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LABORATORY INVESTIGATION

Ocular penetration of caspofungin in a rabbit uveitis model

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Abstract
Background Little is known about the ocular penetration of echinocandin antifungals. We studied the ocular distribution of systemically administered caspofungin in a rabbit uveitis model.
Methods Caspofungin (1 mg/kg per day) was given intravenously to rabbits as a single dose or as repeated daily doses on 7 days starting 24 h after induction of unilateral uveitis by intravitreal endotoxin injection. Caspofungin concentrations were determined by high-performance liquid chromatography in the cornea, aqueous humor, vitreous humor, and serum 4, 8, 16, and 24 h after administration of a single dose and 24 h after the last of seven doses.
Results The mean caspofungin concentration in the aqueous of the inflamed eye 4 and 8 h after single-dose administration was 1.30±0.39 μg/ml and 1.12±0.34 μg/ml, respectively. Drug concentrations decreased to 0.24±0.09 μg/ml at 16 h and 0.26±0.14 μg/ml at 24 h. In the vitreous of inflamed eyes drug levels were undetectable at all time points. No drug was found in the aqueous of inflamed eyes 24 h after the last of seven repeated doses, and the vitreous only contained trace amounts. In the corneas of inflamed eyes concentrations reached 1.64±0.48 μg/g at 4 h, peaked at 2.16±1.14 μg/g at 8 h, and declined to 1.87±0.52 μg/g and 1.49±0.48 μg/g at 16 and 24 h, respectively. After repeated dosing, corneal concentrations of caspofungin were 0.8 and 1.0 μg/g and below the limit of detection in two of four animals. In non-inflamed eyes no drug was detectable in the aqueous and vitreous humor, and the corneas at any time point.
Conclusions In our model, caspofungin reached therapeutically relevant levels in the aqueous and cornea but not in the vitreous humor of inflamed eyes. Intraocular drug deposition was critically dependent on a disrupted blood-eye barrier. These findings suggest a limited role for caspofungin in the treatment of fungal endophthalmitis.

Keywords Caspofungin · Pharmacokinetics · Ocular penetration · Animal model

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Introduction

Caspofungin is a first-in-class echinocandin with potent antifungal activity against *Candida* and *Aspergillus* spp., the dominant human fungal pathogens. In vitro and in vivo

caspofungin is fungicidal against nearly all *Candida* spp. including fluconazole-resistant strains. Randomized clinical trials with caspofungin in patients with candidemia, invasive candidiasis, and *Candida* esophagitis demonstrate equivalent efficacy to amphotericin B (AmB), with substantially fewer toxic effects [1, 5, 16, 24]. In addition, caspofungin is as effective as liposomal AmB in the empiric antifungal treatment of neutropenic patients with persistent fever and has been licensed for the treatment of severe pulmonary aspergillosis in patients who fail to respond or are intolerant to therapy with AmB [26].

Fungal infections of the eyes are important causes of morbidity and blindness; certain ophthalmic mycoses may even be life-threatening [14, 28]. Keratitis is the most frequent manifestation [20], but intraocular structures as well as orbit, lids, lacrimal apparatus, conjunctiva, and sclera may also be infected.

A recently published case series suggests that caspofungin in combination with voriconazole may be useful for the treatment of endogenous *Candida* endophthalmitis [2]. The same drug combination has been used to successfully treat a patient with endophthalmitis due to *Aspergillus fumigatus* [6]. Clinical reports on the utility of caspofungin monotherapy for ocular infections are scarce and conflicting. Cure has been reported for a patient with endogenous *Candida glabrata* endophthalmitis presenting with a single retinal lesion and mild retinitis [18]. In another reported case of an ocular yeast infection, a patient with more advanced endogenous endophthalmitis due to *C. albicans* failed to respond to caspofungin. After 9 days of treatment with standard doses, his intravitreal caspofungin level was undetectable by a sensitive assay [8]. Successful treatment with caspofungin has also been reported in a patient with *Acremonium* endophthalmitis that failed to respond to AmB [4]. In a rabbit model, topical caspofungin was as effective as AmB for the treatment of *Candida* keratitis [9].

Caspofungin concentrations in homogenates of whole non-inflamed rat eyes 0.5 and 24 h after a 2.0-mg/kg i.v. bolus injection of radioactively labeled drug were 0.52 and 0.30 μg Eq/g, respectively [23]. Due to its high molecular weight (1,213 kD) caspofungin may not cross the uninflamed blood-eye barrier that is thought to be impermeable for molecules exceeding 500 kD. Indeed, the degree of rupture of the blood-eye barrier in inflammation may be critical for the intraocular deposition of this and other echinocandins.

Only detailed knowledge of the pharmacokinetics of caspofungin in ocular inflammation will provide a base for rational treatment decisions. The disposition of caspofungin to various ocular compartments after systemic treatment is unknown. The aim of this study was to characterize the ocular distribution of intravenously administered caspofungin in a rabbit uveitis model. For that purpose, high-performance liquid chromatography (HPLC) assays for the determination of caspofungin concentrations in the cornea, aqueous humor, vitreous humor, and serum had to be developed.

Materials and methods

Animals

Twenty adult (nine male) Burgundy fawn rabbits (2.3–4.9 kg) were used in this study. They were provided by an authorized breeding center and were kept in individual cages under well-defined and standardized conditions (in a humidity and temperature controlled room, with a cycle of 13 h of light and 11 h of dark). They received standard dry food and water ad libitum. All eyes were initially examined with a handheld slit lamp. Only animals without any signs of ocular inflammation were included. All experiments were conducted in accordance with the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986), and the US Animal Welfare Act, as amended, and were approved by the Swiss Federal and local Ethics and Agricultural Committees.

Anesthesia

Animals were anesthetized with intramuscular ketamine (35 mg/kg of body weight; Ketalar, Parke-Davis, Ann Arbor, MI, USA) and xylazine (5 mg/kg; Xylapan, Chassot, Bern, Switzerland). Novocaine 0.2% (Inselspital Pharmacy, Bern, Switzerland) eye drops were used for topical anesthesia. All rabbits were anesthetized once for the induction of uveitis and placement of the central venous catheter. This vascular access was established by surgical placement of a subcutaneous silastic central venous catheter that permitted one-time or repeated drug administrations without further anesthesia [25].

Induction of experimental uveitis

After washing the ocular surface with sterile novocaine 0.2%, 100 ng (for animals given a single drug dose) or 50 μg (for animals given multiple drug doses) of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, St. Louis, MO, USA) diluted in 10 μl of sterile saline solution were injected [13]. The higher LPS dose was administered to maintain intraocular inflammation over the treatment period [7]. LPS was injected through the sclera into the vitreous using a 30–1/2 G needle connected to a Hamilton syringe. The injection was performed in the right

eye of each animal, taking care to avoid damage to the lens. The left eyes served as controls. Inflammation was evaluated by clinical observation of iris and conjunctival hyperemia.

Single-dose studies

Single-dose kinetics of caspofungin acetate (Cancidas, Merck, Whitehouse Station, NJ, USA) were studied over 24 h. Immediately before use, caspofungin acetate was dissolved in sterile 0.9% sodium chloride to a concentration of 0.5 mg/ml. Twenty-four hours after induction of uveitis, 16 rabbits were given 1 mg/kg caspofungin over 6 min by steady bolus injection via a central venous catheter. The drug dosage used corresponds to the standard dose recommended for the treatment of human fungal infections and is well tolerated by rabbits. Four animals per time point were sacrificed by cervical dislocation and subsequent exsanguination at 4, 8, 16, and 24 h after drug administration. Blood samples were collected during bleeding, and serum was separated by centrifugation. Aqueous humor was drawn from the freshly enucleated eyes with a tuberculin syringe using a 30-gauge needle. The cornea was excised at the limbus. After sectioning the eyes just behind the lens, vitreous humor was obtained by dissecting it carefully from the retina.

Multiple-dose study

Twenty-four hours after induction of uveitis, four rabbits were given doses of 1 mg/kg caspofungin by steady intravenous bolus via a central venous catheter that were repeated daily for 7 consecutive days (total seven doses). Twenty-four hours after the last dose, the rabbits were sacrificed and samples were prepared for examination as described above.

Analytical procedures

Samples were stored at −70°C until analysis. Blank aqueous humor, blank vitreous humor, and blank corneas were obtained from porcine eyes collected at the abattoir. Blank plasma was prepared from bovine blood. Caspofungin acetate was a kind gift of D. Sanglard, Institute of Microbiology, University of Lausanne, Lausanne, Switzerland, and 4-hexylresorcinol (internal standard) was from Fluka (Buchs, Switzerland). Chemicals used were of analytical or HPLC grade. Caspofungin in serum and plasma was extracted using Sep-Pak C18 extraction cartridges (Waters, Milford, MA, USA) conditioned with 1 ml of methanol and 2 ml of distilled water. Two hundred and fifty µl of serum or plasma mixed with 25 µl of a 12.5 µg/ml internal standard solution and 750 µl of water was centrifuged at 14,000 *g* for 2 min and applied to the cartridge. After rinsing with 2 ml water, the sample was eluted with 1 ml of methanol containing 4% concentrated acetic acid, evaporated to dryness at 40°C under a gentle stream of air, and reconstituted in 225 µl of mobile phase. For analysis of aqueous humor, 100 µl of sample and 20 µl of a 1.25 µg/ml internal standard solution were mixed with 200 µl of methanol containing 4% acetic acid. After centrifugation and evaporation, it was reconstituted in 100 µl of mobile phase prior to ultrasonic mixing (Bransonic 92, Bender & Hobein, Zurich, Switzerland) for 5 min, and in case of turbid samples, centrifugation at 14,000 *g* for 1 min. Vitreous samples were homogenized with an Ultra-Turrax microhomogenizer (IKA-Labortechnik, Staufen, Switzerland) at high speed for 10 s; 125 µl of vitreous, 25 µl of a 1.25 µg/ml internal standard solution, and 250 µl of methanol containing 4% acetic acid were mixed, centrifuged at 14,000 *g* for 1 min, and the clear supernatant was evaporated and redissolved in 125 µl of mobile phase. Pieces of cornea weighing 20–50 mg were added to 100 µl of distilled water, vortex mixed, and centrifuged at 14,000 *g*. The sample was cut into small pieces with scissors, mixed with 20 µl of a 1.25 µg/ml internal standard solution, sonicated for 15 min, diluted with 200 µl of methanol containing 4% acetic acid, and centrifuged. The supernatant was evaporated and reconstituted with 100 µl of mobile phase. Recoveries for caspofungin in serum, aqueous, vitreous, and cornea were determined to be approximately 65, 86, 91, and 87%, respectively.

Drug levels were determined as total caspofungin concentrations by HPLC with fluorescence detection using a protocol adapted from [19]. Briefly, an LC-Module I plus autosampler and pump (Waters, Milford, MA, USA) were connected to an LC 304 fluorescence detector (Linear Instruments, Reno, NV, USA). A 250/8/4.6 Nucleosil 300 wide bore C8 column (Macherey-Nagel, Düren, Germany) packed with 5 micron material was used. The mobile phase comprised two solutions (A and B) that were mixed in a volume/volume ratio (v/v) of 62:38 for vitreous and aqueous and a ratio of 60:40 (v/v) for serum and cornea. Solvent A was 0.1% trifluoroacetic acid (adjusted to pH 3 with diethylamine) and solvent B was acetonitrile. After a run time of 11 min, the column was washed for 4 min with a solution containing solvents A and B in a ratio (v/v) of 50:50 and reequilibrated for 5 min with mobile phase. Elution was performed at a flow rate of 1.4 ml/min for vitreous and aqueous, 1.3 ml/min for cornea, and 1.0 ml/min for serum. The column temperature was maintained at 30°C. Excitation and emission wavelengths were set at 224 and 300 nm, respectively. For all assays, 50 µl of sample was injected. The concentrations of the lowest calibrators, i.e., 0.1 µg/ml for serum, 0.05 µg/ml for

aqueous and vitreous, and 0.025 μg for cornea (for 40 mg cornea: 0.625 μg/g cornea), were taken as quantification limits. Intraday variabilities (n=4) were <7% in all cases. All control samples were stable during the 3-month study period and interday variabilities were below 10% for plasma, aqueous, and vitreous, and below 20% for cornea. Typical electropherograms obtained with aqueous humor are presented in Fig. 1.

Results

Aqueous

To study drug penetration into the aqueous of inflamed and uninflamed control eyes, samples harvested immediately postmortem from eyes with iatrogenic uveitis leading to disruption of blood-eye barriers were analyzed for caspofungin concentration. Results were compared to those obtained from similarly harvested aqueous from contralateral, uninflamed eyes with intact blood-eye barriers.

Four and 8 h after intravenous injection of a single dose of 1 mg/kg caspofungin, the mean drug concentration in the aqueous of the inflamed eye was 1.30±0.39 and 1.12±0.34 μg/ml, respectively. These concentrations are in excess of the minimum inhibitory concentration of most *Candida* and *Aspergillus* spp. Sixteen and 24 h after treatment, the concentration decreased to 0.24±0.09 and 0.26±0.14 μg/ml, respectively (Fig. 2a). Caspofungin was undetectable in the aqueous of contralateral non-inflamed left eyes at all time points.

Fig. 2 **a**–**c** Mean (±SD) caspofungin concentration over time in **a** ▶ aqueous, **b** corneas of LPS-treated eyes, and **c** serum of rabbits given a single intravenous dose of caspofungin at 1 mg/kg of body weight (n=4 per time point)

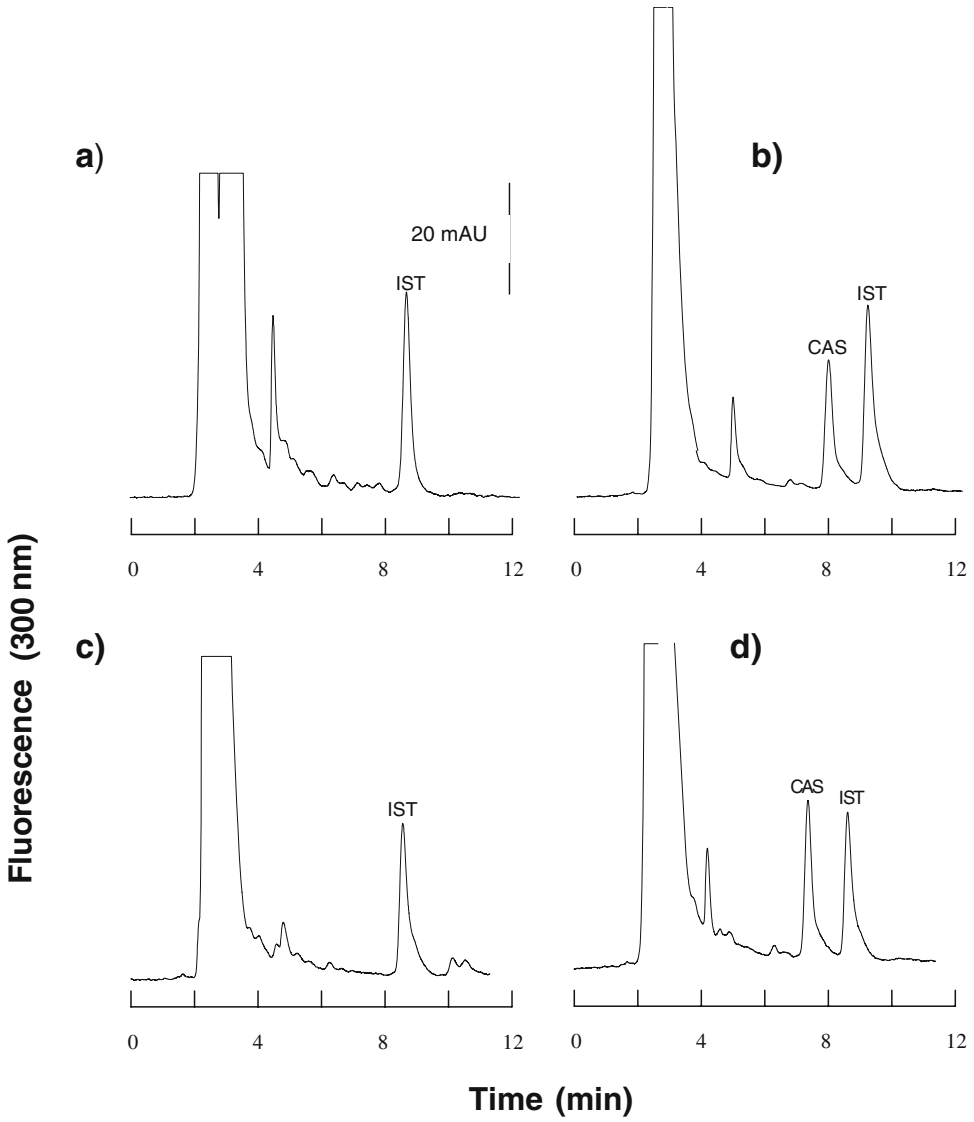
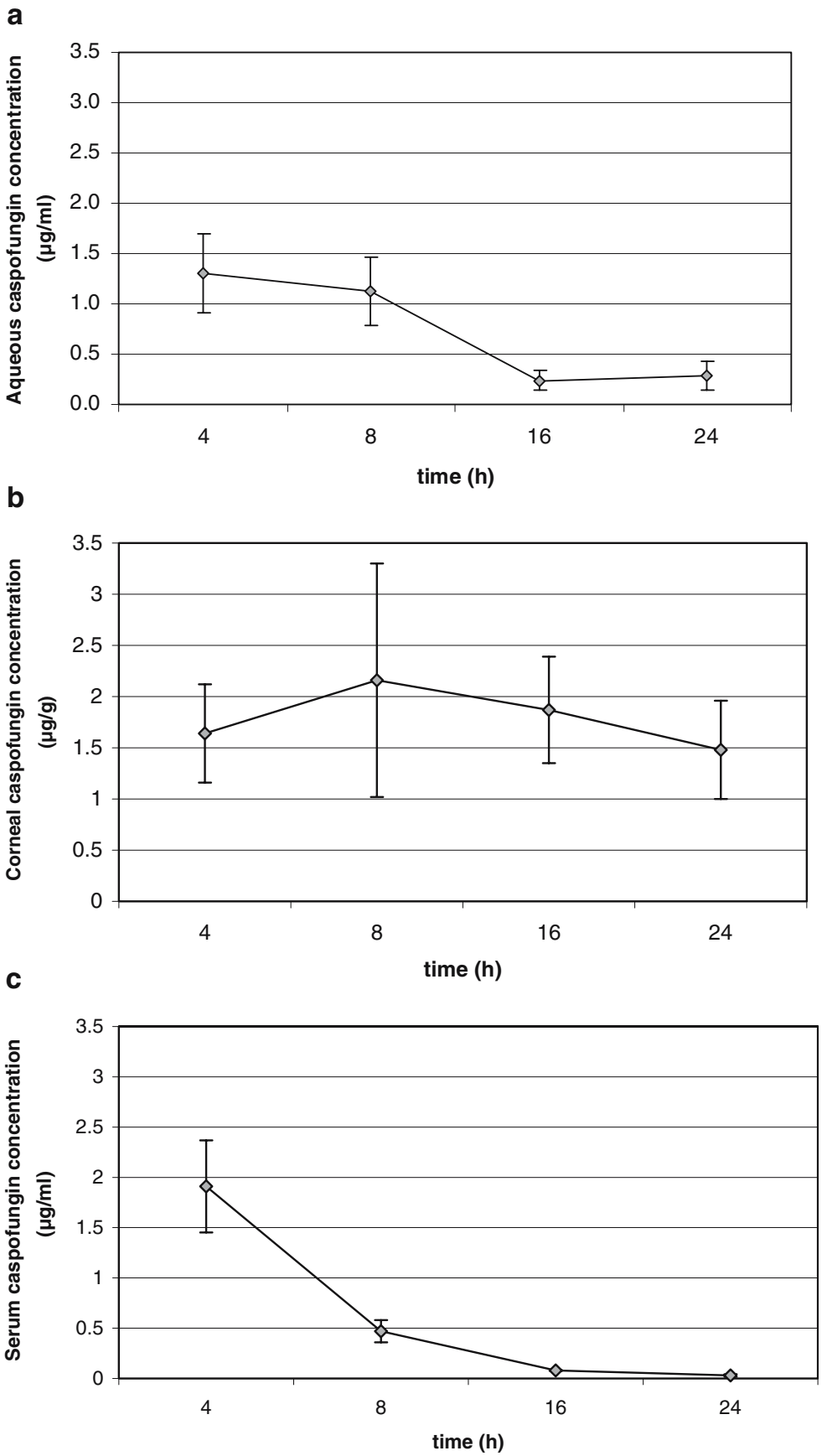


Fig. 1 Typical chromatograms of caspofungin (*CAS*). **a** Blank porcine aqueous fortified with internal standard (*IST*). **b** Four-fold diluted aqueous of an inflamed rabbit eye harvested 4 h after a single-dose injection containing 1.30 μg/ml caspofungin. **c** Aqueous of a non-inflamed eye obtained 8 h after a single dose of caspofungin. **d** Porcine aqueous spiked with caspofungin at a concentration of 0.5 μg/ml


a
Aqueous caspofungin concentration (µg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
8
16
24
time (h)
b
Corneal caspofungin concentration (µg/g)
3.5
3
2.5
2
1.5
1
0.5
0
4
8
16
24
time (h)
c
Serum caspofungin concentration (µg/ml)
3.5
3
2.5
2
1.5
1
0.5
0
4
8
16
24
time (h)

In the multiple-dose study animals were treated with repeated daily doses of 1 mg/kg of body weight for 7 days. Caspofungin concentration was below the level of determination in all the aqueous samples of inflamed and control eyes collected 24 h after the last of seven doses (Fig. 3).

Vitreous

After single-dose administration of 1 mg/kg caspofungin, drug concentrations in all the vitreous samples from both inflamed and non-inflamed eyes were below the level of detection (0.05 μg/ml). Twenty-four hours after the last of seven intravenous doses, caspofungin levels were undetectable in all vitreous samples with one exception: in one inflamed eye a caspofungin concentration of 0.08 μg/ml was found (Fig. 3).

Cornea

Topical antifungals are the treatment of choice for fungal keratitis because high drug concentrations can be achieved in the cornea, particularly when the corneal epithelium is damaged, and systemic toxicity is minimal. In advanced infections with threatening penetration into the anterior chamber, however, systemic antifungals are often added. We were therefore interested in determining the corneal penetration of intravenous caspofungin. Four hours after single-dose treatment, the mean caspofungin concentration in the cornea of inflamed eyes was 1.64±0.48 μg/g. Corneal drug concentrations also exceeded aqueous levels after 8, 16, and 24 h at 2.16±1.14, 1.87±0.52, and 1.49±0.48 μg/g, respectively (Fig. 2b). Caspofungin levels in the corneas of uninflamed eyes were undetectable at all time points, suggesting that disruption of the blood-eye barriers was critical for corneal drug deposition.

Corneal drug concentrations in inflamed eyes of rabbits treated with multiple doses were 0.82 μg/g, and 1.03 μg/g in two animals, and undetectable in the other two (Fig. 3). Caspofungin was below the level of detection (0.625 μg/g) in uninflamed eyes after repeated daily dosing.

Serum

Four hours after single-dose administration of caspofungin, the mean drug concentration in serum was 1.91±0.46 μg/ml (Fig. 2c). It fell to 0.47±0.11, 0.08±0.01, and 0.03±0.01 μg/ml after 8, 16, and 24 h, respectively. Serum level 24 h after the last of seven daily caspofungin doses was 0.17±0.06 μg/ml (Fig. 3)

Discussion

Despite treatment, fungal endophthalmitis results in loss of useful vision in a majority of patients. New antifungal drugs with good ocular penetration would be a welcome addition to our therapeutic armamentarium. In spite of its established efficacy for the treatment of a broad range of infections due to *Candida* and *Aspergillus* spp., caspofungin has only rarely been used to treat fungal infections of the eyes and no clinical studies have been done [1, 16, 24].

In this study we aimed to characterize the penetration into various ocular compartments of systemically administered caspofungin in a rabbit uveitis model. For that purpose, adequate assays had to be at hand. Several HPLC assays for the measurement of caspofungin concentrations in plasma, urine, and various tissues have been described in the literature [3, 11, 12, 17, 19]. No assay for the determination of caspofungin in ocular tissues has been found. The HPLC method of Schwartz et al. [19] was adapted to quantify caspofungin in samples of aqueous, vitreous, cornea, and

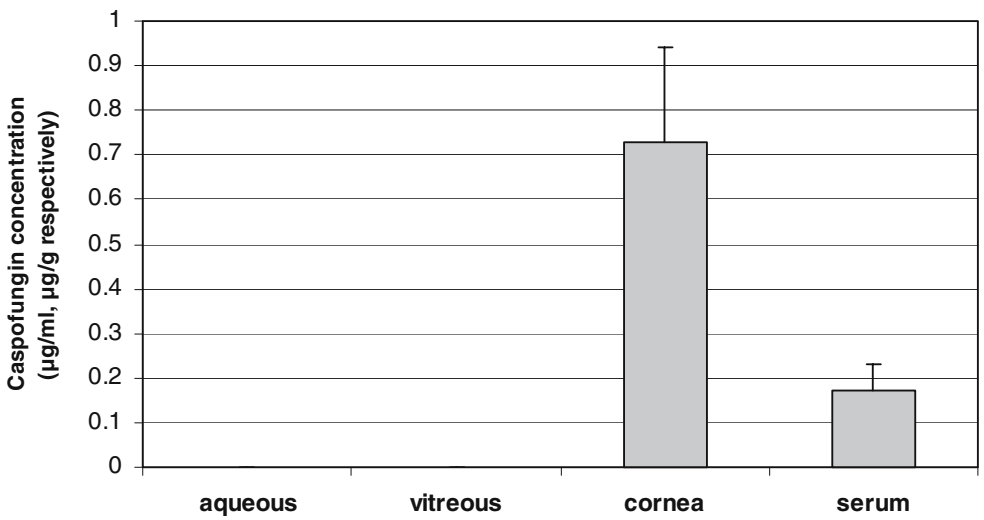


Fig. 3 Mean (+SD) caspofungin concentrations in aqueous, vitreous, and corneas of inflamed eyes as well as serum after repeated-dose administration. Samples were taken 24 h after the last of seven daily doses of 1 mg/kg caspofungin administered via a central venous catheter ($n=4$). Drug levels were undetectable in the aqueous and the vitreous

serum/plasma. The assays, which were found to be sensitive and robust, are all based upon the use of a C8 column and mobile phases composed of an aqueous, 0.1% trifluoroacetic acid solution at pH 3, and acetonitrile, but differ in sample preparation. Solid phase extraction with C18 cartridges is employed for serum analysis, whereas protein precipitation with acidified methanol is used for the determination of caspofungin in aqueous, vitreous, and cornea. The lower limit of quantification (LLQ) of our assay for serum and plasma was 0.1 μg/ml and was significantly lower for ocular tissues. This compares favorably with the sensitivity of published HPLC assays with LLQs for plasma of 0.15 μg/ml [11] and 0.125 μg/ml [17]. In the paper by [19] a diol solid phase extraction column was used to extract caspofungin from human plasma and urine. In our laboratory, the use of such a polar sorbent did not reveal any recovery for the investigated cyclic hexapeptide. With a C18 cartridge, however, a respectable recovery was obtained. Furthermore, there was no difference between plasma and serum.

In inflamed eyes caspofungin concentrations exceeding 1 μg/ml were measured in the aqueous during the first 8 h postdosing and in the cornea for 24 h after single-dose administration. These concentrations exceed the minimal inhibitory concentrations of most *Candida* and *Aspergillus* spp. and are associated with relevant antifungal activity in animal models [15].

Corneal concentrations in inflamed eyes substantially exceeded both serum and aqueous levels: they peaked at 8 h and remained above 1.4 μg/ml for 24 h. The absence of detectable drug levels from corneas of uninflamed eyes suggests that disruption of the blood-eye barrier is as critical for corneal penetration as for other ocular compartments. The same observation also makes it improbable that significant amounts of drug might have penetrated through the tear film although drug concentrations in tears were not established.

In murine models, the kidneys, liver, and large intestine were found to have higher exposure levels to caspofungin than plasma and serum [12, 15]. Differences in the extent of protein binding may explain the high corneal drug concentrations we found. Retention of the water-soluble caspofungin macromolecule in the proteoglycans of the corneal stroma may be another reason for corneal drug accumulation.

Caspofungin levels in the vitreous were undetectable in all animals after single-dose administration and in all but one after repeated dosing. Thus, even in the presence of a disrupted blood-eye barrier caspofungin appears to poorly penetrate into this compartment. A similar finding was recently reported in a patient whose *Candida albicans* endophthalmitis failed to respond to caspofungin. After 9 days of therapy, the level of caspofungin in the vitreous of his infected eye was below 50 ng/ml while his blood level at the time of vitrectomy was 3.3 μg/ml [8].

Using caspofungin at higher doses than currently recommended might result in higher intraocular drug deposition and possibly increased efficacy; reports on paradoxical effects of increased caspofungin doses in animal models, however, warrant caution with this approach [21, 22, 27].

The serum concentrations of caspofungin we measured at 4 and 8 h postdosing are in good agreement with reported results for rabbit plasma [11]. This is also true for the slight drug accumulation we observed in serum following administration over 7 days. Compared to serum drug levels we found ocular caspofungin concentrations to decline more slowly, an observation that is consistent with the reported plasma pharmacokinetics of caspofungin that are largely determined by tissue distribution [12].

The unilateral uveitis model we used allows for the direct observation, in the same animal, of the influence on drug disposition of a disrupted blood-eye barrier. With a sensitive HPLC assay drug levels were undetectable in aqueous, vitreous, and cornea of non-inflamed eyes. Our findings suggest that caspofungin does not penetrate the intact blood-eye barrier. This may be explained in part by the drug's high molecular weight and its level of protein binding in serum of 96% [12].

Repeated dosing was not found to lead to higher intraocular drug concentrations. On the contrary, caspofungin levels were lower in both aqueous and cornea than at the same interval after single-dose administration. This may be due to a decreasing intensity of LPS-induced uveitis over time in spite of the fact that higher endotoxin doses were used in the multiple-dose study [10]. There is no evidence that in the absence of fungal infection caspofungin may directly influence the host's immune response and thus modulate the intensity of, e.g., iatrogenic uveitis.

In established fungal endophthalmitis the treatment of choice is vitrectomy followed by local injection of an antifungal agent, usually amphotericin B. In endogenous endophthalmitis that results from hematogenous fungal dissemination, additional systemic treatment is indicated. In cases where vitrectomy must be postponed or where it may be contraindicated, control of endophthalmitis must rely on the local effects of systemically administered antifungals [2]. The poor vitreal penetration of caspofungin in our experimental system suggests it may be of limited usefulness in the treatment of endogenous endophthalmitis where hematogenously disseminated fungal elements reach the vitreous first, after establishing infection in the choroid.

At present, the i.v. administration of an antifungal agent is not standard for the treatment of fungal keratitis. Nevertheless, many clinicians use adjuvant systemic treatment to prevent spread into the anterior chamber and progression to exogenous endophthalmitis. In this challenging clinical situation there might only be minimal

intraocular inflammation. Because caspofungin only reaches potentially therapeutic concentrations in cornea and aqueous humor when the blood-eye barrier is disrupted, its role as preemptive treatment in exogenous fungal endophthalmitis may be equally limited.

Caution is warranted in extrapolating the results of this rabbit model of iatrogenic uveitis to human fungal endophthalmitis. While caspofungin has similar pharmacokinetics in humans and rabbits and the rabbit model is of established relevance for human eye diseases, the disruption of the blood-eye barrier resulting from endotoxin-induced uveitis may differ from that found in fungal endophthalmitis [17].

In summary, our model suggests that caspofungin ocular penetration is critically dependent on disruption of the blood-eye barrier. This may limit its usefulness in fungal endophthalmitis. Studies in animals with fungal endophthalmitis are needed to further evaluate the drug's potential.

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