is a broad-spectrum drug, structurally unrelated to other classes of antimicrobial agents. It inhibits cell wall synthesis as it interferes with peptidoglycan production at an earlier stage than beta-lactams or glycopeptide antibiotics (Gobernado, 2003; Kahan et al., 1974; Lin, 1976; Popovic et al., 2009). It has a low molecular weight (138.059 Da) and its chemical structure is similar to that of

phosphoenol-pyruvate. When compared with other antibiotics, fosfomycin has a broader *in vitro* spectrum of action than penicillin and semi-synthetic cephalosporins (Mata et al., 1977), and cross-resistance has not been reported (Gobernado, 2003). The use of fosfomycin in animals and humans has been proposed because of its low toxicity and potential efficacy (Gallego et al., 1974), being also widely used in animal production due to its rapid effect, good tolerance and lack of side effects (Aramayona, 1997; Carramiñana, 2004).

The pharmacokinetics of fosfomycin has been described in humans (Gallego et al., 1971; Damaso et al. 1990; Falagas et al., 2008), rabbits (Fernandez Lastra et al. 1986, 1987), broilers (Aramayona et al., 1997, Soraci et al. 2011), cattle (Sumano et al., 2007), horses (Zozaya et al., 2008), dogs (Gutiérrez et al., 2008) and pigs (Soraci et al, 2011, Pérez et al., 2012). Its chemical structure supports different salts: sodium, calcium and tromethamine (Perez-Rodriguez and Chavez Hernandez Velasco, 1997; Serrano, 2002). This is due to its acidic nature that allows the rapid formation of salts. The fosfomycin-calcium salt formulation is used orally, whereas the more water-soluble disodium salt can be used intravenously. Fosfomycin-tromethamine salt is highly hydro-soluble and offers a good oral bioavailability in humans (Borsa et al., 1988; Patel et al., 1997; Popovic et al., 2009). Perez et al. (2011, 2012) have established a fosfomycin withdrawal period of 3 days for broiler chicken muscle, liver and kidney and of 3 days after oral administration and 2 days after intramuscular administration in pig tissues.

It has been demonstrated that, besides being a bacterial inhibitor, fosfomycin has other properties such as, inhibition of bacterial adhesion to epithelial cells, penetration of wells in biofilms of exo-polysaccharide and protection against nephrotoxicity caused by drugs such as, cisplatin, cyclosporine, aminoglycosides, vancomycin, teicoplanin, amphotericin B and polymyxin (Gobernado, 2003). Martínez et al. (2011) demonstrated fosfomycin has a protective effect on HEp-2 cells when they are incubated with the mycotoxin deoxinivalenol. Morikawa et al. (1993) and Honda et al. (1998) have shown that fosfomycin has immunomodulatory effect on lymphocytes. Similarly, Krause et al. (2001) studied the effect of fosfomycin on neutrophil function and showed that destruction of microorganisms is increased when incubated with this antibiotic.

Fosfomycin exhibits a time dependent killing, so it kills bacteria when its concentrations remain constantly above the Minimum Inhibitory Concentration (MIC) (Aliabadi and Lees, 1997; Toutain et al., 2002). In this regard, for an antibiotic to be effective against relevant pathogens, it is essential to reach concentrations higher than the minimum inhibitory concentration (MIC) at the site of action (Nix et al., 1991; Schentag and Ballow, 1991; Toutain et al., 2002). Fosfomycin is indicated for the treatment of a variety of porcine bacterial pathogens (*Haemophilus parasuis*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Staphylococcus hyicus*, *Escherichia coli, Salmonella enterica*) associated with stress and/or several viral diseases (Martineau, 1997) and it MIC_{90} for most of the important pathogens in swine production has been established in 0.25-4 µg/mL (Fernández et al., 1995, Sumano et al., 2007, Ibar et al., 2009). In this regard, Pérez et al. (2012), have demonstrated that fosfomycin is an alternative for the treatment of intracellular respiratory infections in pigs, because it penetrates in HEP-2 cells and reaches concentrations that exceed the MIC_{90} for the most important pathogens in swine respiratory infections.

Although fosfomycin is widely used in clinical practice in the pork industry, its use in pigs is given on the basis of its potential clinical efficacy. However, there are no studies showing that this antibiotic reaches adequate concentrations in the biophase where the most important pathogens

operate. It was hypothesized that the antibiotic reaches concentrations above the MIC_{90} for most of the enteric microorganisms which are relevant in swine production systems. According to this background, the aim of this work was to determine fosfomycin concentration in the intracellular fluid of IPEC-J2 cells, as a model of fosfomycin penetration into intestinal cells and to determine the relationship between intracellular and serum fosfomycin concentrations in weaning piglets.

MATERIALS AND METHODS

This work was performed at the Laboratory of Toxicology of the Faculty of Veterinary Sciences, Tandil Veterinary Research Center, UNICEN, Tandil, Buenos Aires, Argentina.

Antibiotic

Calcium fosfomycin (98.9% of purity) was from Bedson Laboratory, Pilar, Buenos Aires, Argentina.

We use a dose of 580 µg/mL, which was estimated considering a therapeutic dose of 30 mg/kg body weight (b.w.) of calcium fosfomycin for pigs, an average post-weaning piglet b.w. of 15 kg, the intestinal volume where the antibiotic is dissolved (622 mL) (Ruckebusch, 1981) and a bioavailability (F%) of 20 (Pérez et al, 2012, unpublished data), so the 80 % of the drug remains in the gut. Stock solution of fosfomycin calcium salt was prepared in physiological saline solution (PSS).

Cell line

For these experiments, IPEC-J2 cells were used. These cells were kindly provided by Dr. Anthony Blikslager (North Carolina State University, Raleigh, NC). IPEC-J2 is a non-transformed columnar epithelial cell line, from the mid-jejunum of a neonatal piglet. They were originated in 1989, by Helen Berschneider, at the University of North Carolina (Brosnahan and Brown, 2012).Given the high homology in porcine and human intestinal structure and function, and presumably in enterocytes as well (Geens and Niewold, 2011; Brosnahan and Brown, 2012), studies performed with IPEC-J2 cells may provide valuable insights on the penetration of antibiotics in the human intestinal mucosa.

Cells were cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (Ham) (1:1) with GlutaMAX™-I (DMEM/F12) (Invitrogen ™ Life Technologies, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum, 1% insulin-transferring-selenium supplements (Invitrogen), 1% epidermal growth factor (Invitrogen) and 1% penicillin-streptomycin (penicillin 10,000 U/ml and streptomycin 100 mg/ml; Invitrogen) in a humidified atmosphere of 5% CO_2 at 37ºC. IPEC-J2 cells were seeded into 24-well plates at a concentration of 1.2 x 10^{-6}/ml. Cells in a confluent monolayer were used in all experiments. The cells were maintained in an atmosphere of 5% CO_2 at 37ºC for 24 h.

Cell viability

Cell concentration and viability were determined by trypan blue exclusion assay after 24 h of incubation of IPEC-J2 cells with fosfomycin.

Experimental design

The culture medium was removed and cells cultures were washed with PSS to eliminate residual PBS, since phosphates of the buffer might interfere with the detection of fosfomycin by HPLC-MS/MS. Cells were incubated from 5 minutes to 24 hours at 37 º C and 5% CO_2, depending on the experiment, a-) IPEC-J2 cells were incubated with 1 mL of PSS, in absence of fosfomycin (negative control); b-) Cell cultures were incubated with 1 mL of the 580 µg/mL stock solution of calcium fosfomycin. The results were corroborated by six-replicates.

Cell cultures were observed under an inverted microscope at 40X to determine if the monolayer was intact and cell detachment, cytoplasmic 'dots' (sign of intoxication) and loss of cell membrane were not present. In any case, cells were not affected during the incubation period.

Determination of fosfomycin concentrations in IPEC-J2 cells

After each incubation time (15, 30, 45 min, 1, 2, 3, 4, 6, 8, 12, 18 and 24 h, the supernatant was collected and each well containing IPEC-J2 cells was washed twice with HPLC water. Supernatants were centrifuged at 3500 rpm for 6 minutes to obtain the extracellular fosfomycin. Pellet (sloughed cells) was restored to the corresponding well. After the addition of 2 mL of HPLC water to the wells, culture plates were sealed and sonicated for 30 min to break the cells and release fosfomycin to the HPLC water. Then, the content of each well was centrifuged at 10,000 rpm at 4 ° C. One mL was filtered through nylon filters of 0.22 um, placed in vials, and intra and extracellular concentrations of fosfomycin were determined by HPLC-MS / MS.

To estimate the influence of IPEC-J2 cells intracellular water on fosfomycin intracellular concentrations, we based on Kiem and Shentag (2008) work .We have into account the IPEC-J2 cell count in a well plate (1.2×10^{6} cells) and the mean cell volume (2.75×10^{-6}). Therefore, processed data obtained from Xcalibur software were re-calculated considering the intracellular volume of water. The degree of penetration of fosfomycin into IPEC-J2 cells was determined by comparing the AUC_{0-t} of IPEC-J2 cells with the AUC_{0-t} of serum.

Instruments

The HPLC-MS/MS system was from Thermo Electron Corporation (San Jose, CA, USA), consisting of a Finnigan Surveyor auto sampler, a Finnigan Surveyor MS quaternary pump. The detector was a Thermo Quantum Discovery Max triple quadrupole mass spectrometer, equipped with a ESI source. Nitrogen used as nebulizer and sheath gas was obtained through a nitrogen generator from Peak Scientific Ltd. (Inchinnan, Scotland). Data processing was done using Xcalibur software, also from Thermo.

Mass Spectrometer conditions

The mass spectrometer was operated in negative ionization mode. The tuning parameters were optimized with 10 µg/mL individual aqueous solutions of fosfomycin directly infused in the ion source by means of a syringe pump at 10 µl/min, with influence of mobile phase delivered from the LC pump through a T connection to give the corresponding chromatographic flow rate. The spray voltage was set to -3800 eV, the capillary temperature was 350 ºC, and argon 99,999% purity was used for collision induced dissociation (CID) at 1.6 m Torr in the collision cell. Source CID energy was set to -8eV. Fosfomycin detection and quantification were achieved by single reaction monitoring of transitions m/z 137 → 79 with an optimized collision energy of 25.

CHROMATOGRAPHIC CONDITIONS

Separation was achieved on a Phenomenex CN (cyano) stationary phase, 75 mm x 4.6 i.d., 5 µm column. The mobile phase consisted on acetonitrile: water (20:80), working in isocratic mode, at a flow rate of 100 µl/min. The column was maintained at 30 ºC, while the samples in the auto sampler were at 10 ºC. Sample injection volume was 20µl, chromatographic run time was 8 min and fosfomycin retention time was 6 min.

Statistical analysis

Results were analyzed by ANOVA, using InStat3 software.

RESULTS

The viability of the cells incubated with fosfomycin after 24 h of incubation was 100%. The concentrations of intracellular antibiotic in the wells incubated with 580 ppm calcium fosfomycin ranged between 23.48 and 45.81 µg/ml (Table 1). Figure 1 shows fosfomycin mean intracellular concentrations in IPEC-J2 cells. The T_{max}, moment where fosfomycin reached its maximal intracellular concentration, was 4 h. After this time, the concentration of intracellular antibiotic

started to decrease until it reached concentrations of 23.48 µg/ml at 24 h of incubation.

Only a 7.89 % of the administrated dose was able to enter the IPEC-J2 cells, remaining most of the antibiotic in the extracellular fluid.

Insert Table 1

Fosfomycin AUC in serum, C_{max} and T_{max} in weaning piglets were determined by Soraci et al. (2011). Fosfomycin $AUC_{0–t}$ after an oral dose of calcium fosfomycin was 39.6 µg-h/mL. Fosfomycin $AUC_{0–t}$ after the incubation with 580 µg/mL of calcium fosfomycin was 729.60. The ratio of fosfomycin AUC in IPEC-J2 cells compared to AUC in serum ($AUC_{IPEC-J2}$ / AUC_{serum}) was 18.42. The C_{max} was 3.60 µg/mL for the calcium salt orally administered, and 45.81 µg/mL for the 580 µg/mL dose of calcium fosfomycin in IPEC-J2 cells. These concentrations were achieved at different times. T_{max} were: 3 h (fosfomycin calcium salt in serum) and 4 h (580 µg/mL calcium fosfomycin dose in IPEC-J2 cells). Table 2 shows some pharmacokinetics parameters of fosfomycin in serum (after a after a single oral dose of 30 mg/kg b.w.) and in IPEC-J2 cells (after incubation with calcium fosfomycin; 580 µg/mL).

Insert Table 2

DISCUSSION

For many clinically relevant pig enteric pathologies, intestinal cells represent a major site of infection by intracellular pathogens (biophase) (Schentag, 1990; Toutain et al., 2002; Ross, 2006; Došen et al., 2007). We decided to use IPEC-J2 cells as an *in vitro* model to estimate intracellular fosfomycin concentrations in intestinal cells. The capacity of certain antibiotics to penetrate cell membranes represents an important pre-requisite for their *in vivo* efficacy against intracellular bacteria (Mandell, 1973; Johnson et al., 1980; Yourtee and Root, 1982; Jacobs and Wilson, 1983; Höger et al., 1985).

Antibiotics with high lipid solubility are able to penetrate cell membranes (Mandell, 1973; Johnson et al., 1980). However, fosfomycin is known to be a hydrophilic drug and, therefore, passive transmembrane diffusion is an improbable explanation for its uptake (Höger et al., 1985). Apparently, passive transport through the bacterial cell membrane is not a likely mechanism. Nevertheless, intracellular penetration of a small quantity of the drug by this mechanism cannot be rejected. The presence of the antibiotic inside the cells might be explained by active diffusion of transmembrane. Milagre et al. (2011) demonstrated that fosfomycin is uptaken by a proteic transport system. Kahan et al. (1974), shown the existence of two different active transport mechanisms for fosfomycin. In some bacteria, the drug is incorporated via the sn-glycero-3-phosphate transport system. Under certain conditions, the glucosa-6-phosphate system, which can be externally induced, may also be used. We hypothesized that a similar mechanism takes place in animal cells, so it is possible that a similar active transport mechanism for fosfomycin is present in the enterocytes, as described for bacteria.

In the present study, we found that the concentrations of fosfomycin were significantly higher in IPEC-J2 cells than in serum, at all sampling times. Bacteria may survive within cells and remain unaffected by those antimicrobial agents which are unable to reach the intracellular space. There are several antibiotics with known intracellular accumulation and efficacy against intracellular pathogens. However, the clinical applicability might be limited by their adverse effects on important neutrophil functions. Such inhibitory actions of antibiotics on host defense mechanisms might be critical in immunocompromised hosts (Woodruff et al., 1977). This undesirable effect is not present after fosfomycin administration (Gobernado, 2003) and, on the contrary, Morikawa et al. (1993) and Honda et al. (1998) have shown it has immunomodulatory effect on lymphocytes. Krause et al. (2001) have demonstrated an increased power of destruction of

microorganisms when neutrophils are incubated in the presence of fosfomycin.

Besides all this, an antibiotic taken up intracellularly can only be clinically useful if it retains its bactericidal activity. Many infectious diseases are caused by facultative organisms that are able to survive within cells. The intracellular location of these microorganisms protects them from the host defense mechanisms and from antibiotics with poor penetration into phagocytic cells (Briones et al., 2008). In swine production, the intracellular and interstitial fluids are the biophase of *Mycoplasma hyopneumoniae*, *Bordetella bronchisceptica*, *Salmonella cholerasuis*, *Salmonella enterica*, *Pasteurella multocida, Streptococcus sp.* and *Lawsonia intracellularis*. These bacteria would be killed as soon as the antibiotic reaches a bactericidal concentration within the cell (Höger et al., 1985). So, as described, the choice of an antibiotic is dependent on its direct antimicrobial activity. Generally, this property is detected *in vitro* by determination of the MIC (Herbert, 2002). A fosfomycin MIC_{90} of 0.25-4 µg/mL (Fernández et al., 1995, Ibar et al. 2009) has been determined for the most important pathogens in swine production. Our studies in HEP-2 cells with three different doses of fosfomycin demonstrated the penetration of the antibiotic into the cells (Pérez et. al., 2012), but the amounts were lower than in IPEC-J2 cells. We found that the intracellular penetration was around 7.89 % of the incubation dose and concentrations were always higher than the MIC_{90} for the most important enteric pathogens in swine production. When compared with the percentage of the antibiotic that penetrates in HEP-2 cells, it was also higher in the intestinal cells (7.89 vs. 0.25-1.42).

Studies of PK/PD of several antibiotics have demonstrated the importance of measuring the concentrations of antibiotics at the infection sites, taking into account that the distribution of the drug may vary according to the tissues (Nix et al, 1991; Schentag and Ballow, 1991; Toutain et al, 2002). The penetration of drugs into various tissues is best described by the use of the AUC (area under the curve), which responds to variations in concentration with time (Schentag and Ballow, 1991; Kiem and Schentag, 2008). The comparative $AUC_{IPEC-J2}$ vs. AUC_{serum} was considered significant ($p < 0.05$). Orally administered calcium fosfomycin has a low bioavailability (20%) (Pérez et al., 2012, unpublished data), so the 80 % of the drug reaches de gut and is in conditions to penetrate into the enterocites. T_{max} is reached 1 h later in IPEC-J2 than in serum (4 h vs. 3 h).

When the time period exceeding the MIC for the relevant pathogen (t > MIC) is maximized (%T > MIC), optimal bacterial killing by fosfomycin will be achieved (Sumano et al., 2007; Gutierrez et al., 2009; Popovic et al., 2010). In addition, an effective bacterial killing can be expected when the MIC for the pathogen is covered for at least 60-70% of the dosing interval (McKellar et al., 2004). *Salmonella enterica* is considered an important agent in enteric diseases of weaning piglets. Fosfomycin MIC_{90} for this pathogen has been determined at 4 µg/ml, and bacteria have demonstrated to be 100% sensitive to this antibiotic, with no resistance (Ibar et al., 2009). In the present study, fosfomycin concentrations in IPEC-J2 cells were always above the MIC_{90} for *Salmonella enterica* up to 24 h and at the therapeutic dose assayed.

CONCLUSIONS

Fosfomycin MIC_{90} is 0.25-4 µg/mL for the most important pathogens in swine production. As demonstrated in this study, concentrations achieved at the cellular level are remarkably higher. These findings make fosfomycin an excellent alternative for the treatment of enteric intracellular infections in pigs, and taking into account the great amount of the antibiotic which remains in the gut, it is a good drug to treat other pathogens at the extracellular level. To further corroborate our in vitro studies, additional

experiments should be carried out in pigs infected with enteric pathogens.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

Dr. Anthony Blikslager is acknowledged for kindly provide the IPEC-J2 cells and Dra. Sandra Elizabeth Pérez is acknowledged for her help in cell cultures.

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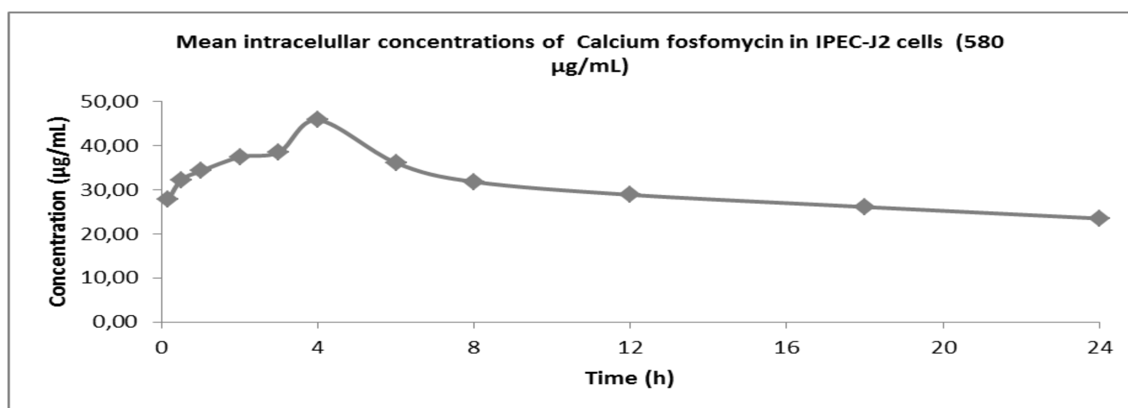

Figure 1: Mean intracellular concentrations of calcium fosfomycin in IPEC-J2 cells after the incubation with a dose of 580 µg/mL.

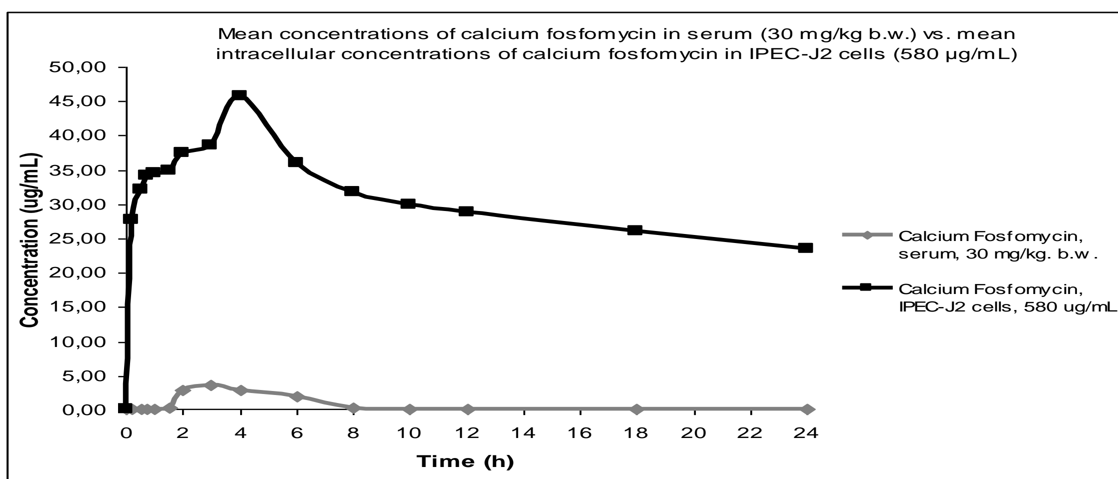

Figure 2: Mean concentrations of calcium fosfomycin in serum (30 mg/kg b.w.) vs. mean intracellular concentrations of calcium fosfomycin in IPEC-J2 cells (580 µg/mL).

Table 1: Penetration of calcium fosfomycin into IPEC-J2 cells (Data are means from experiments performed in sixtuplicate ± SD)

Time	Fosfomycin concentration (µg/ml) in IPEC J2 cells
15 min	27,81 ± 2,71
30 min	32,24 ± 4,89
1 h	34,25 ± 5,88
2 h	37,44 ± 8,45
3 h	38,53 ± 7,67
4 h	45,81± 3,84
6 h	36,09 ± 1,59
8 h	31,79 ± 4,37
12 h	28,86 ± 5,09
18 h	26,10 ± 4,51
24 h	23,48 ± 8,64

Table 2: Calcium fosfomycin pharmacokinetics parameters in serum and IPEC-J2 cells

Parameters	**Serum**	**IPEC-J2 cells**
	Calcium Fosfomycin	Calcium Fosfomycin
	30 mg/kg b.w.	580 µg/mL
AUC_{0-t} µg.h/mL	39.60	729.60
C_{max} µg/mL	3.60	45.81
T_{max} (h)	3.00	4.00