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In-vitro and in-vivo studies of cefpirom using bile salts as absorption enhancers

Yahya Mrestani, Beate Bretschneider, Albert Härtl and Reinhard H. H. Neubert

Abstract

Cephalosporins have to be administered by injection because of the poor intestinal absorption of the orally delivered drugs. Because of the obvious drawbacks of drug delivery by injection, the development of alternatives with enhanced oral bioavailability is receiving much attention in pharmaceutical research. Cefpirom (Cp) is a new semi-synthetic amino-2-thiazolyl-methoxyimino cephalosporin that has been substituted in position 3 with a cyclopenteno-pyridinium group in order to create a zwitterionic compound. It exhibits highly hydrophilic properties, as shown from its extremely low partition coefficient, and therefore its lipophilicity was increased using bile salts. The effect of this on the partition coefficients determined in the n-octanol/buffer system was confirmed using an in-vitro transport model with artificial and biological membranes. The pharmacokinetic properties of Cp were investigated in rabbits after intraduodenal administration with and without bile salts. Furthermore, the physiological compatibility of the bile salts was investigated using active D-glucose transport.

Introduction

Cefpirom (Cp, 3-[(2,3-cyclopenteno-1-pyridinium)methyl]-7-[2-methoxyimino-2-(2-aminothiazole-4-yl)-acetamido]ceph-3-em-4 carboxylate, $pKs = 3.1 \pm 0.13$, Figure 1; Mrestani et al 1998) is a new semi-synthetic cephalosporin antibiotic that has to be administered intravenously or intramuscularly because of its insufficient bioavailability. Cp is a weak inhibitor of β-lactamases, as shown by the high concentration required to achieve a 50% reduction in enzymatic activity (Then & Angehrn 1985; Widemann & Tolxdorff-Neutzing 1993). This weak inhibition is representative of its low affinity for β-lactamases. Cp has high in-vitro activity against many Gram-positive pathogens (Fuchs et al 1985; Arai et al 1987; Horrevorts 1987), offering a potential solution to the therapeutic problems of multiresistant bacteria. In the literature, many possibilities for the improvement of the lipophilicity and bioavailability of hydrophilic drugs are described. These improvements have been obtained through the manufacturing of prodrugs (Auterhoff et al 1991). Drugs have been conjugated to physiological substrates such as peptides and bile salts (Tacnet et al 1993; Lee et al 1996; Smith et al 1996). In recent years, many studies have investigated the influence of absorption enhancers on the intestinal absorption of drugs (Kakemi et al 1969; Van Hoogdalem 1989; Sancho et al 1995). Absorption enhancers are widely used as additives in the preparation of pharmaceuticals. Nishihata et al (1987) studied the effect of absorption enhancers on the rectal absorption of β-lactam antibiotics. In the present study, Cp was selected as the hydrophilic model drug. It is not accepted by the H^+/peptide transporter PEPT1. At the intestinal epithelium, this transport system is responsible for the uptake of many antibiotics, such as cephalosporins and penicillin, into the cell. Bretschneider et al (1999) reported a low affinity of Cp to PEPT1 ($K_i = 40\ mmol\ L^{-1}$). In agreement with this, both the Cp uptake into the cell and the transepithelial flux across Caco-2 monolayers were very low ($flux = 0.1\ nmol\ cm^{-2}\ h^{-1}$) at an extracellular concentration of 1 mM. The effect of the anionic absorption enhancers (bile salts) on the absorption and pharmacokinetics of Cp using in-vitro and in-vivo models was investigated.

Institute of Pharmaceutics and Biopharmaceutics, Department of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, 06120 Halle, Germany

Yahya Mrestani, Beate Bretschneider, Reinhard H. H. Neubert

Hans Knöll Institute for Natural Products Research, Beutenbergstraße 11a, 07745 Jena, Germany

Albert Härtl

Correspondence: Reinhard H. H. Neubert, Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, 06120 Halle/S., Germany. E-mail: neubert@pharmazie.uni-halle.de

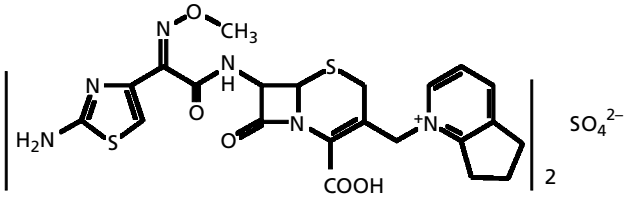

Figure 1 Chemical structure of cefpirom.

Materials and Methods

Materials

Cp was obtained from Hoechst (Germany). Taurodeoxycholate sodium salt (TDOS), deoxycholate sodium salt (DOS), cholate sodium salt (CHS) and dehydrocholate sodium salt (DHS) were obtained from Sigma-Aldrich Chemie (Germany). Collodium 4%, diethyl ether, ethanol, dodecanol and n-octanol were obtained from Caesar & Loretz, Hilden (Germany). The small intestine (Charles River guinea pig) was obtained from the Julius Bernstein Institute for Physiology, Martin Luther University Halle-Wittenberg (Germany). The glucose test set was obtained from Sigma-Diagnostics (Germany).

Sample preparation

Standard solutions of Cp were prepared at 200 μg mL^{-1} in phosphate buffer at pH 7.4 with or without bile salts.

Analytical assays

Capillary zone electrophoresis

The capillary zone electrophoresis (CZE) experiments were performed on a Hewlett Packard G1600A ^{3D}CE system (Waldbronn, Germany). The detection wavelength was 264 nm. Fused-silica capillaries from Hewlett Packard (Waldbronn, Germany) with a total length of 48.5 cm, a length to the detector of 40 cm and an internal diameter of 50 μm were used.

A 20 mM phosphate buffer (pH 7.4), 30 kV, temperature of 25 °C and an injection time of 9 s at 50 mbar were used for the determination of Cp (Mrestani et al 1997).

HPLC

A liquid chromatograph equipped with a diode array detector (Lichrograph, MERCK-Hitachi) was used. For the stationary phase, a reversed-phase column (RP-18, nucleosile) was used. The mobile phase consisted of a mixture of acetonitrile:water:phosphoric acid (15:85:0.5). Cp was determined by measuring the UV absorption at 260 nm (Van Krimpen et al 1987).

In-vitro models

Determination of partition coefficients (P_{ow})

The partition coefficients of Cp with or without absorption enhancers in 1:1, 1:10 and 1:20 molar ratios were determined between water and n-octanol (Table 1). These two phases were saturated with each other. The compounds were dissolved in the water phase (200 μg mL^{-1}). The n-octanol/buffer solutions (phosphate, pH 7.4) were poured into suitable vials and shaken for 12 h at 37 °C. After separation of the samples into the two phases, the drug content was analysed by CZE.

The partition coefficient was calculated using the following equation:

$$P_{ow} = \frac{a_o}{a_w} \quad (1)$$

where a_o and a_w are the concentrations of the drugs in the n-octanol and aqueous phases, respectively.

Permeation model

The transport model system has been described by Neubert & Fürst 1989. The donor and the acceptor compartments were separated by a dodecanol collodium membrane. The effective permeation area of dodecanol collodium membrane was 15.8 cm^2. For permeation, cells were simultaneously used at 37 °C. Twenty millilitres of this solution (200 μg mL^{-1} of drugs) were placed in the donor compartment and 20 mL of the buffer (phosphate, pH 7.4) was poured into the acceptor compartment. The samples (2.0 mL) were periodically removed from the acceptor compartments over 4 h, removed after 4 h from the donor compartments, and the drug content was analysed by HPLC and CZE (Van Krimpen et al 1987; Mrestani et al 1997).

Determination of the content of Cp in the membrane

The membrane was removed from the model after 4 h and shaken in 20 mL water for 30 min. After 30 min of shaking the membrane was removed and repeatedly washed with water. The membrane was then dried and dissolved in 2 mL ethanol:water (90:10). After 30 min the solution was filtered and measured using HPLC.

Mucous membrane model

The mucous membrane model has been described by Bretschneider (1999). A Charles River guinea pig with a

Table 1 Partition coefficients (P_{ow}) of Cp in the system n-octanol/water at pH 7.4 with and without bile salts (n = 8).

	P_{ow}			cmc (mM)
	1:1[a]	1:10[a]	1:20[a]	
Cp	0.02 ± 0.01			
+TDOS	0.16 ± 0.12	0.37 ± 0.22	0.10 ± 0.11	4.2 ± 1.1
+DOS	0.19 ± 0.11	0.23 ± 0.14	0.16 ± 0.12	3.6 ± 1.9
+CHS	0.18 ± 0.13	0.24 ± 0.11	0.22 ± 0.10	10.3 ± 1.1
+DHS	0.12 ± 0.08	0.17 ± 0.13	0.11 ± 0.15	9.2 ± 2

pH = 7.4; 37 °C. [a]Molar ratio of Cp:absorption enhancer.

weight of 300–400 g was killed through cerebral dislocation and 30 cm of small intestine was removed. A buffer consisting of 5 mM KCl, 1 mM KH_2PO_4, 26 mM $NaHCO_3$ and 122 mM NaCl was used for these experiments. Samples of 200 $\mu g\,mL^{-1}$ Cp were investigated alone or in combination with surfactants in ratios of 1:1 and 1:10. All experiments were performed at 37 °C for 3 h.

In-vivo models

Rabbit model

Female rabbits [Chinchilla Bastard and New Zealand White, 3–5 kg body weight (b.w.), Charles River, Kisslegg, Germany] were fasted for 18 h. For the experimental preparation, the rabbits were narcotised by 50 mg kg^{-1} b.w. pentobarbitone sodium (SPOFA, United Pharmaceutical Works Prague, Czech Republic). The animals were fixed onto an operation table that was kept at constant temperature (38 °C). Polyethylene tubes of different diameters were inserted into a carotid artery, the common bile duct, ureters and duodenum. In order to prevent clotting and to stabilize the blood circulation, an infusion of 36 mL h^{-1} of heparinized physiological saline solution was administered using an infusion pump (Program2, Becton Dickinson, France). The investigations were approved by the thüringer Landesverwaltungsamt, 74043-2684.04-52/99.

Drug administration and sampling

Cp with or without absorption enhancers was administered i.d. via an inserted polyethylene tube in the duodenum. The doses of Cp were 100 mg kg^{-1} in phosphate buffer at pH 7.4 with or without surfactants. Bile salts were used as model enhancers to demonstrate basically the absorption increasing ability (247 mg kg^{-1} TDOS, 196 mg kg^{-1} DOS, 100 mg kg^{-1} DHS and 100 mg kg^{-1} CHS). The volume of the solution was 10 mL kg^{-1} ethanol/Soerensen phosphate buffer (1:5 v/v) at a pH of 7.4. For comparison, Cp (100 mg kg^{-1} b.w.) was also injected as bolus intravenously (i.v.) via the femoral vein. Following the Cp administration, 3 mL of blood was withdrawn from the carotid artery with a syringe containing 3 mL of sodium citrate solution (3.13%) at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h. The blood was centrifuged at 3000 rpm for 10 min to obtain plasma, which was kept at −20 °C until analysis. Bile and urine were sampled via the cannulas in ductus choledochus or ureters at different intervals between 0 and 6 h after dosing and the aliquots were kept at −20 °C until further processing.

Calculation of the pharmacokinetic parameters

Where mean results are given the values are shown together with values of standard deviation (s.d.). The pharmacokinetic parameters C_{max}, t_{max} and AUC (area under the plasma concentration–time curve) were calculated using the PC program TOPFIT 2.0 (Heinzel et al 1993).

Calculation of the amount of Cp (%) in bile and urine

The amount of Cp in bile and urine was calculated for every sample using the following equation:

$$m = cV \quad (2)$$

where m and c are the amount and concentration of Cp, respectively, and V is the volume of the sample. The total amount of Cp after 6 h was calculated by the addition of amounts of Cp at different times.

Analytical assays

The determination of Cp in plasma, bile and urine was performed by CZE (Mrestani et al 1997) and HPLC (Van Krimpen et al 1987).

Results

In-vitro models

Partition coefficients (P_{ow})

For the characterization of the hydrophobic/hydrophilic properties of Cp, partition coefficients were measured (Table 1) in the n-octanol/water system. Cp exhibited very small partition coefficients (0.02). It is a very hydrophilic drug and contains a number of polar groups. Its combination with bile salts leads to a considerable improvement in the partition coefficients. The partition coefficients of Cp were investigated using bile salts below the critical micelle concentration (cmc, ion-pair formation) and above the cmc (aggregation form). The lipophilicity of Cp increased 10-fold using the combination with bile salts below the cmc. Above the cmc, the lipophilicity of Cp increased 20-fold.

In-vitro transport model at artificial lipid membranes

Table 2 summarizes data on the influence of bile salts on the permeation of Cp through artificial lipid membranes. Cp alone exhibited no transport via the lipid membranes. The combination with bile salts below and above the cmc led to an improvement of the permeation of Cp via the lipid membranes. In the membrane, Cp was found at all molar ratios between 1.1 and 7.2%. No transport of Cp via the lipid membranes was observed above the cmc. The

Table 2 Amount of Cp (%) in the artificial lipid membranes (n = 8).

	Membrane (%)	Acceptor (%)
Cp	0	0
Cp:TDOS[a]	1.1 ± 0.31	0
Cp:TDOS[b]	4.4 ± 0.45	0
Cp:DOS[a]	3.2 ± 0.15	1.2 ± 0.2
Cp:DOS[b]	7.2 ± 0.25	0
Cp:CHS[a]	1.5 ± 0.12	0
Cp:CHS[b]	2.7 ± 0.25	0
Cp:DHS[a]	1.2 ± 0.12	2.4 ± 0.12
Cp:DHS[b]	2.1 ± 0.17	0

[a]1:1; [b]1:10.

content of Cp in the membrane below the cmc was significantly smaller than that above the cmc (Table 2). The combination with TDOS and CHS led to no transport of Cp across the dodecanol collodium membranes.

In-vitro transport model at native intestinal epithelium (Charles River guinea pig)

Table 3 shows the permeation amount of Cp using the guinea pig small intestine membrane. All bile salts influence the permeation of Cp using biological membranes. The content of Cp in the mucus increases with all bile salts (Table 3). Cp containing TDOS reached the largest dimension with about 6.9%, followed by Cp containing DOS in a ratio of 1:1 with 4.5%. Similar to mucus, in connective tissue the content of Cp in combination with TDOS was the largest, with about 5.2%, followed by Cp in combination with DOS resulting in 3.4%.

Influence of absorption enhancers on the active D-glucose transport

D-Glucose is transported actively via SGLT1. We investigated the influence of absorption enhancers on the active D-glucose transport using guinea pig intestinal epithelium. The concentration of D-glucose was measured in the acceptor by use of a radioactive substance marker. Figure 2 shows that bile salts did not influence the active D-glucose transport at the supported concentrations (5 mM).

In-vivo models

Cumulative bile excretion

Cp was detected in bile after i.d. administration with bile salts. The cumulative biliary excretion of Cp after 6 h was 0.4% of the administered dose with TDOS, 0.9% with DOS, 0.3% with DHS and 0.4% with CHS in comparison to Cp used alone (0.05%).

Cumulative urinary excretion

The combination of Cp with bile salts led to an increase in the amount of Cp in the urine of about 10-fold with TDOS (21% total), about 20-fold with DOS (42% total), about 5-fold with CHS (11% total) and about 2-fold with DHS (4% total) in comparison to Cp when it was used alone (2% total) after 6 h.

Table 3 Influence of bile salts on the permeation of Cp (%) using guinea pig small intestine membrane (n = 8).

	Mucus (%)	Connective tissue (%)	Acceptor (%)
Cp	0.2 ± 0.1	0.4 ± 0.1	0
Cp:TDOS[a]	6.9 ± 0.3	5.2 ± 0.1	3.1 ± 0.1
Cp:TDOS[b]	6.3 ± 0.3	3.1 ± 0.3	15.2 ± 0.4
Cp:DOS[a]	4.5 ± 0.4	3.4 ± 0.3	4.9 ± 0.1
Cp:DOS[b]	2.16 ± 0.9	2.1 ± 0.1	5.5 ± 0.2
Cp:CHS[a]	1.7 ± 0.8	1.7 ± 0.2	3.3 ± 0.3
Cp:CHS[b]	1.4 ± 0.1	1.2 ± 0.4	5.5 ± 0.1
Cp:DHS[a]	2.4 ± 0.2	1.4 ± 0.1	3.2 ± 0.2
Cp:DHS[b]	1.7 ± 0.4	1.2 ± 0.3	6.3 ± 0.1

[a]1:1; [b]1:10.

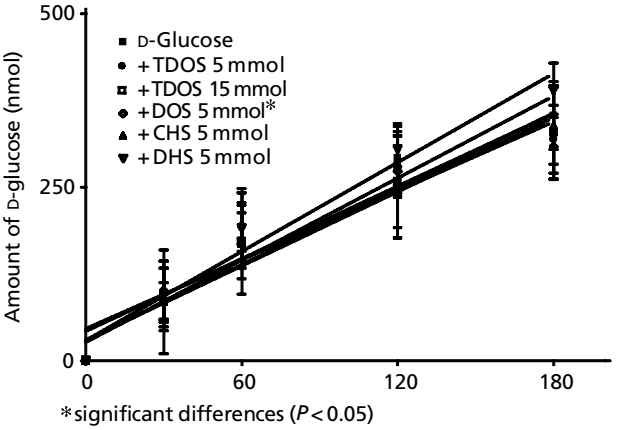

Figure 2 Influence of bile salts on the transport of D-glucose using guinea pig intestinal epithelium ($C_0 = 200\,\mu g\,mL^{-1}$, n = 4, means ± s.d.).

Plasma concentration–time profiles

The pharmacokinetics and absolute bioavailability of Cp were detected after i.v. and i.d. administration (Table 4, Figure 3). The concentration of Cp in plasma was significantly higher than in the case without bile salts. This combination of Cp with bile salts led to an increase in C_{max} of about 22-fold compared to Cp alone. Between 30 and 60 min after dosing, t_{max} was observed. Because of the higher plasma concentration of Cp, a definite increase in the AUC was observed. The AUC of the Cp combination with bile salts was 17 times larger than that when Cp was used alone (Table 4). The absolute bioavailability of Cp when used alone after i.d. administration was 2.8%, for the co-administration with TDOS 32.5%, with DOS 46%, with CHS 14% and with DHS 3%. Table 4 shows the pharmacokinetic parameters of Cp when used alone or co-administered with bile salts.

Table 4 Pharmacokinetic parameters of Cp after separate or simultaneous administration of absorption enhancers.

	t_{max} (min)	C_{max} ($\mu g\,mL^{-1}$)	AUC_{0-300} ($\mu g\,min^{-1}\,mL^{-1}$)
Cp, i.v. (n = 6)			18907 ± 9368
Cp, i.d. (n = 6)	90 ± 1.6	3 ± 1.8	524 ± 403
Cp:TDOS, i.d. (n = 4)	90 ± 2.6	25 ± 2.9	6143 ± 499
Cp:DOS, i.d. (n = 4)	45 ± 1.1	68 ± 3	8648 ± 5576
Cp:CHS, i.d. (n = 3)	45 ± 2.3	26 ± 6	2684 ± 1100
Cp:DHS i.d. (n = 3)	120 ± 3	3 ± 1.4	565 ± 162

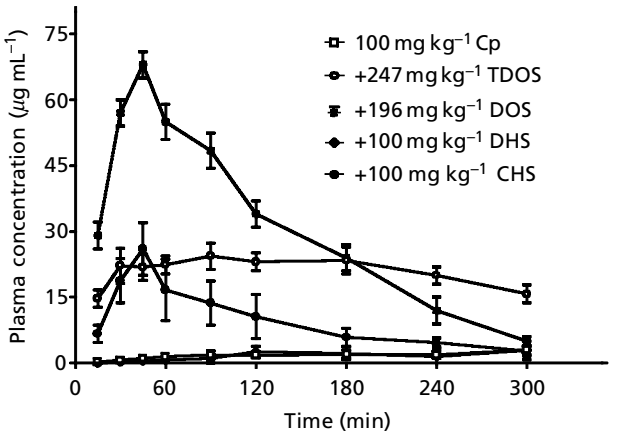

Figure 3 Plasma concentration–time profiles of Cp after i.d. administration (means ± s.d.).

Discussion

The influence of bile salts on the transport of drugs using different transport models has been investigated in many studies. Cp was selected in the present study because it was not accepted as a substrate by the intestinal H^+/peptide symporter PEPT1, and no other active transport mechanism could be shown (Bretschneider et al 1999). The combination of Cp with bile salts was used below and above the cmc. The partition coefficients in the n-octanol/water system were increased both below and above the cmc. We assume that electrostatic interaction (ion-pair formation below the cmc) and hydrophobic interaction (aggregation above the cmc) play an important role. Using a model with artificial lipid membranes, primary enriching of Cp in the membrane by combination with bile salts was observed. Using a model with biological lipid membranes, the same combination leads to an enrichment of Cp in the mucous membrane on the one hand and to permeation into the acceptor on the other. The influence of absorption enhancers on the active D-glucose transport was examined in biological lipid membranes (Brot et al 1986; Burdett & Lauterbach 1994). The results show that the concentration profile of the glucose in the acceptor corresponds to that concentration given in the literature (Kakemi et al 1969; Grass & Sweetana 1998). Our results demonstrate that the viability of the cells stays above 180 min as a minimum in this model. We also showed that bile salts do not influence D-glucose transport through the small intestine layer at the concentration used (5 mM) (Figure 2). A study by Moses (1988) supports our results. No damaging effects on nasal mucosal integrity were observed on acute and subchronic exposure in rats and dogs using bile salts as absorption enhancers. High concentrations of bile salts (4–5%) were proved to enhance the absorption of orally administered phenol red (Feldman et al 1970) and also of heparin (Guarini & Ferrari 1985) in rats. The rabbit model used in this study was found to be very useful for parallel investigation of biliary and renal excretion in comparison to plasma concentration–time profiles of drugs after separated or simultaneous administration. The intestinal absorption of Cp after i.d. administration in combination with TDOS, DOS, CHS or DHS was significantly higher than when Cp was used alone. The absolute bioavailability of Cp was enhanced to 46% through co-administration with DOC in comparison to Cp used alone (2.8%). Although bile salts have been demonstrated to enhance drug uptake to a significant extent, administration of the compounds as safe absorption promoters in humans is not without problems because mucosal damage seems to be correlated to its effect on drug uptake. On the other hand, a two-year therapy of oral administration of chenodeoxycholic acid (350–750 mg day^{-1}) for dissolution of gallstones was associated with mild side effects, for instance increased serum levels of aminotransferase and cholesterol, and diarrhoea (Schoenfield & Lachim 1981). These observations indicate that long-term therapy with formulations containing bile salts may be feasible.

The transport systems used in the present study show several advantages and disadvantages. One advantage of the permeation model is the simple manufacturing of the membrane. Furthermore, this model is simple to handle. On the other hand, the permeation model could only be used for passive transport, which is a disadvantage. The mucous membrane model is simple to prepare and contains physiological cells with mucus, but this model has a relatively small permeation area. By using the rabbit model, both passive and active transport can be tested. Here, physiological cells with mucus are also present. Additionally, it should be possible to determine the pharmacokinetic parameters. The rabbit model is also very time-consuming and expensive.

The ANOVA post-hoc test (Newman–Keuls test, Dunnett's test) and the Mann–Whitney test were used for the determination of significant differences in this work (GraphPad Prism 03, biostatistics, curve fitting and scientific graphing). The ANOVA test exhibited no significant differences for the partitioning coefficients of Cp with absorption enhancers in 1:1, 1:10 and 1:20 molar ratios. The Newman–Keuls test comparing all pairs also showed no significant differences (Table 1). The amount of Cp in the artificial lipid membranes showed significant differences below and above the cmc using the non-parametric test (Mann–Whitney test) (Table 2). No significant differences were also observed for the permeation of Cp below and above the cmc in the guinea pig small membrane model (mucus, connective tissue and acceptor) using the Newman–Keuls test (Table 3). The influence of absorption enhancers on the active D-glucose transport exhibited significant differences (Figure 2). The ANOVA post-hoc test (Dunnett's test) was used to compare all samples with control samples (D-glucose) (Figure 2). Furthermore, the ANOVA post-hoc test (Newman–Keuls test) was used for the comparison of all models with one another (partition coefficients, transport model at artificial lipid membranes, Charles River guinea pig and rabbit models). The transport of Cp using bile salts exhibited significant differences ($P < 0.05$) for all models together. Table 5 shows the results of the Newman–Keuls comparison test.

Table 5 ANOVA post-hoc test (Newman–Keuls test) of significant differences of the in-vivo and in-vitro models.

	< cmc	> cmc
Artificial lipid membranes vs rabbit model	*	*
Artificial lipid membranes vs Charles River guinea pig		
Artificial lipid membranes vs partition coefficients		
Partition coefficients vs rabbit model	*	*
Partition coefficients vs Charles River guinea pig		
Charles River guinea pig vs rabbit model	*	*

*Significant differences ($P < 0.05$).

Conclusion

The influence of bile salts on the transport of the extremely hydrophilic cephalosporin Cp was investigated in in-vitro and in-vivo transport models using artificial lipid membranes, biological membranes and the rabbit model. The combination with bile salts below and above the cmc leads to an improvement of the permeation, lypophilicity properties and bioavailability of Cp. The rabbit model was used because intraduodenal administration and also simultaneous sampling of plasma, bile and urine are possible. Ionic interaction between Cp and bile salts below the cmc (ion-pair formation) and hydrophobic interaction above the cmc (aggregation) have a very strong influence on the permeation in and through the membranes. Up to 46% absolute bioavailability of Cp was reached by its combination with bile salts. The bile salts used here showed no toxicity effects since they did not influence the active D-glucose transport at the supported concentrations (5 mM).

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