Principle *in vitro* microdialysis investigation to characterise the feasibility for antifungals

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
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from Bad Nauheim

2018

Diese Arbeit wurde von März 2014 bis August 2018 unter der Leitung von Frau Prof. Dr. rer. nat. habil. Charlotte Kloft am Institut für Pharmazie der Freien Universität Berlin angefertigt.

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Disputation am	15. November 2018

Für meine Oma Elli

Abstract

Microdialysis represents a minimally invasive sampling technique to collect samples and also to administer drugs at target site, which was first introduced in 1974 by Ungerstedt and Pycock to sample endogenous dopamine from rat brain [1]. Until then, the only methods available to obtain *in vivo* concentrations of analytes were tissue destroying or only possible with a limited number of repetitions of sample taking. In contrast, since the method of microdialysis was introduced, the advantages over more invasive methods due to its minimally invasive character were repeatedly shown in clinical studies. In addition, microdialysis enables the continuous sampling of analytes over a predefined time interval, allowing the determination of concentration-time profiles of analytes over hours and even days without loss of body fluids, associated with other sampling methods. Moreover, samples can be taken directly from the target site (for example of an infection) located in virtually every tissue. Especially in the research field of infectious diseases the method is often used, particularly when investigating the sources of antimicrobial resistance. At present, research tackling antimicrobial resistance is not primarily focussing on bacterial or viral pathogens any more, but gradually shifting its focus also towards fungal infections. These infections are on the rise but still remain underestimated in comparison to the threads caused by bacteria.

Since microdialysis is an important technique for sampling pharmacologically active analytes, the number of *in vivo* clinical studies using microdialyis increased. However, thorough *in vitro* investigations to characterise the analyte and ensure optimal study conditions prior to *in vivo* studies are often missing/not performed. Due to the significance of these *in vitro* investigations and rising importance of antifungal research, the two first-line antifungals anidulafungin and voriconazole were investigated with the static and dynamic *in vitro* microdialysis system in the present thesis. Thus, in a first step a bioanalytical assay for the quantification of anidulafungin samples from *in vitro* microdialysis was developed and validated. A previous bioanalytical assay [2] for quantitative analysis of voriconazole *in vitro* samples from microdialysis, was further revised and validated. Both assays, for anidulafungin and voriconazole, were validated according to the Guideline on bioanalytical method validation [3].

After development of the bioanalytical assay, *in vitro* microdialysis of anidulafungin was performed. As described for *in vitro* microdialysis investigations, the aim was to investigate and proof the feasibility of anidulafungin for microdialysis as a prerequisite for potential *in vivo* clinical studies. Anidulafungin was found to adsorb on catheter material in the static *in vitro* microdialysis system and therefore to bias the forthcoming results. In order to enable *in vivo* investigations despite adsorption, various parameters were investigated, for example catheter design (20 kDa or 100 kDa cut-off),

perfusate composition (different combinations of Ringer's solution and human serum albumin, dextran) and pre-coating of catheters with caspofungin or anidulafungin. The investigations ultimately resulted in the recommendation to perform *in vivo* microdialysis after an equilibration time of at least 3 h in steady state at the target site to allow for equilibration of the adoption process with a perfusate of Ringer's solution and human serum albumin (0.5%) in catheters with 100 kDa cut-off.

Previous investigations on voriconazole using static *in vitro* microdialysis by Simmel *et al.* resulted in a relative recovery of approximately 100% [4]. To verify the results, a shortened *in vitro* microdialysis investigation was performed with the static *in vitro* microdialysis system. The dependence of relative recovery and relative delivery on flow rate and independence of relative recovery and relative delivery on concentration was investigated with the static microdialysis system. Since voriconazole is an easy to handle drug and the results for relative recovery were also close to 100%, it is an ideal drug for the first investigations with the dynamic *in vitro* microdialysis system. Thus, the developed bioanalytical methods and the static *in vitro* microdialysis system were used to develop a dynamic *in vitro* microdialysis system to mimic concentration-time profiles of analytes, to gain further knowledge of the microdialysis specific characteristics of the analyte *in vivo*. The validation of the system demonstrated that it is possible to perform *in vitro* microdialysis with this model. First, concentration-time profiles of anidulafungin and voriconazole were predicted *in silico,* based on *in vivo* data from clinical studies. Then, the concentration-time profiles were mimicked *in vitro* with the dynamic microdialysis system. Finally, microdialysis with subsequent calibration by retrodialysis of the single drugs was performed. The resulting microdialysis concentration-time profiles for voriconazole had a high accuracy compared to the concentration in the medium mimicking the tissue, whereas experiments with anidulafungin resulted in a misleading prediction of concentrations due to adsorption of the analyte on catheter material.

During this work, it was emphasised that it is indispensable to perform adequate *in vitro* microdialysis before starting *in vivo* studies. Apart from experiments with the static *in vitro* microdialysis system on flow rate, concentration and perfusate also investigations based on concentration-time profiles of the analyte (i.e. decision about calibration technique) should be conducted for a more detailed characterisation of the analyte of interest and thus reliable clinical results.

Zusammenfassung

Bei der Mikrodialyse handelt es sich um eine minimalinvasive Technik zur Probennahme sowie zur Applikation von Arzneistoffen am Wirkort, die erstmals 1974 von Ungerstedt und Pycock vorgestellt wurde, um Proben von endogenem Dopamin im Gehirn von Ratten zu gewinnen [1]. Bis zu diesem Zeitpunkt waren die einzigen zur Verfügung stehenden Methoden, um *in vivo* Konzentrationen der Analyten zu erhalten, invasiver Natur bzw. Gewebe zerstörend oder die Probennahme war nur begrenzt wiederholbar. Seit die Methode der Mikrodialyse eingeführt wurde, wurde der Vorteil über invasivere Methoden durch ihren minimal invasiven Charakter wiederholt in klinischen Studien bewiesen. Zusätzlich ermöglicht die Mikrodialyse das kontinuierliche Sammeln von Analyten über ein vorher definiertes Zeitintervall, und erlaubt die Bestimmung von Konzentrations-Zeit Profilen von Analyten über Stunden und sogar Tage ohne gleichzeitigen Flüssigkeitsverlust, welcher beispielweise mit anderen Probenahme-Methoden assoziiert ist. Darüber hinaus können Proben direkt am Wirkort (z.B. einer Infektion) aus nahezu jedem Gewebe gewonnen werden. Insbesondere auf dem Forschungsgebiet der Infektionskrankheiten wird die Methode häufig eingesetzt, vor allem wenn es um die Erforschung der Herkunft antimikrobieller Resistenzen geht. Zurzeit richtet sich der Fokus der Forschung zur Bekämpfung der antimikrobiellen Resistenz nicht mehr nur auf bakterielle oder virale Krankheitserreger, sondern verschiebt sich auch in Richtung Pilzinfektionen. Diese Infektionen werden aber noch immer unterschätzt im Vergleich zu den Bedrohungen verursacht durch Bakterien.

Infolge der gewachsenen Bedeutung der Mikrodialyse als wichtige Technik zum Sammeln von pharmakologisch aktiven Analyten ist die Zahl der *in vivo* klinischen Studien gestiegen, die die Mikrodialyse nutzen. Allerdings wurden vor dem Start der *In-vivo*-Studien gründliche *In-vitro*-Experimente, um den Analyten zu charakterisieren und optimale Studienbedingungen zu gewährleisten, oftmals nicht durchgeführt.

Aufgrund der Unerlässlichkeit dieser *In-vitro*-Experimente und der zunehmenden Bedeutung antimykotischer Forschung wurden die zwei Antimykotika zur Primärbehandlung, Anidulafungin und Voriconazol, mit dem statischen und dem dynamischen *In-vitro*-Mikrodialysesystem in der vorliegenden Dissertation untersucht. Dazu wurde in einem ersten Schritt ein bioanalytischer Assay für die Quantifizierung von Anidulafungin *In-vitro*-Mikrodialyseproben entwickelt und validiert. Ein vorhergehender bioanalytischer Assay [2] für die Quantifizierung von Voriconazol *in vitro* Mikrodialyseproben wurde überarbeitet und validiert. Beide Assays, für Anidulafungin und Voriconazol, wurden gemäß der Guideline für bioanalytische Methodenvalidierung [3] validiert. Nach der Entwicklung des bioanalytischen Assays für Anidulafungin wurde die neue *in vitro* Mikrodialyse-Methode durchgeführt.

Das Ziel war, die Durchführbarkeit der Mikrodialyse von Anidulafungin als Voraussetzung für mögliche *in vivo* klinische Studien zu untersuchen. Es wurde festgestellt, dass Anidulafungin an Katheter-Material des statischen *In-vitro*-Mikrodialysesystems adsorbiert und dadurch die zukünftigen Ergebnisse verfälschen würde. Um *In-vivo*-Untersuchungen trotz der Adsorption zu ermöglichen, wurden eine Vielzahl an Parametern untersucht, z.B. das Design des Katheters (20 kDa oder 100 kDa Cut-off), die Zusammensetzung des Perfusats (verschiedene Kombinationen von Ringer-Lösung mit humanem Serumalbumin, Dextran) und die Vor-Benetzung der Katheter mit Caspofungin oder Anidulafungin. Die Untersuchungen resultierten schließlich in der Empfehlung, die *In-vivo*-Mikrodialyse nach einer Equilibrierung von mindestens 3 Stunden im Konzentrations-Gleichgewicht (steady state) am Wirkort durchzuführen, um die Anpassungsprozesse des Perfusats aus Ringer-Lösung und humanem Serumalbumin (0.5%) in Kathetern mit 100 kDa Cut-off ins Gleichgewicht zu bringen.

Vorhergehende Untersuchungen von Voriconazol mit der statischen *In-vitro*-Mikrodialyse von Simmel *et al.* ergaben eine Relative Recovery von annähernd 100% [4]. Um die Ergebnisse zu überprüfen, wurde eine komprimierte *In-vitro*-Mikrodialyse-Untersuchung mit dem statischen *In-vitro*-Mikrodialysesystem durchgeführt. Die Abhängigkeit der Relative Recovery und Relative Delivery von der Flussrate und die Unabhängigkeit der Relative Recovery und Relative Delivery von der Konzentration wurden mit dem statischen Mikrodialysesystem untersucht. Aufgrund der einfachen Handhabung von Voriconazol und dem Ergebnis der Relative Recovery von nahezu 100%, ist Voriconazol ein idealer Arzneistoff für die ersten Untersuchungen mit dem dynamischen *In-vitro*-Mikrodialysesystem. Die entwickelten bioanalytischen Methoden und das statische *In-vitro*-Mikrodialysesystem wurden genutzt, um ein dynamisches *In-vitro*-Mikrodialysesystem zu entwickeln, welches die Konzentrations-Zeit-Profile von Analyten nachahmen kann, damit zusätzliches Wissen über die *In-vivo*-Mikrodialyse spezifischen Charakteristika des Analyten gewonnen werden können. Die Validierung des Systems zeigte, dass es möglich ist, mit diesem dynamischen Modell *In-vitro*-Mikrodialyse durchzuführen. In einem ersten Schritt wurden basierend auf *In-vivo*-Daten klinischer Studien, Konzentrations-Zeit-Profile von Anidulafungin und Voriconazol *in silico* simuliert. Im nächsten Schritt wurden die Konzentrations-Zeit-Profile *in vitro* mit dem dynamischen Mikrodialysesystem nachgeahmt. Schließlich wurden Mikrodialyse-Untersuchungen mit anschließender Kalibrierung mittels Retrodialyse der jeweils einzelnen Arzneistoffe durchgeführt. Die resultierenden Konzentrations-Zeit-Profile von Voriconazol hatten eine hohe Übereinstimmung verglichen mit der Konzentration im Medium, die die Gewebskonzentrationen nachahmte, wohingegen Experimente mit Anidulafungin in einer falschen Vorhersage der Mediumskonzentration resultierten.

In dieser Arbeit wurde die Notwendigkeit der Durchführung von entsprechenden *In-vitro*-Mikrodialyse-Untersuchungen gezeigt, welche unbedingt vor *In-vivo*-Studien mit der Mikrodialyse stattfinden sollten; neben den Experimenten des statischen *In-vitro*-Mikrodialysesystems zur Flussrate, Analyt-Konzentrationen und zum Perfusat, sollten für eine detailliertere Charakterisierung des Analyten auch Untersuchungen basierend auf Konzentrations-Zeit-Profilen des Analyten (d.h. die Entscheidung über die Kalibriertechnik) durchgeführt werden, um anschließend zuverlässige klinische Ergebnisse zu erhalten.

Contents

Abbreviations

Abbreviation	Definition
ACN	Acetonitrile
AFG	Anidulafungin
AU	Arbitrary unit
BSA	Bovine serum albumin
C_{AFG}	Anidulafungin concentration
Cal	Calibration sample
CFG	Caspofungin
CI	Confidence interval
C_{ISF}	Interstitial space fluid concentration
$C_{IV\ bolus\ injection}$	Concentration of intravenous bolus injection
CL	Clearance
C_{max}	Maximum concentration
$C_{max,total}$	Maximum total concentration
$C_{max,total/unbound}$	Maximum total or unbound concentration
$C_{max,unbound}$	Maximum unbound concentration
C_{Medium}	Medium concentration
$C_{\mu Dialysate}$	Microdialysate concentration
C_{nom}	Nominal concentration
C_{obs}	Observed concentration
$C_{Perfusate}$	Perfusate concentration
CRRT	Continuous renal replacement therapy
CV	Coefficient of variation
C_{VOR}	Voriconazole concentration
dIVMS	Dynamic *in vitro* microdialysis system
EMA	European Medicine Agency
FDA	Food and Drug Administration
FEP	Fluorinated ethylene propylene
FR	Flow rate
HPLC	High-performance liquid chromatography

HSA	Human serum albumin
IFI	Invasive fungal infection
IL	Interleukin
ISF	Interstitial space fluid
i.v.	Intravenous
IVMS	*In vitro* microdialysis system
KDR	Kinase insert Domain-containing Receptor
LLOQ	Lower limit of quantification
m	Mass
M_r	Relative molecular mass
$m_{\mu Dialysate}$	Microdialysate mass
µDialysate	Microdialysate
µDialysis	Microdialysis
n	Number
n_{AFG}	Number of anidulafungin samples
$n_{in+outflow}$	Number of in- and out-flow samples
n_{tubing}	Number of tubings
n_{VOR}	Number of voriconazole samples
p	Probability value
PAES	Polyarylethersulfone
PD	Pharmacodynamic(s)
PK	Pharmacokinetic(s)
PP	Polypropylen
PPB	Plasma protein binding
PR	Pump rate
PUR	Polyurethane
PVC	Polyvinyl chloride
QC	Quality control sample
R	Range
R^2	Coefficient of determination
rD	Relative delivery
RE	Relative error
RR	Relative recovery

RR_{max}	Maximum relative recovery
RR_t	RR at a specific time point t
$RR_{Volume\text{-}corrected}$	Volume-corrected relative recovery
RS	Ringer´s solution
SD	Standard deviation
sIVMS	Static *in vitro* microdialysis system
SP-D	Surfactant Protein D
TDM	Therapeutic drug monitoring
TNF	Tumor necrosis factor
$t_{RR,50}$	Time until 50% of RR_{max} is reached
$t_{RR,50}$	Time until 90% of RR_{max} is reached
UF	Ultrafiltration
ULOQ	Upper limit of quantification
$V_{\mu Dialysate}$	Volume of microdialysate
$V_{IV\ bolus\ injection}$	Volume of intravenous bolus injection
V_{medium}	Volume of medium
VOR	Voriconazole

Symbols	Definition
k_e	Elimination rate constant
ρ_{H2O}	Density of water
$t_{1/2}$	Half-life
$\bar{x}$	Arithmetic mean
x_i	Independent variable
y_i	Dependent variable

1 Introduction

1.1 The fight against fungal infections starts *in vitro*

Today, the most important global health challenge constitutes of fighting antimicrobial resistance, which is supported by the recent UK Review on Antimicrobial Resistance, predicting kills from antimicrobials to be the leading cause of death in 2050 [5]. When talking about antimicrobial resistance, it is nowadays not only sufficient to mention bacteria or viruses, but also fungi have to be taken into account [6]. Fungal infections are on the rise but still remain underestimated in comparison to the threads caused by bacteria. In addition, the treatment of fungal infections often starts delayed due to complicated diagnosis: Fungal infections reveal only unspecific symptoms and detection of fungi based on blood cultures takes often 2-5 days [7]. Inappropriate use of antifungal drugs has contributed to the increase in global antifungal resistance [8]. Furthermore, the extended use of antifungal therapy may also contribute to increased toxicity associated with unnecessary medication exposure but also rising costs [8]. Especially older-generation antifungals like amphotericin B and azoles are questioned regarding their toxicity to the host, the patient [9]. Recently there are proposals for establishing antifungal stewardship, which does not only aim at the improvement of diagnosis, but also promotes the use of therapeutic drug monitoring (TDM) for applied antifungals [8]. Routinely, TDM samples are taken from the patient's blood circulation and plasma concentrations are determined. Of course, plasma does not always reflect the target site of the respective drug, or in case of fungal infections more precisely, where the fungal infection occurs. For this reason, more sophisticated methods such as the minimally invasive technique of microdialysis (µDialysis) should be used to collect analytes at the site of action. Just recently, the European Medicines Agency (EMA) recommended to assess, in addition to plasma concentrations, the total and unbound drug concentrations of antibiotics in non-homogenate tissues, which can be implemented with µDialysis [10]. Crucial for the clinical application of the method is the previous investigation of the drug of interest in an *in vitro* µDialysis setting. Working groups specialised in the µDialysis technique extensively investigate the feasibility of µDialysis for a broad range of drugs to ultimately gain more insight into target-site exposure. This insight can contribute to gain more understanding of target-site pharmacokinetics (PK) and pharmacodynamics (PD) and to foster the fight against fungal infections and fungal resistance, to lower the patients' burden such as toxicity associated with antifungal therapy and overall to contain the expansion of hospitalisation, morbidity and mortality.

1.2 Microdialysis

The technique of µDialysis was the first minimally invasive technique for sampling analytes at the target site (possible site of infection) [1]. The first samples were taken of dopamine, a neurotransmitter, in rat brain in 1974 [1].

Additionally, the technique of µDialysis can be used for delivery of drugs. One of the first experiments was the delivery of quinolinic acid to the striatum in rats [11]. Before the minimally invasive technique of µDialysis was invented and disseminated, widespread techniques for tissue sampling were available: (i) tissue biopsy, an invasive method, which can only determine the analyte concentration at one single time point from a tissue sample after homogenisation; (ii) skin blister fluid, a method which uses a compartment, that is created by separating the epidermis from the dermis and filled with fluid, as surrogate for interstitial concentration of the dermis; (iii) complex non-invasive imaging techniques such as positron emission tomography or magnetic resonance spectroscopy [12–15]. By now, the American Food and Drug Administration (FDA) addressed the importance of gaining more insight regarding the drug's target sites [16] and the EMA recommended to assess drug concentrations in non-homogenate tissue using µDialysis (see chapter 1.1).

1.2.1 Principle of microdialysis

The technique of µDialysis is based on the principle that unbound analytes (e.g. endogenous substances, such as neurotransmitters, or drugs) are able to diffuse, driven by a concentration gradient, from the interstitial space fluid (ISF) at the target site through a semi permeable membrane (with a defined cut-off, which defines the approximate pore size of the membrane) into a µDialysis catheter containing analyte-free perfusate (Fig. 1.2-1). Samples are collected over a defined sampling interval. Due to the continuous flow, (caused by a pump and adjusted by flow rate (FR)) of perfusate inside the catheter, the drug concentration will never reach an equilibrium on both sides of the membrane. Hence, only a fraction of the target-site concentration is collected and sample concentrations only represent a fraction of the actual concentration of the respective analyte at the target site. Actual target-site concentrations can be obtained by different calibration methods of the catheter (see chapter 1.2.2). Due to the size-restrictions of the membrane (mainly M_r of 20 kDa), only the unbound (protein-free) analyte is sampled, which is a major advantage as the unbound analyte, or in this case, the unbound drug, is the pharmacodynamic active compound [12]. There are several different catheter types with varying cut-offs (6 kDa – 1000 kDa) [17,18], varying materials and different areas of application, e.g. for pre-clinical or clinical use, and for a multitude of tissues (brain, heart, eye, liver, skin, skeletal muscle, subcutaneous adipose tissue, blood etc.) [12–15]. In general, it is assumed that only molecules smaller than the molecular weight cut-off of the semi permeable

membrane can pass the membrane, whereby the cut-off is defined as the molecular mass of a molecule at which 80% of the molecules are prevented from diffusing through the membrane [15]. To assure diffusion of a specific molecule, the molecular mass of the analyte molecule should be smaller than one-fourth of the membrane cut-off [14].

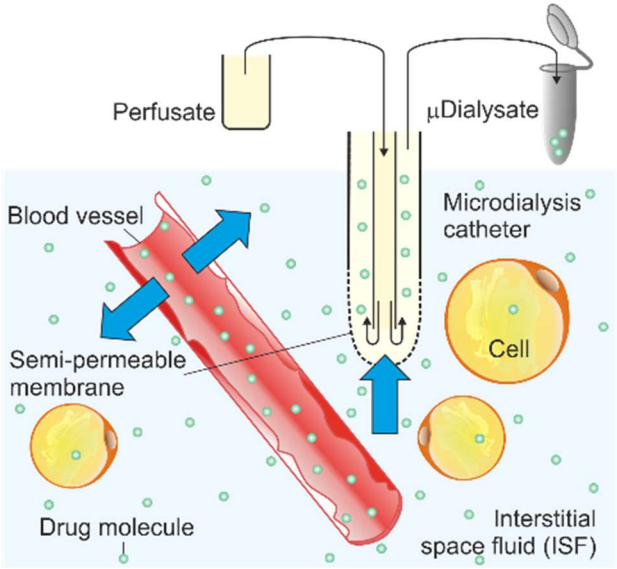

Fig. 1.2-1: The principle of microdialysis is based on the diffusion of a drug molecule, driven by a concentration gradient, through a semi permeable membrane into a catheter containing analyte-free perfusate, representing an analyte fraction of the interstitial space fluid.

1.2.2 Calibration of microdialysis catheters

To determine the analyte concentration at the target site via µDialysis an adequate calibration method is needed. There are several calibration methods used for clinical studies like the flow-rate variation method, the no-net-flux method, the dynamic-no-net-flux method, the retrodialysis method and the calibration method using endogenous compounds [14,19]. The **flow-rate variation method** is based on the variation of the perfusate flow-rate. Since the relative recovery (RR) is dependent on the flow-rate and a flow-rate of zero is considered to yield a RR of 100%, the concentration of the sample (µDialysate) represents the concentration at the target site. For *in vivo* calibration different flow rates and the respective concentrations in µDialysate are plotted and extrapolated to zero flow [20]. The **no-net-flux method** makes use of differing perfusate concentrations of the analyte of interest. In case, perfusate and target site concentrations are similar, there is no concentration gradient at the semi permeable membrane and concentrations in µDialysate are equal to perfusate concentrations. If the concentration in perfusate is higher than at the target site, analyte molecules will diffuse from perfusate to the target site and the analyte concentration in µDialysate will decrease. In contrast, if the analyte concentration at the target site is higher than in perfusate, the concentration in µDialysate will increase [21]. To determine the target-

site concentration, the difference of concentration between µDialysate and perfusate is plotted against the concentration in perfusate and the function will cross the x-axis where the perfusate concentration equals the ISF concentration. A more advanced approach is the **dynamic-no-net-flux method**. Using this calibration method, catheters of three to four individuals are perfused with different concentrations, only a single concentration per individuum, in perfusate and the corresponding data is pooled afterwards [22]. A common and widely-used calibration method is the **retrodialysis,** which is based on the principle that diffusion of equal concentrations of an analyte through the semi permeable membrane in both directions is considered to be equivalent [23]. It is important to emphasise that the retrodialysis must be performed for each µDialysis catheter separately, yielding a unique calibration factor per catheter. In the µDialysis setting, the fraction of the analyte concentration at the target site that is afterwards determined in the µDialysate-containing microvial ($C_{\mu Dialysate}$), is referred to as the relative recovery, usually expressed as RR. The *in vitro* RR is calculated using the following equation, taking the actual target-site concentrations into account (Eq. 1-1):

$$RR\ (in\ vitro), \% = \frac{C_{\mu Dialysate}}{C_{Medium}} \cdot 100 \qquad \text{Eq. 1-1}$$

It is assumed that the relative delivery (rD), as described in Eq. 1-2, is the equivalent to the RR in the retrodialysis setting and represents the fraction of a known total analyte concentration, which diffuses from analyte-containing perfusate ($C_{Perfusate}$) through the semi permeable membrane to the drug-free target site (Fig. 1.2-2).

$$rD\ (in\ vitro), \% = 100 - \left(\frac{C_{\mu Dialysate}}{C_{Perfusate}} \cdot 100\right) \qquad \text{Eq. 1-2}$$

As a prerequisite for the conduction of retrodialysis experiments for catheter calibration, it must be ensured that the target site is free of analyte before the calibration procedure starts. This means that calibration should be performed before the actual investigation starts. In case that calibration should be performed during retrodialysis at steady-state it is recommended that, $C_{Perfusate}$ is at least a tenfold of desired steady-state C_{ISF} during retrodialysis. Accordingly, based on the assumption of an *in vivo* steady-state concentration, the concentration of the analyte in perfusate during retrodialysis should be verified before in an *in vitro* setting [14].

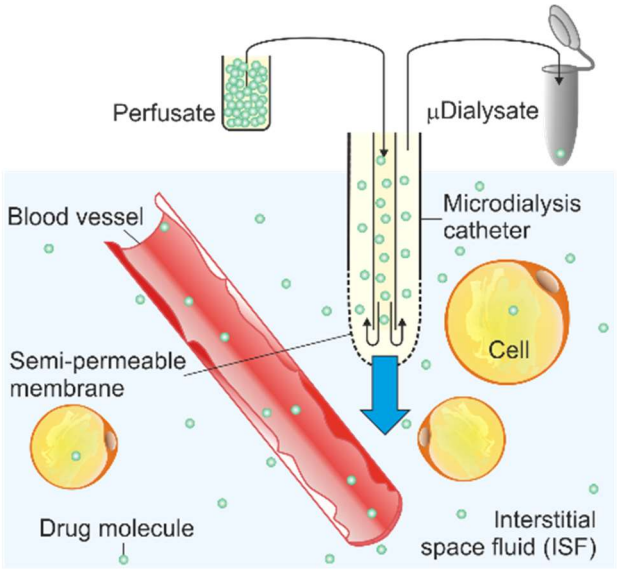

Fig. 1.2-2: The principle of retrodialysis is based on the diffusion of a drug molecule, driven by a concentration gradient, through a semi permeable membrane from analyte containing perfusate into the analyte-free interstitial space fluid.

For *in vivo* catheter calibration in the retrodialysis setting, where the actual target-site C_{ISF} is unknown, the following equation is applied to determine the RR (Eq. 1-3):

$$RR\ (in\ vivo), \% = 100 - \left(\frac{C_{\mu Dialysate}}{C_{Perfusate}} \cdot 100\right) \qquad \text{Eq. 1-3}$$

After determining the RR using retrodialysis *in vivo*, the actual C_{ISF} of analyte can be calculated with Eq. 1-4.

$$C_{ISF} = 100 \cdot \left(\frac{C_{\mu Dialysate}}{RR, \%}\right) \qquad \text{Eq. 1-4}$$

Another, but controversially discussed approach is the calibration with an **endogenous reference substance**. RR is not obtained for the analyte of interest, but the RR of an endogenous reference substance is measured, which has comparable physicochemical properties to the analyte of interest. The endogenous reference substance was previously analysed *in vitro* and *in vitro* results are being transferred to *in vivo* (behaviour of reference substance is required to be the same *in vitro* as *in vivo*). Controversial results on urea as reference substance are discussed in the literature [24,25].

Regardless of the method of choice for determining RR, there are several factors affecting the RR, as described in a review of Plock *et al.* [14]. **Velocity of diffusion processes**, **flow rate**, **composition of perfusate** and **tortuosity of sample matrix** (e.g. ISF) should be mentioned in particular. The target-site conditions, such as tortuosity, can only be influenced to some extent, whereas the velocity of the diffusion process of the analyte is dependent on easily adaptable factors, such as temperature (ideally body temperature of 37°C), the molecular weight cut-off of the semi permeable membrane, its length and surface area and the concentration gradient at the membrane. Moreover, also the FR

and the composition of the perfusate can be modified. However, at least for *in vivo* studies, the composition of the perfusate is strictly limited due to physiological compatibility. In addition physical interactions between the analyte and the membrane can occur, and endogenous influences, such as the blood flow rate to and from the respective tissue and possible metabolism, have to be taken into account [13]. Furthermore, the uptake of the analyte into cells and also to an extent tissue vascularisation can have high impact on RR [26]. Ståhle *et al.* concluded that RR from *in vitro* investigations cannot be transferred to *in vivo* studies [27], which is a prominent question, since calibration is a time-consuming step and *in vitro* calibration could minimise the efforts during *in vivo* µDialysis studies.

1.2.3 Static *in vitro* microdialysis system

The static *in vitro* µDialysis system (sIVMS) is composed of pumps used *in vitro* or *in vivo*, microsyringes, µDialysis catheters (usually in *in vitro* settings called "probe" and in *in vivo* settings called "catheter") and medium containers, containing the membranes during investigations. To enable the comparison of results from *in vitro* investigations with *in vivo* studies, physiological conditions were as much as possible mimicked during *in vitro* investigations using tempered medium vessels, stirring of medium fluid and a physiological-like medium fluid. The temperature of the medium fluid was generally set to 37°C body temperature. To mimic the flow of analytes at the target site *in vivo*, the medium was stirred *in vitro* with a magnetic stir bar and a magnetic stirrer. As the exact composition of the target-site fluids is mostly unknown, the physiological-like composition of the medium is only an approximation of the *in vivo* composition and due to the limited exemplary nature of *in vitro* investigations, only fluid liquids were used for the simulation of the target site [14,26,28]. Still, a major advance for this work was the preliminary development of a standardised sIVMS by Simmel *et al.* [28], i.e. a standardised experimental setup and all previously discussed physiological-like features to guarantee reproducible results of *in vitro* investigations.

To perform *in vitro* µDialysis prior to *in vivo* clinical studies was found to be crucial [29], especially if specific analytes had not been characterised by any µDialysis investigations before. First, it is an essential prerequisite to find out whether the analyte can pass the semi permeable membrane [30], otherwise *in vivo* results from clinical studies are interpreted incorrectly. Second, it can be investigated if adsorption of the analyte on catheter material (tubing, membrane) occurs, if there are any time delays in analyte movement and if for example retrodialysis would be an adequate calibration technique, by examining and comparing RR and rD of the analyte. Although results from *in vitro* investigations yield important information for the proceeding *in vivo* µDialysis experiments, it has to be emphasised that the results of *in vitro* investigations, especially for retrodialysis and calibration methods in general, cannot directly be transferred to *in vivo* behaviour of the analyte.

Especially the fact that the diffusion processes at the target site in the respective tissue fluid are not comparable to *in vitro* medium fluid movements should be considered. The effect of an altered diffusion (diffusional resistance) of the analyte in extracellular matrix, strongly affects to a large extent RR and rD in comparison to *in vitro* results [31]. Furthermore, the composition of the perfusate and the *in vitro* membrane-surrounding medium remains challenging. Still, Ringer's solution (RS) or phosphate buffered saline are used as aqueous solutions for perfusate and medium, limiting the technique to drugs sufficiently soluble in these fluids [15]. Finally, a major limitation of the sIVMS is the fact that the concentration over time in medium is constant, opposite and therefore not comparable to the pharmacokinetic profiles *in vivo*. Hence, a dynamic experimental model for µDialysis should be initiated.

1.2.4 *In vitro* microdialysis of experimentally challenging drugs

A major challenge next to handling analytes (e.g. collecting, quantifying) is the interpretation of the bioanalytical results. In general, it is crucial to exclude potential experimental bias prior to *in vivo* studies. Hence, as previously described before *in vitro* µDialysis investigations should be conducted prior to *in vivo* experiments. Especially before starting *in vivo* µDialysis of a new analyte, there should be prior *in vitro* investigation performed to study the influence of FR or differing analyte concentrations, to choose the appropriate calibration method, to determine the range of relative recovery of the analyte and to detect potential adsorption, e.g. to surfaces of catheters. In the literature, different methods to reduce adsorption have been previously described, such as addition of adjuvants like albumin [32], beta-cyclodextrin [33], polysorbate [34,35] or of the analyte of interest itself, which was used for saturation of the catheter material prior to recovery investigations [36]. Frequently encountered problems during µDialysis, including low recovery of analytes or biased results due to adsorption are commonly found throughout the literature and not only seen in the field of µDialysis. For instance complications due to low recovery of analytes altering the quantification of analytes from urine samples are frequently discussed [37], which require similar solution strategies like in µDialysis.

An exemplary case described in literature is a Kinase insert Domain-containing Receptor (KDR) kinase inhibitor with a low analyte recovery - due to adsorption to sampling and handling material - from urine samples [37]. It seems that in plasma, the plasma proteins (6%-8%) were able to prevent adsorption and therefore the recovery in plasma samples was higher [37]. Therefore, the experimental setup for urine samples had to be adjusted, either by exchanging containers from polypropylene (PP) to glass or using additives as bovine serum albumin (BSA) or the non-ionic surfactant Tween®-20 (polysorbate 20) [37]. Recovery of the KDR kinase inhibitor increased up to 80% by changing containers to glass and adding BSA, but the addition of 0.2% Tween®-20 increased

the recovery up to 95%. Tween®-20 improved recovery of the analyte by reducing adsorption. At the same time, it did not denature proteins or disturb the specific protein-protein interaction [37]. The increased recovery by addition of BSA may be due to stronger interactions of the analyte with BSA than with the container surface, such as hydrogen bonding and hydrophobic attractions (e.g. protein binding). Disadvantages of BSA usage are the availability in European clinical sites, the costs and sample handling due to the high viscosity [37].

A similar problem of adsorption, as described for KDR kinase inhibitor, is described for Surfactant Protein D (SP-D) to PP tubes. SP-D is a multifunctional protein, which is present in the lung and in respiratory secretions [38]. The usage of special "low-binding" tubes (produced by several manufacturers), which should minimise analyte loss due to a hydrophile inner surface, increased the amount of SP-D binding to the tubes. Coating of the tubes' surface with BSA reduced the binding of SP-D to PP [38].

Comparable issues with catheter adsorption are often described in µDialysis. In a study, all parts of the µDialysis catheters were investigated separately for adsorption of several analytes [39]. The soft polyurethane (PUR) outlet tubing, the hard PUR inlet tubing and the µDialysis membrane were investigated with the antiepileptic drugs phenytoin, carbamazepine and phenobarbital. Phenytoin was found to extensively bind to the soft PUR outlet and lower binding was observed for carbamazepine and phenobarbital [39]. None of the analytes bound to the hard PUR inlet tubing while only a small amount of carbamazepine bound to the membrane. Here should be mentioned, that temperature and pressure during the manufacturing process of the soft outlet tubing are differing from the process for the hard inlet tubing. Thus, surface properties of plastic material are different and the replacement of the outlet tubing by an inlet tubing would imply an improved outcome [39]. In case of adsorption to catheter material, µDialysis investigations concerning recovery and retrodialysis (delivery) are not feasible due to adsorption of the analytes to parts of catheter material. Previously, instead of changing the catheter or catheter material, albumin and lipid emulsions were applied to improve recovery of fatty acids [32] or cyclodextrin to increase recovery of ibuprofen [33] to prevent adsorption to catheter material.

Another example is doxorubicin, a lipophilic cytostatic, which was investigated with *in vitro* µDialysis [40] and could be monitored also by its red colour, which was visible on the catheter material. Mainly recovery and delivery investigations were performed, and the RR was calculated for both, showing incomparability of results, which was underlined by the colouring of catheters, showing that doxorubicin adsorbed on tubings and membranes [40]. In another study, adsorption was investigated during µDialysis experiments (recovery and delivery settings) with seven structurally diverse analytes [35]. First, BSA was added to artificial cerebrospinal fluid and the analyte of interest in a fluorinated

ethylene propylene (FEP)-tubing. In case the setting failed to prevent adsorption, a less flexible polyetheretherketone was used instead of FEP [35]. The result was that lipophilicity was not only an indicator for adsorption of compounds to tubing materials but that other drug specific mechanisms of binding might play a role and adsorption should be evaluated *in vitro* with appropriate drug concentrations before the *in vivo* application. Additionally, a highly flexible polyimide tubing (Microlumen, Oldsmar, FL, USA) for pre-clinical use is on the market and considered to be related with less adsorption [35].

Another cytostatic drug, docetaxel, was investigated with µDialysis. It is of note that docetaxel is a highly lipophilic compound and highly protein bound, mostly to albumin and α-1-acid glycoprotein [41]. One group experienced adsorption of the drug to catheter material [34] and another did not detect any adsorption after 3 h of equilibration [42]. RR of docetaxel was rather low during the *in vitro* recovery experiments, which was assumed to result from adsorption to catheter material in RS. An option to increase RR of docetaxel by diminishing adsorption was the use of polysorbate 80. A PUR- and a FEP-tubing were perfused with different concentrations of polysorbate 80 and µDialysis was performed in the recovery and delivery setting. RR of docetaxel calculated from recovery investigation was higher compared to the delivery setting [34]. Docetaxel seems to have a high affinity to polysorbate 80, which leads to the formation of a gradient with docetaxel diffusing from medium into perfusate in the recovery setting. Human serum albumin (HSA) was previously investigated to prevent adsorption of docetaxel but only polysorbate 80 was able to prohibit adsorption to catheter material [34]. Not only lipophilicity of the analyte is important with regards to adsorption on catheter material, but also the material itself, as lipophilicity alone may not predict the extents of adsorption. By comparing adsorption to PUR- and FEP-tubings, no adsorption of docetaxel was observed with the FEP-tubings but with the PUR-tubings. Since FEP-tubings were only used in pre-clinical and PUR-tubings in clinical settings, adsorption of docetaxel would occur during *in vivo* studies using PUR-tubings. After µDialysis of docetaxel with a FEP-tubing, recovery was only stable after long equilibration time, which was assumed to be due to adsorption to membrane material. Unfortunately, docetaxel was not recommended for µDialysis investigations *in vivo*, since neither polysorbate 80 nor FEP-tubing were sufficient to prevent adsorption [34].

Caspofungin (CFG), an echinocandin, was investigated *in vitro* during a recovery experiment with a perfusate consisting of RS and 1% HSA. Here, adsorption of CFG was not entirely prevented with HSA, but after a previous coating process over 2 hours with HSA and CFG, investigated CFG concentrations were stable during recovery investigation [36]. In addition to the prevention of adsorption of CFG [43], HSA was also added as osmotic agent to limit ultrafiltration (UF) and fluid loss [44] through the 100 kDa cut-off semi permeable membrane of the catheter [36].

Furthermore, UF was an issue when performing µDialysis of macromolecules, like cytokines due to the use of high cut-off semi permeable membranes [45]. The application of HSA instead of the previously described BSA [46] was a comparable alternative, which also reduces UF due to an osmotic gradient. Additionally, HSA was added to the perfusate to guarantee protein stability and reduce adsorption to surfaces. Treatment of catheter material with HSA during perfusion, prevented adsorption of cytokines [45]. HSA as add-on in the perfusate was previously described [47]. It was assumed that HSA binds to adsorption sites on the membrane and tubing surfaces and was potentially forming a complex with cytokines, which had a higher molecular mass and hence, inhibited the diffusion back to the catheter-surrounding medium. An important aspect was that HSA is approved for clinical use and can be applied for *in vivo* µDialysis [45]. In an *in vitro* study, investigating recombinant rat tumor necrosis factor- (TNF) α (M_r= 53 kDa) and interleukin- (IL) 1B (M_r= 17.3 kDa), a 100 kDa cut-off membrane was used, which resulted in an increased fluid loss from perfusate through the membrane [46]. Thus, analyte recovery and the physiology of the tissue compartment were influenced. In the presence of BSA, the loss of perfusate fluid through the semi permeable membrane was minimised and recovery of the analyte increased. Assumed effects of the loss of fluid into the catheter-surrounding medium were the dilution of the so called microenvironment surrounding the catheter membrane. Hence, underestimation of the concentrations at the target site or overestimation due to loss of volume in µDialysate could be possible. Without BSA, the recovery of recombinant rat TNF-α and IL-1B in µDialysate was below the limits of detection. Sufficient, but low amounts of the two analytes were found after the addition of BSA to the perfusate [46]. The major drawback of results gained by using additives, is the handling of these results. These results do often not reflect the actual extracellular concentrations [35].

1.3 Invasive fungal infections and antifungal model drugs

Overall, worldwide 1.2 billion people are considered to be infected by fungi [48]. Of these 1.2 billion patients, only a minority is estimated to suffer from invasive fungal infections (IFIs), from which at least 1.5 to 2 million people die each year. With this compared to other infections, deaths caused by e.g. malaria or tuberculosis are tremendously outnumbered by invasive fungal infections. Despite high mortality rates, antifungal drug development since the 1990s is assumed to be widely on hold compared to other disease areas [48].

1.3.1 Invasive fungal infections

Causative organisms of IFIs can be classified as primary pathogenic fungi and opportunistic fungal pathogens. Belonging to the last-mentioned group, worldwide every year more than 200,000

patients are infected with aspergillosis [49] and >400,000 patients are estimated to be infected with candidiasis [50], with mortality rates of 30%-95% and of 46%-75%, respectively. Life-threatening invasive fungal infections caused by opportunistic pathogens like *Candida spp*., *Aspergillus spp.* and other fungi are mainly causing infections in immunocompromised patients [51,52]. It was estimated that approximately 1.5%-10% of immunocompromised patients are at high risk for fungal infections worldwide. Patients with haematological malignancies or after haematopoietic stem cell transplantation as well as critically ill patients suffering from severe liver cirrhosis or advanced chronic obstructive pulmonary disease are mainly at risk of invasive aspergillosis. In contrast, invasive candidiasis affects critically ill patients on broad-spectrum antibacterial treatment, on total parenteral nutrition, renal replacement therapy and patients receiving immunosuppressive agents (e.g. corticosteroids) [51,52]. However, also other rare mould species can cause IFI, e.g. a typical opportunistic infection of HIV patients or solid organ transplant recipients is *Cryptococcosis* [51,52].

The diagnosis of IFI is often difficult and also delayed due to time-consuming blood culture [49,51]. Hence, empirical or preventive therapy is in many cases mandatory and should follow a prompt and aggressive procedure, which is crucial for successful outcome of IFI therapy. There are not only cases of IFI demanding for immediate treatment but also cases requiring antifungal prophylaxis, like in patients with prolonged neutropenia after start of chemotherapy or those receiving aggressive immunosuppression for graft versus host disease after haematopoietic stem cell transplantation [51].

A major threat for antifungal therapy is emerging resistance against established antifungal agents. An indispensable group of antifungal agents is the class of azoles (e.g. ketoconazole, fluconazole, itraconazole, voriconazole, posaconazole and ravuconazole) [53]. Hence, it is a crucial task in modern medicine to restrain resistance against these drugs, i.e. to detect emerging resistances and to immediately (and subsequently) rearrange antifungal therapy to ultimately ensure patients' survival. Notably the frequency of azole resistance in *Aspergillus fumigatus* has increased over the last decades [53]. Most developed countries reported itraconazole resistance and a large proportion of these isolates are cross-resistant to voriconazole (VOR) [54]. As an example for the severity of azole resistance, patients infected with a susceptible *Aspergillus* isolate show mortality rates of 30%-50%, whereas patients infected with a special multi-azole-resistant isolate of *Aspergillus*, have a mortality rate of 88% [53]. Today different causes of resistance development are thoroughly investigated. A frequently discussed threat is the use of pesticides, which are sprayed on crops. Of these, 40% are fungicides and more than 25% of fungicides are azoles, most of them triazoles, which resemble the triazoles for human use [53]. Interestingly, more than 60% of the global market shore of agricultural fungicides are used in Western European and Asia Pacific areas, and the prevalence of the resistance mutation TR_{34}/L98H (mutations in the cyp51A-gene, which encodes the target enzyme of azoles [49])

seems to correlate with the geographical usage of these fungicides [55]. Resistances may also occur during antifungal treatment, which is mostly the case for long-term treatment in patients with allergic or chronic forms of aspergillosis [56]. A conservative estimate of the global prevalence of azole resistance in *Aspergillus ssp.* was considered to be 3%-6% in 2014 [56]. However, a further increase in resistance has a direct impact on morbidity and mortality caused by fungal pathogens and consequently increases costs for the health care system [53].

1.3.2 Anidulafungin

Anidulafungin (AFG), an echinocandin, was approved in the US in 2006 after CFG, which already came to the market in 2001, and in Europe in 2007 [57,58]. Since in high risk patients the burden of concomitant nephrotoxic drugs and drug-drug interactions should be avoided, it is preferable to use antifungals, which are equally effective and less toxic as the potential alternatives, like amphotericin B and azoles [59].

Mode of action: target, molecular structure, resistances

AFG is an echinocandin antifungal drug with an activity against most *Candida spp.* [57], which is gained from naturally-occurring fermentation of *Aspergillus nidulans* and *Zalerion arbicola* [58–60]. AFG is a semi-synthetic lipopeptide, which selectively and non-competitively inhibits beta-(1,3)-glucan synthase embedded in the cell membrane [59–61], an enzyme crucial to fungal cell wall synthesis (see Fig. 1.3-1) [57,60,62]. Due to that, the production of beta-(1,3)-glucan is inhibited, which is a key structural component of the fungal cell wall [58,59]. Following, the depletion of beta-(1,3)-glucan leads to osmotic instability and eventual lysis of the cell [58–60,62]. Amongst others, AFG has an activity gap against *Zygomycetes spp.* due to their cell wall structure of α-(1,3)-glucans instead of beta-(1,3)-glucans [59]. As fungi are eukaryotes, like mammalian cells, they are good targets for antifungal therapy (e.g. interference with RNA and DNA synthesis, proteins), but this also holds a greater potential for toxicity to mammalian hosts. However, for AFG the target is the fungal cell wall, which is not present in mammalian cells, therefore limiting potential toxicities.

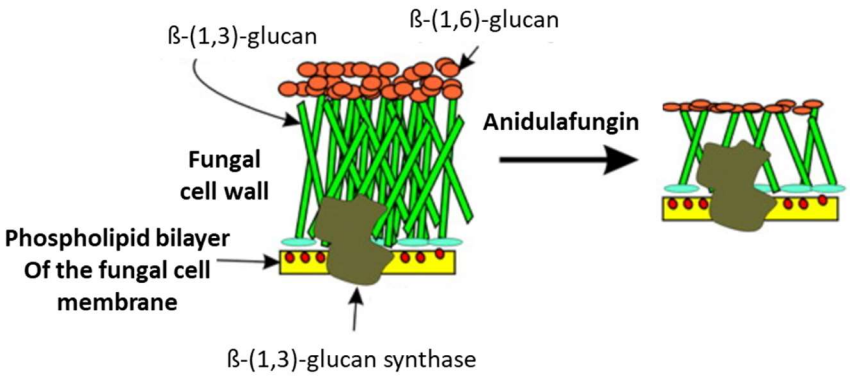

Fig. 1.3-1: Mode of action of echinocandins (anidulafungin) (modified from [63]).

The structure of echinocandins comprises a cyclic peptide with a *N*-aryl group and different hydroxylation patterns [59]. AFG has a alkoxytriphenyl (terphenyl) side-chain (see Fig. 1.3-2), which is assumed to intercalate with the phospholipid bilayer of the cell membrane and therefore plays a critical role in the potency and toxicity of echinocandins [64]. It is pervasively used for the treatment of disseminated candidiasis in non-neutropenic adult patients [57]. Still, AFG displays *in vitro* activity not only against *Candida spp.* but also against *Aspergillus spp.*, including azole resistant strains [61,65]. For echinocandins, lysis of *Candida spp.* cells is easy due to the synthase inhibition during active growth, whereas *Aspergillus spp.* have the synthase localised in the apical tips of their growing hyphae and treatment with echinocandins results in swollen apical tips, which bursting, only leaves short hyphae without further consequence. Echinocandins show a concentration-dependent activity in *Candida spp.* and *Aspergillus spp.*. An “eagle-like” effect is shown *in vitro* with high echinocandin concentrations, meaning a paradoxical loss of antifungal activity [58,59]. This effect is assumed to be derived from drug solubility, micelle formation, slow metabolic activity, transition into a “persistent phenotype” and also altered distribution and elimination of AFG [59]. The clinical relevance of the effect is unknown but occurs to be insignificant [58].

Cross-resistances within the echinocandin-group is already described, assumed to result after prolonged exposure to echinocandins. One of the most often described resistance mechanisms includes the genes FKS1 and FKS2, which encode for the catalytic subunit of beta-(1,3)-glucan synthase. Other resistance mechanisms are assumed to be overproductions of beta-(1,3)-glucan synthase and the expression of efflux pumps [62].

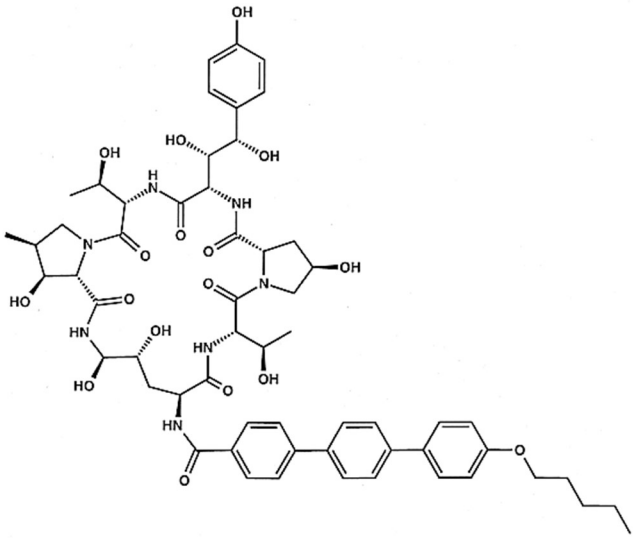

Fig. 1.3-2: Chemical structure of anidulafungin [66].

Indication and dosing

Echinocandins are broad-spectrum antifungal agents against *Candida spp.*, *Aspergillus* and mycelial forms of endemic fungi but lack activity against *Cryptococcus neoformans*, *Fusarium spp.* and *Zygomycetes* [59]. In the US, AFG is indicated for the treatment of candidemia and other forms of *Candida* infections, like intra-abdominal abscess and peritonitis and oesophageal candidiasis [67]. In addition to that it is indicated in Europe for the treatment of invasive candidiasis in adult patients [68]. More specifically, Echinocandins are recommended as first-line therapy for candidemia in non-neutropenic and neutropenic patients and first- as well as second-line treatment for other *Candida* infections [58]. Additionally, they have activity against biofilm-embedded *Candida spp.*, which is of special interest, since biofilms are a major cause of resistance due to their cells with increased resistance to antimicrobial agents [59]. *In vitro*, AFG is active against *Candida spp.*, including strains resistant to fluconazole or amphotericin B [62]. Additionally, *in vitro* it is active against *Aspergillus spp.* including *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* and many of the emerging molds (e.g. *Bipolaris spicifera*, *Exophiala jeanselmei*). AFG also demonstrated additive effects in combination with amphotericin B against *Aspergillus* and *Fusarium spp.*. AFG shows a fungistatic effect *in vivo* against *Aspergillus spp.* and fungicidal activity against *Candida spp. in vitro* and *in vivo* [62].

In 2014, Muldoon *et al.* published a study about the prophylactic use of echinocandins, concluding that prophylactic antifungal therapy in general should be limited to intensive care unit patients. Specific populations in which prophylactic treatment has already been proven useful include patients with post–gastrointestinal perforation, severe pancreatitis, liver, pancreas, and small bowel transplant and extremely low birth-weight neonates [69]. Still, especially recognising an emergence

of echinocandin resistance of 12% in already fluconazole-resistant *Candida glabrate* clinical isolates published in 2014, the use of antifungals should be limited [69].

Combinations of echinocandins with azoles or polyenes are currently used as alternative treatment of persistent infections and are being discussed for first-line therapy [70]. For example a combined therapy against invasive aspergillosis with VOR and AFG showed improved clinical outcome compared to the VOR monotherapy [49].

AFG is available as intravenously (i.v.) administered formulation. Adult patients with candidemia and other forms of *Candida* infections receive a loading dose of 200 mg on day 1, followed by a maintenance dose of 100 mg daily for at least 14 days after the last positive culture [67,68]. Patients with oesophageal candidiasis receive lower doses, comprising a loading dose of 100 mg on day 1, followed by a maintenance dose of 50 mg daily for a minimum of 14 days and for at least 7 days following resolution of symptoms [67]. Here, the duration of treatment should be based on the patient's clinical response. The rate of infusion should not exceed 1.1 mg/minute [67,68]. Patients with hepatic or renal impairment do not need dose adjustment and AFG is also not dialysed during continuous renal replacement therapy (CRRT) [62,67]. For children of 2 years and older, AFG should be dosed based on body weight instead of age [58].

Physicochemical and pharmacokinetic properties

AFG is almost insoluble in water and only slightly soluble in ethanol. The molecular mass is 1140.3 Da [58,64,67]. The lipophilicity of a molecule can be described by logP and the octanol/water partition coefficient, which is known to be high (concrete numbers are not published in literature) [71].

Oral bioavailability of AFG is low (less than 10% [62]) and very variable [58], and as a consequence the formulation of AFG was developed as i.v. administration [59]. The i.v. application shows predictable concentrations, which increase linearly with the dose [58]. More than 90% of AFG undergoes slow chemical degradation to an *in vitro* inactive product, which is degraded by plasma peptidase [58,72] to an open hexapeptide ring and further metabolised [62]. AFG molecules are not metabolised hepatically and do not interact with cytochrome P450 enzymes [58,62]. Less than 10% of the unchanged drug and its degradation products are eliminated via faeces and biliary excretion [58,62]. Renal elimination is insignificant [58]. The volume of distribution is 30-50 L and the terminal elimination half-life is 25 h [62]. Plasma protein binding is assumed to be approximately 98%-99% [73]. Steady state concentrations are achieved within 24 h after administering the loading dose [62]. The PK of AFG in patients suffering from IFI is best described by a two-compartment model with first-order elimination and the estimated PK parameters were similar to the observed PK parameters in healthy volunteers [58]. Moreover, the total volume of distribution increased with higher body

weight and clearance was increased in male patients, patients with increased body weight, older patients and acutely ill patients. Substrates, inducers and inhibitors of the cytochrome P450 enzyme family, like rifampin, were given as concomitant drugs and as expected resulted in non-significant impact on PK parameters [58], due to the lack of interaction with cytochrome P450 enzymes. A significant decrease in maximum concentration (C_{max}) was seen in patients with severe hepatic impairment compared to a healthy control group, which is assumed to be caused by ascites, oedema, and an increase in clearance and volume of distribution. The drug half-life is similar to the healthy volunteer control group. No significant changes in PK were found in patients with mild or moderate hepatic impairment. No dosage adjustment is recommended for all degrees of hepatic impairment [58]. Furthermore, patients with renal insufficiency (mild, moderate and severe renal impairment or end-stage renal disease) were studied and the PK profiles were similar to the healthy control group. PK determined in patients receiving continuous venovenous haemodiafiltration was similar to previously determined PK in healthy volunteers and patients with fungal infections [61]. Therapeutic AFG concentrations were achieved throughout the study and CRRT seems to have only an negligible effect on AFG elimination, therefore no dose adjustments for patients during CRRT are recommended [61].

Adverse drug reactions and interactions

Only a few adverse drug reactions were reported for AFG in clinical trials. Hence, it seems to be a well-tolerated antifungal drug [59]. The most common adverse drug reactions include headache and nausea, reversible abnormal liver function test, hypokalaemia, vomiting and diarrhoea [58,62]. The infusion rate should not exceed 1.1 mg/min, otherwise histamine-related reactions like rash, urticaria, flushing, pruritus, dyspnoea and hypotension could occur [67], though quickly declining [62]. Due to the well tolerability of AFG, a specific routine monitoring in patients treated with AFG is not recommended [62]. As described before, AFG is no substrate of the cytochrome P450 system or of P-glycoproteins and at the same time not inducing or inhibiting either of them [58]. Thus, it is improbable that AFG will interact with the PK of concomitant drugs, metabolised via CYP enzymes [58,62]. This was demonstrated during the co-administration of cyclosporine, a CYP3A4 substrate, which showed no significant difference in the PK of both drugs [74] and the co-administration of VOR, which showed also no changes in PK [75].

1.3.3 Voriconazole

VOR is on the World Health Organisation's Model List of Essential Medicines [76]. Furthermore, the EMA points out that if VOR is used for treatment of fungal infections, it will be intended mainly for patients with worsening and possibly life-threatening infections [77].

Mode of action: target, molecular structure, resistance

The molecular structure of the triazole antifungal agent VOR was derived from the structure of its predecessor fluconazole (see Fig. 1.3-3). A methyl group was added to the propranolol structure and the triazole structure was exchanged with a fluoropyrimidine ring [78–80], which provided fungicidal activity against *Aspergillus spp.* and other moulds [78,80] and fungistatic activity in *Candida spp.* [80,81].

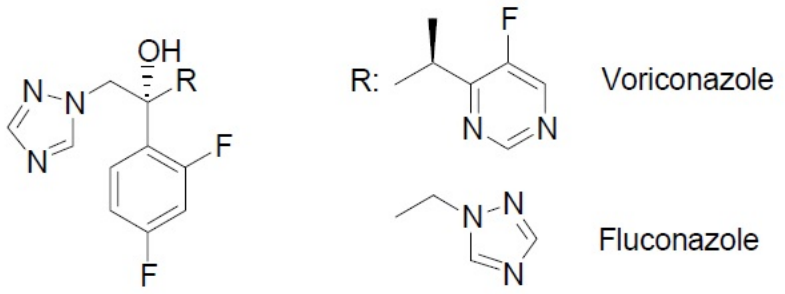

Fig. 1.3-3: Chemical structure of voriconazole and fluconazole from Simmel *et al.* [28].

As a triazole antifungal agent, VOR reversibly inhibits the 14-α-lanosterol-demethylase [79,81] by binding to its haem group [51,78]. During normal ergosterol biosynthesis, the 14-α-lanosterol-demethylase (defined as CYP51A1 [51]), which is cytochrome P450-mediated [78], removes the methyl group on C-14-lanosterol for the conversion to ergosterol [51,81]. Hence, the subsequent accumulation of toxic [51,79] 14-α-methyl sterols is responsible for the lack of ergosterol in the fungal cell wall [51,78,79] and a decrease of ergosterol leads to cell death [81]. VOR does not interfere with the human metabolism and biosynthesis, but it shows fungistatic activity against yeasts and also fungicidal activity against moulds. *In vitro*, VOR shows an immunomodulatory effect of susceptibility of the cell wall to oxidative and non-oxidative phagocytic damage [78]. A significant advantage of VOR is the broad antifungal activity against yeasts and filamentous fungi and with *Zygomycetes* the activity spectrum has only a single gap [78]. Since the 14-α-lanosterol-demethylase (CYP51A1) belongs to the CYP family, mutations on its gene can lead to azole resistance [51].

Indication and dosing

VOR has a wide range of antifungal activity and is approved for the treatment of a multitude of fungal infections. The indication includes in adults and children aged 2 years and above, the treatment of invasive aspergillosis, invasive candidiasis, candidemia in non-neutropenic patients and infections caused by *Scedosporium apiospermum* and *Fusarium spp.* in patients intolerant of or

refractory to other therapies [52,78,82,83]. Further, it should be administered primarily to patients with progressive, potentially life-threatening infections and as prophylaxis of invasive fungal infections in high risk allogenic hematopoietic stem cell transplant recipients [82]. VOR is recommended as first-line therapy for invasive aspergillosis since it achieved a better clinical outcome than amphotericin B deoxycholate [51,80].

Therapy with VOR starts with an i.v. standard dose of 6 mg/kg bid (*bis in die*: twice daily) as loading dose on day 1. Subsequently maintenance doses of 4 mg/kg bid are given [51]. Due to the high bioavailability, an oral dose can be applied as an alternative to the i.v. dose. For adult patients it is 400 mg bid on day 1 followed by 200 mg bid. For adult patients with a body weight of less than 40 kg, a per oral dose reduction by 50% for loading and maintenance dose is recommended [51]. Dose adjustment is required in case of hepatic failure, but not necessarily during renal failure using the oral formulation, due to the lack of nephrotoxicity [51,78]. Since the i.v. formulation contains cyclodextrin (sulpho-butyl-ether-cyclodextrin) to increase solubility [79], it may accumulate in case of renal insufficiency, but long-term effects on renal function are unknown [51,78]. Hence, the treatment with the i.v. formulation should be avoided in case the creatinine clearance is ≤50 mL/min unless the benefit predominates the risk [51,78,79].

Physicochemical and pharmacokinetic properties

The lipophilicity of VOR was described by logP and the octanol/water partition coefficient, which for the lipophile VOR molecules was 1.8 [84] and 64.7 [28], respectively. With a molecular mass of 349.3 Da and as a weak base with pKa 1.76 [84–86], VOR is a molecule of high permeability and low solubility [84,86], representing a Class 2 compound in the Biopharmaceutical Classification System [87].

The oral formulation of VOR has an excellent bioavailability of 96% [51,79,80] and a C_{max} is achieved 1-2 h after administration [78–80]. The high oral bioavailability enables switching between i.v. and oral administration. Absorption is not affected by gastric pH, but is decreased by simultaneous presence of food [78,79]. Hence, the oral formulations should be administered 1 h before or 2 h after a meal, otherwise the bioavailability may be reduced by 20% [78]. Steady state concentration in plasma can be achieved after an i.v. loading dose followed by a maintenance dose within 24 h. After i.v. administration of 3-6 mg/kg bid, a total C_{max} of VOR in plasma at steady-state was 2.7-6.0 µg/ml [79]. In case of oral administration, steady state is achieved after 5-7 days [78–80] with a C_{max} of 2 µg/mL and C_{min} of 0.5 µg/mL on day 7 [51]. Hence, critically ill patients should receive an initial i.v. therapy to achieve therapeutic VOR concentrations as early as possible, to rapidly diminish the fungal infection and prevent complications. Although gender, weight, age and critically ill patients are

associated with differences in the PK of oral administered VOR, no dose adjustment is needed [78]. VOR has a linear PK in children treated with 3-4 mg/kg of the i.v. dose [78,80]. However, the VOR PK was found to be nonlinear if treated with an i.v. dose of ≥7 mg/kg [51,78–80]. This is considered to be due to saturation of its own metabolism [78,79] and therefore PK is dependent on the administered dose [80]. Plasma protein binding (PPB) of VOR is 58%-60% and is linked to high unbound plasma concentrations and therefore, a good tissue distribution [51,78,79] and is independent of dose or plasma concentrations [80]. Extensive distribution is emphasised by the volume of distribution of 2-4.6 L/kg [51,78–80] and a clearance (CL) of 7 L/h in healthy volunteers [51]. VOR is a substrate of the CYP enzymes 2C19, 2C9 and 3A4, which explains the variability in plasma concentrations due to interactions with co-administered drugs and the CYP enzymes or genetic polymorphisms (ultra rapid to poor metabolisers) [51,79,80]. In addition, VOR is a strong inhibitor of CYP2C19 and a moderate inhibitor of CYP2C9 and CYP3A4 [51,78]. Homozygous CYP2C19 poor metabolisers have a three- to fourfold increase in plasma concentrations compared to their homozygous extensive metaboliser counterparts, whereas heterozygous poor metabolisers have only a twofold increase [51,78]. Due to variable and unpredictable metabolism related to i.a. genetic factors, which may result in insufficient drug exposure, VOR concentrations and therefore VOR exposure of fungi to VOR can only monitored by TDM [78].

The elimination half-life is 6-9 h (up to 12 h [79]) after single dose and an i.v. or oral dose of VOR is excreted within 48 h [28,51,78,80]. The elimination half-life increases with further VOR administrations due to nonlinear PK [28]. Mostly metabolites (98%) are excreted in urine and faeces [51,78,79]. VOR is mainly metabolised to a *N*-oxide metabolite, which has a negligible antifungal activity compared to VOR [78]. The main elimination of VOR occurs via the liver and less than 5% of the active drug is found in urine [78,79].

Adverse drug reactions and interactions

The most important and most frequently described adverse drug reactions include visual disturbances, neurological adverse drug reactions like hallucinations, confusion, neuropathy and paraesthesia. In addition, hepatic toxicity, nausea and vomiting and QT interval prolongation (especially for haemato-oncologic patients [88]) are observed [78,79]. Visual disturbances are related to high serum concentrations of VOR and diminish after the first days of treatment [78]. The same applies for central nervous system adverse drug reactions and liver toxicity, which are also associated to high serum concentrations of VOR (C_{VOR} > 5 mg/L) [89]. In general, a target trough concentration of 2-5 mg/L VOR should be aimed for [89]. Hence, routine dose adjustment based on TDM is highly recommended to reduce incidence and degree of severity [78]. Exfoliative cutaneous reactions like Stevens-Johnson syndrome and phototoxicity are rare, but rash is very common as adverse drug

reaction [78,79]. A major thread is the development of skin cancer under treatment with VOR and simultaneously with immunosuppressants during long-term therapy. Phototoxicity reactions and pre-neoplastic lesions regressed or significantly improved after VOR discontinuation [78]. Further, increased VOR concentrations are associated with liver toxicity, since VOR is extensively metabolised by liver enzymes [78,79]. However, also this adverse drug reaction is reversible after discontinuation of the drug treatment [79]. Liver function tests should be performed for high risk patients before starting and during VOR treatment. A special regard should be given to the co-administration of cyclosporine A [79] and tacrolimus due to increased cyclosporine A and tacrolimus concentrations, which have been associated with nephrotoxicity [83]. In this case doses of immunosuppressants must be reduced by 50% and 66%. The risk of severe haemorrhage is given by enhanced plasma concentrations of vitamin K antagonists due to VOR co-administration. The sedative effect of benzodiazepines is prolonged and together with sulfonylureas, VOR may cause hypoglycaemia. Statin levels can be enhanced with the risk of rhabdomyolysis and for omeprazole, a dose reduction by 50% is advised when VOR is co-administered [78,79]. Together with histamine blockers, cimetidine or quinidine, VOR may not only cause prolongation of the QT interval but result in torsades de pointes [51]. Phenytoin induces CYP3A4, which results in a decrease of VOR concentration. Since rifampin is an inducer of CYP3A4, it is suggested to switch therapy to prevent giving rifampin and VOR concomitantly. Also prednisolone is metabolised by CYP3A4 and therefore has a potential for interactions with VOR [79].

1.4 Objectives

Deaths and hospitalisation due to IFIs are constantly increasing and the rise of antifungal resistance to the respective antifungal drugs is alarming. This emphasises how important it is to prevent resistances and guarantee an easy, fast and accurate routine in hospitals to determine not only the fungal pathogen but also antifungal drug concentrations at the target site. Since meanwhile the EMA recommended in the Guideline on the use of Pharmacokinetics and Pharmacodynamics in the Development of Antibacterial Medicinal Products to assess, in addition to plasma concentrations, the total and unbound drug concentrations in non-homogenate tissue with i.e. µDialysis [10], it is of high priority to expand the field of drugs monitored with µDialysis and continuously improve the method. This technique is mainly recommended for its unlimited access to all parts of the human body and the unbound and therefore active drug at the target-site can be determined. To ensure these requirements, valid bioanalytical assays and *in vitro* µDialysis investigations for the respective drugs are needed, as each drug molecule is unique and therefore has differing chemical and physicochemical properties which affect the behavior of the drug during µDialysis. Previously, it was only possible to investigate analytes during steady state concentrations in the sIVMS. The dependence on FR and concentration of the analyte, as well as potential adsorption to catheters could be described. Thus, the objective of this thesis was to create a more physiological surrounding for the analyte by mimicking *in vivo* PK profiles with the dynamic *in vitro* microdialysis system (dIVMS). The time delay of a drug from medium to µDialysate, feasibility of calibration methods and the effect of the calculated on the observed concentration-time profile may be observed.

The structure of this thesis emphasises the logical development of the conducted investigations.

- Bioanalytical assay of AFG and VOR: Development of a new assay for AFG and update of a previously developed assay for VOR, with a focus on small sample volume, simpler sample preparation and coverage of *in vivo* concentration ranges.
- *In vitro* investigation of AFG with µDialysis: Conduction of recovery and retrodialysis experiments with the sIVMS with a special focus on adsorption reduction.
- *In vitro* investigation of VOR with µDialysis: Conduction of *in vitro* µDialysis with a reduced set of experimental parameters in the sIVMS. These parameters are FR and concentrations, both investigated in a recovery and delivery setting with an additional set of retrodialysis investigations.
- Application of the newly developed dIVMS for AFG and VOR: Development and validation of the dIVMS. Subsequent mimicry of pharmacokinetic concentration-time profiles of VOR and AFG.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals, drugs and pharmaceutical products

Ammonium dihydrogen phosphate	Carl Roth, Karlsruhe, Germany
Caspofungin acetate LOT No.02, Batch No. S307302 purity >99.63%	Selleckchem, München, Germany
Dextran (Dextran 40, 10%)	AlleMan Pharma GmbH, Rimbach, Germany
Ecalta® (Anidulafungin)	Pfizer, Kent, UK
Ethanol (gradient grade)	VWR Prolabo, Fontenay-sous-Bois, France
Human serum albumin (Albiomin® 5%)	Biotest, Dreieich, Germany
Human serum albumin (Human-Albumin Kabi 20%)	Octapharma, Langenfeld, Germany
Methanol (gradient grade)	VWR Prolabo, Fontenay-sous-Bois, France
Milli-Q water obtained from Millipak® system	Merck Millipore, Darmstadt, Germany
Ringer's solution	BERLIN-CHEMIE AG (Menarini), Berlin, Germany
Ringer's solution	Braun, Melsungen, Germany
Voriconazole LOT No. 052301-008-09 potency 98%	Pfizer, Kent, UK

2.1.2 Laboratory and study equipment

Balance AT 250	Mettler, Greifensee, Switzerland
Balance Sartorius BP 221 S	Sartorius AG, Göttingen, Germany
Centrifuge 5430 R	Eppendorf, Hamburg, Germany
Laboratory shaker REAX 2000	Heidolph instruments, Schwabach
NUNC 96-well plates	Thermo Fisher Scientific, Waltham, USA
NUNC 96-well cap mats	Thermo Fisher Scientific, Waltham, USA

pH/mV Meter	Knick Elektronische Messgeräte, Berlin, Germany
Pipettes and pipette tips (20-1000 µL)	Eppendorf, Hamburg, Germany
Safe lock vials (0.5-1.5 mL), PP	Eppendorf, Hamburg, Germany
Ultrasonic bath Sonorex Digitec, Typ: DT 106	Bandelin Electronic, Berlin, Germany

HPLC columns

LiChrospher® 100 RP18 (125x4 mm; 5 µm)	Merck, Darmstadt, Germany
Symmetry® C18 (4.6x75 mm; 3.5 µm)	Waters, Milford, USA
XBridge™ BEH C18 (3.0x50 mm; 2.5 µm)	Waters, Milford, USA

HPLC system (modular)

Thermo Scientific Dionex Ultimate 3000	Thermo Fisher Scientific, Waltham, USA
Controller (LC-Net II/ ADC)	
Pump (HPG-3200SD)	
Autosampler (WPS-3000TSL)	
Column compartment (TCC-3000SD column thermostat)	
Diode array detector (DAD3000)	

Microdialysis equipment

CMA102® Pumps	CMA Microdialysis AB, Solna, Sweden
BD® Syringe 1 mL Luer Lock® Tip	Becton, Dickinson and Company, New Jersey, US
CMA71® microdialysis catheter	M Dialysis AB, Solna, Sweden
Concentric catheter design	
Material membrane: PAES	
Material inlet-outlet tubing: PUR	
Length [mm] membrane: 20	
Membrane cut-off [kDa]: 100 kDa	
CMA63® microdialysis catheter	M Dialysis AB, Solna, Sweden
Concentric catheter design	
Material membrane: PAES	
Material inlet-outlet tubing: PUR	

Length [mm] membrane: 30

Membrane cut-off [kDa]: 20 kDa

CMA60® microdialysis catheter — M Dialysis AB, Solna, Sweden

Concentric catheter design

Material membrane: PAES

Material inlet-outlet tubing: PUR

Length [mm] membrane: 30

Membrane cut-off [kDa]: 20 kDa

Microdialysis catheters and accessories:

All catheters used were CE marked according to the Medical Device Directive, 93/42/EEC, sterilised by β-radiation, stored according to the requirements in the product sheet (4° C – 25° C) and were purchased from M Dialysis AB, Stockholm and Solna, Sweden.

Dynamic system

Pump ISMATEC MV-CA 4 — Cole-Parmer GmbH, Wertheim, Germany

Tygon LMT — Cole-Parmer GmbH, Wertheim, Germany

- **"2 Color Code Stopper Schläuche"**
  Material: Polyvinyl chloride (PVC) based material with platicisers
  Diameter [mm]: 0.51 (in), 2.33 (out)
  Length [mm]: 381
  Colour code: orange-yellow

- **Extension tubing**
  Material: PVC based material with platicisers
  Diameter [mm]: 0.51 (in), 2.21 (out)
  Length [mm]: 300 mm (inlet), 900 mm (outlet)

Magnetic stirrer RCT basic — IKA Labortechnik GmbH, Staufen, Germany

Water heating HAAKE 001-7983 — HAAKE, Karlsruhe, Germany (new: Thermo Fisher Scientific, Waltham, USA)

2.2 Bioanalytical methods for quantification of anidulafungin and voriconazole in microdialysate

2.2.1 Method development

Several matrices were investigated to find a suitable matrix for the *in vitro* µDialysate, medium, perfusate solutions, calibration and quality control samples of AFG from Ecalta® (pharmaceutical formulation). Potential matrices were 0.9% NaCl solution, mixtures of 0.9% NaCl solution and methanol or ethanol (20%-90% each), and a mixture of RS and HSA (RS/HSA (0.5%,1%)). Additional investigations regarding sample preparation (precipitation of HSA), separation (Accucore™ Phenyl-Hexyl, LiChrospher® 100 RP-18; XBridge® BEH C18), elution of analyte (gradient and isocratic methods; methanol and an ammonium dihydrogen phosphate buffer in varying ratios, column temperature: 15°C to 45°C) and sample injection (injection volume, sample containers of glass or PP) were conducted. In contrast, an already existing HPLC assay for VOR from Simmel/Kirbs *et al.* [2,90] was used and improved for *in vitro* µDialysate samples of VOR in RS for this work. Here, the focus was on separation (LiChrospher® 100 RP-18; Symmetry® C18) and elution of VOR (acetonitrile, methanol and an ammonium dihydrogen phosphate buffer in varying combinations and ratios, column temperature: 25°C to 40°C).

During HPLC runs, AFG and VOR samples were stored in a 96-well-plate on an automatic tray in the autosampler compartment. The cavities of the 96-well-plate were closed tightly with a cap mat.

Identification and quantification of AFG or VOR after HPLC was realised with UV detection at a wavelength of 310 nm or 254 nm, respectively. Peak areas of unknown concentrations were calculated using the calibration function obtained by weighted linear regression ($1/concentration^2$).

2.2.2 Preparation of stock solutions, calibration samples and quality control samples

The AFG pharmaceutical formulation Ecalta® was diluted with 100 mL milli-Q water to obtain a final concentration of 1 mg/mL and 50.0 mg VOR analytical reference substance were diluted with 50 mL ethanol to obtain a final concentration of 1 mg/mL. Aliquots of 1 mL for both stock solutions were stored at -80°C. Two aliquots of each stock solution, instead of two independently prepared stock solutions (see chapter 4.1), were used to spike either calibration (Cal) or quality control (QC) samples. The sample matrices were RS/HSA (0.5%) for AFG and RS for VOR. The stock solutions for Cal samples were diluted with the respective matrix to obtain working solutions of 0.1, 0.5, 1.0, 5.0, 10, 20 µg/mL for AFG and of 1.5, 5.0, 10, 20, 60, 100 µg/mL for VOR. Subsequently, 10 µL of each

working solution of VOR were diluted with 90 µL water directly before analysis to reach the final concentrations of 0.15, 0.5, 1.0, 2.0, 6.0 and 10 µg/mL VOR.

For QC samples, the stock solutions were diluted with RS/HSA (0.5%) for AFG and RS for VOR to obtain solutions of 0.1, 0.3, 10, 15 µg/mL for AFG and 0.15, 0.45, 5.0, 7.5 µg/mL for VOR.

2.2.3 Method validation

The bioanalytical HPLC assays for the quantification of AFG and VOR in the respective *in vitro* µDialysate matrix were developed and validated according to the criteria of the EMA Guideline on bioanalytical method validation [3]. The validation included determination of selectivity, carry-over, lower limit of quantification (LLOQ), linearity (calibration function), accuracy, precision and stability of AFG and VOR.

Selectivity of the analytical method

To avoid possible interferences of substances in the matrix or additives in perfusate with the AFG peak during HPLC, the assay was performed with the blank matrix of RS/HSA (0.5%) or dextran 40/HSA (5 mg/mL / 0.5%) in water and with CFG as additive in perfusate. Chromatograms of VOR were also analysed for interferences of substances in the matrix of RS.

The respective blank matrix, RS/HSA (0.5%) or dextran 40/HSA (5 mg/mL / 0.5%) in water for AFG and RS for VOR, was repeatedly analysed and chromatograms were evaluated and compared to chromatograms from samples of AFG or VOR in the respective matrix.

To verify the selectivity of the AFG assay for CFG, CFG stock solution (1 mg/mL in milli-Q water, stored at -80°C in 1 mL aliquots) was diluted to a concentration of 10 µg/mL and added to AFG calibration samples. Results were compared to CFG-free calibration samples.

Carry-over effects

The possible emergence of AFG or VOR carry-over effects to the following experimental run was investigated by successively injecting AFG or VOR samples concentrated at the pre-defined upper limit of quantification (ULOQ) (AFG: 20 µg/mL; VOR: 10 µg/mL) and blank matrix (n_{AFG}=6; n_{VOR}=5).

Accuracy and precision

Within- and between-day accuracy and precision were evaluated from QC samples analysed against the calibration curve, the obtained concentrations were compared to the nominal value. For validation of AFG and VOR, six QC samples per concentration (0.10, 0.30, 10.0 and 15.0 µg/mL for AFG and 0.15, 0.45, 5.00 and 7.50 µg/mL for VOR) were analysed on four different days. Accuracy

was calculated as the percentage deviation of C_{obs} (relative error, RE (%)) of QC samples from C_{nom} (Eq. 2-1). Precision was described by the coefficient of variation (CV, %) from multiple determinations (n=6 per concentration) (Eq. 2-2).

$$RE, \% = \frac{C_{obs} - C_{nom}}{C_{nom}} \cdot 100 \qquad \text{Eq. 2-1}$$

$$CV, \% = \frac{Standard\ deviation\ (\mathrm{C_{obs}})}{Mean\ (\mathrm{C_{obs}})} \cdot 100 \qquad \text{Eq. 2-2}$$

Lower limit of quantification and assay linearity

The LLOQ was assessed by comparing the signal-to-noise-ratio (S/N) of the spiked µDialysate samples and the blank matrix. According to the EMA Guideline on bioanalytical method validation, the analyte signal (height of peak) of the LLOQ should be at least five times higher than the signal of the blank matrix [3]. Hence, AFG and VOR solutions were added to blank RS/HSA (0.5%) or RS, respectively, yielding concentrations from 0.05 µg/mL to 0.30 µg/mL for both analytes (n=5 per analyte). The lowest concentration of each analyte was defined as LLOQ which was quantified with acceptable accuracy (±20% RE) and precision (20% CV). Calibration curves consisting of six calibrator concentrations (nominal) were analysed by weighted linear regression (1/concentration^2) against the respective peak areas. Linearity was evaluated in a concentration range from 0.10 µg/mL – 20.0 µg/mL of AFG in RS/HSA (0.5%) (n=4) and from 0.15 µg/mL – 10.0 µg/mL of VOR in RS (n=4).

Dilution integrity

During experiments, if µDialysate samples had a higher concentration than the ULOQ, they were diluted with the respective matrix to concentrations in the calibration range. Therefore, dilution integrity was investigated before with the AFG and VOR stock solutions (1 mg/mL each). The stock solutions were serially (lower concentrations resulted from diluting the respective higher concentration) and non-serially (all dilutions were based on one stock or working solution) diluted. For serial dilution, AFG stock solution was diluted with RS/HSA (0.5%) to a concentration of 40 µg/mL, which was outside the calibration range. Subsequently, the resulting solution was serially diluted to AFG concentrations of 20 µg/mL, 10 µg/mL and 4 µg/mL (n=5 each). VOR stock solution was diluted with RS to 200 µg/mL and afterwards with RS to 10 µg/mL, 5 µg/mL and 0.5 µg/mL VOR (n=3 each).

Non-serial dilution was investigated by diluting the respective stock solution with the respective matrix to 40 µg/mL for AFG or 200 µg/mL for VOR. Following, the resulting solutions were non-serially diluted with RS/HSA (0.5%) to 20 µg/mL, 10 µg/mL, and 4 µg/mL AFG (n=5 each) and with RS

to 10 µg/mL and 5 µg/mL VOR (n=3 each). Resulting serial or non-serial dilutions were analysed with the previously described bioanalytical method. For accuracy and precision of the resulting concentrations, a criterion of below ±15% was set [3].

Drug stability in microdialysate

Different processes, such as the freeze and thaw, short-term at room temperature, autosampler (10°C) and long-term stability of the QC samples and stock solution of AFG were investigated. The QC samples and stock solution were spiked into RS/HSA (0.5%) and the stability of AFG in the matrix was evaluated using low (0.3 µg/mL) and high (15 µg/mL) concentrations of QC samples. To prove the stability of the stock solution, the 1 mg/mL AFG stock solution was spiked into RS/HSA (0.5%), resulting in a concentration of 15 µg/mL shortly before analysis.

For the determination of the freeze and thaw stability of AFG, aliquots of each of the two QC concentrations (n=6 per cycle) and stock solution (n=5 per cycle) were analysed immediately after preparation and the 1st, 2nd and 3rd freeze and thaw cycle (freezing at -80°C for at least 12 h before they were thawed [3], completely thawing unassisted at room temperature, then refreezing for at least 12 h) and evaluated as described in Eq. 2-3. Long-term stability of QC samples of low and high concentrations of AFG (n=5) were analysed immediately and after storing the samples in the ultrafreezer (-80°C) for 7 months. Samples were evaluated as described in Eq. 2-3.

Short-term and autosampler stability of AFG QC samples were investigated in sextuple and triplicate, respectively. For the determination of room temperature stability of AFG, QC samples were prepared and analysed immediately and after 4 and 24 h and the stock solution after 6 h (n=3) at room temperature and evaluated as described below. To investigate autosampler stability of AFG, QC samples were analysed immediately and after 6 and 24 h in the autosampler, which was tempered at 10°C. Samples were evaluated as described in Eq. 2-3.

The means of the peak areas per cycle were compared to the mean of the peak areas of the freshly prepared samples by calculating the recovery of the analyte after each cycle and storing condition (Eq. 2-3). Recovery should range from 85% to 115%.

$$Recovery, \% = \frac{Peak\ area_{\text{ storing condition}}}{Peak\ area_{\text{ freshly prepared}}} \cdot 100 \qquad \text{Eq. 2-3}$$

Due to previous stability investigations of VOR in RS, performed by Simmel/Kirbs *et al.* [2,90], only autosampler (10°C) stability of QC samples of VOR in RS was investigated. The QC samples were spiked into RS and the stability of AFG in the matrix was evaluated using low (0.45 µg/mL) and high (7.5 µg/mL) concentrations of QC samples.

To investigate autosampler stability of VOR, QC samples were analysed in triplicate immediately and after 6 and 48 h in the autosampler, which was tempered at 10°C. Samples were evaluated as described in Eq. 2-3.

2.3 Anidulafungin investigations in the static *in vitro* microdialysis system

2.3.1 Static *in vitro* microdialysis system

To gain reproducible results of basic investigations on feasibility of µDialysis for the analyte of interest, including FR, concentration range, composition of perfusate, sampling intervals and catheter design, a standardised sIVMS was used to perform *in vitro* µDialysis investigations as shown in Fig. 2.3-1, based on previous work by Simmel *et al.* [28]. Additionally, investigations on interactions of the analyte with µDialysis equipment and different catheter designs were conducted with the sIVMS.

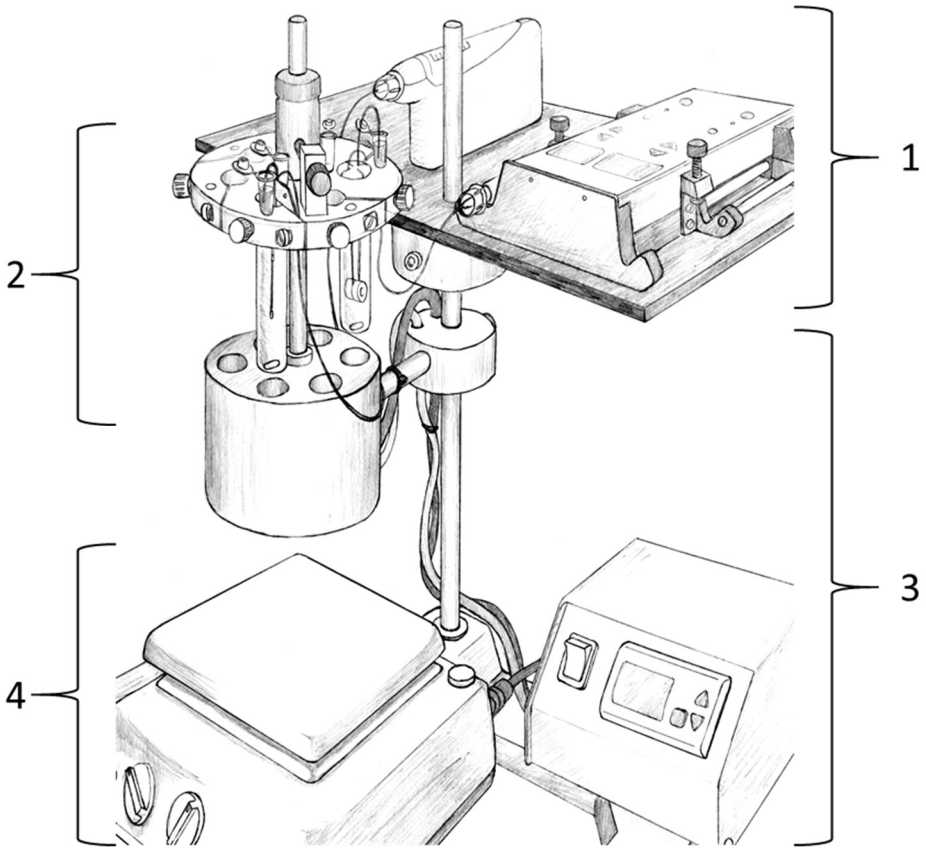

Fig. 2.3-1: The static *in vitro* microdialysis system (sIVMS). Standardised experimental conditions: (1) pump module, (2) catheter module, (3) thermo module, (4) stirring module [28].

In the pump module of the sIVMS, microsyringes were placed in the µDialysis pumps, located on a platform. The platform was installed above a heating compartment for medium containers (thermo module). Catheters were connected with the microsyringes in the µDialysis pumps and the membranes of catheters were fixed in the medium containers (catheter module), surrounded by the thermo module. An adjustable rack was installed on top of the thermo module to store the sampling vials during investigations. Due to technical standardisation, heights and distances between the platform of the pump module and thermo module were constant, which was crucial for reproducibility of results. Medium vessels in the thermo module were tempered at the physiological body temperature of 37°C. Additionally, a magnetic stir bar was placed into the medium container, which was set in motion with a magnetic stirrer underneath the thermo compartment (stirring module), to mimic the movement of body fluids in the tissue.

To get a better understanding of the conducted investigations, the experimental set-up is shown schematically in Fig. 2.3-2. Medium was sampled with a syringe and µDialysate was collected in a vial. Furthermore, the figure depicts the membrane placed in the medium and a microsyringe, filled with perfusate, perfusing the catheter.

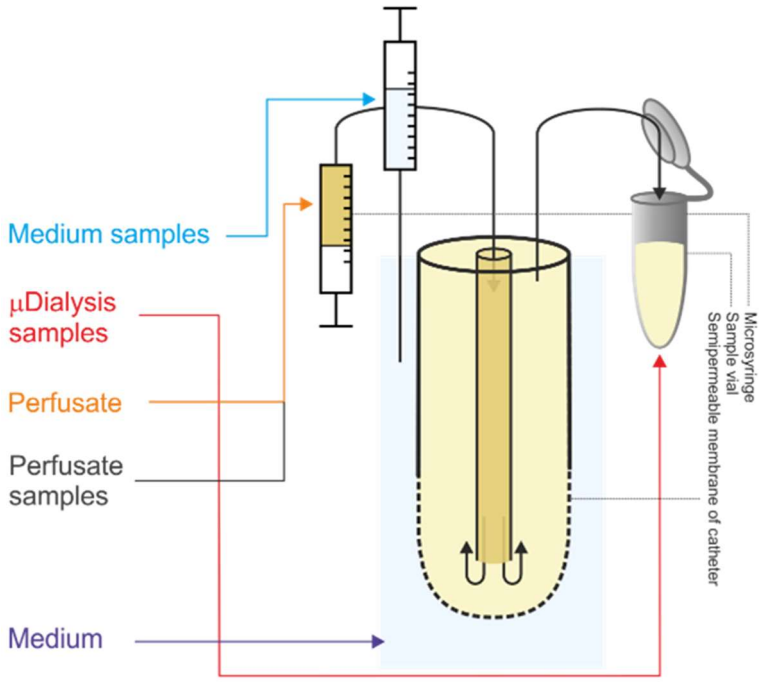

Fig. 2.3-2: Detailed close-up of the microdialysis catheter. Medium samples were taken with a syringe and the microdialysate samples collected in a vial. A microsyringe is filled with perfusate, which is perfused through the catheter and its membrane. The catheter membrane is placed in medium.

2.3.1.1 Microdialysis system settings

As described in chapter 2.3.1, a standardised sIVMS was utilised for *in vitro* µDialysis investigations mimicking physiological conditions. These physiological conditions were mainly the mimicry of body temperature and fluid movement outside the membrane. Hence, temperature in the thermo compartment was always set to 37°C and stirring to 700 min^{-1}. The FR was adjustable, but only FR of 1 and 2 µL/min were investigated in the following due to their typical usage during *in vivo* clinical studies.

2.3.1.2 Preparation of solutions used as perfusate or medium

Solutions of AFG were prepared by diluting the Ecalta® stock solution (1 mg/mL AFG in milli-Q water) with a mixture of RS/HSA (0.5%) to the respective concentrations. CFG stock solution (1 mg/mL in milli-Q water) was diluted with a mixture of RS/HSA (0.5%) to the respective concentrations.

VOR stock solution of 1 mg/mL in ethanol was diluted with RS to final concentrations.

The composition of perfusate and medium was individually adapted for the respective investigations.

2.3.2 Static *in vitro* microdialysis of anidulafungin

Before start of µDialysis investigations, catheters were equilibrated with the respective perfusate in medium for at least 15 min. A minimal sample volume of 20 µL was needed for the bioanalytical quantification of AFG. Thus, a sample volume of 40 µL was aimed at to ensure quantification due to a loss of perfusate volume in the µDialysate samples.

2.3.3 Feasibility of microdialysis for anidulafungin: Behaviour of anidulafungin in microsyringes

First, two sets of microsyringes were studied: (i) three new microsyringes and (ii) three microsyringes which were washed with RS/HSA after containing 50 µg/mL AFG in RS/HSA (0.5%) for several hours. AFG perfusate concentration in microsyringes was set to 0.2 µg/mL. After the microsyringes were filled with 0.2 µg/mL AFG in RS/HSA (0.5%), samples of 40 µL (1 drop) were collected (n=9 samples per microsyringe) from microsyringes and the production vial (here, drug solution and matrix were mixed, n=8-9 samples per production vial). Samples were taken in 40 min intervals (corresponding to µDialysate sampling intervals) for 320 min. The first drop from microsyringes was discarded before taking the sample. The two sets of microsyringes were compared regarding precision, calculated as CV (%), and accuracy, calculated as percentage deviation of C_{obs} from C_{nom} (RE, (%), of AFG concentrations (C_{AFG}) in samples and the course of C_{AFG} over time per microsyringe.

2.3.4 Feasibility of microdialysis for anidulafungin: Comparison of microdialysis catheters

First, two CMA63® and two CMA71® catheters with a cut-off of 20 kDa and 100 kDa, respectively, were perfused with RS/HSA (0.5%) at a FR of 2 µL/min in a medium containing 10 µg/mL AFG in RS/HSA (0.5%). Sampling of µDialysate and medium (n=1 per catheter/medium and time point) started after 15 min of equilibration in intervals of 30 min for 90 min (Fig. 2.3-3). Subsequently, the samples were quantified with the previously validated HPLC method (see chapter 2.2.3) and RR was calculated (Eq. 1-1). The catheter design, which resulted in a higher RR was chosen for further investigations.

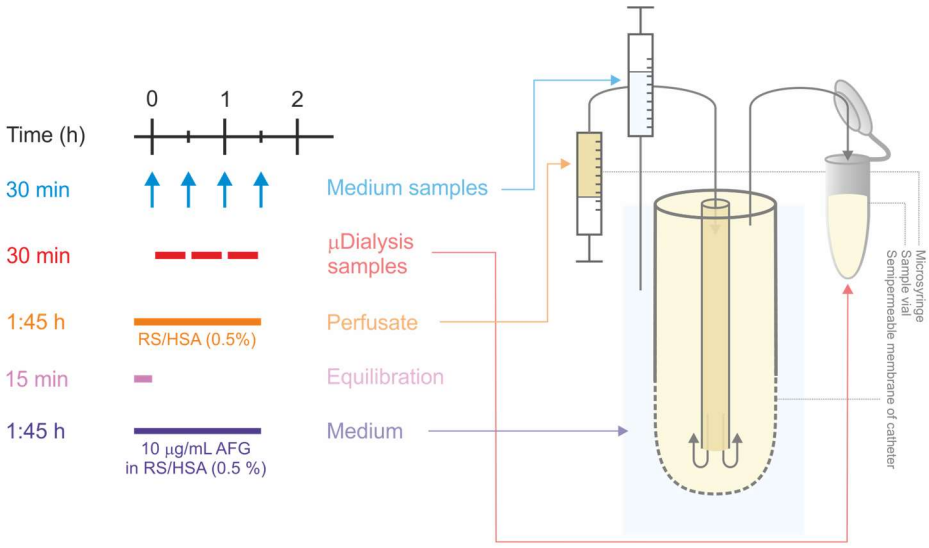

Fig. 2.3-3: Sampling schedule (microdialysate, perfusate and medium) of feasibility investigation with CMA63® and CMA71® catheters for anidulafungin in the static *in vitro* microdialysis system.

2.3.5 Feasibility of microdialysis for anidulafungin: Recovery investigation of anidulafungin with microdialysis catheters

Three CMA71® catheters were perfused with RS/HSA (0.5%) in a medium containing 1 µg/mL AFG in RS/HSA (0.5%). Catheters were perfused at a FR of 1 µL/min for 480 min (=8 h) and µDialysate and medium samples (n=1 per catheter/medium and time point) were taken in 40 and 80 min intervals, respectively (Fig. 2.3-4).

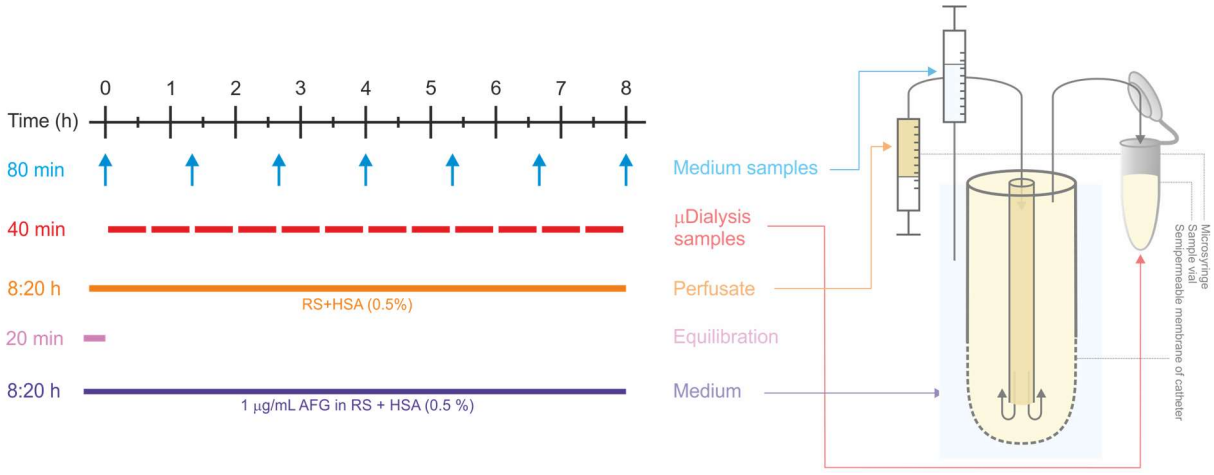

Fig. 2.3-4: Sampling schedule (microdialysate, perfusate and medium) of recovery investigation with CMA71® catheters for anidulafungin (AFG) in Ringer's solution (RS) and human serum albumin (HSA (0.5%)) containing medium with AFG-free perfusate consisting of RS and HSA (0.5%) in the static *in vitro* microdialysis system.

Samples were quantified with the bioanalytical method for AFG (see chapter 2.2.3). RR (Eq. 1-1) was calculated over time. Vials were weighed before and after sample taking. The difference in weight from full to empty vial was determined. The volume of the sample was calculated by using Eq. 2-4 with the assumption that RS/HSA containing perfusate had the same density as water. The sample volume in µDialysate ($V_{\mu Dialysate}$) was determined with the sample weight ($m_{\mu Dialysate}$) and density of water (ρ_{H2O}):

$$V_{\mu Dialysate} = \frac{m_{\mu Dialysate}}{\rho_{H2O}} \qquad \text{Eq. 2-4}$$

The nominal volume was 40 µL (1 µL/min in 40 min). The RE of observed compared to nominal volume was calculated.

In the following, the RR was calculated with the observed C_{AFG} in µDialysate and additionally with a factor for volume correction (Eq. 2-5). The volume-corrected relative recovery ($RR_{Volume\text{-}corrected}$) was determined with the RR calculated from observed concentrations in µDialysate and the RE of the volume in µDialysate:

$$RR_{Volume\text{-}corrected}, \% = RR \cdot \left(1 + \frac{RE}{100}\right) \qquad \text{Eq. 2-5}$$

The results for RR and $RR_{Volume\text{-}corrected}$ were used to estimate its time evolvement, the maximum RR (RR_{max}) and its confidence interval (CI). RR_{max} and $t_{RR,50}$ (and $t_{RR,90}$) were estimated with a mathematical model assuming Michaelis-Menten kinetics (Eq. 2-6) derived from the Langmuir adsorption isotherme [91] in R (version 3.4.1) and R Studio (version 1.0.143). RR_t (%) is the RR at a specific time point t, t (min) is the time point, $t_{RR,50}$ (min) is the time at which 50% of RR_{max} is achieved and RR_{max} is the maximum RR:

$$RR_t, \% = \frac{RR_{max} \cdot t}{t_{RR50} + t} \qquad \text{Eq. 2-6}$$

2.3.6 Feasibility of microdialysis for anidulafungin: Influence of dextran on ultrafiltration

Two CMA71® catheters were perfused with a solution of 5 mg/mL dextran 40 and HSA (0.5%) in milli-Q water in a medium containing 1 µg/mL AFG in RS/HSA (0.5%).

Catheters were perfused with a FR of 1 µL/min for 480 min and µDialysate and medium (n=1 per catheter and medium) were taken in 40 and 80 min intervals, respectively (Fig. 2.3-5).

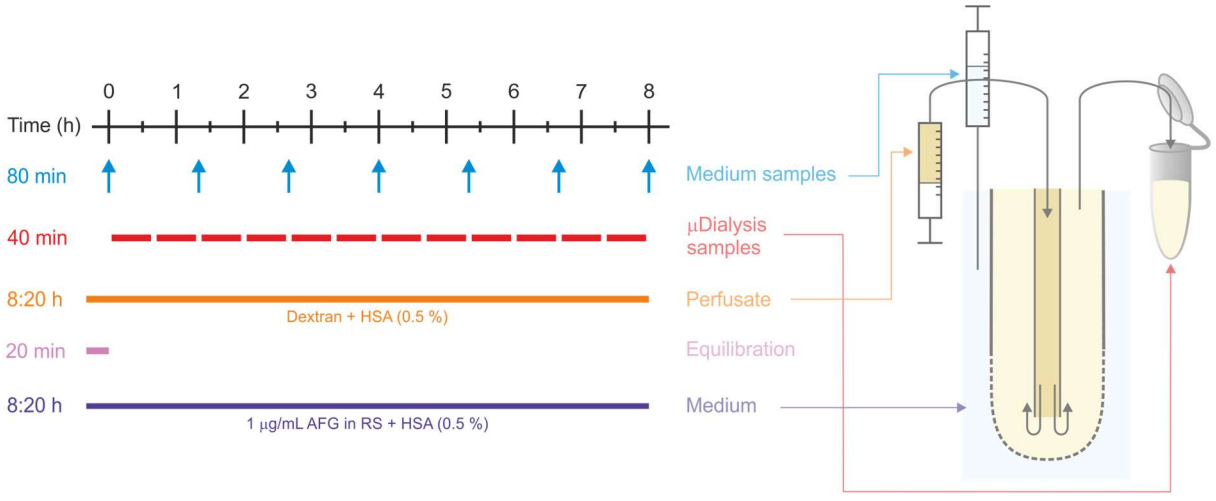

Fig. 2.3-5: Sampling schedule (microdialysate, perfusate and medium) for microdialysis recovery investigation of 1 µg/mL anidulafungin (AFG) in Ringer's solution (RS) and human serum albumin (HSA (0.5%)) containing medium with AFG-free perfusate consisting of dextran (5 mg/mL) and HSA (0.5%).

The volume in µDialysate samples was determined by weighing vials prior and after sample collection. The percentage deviation (RE, %) of of the recovered volume to the nominal volume of 40 µL in µDialysate was calculated. Samples (µDialysate and medium) were quantified with the bioanalytical method for AFG (see chapter 2.2). In the following, RR was calculated (Eq. 1-1) from observed C_{AFG} and with a volume correcting factor ($RR_{Volume\text{-}corrected}$, Eq. 2-5). Additionally, RR_{max} was estimated with a mathematical model (Eq. 2-6) and compared to results from chapter 2.3.5.

2.3.7 Feasibility of microdialysis for anidulafungin: Influence of catheter pre-coating on adsorption

The following investigations were performed with CFG and AFG for pre-coating of catheters before start of µDialysis investigations.

2.3.7.1 Catheter pre-coating with caspofungin

Three different approaches were investigated for AFG recovery from the medium. The first investigation (i) examined a perfusate of 50 µg/mL CFG in RS/HSA (0.5%) and pre-coating of 2 h, the second (ii) investigated also a perfusate of 50 µg/mL CFG in RS/HSA (0.5%) and a prolonged pre-coating of 14 h (overnight), the third (iii) studied 200 µg/mL CFG in RS/HSA (0.5%) in perfusate and pre-coating overnight (15 h). For each investigation, three CMA71® catheters were perfused with the respective perfusate of (i), (ii) or (iii) for the respective time (2 h or overnight). The CFG-containing perfusate was also perfusing catheters during the recovery investigation to ensure continuous coating of the surface. The medium contained (i) RS/HSA, (ii) 50 µg/mL and (iii) 200 µg/mL CFG during catheter pre-coating and 1 µg/mL AFG in RS/HSA (0.5%) during the sampling period. µDialysate and medium (n=1 per catheter/medium and time point) were collected at a FR of 1 µL/min (i) in 80 min intervals for 400 min (Fig. 2.3-6), (ii) in 40 min intervals for 480 min (Fig. 2.3-7), and (iii) in 40 min intervals for 480 min (Fig. 2.3-8). Samples (µDialysate and medium) were quantified with the bioanalytical HPLC assay for AFG, which was validated for the selectivity of the assay for separation of AFG and CFG (see chapter 2.2.3). RR (without volume correction) was calculated from µDialysate (Eq. 1-1) and RR_{max} was estimated by nonlinear regression (Eq. 2-6) and compared to RR_{max} from the investigation with RS/HSA (0.5%) containing perfusate without CFG (see chapter 2.3.5).

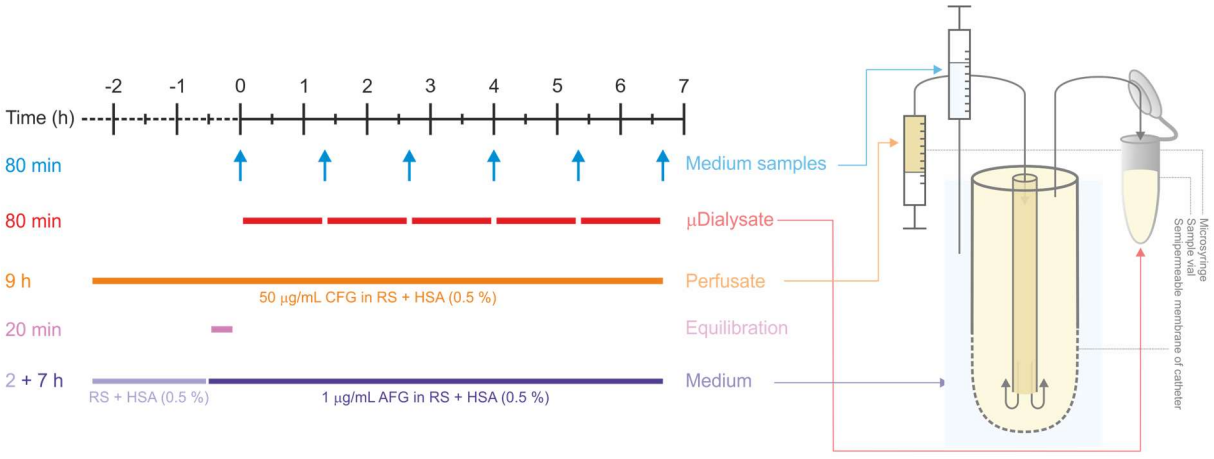

Fig. 2.3-6: Sampling schedule (microdialysate, perfusate and medium) of (i): (pre-) coating of the catheter with 50 µg/mL caspofungin (CFG) for 2 h prior (in CFG-free medium) and during microdialysis recovery investigation of 1 µg/mL anidulafungin (AFG) in Ringer's solution (RS) and human serum albumin (HSA (0.5%)) containing medium.

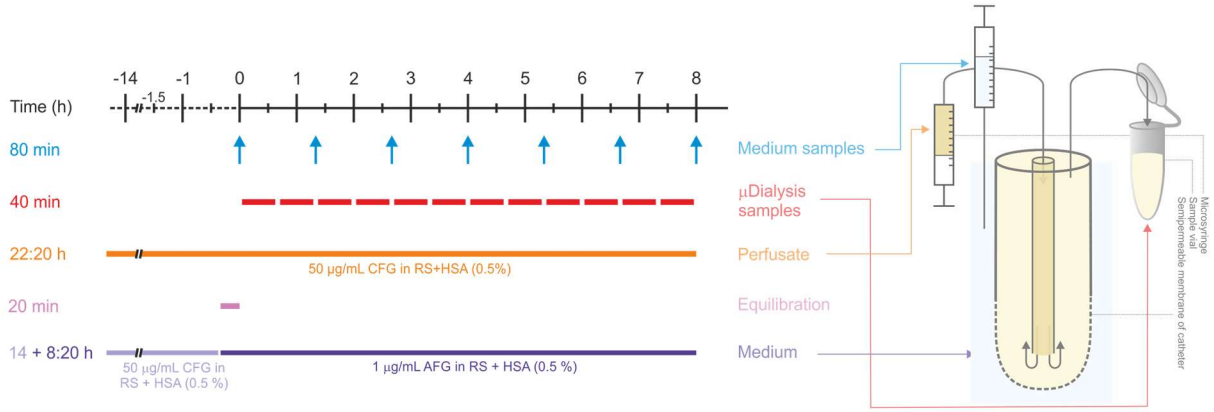

Fig. 2.3-7: Sampling schedule (microdialysate, perfusate and medium) of (ii): (pre-) coating of the catheter with 50 µg/mL caspofungin (CFG) prior (in CFG-containing medium), overnight, and during microdialysis recovery investigation of 1 µg/mL anidulafungin (AFG) in Ringer's solution (RS) and human serum albumin (HSA (0.5%)) containing medium.

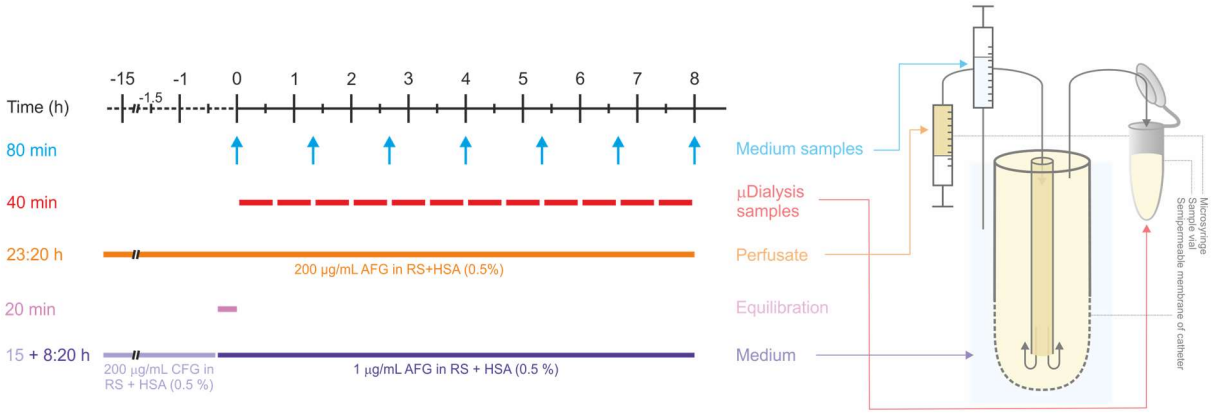

Fig. 2.3-8: Sampling schedule (microdialysate, perfusate and medium) of (iii): (pre-) coating of the catheter with 200 µg/mL caspofungin (CFG) prior (in CFG-containing medium), overnight, and during microdialysis recovery investigation of 1 µg/mL anidulafungin (AFG) in Ringer's solution (RS) and human serum albumin (HSA (0.5%)) containing medium.

2.3.7.2 Catheter pre-coating with anidulafungin

Three CMA71® catheters were perfused with 0.2 µg/mL AFG containing RS/HSA (0.5%) perfusate during the process of overnight pre-coating in an AFG-free RS/HSA (0.5%) medium. After pre-coating was completed, catheters were transferred into a medium containing 1 or 8 µg/mL AFG in RS/HSA (0.5%) and were further perfused with 0.2 µg/mL AFG in RS/HSA (0.5%) (see Fig. 2.3-9 and Fig. 2.3-10). Samples of medium, µDialysate and perfusate (n=1 per catheter/medium and time point) were quantified with the bioanalytical assay for AFG (see chapter 2.2).

C_{AFG} in µDialysate samples during the pre-coating phase in AFG-free medium were quantified (mean and CV were calculated) and used to calculate the relative delivery (rD) (Eq. 1-2). The RR (without volume correction) during the recovery investigation was calculated by using Eq. 2-7. Here, C_{AFG} from µDialysate ($C_{\mu Dialysate}$), mean C_{AFG} in perfusate ($C_{Perfusate}$) and medium (C_{Medium}) were implemented.

RR_{max} was estimated by nonlinear regression with Eq. 2-6. RR_{max} of both investigations and the standard investigation with RS/HSA (0.5%) containing perfusate and 1 µg/mL AFG in medium (without pre-coating) were compared (see chapter 2.3.5).

$$\mathrm{RR}, \% = \frac{C_{\mu Dialysate} - C_{Perfusate}}{C_{Medium} - C_{Perfusate}} * 100 \qquad \text{Eq. 2-7}$$

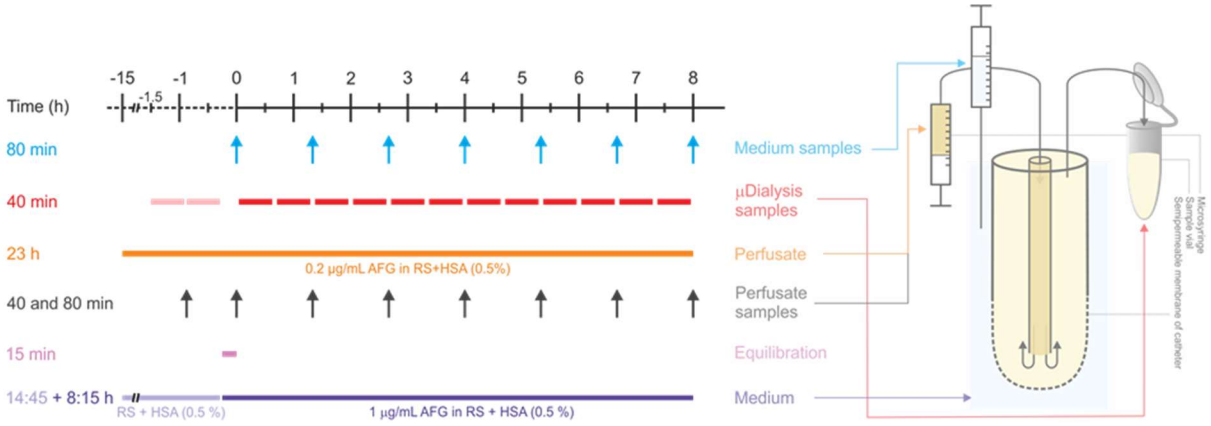

Fig. 2.3-9: Sampling schedule of medium, microdialysate and perfusate samples during recovery investigations of anidulafungin (AFG) with additional pre-coating of the catheter. Perfusion of catheters with 0.2 µg/mL AFG containing Ringer's solution (RS) and human serum albumin (HSA) overnight and microdialysis of **1 µg/mL** AFG containing medium for 8 h.

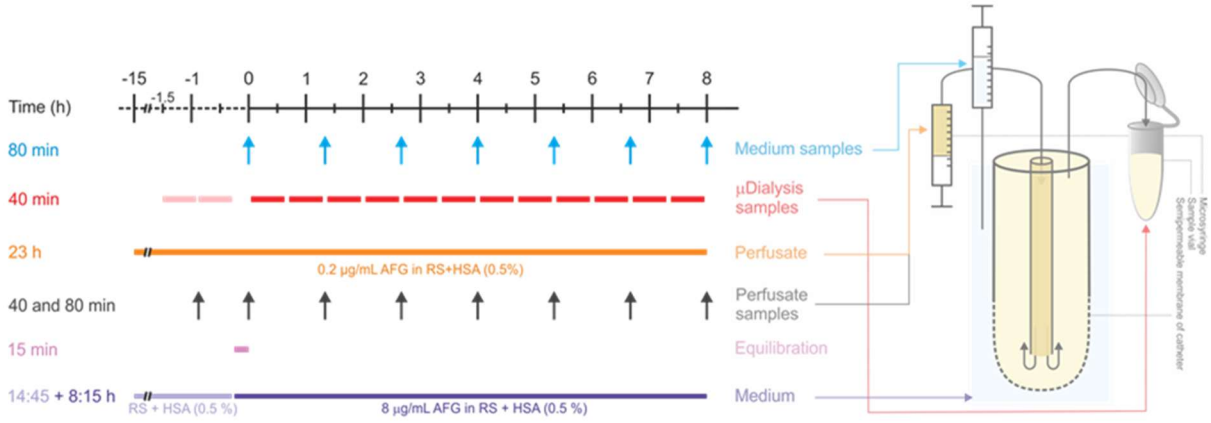

Fig. 2.3-10: Sampling schedule of medium, microdialysate and perfusate samples during recovery investigation of anidulafungin (AFG) with additional pre-coating of the catheter. Perfusion of catheters with 0.2 µg/mL AFG containing Ringer's solution (RS) and human serum albumin (HSA) overnight and microdialysis of **8 µg/mL** AFG containing medium for 8 h.

2.3.8 Feasibility of microdialysis for anidulafungin: Investigation of retrodialysis

Apart from the recovery of AFG, the delivery in a retrodialysis calibration setting was investigated. The design of the delivery (or retrodialysis) investigation was reduced to investigations with a single C_{AFG} of 200 µg/mL in perfusate and different medium concentrations. Here, rD was determined.

Three CMA71® catheters were perfused with 200 µg/mL AFG in RS/HSA (0.5%) containing perfusate. The investigations took place in an (i) AFG-free or a (ii) 10 µg/mL AFG in RS/HSA (0.5%) medium. An equilibration period of 15 min was performed before each delivery investigation started. µDialysate, perfusate and medium samples (n=1 per catheter/perfusate/medium per time point) were taken during the investigation (Fig. 2.3-11). The rD was calculated according to equation Eq. 1-2.

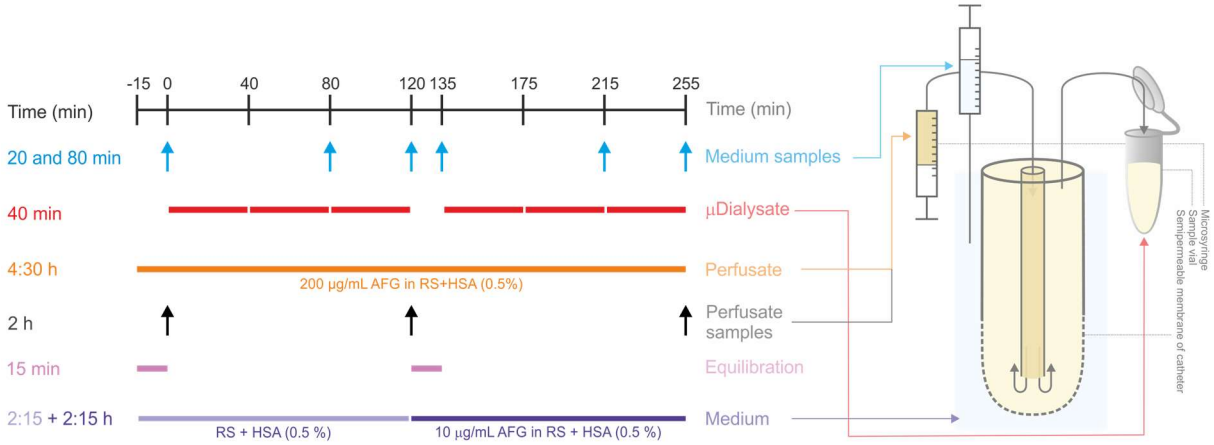

Fig. 2.3-11: Sampling schedule of medium, microdialysate and perfusate samples during delivery investigation of anidulafungin (AFG). Perfusion of catheters with 200 µg/mL AFG containing Ringer's solution (RS) and human serum albumin (HSA) in AFG-free or 10 µg/mL AFG containing medium.

2.4 Voriconazole static *in vitro* microdialysis

2.4.1 Dependence of relative recovery and relative delivery on flow rate or concentration

Three CMA60® catheters (20 kDa cut-off) were placed in a VOR containing (recovery) or VOR-free medium of RS (delivery, retrodialysis) and perfused with either VOR-free RS (recovery) or VOR containing RS (delivery, retrodialysis).

The dependency of RR and rD on FR and concentration were investigated using flow rates of 1 µL/min or 2 µL/min and 10 or 20 min sampling intervals, respectively. VOR concentrations in medium (for recovery) or perfusate (for delivery) were 0.5-4.0 µg/mL (n=4 samples per concentration, FR and catheter) (Tab. 2.4-1). RR was calculated with Eq. 1-1 and rD with Eq. 1-2.

Tab. 2.4-1: Experimental settings for recovery and delivery investigations with changing voriconazole concentrations (C_{VOR}) and flow rates.

Experimental setting	Recovery investigation	Delivery. investigation
Stirring [min^{-1}]	700	
Temperature [°C]	37	
C_{VOR} in medium [µg/mL]	0.5, 1.5, 3.0, 4.0	-
C_{VOR} in perfusate [µg/mL]	-	0.5, 1.5, 3.0, 4.0
Flow rate [µL/min]	1.0, 2.0	1.0, 2.0

2.4.2 Investigation of retrodialysis

For a further investigation of retrodialysis (delivery), three CMA60® catheters were perfused with 20 or 200 µg/mL VOR in RS. Investigations were conducted in VOR-free medium (i), medium with 1 µg/mL VOR in RS (ii) and 10 µg/mL VOR in RS (iii). Sampling intervals for µDialysate were 10 min (n=3 samples per medium, perfusate and catheter) at a flow rate of 2 µL/min (Tab. 2.4-2). rD was calculated with Eq. 1-2.

Tab. 2.4-2: Experimental settings for retrodialysis with steady state conditions and changing voriconazole concentrations (C_{VOR}) in medium.

Experimental settings	Retrodialysis		
	(i)	(ii)	(iii)
Stirring [min^{-1}]		700	
Temperature [°C]		37	
C_{VOR} in medium [µg/mL]	-	1	10
C_{VOR} in perfusate [µg/mL]	20, 200	20, 200	20, 200
Flow rate [µL/min]		2	

2.5 Antifungals in a dynamic *in vitro* microdialysis system

2.5.1 Development of the dynamic *in vitro* microdialysis system

In this thesis, the development of a prototype of the dIVMS was described, which was suitable to simulate pharmacokinetic profiles appearing *in vivo*. A prototype of the experimental dIVMS was developed. The experimental model should contain a medium vessel, in which µDialysis catheters could be placed and medium samples could be taken, a pump to control drug concentrations in the medium by pumping drug-free or drug containing medium into and from the medium vessel and a thermo-system to enable physiological temperatures in the medium.

2.5.2 Validation of the dynamic *in vitro* microdialysis system

During the validation of the dIVMS, investigated parameters were temperature in the flask, continuous pumping of medium fluid into and from the flask and continuous stirring of the medium in the flask.

The temperature of the water bath was set to a physiological body temperature of 37.4°C [92] and the temperature-time profile was monitored for 23 h. Therefore, digital thermometers were placed in the water of the inner chamber of the flask and in the heated water bath. The mercury thermometer was also placed in the heated water bath. The temperature in the flask and in the water bath was documented in 5 or 10 min intervals during the first hour, followed by 1 h intervals up to 7 h and after 23 h of the investigation.

Continuous pumping was investigated with water in a (new) pair of tubings at speed of 23 rpm (only adjustment by rpm possible). The set speed was comparable to the speed during the following µDialysis investigations in the dIVMS. The resulting pump rate (PR) was measured as volume per minute with samples of in- and outflow of two tubings fixed in the peristaltic pump (ISMATEC). Sampling ($n_{in+outflow}$=31-32 samples per interval) of in- and outflow was performed at three different intervals: 0-1 h, 5-6 h and 24-25 h. Empty or water containing vials were weighed before and after taking in- and outflow samples. The difference in mass was converted to volume (Eq. 2-8) per time = PR (Eq. 2-13).

$$V[mL] = \frac{\Delta m[g]}{\rho[\frac{g}{mL}]} \qquad \text{Eq. 2-8}$$

Continuous stirring of the membrane-surrounding medium in the inner chamber was performed with a magnetic stir bar (oval shape; max. diameter 10 mm) in and a magnetic stirrer below the glass flask. Homogenous distribution of the membrane-surrounding medium was investigated at a stirring

velocity of 700 rpm, testing whether the homogenous distribution of 50 µL methylene blue solution (1%) was visually achieved within 2 min (n=10).

2.5.3 *In silico* simulations and *in vitro* mimicry of concentration-time profiles of antifungals in the dynamic *in vitro* microdialysis system

2.5.3.1 *In silico* simulation of pharmacokinetic profiles of antifungals

For the investigation of *in vitro* pharmacokinetic profiles with µDialysis, first pharmacokinetic parameters from literature were required to simulate *in silico* PK profiles of VOR and AFG. Afterwards, pharmacokinetic profiles were mimicked with the dIVMS.

For simulating an *in vitro* PK profile with the dIVMS, simulations should be based on *in vivo* profiles of the respective drug. For VOR and AFG, the applied PK profiles were founded on steady state plasma (VOR [80], AFG [73]) and ISF concentrations (VOR [2]). Simulations were derived from the *in vivo* clearance. A one-compartment model was used for *in silico* simulations of the dIVMS. *In vivo*, VOR and AFG were administered as i.v. infusions, but for the experimental setup the bolus application was chosen. The applied PK characteristics from literature are listed in Tab. 2.5-1.

Tab. 2.5-1: Pharmakokinetic characteristics of voriconazole (VOR) in plasma [80] after infusion at steady-state and interstitial space fluid (ISF) [2] and of anidulafungin (AFG) in plasma [73] after infusion at steady-state: half-life ($t_{1/2}$), maximum concentration (C_{max}), plasma protein binding (PPB).

Pharmacokinetic characteristics	VOR plasma	VOR ISF	AFG plasma
Drug half-life ($t_{1/2}$) [h]	6.0	8.5	20.8
C_{max} [µg/mL]	4.7 (total)	0.94 (unbound)	6.6 (total)
PPB, %	58	unbound	not specified

The PK characteristics were used to calculate the experimental conditions for the dIVMS. Since investigations in the dIVMS with AFG were conducted in HSA-containing and VOR in protein-free medium, the maximum unbound VOR concentration ($C_{max,unbound}$) was calculated according to Eq. 2-9. The percentage of unbound molecules was multiplied with the maximum total drug concentration in plasma ($C_{max,total}$). For AFG, total plasma concentrations were used. To calculate the drug concentration in the i.v. bolus injection ($C_{IV\ bolus\ injection}$), first the dose of the analyte (D) was calculated by multiplying the maximum total or unbound drug concentration ($C_{max,total/unbound}$) with the volume of the medium fluid in the glass flask (V_{Medium}), which was 100 mL, shown in Eq. 2-10. $C_{IV\ bolus\ injection}$ was calculated in Eq. 2-11 by dividing the dose of the analyte with the volume of the i.v. bolus injection ($V_{IV\ bolus\ injection}$). For the elimination rate constant (k_e), ln(2) was divided by the drug half-life ($t_{1/2}$) in

Eq. 2-12 and the PR by multiplying k_e with V_{Medium} and dividing it by the factor 60 to get the unit of PR in minutes instead of hours (see Eq. 2-13).

$$C_{max,unbound}\left[\frac{\mu g}{mL}\right] = C_{max,total} \cdot \left(\frac{100 - PPB}{100}\right) \quad \text{Eq. 2-9}$$

$$D\ [\mu g] = C_{max,total/unbound} \cdot V_{Medium} \quad \text{Eq. 2-10}$$

$$C_{IV\ bolus\ injection}\left[\frac{\mu g}{mL}\right] = \frac{D}{V_{IV\ bolus\ injection}} \quad \text{Eq. 2-11}$$

$$k_e[h^{-1}] = \frac{\ln(2)}{t_{1/2}} \quad \text{Eq. 2-12}$$

$$PR\ \left[\frac{mL}{min}\right] = \frac{k_e \cdot V_{medium}}{60} \quad \text{Eq. 2-13}$$

The total (AFG) or unbound (VOR) drug concentration at the different time points t was calculated with Eq. 2-14. Here, a one-compartment model with first order kinetics and drug administration via i.v. bolus was described: C(t): total/unbound drug concentration at time point t [µg/mL]; C_{max}: maximum total/unbound drug concentration [µg/mL]; k_e: elimination rate constant [h^{-1}]; t: time point [h]

$$C(t)\left[\frac{\mu g}{mL}\right] = C_{max} \cdot e^{-k_e \cdot t} \quad \text{Eq. 2-14}$$

In the following, concentration-time profiles were simulated for different time periods.

2.5.3.2 *In vitro* mimicry of pharmacokinetic profiles

After calculating the concentration-time profile for the antifungal drugs *in silico*, the experimental investigation was performed with the dIVMS. Preliminary to performing µDialysis investigations, the mimicry of the respective concentration-time profiles was investigated.

Voriconazole

Mimicry of human concentration-time profiles of VOR were based on plasma data from Theuretzbacher *et al.* [80] (i) or ISF data from Simmel *et al.* [2] (ii). The PR was set to 193 µL/min for (i) and to 136 µL/mL for (ii). Injection solutions had a C_{nom} of 394.8 µg/mL for a C_{max} of 1.97 µg/mL (i) in medium or 188 µg/mL for a C_{max} of 0.940 µg/mL (ii) VOR in RS containing medium. In the inner chamber of the glass flask was a volume of 99.5 mL RS. The temperature of the water bath was set to 37°C and the stirring velocity to 700 min^{-1}. Two tubings were placed into the pump and connected to the medium in the flask. The investigations started after injection of 500 µL VOR bolus solution into the medium to reach C_{max}. Samples of VOR from the medium were collected after 1, 10 and 30 min after injection, followed by 30 or 60 min intervals up to 8 h (n=1 sample per time point for (i) and n=3 sample per time point for (ii)). Based on the data from Simmel *et al.*, a long-term investigation of 55 h was performed for VOR (iii). Samples were taken in 30 to 60 min intervals during 55 h (n=1 sample per time point).

Anidulafungin

Mimicry of concentration-time profiles of AFG was based on plasma data from Crandon *et al.* [73]. The PR was set to 55.5 µL/min for AFG. The injection solution had a C_{nom} of 660 µg/mL AFG for a C_{max} of 6.60 µg/mL in RS/HSA (0.5%) containing medium. In the inner chamber of the glass flask was a volume of 99.0 mL RS/HSA (0.5%). The temperature of the water bath was set to 37°C and the stirring velocity to 700 min^{-1}. Two tubings were placed into the pump and connected to the medium in the flask. The investigations started after injection of 1 mL AFG bolus solution into the medium to reach C_{max}. Samples of AFG from medium were collected after 2, 10 and 30 min after injection, afterwards in 30 min intervals up to 2 h and followed by 10 to 20 min intervals up to 6 h (n=1 sample per time point).

Pump rate settings and bioanalytical quantification

After starting the pump, empty vials were weighed and subsequently 3 samples of the outflow of each tubing (n_{tubing}=2) were taken per minute and weighed consecutively. The PR was determined (with Eq. 2-8 for volume and the PR=volume per minute) and adjusted if necessary. The investigation started after the nominal PR was achieved. After the end of the investigation, the procedure for determining the PR was performed again. Here, the PR was determined and potential deviations from previously determined PR were recorded.

Medium Samples from investigations with VOR and AFG were quantified with the respective HPLC assays (see chapter 2.2). The *in silico* concentration-time profile was based on C_{obs} of AFG or VOR in

the injection solution of the syringe. To describe the variability of the analyte concentration in medium, the variability, expressed as CV (%), of the pump in the dIVMS and of the bioanalytical method (precision of QC samples) of the respective analyte, was plotted around the *in silico* calculated concentration-time profiles of the analyte.

2.5.4 *In vitro* microdialysis investigations with the dynamic *in vitro* microdialysis system

The dIVMS was validated previously and various concentration-time profiles of VOR and AFG were recorded as described in the previous paragraph. Hence, µDialysis investigations with the model drugs VOR and AFG using retrodialysis as calibration method were enabled with the dIVMS.

Voriconazole

µDialysis investigation of VOR in the dIVMS was performed with 3 CMA60® catheters (20 kDa cut-off). Catheters were perfused with VOR-free RS at a FR of 2 µL/min. The investigation started after the i.v. bolus injection of C_{nom} 9.4 µg/mL VOR into the RS in the inner chamber of the flask (V_{flask}=100 mL). To mimic the concentration-time profile, the same volume of fresh RS was pumped into the inner chamber of the flask as VOR in RS was pumped from the flask at a PR of 136 µL/min. µDialysate samples (n=1 sample per catheter and time point) were taken in 10 or 30 min intervals for 5 h. Medium samples (n=1 per time point) were taken after 2, 10 and 30 min followed by 30 min intervals up to 5 h. The µDialysis investigation started after an equilibration phase of 15 min. Subsequently to the µDialysis investigation, the retrodialysis investigation was performed with a perfusate containing 20 µg/mL (i) or 200 µg/mL (ii) VOR in RS. Here, after a 15 min equilibration phase, samples (n=1 sample per microsyringe) were taken from perfusate in 10 min intervals for 30 min.

Experimental settings for investigations (i) and (ii) are listed in Tab. 2.5-2.

Tab. 2.5-2: Experimental settings of microdialysis investigations in the dynamic *in vitro* microdialysis system with 20 and 200 µg/mL voriconazole (VOR) in perfusate during retrodialysis. C_{max}: maximum concentration; RS: Ringer's solution.

Experimental settings	Retrodialysis with 20 µg/mL VOR	Retrodialysis with 200 µg/mL VOR
Catheter, membrane length	CMA60®, 30 mm	
Perfusate	RS	
Medium	VOR in RS	
Flow rate [µL/min]	2	
C_{max} in medium [µg/mL]	0.94	
Pump rate [µL/min]	136	
Sampling intervals microdialysate [min]	10 and 30 min	
Sampling intervals medium [min]	2, 10, 30 min and in 30 min intervals	

Anidulafungin

µDialysis investigation of AFG in the dIVMS was performed with 3 CMA71® catheters (100 kDa cut-off). Catheters were perfused with AFG-free RS/HSA (0.5%) at a FR of 1 µL/min. The investigation started after the i.v. bolus injection of C_{nom} 660 µg/mL AFG into the RS/HSA (0.5%) in the inner chamber of the flask. To mimic the concentration-time profile, the same volume of fresh RS/HSA (0.5%) was pumped into the inner chamber of the flask as AFG in RS/HSA (0.5%) was pumped from the flask at a PR of 55.5 µL/min. µDialysate samples (n=1 sample per catheter and time point) were taken in 40 min intervals for 6 h. Medium samples (n=1 per time point) were taken 1 min before and 2, 10 and 30 min after start of investigation followed by 30 min intervals up to 6 h. The µDialysis investigation started after an equilibration phase of 15 min. Subsequently to the µDialysis investigation, the retrodialysis investigation was performed with a perfusate containing 200 µg/mL AFG in RS/HSA (0.5%). Here, after a 15 min equilibration phase, samples (n=1 sample per microsyringe) were taken from perfusate in 40 min intervals for 120 min.

Experimental settings for the investigation are listed in Tab. 2.5-3.

Tab. 2.5-3: Experimental settings of microdialysis investigations with the dynamic *in vitro* microdialysis system with 200 µg/mL anidulafungin (AFG) in perfusate during retrodialysis. C_{max}: maximum concentration; RS: Ringer's solution; HSA: human serum albumin.

Experimental settings	Retrodialysis with 200 µg/mL AFG
Catheter, membrane length	CMA71®, 20 mm
Perfusate	RS/HSA (0.5%)
Medium	AFG in RS/HSA (0.5%)
Flow rate [µL/min]	1
C_{max} in medium [µg/mL]	6.60
Pump rate [µL/min]	55.5
Sampling intervals microdialysate [min]	40 min
Sampling intervals medium [min]	-1, 2, 10, 30 min and in 30 min intervals

Pump rate settings, bioanalytical quantification and calculations

The PR was adjusted as explained in chapter 2.5.3.2.

The AFG and VOR sample vials of µDialysate were weighed before and after the sampling intervals. The weight of the samples was determined, and the respective volume calculated (Eq. 2-4). The accuracy expressed as the percentage deviation of the nominal volume (RE, %) of the µDialysate sample from the nominal volume of 40 µL was calculated.

Samples of injection solution, medium, perfusate and µDialysate were quantified with the validated HPLC assay for AFG or VOR (see chapter 2.2.3).

The *in silico* concentration-time profile was based on C_{obs} of AFG or VOR in the i.v. injection solution of the syringe. To describe the variability of the analyte concentration in medium, the variability, expressed as CV (%), of the pump in the dIVMS and of the bioanalytical method (precision of QC samples) of the respective analyte, was plotted around the *in silico* calculated concentration-time profiles of the analyte. The CV of the pump in the dIVMS was 4.50% (rounded up from 4.38% (Tab. 3.4-2) after equilibration (5-6 h interval). A maximum CV (precision of QC samples) of the bioanalytical method of VOR was 9.32%, which was rounded up to 9.50% (Tab. 3.1-3), and of AFG 8.00%.

After analysing AFG or VOR in µDialysate samples from retrodialysis, the RR was calculated (Eq. 1-3). A mean RR was determined per catheter (n=3 samples per catheter) and AFG or VOR medium concentrations, based on the µDialysate concentrations, were calculated according to Eq. 1-4. To compare the observed concentration in medium to the calculated medium concentrations from

µDialysate, the variability of the pump and the respective bioanalytical method was plotted around the observed medium concentration.

2.6 Descriptive and explorative statistics

For statistical analysis, different software as described in section 2.7 was applied. In Tab. 2.6-1, description and calculation of standard localisation and dispersion parameters are given [93]. Throughout the work, a central tendency was described by localisation parameters and the variability of a distribution by dispersion parameters. The CI was used as an interval estimate of a parameter, which likely contained the parameter.

Tab. 2.6-1: Statistical parameters for localisation and dispersion.

Parameter	Expression
Localisation parameters (continuous data)	
Arithmetic mean ($\bar{x}$)	$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$
Relative error (RE)	$\mathrm{RE}, \% = \frac{C_{obs} - C_{nom}}{C_{nom}} \cdot 100$
Dispersion parameters (continuous data)	
Range (R)	Minimum (Min) – Maximum (Max)
Standard deviation (SD)	$SD = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$
Coefficient of variation (CV)	$CV, \% = \frac{SD}{\bar{x}} \cdot 100$

In addition, explorative statistical analysis was also accomplished using appropriate statistical tests shown in Tab. 2.6-2 [93]. The choice of the appropriate statistical test was dependent on the statistical problem, the distribution of data (normally and not normally distributed), the homogeneity of variance of the parameter (homogeneous or heterogeneous) and the sample size (n). The significance level α was set to 0.05, implying statistically significant differences (rejection of null hypothesis) with probability values (p) of less than 5% ($p<0.05$).

Tab. 2.6-2: Statistical hypothesis tests.

Statistical test	Description
Test on normal distribution	
Shapiro-Wilk normality test	Test of normality of the data (n<50)
Test on homogeneity of variance	
F-test	Test of homogeneity of two variances
Levene's test	Test of homogeneity of variances of more than two groups
Parametric test (applied for normally distributed data and homogeneous variances)	
One-way ANOVA (analysis of variance)	Test to compare the means of more than two groups (unpaired samples)
Student's t-test	Test of the significance of the difference between two sample means (two unpaired samples)
Non-parametric test (applied for not normally distributed data)	
Kruskal-Wallis test	One-way analysis of variance by ranks to compare multiple unpaired samples
Wilcoxon-Mann-Whitney test (rank-sum test)	Rank test to compare two unpaired samples

Linear regression analysis was used to describe the relationship between values of a dependent variable (outcome of a measurement, y_i) and one or more independent variables (x_i). The relation was calculated using the regression equation y=f(x, ß), representing y as a function of the independent variables x, the unknown model parameters ß, and corresponding constants. The aim of the regression analysis was to estimate model parameters, which best described the relation among variables. Estimation was achieved by using least square method, which meant reduction of squared distances between observed and predicted values of the dependent variable y. The goodness of fit was characterised and confirmed by the coefficient of determination R^2 ($0 \leq R^2 \leq 1$). An R^2 of 1 implies that the regression line perfectly fits the data. With decreasing R^2, data is less well described by the regression line. By plotting calculated values against predicted values, the model fit was also assessed graphically (goodness-of-fit plots).

A nonlinear regression model was used to describe the RR over time. Hence, RR_{max}, $t_{RR,50}$ and $t_{RR,90}$ were estimated with a mathematical model assuming Michaelis-Menten kinetics (Eq. 2-6).

2.7 Software

Microsoft® Office Excel 2016	Microsoft Corporation, Redmond, Washington, USA
R, version 3.4.1	R Core Team (2017), Vienna, Austria URL http://www.R-project.org/
RStudio, version 1.0.143	RStudio Team (2016), Inc., Boston, MA, USA
Chromeleon®	ThermoFisher SCIENTIFIC Chromatography Data System, Waltham, Massachusetts, USA, Version 7.2, 2016

For calculation of AFG and VOR concentrations from analysed µDialysate, medium and perfusate samples, Microsoft® Office Excel 2016 and R 3.4.1 together with RStudio 1.0.143 were used. Statistical analysis was performed using R 3.4.1, RStudio and Microsoft® Office Excel 2016, statistical tests were performed in R 3.4.1 and RStudio 1.0.143. HPLC system control, data acquisition, and processing (integration of HPLC signals) were carried out with Chromeleon®.

3 Results

3.1 Bioanalytical methods for quantification of anidulafungin and voriconazole in microdialysate

3.1.1 Method development

After successful preliminary method development, the experimental settings were determined. The HPLC settings of experimental parameters of AFG and VOR were listed in Tab. 3.1-1.

Tab. 3.1-1: Experimental parameters of anidulafungin and voriconazole bioanalytical HPLC assays.

Experimental parameters	Anidulafungin	Voriconazole
Column	XBridge® BEH C18 (3.0 x 50 mm; 2.5 µm)	Symmetry® C18 (4.6 x 75 mm; 3.5 µm)
Pre-column	Accucore® C18 (10x2.1 mm; 2.6 µm)	Accucore® C18 (10x4.6 mm; 2.6 µm)
Mobile phase	Methanol/ $NH_4H_2PO_4$ (pH 4.6) (80:20, v:v)	Methanol/ $NH_4H_2PO_4$ (pH 4.6) (55:45, v:v)
Flow rate [mL/min]	0.40	1.00
Run time [min]	6.00	8.00
Injection volume [µL]	20.0	
Pressure (upper limit) [bar]	400	
Temperature sampler [°C]	10.0	
Temperature column oven [°C]	40.0	
Wavelength UV lamp [nm]	310	254

Sample (Cal samples, QC samples and *in vitro* µDialysate samples) preparation for AFG in RS/HSA (0.5%) included a precipitation step for protein separation. Precipitation was realised by adding 70 µL methanol to 20 µL of AFG containing samples. Afterwards the methanol containing sample was vortex mixed for 30 s and centrifuged at 5000 g for 10 min at 22°C. Finally, the clear supernatant was transferred into a cavity of a 96-well-plate.

For preparation of VOR samples, 16 µL of VOR containing samples in RS (Cal samples, QC samples and *in vitro* µDialysate samples) were diluted with 24 µL water in the respective well of the 96-well-plate before analysis.

3.1.2 Method validation

The two developed bioanalytical assays for AFG and VOR, described in chapter 3.1.1, were validated according to EMA Guideline on bioanalytical method validation [3].

Selectivity of the analytical method

Chromatograms resulting from injections of blank matrix (RS+HSA (0.5%) or dextran 40 (5 mg/mL)/HSA (0.5%) in water) showed no interferences with the AFG peak (C_{nom}=0.10-20 µg/mL). The same was true for chromatograms of blank RS and the peak of VOR (C_{nom}=0.15-10 µg/mL), also there no interferences were recorded.

After injecting the CFG containing solution (C_{nom}=10 µg/mL), no peak was visible in the chromatogram. Using only AFG (C_{nom}=0.10-20 µg/mL) or the combination with CFG (C_{nom}=10 µg/mL) containing samples resulted in one peak for AFG and no visible peak for CFG (Fig. 3.1-1).

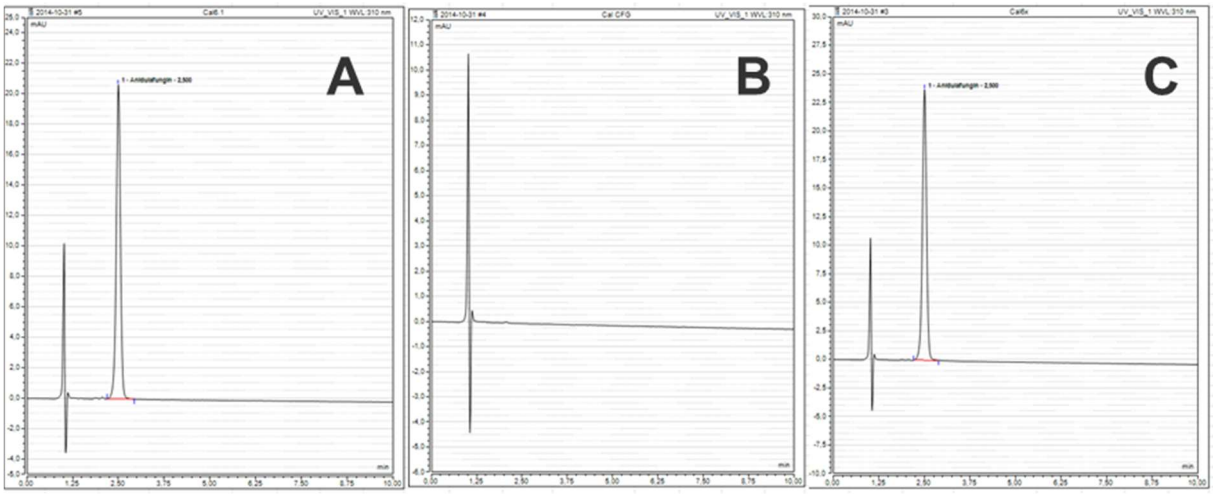

Fig. 3.1-1: (A) Chromatogram of anidulafungin (AFG) (nominal concentration (C_{nom})= 20 µg/mL) in Ringer's solution and human serum albumin (RS/HSA) (0.5%); (B) chromatogram of caspofungin (CFG) (C_{nom}= 10 µg/mL) in RS/HSA (0.5%); (C) chromatogram of AFG (C_{nom}= 20 µg/mL) and CFG (C_{nom}= 10 µg/mL) in RS/HSA (0.5%).

Carry-over

After subsequent injection of samples concentrated at the ULOQ of AFG or VOR and blank matrix, no analyte was detected in the blank matrix following the respective ULOQ injection (n_{AFG}=6; n_{VOR}=5).

Accuracy and precision

Within- and between-day accuracy and precision were within the limits of RE ≤±20% for samples with concentrations below the LLOQ and RE ≤±15% for QC samples concentrated higher than LLOQ. Precision and accuracy of AFG samples ranged between 4.10% to 6.07% for within-day and from 4.40% to 8.00% for between-day precision as well as from -9.38% to +1.41% for within-day and from -6.69% to +0.519% for between-day accuracy (Tab. 3.1-2). Samples of VOR ranged from 1.63% to

6.51% for within-day and from 5.00% to 9.32% for between-day precision and from -4.01% to +2.15% for within-day and from +2.58% to +14.2% for between-day accuarcy (Tab. 3.1-3).

Tab. 3.1-2: Within-day and between-day accuracy (as mean percentage deviation, RE, %) and precision (as coefficient of variation, CV, %) of determined anidulafungin concentrations [µg/mL] in Ringer's solution and human serum albumin (0.5%) quality control samples.

C_{nom} [µg/mL]	x_{mean} ± SD [µg/mL]	CV, %	RE, %
Within-day variability (n=6), day 1			
0.10	$0.101 \pm 4.15\ 10^{-3}$	4.10	+1.41
0.30	$0.272 \pm 1.64\ 10^{-2}$	6.02	-9.38
10.0	9.69 ± 0.589	6.07	-3.10
15.0	13.9 ± 0.806	5.81	-7.55
Between-day variability (n=24)			
0.10	$9.8\ 10^{-2} \pm 7.33\ 10^{-3}$	7.50	-1.60
0.30	$0.280 \pm 1.48\ 10^{-2}$	5.28	-6.69
10.0	10.1 ± 0.439	4.40	+0.519
15.0	14.8 ± 1.19	8.00	-1.28

Tab. 3.1-3: Within-day and between-day accuracy (as mean percentage deviation, RE, %) and precision (as coefficient of variation, CV, %) of determined voriconazole concentrations [µg/mL] in Ringer's solution quality control samples.

C_{nom} [µg/mL]	x_{mean} ± SD [µg/mL]	CV, %	RE, %
Within-day variability (n=6), day 2			
0.15	$0.153 \pm 9.98\ 10^{-3}$	6.51	+2.15
0.45	$0.432 \pm 1.64\ 10^{-2}$	3.79	-4.01
5.00	$4.93 \pm 8.05\ 10^{-2}$	1.63	-1.31
7.50	7.54 ± 0.242	3.21	+0.559
Between-day variability (n=24)			
0.15	$0.171 \pm 1.60\ 10^{-2}$	9.32	+14.2
0.45	$0.462 \pm 3.17\ 10^{-2}$	6.87	+2.58
5.00	5.39 ± 0.297	5.51	+7.80
7.50	8.06 ± 0.404	5.00	+7.50

Lower limit of quantification and assay linearity

The LLOQ was determined as 0.1 µg/mL for AFG in *in vitro* µDialysate sample matrix of RS/HSA (0.5%) and as 0.15 µg/mL VOR in RS, respectively. Concentrations (0.05 µg/mL to 0.30 µg/mL were

investigated for both analytes) lower than 0.1 µg/mL or 0.15 µg/mL were outside the acceptable limits for accuracy and precision (CV ≤20% and RE ≤±20%,). The observed concentrations (n=5) for LLOQ of AFG or VOR were determined with a precision of 6.48% and 9.94% and an accuracy (calculated as mean percentage deviation) of +2.20% and +0.815%, respectively. Assay linearity was demonstrated by using weighted linear regression analysis (weighting factor: 1/concentration^2) of the peak areas against the nominal AFG or VOR concentrations in the respective matrix. Calibration functions showed linearity over the calibration range of 0.10-20.0 µg/mL for AFG and 0.15-10.0 µg/mL for VOR. Linearity was indicated by the coefficient of determination $R^2 \geq 0.996$ for all functions of AFG (n=4) and $R^2 \geq 0.997$ for all functions of VOR (n=4). Mean calibration functions were determined on four validation days in two weeks (Tab. 3.1-4 and Tab. 3.1-5).

Tab. 3.1-4: Regression parameters for anidulafungin in Ringer's solution and human serum albumin (0.5%). AU: arbitrary unit.

Day	n	Slope [AU·mL/µg]	Intercept [AU]	Coefficient of determination
1	1	0.446	$-5.55\ 10^{-3}$	0.996
2	1	0.407	$1.92\ 10^{-4}$	0.998
4	1	0.360	$7.57\ 10^{-4}$	0.997
5	1	0.373	$1.47\ 10^{-3}$	0.999
$\bar{x}$ ± SD	4	$0.397 \pm 3.85\ 10^{-2}$	$-7.82\ 10^{-4} \pm 3.22\ 10^{-3}$	$0.998 \pm 1.60\ 10^{-3}$
(CV, %)		(9.70)	(411)	(0.161)

Tab. 3.1-5: Regression parameters for voriconazole in Ringer's solution. AU: arbitrary unit.

Day	n	Slope [AU·mL/µg]	Intercept [AU]	Coefficient of determination
2	1	0.159	$8.65\ 10^{-4}$	0.998
3	1	0.160	$-3.59\ 10^{-3}$	0.997
4	1	0.146	$-3.71\ 10^{-3}$	0.997
5	1	0.147	$-2.32\ 10^{-3}$	0.997
$\bar{x}$ ± SD	4	$0.153 \pm 7.51\ 10^{-3}$	$-2.19\ 10^{-3} \pm 2.13\ 10^{-3}$	$0.997 \pm 5.69\ 10^{-4}$
(CV, %)		(4.90)	(97.4)	($5.70\ 10^{-2}$)

Dilution integrity

The determined diluted concentrations fulfilled the requirements of the Guideline on bioanalytical method validation [3] concerning accuracy and precision (RE≤± 15%, CV≤15%). Accuracy and precision for serial and non-serial dilution of the respective concentrations are presented in Tab.

3.1-6 for AFG and in Tab. 3.1-7 for VOR. Accuracy of the serial dilution of AFG ranged from -11.9 to -10.5% and of VOR from -7.10% to -1.03%. Precision of serial dilution was given with a CV up to 5.58% for AFG and 6.75% for VOR. The non-serial dilution of AFG samples had an accuracy from -8.43% to -0.053% and of VOR from +6.88% to 10.6%. Precision of non-serial dilution was up to 4.68% for AFG and 3.86% for VOR.

Tab. 3.1-6: Accuracy (as mean percentage deviation, RE, %) and precision (as coefficient of variation, CV, %) of serial and non-serial dilution of anidulafungin with Ringer's solution and human serum albumin (0.5%).

Dilution design	C_{nom} [µg/mL]	x_{mean} ± SD [µg/mL]	RE, %	CV, %
Serial	20 (n=5)	17.9 ± 0.998	-10.5	5.58
	10 (n=5)	8.81 ± 0.355	-11.9	4.03
	4.0 (n=5)	3.54 ± 0.118	-11.5	3.33
Non-serial	20 (n=5)	18.4 ± 0.860	-8.19	4.68
	10 (n=5)	9.16 ± 0.232	-8.43	2.54
	4.0 (n=7)	3.99 ± 0.153	-0.053	3.83

Tab. 3.1-7: Accuracy (as mean percentage deviation, RE, %) and precision (as coefficient of variation, CV, %) of serial and non-serial dilution of voriconazole with Ringer's solution.

Dilution design	C_{nom} [µg/mL]	x_{mean} ± SD [µg/mL]	RE, %	CV, %
Serial	10 (n=3)	9.90 ± 0.140	-1.03	1.41
	5.0 (n=3)	4.87 ± 0.256	-2.55	5.25
	0.50 (n=3)	0.464 ± 0.031	-7.10	6.75
Non-serial	10 (n=3)	11.1 ± 0.041	+10.6	0.371
	5.0 (n=3)	5.34 ± 0.206	+6.88	3.86

Drug stability in microdialysate

For low and high QC samples of AFG in RS/HSA (0.5%), the freeze and thaw, short-term at room temperature, autosampler and long-term stability of stock solution were analysed and showed no degradation or enrichment tendencies. Results for recovery were in agreement with the limits of the Guideline on bioanalytical method validation [3].

Recovery of AFG in *in vitro* µDialysate matrix after 1 - 3 freeze-thaw cycles ranged from 90.2% (CV: 3.88%) to 93.8% (CV: 3.35%) for low QC sample (n=6 per cycle) compared to freshly prepared samples. For high QC samples, recovery of freeze-thaw-samples ranged from 91.1% (CV: 2.31%) to 97.1% (CV: 2.56%) after 1 - 3-cycles, compared to peak areas before freezing (n=6 per cycle). For the stock solution (n=5; analysed as high QC concentration) 105% (CV: 2.69%) of AFG was recovered after

the first freeze-thaw cycle, 99.7% (CV: 2.04%) after the second freeze-thaw cycle and 111% (CV: 3.81%) after the third freeze-thaw cycle.

For stock solution of AFG (n=3; analysed as high QC concentration) 93.4% (CV: 5.67%) was recovered after 6 h at ambient temperature compared to freshly prepared samples.

Under storage conditions at room temperature after 4 and 24 h, recovery of AFG ranged from 99.5% (CV: 2.03%) after 4 h to 102% (CV: 1.85%) after 24 h for low QC sample concentrations (n=6 per cycle) compared to freshly prepared samples. For high QC samples, recovery (n=6 per cycle) ranged from 95.3% (CV: 4.63%) after 4 h to 102% (CV: 4.40%) after 24 h.

Autosampler stability of AFG was investigated by storing QC samples of low and high concentration in the 96-well-plate in the tempered (10°C) autosampler up to 24 h. The mean recovery of low concentrations (n=3 per cycle) resulted in 101.2% (CV: 1.99%) after 6 h, and 101.4% (CV: 2.65%) after 24 h. For QC samples of high concentration (n=3 per cycle) the mean recovery was 98.4% (CV: 1.22%) after 6 h, and 97.8% (CV: 1.68%) after 24 h.

Long-term stability of QC samples with AFG of low and high concentration stored in the ultrafreezer (-80°C) was investigated. The mean recovery of QC samples of low concentrations (n=5) was 89.8% (CV: 2.21%), and 94.8% (CV: 3.34%) for QC samples of high concentration (n=5).

The autosampler stability of low and high QC samples of VOR in RS showed no degradation or enrichment tendencies. Results for recovery were also in agreement with the limits of the Guideline on bioanalytical method validation [3].

Autosampler stability of VOR was investigated by storing QC samples of low and high concentration in the 96-well-plate in the tempered (10°C) autosampler up to 48 h. The mean recovery of QC samples of low concentrations (n=3 per cycle) was 96.9% (CV: 2.60%) after 6 h, and 96.8% (CV: 2.67%) after 48 h. For QC samples of high concentration (n=3 per cycle) the mean recovery was 95.6% (CV: 0.84%) after 6 h, and 96.0% (CV: 1.87%) after 48 h.

3.2 Anidulafungin investigations in the static *in vitro* microdialysis system

3.2.1 Feasibility of microdialysis for anidulafungin: Behaviour of anidulafungin in microsyringes

During investigation (i) C_{AFG} in microsyringes (n=9 per microsyringe) and production vial (n=8) were constant over time and ranged from 0.145 to 0.184 µg/mL and from 0.132 to 0.188 µg/mL, respectively, for 320 min (Fig. 3.2-1). Mean, accuracy of single concentrations against the concentration at t=0 min per sample and precision of C_{AFG} for microsyringes and the production vial are shown in Tab. 3.2-1. CV and RE range of all microsyringes were smaller than the CV of 11.6% and the RE range of -30.0% to -11.7% of the production vial. The profile displayed no increase or decrease of C_{AFG} during the investigated time.

C_{AFG} in the production vial (n=9) of investigation (ii) was constant (CV, %: 2.69). In microsyringes of investigation (ii), C_{AFG} (n=9 per microsyringe) increased from 0.196 to 0.432 µg/mL (Fig. 3.2-1). Mean, accuracy of single concentrations against the concentration at t=0 min per sample and precision was calculated per microsyringe as shown in Tab. 3.2-1. The mean C_{AFG} of the three microsyringes per time point increased from 0.202 µg/mL (CV, %: 2.58) at time point 0, to 0.270 µg/mL (CV, %: 0.996) after 40 min and 0.370 µg/mL (CV, %: 14.6) after 320 min. The overall accuracy of concentrations over time in microsyringes of investigation (i) ranged from RE -9.89% to +14.6% and of investigation (ii) from RE +31.6% to +110%.

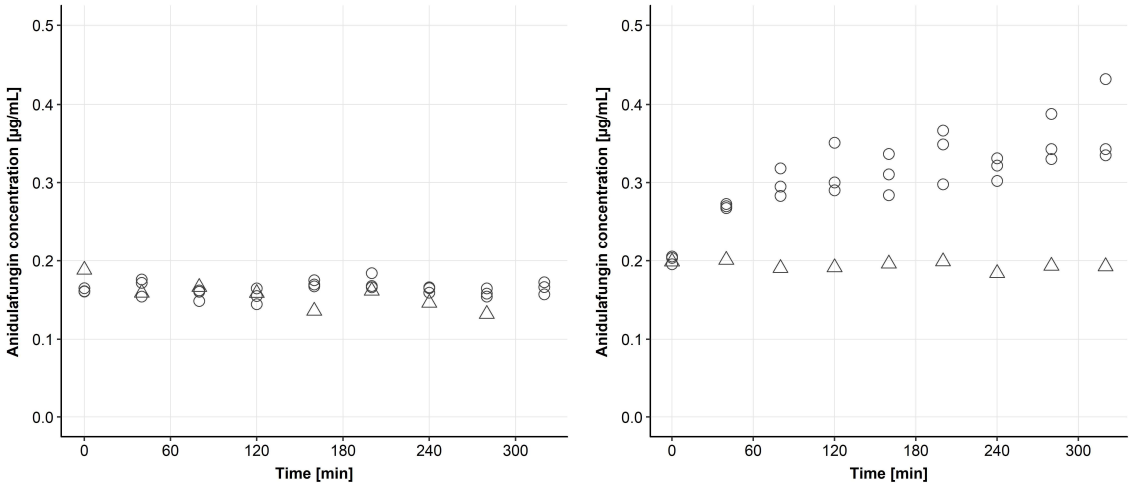

Fig. 3.2-1: Anidulafungin concentration in investigation (i) new microsyringes (left) and in investigation (ii) previously used microsyringes (right). Microsyringes (n=9 per microsyringe) are presented as circles and production vials (n=8/9 per production vial) as triangles over 320 min.

Tab. 3.2-1: Means of anidulafungin concentrations, coefficient of variation (CV) and range of relative error (RE) from investigation (i) new or investigation (ii) previously used microsyringes. n=9 samples per microsyringe; n=8/9 for production vial (i)/(ii).

Microsyringe/ Production vial	Mean C_{AFG} [µg/mL] CV, % RE range, % (i)	Mean C_{AFG} [µg/mL] CV, % RE range, % (ii)
1	0.160 4.84 (-7.68) – +8.72	0.330 20.3 +31.6 - +110
2	0.164 6.80 (-4.25) – +6.68	0.306 14.9 +36.9 – +71.0
3	0.167 3.20 (-9.98) – +14.6	0.288 14.2 +34.2 – +71.1
Production vial	0.156 11.6 (-30.0) – (-11.7)	0.194 2.69 (-7.33) – +1.11

3.2.2 Feasibility of microdialysis for anidulafungin: Comparison of microdialysis catheters

C_{AFG} in medium was constant over time in all medium containers with a mean of 8.65 µg/mL (n=15; CV: 5.57%). Data is presented in Fig 8-1. Catheters (CMA63® and CMA71®) were compared regarding the respective RR (n=1 sample per time point and catheter). RR was presented as midtime (the time point in the middle of the sampling interval). C_{AFG} was below LLOQ (0.1 µg/mL, see chapter 2.2.3) in all µDialysate samples collected from CMA63® catheters (Fig. 3.2-2). C_{AFG} in µDialysate of CMA71® catheters was increasing for 90 min, apart from 1 µDialysate sample taken after 45 min, which had a considerable low concentration. Over 90 min the RR increased for catheters of CMA71® from 11.7% to 18.4% and from 8.62% to 18.4%, respectively (Fig. 3.2-2). Due to these results, CMA71® catheters were used throughout the following investigations.

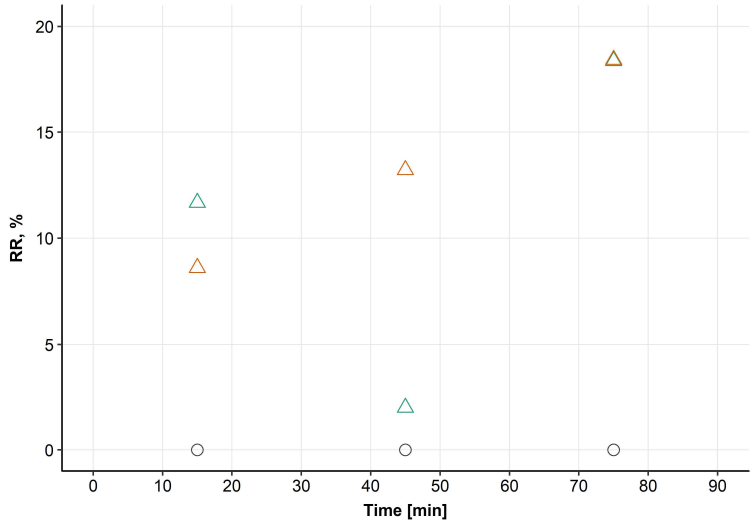

Fig. 3.2-2: Relative recovery (RR) over time (time shown as midtime) for CMA63® (n=2 catheters, purple and black circles) and CMA71® (n=2 catheters, green and orange triangles) from a medium of 10 µg/mL anidulafungin (AFG). The AFG concentration is below the lower limit of quantification in all µDialysate samples collected from CMA63® catheters. All RR values of CMA63® are overlapping at each time point. RR values of both CMA71® catheters are overlapping at 75 min.

3.2.3 Feasibility of microdialysis for anidulafungin: Recovery investigation of anidulafungin with microdialysis catheters

The percentage deviation (RE, %) of observed compared to nominal µDialysate volume was calculated. The range of RE is shown in Tab. 3.2-2. RE per catheter is shown in Fig. 3.2-3 and RE over time in Fig. 3.2-4. The RE of µdialysate volume ranged from -24.6% to -17.3% for all catheters. A mean volume (n=12 per catheter) of 31.2 µL (CV: 1.81%) was calculated for catheter 1, 31.9 µL (CV: 1.81%) for catheter 2 and 32.0 µL (CV: 1.50%) for catheter 3.

Tab. 3.2-2: Minimum and maximum relative errors (RE) of recovered compared to nominal microdialysate volume for the three catheters (n=12 samples per catheter) during the recovery investigation in medium containing anidulafungin (1 µg/mL).

Catheter	RE_{min}, %	RE_{max}, %
1	-18.8	-24.6
2	-17.8	-22.3
3	-17.3	-21.5

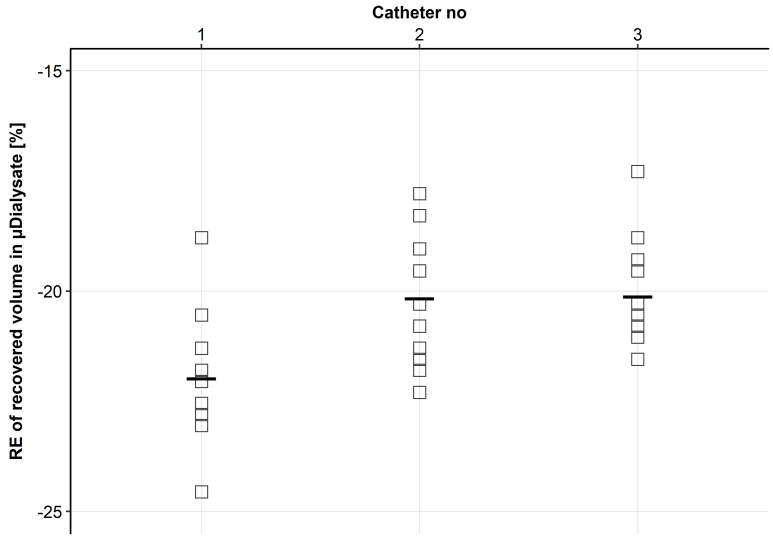

Fig. 3.2-3: Relative error (RE) and mean RE (n=12 per catheter) of recovered volume in microdialysate for three CMA71® catheters perfused with Ringer's solution and human serum albumin in perfusate and a medium containing 1 µg/mL anidulafungin, presented per catheter. Individual RE presented as symbols and means as bars.

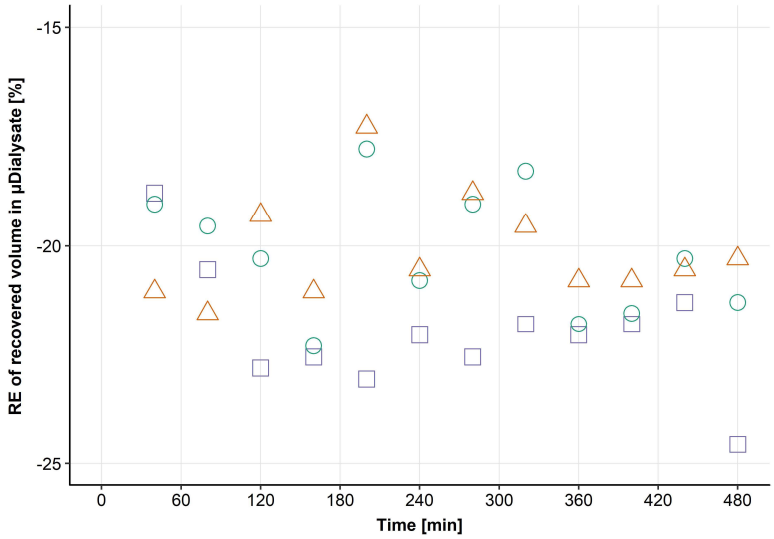

Fig. 3.2-4: Relative error (RE) of recovered volume in microdialysate for three CMA71® catheters during 480 min of the investigation with Ringer's solution and human serum albumin in perfusate and a medium containing 1 µg/mL anidulafungin. Catheter 1 presented as violet squares, catheter 2 as green circles and catheter 3 as orange triangles.

Results for RR and $RR_{Volume\text{-}corrected}$ are shown in Tab. 3.2-3. The range of RR was from < LLOQ/ 7.47% to 34.2% and of $RR_{Volume\text{-}corrected}$ from < LLOQ/ 6.07% to 26.9%. The profile of RR and $RR_{Volume\text{-}corrected}$ over time is presented in Fig. 3.2-5.

Tab. 3.2-3: Minimum and maximum relative recovery (RR_{min}, RR_{max}) for recovery of 1 µg/mL anidulafungin in medium from observed anidulafungin concentrations in microdialysate and RR_{min}, RR_{max} with volume correction (n=12 per catheter). < LLOQ: concentration in microdialysate below the lower limit of quantification (LLOQ).

Catheter	RR_{min}, %	RR_{max}, %
No volume correction		
1	7.47	31.8
2	8.41	34.2
3	< LLOQ	29.1
Volume correction		
1	6.07	24.0
2	6.81	26.9
3	< LLOQ	23.2

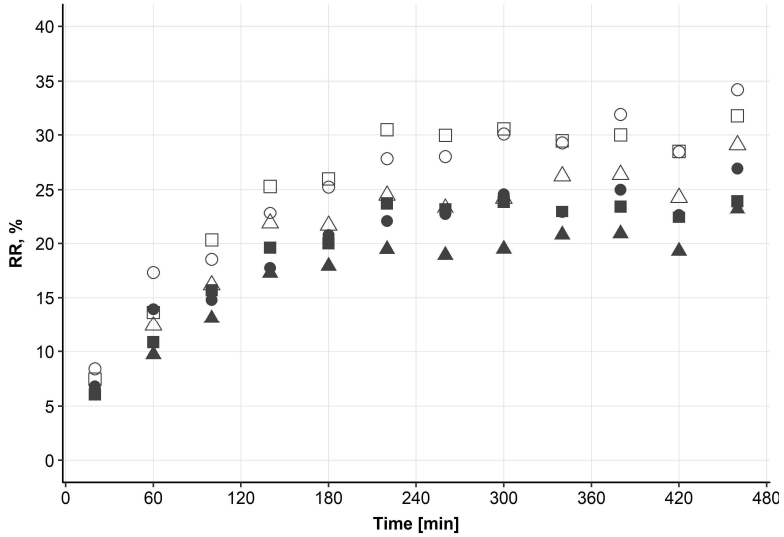

Fig. 3.2-5: Relative recovery (RR) without volume correction (open symbols) and RR with volume correction (filled symbols) during 480 min (time shown as midtime) of recovery investigation for catheter 1 (squares), catheter 2 (circles) and catheter 3 (triangles) in 1 µg/mL AFG containing medium.

Based on the results of RR during the recovery investigation, RR_{max} and the confidence intervals as well as the time until 50% ($t_{RR,50}$) and 90% ($t_{RR,90}$) of RR_{max} was reached were determined. Model-based results of RR_{max} from values for RR and $RR_{Volume\text{-}corrected}$ are compared in Tab. 3.2-4. RR_{max} was 35.6% without volume correction and 27.9% with volume correction. Confidence intervals of RR_{max} without volume correction were from 32.8% to 38.4% and from 25.9% to 30.0% for RR_{max} with volume correction. The confidence intervals were not overlapping and therefore indicated a significant difference between investigations. $t_{RR,50}$ and $t_{RR,90}$ were similar for both investigations with and without volume correction. $t_{RR,50}$ and $t_{RR,90}$ were 81.7 min and 735 min for RR values without

volume correction, respectively. For volume corrected RR $t_{RR,50}$ and $t_{RR,90}$ were 79.5 min and 716 min, respectively.

The time-course of RR and $RR_{Volume\text{-}corrected}$ is shown in Fig. 3.2-6, including RR_{max} and $t_{RR,50}$.

Tab. 3.2-4: The estimated parameters and confidence intervals (CI, upper limit 97.5% and lower limit 2.5% percentile) of the maximum relative recovery (RR_{max}) from RR and $RR_{Volume\text{-}corrected}$ for the investigation with 1 µg/mL AFG in medium; obtained from the adsorption model based on nonlinear regression.

Parameter	Estimated results [95% CI]
No volume correction	
RR_{max}, %	35.6 [32.8-38.4]
$t_{RR,50}$ [min]	81.7
$t_{RR,90}$ [min]	735
Volume correction	
RR_{max}, %	27.9 [25.9-30.0]
$t_{RR,50}$ [min]	79.5
$t_{RR,90}$ [min]	716

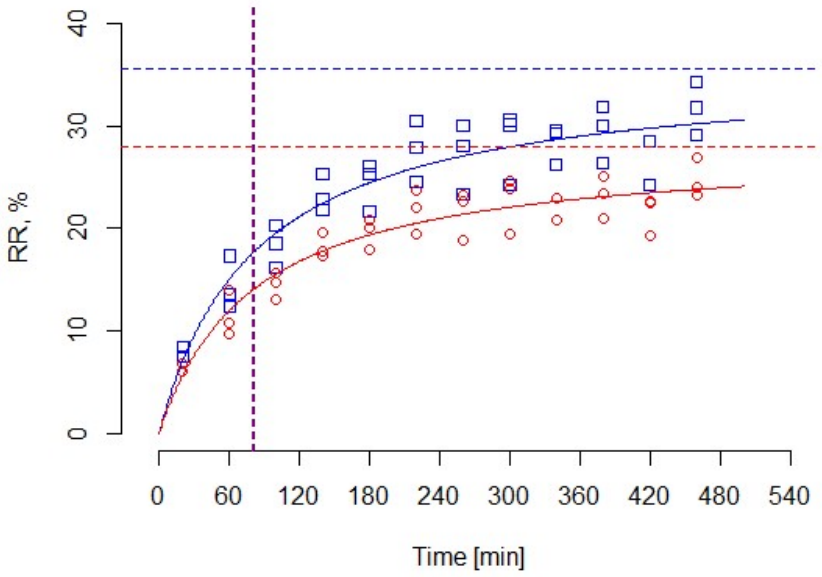

Fig. 3.2-6: Time-course (time shown as midtime) of relative recovery (RR) during the investigated time interval from RR (blue squares) and $RR_{Volume\text{-}corrected}$ (red circles) in a medium containing 1 µg/mL anidulafungin (AFG) in Ringer's solution (RS) and human serum albumin (HSA, 0.5%) with a perfusate of RS+HSA (0.5%); individual RR values (symbols) from AFG concentrations in microdialysate of three catheters; maximum RR (RR_{max}) as blue and red dashed line, respectively. The time of 50% of RR_{max} ($t_{RR,50}$) is presented as vertical red and blue dashed line, respectively.

3.2.4 Feasibility of microdialysis for anidulafungin: Influence of dextran on ultrafiltration

The accuracy (RE, %) of the recovered volume in µDialysate was calculated and results are shown in Tab. 3.2-5 and Fig. 3.2-7. Accuracy of catheter 1 ranged from RE -23.8% to +0.68% and of catheter 2 from RE -9.51% to -4.54%. RE of recovered µDialysate volume over time was constant (Fig. 3.2-8) for the two catheters with a CV of 11.5% for catheter 1 (n=12) and 9.08% for catheter 2 (n=12).

Tab. 3.2-5: Minimum and maximum relative error (RE_{min}, RE_{max}) of the recovered to the nominal µDialysate volume for the two catheters during recovery investigation with dextran and human serum albumin in perfusate (n=12 per catheter).

Catheter	RE_{min}, %	RE_{max}, %
1	+0.68	-23.8
2	-4.54	-9.51

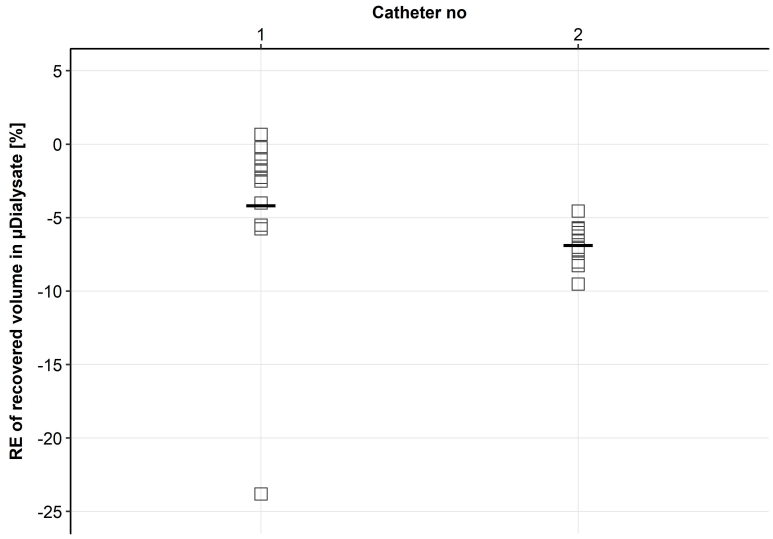

Fig. 3.2-7: Relative error (RE) and mean RE (n=12 per catheter) of recovered volume in microdialysate (µDialysate) for two CMA71® catheters perfused with dextran containing perfusate in a medium containg 1 µg/mL anidulafungin, presented per catheter. Individual RE presented as symbols and means as bars.

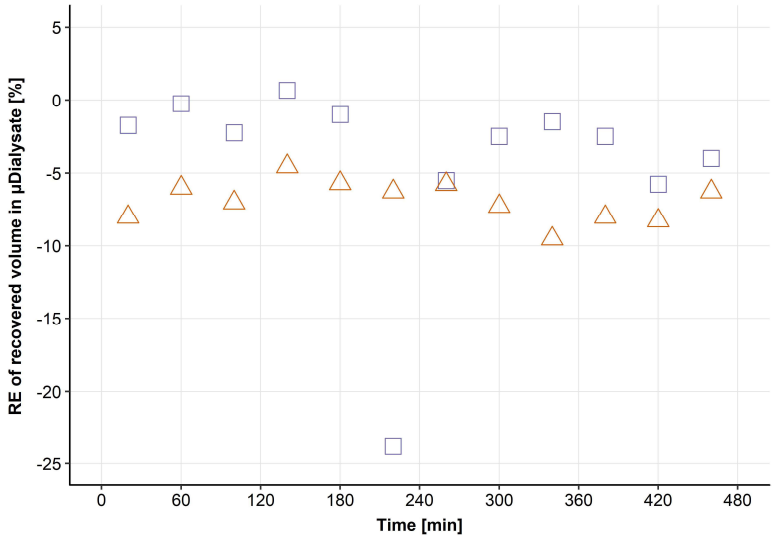

Fig. 3.2-8: Relative error (RE) of recovered volume in microdialysate (µDialysate) for two CMA71® catheters during 480 min of the investigation with dextran in perfusate in a medium containg 1 µg/mL anidulafungin. Catheter 1 presented as violet squares and catheter 2 as orange triangles.

C_{AFG} in medium of the respective catheters was constant for the investigated 480 min. The mean C_{AFG} (n=7 samples per medium) was 0.983 µg/mL (CV: 4.50%) for catheter 1 and 1.50 µg/mL (CV: 4.83%) for catheter 2.

In the following, RR was calculated without volume correction, with the observed C_{AFG} (Eq. 1-1), and with volume correction (Eq. 2-5). RR was increasing over time from 1.58% to 35.9% of catheter 1 and from 5.49% to 26.7% of catheter 2 (Tab. 3.2-6). In Fig. 3.2-9 data on RR and $RR_{Volume\text{-}corrected}$ over time (shown as midtime) is shown.

Tab. 3.2-6: Minimum and maximum relative recovery (RR_{min}, RR_{max}) for recovery of 1 µg/mL AFG in medium with perfusate containing dextran and human serum albumin from C_{AFG} in microdialysate (n=11-12 per catheter).

Catheter	RR_{min}, %	RR_{max}, %
1	1.58	35.9
2	5.49	26.7

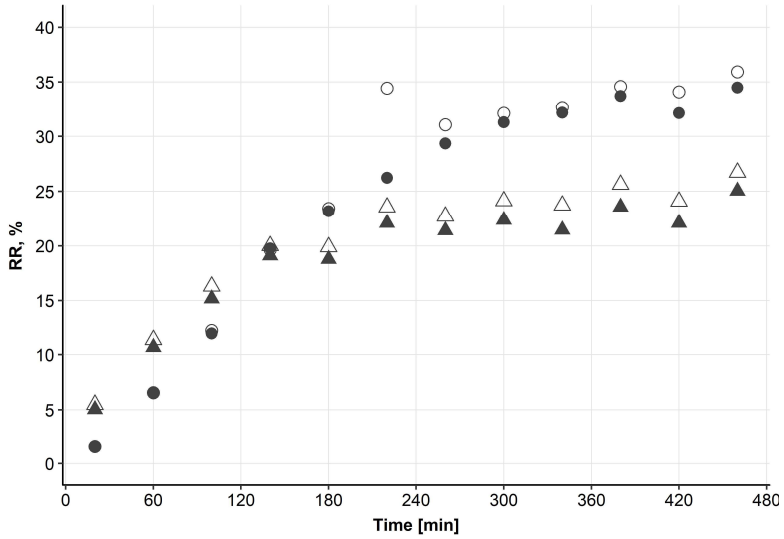

Fig. 3.2-9: Relative recovery (RR) without volume correction (open symbols) and $RR_{Volume\text{-}corrected}$ (filled symbols) during 480 min (time shown as midtime) of recovery investigation for catheter 1 (circles) and 2 (triangles) with a perfusate containing dextran in 1 µg/mL AFG containing medium.

RR values were calculated (Eq. 2-6) from observed C_{AFG} in µDialysate and based on these results, RR_{max} and the respective confidence interval and $t_{RR,50}$ and $t_{RR,90}$ were determined. Model-based results of RR_{max} of the investigation with dextran and HSA in perfusate were compared to the previous investigation with HSA containing perfusate (see chapter 3.2.3) in Tab. 3.2-7. RR_{max} based on volume corrected RR values was 44.5% for the investigation with the dextran containing perfusate and 35.6% for the investigation with HSA containing perfusate. Since confidence intervals of RR_{max} were overlapping, differences between the investigations were not significant. $t_{RR,50}$ was 183 min for the investigation with the dextran containing perfusate but only 81.7 min with the RS/HSA (0.5%) perfusate. It was the same for $t_{RR,90}$ with 1647 min and only 735 min, respectively.

RR_{max} based on volume corrected RR values was 42.0% for the investigation with the dextran containing perfusate and 27.9% for the investigation with HSA containing perfusate. The confidence intervals of RR_{max} were not overlapping and therefore, there was a significant difference between the volume corrected RR values of the two investigations. $t_{RR,50}$ and $t_{RR,90}$ were 185 min and 1663 min for the investigation with the dextran containing perfusate and 79.5 min and 716 min for the investigation with HSA containing perfusate. The RR_{max}, $t_{RR,50}$ and $t_{RR,90}$ of volume corrected RR values were similar to the RR values without volume correction.

Fig. 3.2-10 presents the time-course of RR for the two investigations with perfusate containing dextran and HSA (0.5%) or RS and HSA (0.5%) and the (estimated) RR_{max} and $t_{RR,50}$ for two investigated perfusion media and in Fig. 3.2-11 the time-course of volume corrected RR, RR_{max} and $t_{RR,50}$ of the two investigations.

Tab. 3.2-7: The estimated parameters and confidence intervals (CI, upper limit 97.5% and lower limit 2.5% percentile) of RR_{max} anidulafungin (AFG) concentrations in microdialysate for the investigation with a perfusate containing dextran and human serum albumin (HSA) or Ringer's solution (RS) and HSA in 1 µg/mL AFG in medium; from the adsorption model based on nonlinear regression.

Parameter	**Estimated results [95% CI]** Dextran and HSA (n=2 catheters)	**Estimated results [95% CI]** RS and HSA (n=3 catheters)
No volume correction		
RR_{max}, %	44.5 [33.9-55.2]	35.6 [32.8-38.4]
$t_{RR,50}$ [min]	183	81.7
$t_{RR,90}$ [min]	1647	735
Volume correction		
RR_{max}, %	42.0 [31.9-52.0]	27.9 [25.9-30.0]
$t_{RR,50}$ [min]	185	79.5
$t_{RR,90}$ [min]	1663	716

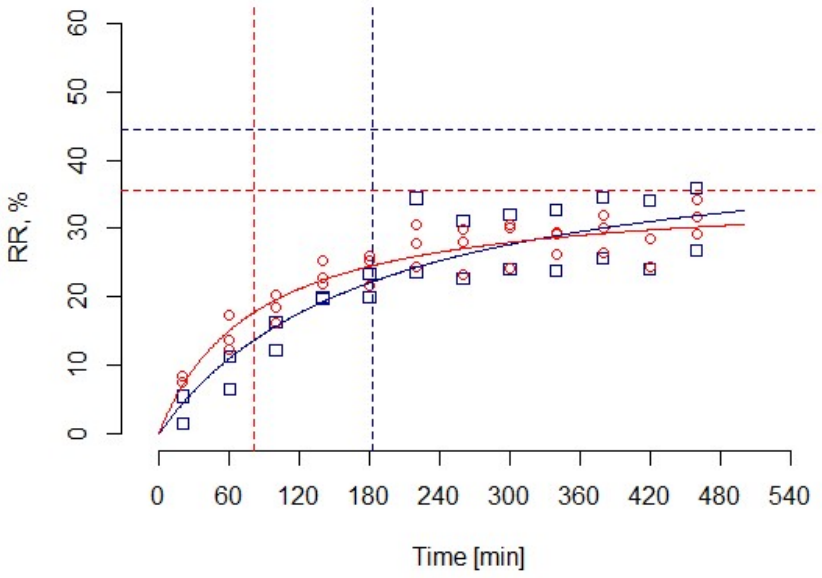

Fig. 3.2-10: Time-course (time shown as midtime) of relative recovery (RR) during the investigated time interval; individual RR values from anidulafungin (AFG) concentrations of two catheters from dextran and human serum albumin (0.5%, HSA) containing perfusate (blue squares) and from Ringer's solution and HSA (0.5%) containing perfusate (red circles) each in a medium containing 1 µg/mL AFG; maximum RR (RR_{max}) is presented as blue and red dashed lines, respectively. The time of 50% of RR_{max} ($t_{RR,50}$) is presented as vertical red and blue dashed line, respectively.

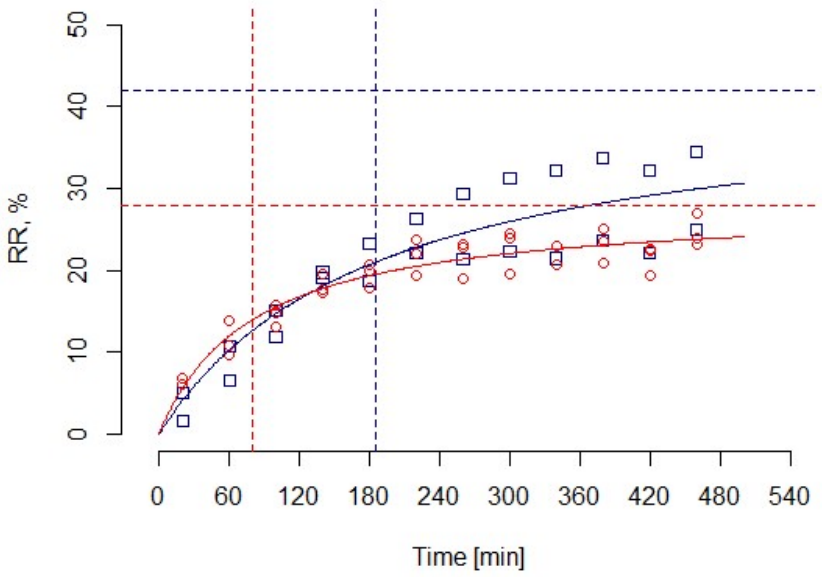

Fig. 3.2-11: Time-course (time shown as midtime) of volume corrected relative recovery (RR) during the investigated time interval; individual volume corrected RR values from anidulafungin (AFG) concentrations of two catheters from dextran and human serum albumin (0.5%, HSA) containing perfusate (blue squares) and from Ringer's solution and HSA (0.5%) containing perfusate (red circles) each in a medium containing 1 µg/mL AFG; maximum RR (RR_{max}) is presented as blue and red dashed lines, respectively. The time of 50% of RR_{max} ($t_{RR,50}$) is presented as vertical red and blue dashed line, respectively.

3.2.5 Feasibility of microdialysis for anidulafungin: Influence of catheter pre-coating on adsorption

3.2.5.1 Catheter pre-coating with caspofungin

Anidulafungin concentration in medium

Medium samples (n=6-7 per medium) were analysed and the mean and CV of all media over the whole investigation period (400 min and 480 min, respectively) in the respective investigation was calculated. The mean C_{AFG} in medium was 1.14 µg/mL for investigation (i), 0.961 µg/mL for investigation (ii) and 0.927 µg/mL for investigation (iii) as shown in Tab. 3.2-8.

Tab. 3.2-8: Summary of mean and coefficient of variation (CV, %) from anidulafungin concentrations in medium (nominal concentration of 1 µg/mL) during the respective investigations (i), (ii) and (iii).

Pre-coating investigation with CFG	Mean C_{AFG} in medium [µg/mL]	CV, %
(i)	1.14 (n=18)	8.42
(ii)	0.961 (n=21)	3.63
(iii)	0.927 (n=21)	5.28

Relative recovery

RR is summarised in Tab. 3.2-9 by presenting the minimum and maximum RR (RR_{min}, RR_{max}). The RR range of investigation (i) ranged from 2.93% to 43.1%, of investigation (ii) from < LLOQ /6.74% to 36.5% and of investigation (iii) from < LLOQ /6.56% to 56.4%. Overall, RR from µDialysate samples of AFG in all catheters of (i), (ii) and (iii) were increasing over time (Fig. 3.2-12, Fig. 3.2-13 and Fig. 3.2-14).

Tab. 3.2-9: Minimum and maximum relative recovery (RR_{min}, RR_{max}) for (i), (ii) and (iii) of pre-coating investigations with CFG. < LLOQ: concentration in microdialysate below the lower limit of quantification (LLOQ).

Catheter	RR_{min}, %	RR_{max}, %
(i) n=5 per catheter		
1	2.93	25.1
2	7.04	43.1
3	7.70	28.8
(ii) n=12 per catheter		
1	8.27	36.5
2	< LLOQ /18.1	34.0
3	6.74	34.3
(iii) n=12 per catheter		
1	6.56	24.1
2	< LLOQ /6.87	18.2
3	6.62	56.4

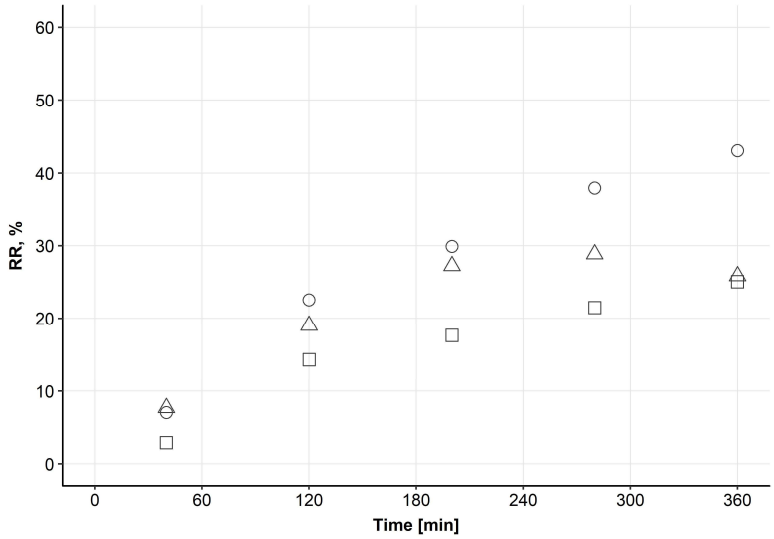

Fig. 3.2-12: Relative recovery (RR) from 1 µg/mL anidulafungin in medium during 360 min in setting (i) with pre-coating of 50 µg/mL caspofungin in perfusate starting 2 h prior to investigation (time shown as midtime). Catheter 1 presented as squares, catheter 2 as circles and catheter 3 as triangles.

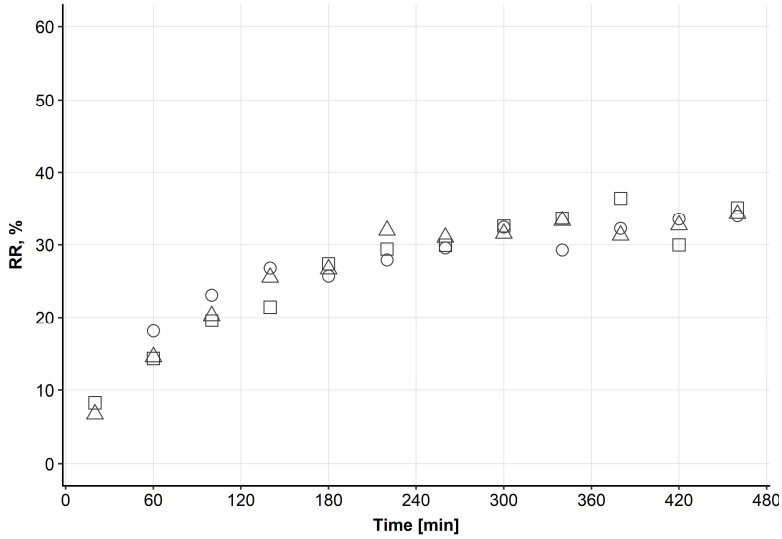

Fig. 3.2-13: Relative recovery (RR) from 1 µg/mL anidulafungin (AFG) in medium during 480 min in setting (ii) with pre-coating of 50 µg/mL caspofungin in perfusate overnight (time shown as midtime). Catheter 1 presented as squares, catheter 2 as circles and catheter 3 as triangles. The AFG concentration in the first sample of catheter 2 was lower then the lower limit of quantification.

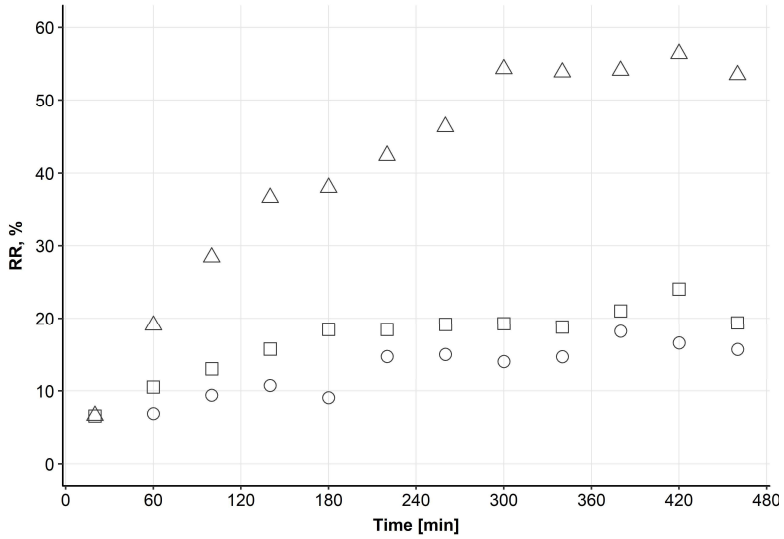

Fig. 3.2-14: Relative recovery (RR) from 1 µg/mL anidulafungin (AFG) in medium during 480 min in setting (iii) with pre-coating of 200 µg/mL caspofungin in perfusate overnight (time shown as midtime). Catheter 1 presented as squares, catheter 2 as circles and catheter 3 as triangles. The AFG concentration in the first sample of catheter 2 was lower then the lower limit of quantification.

RR_{max}, $t_{RR,50}$ and $t_{RR,90}$ were estimated from RR values of C_{AFG} in µDialysate, and the confidence intervals of RR_{max} of (i), (ii) and (iii) were determined. Model-based results of RR_{max} from three individual investigations with CFG pre-coated catheters were compared to each other and the standard investigation with RS/HSA (0.5%) in perfusate (see chapter 3.2.3) in Tab. 3.2-10. RR_{max} of investigation (i) was 53.5%, (ii) was 41.2%, (iii) was 41.4% and for the investigation with RS/HSA (0.5%) in perfusate RR_{max} was 35.6%. Confidence intervals of RR_{max} for the four investigated experimental designs with pre-coating with CFG and without pre-coating (RS/HSA (0.5%) in perfusate) were overlapping (no significant differences between investigations). Confidence intervals of RR_{max} of (i) and (iii) were with 23.2%-83.7% and 16.7%-66.0%, respectively, much larger compared to (ii) and the investigation with RS/HSA (0.5%) containing perfusate (39.0%-43.3% and 32.8%-38.4%, respectively).

$t_{RR,50}$ was 238 min for (i), 94.2 min for (ii), 140 min for (iii) and 81.7 min for RS/HSA (0.5%) containing perfusate. $t_{RR,90}$ was 2142 min for (i), 848 min for (ii), 1264 min for (iii) and 735 min for RS/HSA (0.5%) containing perfusate. $t_{RR,50}$ and $t_{RR,90}$ values of investigation (ii) and of the investigation with RS/HSA (0.5%) in perfusate had the shortest times and had similar values.

The RR-time profile of CFG-pre-coated catheters and of recovery investigation with RS/HSA (0.5%) in perfusate are presented in Fig. 3.2-15. RR_{max} and $t_{RR,50}$ are presented as dashed lines for the respective investigations.

Tab. 3.2-10: The estimated parameters and confidence intervals (CI, upper limit 97.5% and lower limit 2.5% percentile) of RR_{max} from anidulafungin concentrations in microdialysate during investigations (i), (ii) and (iii) of 1 µg/mL AFG in medium and for the investigation with 1 µg/mL AFG in medium (see Tab. 3.2-4); from the adsorption model based on nonlinear regression.

Parameter	Estimated results [95% CI]			Estimated results [95% CI]
	(i)	(ii)	(iii)	RS and HSA
	(n=3 catheter)	(n=3 catheter)	(n=3 catheter)	(n=3 catheter)
RR_{max}, %	53.5 [23.2-83.7]	41.2 [39.0-43.3]	41.4 [16.7-66.0]	35.6 [32.8-38.4]
$t_{RR,50}$ [min]	238	94.2	140	81.7
$t_{RR,90}$ [min]	2142	848	1264	735

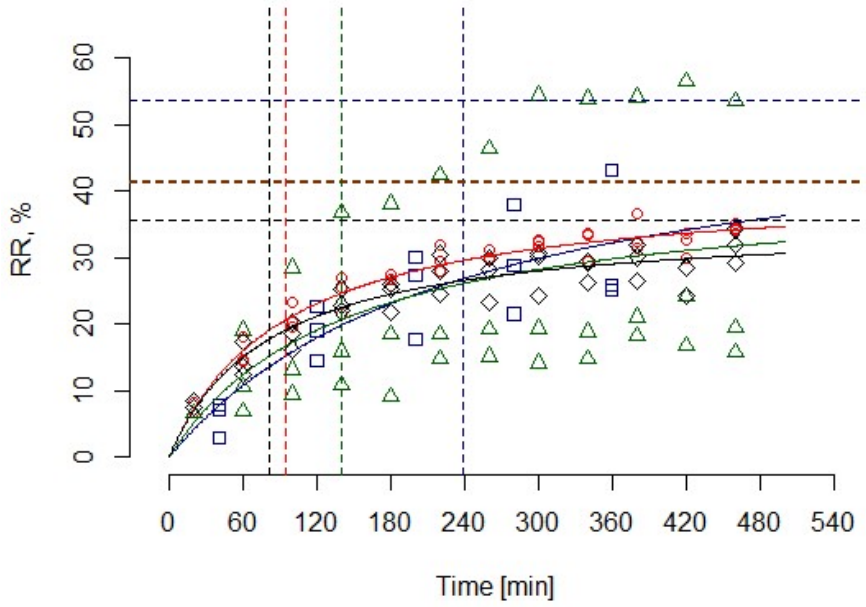

Fig. 3.2-15: Time-course (time shown as midtime) of relative recovery (RR) during the investigated time interval from anidulafungin (AFG) concentrations in microdialysate of caspofungin pre-coated catheters: (i) blue squares, (ii) red circles and (iii) green triangles; RR for recovery investigation with Ringer's solution and human serum albumin in perfusate and a 1 µg/mL AFG containing medium presented as black diamonds. Estimated RR_{max} presented as blue (i), red (ii), green (iii) and black dashed lines. The time of 50% of RR_{max} ($t_{RR,50}$) is presented as vertical blue (i), red (ii), green (iii) and black dashed line, respectively.

3.2.5.2 Catheter pre-coating with anidulafungin

Anidulafungin concentration in medium

C_{AFG} in medium, over the whole investigated period (480 min per investigation), was constant during the investigation in 1 and 8 µg/mL AFG containing medium (Tab. 3.2-11) with C_{AFG} of 1.01 µg/mL and 8.04 µg/mL, respectively.

Tab. 3.2-11: Mean and coefficient of variation (CV) of anidulafungin concentrations (C_{AFG}) in medium (n=21 per investigation).

C_{AFG} in medium	Mean C_{AFG} [µg/mL]	CV, %
1 µg/mL	1.01	4.17
8 µg/mL	8.04	7.87

Anidulafungin concentration in perfusate

Data for mean and CV of C_{AFG} in perfusate (n=7 per catheter and investigation) during the investigations is shown in Tab. 3.2-12. The mean C_{AFG} was 0.393 µg/mL (CV: 4.15%) in the investigation with 1 µg/mL AFG in medium and 0.160 (CV: 7.35%) in the investigation with 8 µg/mL AFG in medium. Fig 8-2 and Fig 8-3 additionally present data at the end of pre-coating, respectively. C_{AFG} in perfusate was constant at the end of pre-coating and during the investigated time.

Anidulafungin concentration in microdialysate

Mean C_{AFG} in µDialysate samples (n=2 per catheter), taken during the process of pre-coating in 1 µg/mL AFG investigation (medium of RS/HSA (0.5%) and perfusate 0.2 µg/mL AFG in RS/HSA (0.5%)), was 0.300 µg/mL (CV: 4.07%) for catheter 1, 0.352 µg/mL (CV: 4.74%) for catheter 2, and 0.352 µg/mL (CV: 0.400%) for catheter 3. Delivery, rD, of C_{AFG} in µDialysate was 23.1%, 12% and 11.1% from C_{AFG} in perfusate during pre-coating, respectively.

Mean C_{AFG} in µDialysate (n=2 per catheter) during the pre-coating of the 8 µg/mL AFG investigation (medium of RS/HSA (0.5%) and perfusate 0.2 µg/mL AFG in RS/HSA (0.5%)) was 0.0705 µg/mL (CV: 10.9%) for catheter 1, 0.0831 µg/mL (CV: 8.08%) for catheter 2, and 0.0829 µg/mL (CV: 16.8%) for catheter 3. Here the percentage of delivered AFG, rD, from perfusate to medium during pre-coating was 55.0%, 47.1% and 48.7%, respectively.

Results are shown in Tab. 3.2-13.

Tab. 3.2-12: Mean and coefficient of variation (CV) of anidulafungin concentrations (C_{AFG}) in perfusate; overall (n=21 per investigation) and per catheter (n=7 per investigation) during recovery investigation.

Perfusate	Mean C_{AFG} [µg/mL]	CV, %
1 µg/mL AFG in medium		
investigation		
1	0.387	2.57
2	0.403	3.22
3	0.388	5.33
Overall	0.393	4.15
8 µg/mL AFG in medium		
investigation		
1	0.158	6.73
2	0.157	7.58
3	0.165	7.63
Overall	0.160	7.35

Tab. 3.2-13: Mean, coefficient of variation (CV) and relative delivery (rD) of anidulafungin (AFG) concentrations (C_{AFG}) in microdialysate during pre-coating (n=2 per catheter) in a medium of Ringer's solution (RS) and human serum albumin (HSA, 0.5%) with a perfusate of 0.2 µg/mL AFG in RS/HSA (0.5%).

Catheter	Mean C_{AFG} [µg/mL]	CV, %	rD, %
1 µg/mL AFG in medium			
investigation			
1	0.300	4.07	23.1
2	0.352	4.74	12.0
3	0.352	0.400	11.1
8 µg/mL AFG in medium			
investigation			
1	0.0705	10.9	55.0
2	0.0831	8.08	47.1
3	0.0829	16.8	48.7

C_{AFG} in µDialysate during pre-coating and the recovery investigation is presented in Fig 8-4 and Fig 8-5. The figures show, that C_{AFG} in µDialysate increased during both investigations.

The course of C_{AFG} in µDialysate, perfusate and medium is shown in Fig. 3.2-16 for both investigations during the recovery investigation. Here, C_{AFG} in medium and perfusate was constant during the time investigated (480 min) while C_{AFG} in µDialysate samples were increasing. C_{AFG} in µDialysate increased partly up to the medium concentration during the investigation with 1 µg/mL AFG in medium (left plot in Fig. 3.2-16) but not for the 8 µg/mL AFG in medium investigation (right plot in Fig. 3.2-16).

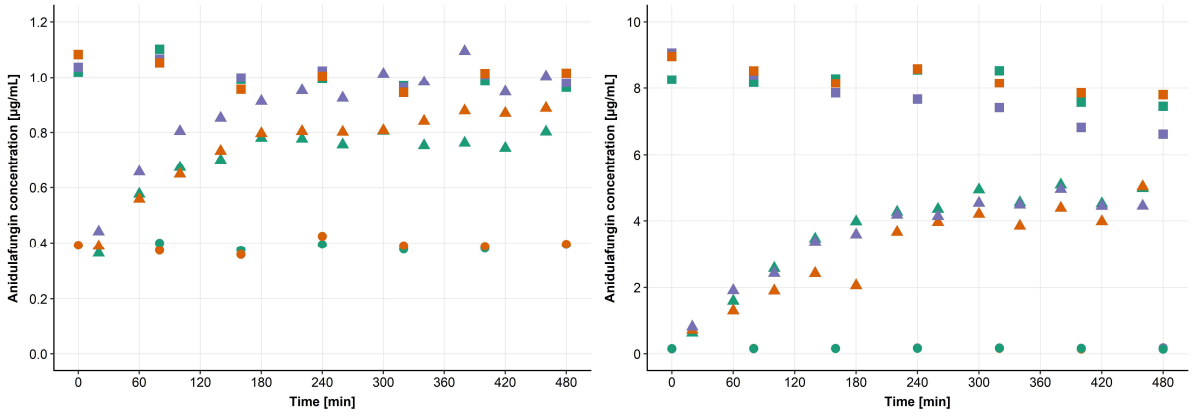

Fig. 3.2-16: Anidulafungin (AFG) concentration (C_{AFG}) in microdialysate (triangles), time shown as midtime, C_{AFG} in perfusate (circles) and C_{AFG} in medium (squares). Recovery investigation from 1 µg/mL (left) and 8 µg/mL (right) containing medium with a perfusate of 0.2 µg/mL AFG in Ringer's solution and human serum albumin (0.5%).

Relative recovery of anidulafungin

As described previously, RR was calculated from C_{AFG} of µDialysate, perfusate and medium using Eq. 2-7. RR values (n=12 per catheter and investigation) of both investigations were increasing over time (presented as midtime) and ranged from -3.94% to 113% (1 µg/mL AFG in medium investigation) and from 5.89% to 63.5% (8 µg/mL AFG in medium investigation). RR values of 5 catheters from both investigations were in a similar RR-range of -3.94% to 79.8%. Only catheter 3 of the investigation with 1 µg/mL AFG in medium had RR values up to 113%. Results are presented in Fig. 3.2-17 and summarised in Tab. 3.2-14 for both investigations.

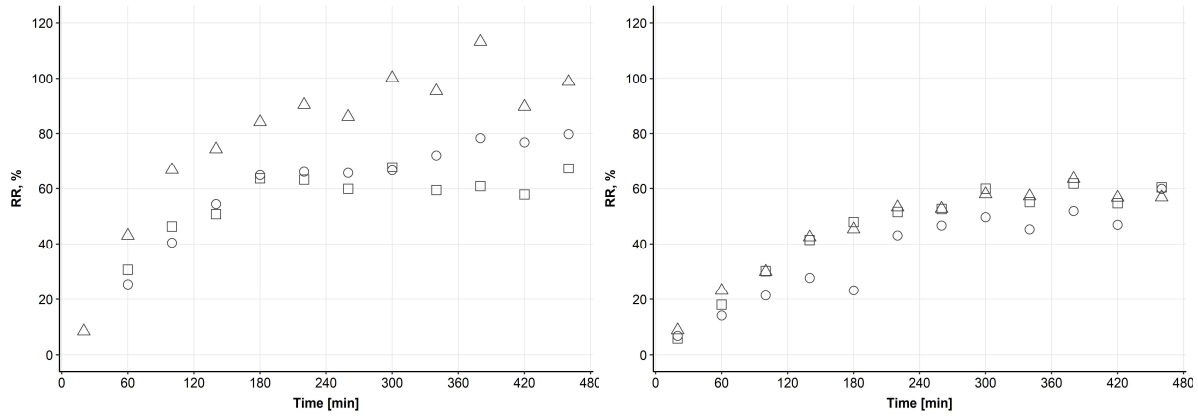

Fig. 3.2-17: Relative recovery (RR) from anidulafungin concentrations (C_{AFG}) in microdialysate (symbols) over time (time shown as midtime). Pre-coating investigation with anidulafungin (AFG), 1 µg/mL (left) and 8 µg/mL (right) AFG in medium. Catheter 1 presented as squares, catheter 2 as circles and catheter 3 as triangles. Values for the first sampling interval (20 min) of catheter 1 and 2 are missing due to negative values for RR.

Tab. 3.2-14: Minimum and maximum relative recovery (RR_{min}, RR_{max}) per catheter (n=12) and the overall range of RR per setting (n=36). RR for 1 and 8 µg/mL AFG in medium.

Catheter	RR_{min}, %	RR_{max}, %
1 µg/mL AFG in medium		
1	-3.94	67.8
2	-2.59	79.8
3	8.46	113
8 µg/mL AFG in medium		
1	5.89	61.8
2	6.81	59.8
3	8.90	63.5

Model-based estimations and confidence intervals of RR_{max}, $t_{RR,50}$ and $t_{RR,90}$ are presented in Tab. 3.2-15 for the two AFG pre-coating recovery investigations. Confidence intervals of RR_{max} of the pre-coating investigations with 1 and 8 µg/mL AFG in medium (85.8%-125% and 71.2%-97.2%, respectively) were overlapping with each other but not with the confidence intervals of the standard investigation without pre-coating (32.8%-38.4%) in 1 µg/mL AFG containing medium (see chapter 3.2.3), which implied that there was a significant difference between the standard investigation without pre-coating and the two pre-coating investigations.

$t_{RR,50}$ was 116 min of the pre-coating investigation with 1 µg/mL AFG in medium and 1047 min for $t_{RR,90}$. $t_{RR,50}$ and $t_{RR,90}$ of the pre-coating investigation with 8 µg/mL AFG in medium was 187 min and

1686 min, respectively. For the investigation without pre-coatung the $t_{RR,50}$ and $t_{RR,90}$ were 81.7 min and 735 min, respectively.

The RR-time course is presented in Fig. 3.2-18 with RR_{max} and $t_{RR,50}$ plotted as dashed lines for both investigations. Here, it is graphically displayed that the investigation with 1 µg/mL AFG in medium had the highest RR_{max} and the shortest $t_{RR,50}$.

Tab. 3.2-15: The estimated parameters and confidence intervals (CI, upper limit 97.5% and lower limit 2.5% percentile) of RR_{max} from anidulafungin (AFG) concentrations (C_{AFG}) for the AFG pre-coating investigations with 1 or 8 µg/mL AFG in medium and with 1 µg/mL AFG in medium without pre-coating (perfusate containing RS/HSA); from the adsorption model based on nonlinear regression in 'R'.

Parameter	**Estimated results [95% CI]** 1 µg/mL AFG in medium (n=3 catheter)	**Estimated results [95% CI]** 8 µg/mL AFG in medium (n=3 catheter)	**Estimated results [95% CI]** RS and HSA (n=3 catheter)
RR_{max}	105 [85.8-125]	84.2 [71.2-97.2]	35.6 [32.8-38.4]
$t_{RR,50}$ [min]	116	187	81.7
$t_{RR,90}$ [min]	1047	1686	735

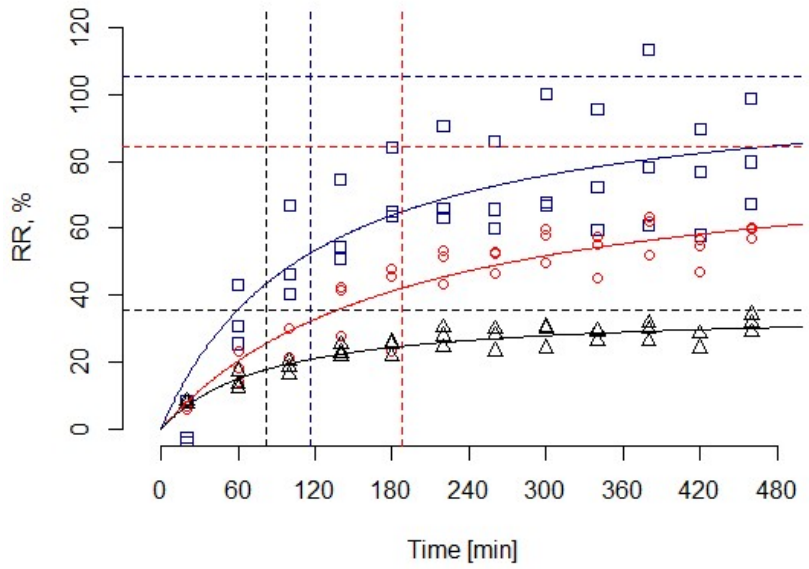

Fig. 3.2-18: Time-course (time shown as midtime) of relative recovery (RR) during the investigated time interval with individual RR values (symbols) for pre-coating investigations with 1 µg/mL anidulafungin (AFG) in medium (blue squares), 8 µg/mL AFG in medium (red circles) and with 1 µg/mL AFG in medium without coating (black triangles); RR_{max} as blue, red and black dashed lines, respectively. The time of 50% of RR_{max} ($t_{RR,50}$) is presented as vertical blue, red and black dashed line, respectively.

3.2.6 Feasibility of microdialysis for anidulafungin: Investigation of retrodialysis

C_{AFG} in perfusate was constant during the investigated time of investigation (i) with AFG-free and investigation (ii) with 10 µg/mL AFG in RS/HSA (0.5%) medium. C_{nom} of perfusate was 200 µg/mL AFG in RS/HSA (0.5%) resulting in the mean C_{AFG} (n=3 per catheter) of 215 µg/mL (CV: 2.56%) for catheter 1, 222 µg/mL (CV: 7.19%) for catheter 2 and 212 µg/mL (CV: 4.77%) for catheter 3 (Tab. 3.2-16). Hence, C_{AFG} in perfusate was constant during the investigated time.

Tab. 3.2-16: Mean and coefficient of variation (CV) of anidulafungin concentrations (C_{AFG}) in perfusate (n=3 per catheter) with a nominal concentration of 200 µg/mL AFG during delivery investigation.

Perfusate	Mean C_{AFG} [µg/mL]	CV, %
1	215	2.56
2	222	7.19
3	212	4.77

In medium, the mean C_{AFG} of (i) was determined at time point 0 min and no AFG was detected. C_{AFG} in medium increased over time. After 80 min, the mean C_{AFG} was 0.094 µg/mL (CV: 8.85%), hence <LLOQ, and after 120 min 0.153 µg/mL (CV: 17.2%) (n=3 per time point of three catheters). In (ii), the mean C_{AFG} of 10 µg/mL AFG containing medium (n=3 per catheter) was 9.84 µg/mL (CV: 3.31%), 9.91 µg/mL (CV: 1.71%), and 9.77 µg/mL (CV: 2.90%) during the time investigated. In (ii) C_{AFG} in medium was constant during the investigated time.

After the equilibration phase of 15 min, C_{AFG} in µDialysate was constant during the investigated time for both investigations (Fig. 3.2-19). Only the first sample from catheter 2 and catheter 3, in the beginning of the investigation with AFG-free medium (i), had a low concentration of 109 µg/mL and 149 µg/mL AFG in µDialysate, respectively. Besides, the range of C_{AFG} was from 172 to 222 µg/mL during subsequent investigations for all catheters.

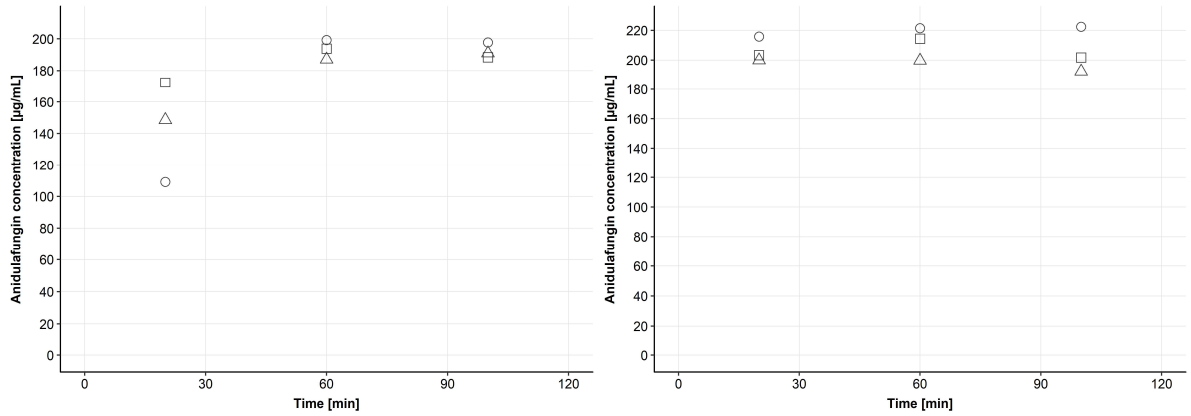

Fig. 3.2-19: Anidulafungin (AFG) concentrations in microdialysate (n=3 per catheter and investigation) during (i) in AFG-free medium (left) and (ii) in 10 µg/mL AFG containing medium (right) over time (time shown as midtime). Catheters of (i) and (ii) were perfused with 200 µg/mL AFG. Catheter 1 presented as squares, catheter 2 as circles and catheter 3 as triangles.

rD in (i), calculated from C_{AFG} in µDialysate and perfusate, ranged from 9.82% to 50.8%. In (ii) rD ranged from -0.205% to 9.42%. Data on rD is presented in Tab. 3.2-17. The rD-time profile during investigation (i) and (ii) is shown in Fig. 3.2-20. rD of (i) was decreasing over time, whereas rD of (ii) was constant during the investigated time.

Tab. 3.2-17: Minimum and maximum relative delivery (rD_{min}, rD_{max}) for (i) anidulafungin (AFG)-free and (ii) 10 µg/mL AFG in medium with 200 µg/mL AFG in perfusate calculated from C_{AFG} in microdialysate (n=3 per catheter).

Catheter	rD_{min}, %	rD_{max}, %
AFG-free medium		
1	9.82	19.8
2	10.3	50.8
3	10.1	30.0
10 µg/mL AFG in medium		
1	0.252	6.12
2	-0.205	2.77
3	5.85	9.42

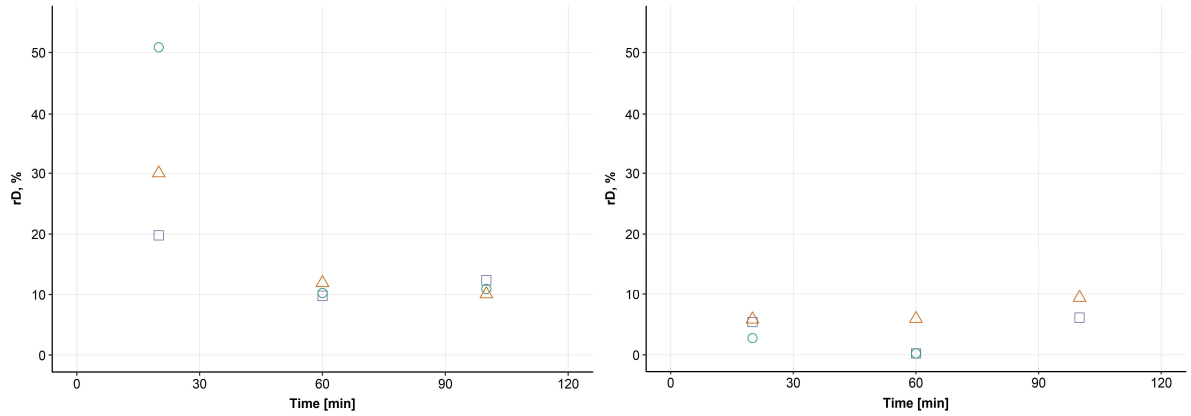

Fig. 3.2-20: Relative delivery (rD) of anidulafungin (AFG) concentrations (C_{AFG}) in microdialysate presented as midtime. Investigation (i) with AFG-free medium (left) and (ii) with 10 µg/mL AFG in the medium (right). 200 µg/mL AFG is in perfusate during both investigations. rD of catheter 1 presented as violet squares, catheter 2 as green circles and catheter 3 as orange triangles. In (ii), the rD of catheter 1 and catheter 2 are overlapping at 60 min and the rD of catheter 2 has a negative value, hence, the value is missing at 100 min.

3.3 Voriconazole static *in vitro* microdialysis

3.3.1 Dependence of relative recovery and relative delivery on flow rate or concentration

3.3.1.1 Dependence of relative recovery and relative delivery on flow rate

Flow rates of 1 and 2 µL/min were compared in terms of differences in resulting RR or rD. C_{VOR} ranging from 0.5 µg/mL to 4.0 µg/mL in perfusate or medium were combined with respective flow rates. The mean RR/rD of 1 µL/min was 88.3% (CV: 7.56%; n=94) and for 2 µL/min 88.4% (CV: 6.87%; n=96). Hence, there were no significant differences between a FR of 1 and 2 µL/min (p=0.778, Wilcoxon-Mann-Whitney test).

During recovery investigations the mean RR (n=48 per FR) of 1 µL/min was 87.1% (CV: 4.89%) and of 2 µL/min 87.0% (CV: 4.44%). Delivery investigations showed a mean rD of 90.2% (CV: 8.73%) for 1 µL/min (n=46, two samples were excluded due to flushing) and 89.8% (CV: 8.31%) for 2 µL/min (n=48). Between recovery and delivery setting, a significant difference in RR/rD was determined (p=1.71e-05, Wilcoxon-Mann-Whitney test). Results are shown in Fig. 3.3-1 for the combinations of flow rates and experimental setting (recovery or delivery). Individual results are presented in Tab 8-1 for the recovery setting and Tab 8-2 for the delivery setting.

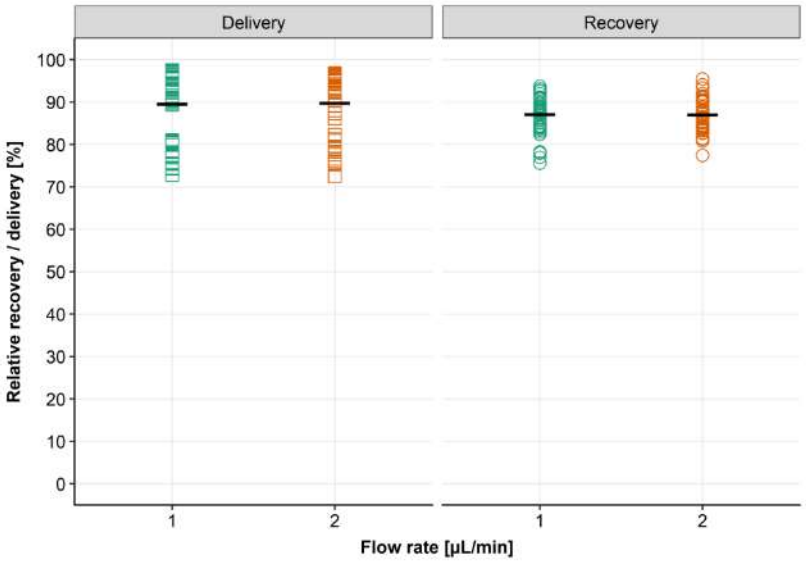

Fig. 3.3-1: Comparing flow rates of 1 µL/min (green) and 2 µL/min (orange) with relative delivery resulting from delivery (squares) and relative recovery from recovery (circles) investigations, mean is presented as black bar (n=46-48 per combination of flow rate and recovery/delivery setting).

3.3.1.2 Dependence of relative recovery and relative delivery on concentration

The mean RR and rD from C_{VOR} of 0.5, 1.5, 3 and 4 µg/mL in perfusate or medium were compared with each other. The recovery investigation of 0.5 µg/mL VOR in medium had a mean RR (n=24 per concentration of FR 1 and 2 µL/min) of 87.6% (CV: 6.10%), 87.4% (CV: 4.09%) for 1.5 µg/mL, 85.9% (CV: 4.49%) for 3.0 µg/mL, and 87.3% (CV: 3.52%) for 4.0 µg/mL. There was no statistical difference of rD between the concentrations of the recovery investigation (p=0.44, one-way ANOVA). In comparison, the mean rD (n=23-24 per concentration of FR 1 and 2 µL/min) during the delivery investigations was 78.0% (CV: 3.41%) for 0.5 µg/mL VOR in perfusate, 90.8% (CV: 2.35%) for 1.5 µg/mL, 95.8% (CV: 1.21%) for 3.0 µg/mL, and 95.6% (CV: 2.14%) for 4.0 µg/mL. Significant differences in rD were seen between all the single investigated concentrations during the delivery setting ($p<2.2e-16$, Kruskal-Wallis test; a pairwise comparison of combinations was performed using the Wilcoxon-Mann-Whitney test which resulted for all combinations in $p < 0.05$). Overall, the mean RR (n=96) was 87.0% (CV: 4.65%) and rD (n=94) was 90.0% (CV: 8.48%). Means of RR, SD and CV are listed for the individual concentrations and experimental settings in Tab. 3.3-1.

Individual results are shown in Tab 8-1 for the recovery setting and in Tab 8-2 for the delivery setting. Results for the combinations of concentrations and experimental setting (recovery or delivery) are shown in Fig. 3.3-2.

Tab. 3.3-1: Mean, standard deviation (SD) and coefficient of variation (CV) (n=23-24 per concentration and experimental setting) of relative recovery (RR) and relative delivery (rD) for the individual concentrations and experimental settings of voriconazole (VOR).

VOR concentration in	Recovery		Delivery	
medium/perfusate [µg/mL]	Mean RR ± SD, %	CV, %	Mean rD ± SD, %	CV, %
0.5	87.6 ± 5.35	6.10	78.0 ± 2.66	3.41
1.5	87.4 ± 3.57	4.09	90.8 ± 2.14	2.35
3	85.9 ± 3.86	4.49	95.8 ± 1.16	1.21
4	87.3 ± 3.07	3.52	95.6 ± 2.04	2.14
Overall	87.0 ± 4.05	4.65	90.0 ± 7.63	8.48

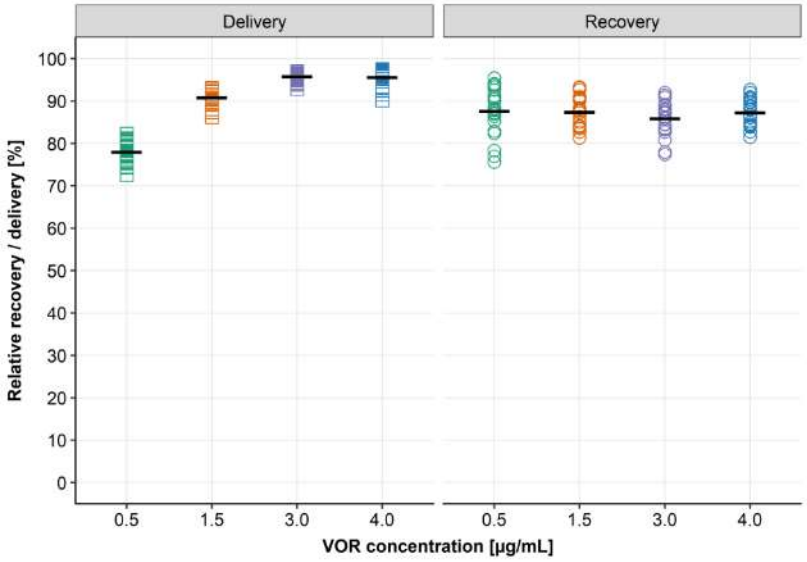

Fig. 3.3-2: Comparing the individual concentrations (0.5 µg/mL (green), 1.5 µg/mL (orange), 3.0 µg/mL (purple) and 4.0 µg/mL (blue)) with relative recovery and relative delivery resulting from delivery (squares) and recovery (circles) investigations, mean is presented as black bar (n=24 per combination of concentration and recovery/delivery setting).

3.3.2 Investigation of retrodialysis

During the retrodialysis investigation (i) with 20 µg/mL VOR in RS in perfusate and VOR-free medium of RS, the overall mean rD was 95.3% (CV: 1.51%) (n=9). The overall mean rD for 200 µg/mL VOR in RS in perfusate was 96.0% (CV: 1.21%) (n=9). The investigation (ii) with 20 µg/mL VOR in perfusate and 1 µg/mL VOR in medium had an overall mean of 93.0% (CV: 0.416%). 200 µg/mL VOR in perfusate led to a mean rD of 96.4% (CV: 1.79%). The perfusate containing 20 µg/mL in a 10 µg/mL VOR medium in investigation (iii) resulted in a mean rD of 54.7% (CV: 2.79%) and 92.5% (CV: 1.34%) for 200 µg/mL VOR in perfusate. There was a significant difference in rD between the three settings of retrodialysis with 20 µg/mL VOR in perfusate and different medium concentrations (p=1.09e-05,

Kruskal-Wallis test; a pairwise comparison of combinations was performed using the Wilcoxon-Mann-Whitney test: 10 µg/mL and VOR-free medium with a p=6.2e-05, 10 µg/mL and 1 µg/mL with a p=6.2e-05, 1 µg/mL and VOR-free medium with a p= 8.2e-05). For the setting with 200 µg/mL VOR in perfusate, only the rD in the investigation with 10 µg/mL VOR in medium was significantly different (p=3.00e-04, Kruskal-Wallis test; a pairwise comparison of combinations was performed using the Wilcoxon-Mann-Whitney test: 10 µg/mL and VOR-free medium with a p=1.2e-04, 10 µg/mL and 1 µg/mL with a p=4.3e-04). Data on rD from retrodialysis is presented in Fig. 3.3-3. Individual data on rD and CV are shown in Fig 8-3.

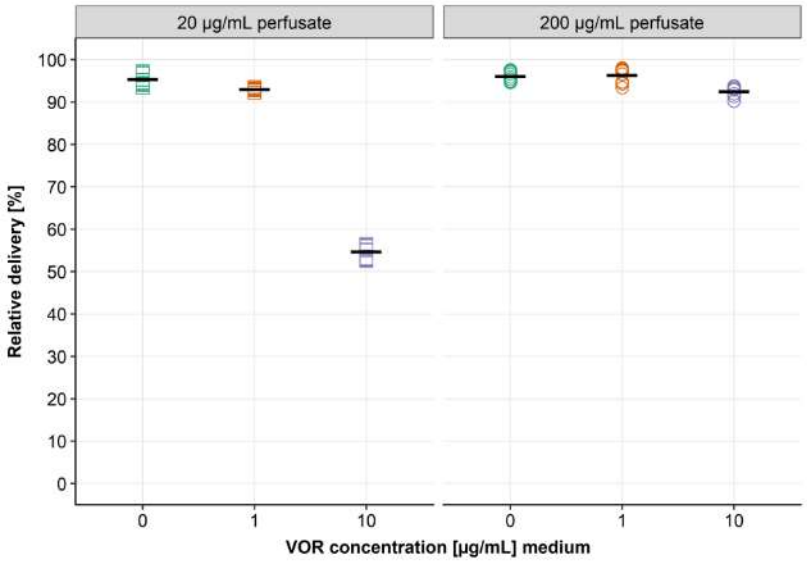

Fig. 3.3-3: Relative delivery determined during retrodialysis with 20 (squares) or 200 µg/mL (circles) voriconazole (VOR) in Ringer's solution in perfusate in (i) VOR-free (green), (ii) 1 µg/mL VOR (orange) and (iii) 10 µg/mL VOR (purple) containing medium (n=9 per perfusate and medium combination).

3.4 Antifungals in a dynamic *in vitro* microdialysis system

3.4.1 Development of the dynamic *in vitro* microdialysis system

The structure of the experimental dIVMS is shown in Fig. 3.4-1. The dIVMS consisted of a double layer (spinner) glass flask, a magnetic stirrer and a stir bar, a peristaltic pump, a heated water bath, reservoir and waste containers, the µDialysis catheters and pumps. A spinner flask was used as medium vessel for the preliminary tests. The flask was made of glass and contained a double layer (water jacket): an inner compartment for the medium and an outer compartment for the tempered water. The outer layer was connected to a water bath, which assured constant temperature in the medium of the inner compartment. A magnetic stir bar in the medium fluid was activated by a magnetic stirrer underneath the flask. The magnetic stir bar guaranteed continuous fluid movement. The inner compartment was connected with a peristaltic pump to a reservoir and a waste container. In the peristaltic pump, tubings were fixed over a roller. When the roller pressed the tubings, the medium in the tubings was transported. Thus, the volume of medium was passed through the tubings in intervals.

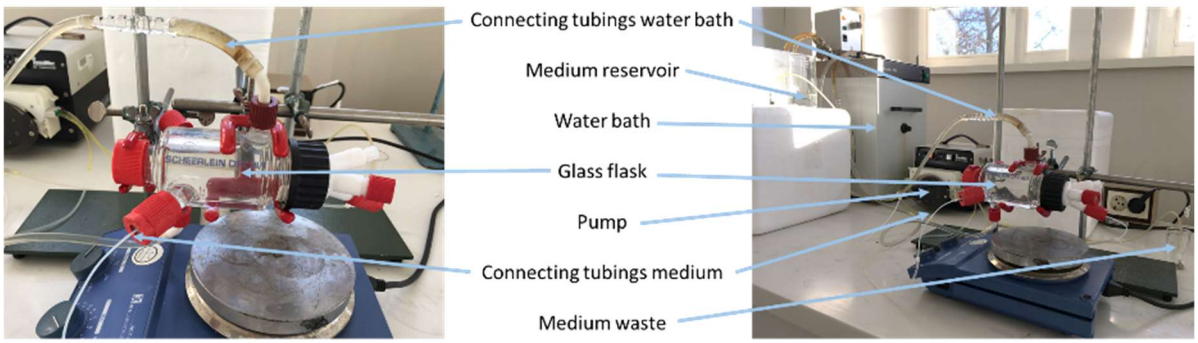

Fig. 3.4-1: Dynamic *in vitro* microdialysis system. Glass flask with a magnetic stir bar in the medium fluid in the inner compartment and the warm water stream in the outer compartment, respectively (left). Glass flask, heated water bath and respective connecting tubings, pump and connecting tubings to medium reservoir and waste containers (right).

For the fixation of µDialysis catheters, a guiding cannula (glass tube) was pierced through a rubber plug which prevented the medium from leaking. Medium samples were taken via a fixed long cannula leading into the medium fluid. The medium was filled into the inner chamber of the flask and warm water was perfused through the outer chamber (connected to the water bath). In- and outflow for medium was guaranteed with tubings guided through the “arms” of the flask into the medium. The flask is shown in detail in Fig. 3.4-2 and an overview of the experimental dIVMS is given in Fig. 3.4-3.

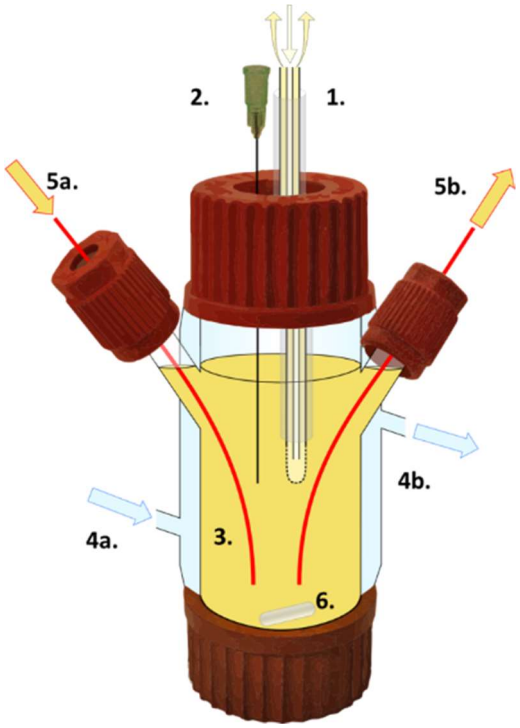

Fig. 3.4-2: Dynamic *in vitro* microdialysis system: 1. concentric microdialysis catheter in guiding cannula; 2. cannula; 3. inner chamber; 4a. Inflow of water into the outer chamber; 4b. Outflow of water out of the outer chamber; 5a. inflow tubing for medium; 5b. outflow tubing for medium; 6. magnetic stir bar.

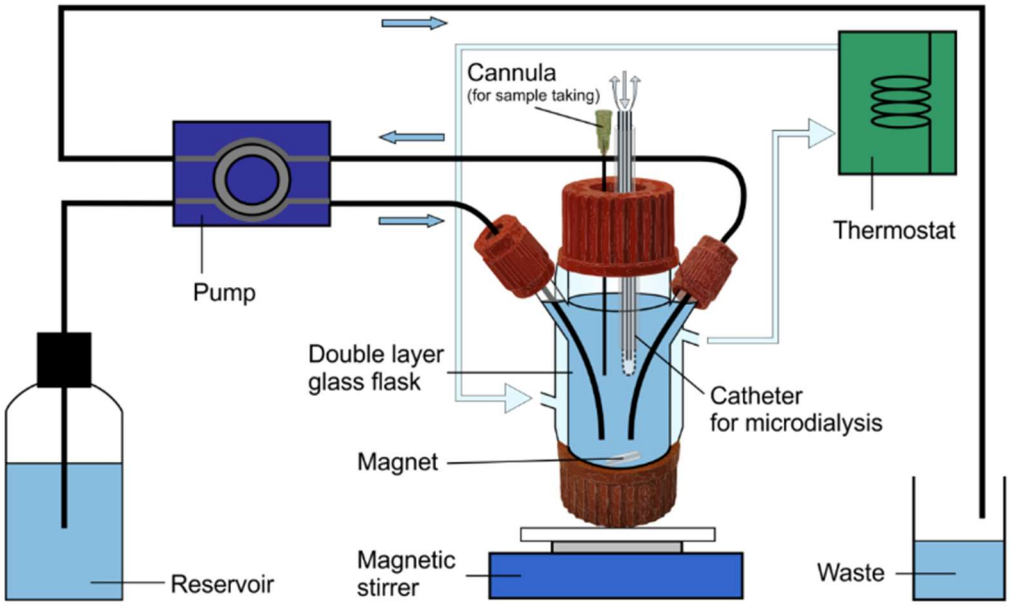

Fig. 3.4-3: Structure of the experimental model prototype of the dynamic *in vitro* microdialysis system. The double layer glass flask contains the medium. Microdialysis catheters and a long cannula for sample taking are placed into the medium. The medium is pumped by a peristaltic pump from the medium reservoir container into the flask and from there into the waste container. Warm water from the water bath (thermostat) is perfused through the outer layer of the glass flask. A magnetic stir bar (magnet) in the medium is activated by a magnetic stirrer below the glass flask.

3.4.2 Validation of the dynamic *in vitro* microdialysis system

From 20.5°C at the start of the experiment, the temperature in the glass flask and in the water bath, measured by digital thermometers, rose and achieved 37.3°C (CV: 0.615%) and 37.5°C (CV: 0.133%) after 10 min, respectively. The mercury thermometer continuously measured a temperature of 38.0°C, 10 min after start of investigation. Results of temperature measurements are shown in Tab. 3.4-1.

Tab. 3.4-1: Mean, standard deviation (SD) and coefficient of variation (CV) of temperature monitored in the flask or water bath with digital or mercury thermometers. n=14 measurements per thermometer after 10 min up to 23 h; only decimal numbers of .0 and .5 were displayed on mercury thermometer.

Thermometer	Mean [°C]	SD [°C]	CV, %
Digital (flask)	37.3	0.229	0.615
Digital (water bath)	37.5	$4.97\ 10^{-2}$	0.133
Mercury (water bath)	38.0	0.00	0.00

The pump worked continuously without interruption during the investigated time. In the first hour after the start of pumping with a new pair of tubings at a speed of 23 rpm, the mean PR was 153 µL/min (CV: 6.78%; $n_{in+outflow}$=32). The PR increased from the first sampling interval (0-1 h) to the second interval (5-6 h) and was stable during the following investigation. The average of samples taken from 5-6 h and 24-25 h were 166 µL/min (CV: 4.60%, $n_{in+outflow}$=32) and 168 µL/min (CV: 3.73%, $n_{in+outflow}$=31), respectively (Fig. 3.4-4).

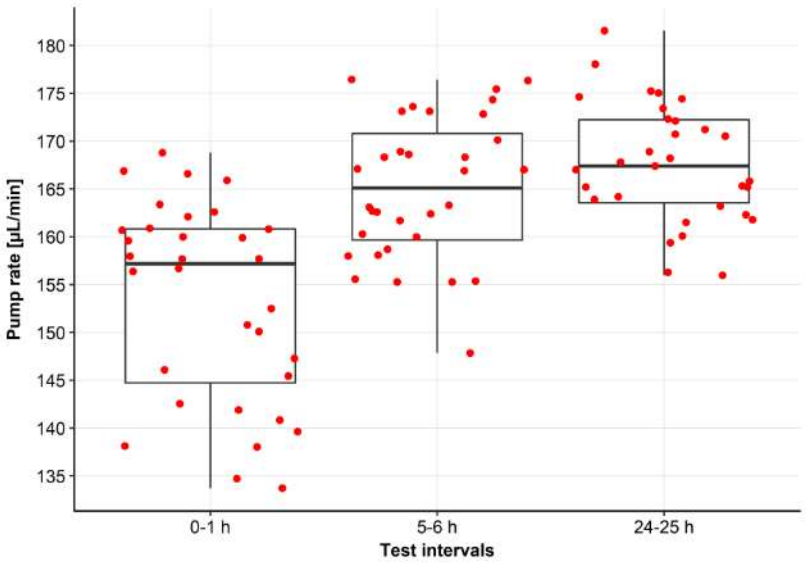

Fig. 3.4-4: Boxplots of measured pump rate (PR) from samples of in- and outflow of two tubings fixed in the pump of the dynamic *in vitro* microdialysis system. Samples were taken from 0-1 h, 5-6 h and 24-25 h. Boxes: inter-quartile range (IQR), including median; whiskers: 1.5 · IQR; red points: randomly scattered single PR values.

The observed PR for inflow samples was smaller than for outflow samples throughout the investigated time. The mean PR during the first interval (0-1 h) was 149 µL/min (CV: 7.42%) for in- and 157 µL/min (CV: 5.27%) for outflow. Inflow during the second sampling interval (5-6 h) was 163 µL/min (CV: 4.38%) and 167 µL/min (CV: 4.29%) for outflow. In the last sampling interval, the PR for inflow was 167 µL/min (CV: 3.11%) and 169 µL/min (CV: 4.26%) for outflow. Results for in- and outflow are shown in Tab. 3.4-2. Hence, the pump with new tubings should be started at least 5-6 h before start of actual investigation.

Tab. 3.4-2: Mean, standard deviation (SD) and coefficient of variation (CV) of in- and outflow samples during the three investigated intervals (0-1 h, 5-6 h and 24-25 h).

Investigated parameter	**0-1 h** Mean [µL/min] (n) SD [µL/min] CV, %	**5-6 h** Mean [µL/min] (n) SD [µL/min] CV, %	**24-25 h** Mean [µL/min] (n) SD [µL/min] CV, %
Inflow	149 (16) 11.1 7.42	163 (16) 7.13 4.38	167 (15) 5.19 3.11
Outflow	157 (16) 8.28 5.27	167 (16) 7.18 4.29	169 (16) 7.18 4.26

The stirring of the catheter surrounding medium was continuous and constant with the speed of the magnetic stirrer at 700 rpm. 50 µL of methylene blue solution (1%), administered via 2. (Fig. 3.4-2), were homogenously distributed within 2 min (n=10).

3.4.3 *In silico* simulations and *in vitro* mimicry of concentration-time profiles of antifungals in the dynamic *in vitro* microdialysis system

3.4.3.1 *In silico* simulation of pharmacokinetic profiles of antifungals

Results of the technical parameters for the dIVMS of VOR and AFG are listed in Tab. 3.4-3. The maximum drug concentration in the medium fluid was 1.97 µg/mL VOR for the plasma and 0.940 µg/mL VOR for the ISF mimicry (unbound). A total AFG concentration in medium fluid was 6.60 µg/mL for the plasma profile. Hence a mass of 197 µg and 94.0 µg of VOR and 660 µg of AFG were to be diluted in 0.5 mL (VOR) or 1.0 mL (AFG) drug-free medium ($V_{IV\ bolus\ injection}$), resulting in $C_{IV\ bolus\ injection}$ of 395 µg/mL and 188 µg/mL VOR and 660 µg/mL AFG, respectively. The calculated k_e was 0.1155 h^{-1} and 0.0815 h^{-1} for VOR profiles and 0.0333 h^{-1} for AFG. Pump rates of 193 µL/min

were determined for the plasma profile of VOR and 136 µL/min for the VOR ISF profile. The AFG plasma profile had a PR of 55.5 µL/min. All concentration-time profiles were simulated for 24 h. Simulated profiles are shown in Fig. 3.4-5 for AFG plasma concentrations, in Fig. 3.4-6 for plasma and in Fig. 3.4-7 for VOR ISF concentrations.

Tab. 3.4-3: Technical parameters of voriconazole (VOR) and anidulafungin (AFG) pharmacokinetic profile mimicry of plasma and interstitial space fluid (ISF): maximum total/unbound drug concentration (C_{max}), dose of the analyte (D), volume of IV bolus injection ($V_{IV\ bolus\ injection}$), drug concentration in IV bolus injection ($C_{IV\ bolus\ injection}$), elimination rate constant (k_e), PR (pump rate).

Technical parameters	VOR plasma	VOR ISF	AFG plasma
C_{max} [µg/mL]	1.97 (unbound)	0.940 (unbound)	6.60 (total)
D [µg]	197	94.0	660
$V_{IV\ bolus\ injection}$ [mL]	0.5	0.5	1.0
$C_{IV\ bolus\ injection}$ [µg/mL]	395	188	660
k_e [h^{-1}]	0.1155	0.0815	0.0333
PR [µL/min]	193	136	55.5

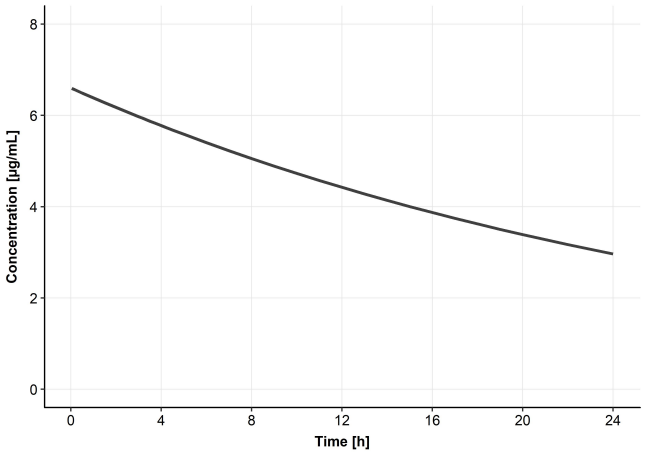

Fig. 3.4-5: Simulation of the concentration-time profile of anidulafungin based on data from Crandon *et al.* [73] for 24 h.

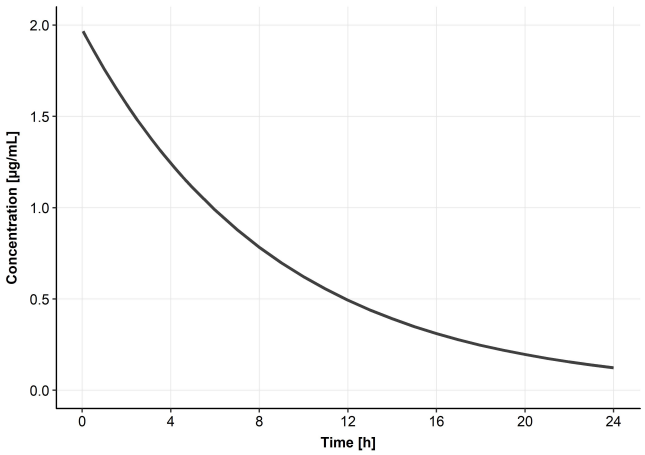

Fig. 3.4-6: Simulation of the concentration-time profile of voriconazole based on data from Theuretzbacher *et al.* [80] for 24 h.

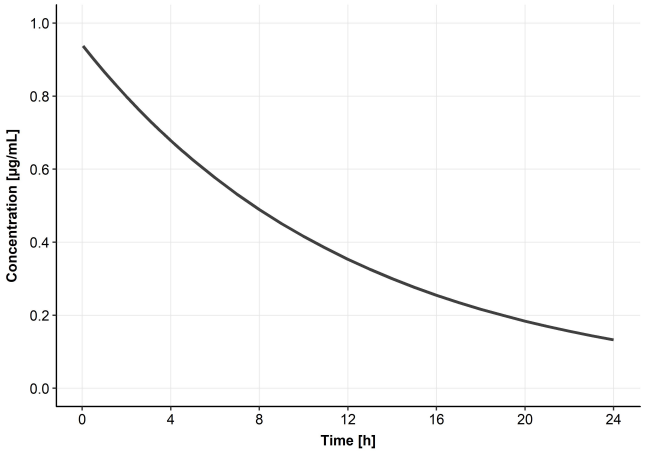

Fig. 3.4-7: Simulation of the concentration-time profile of voriconazole based on data from Simmel *et al.* [2] for 24 h.

3.4.3.2 *In vitro* mimicry of pharmacokinetic profiles

The drug concentration of i.v. bolus injection in the syringe for the respective VOR or AFG investigations is listed in Tab. 3.4-4. *In silico* calculated drug concentrations of i.v. bolus injection solution for VOR was 395 µg/mL (i) and 188 µg/mL (ii) in the syringe. VOR concentrations in injection solution observed *in vitro* were 379 µg/mL and a mean of 189 µg/mL (n=1 (i) and n=3 (ii); RE: -4.13% (i), +0.410% (ii)). The calculated AFG concentration of the injection solution was 660 µg/mL and C_{obs} was 723 µg/mL (RE: +9.39%).

Tab. 3.4-4: *In silico* calculated and *in vitro* observed drug concentrations of voriconazole (VOR) and anidulafungin (AFG) of i.v. bolus injection solution in syringes (n_{VOR}=1 sample (ii), n_{VOR}=3 samples (ii), n_{AFG}=1 sample) and accuracy of observed to calculated concentrations expressed as percentage deviation (RE, %).

Drug in i.v. bolus injection	Drug concentration [µg/mL] Calculated	Drug concentration [µg/mL] Observed	RE, %
VOR (i)	395	379	-4.13
VOR (ii)	188	189 (mean)	+0.410
AFG	660	723	+9.39

Voriconazole

The variability of VOR concentrations in medium was presented as CV around the *in silico* calculated VOR concentrations. The coefficient of variation (CV, %) of the pump in the dIVMS was 4.50% (rounded up from 4.38% (Tab. 3.4-2)) after equilibration (5-6 h interval). A maximum CV (precision of QC samples) of the bioanalytical method of VOR was 9.32%, which was rounded up to 9.50% (Tab. 3.1-3). The concentration-time profile for (i) of *in silico* calculated and *in vitro* observed VOR concentrations in medium is shown in Fig. 3.4-8. The observed VOR concentrations showed a decrease of VOR concentration over time similar to the calculated concentrations.

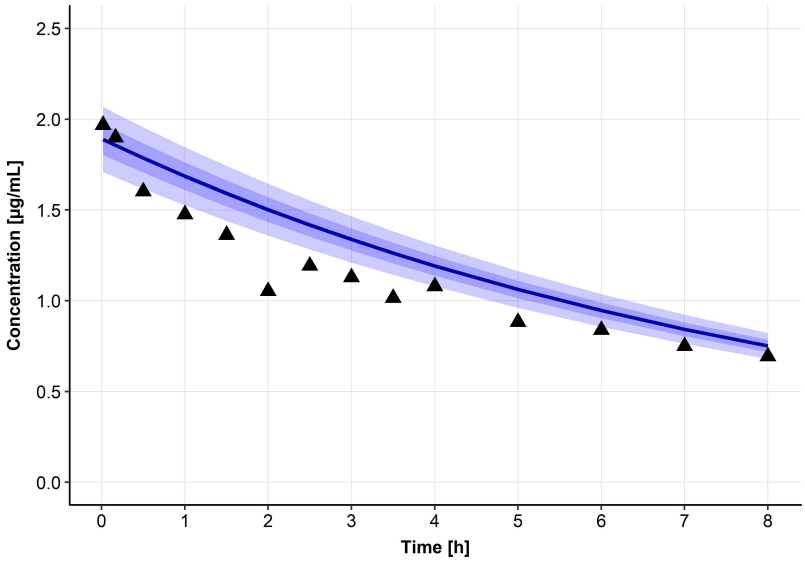

Fig. 3.4-8: Concentration-time profile based on data of Theuretzbacher *et al.* of voriconazole (VOR) in medium during 8 h; *in silico* calculated (blue line) and *in vitro* observed (triangles) VOR concentrations. The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for VOR (CV: 9.50%) are presented as dark blue and light blue ribbons around the *in silico* concentrations of VOR, respectively.

The concentration-time profile of (ii) is shown in Fig. 3.4-9. The observed VOR concentrations of (ii) showed a concentration-time profile of VOR concentrations over time similar to the calculated concentrations.

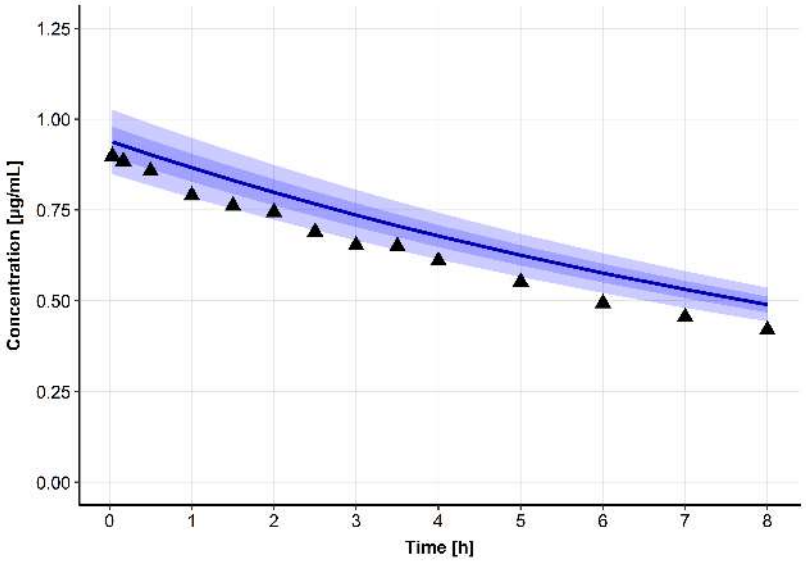

Fig. 3.4-9: Concentration-time profile based on data from Simmel *et al.* of voriconazole (VOR) in medium during 8 h; *in silico* calculated (blue line) and *in vitro* the mean observed (triangles) concentrations. The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for VOR (CV: 9.50%) are presented as dark blue and light blue ribbons around the *in silico* concentrations of VOR, respectively.

For (iii), the concentration-time profile is shown in Fig. 3.4-10. Samples taken after 45 h of investigation had concentrations <LOD. The observed VOR concentrations above the LLOQ were similar to the calculated VOR concentrations.

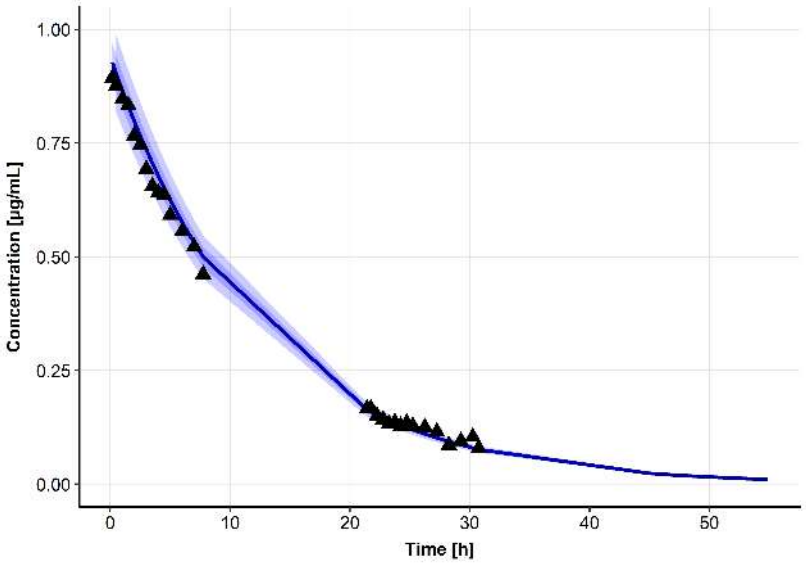

Fig. 3.4-10: Concentration-time profile based on data from Simmel *et al.* of voriconazole (VOR) in medium during 55 h; *in silico* calculated (blue line) and *in vitro* observed (triangles) concentrations. Medium samples taken after 45 h of investigation had concentrations <LOD. The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for VOR (CV: 9.50%) are presented as dark blue and light blue ribbons around the *in silico* concentrations of VOR, respectively.

Anidulafungin

The variability of AFG concentrations in medium was presented as CV around the *in silico* calculated AFG concentrations. The coefficient of variation (CV, %) of the pump in the dIVMS was 4.50% (rounded up from 4.38% (Tab. 3.4-2)) after equilibration (5-6 h interval). A maximum CV (precision of QC samples) of the bioanalytical method of AFG was 8.00% (Tab. 3.1-2). The concentration-time profile of *in silico* calculated and *in vitro* observed AFG concentrations in medium is shown in Fig. 3.4-11. Here, it was shown that the concentration-time profile of observed AFG concentrations decreased similar to the calculated profile of AFG.

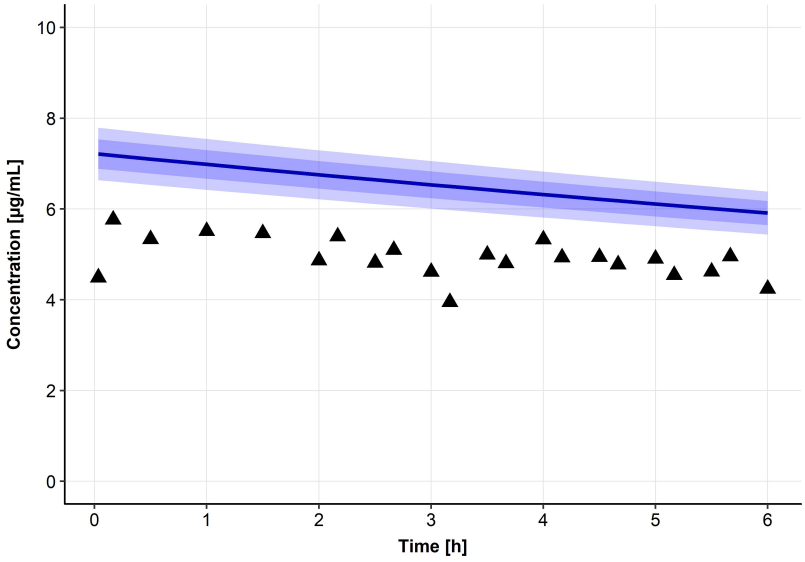

Fig. 3.4-11: Concentration-time profile based on data from Crandon *et al.* of anidulafungin (AFG) in medium during 6 h; *in silico* calculated (blue line) and *in vitro* observed (triangles) concentrations. The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for AFG (CV: 8.00%) are presented as dark blue and light blue ribbons around the *in silico* concentrations of AFG, respectively.

3.4.4 *In vitro* microdialysis investigations with the dynamic *in vitro* microdialysis system

Voriconazole

The *in vitro* observed and *in silico* calculated VOR concentrations in the medium are shown in Fig. 3.4-12 for investigation (i) and with a perfusate containing 20 µg/mL VOR and in Fig. 3.4-13 for investigation (ii) with a perfusate containing 200 µg/mL VOR.

The observed VOR concentrations of (i) and (ii) showed a concentration-time profile of VOR similar to the calculated profile with most concentrations within the variability ranges of the pump (CV: 4.50%) and the bioanalytical method (CV: 9.50%).

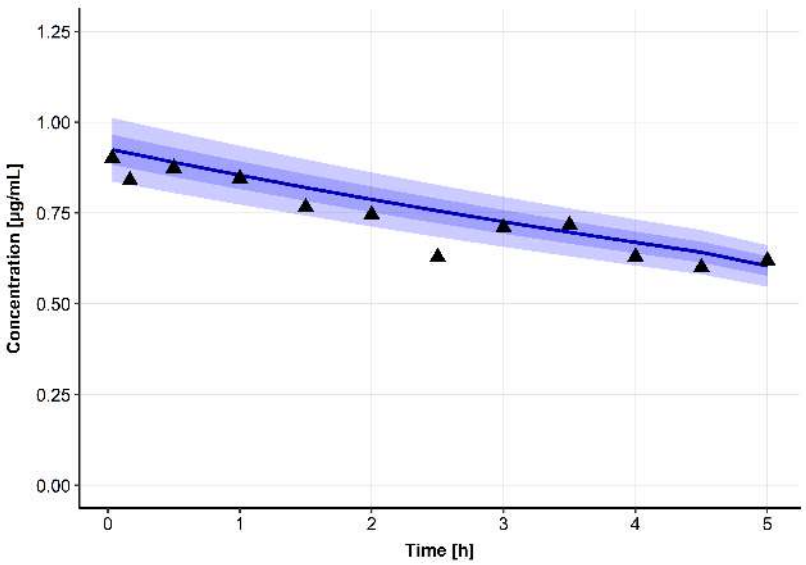

Fig. 3.4-12: Calculated (blue line) and observed (triangles) voriconazole (VOR) concentrations in medium of (i). The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for VOR (CV: 9.50%) are presented as dark blue and light blue ribbons around the calculated concentrations of VOR, respectively.

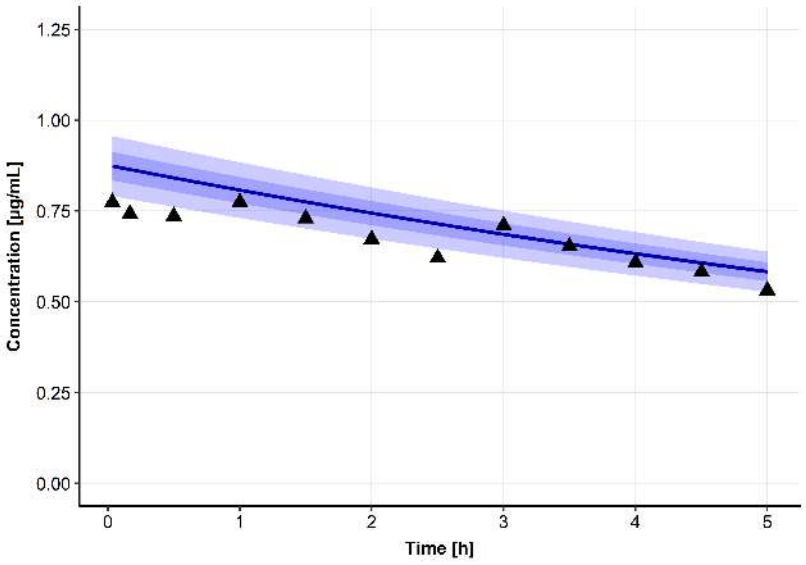

Fig. 3.4-13: Calculated (blue line) and observed (triangles) voriconazole (VOR) concentrations in medium of (ii). The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for VOR (CV: 9.50%) are presented as dark blue and light blue ribbons around the calculated concentrations of VOR, respectively.

The VOR concentration in perfusate was decreasing during retrodialysis. During (i) C_{VOR} slightly decreased from a mean concentration of 20.5 µg/mL to 19.0 µg/mL (SD ≤0.463 µg/mL, CV ≤2.26%) and during (ii) from 212 µg/mL to 201 µg/mL (SD ≤3.06 µg/mL, CV ≤1.52%). Results are shown in Tab. 3.4-5 and Fig 8-6.

Tab. 3.4-5: Mean, standard deviation (SD) and coefficient of variation (CV) of voriconazole concentration (C_{VOR}) in perfusate during retrodialysis of (i) and (ii).

Time point of investigations	Mean C_{VOR} [µg/mL]	SD [µg/mL]	CV, %
(i)			
t=0 min (n=3)	20.5	0.463	2.26
t=30 min (n=3)	19.0	0.146	0.770
Overall (n=6)	19.7	0.875	4.44
(ii)			
t=0 min (n=3)	212	0.677	0.319
t=30 min (n=3)	201	3.06	1.52
Overall (n=6)	207	6.06	2.93

RR values of (i) and (ii) were constant during retrodialysis (see Fig. 3.4-14). The mean RR (n=3) for the catheters of (i) ranged from 94.6% to 95.7% (SD ≤0.352%, CV ≤0.369%) and in (ii) from 96.6% to 97.6% (SD ≤0.644%, CV ≤0.662%), which is presented in Tab. 3.4-6.

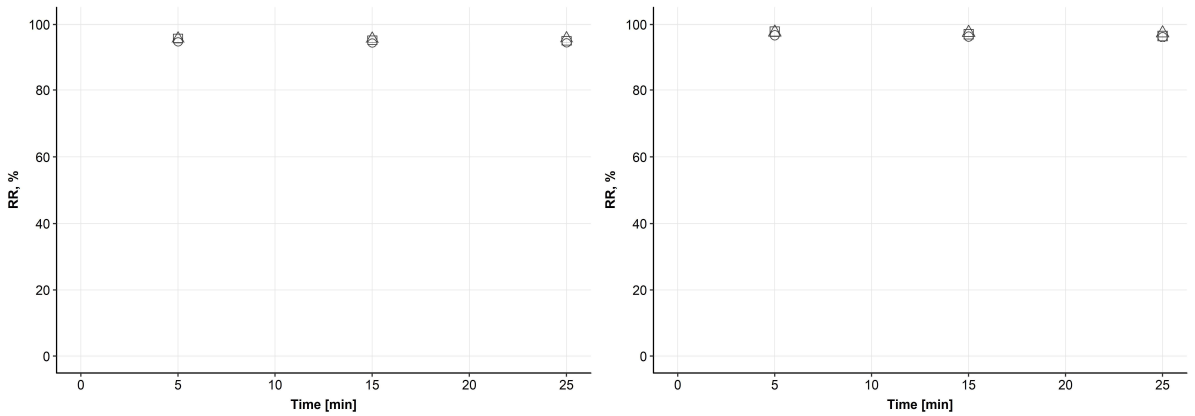

Fig. 3.4-14: Relative recovery (RR) during the 30 min (shown as midtime points) of retrodialysis for catheter 1 (squares), catheter 2 (circles) and catheter 3 (triangles) in (i) on the left and in (ii) on the right (n=3 per midtime point).

Tab. 3.4-6: Mean, standard deviation (SD) and coefficient of variation (CV) of relative recovery (RR) from retrodialysis of catheters from (i) and (ii), n=3 per catheter.

Catheter	Mean RR, %	SD, %	CV, %
(i)			
1	95.4	0.352	0.369
2	94.6	0.168	0.177
3	95.7	0.0441	0.0460
(ii)			
1	97.2	0.644	0.662
2	96.6	0.307	0.296
3	97.6	0.126	0.123

Accuracy of the volume in µDialysate samples ranged from RE (%) -15.3% to +3.27% for (i) and from -10.3% to +5.78% for (ii). The recovered volume in µDialysate samples was constant and high enough, hence, no volume correction of RR was performed.

The observed VOR concentration in medium was plotted together with the VOR medium concentration calculated of the VOR concentration in µDialysate samples (of the three catheters) against midtime, shown in Fig. 3.4-15. The first µDialysate samples of all three catheters (of (i) and (ii)) had VOR concentrations considerably below the observed medium concentration. Whereas the other medium concentrations calculated of the VOR concentration in µDialysate samples were similar to the observed medium concentration.

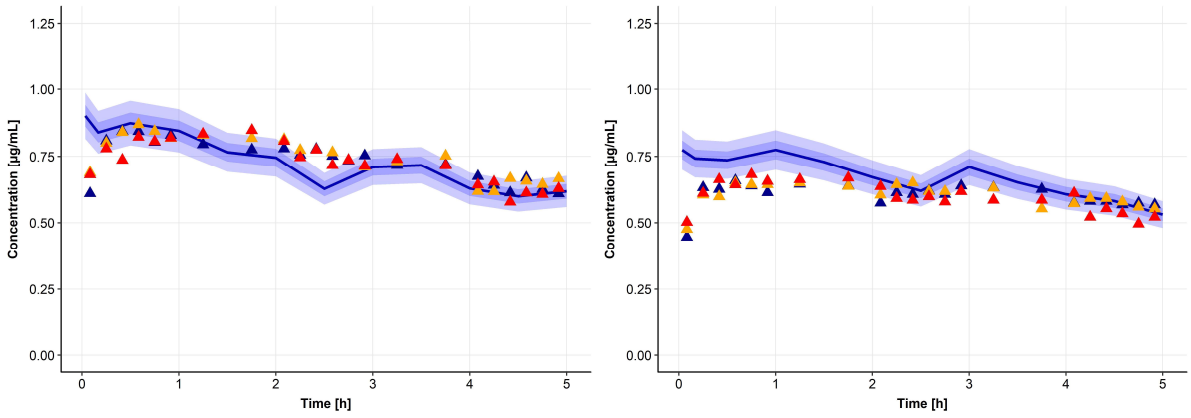

Fig. 3.4-15: Observed voriconazole (VOR) concentrations in medium (blue line) and the VOR medium concentration calculated of the VOR concentration in microdialysate samples (triangles, shown as midtime) are plotted against time: catheter 1 (blue), catheter 2 (orange), catheter 3 (red). Plot of (i) is on the left and of (ii) on the right. The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for VOR (CV: 9.50%) are presented as dark blue and light blue ribbons around the observed VOR concentrations in medium, respectively.

Anidulafungin

The *in vitro* observed and *in silico* calculated AFG concentrations in the medium are shown in Fig. 3.4-16. Here, the variability of AFG concentrations in medium was presented as CV around the *in silico* calculated AFG concentrations. In Fig. 3.4-16, it was shown that the concentration-time profile of observed AFG concentrations decreased similar to the calculated profile of AFG but observed concentrations were not withing the variability ranges of the pump (CV: 4.50%) and the bioanalytical method (CV: 8.00%). The observed concentrations of AFG were below the calculated concentrations.

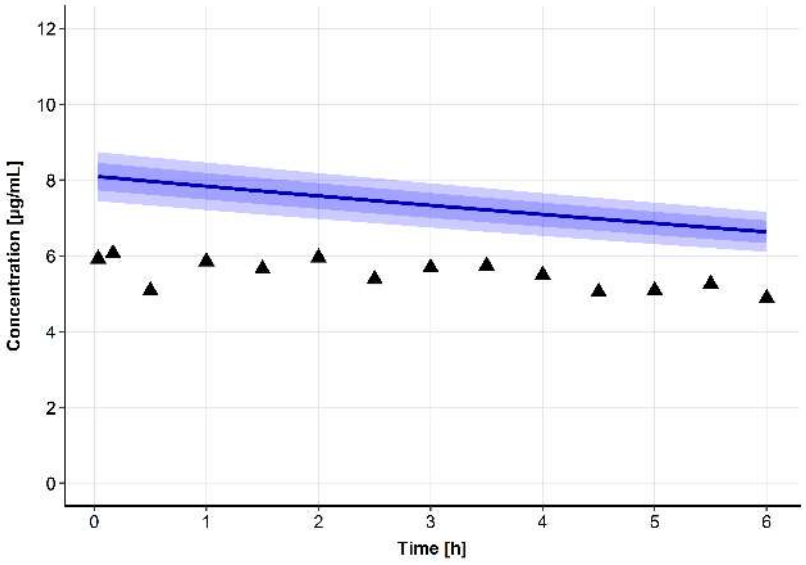

Fig. 3.4-16: Calculated (blue line) and observed (triangles) anidulafungin (AFG) concentrations in medium. The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for AFG (CV: 8.00%) are presented as dark blue and light blue ribbons around the calculated concentrations of AFG, respectively.

The AFG concentration in perfusate was increasing during retrodialysis (see Tab. 3.4-7 and Fig 8-7), from a mean concentration of 202 µg/mL to 219 µg/mL (SD ≤25.7 µg/mL, CV ≤11.8%).

Tab. 3.4-7: Mean, standard deviation (SD) and coefficient of variation (CV) of anidulafungin concentrations in perfusate during retrodialysis.

Sampling time	Mean [µg/mL]	SD [µg/mL]	CV, %
t=0 min (n=3)	202	3.30	1.63
t=120 min (n=3)	219	25.7	11.8
Overall (n=6)	210	18.6	8.86

Accuracy of the volume in µDialysate samples (nominal volume: 40 µL) ranged from RE (%) -46.1% to -11.0% (Fig 8-8). Thus, results on RR during retrodialysis and on AFG concentrations in µDialysate during recovery investigation were corrected with the respective recovered volume in µDialysate.

In general, RR was decreasing over time during retrodialysis (see Fig. 3.4-17). In Tab. 3.4-8 the mean RR per midtime point (n=3 per midtime point) are presented. The mean RR decreased from 41.0% (SD: 5.24%, CV: 12.8%) after the first sampling interval to 8.14% (SD: 5.70%, CV: 70.0%) after the third interval of observed RR and from 51.3% (SD: 3.10%, CV: 6.04%) to 20.8% (SD: 3.98%, CV: 19.2%) of volume-corrected RR.

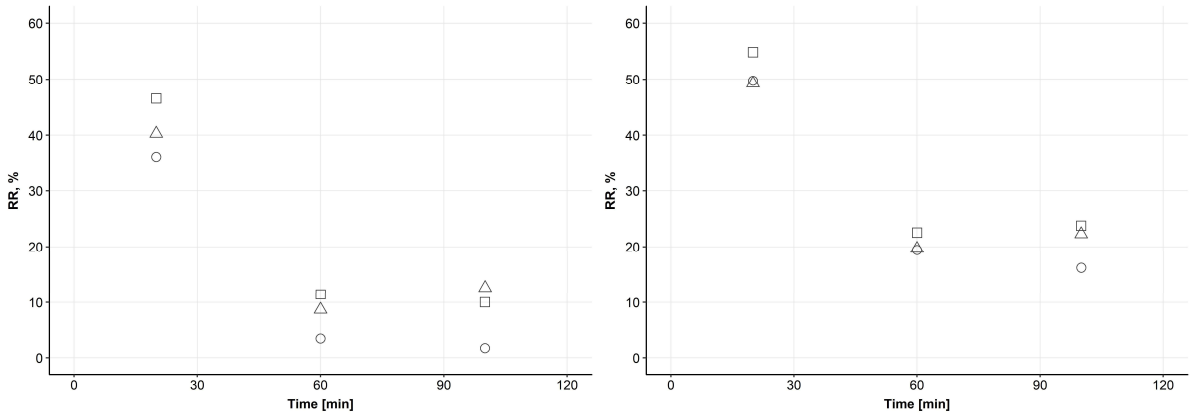

Fig. 3.4-17: Relative recovery (RR) during retrodialysis (shown as midtime) calculated from observed anidulafungin concentrations in microdialysate (left) and with volume correction (right). Catheter 1: squares; catheter 2: circles; catheter 3: triangles.

Tab. 3.4-8: Mean, standard deviation (SD) and coefficient of variation (CV) of relative recovery (RR) from anidulafungin (AFG) containing retrodialysate per **midtime point** (n=3 per midtime point). Observed: RR calculated from AFG in µDialysate; Volume-corrected: RR adjusted by volume correction.

Time point	Mean RR, %	SD, %	CV, %
Observed			
Time=20 min	41.0	5.24	12.8
Time=60 min	7.88	4.06	51.6
Time=100 min	8.14	5.70	70.0
Volume-corrected			
Time=20 min	51.3	3.10	6.04
Time=60 min	20.6	1.73	8.44
Time=100 min	20.8	3.98	19.2

Results

For µDialysis samples during retrodialysis, the mean RR (n=3 per catheter) of observed RR was 20.5% (CV: 83.7%) for catheter 1, 13.8% (CV: 141%) for catheter 2 and 22.7% (CV: 91.2%) for catheter 3 (see Tab. 3.4-9). The mean volume-corrected RR was 30.4% (CV: 53.9%) for catheter 1, 28.5% (CV: 64.9%) for catheter 2 and 33.7% (CV: 54.3%) for catheter 3 (see Tab. 3.4-9).

Tab. 3.4-9: Mean, standard deviation (SD) and coefficient of variation (CV) of relative recovery from anidulafungin containing retrodialysate per **catheter** (n=3 per catheter). Observed: RR calculated from AFG in µDialysate; Volume-corrected: RR adjusted by volume correction.

Catheter	Mean RR, %	SD, %	CV, %
Observed			
Catheter1	20.5	17.2	83.7
Catheter2	13.8	19.4	141
Catheter3	22.7	20.7	91.2
Volume-corrected			
Catheter1	30.4	16.4	53.9
Catheter2	28.5	18.5	64.9
Catheter3	33.7	18.3	54.3

During the first sampling interval of the recovery investigation, AFG was not detected with the bioanalytical HPLC assay (< LLOQ, see chapter 2.2.3) in µDialysate samples of catheter 1, 2 and 3.

The observed AFG concentrations in µDialysate and in medium during recovery investigation were plotted against (mid-) time in Fig. 3.4-18. The AGF medium concentration was constantly decreasing as shown in Fig. 3.4-16 while C_{AFG} in µDialysate was increasing during the time investigated. After the 6 h of investigation, C_{AFG} in µDialysate reached a plateau phase.

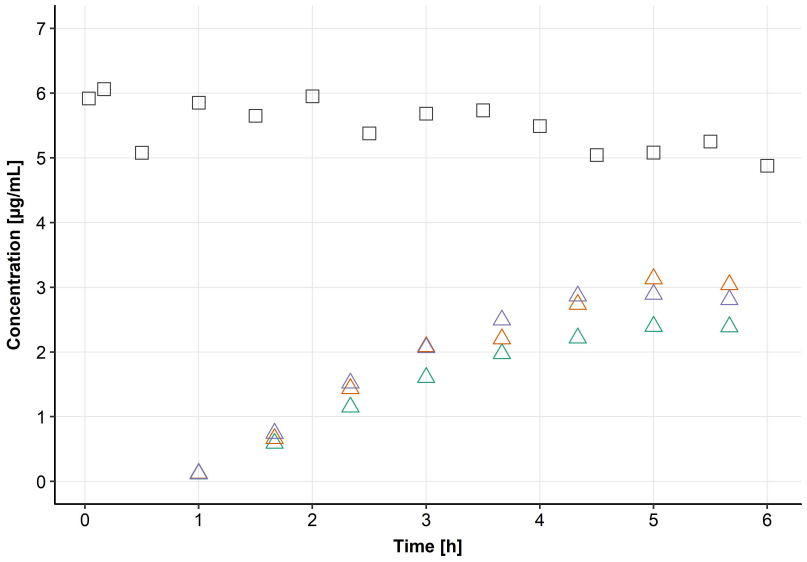

Fig. 3.4-18: Observed anidulafungin concentrations in microdialysate (triangles) presented at midtime and medium samples (squares) against time in the dynamic *in vitro* microdialysis system.

The observed AFG concentration in medium was plotted together with the AFG medium concentration calculated of the AFG concentration in µDialysate samples (of the three catheters) against midtime (observed concentrations in Fig. 3.4-19 and the volume-corrected concentrations in Fig. 3.4-20).

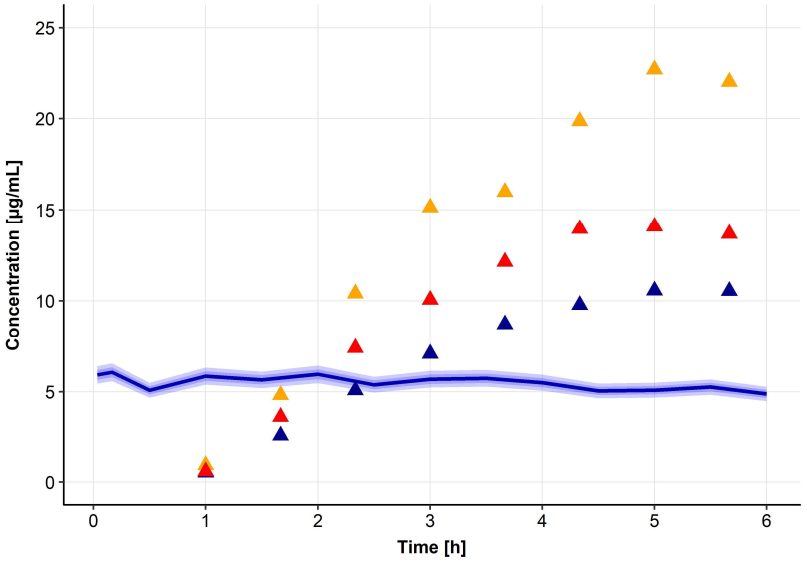

Fig. 3.4-19: Observed anidulafungin (AFG) concentrations in medium (blue line) and the AFG medium concentration calculated of the AFG concentration in microdialysate samples (triangles, shown as midtime) are plotted against time: catheter 1 (blue), catheter 2 (orange), catheter 3 (red). The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for AFG (CV: 8.00%) are presented as dark blue and light blue ribbons around the observed AFG concentrations in medium, respectively.

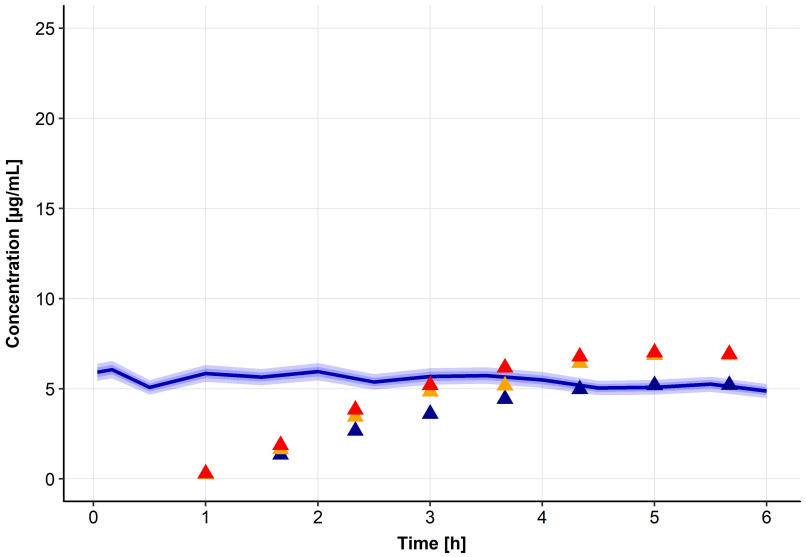

Fig. 3.4-20: Observed anidulafungin (AFG) concentrations in medium (blue line) and the AFG medium concentration calculated of the AFG concentration with **volume correction** in microdialysate samples (triangles, shown as midtime) are plotted against time: catheter 1 (blue), catheter 2 (orange), catheter 3 (red). The coefficient of variation (CV, %) of the pump (dynamic *in vitro* microdialysis system) after equilibration (CV: 4.50%) and the maximum CV of the bioanalytical assay for AFG (CV: 8.00%) are presented as dark blue and light blue ribbons around the observed AFG concentrations in medium, respectively.

4 Discussion

4.1 Bioanalytical quantification of anidulafungin and voriconazole

The validation results of the developed HPLC methods were in accordance with the EMA Guideline on bioanalytical method validation [3]. Many different assays for the quantification of AFG [60,65,70,73,94,95] and VOR [2,52,70,73,75,84,94,96,97], primarily in plasma, have been established previously. A major advantage of the bioanalytical assays established in the present thesis, was the suitability for µDialysis samples, i.e. to quantify analyte concentrations in very small volumes, which are typical for µDialysis samples [12,24,34,36]. Given the sampling intervals and flow rates in the *in vitro* µDialysis investigations, sample volumes resulted in 20 µL for AFG and 16 µL for VOR. Apart from the possibility to handle small sample volumes, the final methods should also be as simple as possible to be implemented in routine measurements.

The major optimisation of the bioanalytical assay for VOR compared to the previously established method [90] was the modified sample dilution in the 96-well-plate, using water instead of acetonitrile (ACN) during sample preparation. In the prior quantification method, the mobile phase contained ACN to achieve sufficient separation of the VOR peak from potentially interfering peaks. Therefore it was required to use ACN also for dilution of samples [90]. Due to an altered composition of the mobile phase in the new method, i.e. using methanol instead of ACN, milli-Q water could be used for dilution instead of ACN. Hence, using water for dilution instead of ACN improved the method, because accurate pipetting of water compared to ACN was much easier due to the lower vapour pressure of water (water at 25°C of 31.7 hPa [98] compared to ACN at 25°C of 118 hPa [99]). The new sample preparation increased precision of the assay as a result of increased accuracy in preparation.

Since the here presented µDialysis study of AFG was the first one investigating AFG, a completely new bioanalytical assay for *in vitro* µDialysate samples had to be developed. A standard matrix for µDialysis samples like water or RS was not applicable because of occurring adsorption of the drug to laboratory material, which had already been stated in literature [100]. AFG was detectable with HPLC from samples containing AFG in a matrix of water or NaCL solution (0.9%) only with high analyte concentrations [101]. A matrix composed of RS and 0.5% HSA was found to reduce adsorption by providing potential binding to albumin instead of surfaces of the laboratory material [32] and hence, allowed the detection of lower concentrations (LLOQ: 0.10 µg/mL, see chapter 3.1.1). A disadvantage of this matrix was the more complex sample preparation due to the proteins in the matrix. Usually,

an advantage of µDialysis is the easy sample preparation of protein-free samples, which requires only few working steps or allows even direct injection of the sample [12] as for VOR. During the development of the bioanalytical quantification assay for AFG, a necessary step in the process of sample preparation was the precipitation of HSA molecules prior to quantification using an approach based on Sutherland *et al.* [65], which involved the use of methanol for precipitation. Samples treated with methanol for HSA precipitation were determined less precisely as a result of the higher vapour pressure of 169 hPa at 25°C [102] and loss of volume of methanol. Thus, tight closure of the wells using a cap mat was crucial. Otherwise concentrations might have increased as a result of volume loss by evaporation.

A major challenge during development and validation of the bioanalytical assay as well as during the *in vitro* µDialysis investigations was the unavailability of AFG analytic reference substance. Instead, the AFG formulation Ecalta® was used to prepare the stock solution. Due to the availability of one package of Ecalta®, independent preparation of calibration solutions and quality control samples from two independently prepared stock solutions of analytical reference substances (or at least of two independent packages of Ecalta®) was not possible as required by the EMA Guideline on bioanalytical method validation [3]. In addition, Ecalta® did not only contain AFG but also excipients, e.g. fructose, mannitol, polysorbate 80, tartaric acid, sodium hydroxide and hydrochloric acid [103]. The exact amount of AFG in the formulation can slightly differ and the exact amount of AFG in the formulation can only be quantified against the analytical reference substance. The EMA restricts the maximum acceptable deviation in the active substance content of the pharmaceutical products to ±5% [104], therefore the potential bias was negligible for all *in vitro* investigations (bioanalytical quantification and µDialysis), which were performed with aliquots of the stock solution.

Selectivity of the analytical method: There were no interferences with the investigated matrices for AFG: RS+HSA (0.5%) or dextran 40 (5 mg/mL)/HSA (0.5%) in water. There was also no interference with VOR in the RS matrix. As shown in the chromatogram (see chapter 3.1.1), CFG used for pre-coating was not detected with the applied bioanalytical assay for AFG. A potential CFG peak would presumably have appeared in front of the AFG peak due to higher hydrophilicity of the amine moiety of CFG. It could not be excluded that CFG was adsorbing on PP vials after it had been diluted with water for preparation of the stock solution. When the pharmaceutical formulation of CFG (Cancidas™) was diluted, no adsorption of drug to laboratory material was detected [36]. The resulting solution from the pharmaceutical formulation contained CFG, acetic acid, sodium hydroxide, sucrose and mannitol [105]. Since only a change in pH is conceivable from the composition of Cancidas™ compared to the analytical reference substance in water, a change in the ionic structure of the molecule could result. This may have affected adsorption, but data is lacking. In

the future, aqueous solutions of CFG with different pH values may be tested on their ability to adsorb on laboratory material.

Carry-over effect: No carry-over was observed after injection of AFG or VOR samples at the ULOQ subsequently to blank matrix. Here, an absence of carry-over was especially important, considering AFG was known to adsorb on laboratory surfaces [100].

Lower limit of quantification and assay linearity: The investigated range of AFG in RS/HSA (0.5%) was 0.10-20.0 µg/mL and of VOR 0.15-10.0 µg/mL. The investigated concentration ranges of both drugs covered *in vivo* therapeutic concentration ranges. For the treatment of IFIs, a therapeutic range of total (i.e. bound and unbound) concentrations from 1.5 µg/ml to 4.5 µg/ml in plasma is recommended for VOR [106]. For oesophageal or oropharyngeal candidiasis, a total AFG concentration at steady state higher than 1.5 µg/mL, and minimum concentration higher than 1.0 µg/mL of AFG have been associated with therapeutic success [58]. Thus, target-site concentrations were in a comparable range to the *in vitro* µDialysis concentrations and the assay was therefore considered to be also adequate.

Accuracy and precision: Accuracy and precision of both methods concerning AFG or VOR with their respective matrices corresponded to the Guideline on bioanalytical method validation [3] with RE and CV ≤20% for LLOQ and ≤±15% for other concentrations investigated up to the ULOQ (see chapter 3.1.1).

Dilution integrity: Stock solutions of AFG and VOR of 1 mg/mL each were diluted to 40 µg/mL and 200 µg/mL for AFG and VOR, respectively. Both concentrations were above the ULOQ and were subsequently diluted to obtain concentration ranges occurring during *in vitro* investigations. Accuracy and precision of diluted concentrations corresponded to the Guideline on bioanalytical method validation (≤±15% RE and CV ≤15%, see chapter 3.1.1) [3].

Stability in µDialysate: Stability of VOR solutions in RS had already been investigated [28]. Due to the new sample preparation method, only the stability in the autosampler was investigated at 10°C up to 48 h and stable concentrations were shown (see chapter 3.1.1). For samples of AFG in RS/HSA (0.5%), a full stability investigation was successfully performed. Freeze-thaw, short-term-stability at room temperature, autosampler and long-term stability were investigated in accordance with the scenarios in daily laboratory-routine. Stability of AFG samples was confirmed for the investigated durations and temperatures (see chapter 3.1.1).

4.2 Anidulafungin in the static *in vitro* microdialysis system

AFG concentrations used for *in vitro* investigations were based on a nonlinear mixed-effects PK model for plasma data [67]. C_{min} and C_{max} values had been estimated at steady state after different dosing regimens of AFG i.v. infusions in patients with fungal infections. C_{min} was 1.6 µg/mL after a dosing regimen of 100/50 mg (loading/daily maintenance dose) and C_{max} was 7.2 µg/mL of 200/100 mg. Hence, C_{AFG} between 1 and 10 µg/mL AFG in medium were analysed in the static and dynamic IVMS projects, covering the whole range of therapeutic concentrations.

4.2.1 Adsorption and release of anidulafungin on catheter material: Behaviour of AFG in microsyringes

AFG perfusate concentration in microsyringes was set to 0.2 µg/mL. This setting was supposed to be sensitive also for small concentration changes resulting from adsorption and release processes. Additionally, the AFG concentration in microsyringes was supposed to be low, but still quantifiable (LLOQ: 0.1 µg/mL, see chapter 2.2.3) throughout the investigation. Since a maximum CV of <6.80% (n=9) of AFG in new microsyringes was in accordance with the between-day variability (CV: 7.50%, see chapter 3.1.1) of QC samples in the bioanalytical assay at the level of LLOQ (0.1 µg/mL), variability in C_{AFG} in the new microsyringes was in an acceptable range over the investigated time.

For the investigation with previously used microsyringes a much higher concentrated AFG solution (50 µg/mL AFG) was filled in the microsyringes and subsequently washed out and dried, hence, C_{AFG} in the perfusate of previously used microsyringes increased during the investigated time and significantly exceeded the nominal concentration (Tab. 3.2-1). Considering that the microsyringe had previously contained a solution of 50 µg/mL AFG, only a short exposure time was sufficient for AFG molecules to be released from the inner surface of the microsyringe into a solution of 0.2 µg/mL AFG (see chapter 3.2.1). Thus, C_{AFG} in perfusate increased over time with final concentrations higher than the concentration in the beginning. It can be concluded that adsorption of AFG is reversible and for each *in vitro* µDialysis investigation, new microsyringes should be used. Otherwise, AFG adsorbed on the inside of microsyringes from previous investigations, e.g. delivery investigations, could increase C_{AFG} in perfusate and result in biased assumptions regarding the behaviour of AFG in µDialysis. Hence, the hypothesis of adsorption of AFG to microsyringe material and subsequent release from the surface into lower concentrated solutions was confirmed (see chapter 3.2.1).

Furthermore, these results lead to the hypothesis that also catheters previously used with AFG are at risk of releasing AFG from catheter surfaces, contributing to higher concentrations in µDialysate. The shown reversible adsorption behaviour requires special arrangements for further µDialysis

investigations, i.e. additional equilibration time of 3-4 h after concentration changes in medium and continuous perfusion of catheters during catheter coating with AFG even during recovery investigations.

4.2.2 Comparison of microdialysis catheters

AFG was quantified in µDialysate of CMA71® catheters with increasing RR over time. Since AFG was able to diffuse through the semi permeable membrane of a 100 kDa cut-off, CMA71® catheters with 100 kDa cut-off were used for further investigations. It was assumed that maximum 1% of AFG molecules were not bound to albumin, due to the plasma protein binding of >99% [67]. Hence, maximum 1% of AFG molecules would have been able to diffuse through the semi permeable membrane with 20 kDa cut-off (e.g. CMA63®) according to their molecular mass of 1.14 kDa. Quantification of 1% at a concentration of 10 µg/mL would have resulted in concentrations of 0.1 µg/mL, close to the LLOQ. Thus, collecting AFG molecules with a CMA63® catheter from an AFG-containing medium was not feasible. HSA has a molecular mass of 66.5 kDa [107], hence the complex of AFG and HSA was assumed to have a molecular mass of 67.64 kDa. HSA-bound AFG was not able to diffuse through the membrane of the CMA63® catheter considering the higher molecular mass of the albumin-AFG complex compared to the membrane cut-off. In general, only analytes with a molecular mass of one-fourth of the membrane cut-off can diffuse freely through the semi permeable membrane [14]. Thus, the complex of AFG and HSA was not only too large to pass a membrane with a cut-off of 20 kDa, but also to pass freely one with a cut-off of a 100 kDa. Since the cut-off of the CMA71® membrane was larger than the AFG-HSA complex, it was expected that the complex was partially diffusing through the 100 kDa membrane.

A possible reason for the increase of C_{AFG} in µDialysate during recovery investigations over time was adsorption of AFG molecules to catheter material. Adsorption of AFG had been described previously [100] and had also been observed during the development of the bioanalytical HPLC method for AFG (see chapter 3.1.1). Addition of HSA (0.5%) during quantification sufficiently prevented adsorption. Here, binding of AFG to catheter material (PUR, PAES) was stronger compared to sample vial and tip material (PP). The addition of HSA did not have a significant effect on adsorption during µDialysis (see chapter 3.2.2).

4.2.3 Ultrafiltration: Recovery investigation of anidulafungin with microdialysis catheters

UF of perfusate through the semi permeable membrane into the medium caused a reduced volume in µDialysate. Potential explanations included the hydrostatic pressure of the pumping system on the

membrane combined with the relatively high molecular mass cut-off of 100 kDa [46]. Whereas, UF was not observed with 20 kDa cut-off catheters. Evaporation of fluid (e.g. RS, HSA, water) in the µDialysate could also be a reason. However, investigations on evaporation from µDialysate showed only negligible volume loss through evaporation. The osmotic pressure on the membrane caused by the surrounding medium and the pumps might influence fluid movement through a semi permeable membrane [46,108,109]. In general, semi permeable membranes with a cut-off of 100 kDa are associated with a fluid loss of at least 20% [110]. Assuming that the loss of perfusate through the membrane into the medium occurred without changes in analyte diffusion from medium into catheter, an adjustment of the resulting RR by the recovered smaller volume could be taken into account with respect to analyte concentration in µDialysate. Here, the corrected RR would be smaller than the calculated RR from µDialysate. There was no evidence so far about the use of volume correction on RR. Still, further research is required to overcome UF, which would be a beneficial improvement of µDialysis. Without UF the µDialysate volume could be increased and simultaneously sampling intervals shortened to ensure reliable bioanalytical quantification and a high concentration-time profile resolution [111].

A well-known approach to overcome UF is the addition of osmotic agents, such as large molecular mass dextran or albumin, to the perfusate [45,109,110,112–115]. The perfusate used in the previously described experiments already contained HSA and still, a fluid loss of up to 24.6% was observed (see chapter 3.2.3). Hence, dextran was added to the perfusate to further reduce fluid loss. The dextran containing perfusate led to a constant and higher recovery of µDialysate volume, with a volume loss of up to 9.51% (see chapter 3.2.4: an outlier had a volume loss up to 23.8%). Another hypothesis was that UF from perfusate to medium altered diffusion of the analyte from medium to µDialysate during recovery investigations. According to this hypothesis, the diffusion of analyte into µDialysate and ultimately RR would increase, if UF could be eliminated. RR_{max} values during the investigation with RS/HSA (0.5%) were 35.6% without volume correction and 27.9% with volume correction (see chapter 3.2.3). Higher results were achieved during the investigation with dextran added to RS/HSA (0.5%): RR_{max} was 44.5% without volume correction and 42.0% with volume correction (see chapter 3.2.4). The confidence intervals of estimated RR_{max} values obtained from investigations with and without dextran (without volume correction) were overlapping. The confidence intervals of estimated RR_{max} of the two investigations with volume correction were just not overlapping. Overall, the overlapping and almost overlapping confidence intervals indicated that there was no significant difference between the investigations and volume correction or no volume correction, and leading to the conclusion that the addition of dextran to perfusate overcame UF but did not improve the increasing RR_{max} values during recovery investigations. Since a sufficiently high

value for RR was crucial to perform *in vivo* µDialysis [45], only additives to the perfusate leading to higher and constant RR values should be used.

Additionally, the time to reach 50% or 90% of RR_{max} was estimated. The investigation with RS/HSA (0.5%) containing perfusate had a $t_{RR,50}$ of 81.7 min (=1 h 21.7 min), whereas the investigation with the dextran containing perfusate had a much longer $t_{RR,50}$ of 183 min (=3 h 3 min). $t_{RR,90}$ was 735 min (=12 h 15 min) and 1647 min (=27 h 27 min),respectively. These numbers give an impression of how long it would take to get an equilibrium of adsorption of AFG on catheter material until stable concentrations in medium could be measured. $t_{RR,50}$ and $t_{RR,90}$ of volume corrected RR_{max} showed similar results.

In general, adding osmotic agents or substances in order to prevent unspecific binding to laboratory material, e.g. HSA, polysorbate (Tween®) [34], or (cyclic) dextran, introduces an additional bias due to a hindered diffusion. Furthermore, most of the additives have not been approved for *in vivo* application so far. The bias could be caused by an extra compartment, which would be created by "pulling" analytes from medium to perfusate. In the delivery setting, the extra compartment would lead to a decrease in diffusion of AFG from perfusate into medium. It can be recommended to use additives like dextran in cases if a higher µDialysate volume is needed e.g. for quantification purposes. For PK studies, only analytes, which could be demonstrated to be applicable with standard perfusate (e.g. RS) in *in vitro* µDialysis investigations, should be used.

4.2.4 Influence of catheter pre-coating on the adsorption of anidulafungin: Catheter coating with caspofungin

The successful pre-coating approach using CFG, performed by Traunmüller *et al.* [36], was the basis for the *in vitro* investigations with CFG as pre-coating substance for AFG recovery investigations. The pre-coating with CFG was assumed to block potential binding-sites before the recovery investigation started. The investigation performed by Traunmüller *et al.* was a pre-coating approach of 50 µg/mL CFG in perfusate of RS/HSA (1.0%) for 120 min in a medium of RS/HSA (1.0%) at a FR of 1 µL/min. In the work of Traunmüller *et al.*, CFG was added to the perfusate and catheters were pre-coated, before the main investigation with CFG started. After pre-coating was finished, the recovery investigation with CFG-containing medium and CFG-free perfusate was performed. Without pre-coating, RR increased from the start, but with prior pre-coating, RR values were constant over the whole investigation time and generally higher. µDialysate samples contained only negligible amounts of CFG after a wash-out phase of 30 min. In conclusion, CFG adsorbed irreversibly at the catheter material binding sites.

Discussion

The pre-coating setting developed by Traunmüller *et al.* was applied for the pre-coating approach during this work, using a perfusate containing 50 µg/mL CFG in HSA with 2 h pre-coating before start of the investigation (see chapter 2.3.7.1). Different CFG concentrations in perfusate and medium during pre-coating were investigated as well as a prolonged pre-coating phase. To ensure blocking of AFG binding sites with CFG, the perfusate during recovery investigations also contained CFG. The investigations on pre-coating with CFG should show if RR values of AFG would be constant (no increase) and also higher than results of investigations without previous CFG pre-coating.

Before the pre-coating approach with CFG was carried out, a potential PD effect of CFG at the target site had to be discussed. The potential diffusion of CFG during *in vivo* µDialysis from perfusate into the catheter-surrounding tissue and the effect of the drug at the target site is of minor clinical relevance: Since CFG is highly protein bound [64] and shows adsorption phenomena like AFG [36,43], it is unlikely that larger amounts of CFG diffuse unhindered through the membrane into medium. As it was not possible to quantify CFG with the bioanalytical assay for AFG (see chapter 3.1.1), it was not feasible to determine the concentrations of CFG in µDialysate and thus in the medium. Hence, any interference of CFG and AFG during quantification was excluded.

During µDialysis investigations, a prolonged pre-coating time was performed, to ensure saturated adsorption of CFG to corresponding binding sites, which should result in high and constant RR values. RR_{max} values, estimated with the formula from the Langmuir adsorption isotherms (Eq. 2-6), did not support this hypothesis. The estimated RR_{max} of the investigation with 2 h pre-coating and 50 µg/mL CFG in perfusate (i) was 53.5%, RR_{max} of the investigation with 14 h pre-coating and 50 µg/mL CFG in perfusate (ii) was 41.2% and RR_{max} of the investigation with 15 h pre-coating and 200 µg/mL CFG in perfusate (iii) was 41.4%. These values for RR_{max} were higher compared to the estimated RR_{max} of 35.6% from the investigation without pre-coating and without CFG in perfusate but RS/HSA (0.5%) in perfusate. These results would support the theory that pre-coating of catheters with CFG led to higher values for RR_{max}. In Fig. 3.2-15 it was shown that the observed RR values of the three catheters from investigation (i) and (ii) were very variable over time, which was also seen in the large confidence intervals of 23.2% to 83.7% of (i) and 16.7% to 66.0% of (ii). Whereas investigation (ii) and the investigation with RS/HSA (0.5%) in perfusate had only a narrow confidence interval from 39.0% to 43.3% and from 32.8% to 38.4%, respectively. Investigation (i) and (iii) should be repeated to investigate if these strategies lead to reproducible RR-time profiles and smaller confidence intervals. The confidence intervals of (ii) and the investigation with RS/HSA (0.5%) in perfusate were not overlapping but were so close together that a significant difference between these investiagtions was not considered. Additionally, with the CFG-pre-coating it was tried to achieve a shortening of the time to reach 50% and 90% of RR_{max}. The estimated $t_{RR,50}$ of (i), (ii) and (iii) was 238 min (=3 h 58 min),

94.2 min (=1 h 34.2 min) and 140 min (=2 h 20 min) and overall higher than $t_{RR,50}$ of 81.7 min (=1 h 21.7 min) of the investigation with RS/HSA (0.5%) in perfusate. The same was true for $t_{RR,90}$. Estimated values of $t_{RR,90}$ were 2142 min (=35 h 42 min), 848 min (=14 h 8 min) and 1264 min (=21 h 4 min) for (i), (ii) and (iii). Again, the $t_{RR,90}$ value for the investigation with RS/HSA (0.5%) in perfsate was with 735 min (=12 h 15 min) much shorter. An explanation for the higher $t_{RR,50}$ and $t_{RR,90}$ of CFG containing perfusate, was the estimated higher RR_{max} taking into account that the increase of RR over time is the same. The theory that with a higher concentration of CFG in perfusate, higher RR values would be reached was disproved. The highest CFG concentration in perfusate of 200 µg/mL was not leading to the highest RR_{max} or the shortest $t_{RR,50}$ or $t_{RR,90}$.

Hence, it was assumed that pre-coating with CFG, independent of perfusate concentration and pre-coating time, did not lead to a significant improvement compared to the basic investigation with RS/HSA (0.5%) in perfusate. Neither shorter equilibration time until steady-state, nor higher RR_{max} values were reached. Ultimately, a constant and reproducible RR was not achieved by pre-coating with CFG.

4.2.5 Influence of catheter pre-coating on the adsorption of anidulafungin: Catheter coating with anidulafungin

The AFG concentrations in medium of 1 and 8 µg/mL were within the range of the minimum and maximum concentrations in plasma samples during steady-state [67]. The nominal concentration in the perfusate was 0.2 µg/mL AFG. A pre-coating concentration of 0.2 µg/mL should be small enough to allow detecting significant changes in µDialysate concentrations during recovery investigations. Additionally, the concentration should be above the LLOQ of 0.1 µg/mL to ensure quantification. The mean C_{AFG} in perfusate for the three catheters during pre-coating and recovery investigation was, however, 0.393 µg/mL AFG for the investigation with 1 µg/mL in medium and 0.160 µg/mL AFG for the investigation with 8 µg/mL AFG in medium (Tab. 3.2-12). Perfusate for the two investigations was prepared separately. A bias caused by the independently prepared perfusate solutions could not be excluded. Potentially, the perfusate concentration had a greater influence on diffusion of AFG molecules from medium (1 µg/mL AFG) into perfusate (0.2 µg/mL). A medium with a C_{AFG} of 8 µg/mL leads to a steeper concentration gradient compared to 1 µg/mL AFG, while smaller perfusate concentrations had negligible effects on diffusion. Hence, a higher flux and higher RR could be expected. Since adsorption of AFG to laboratory material was reversible (see chapter 3.2.1), a continuous perfusion of catheters with AFG-containing perfusate was recommended before and during the recovery investigation.

Discussion

After the overnight pre-coating phase, C_{AFG} was quantified in perfusate and µDialysate. The calculated rD of AFG of the investigation of 1 µg/mL AFG in medium ranged from 11.1% to 23.1% and of the investigation of 8 µg/mL AFG in medium calculated rD ranged from 47.1% to 55.0%. Results for rD of the investigation of 1 µg/mL AFG containing medium (Tab. 3.2-13) were closer to results of retrodialysis of AFG-free medium and 200 µg/mL AFG in perfusate of approximately 10% (Fig. 3.2-19) than to rD of the investigation of 8 µg/mL AFG in medium (Tab. 3.2-13). The delivered amount of AFG was much higher for the investigation of 8 µg/mL AFG in medium compared to 1 µg/mL AFG. A reason for the observation could be the lower AFG concentration in perfusate during pre-coating leading to a prolonged saturation time due to adsorption of AFG molecules on catheter material during the recovery investigation. An apparently high rD could have been caused by adsorption and not caused by diffusion through membrane. Additionally, it is very likely that only small amounts of AFG molecules were diffusing through the membrane into medium, resulting in higher concentrations in µDialysate for higher perfusate concentrations.

For the investigations with AFG pre-coating, the perfusate concentration was included into the calculation of RR during recovery investigation (Eq. 2-7). Thus, the first sample of two catheters, in the setting of 1 µg/mL AFG in medium, showed negative results for RR (Tab. 3.2-14) which was caused by analytical accuracy combined with subtraction of $C_{Perfusate}$ from $C_{\mu Dialysate}$. The observed RR_{max} in the setting of 1 µg/mL AFG in medium was 113% and in the setting of 8 µg/mL AFG in medium it was 63.5% (Tab. 3.2-14). The estimated RR_{max} resulted in 105% and 84.2% (Tab. 3.2-15), respectively. RR values exceeding 100% are assumed to result from the AFG concentration in perfusate, which cannot be adequately removed from the total µDialysate concentration. Since AFG in the 1 µg/mL AFG investigation had a higher AFG concentration in perfusate and a smaller rD compared to the 8 µg/mL AFG investigation, an increased amount of AFG from perfusate was still found in µDialysate leading to RR values exceeding 100%. Confidence intervals of RR_{max} of the two investigations ranged from 85.8% to 125% for the investigation with 1 µg/mL AFG in medium and from 71.2% to 97.2% for the investigation with 8 µg/mL AFG in medium. Since confidence intervals were overlapping, there was no significant difference in the estimated RR_{max} values. The confidence interval of estimated RR_{max} from the investigation of RS/HSA (0.5%) in perfusate was not overlapping with the confidence intervals of RR_{max} of the AFG-pre-coated catheters, thus, leading to a significant difference between µDialysis with and without the pre-coating approach. An explanation for the significantly higher RR_{max} values of the investigation with AFG-pre-coated catheters could be the amount of AFG in µDialysate, which originated from perfusate and was not adequately removed from the total µDialysate concentration. Additionally, the time to reach 50% or 90% of RR_{max} should be shorter with the approach of pre-coating with AFG. In general, $t_{RR,50}$ and $t_{RR,90}$ of 1 µg/mL AFG in medium were 116 min (=1 h 56 min) and 1047 min (=17 h 27 min), respectively, and shorter than the

results for 8 µg/mL AFG in medium with 187 min (=3 h 7 min) and 1686 min (=28 h 6 min), respectively. Much shorter $t_{RR,50}$ and $t_{RR,90}$ values were estimated for the investigation with RS/HSA (0.5%) in perfusate with 81.7 min (=1 h 21.7 min) and 735 min (=12 h 15 min), respectively. Again, smaller RR_{max} values led to shorter $t_{RR,50}$ and $t_{RR,90}$.

After all, no significant shortening of the time was achieved until RR_{max} was reached compared to other approaches without pre-coating with AFG. A stable RR_{max} could also not be guaranteed due to the insufficient removing of AFG concentrations in perfusate from the total µDialysate concentration. Hence, catheter pre-coating with AFG was no improvement to the investigation with RS/HSA (0.5%) as perfusate and the concentration-dependence of RR, which is a prerequisite for *in vivo* determination of AFG, was not met.

4.2.6 Investigation of anidulafungin retrodialysis

Retrodialysis is a calibration method, which is based on the theory that diffusion of a molecule is depending on the concentration gradient and therefore the flux caused by the gradient is equal on both sides of the semi permeable membrane: from medium to perfusate and from perfusate to medium [23]. Usually, to determine retrodialysis as calibration technique, clinically relevant analyte concentrations would be investigated. The investigation of retrodialysis was used to evaluate feasibility of this calibration method for an investigation under *in vivo* conditions in the dIVMS. A perfusate concentration of 200 µg/mL AFG was determined to build a concentration gradient from perfusate to medium while medium concentrations of AFG were much smaller or < LLOQ. In case of unknown ISF concentrations of AFG, the concentration of 200 µg/mL AFG would be high enough to be a minimum of 10-fold the medium concentration [14] (plasma steady state: C_{min} of 1 µg/mL, C_{max} of 8 µg/mL [67]). Two different settings were investigated, which covered the range of steady state plasma concentrations: AFG-free medium and 10 µg/mL AFG in medium (close to C_{max} [67]). Samples were collected during three sampling intervals of 40 min each in both settings, equally to an *in vivo* retrodialysis scenario. Both investigations started with an equilibration phase of 15 min, first in AFG-free medium and subsequent in 10 µg/mL AFG containing medium 2.3.8).

C_{AFG} during the investigation in AFG-free medium increased from 0 µg/mL to 0.153 µg/mL after 120 min. Since C_{AFG} in medium after 120 min had approximately the same concentration as the LLOQ (0.1 µg/mL, see chapter 3.1.2), the concentration was negligible and therefore not included in calculations. C_{AFG} in the medium of 10 µg/mL did not change during the investigation.

In µDialysate, C_{AFG} was constant from 149 µg/mL to 222 µg/mL in both investigations with AFG-free medium and a medium containing 10 µg/mL AFG (see chapter 3.2.6). An exception was the first sample of AFG-free medium with 109 µg/mL AFG in µDialysate (Fig. 3.2-19). In AFG-free medium,

resulting values for rD were decreasing after the first sampling interval. Values during the first interval had a rD ranging from 19.8% to 50.8%. During the second and third interval, the minimum values for rD ranged between 9.82% and 10.3%. The high rD values during the first sampling interval did probably not arise from higher diffusion of AFG molecules through the semi permeable membrane, but from continuing adsorption processes on catheter material and diffusion. During the last two sampling intervals, adsorption and desorption were in steady state and only diffusion defined rD. Hence, after one sampling interval for equilibration the proportion of AFG molecules diffusing from perfusate into medium was 10%. During the investigation of 10 µg/mL AFG in medium, the values for rD were constant during the three sampling intervals. Here, rD ranged between -0.205% and 9.42% (Tab. 3.2-17) (negative values due to analytical accuracy). Less molecules seemed to diffuse from perfusate to medium compared to the investigation in AFG-free medium (<10%). The increase of medium concentration from AFG-free to 10 µg/mL AFG was supposed to lead to a change in the concentration gradient. Still, the perfusate concentration was more than 10-fold higher, hence, an increase in AFG concentration in medium should not influence the concentration gradient.

In contrast to previously performed recovery investigations of AFG (see chapter 3.2.2 - 3.2.5), the number of molecules diffusing from perfusate to medium was considerably smaller (≤10.3%, Tab. 3.2-17) compared to the amount diffusing from medium to perfusate (≥18.2%, see chapter 3.2.2 - 3.2.5).

It can be concluded that retrodialysis as calibration method may not be recommended for the µDialysis of AFG. To confirm these results, further recovery and delivery settings with equal concentrations in perfusate and medium should be investigated. Besides, if retrodialysis with 200 µg/mL AFG in perfusate should be applied in an *in vivo* setting, it requires a longer equilibration phase of at least one additional sampling interval (40 min) compared to the standard equilibration phase of 15-20 min.

4.3 Voriconazole in the static *in vitro* microdialysis system

Investigations of VOR in the sIVMS were performed prior to feasibility investigations in the dIVMS. Catheters were used for the preliminary investigations in the sIVMS and the feasibility investigations in the dIVMS, which had an earlier expiry date than the date of investigation (investigations 6-7 years later). In general, expiration only implies that sterility of catheters is not assured. These catheters were sufficient for the development stage of the dIVMS. It was assumed that catheters work equally in the static and dynamic setting.

µDialysis investigations on VOR were performed previously by Simmel *et al.* [4,28]. A partial investigation of different flow rates, concentrations and retrodialysis was conducted in this work. The

sIVMS was used for the investigations of dependency of RR and rD on FR and concentration as well as under steady state conditions (retrodialysis). It was concluded by Simmel *et al.* that µDialysis of VOR was FR dependent (RR increased with decreasing FR) and concentration independent (RR was the same with different concentrations). Furthermore, a sufficient drug concentration in perfusate during retrodialysis was investigated by Simmel *et al.*. It was found that a C_{VOR} of 100 µg/mL to 200 µg/mL during *in vitro* retrodialysis was sufficient to neglect the influence of remaining VOR at the sampling site. The pharmaceutical formulation Vfend® was applied during retrodialysis in the *in vivo* study, since the analytical reference substance should not be used *in vivo*. Prior to the *in vivo* study, VOR analytical reference substance and the pharmaceutical formulation Vfend® were compared in *in vitro* µDialysis and resulted in similar RR values [28]. Hence, the possibility of substitution was regarded feasible during the previously performed investigation, thus the analytical reference substance was used during the present investigations. First, the dependence of RR and rD on FR was investigated to determine appropriate FR for the following investigations. Since flow rates from 0.4 to 10 µL/min were previously investigated for CMA60® catheters [4], only flow rates of 1 and 2 µL/min with the potentially highest RR and rD were investigated with the available CMA60® catheters. The mean RR of recovery and delivery investigations of 1 µL/min was 88.3% (CV: 7.56%; n=94) and for 2 µL/min 88.4% (CV: 6.87%; n=96). There were no significant differences between a FR of 1 and 2 µL/min (p=0.778, Wilcoxon-Mann-Whitney test). The mean RR and rD for the FR of 1 µL/min was 87.1% (CV: 4.89%) and 90.2% (CV: 8.73%), respectively. The FR of 2 µL/min resulted in 87.0% (CV: 4.44%) and 89.8% (CV: 8.31%), respectively. The results of recovery and delivery investigations showed a statistically significant difference. The results as mean RR of recovery investigations conducted by Simmel *et al.* [28] were 98.9% (CV: 0.30%) for 1 µL/min and 91.5% (CV: 1.80%) for 2 µL/min. Obviously, there was a difference in RR between 1 and 2 µL/min. In contrast to previous results with a wider range of FR, present results showed no influence of FR on RR. RR was similar to previous results at a FR of 2 µL/min, but not for a FR of 1 µL/min. A potential reason for these different results compared to the previous findings [28] could be the age of the catheters (expiring date exceeded), possibly leading to an altered performance. The shaft, inlet and outlet tubings were made of PUR and the membrane of PAES. Catheters were sterilized by beta-radiation and had a shelf life of 2 years from the manufacturing date. No information about long-term stability of the catheter material could be found in literature. Sterility could be influenced after expiring, but a loss of stability of membranes and tubings was rather unexpected. However, results from µDialysis investigations in the dIVMS with aged CMA60® catheters with a FR of 2 µL/min resulted in an even higher rD of 95% and 97% during retrodialysis with 20 or 200 µg/mL VOR in perfusate (see chapter 3.4.4). Additionally, deviations in RR could be due to analytical reasons, since the analytical precision of the between-day variability of QC samples (LLOQ: 0.15 µg/mL) was up to 9.32%.

Discussion

The concentration independence of RR was investigated previously by Simmel *et al.* [28] over a concentration range between 1 and 50 µg/mL at a FR of 1.5 µL/min, concluding that RR and rD were independent of VOR concentrations. RR from recovery investigations ranged from 95.3% to 99.4% and from delivery investigations from 96.9% to 97.4% [28]. Since the investigations in the sIVMS served as prerequisite for the dynamic investigations, only a part of the previously investigated concentration range was conducted. Here, a range between 0.5 and 4 µg/mL VOR was investigated at a FR of 1 and 2 µL/min. Since the two flow rates resulted in similar RR values, RR values of both flow rates were merged for the single concentrations. Additionally, a concentration of 0.5 µg/mL was investigated to take early and late-phase concentrations of VOR below 1 µg/mL during dIVMS investigations into account. The results of recovery investigations were not significantly different and had an overall mean RR of 87.0% (CV: 4.65%), whereas the results observed in the delivery investigations showed significant differences between the different concentrations, which can be seen in Fig. 3.3-2, with an overall mean rD of 90.0% (CV: 8.48%). Here, results from recovery and delivery investigations were lower compared to results from Simmel *et al.* (see above). A potential reason might be adsorption of VOR molecules on catheter material, which was described by Araujo *et al.* [97], but this would also have influenced previous recovery investigations. The adsorption process of VOR described by Araujo *et al.* may only be partly applied to the present investigation. The adsorption process was found for VOR concentration between 0.5 µg/mL and 2.0 µg/mL in perfusate of CMA20® catheters (same catheter material, PAES and PUR, and cut-off as CMA60® catheters) by Araujo *et al*.. A more reasonable explanation is a flatter concentration gradient in combination with high FR of 1 µL/min and 2 µL/min had influenced the rD for low VOR concentrations. Overall, the consistent results from the recovery setting indicate that VOR has a concentration-independent recovery and hence, is a suitable drug for µDialysis investigations, as previously described by Simmel *et al.*.

Retrodialysis investigations during steady state conditions were performed as a prerequisite for the subsequent mimicry of a concentration-time profile of VOR in the dIVMS. Hence, different perfusate concentrations were investigated with changing VOR concentrations in medium of the sIVMS (mimicking the potential concentrations of VOR in ISF before drug administration (VOR-free) and after recovery investigation (1 and 10 µg/mL VOR)). Combinations of 200 µg/mL VOR in perfusate with VOR-free, 1 µg/mL VOR and 10 µg/mL VOR containing medium resulted in mean rD values of 92.5% to 96.4%. Only the rD of the setting with 10 µg/mL VOR in medium was significantly different compared to the two other settings. Still, rD values were in a comparable range as can be seen from Fig. 3.3-3. 20 µg/mL VOR in perfsuate resulted in mean rD values between 93.0% and 95.3% for VOR-free and 1 µg/mL VOR containing medium. The combination of 20 µg/mL VOR in perfusate with 10 µg/mL VOR in medium led to a rD of 54.7%. There was a statistically significant difference

between all three settings of 20 µg/mL VOR in perfusate. A flat concentration gradient between medium and perfusate would be the explanation for the significantly lower rD results of 20 µg/mL VOR in perfusate and 10 µg/mL VOR in medium as described above (delivery investigations from 0.5 to 4 µg/mL VOR in perfusate). A similar result was obtained at a combination of 50 µg/mL VOR in perfusate and 10 µg/mL VOR in medium by Simmel *et al*. [28]. Here, a rD of 78.1% was calculated. The concentration gradient was less pronounced compared to combinations with larger differences in the analyte concentration between perfusate and medium. As previously described, the drug concentration in perfusate should be at least 10-fold higher than the concentration in medium or ISF [14], if retrodialysis is performed after the recovery investigation (lower concentrations may be used in perfusate during retrodialysis prior to the main recovery investigation). As described in chapter 3.4.4, the results from retrodialysis investigations with the dIVMS had relative deliveries of 95% and 97% with 20 µg/mL or 200 µg/mL VOR in perfusate, respectively. Comparing results of present sIVMS, sIVMS results by Simmel *et al.* (see above) and dIVMS investigations, it can be concluded that a higher perfusate concentration, above the previously investigated 0.5 µg/mL to 4.0 µg/mL, e.g. of 20 µg/mL and 200 µg/mL, would increase the rD due to a steeper concentration gradient between medium and perfusate.

After all, it is crucial to perform *in vitro* µDialysis and in particular retrodialysis prior to *in vivo* studies. The requirement to use retrodialysis as calibration method is that RR and rD of the analyte are the same and that the concentration of VOR in perfusate during retrodialysis should be sufficiently high (a minimum of 10-fold higher than the concentration in medium or ISF). Overall, this requirement was fulfilled for VOR in the specified settings.

4.4 The dynamic *in vitro* microdialysis system

4.4.1 Development of the dynamic *in vitro* microdialysis system

With the established sIVMS [28], investigations of several antiinfectives and biomarkers were previously performed [4,45,116–119]. In order to continuously adapt drug concentrations for concentration-time profiles from *in vivo* data, the sIVMS, which was developed by Simmel *et al.* [28], had to be further developed to a dIVMS. The aim of the dIVMS was to mimic the changing drug concentrations over time without replacement of medium containers with different drug concentrations. The most important advantage was the mimicry of PK profiles for better prediction of µDialysis behaviour of the respective drug *in vivo*. In literature, an experimental dynamic *in vitro* infection model (dilution model) to investigate the PD effects of antibiotics against bacteria under physiological conditions was described by Löwdin *et al.* [120]. A comparable experimental flask model for bacteria was previously developed by Scheerans and Gloede *et al.* [121] and further

developed by Bartels and Goebgen *et al.* [122]. By now, no dynamic experimental models for µDialysis investigations have been found in literature.

4.4.2 Validation of the dynamic *in vitro* microdialysis system

The temperature in the glass flask of 37.3°C corresponded well with body temperature of 37°C [92]. The water bath ensured a constant temperature in the inner chamber of the flask after 10 min for at least 23 h. The digital and mercury thermometer showed similar results of 37.5°C and 38.0°C, respectively (see chapter 3.4.2). For following investigations, it would be sufficient to measure the temperature in the water bath due to the analogy of temperatures in water bath and glass flask. Previously, temperatures in dynamic systems were measured with digital thermometers [122]. Advantages of digital thermometers were their material robustness and harmlessness, due to their lack of mercury. Disadvantages were the regular change of batteries accompanied with precise temperature measurements with low batteries and previous calibration with only mercury thermometers. Still, a digital thermometer was utilised in the water bath during the following investigations. The stirring of medium was constant for the investigated settings, allowing homogenous distribution of the drug in the medium (see chapter 3.4.2). Tubings for in- and outflow of medium were made of PVC and plasticisers. The material gets more flexible with increasing temperatures. It can be assumed that with progressing rolling of tubings in the pump, tubing material became more flexible and therefore a larger volume of medium was pumped per time. Thus, the measured pump rates during the first hour after starting the pump were smaller compared to the pump rates measured from 5-6 h and 24-25 h (see chapter 3.4.2). It was required to roll new tubings in the pump until a constant PR after 5 h could be assured, which was in accordance to previously published results [123]. Additionally, earlier time points between 1 and 5 h should be investigated due to a potentially constant PR before 5 h. Finally, it can be concluded that a dIVMS was successfully developed and the performance of the described parameters was validated over 24 h. The validation procedure showed the potential of the dIVMS to perform *in vitro* µDialysis under physiological-like conditions considering the pharmacokinetics of the investigated drug.

4.4.3 *In silico* simulation of pharmacokinetic profiles of antifungals

In chapter 3.4.3.1, the two antifungal drugs VOR and AFG were investigated in the novel developed dIVMS. For VOR, one- and two-compartment models [124–127] and for AFG, two- and three-compartment-models [58,67,125,128,129] are described in literature. Nevertheless, a one-compartment-model promised the best applicability in the dIVMS. Here, the use of one pump for in- and out-flow at the same time and a constant PR was most important. Since a one-compartment-model with i.v. bolus drug administration was assumed for both antifungals, the CL was equal to the

PR [121]. For testing routine, an i.v. bolus injection was chosen instead of administration routes like oral administration or i.v. infusions [28]. The calculated PR for VOR and AFG were pumped with tubings of the same inner diameter, which reduced technical efforts. The inner diameter of the tubing is crucial for the volume pumped through the tubing. For low pump rates, only tubings with a smaller inner diameter are applicable.

Plasma data (total C_{max}) of steady state samples [73] was used for simulations of the AFG concentration-time profile *in vitro* (Tab. 3.4-3), since ISF concentrations of AFG have not been determined until today. For VOR, PK simulations for *in vitro* investigations at steady state were based on plasma [80] and ISF data [28]. For plasma, an average C_{max} and the PPB for VOR were given [80]. Drug concentrations in plasma were equal to total drug concentrations. Since *in vitro* investigations of VOR with the dIVMS were performed in protein-free medium, the unbound concentrations were determined based on total concentrations using the PPB (Eq. 2-9). Data of VOR in ISF was already given as maximum unbound concentration [28] and no further adjustments had to be made for the protein-free medium. For *in vitro* investigations of AFG, HSA was added to medium and perfusate fluid to prevent adsorption of drug molecules to laboratory material (sampling vial, tubings and membranes) [34]. Since bioanalytical quantification and µDialysis investigations of AFG were performed in HSA containing medium, also simulations of *in vitro* PK profiles were based on total C_{max} in plasma (Tab. 3.4-3). All concentration-time profiles were simulated *in silico* and the profiles of VOR over 24 h resembled a first-order kinetic in a one-compartment model (Fig. 3.4-6 and Fig. 3.4-7). Because of its long elimination half-life (20.8 h [73]), the AFG PK profile had a linear profile during the first 6 h of investigation (Fig. 3.4-5).

4.4.4 *In vitro* mimicry of pharmacokinetic profiles of antifungals

Observed *in vitro* concentrations in medium over time were compared to the corresponding *in silico* simulated concentration-time profiles (Fig. 3.4-8 - Fig. 3.4-11). The *in silico* concentration-time profile was based on C_{obs} of AFG or VOR in the injection solution of the syringe.

To describe the variability of the analyte concentration in medium, the variability, expressed as CV (%), of the pump in the dIVMS and of the bioanalytical method (precision of QC samples) of the respective analyte, was plotted around the *in silico* calculated concentration-time profiles of VOR and AFG. The variability of AFG and VOR concentrations in medium was presented as CV of the pump in the dIVMS of 4.50%. The CV was derived from 4.38%, which was the precision of the pump after an equilibration time of 5-6 h (Tab. 3.4-2). For easier presentation, 4.38% was rounded up to 4.50%. Another factor to express variability was the maximum precision of QC samples of the bioanalytical

methods of AFG and VOR, respectively. AFG had a CV of 8.00% (Tab. 3.1-2) and VOR of 9.32%, which was rounded up to 9.50% (Tab. 3.1-3).

The observed VOR concentrations and the in silico calculated concentrations showed similar profiles. The observed concentrations based on *in silico* data from Theuretzbacher *et al.* [80], as well as the observed concentrations based on ISF data analysed by Simmel *et al.* [28], were not completely within the variability (plotted as ribbons) of the pump and the bioanalytical method. A similar pattern was found for AFG, which was based on plasma data from Crandon *et al.* [73]. Observed and in silico calculated concentrations had a similar profile but only a few of the observed concentrations were within the variability range of pump and bioanalytical method. Since the dIVMS was newly invented, there were no acceptance criteria for the observed concentration-time profiles in literature. Here it was demonstrated that the pump and the bioanalytical method could have an impact but these two variables can not fully explain the variability of observed medium concentrations in the dIVMS from in silico calculated concentrations. Other factors influencing the observed concentrations were e.g. the temperature insight the flask or unsteady volume of medium in the flask (i.a. decrease of volume through medium sampling) or unbalanced stirring of medium. Finally, to get a better idea of the actual variability of the medium concentration in the flask, more investigations of the same PK profiles should be performed.

4.4.5 *In vitro* microdialysis investigations of antifungals with the dynamic *in vitro* microdialysis system

Initially, a proof-of-concept and feasibility investigation using the model drugs VOR and AFG was performed in the dIVMS. The investigations were conducted to determine whether retrodialysis as calibration method for VOR and AFG results in the true medium concentrations. To evaluate the robustness of the calibration method, two approaches with perfusate concentrations of 20 and 200 µg/mL were explored for VOR (see chapter 3.4.4).

Initial investigations were based on i.v. bolus administration, although both model drugs, VOR and AFG, are not administered as i.v. bolus injections in clinical routine. The concentration-time profiles represent drug exposure at the target site after distribution of drug molecules into the compartments. AFG [67] and VOR [130] are both administered as i.v. infusions and VOR additionally as oral formulation [130]. Additionally to the i.v. bolus administration of the drug, the elimination from one compartment (the glass flask) was chosen for the following investigations. Pump rates were determined by adjusting the speed of the pump until the required volume was pumped. This was conducted by weighing the sampling vials before and after the sampling interval. The resulting PR depended on the diameter of the tubing, the temperature and the time length of the previous usage

of the tubing (Fig. 3.4-4). Since calibration of PR was not possible during the running investigation, the PR was not changed after start of investigation.

4.4.5.1 Voriconazole and anidulafungin in the medium of the dynamic *in vitro* microdialysis system

For VOR and AFG, concentrations of *in vitro* i.v. bolus injection solution of the two investigations were different from the nominal concentration. Hence, the observed concentrations of VOR and AFG in the i.v. injection solution were used to calculate the *in silico* concentration-time profiles.

In chapter 3.4.4 the observed VOR medium concentrations of both investigations were well within the variability range plotted around the *in silico* concentrations. Meaning that the variability of concentrations could be described by the variability of the pump or the bioanalytical method. In contrast, the observed medium concentrations of VOR were not fully explained by the variability of pump or bioanalytical method (see previous chapter 4.4.4). A described in chapter 4.4.4, more investigations on medium concentrations over time in the dIVMS should be performed.

The observed medium concentrations of AFG were not within the variability ranges of the pump and the bioanalytical method, but the concentration-time profile showed decrease over time a similar to the *in silico* concentrations of AFG. The higher variability of observed AFG concentrations in medium could be explained by the adsorption of AFG on laboratory equipment (glass flask, tubings) as previously described (chapter 4.2). Especially, since the observed AFG concentrations were much lower than the calculated concentrations, demonstrating a decrease of concentration. Whilst the concentration-time profiles were equivalent in their orientation, the here observed concentration-time profile was acceptable for the following investigations.

4.4.5.2 Retrodialysis of voriconazole and anidulafungin

Several pre-investigations of retrodialysis as calibration method for VOR were performed with the sIVMS (chapter 3.3). Here, it was confirmed that for VOR the behaviour of molecules was the same when comparing recovery and delivery (see chapter 3.3.1 and 3.3.2), which is a requirement for performing retrodialysis. Hence, it was possible to use retrodialysis as calibration method for µDialysis investigations in the dIVMS with VOR. During retrodialysis, VOR concentrations in perfusate were decreasing during 30 min (Tab. 3.4-5 and Fig. 3.4-14), but the concentration differences were only negligible and assumed to be based on bioanalytical variability. Resulting RR ranged from 94.6% to 95.7% for (i) and from 96.6% to 97.2% for (ii). The RR results were constant over time and comparable to results from sIVMS, demonstrating that retrodialysis is a suitable calibration technique for the dIVMS.

AFG concentrations in perfusate were more variable after 120 min compared to the start of retrodialysis (Tab. 3.4-7 and Fig. 3.4-17). A decrease of concentration in perfusate might be explained by adsorption of molecules to plastic surfaces [100]. The increase of AFG concentration could either be caused by instable PPB or by adsorbed AFG molecules on dIVMS material (glass flask, tubing), which were desorbing over time (see chapter 3.2.1). Values for RR were decreasing from the first and second sampling interval and were constant after the third interval of retrodialysis. The RR was derived from rD of AFG in µDialysate, hence, a decreasing RR would result from an increasing rD. Usually, an increasing rD would be based on an increase of diffusing molecules from perfusate to medium. Here, a plausible explanation was that the molecules did not diffuse into medium, but that molecules were adsorbing to catheter material. The decrease of RR during the first sampling interval could be interpreted as adsorption of AFG to surfaces and the constant RR during the second and third interval by the saturation of surfaces with AFG.

4.4.5.3 Comparison of observed and calculated medium concentrations of voriconazole and anidulafungin

Based on VOR concentrations in µDialysate and the respective RR per catheter, medium concentrations were calculated (Eq. 1-4) and compared to the observed VOR medium concentrations. The calculated VOR medium concentrations from µDialysate after the first sampling interval for (i) and (ii) were smaller compared to the observed medium concentration (Fig. 3.4-15). Hence, it can be concluded that a prolonged equilibration time after injection of the drug is needed to reach reasonable µDialysate concentrations after the first sampling interval of a minimum of 15 min.

Although calibration was performed with different VOR concentrations (both concentrations were 10-fold higher than the concentration in medium) for the two investigations, the RR from retrodialysis of both investigations was similar. Hence, both investigational settings resulted in medium concentrations, which were calculated from µDialysate, partly located within the variability ranges of the pump and the bioanalytical method around the observed medium concentrations. Calculated VOR concentrations not within the variability ranges could be explained by the higher variability of the handmade catheters used in µDialysate investiagtions [14]. It can be concluded that retrodialysis as calibration method for VOR was able to give a close approximation of “target site” concentrations in medium of the dIVMS.

Resulting AFG concentrations in medium calculated from µDialysate concentrations did not correspond to the concentrations observed in medium (Fig. 3.4-19). Regarding the medium concentrations calculated from the µDialysis data and RR, the resulting concentration-time profile

was not matching with the profile of observed concentrations in medium (Fig. 3.4-19). AFG concentrations were increasing for four hours after start of recovery investigation until a steady-state like profile was reached. After volume correction, the calculated concentrations from µDialysate data during the steady-state like profile (after 4 h) were matching the observed concentrations in medium (Fig. 3.4-20). The implementation of volume correction of RR with the recovered sample volume in µDialysate is not approved for the calculation of *in vivo* ISF concentrations. It might be concluded that due to the volume loss through the 100 kDa cut-off membrane, an increase in drug concentration in µDialysate takes place due to less volume in samples. During the investigations performed in the current thesis, no references about the inclusion of volume correction in calculations were found in literature. Longer investigation times would be required to make a statement about the orientation of the concentration-time profile of calculated medium concentrations (by µDialysate) during steady-state. Other calibration methods were not recommended for a better outcome of AFG µDialysis investigations. Due to the potential adsorption of AFG molecules on catheter material, also calibration techniques based on FR, concentrations or reference substances would not lead to unbiased values without sufficiently long equilibration times of at least 3-4 h.

5 Conclusion and perspectives

Until now, it was not possible to perform *in vitro* microdialysis investigations in a system enabling adjustable concentration-time profiles of the respective analyte. With the dynamic *in vitro* microdialysis system developed during the present thesis, a novel link between static *in vitro* investigations and *in vivo* clinical studies was established.

Prior to the start of *in vitro* microdialysis investigations, the bioanalytical HPLC quantification methods for anidulafungin and voriconazole were successfully developed, technically feasible for *in vitro* samples with minimal sample volumes of 20-40 µL. The lower limit of quantification for anidulafungin was 0.1 µg/mL and for voriconazole 0.15 µg/mL, i.e. low enough to quantify most of the *in vitro* microdialysis samples from the static and dynamic microdialysis investigations. However, mass spectrometers (e.g. LC-MS/MS) may be used to quantify even concentrations below LLOQ of anidulafungin and voriconazole in the nanogram range from *in vivo* studies.

Anidulafungin was first characterised with the static *in vitro* microdialysis system. These static investigations came to the conclusion that an *in vivo* microdialysis study would be only recommended with a sufficiently long equilibration time of at least 180 min (3 h) at steady state at the target site. Results from this project demonstrate that anidulafungin reversibly adsorbs on catheter material, which produces a bias on microdialysate concentrations and thus on the calculated drug concentrations at the target site. The use of additives in perfusate or pre-coating of catheters with caspofungin or anidulafungin was not able to prevent adsorption or reduce the equilibration time significantly. Hence, the basic experimental setup with a perfusate composition of Ringer's solution and human serum albumin was preferred for *in vitro* as well as *in vivo* microdialysis. With this analysis of anidulafungin in the static *in vitro* microdialysis system, a systematic investigation of different methods to handle adsorption of the analyte to the catheter material was performed. New knowledge was not only gained for anidulafungin, but also for other adsorbing compounds like other echinocandins (e.g. caspofungin). In the future, other types of catheter materials such as hard polyurethane, polyimide and fluorinated ethylene propylene should be investigated to prevent adsorption of sticky analytes, thus enhancing clinical application and ultimately allowing for more precise microdialysis results.

Furthermore, voriconazole was investigated with the static *in vitro* microdialysis system resulting in overall high and stable values for relative recovery. Since voriconazole had also a concentration-independent recovery additionally to the high and stable values for relative recovery, it was concluded that voriconazole could be used as a model drug for the following experiments with the dynamic microdialysis system. In the current work, relative recovery was not dependent on the two

investigated flow rates of 1 µL/min and 2 µL/min during investigations with the static system, which was not in accordance with previously established results of Simmel *et al.* [28]. Varying pore sizes in catheter membranes and the usage of catheters from aged, i.e. altered, material could explain the differing results. To rule out this bias, dynamic microdialysis investigations were performed with the same catheters as for static investigations. Further investigations with voriconazole in the static system are recommended with expired and new catheters to gain important knowledge about aging of catheter material and thus the usability of expired catheters *in vitro*.

The behaviour of analytes is most often different *in vitro* compared to *in vivo* studies. This is especially the case for microdialysis. *In vitro* microdialysis is an indispensable tool to characterise analytes prior to the clinical studies. Still, there is a clear distinction and *in vitro* results from static experiments may not be transferred to *in vivo*. Hence, the usage of a dynamic model for microdialysis, which mimics a concentration-time profile of the analyte, contributes to narrowing this distinction. The experimental dynamic model was developed and successfully validated to mimic concentration-time profiles under physiological conditions. A pilot investigation was conducted with the aim to describe the suitability of retrodialysis as calibration technique by comparing the observed with the by microdialysis determined medium concentration over time. Voriconazole and anidulafungin were individually investigated in the dynamic *in vitro* microdialysis system with changing medium concentrations over time and subsequent retrodialysis. Voriconazole concentrations in medium resulting from microdialysate samples were in good agreement with the actual concentrations in medium, whereas this was not the case for anidulafungin. While the observed anidulafungin concentrations in medium decreased, the (measured) microdialysis concentrations increased over time. The overall conclusion of the dynamic *in vitro* experiments with anidulafungin was, that *in vivo* concentration-time profiles of anidulafungin determined by microdialysis, would not display the true concentrations at the target site. This result is consistent with previous results from the static model, showing that microdialysis of anidulafungin is only possible under certain circumstances, including steady state conditions. In contrast, for voriconazole it was concluded that *in vitro* studies are useful to determine *in vivo* characteristics and settings. This was already shown in the literature, that *in vitro* feasibility studies of voriconazole [4] led to successful clinical studies [28,90].

Future research in the field of dynamic microdialysis should focus on the implementation of the dynamic system in *in vitro* routine investigations of analytes. To even better reflect physiological conditions within the dynamic system, tissue imitations like medium consisting of collagen, skin or fat should be explored. Additionally, a combination with the dynamic infectious disease model is conceivable, which is used to investigate *in vitro* the effect of changing drug concentrations on

bacteria over time [123,131]. The combination of the two experimental models would enable the simultaneous investigation of PK and PD of various drugs and pathogens (e.g. bacteria, fungi) with microdialysis. An interesting and important question to answer with such a combined model, is how the usage of high drug concentrations in perfusate during retrodialysis effects PD at target site.

This work clearly demonstrated that experimental *in vitro* models are of indispensable importance. Right now, we are not at the point where *in vitro* investigations may replace *in vivo* studies in general, but the gap between *in vitro* and *in vivo* is getting smaller due to sophisticated physiological experimental systems, their unremitting usage and further development.

6 Bibliography

[1] U. Ungerstedt, C. Pycock. Functional correlates of dopamine neurotransmission. Bull. Schweiz Akad Med Wiss., 30: 44–55 (1974).

[2] F. Simmel, J. Soukup, A. Zoerner, J. Radke, C. Kloft. Development and validation of an efficient HPLC method for quantification of voriconazole in plasma and microdialysate reflecting an important target site. Anal. Bioanal. Chem., 392: 479–88 (2008).

[3] European Medicines Agency. Guideline on bioanalytical method validation. (2012). http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf. (last access 06 August 2018).

[4] F. Simmel, C. Kloft. Microdialysis feasibility investigations with the non-hydrophilic antifungal voriconazole for potential applications in nonclinical and clinical settings. Int. J. Clin. Pharmacol. Ther., 48: 695–704 (2010).

[5] J. O'Neill. Tackling drug-resistant infections globally: final report and recommendations. (2016). https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf. (last access 06 August 2018).

[6] World Health Organization. Antimicrobial resistance: global report on surveillance. (2014). http://apps.who.int/iris/bitstream/handle/10665/112642/9789241564748_eng.pdf;jsessionid=825FFAD03DF1CDC787FF83C55B56ADE7?sequence=1. (last access 06 August 2018).

[7] E. Mylonakis, C.J. Clancy, L. Ostrosky-Zeichner, K.W. Garey, G.J. Alangaden, J.A. Vazquez, J.S. Groeger, M.A. Judson, Y.M. Vinagre, S.O. Heard, F.N. Zervou, I.M. Zacharioudakis, D.P. Kontoyiannis, P.G. Pappas. T2 magnetic resonance assay for the rapid diagnosis of candidemia in whole blood: A clinical trial. Clin. Infect. Dis., 60: 892–899 (2015).

[8] P. Munoz, M. Valerio, A. Vena, E. Bouza. Antifungal stewardship in daily practice and health economic implications. Mycoses, 58: 14–25 (2015).

[9] L. Jeu, F.J. Piacenti, A.G. Lyakhovetskiy, H.B. Fung. New Drugs Voriconazole. Clin. Ther., 25: 1321–1381 (2003).

[10] European Medicines Agency. Guideline on the use of pharmacokinetics and pharmacodynamics in the development of antibacterial medicinal products. (2016). http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC500210982.pdf. (last access 06 August 2018).

[11] T.J. Bazzett, J.B. Becker, R.L. Albin. A novel device for chronic intracranial drug delivery via microdialysis. J. Neurosci. Methods, 40: 1–8 (1991).

[12] M. Brunner, O. Langer. Microdialysis versus other techniques for the clinical assessment of in vivo tissue drug distribution. AAPS J., 8: E263-71 (2006).

[13] A. De La Peña, P. Liu, H. Derendorf. Microdialysis in peripheral tissues. Adv. Drug Deliv. Rev., 45: 189–216 (2000).

[14] N. Plock, C. Kloft. Microdialysis--theoretical background and recent implementation in applied life-sciences. Eur. J. Pharm. Sci., 25: 1–24 (2005).

[15] C. Joukhadar, M. Müller. Current applications in clinical pharmacokinetic studies and its potential role in the future. Clin. Pharmacokinet., 44: 895–913 (2005).

[16] Food and Drug Administration. Guidance for Industry. Developing Antimicrobial Drugs -

General Considerations for Clinical Trials. (1998).

[17] M Dialysis AB. Catalogue: Microdialysis for Clinical Use. (2017).

[18] CMA. Catalogue: Products for microdialysis research. (2017).

[19] E.C. de Lange, A.G. de Boer, D.D. Breimer. Methodological issues in microdialysis sampling for pharmacokinetic studies. Adv. Drug Deliv. Rev., 45: 125–48 (2000).

[20] I. Jacobson, M. Sandberg, A. Hamberger. Mass transfer in brain dialysis devices - a new method for the estimation of extracellular amino acids concentration. J. Neurosci. Methods, 15: 163–168 (1985).

[21] P. Lonnroth, P.A. Jansson, U. Smith. A microdialysis method allowing characterization of intercellular water space in humans. Am. J. Physiol., 253: E228-31 (1987).

[22] R.J. Olson, J.B. Justice. Quantitative Microdialysis Under Transient Conditions. Anal. Chem., 65: 1017–1022 (1993).

[23] L. Stahle, P. Arner, U. Ungerstedt. Drug Distribution Studies With Microdialysis. Life Sci., 49: 1853–1858 (1991).

[24] M. Brunner, C. Joukhadar, R. Schmid, B. Erovic, H.G. Eichler, M. Müller. Validation of urea as an endogenous reference compound for the in vivo calibration of microdialysis probes. Life Sci., 67: 977–984 (2000).

[25] O. Schwalbe, C. Buerger, N. Plock, C. Joukhadar, C. Kloft. Urea as an endogenous surrogate in human microdialysis to determine relative recovery of drugs: Analytics and applications. J. Pharm. Biomed. Anal., 41: 233–239 (2006).

[26] C.S. Chaurasia. In vivo microdialysis sampling: theory and applications. Biomed. Chromatogr., 13: 317–32 (1999).

[27] L. Stahle. Drug distribution studies with microdialysis: I. Tissue dependent difference in recovery between caffeine and theophylline. Life Sci., 49: 1835–1842 (1991).

[28] F. Simmel. Development, optimisation and application of in vitro and in vivo microdialysis methods contributing to characterise drug disposition processes. Martin-Luther-Universitaet Halle-Wittenberg. (2010).

[29] I. Minichmayer, C. Weiser. In vitro and in silico approaches to improve clinical drug monitoring and drug development. International Clinical Microdialysis Symposium, Berlin, Germany, 12 December 2014.

[30] C.S. Chaurasia. In vivo microdialysis sampling: Theory and applications. Biomed. Chromatogr., 13: 317–332 (1999).

[31] C.S. Chaurasia, M. Müller, E.D. Bashaw, E. Benfeldt, J. Bolinder, R. Bullock, P.M. Bungay, E.C.M. DeLange, H. Derendorf, W.F. Elmquist, M. Hammarlund-Udenaes, C. Joukhadar, D.L. Kellogg, C.E. Lunte, C.H. Nordstrom, H. Rollema, R.J. Sawchuk, B.W.Y. Cheung, V.P. Shah, L. Stahle, U. Ungerstedt, D.F. Welty, H. Yeo. AAPS-FDA workshop white paper: microdialysis principles, application and regulatory perspectives. Pharm. Res., 24: 1014–25 (2007).

[32] C. Carneheim, L. Ståhle. Microdialysis of Lipophilic Compounds: A Methodological Study. Pharmacol. Toxicol., 69: 378–380 (1991).

[33] A.N. Khramov, J.A. Stenken. Enhanced microdialysis recovery of some tricyclic antidepressants and structurally related drugs by cyclodextrin-mediated transport. Analyst, 124: 1027–1033 (1999).

[34] W.J. Loos, W.C. Zamboni, F.K. Engels, P. de Bruijn, M.H. Lam, R. de Wit, J. Verweij, E.A.C. Wiemer. Pitfalls of the application of microdialysis in clinical oncology: controversial findings with docetaxel. J. Pharm. Biomed. Anal., 45: 288–94 (2007).

[35] R. Nirogi, V. Kandikere, G. Bhyrapuneni, V. Benade, R. Saralaya, S. Irappanavar, N. Muddana, D.R. Ajjala. Approach to reduce the non-specific binding in microdialysis. J. Neurosci. Methods, 209: 379–87 (2012).

[36] F. Traunmüller, I. Steiner, M. Zeitlinger, C. Joukhadar. Development of a high-performance liquid chromatography method for the determination of caspofungin with amperometric detection and its application to in vitro microdialysis experiments. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., 843: 142–6 (2006).

[37] Y. Xu, L. Du, M.J. Rose, I. Fu, E.J. Woolf, D.G. Musson. Concerns in the development of an assay for determination of a highly conjugated adsorption-prone compound in human urine. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., 818: 241–8 (2005).

[38] P.E. Bratcher, A. Gaggar. Characterization and prevention of the adsorption of surfactant protein D to polypropylene. PLoS One, 8: e73467 (2013).

[39] M. Lindberger, T. Tomson, S. Lars. Microdialysis sampling of carbamazepine, phenytoin and phenobarbital in subcutaneous extracellular fluid and subdural cerebrospinal fluid in humans: an in vitro and in vivo study of adsorption to the sampling device. Pharmacol. Toxicol., 91: 158–65 (2002).

[40] G. Whitaker, C.E. Lunte. Investigation of microdialysis sampling calibration approaches for lipophilic analytes: doxorubicin. J. Pharm. Biomed. Anal., 53: 490–6 (2010).

[41] W.J. Loos, S.D. Baker, J. Verweij, J.G. Boonstra, A. Sparreboom. Clinical pharmacokinetics of unbound docetaxel: Role of polysorbate 80 and serum proteins. Clin. Pharmacol. Ther., 74: 364–371 (2003).

[42] V.J.A. Schuck, I. Rinas, H. Derendorf. In vitro microdialysis sampling of docetaxel. J. Pharm. Biomed. Anal., 36: 807–813 (2004).

[43] M. Schwartz, W. Kline, B. Matuszewski. Determination of a cyclic hexapeptide (L-743 872), a novel pneumocandin antifungal agent in human plasma and urine by high-performance liquid chromatography with fluorescence detection. Anal. Chim. Acta, 352: 299–307 (1997).

[44] R. Hajdu, R. Thompson, J.G. Sundelof, B.A. Pelak, F.A. Bouffard, J.F. Dropinski, H. Kropp. Preliminary animal pharmacokinetics of the parenteral antifungal agent MK-0991 (L-743,872). Antimicrob. Agents Chemother., 41: 2339–44 (1997).

[45] C. Kirbs, C. Kloft. In vitro microdialysis recovery and delivery investigation of cytokines as prerequisite for potential biomarker profiling. Eur. J. Pharm. Sci., 57: 48-59 (2014).

[46] W.J. Trickler, D.W. Miller. Use of Osmotic Agents in Microdialysis Studies to Improve the Recovery of Macromolecules. J. Pharm. Sci., 92: 8–13 (2003).

[47] G.F. Clough. Microdialysis of large molecules. AAPS J., 7: E686-92 (2005).

[48] D.W. Denning, M.J. Bromley. How to bolster the antifungal pipeline. Science, 347: 1414–1416 (2015).

[49] P.E. Verweij, M. Ananda-Rajah, D. Andes, M.C. Arendrup, R.J. Brüggemann, A. Chowdhary, O.A. Cornely, D.W. Denning, A.H. Groll, K. Izumikawa, B.J. Kullberg, K. Lagrou, J. Maertens, J.F. Meis, P. Newton, I. Page, S. Seyedmousavi, D.C. Sheppard, C. Viscoli, A. Warris, J.P. Donnelly. International expert opinion on the management of infection caused by azole-resistant

Aspergillus fumigatus. Drug Resist. Updat., 21–22: 30–40 (2015).

[50] G.D. Brown, D.W. Denning, N.A.R. Gow, S.M. Levitz, M.G. Netea, T.C. White. Hidden Killers: Human Fungal Infections. Sci. Transl. Med., 4: 1–9 (2012).

[51] R. Bellmann, P. Smuszkiewicz. Pharmacokinetics of antifungal drugs: practical implications for optimized treatment of patients. Infection, 45: 1–43 (2017).

[52] H.M. Lazarus, J.L. Blumer, S. Yanovich, H. Schlamm, A. Romero. Safety and pharmacokinetics of oral voriconazole in patients at risk of fungal infection: a dose escalation study. J. Clin. Pharmacol., 42: 395–402 (2002).

[53] D.W. Denning, P. Bowyer. Voriconazole resistance in Aspergillus fumigatus: Should we be concerned? Clin. Infect. Dis., 57: 521–523 (2013).

[54] S.J. Howard, M.C. Arendrup. Acquired antifungal drug resistance in *Aspergillus fumigatus:* epidemiology and detection. Med. Mycol., 49: S90–S95 (2011).

[55] C.R. Stensvold, L.N. Jorgensen, M.C. Arendrup. Azole-Resistant invasive aspergillosis: Relationship to agriculture. Curr. Fungal Infect. Rep., 6: 178–191 (2012).

[56] M.C. Arendrup. Update on antifungal resistance in children: epidemiology and recommendations. Pediatr. Infect. Dis. J., 32: 1–10 (2013).

[57] M.C. Arendrup, J.-L. Rodriguez-Tudela, C. Lass-Flörl, M. Cuenca-Estrella, J.P. Donnelly, W. Hope. EUCAST technical note on anidulafungin. Clin. Microbiol. Infect., 17: E18-20 (2011).

[58] D.M. Cappelletty, R. Jung. Anidulafungin and its role in candida infections. Infect. Drug Resist., 2: 51–60 (2009).

[59] N.P. Wiederhold, R.E. Lewis. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. Expert Opin. Investig. Drugs, 12: 1313–33 (2003).

[60] T. Alebic-Kolbah, M.S. Modesitt. Anidulafungin--challenges in development and validation of an LC-MS/MS bioanalytical method validated for regulated clinical studies. Anal. Bioanal. Chem., 404: 2043–55 (2012).

[61] G. Aguilar, J.R. Azanza, J.A. Carbonell, C. Ferrando, R. Badenes, M.A. Parra, B. Sadaba, D. Navarro, J. Puig, A. Miñana, C. Garcia-Marquez, G. Gencheva, A. Gutierrez, F.J. Marti, F.J. Belda. Anidulafungin dosing in critically ill patients with continuous venovenous haemodiafiltration. J. Antimicrob. Chemother., 69: 1620–3 (2014).

[62] S.L. Davis, J.A. Vazquez. Place in therapy review Anidulafungin : an evidence-based review of its use in invasive fungal infections. Core Evid., 2: 242–249 (2008).

[63] https://www.researchgate.net/ publication/8077247_Combination_Treatment_ of_Invasive_ Fungal_ Infections ?_ sg=J6GewzzL p Xk5eAlQYQ2 -rMpOTmg Wnsa8Xz W GR h9Gnp 1x7O2a LiA O5M 4vfemDmmWodbRYHLBq- Q. (last access 06 August 2018).

[64] D.W. Denning. New drug classes Echinocandin antifungal drugs. Lancet, 362: 1142–1151 (2003).

[65] C.A. Sutherland, D.P. Nicolau, J.L. Kuti. Development of an HPLC method for the determination of anidulafungin in human plasma and saline. J. Chromatogr. Sci., 49: 397–400 (2011).

[66] https://www.pharmazeutische-zeitung.de/index.php?id=4353. (last access 06 August 2018).

[67] Pfizer. Eraxis: Summary of product characteristics. (2013).

[68] Pfizer. Ecalta Gebrauchsinformation: Information für den Anwender. (2013).

[69] E.G. Muldoon, D.W. Denning. Editorial commentary: Prophylactic echinocandin: is there a subgroup of intensive care unit patients who benefit? Clin. Infect. Dis., 58: 1227–9 (2014).

[70] L.A. Decosterd, B. Rochat, B. Pesse, T. Mercier, F. Tissot, N. Widmer, J. Bille, T. Calandra, B. Zanolari, O. Marchetti. Multiplex ultra-performance liquid chromatography-tandem mass spectrometry method for simultaneous quantification in human plasma of fluconazole, itraconazole, hydroxyitraconazole, posaconazole, voriconazole, voriconazole-N-oxide, anidulafungin, and caspofungin. Antimicrob. Agents Chemother., 54: 5303–15 (2010).

[71] M.A. Ghannoum, M. D'Angelo. Anidulafungin: A Potent Antifungal that Targets Candida and Aspergillus. Infect. Dis. Clin. Pract., 13: 165–178 (2005).

[72] J.A. Vazquez. Anidulafungin: a novel echinocandin. Therapy, 3: 39–54 (2006).

[73] J.L. Crandon, M.A. Banevicius, A.F. Fang, P.H. Crownover, R.F. Knauft, J.S. Pope, J.H. Russomanno, E. Shore, D.P. Nicolau, J.L. Kuti. Bronchopulmonary disposition of intravenous voriconazole and anidulafungin given in combination to healthy adults. Antimicrob. Agents Chemother., 53: 5102–7 (2009).

[74] J.A. Dowell, M. Stogniew, D. Krause, T. Henkel, I.E. Weston. Assessment of the Safety and Pharmacokinetics of Anidulafungin When Administered With Cyclosporine. J. Clin. Pharmacol., 45: 227–233 (2005).

[75] J.A. Dowell, J. Schranz, A. Baruch, G. Foster. Safety and pharmacokinetics of coadministered voriconazole and anidulafungin. J. Clin. Pharmacol., 45: 1373–82 (2005).

[76] World Health Organization. WHO Model List of Essential Medicines. (2017). http://www.who.int/medicines/publications/essentialmedicines/20th_EML2017_FINAL_amendedAug2017.pdf?ua=1. (last access 06 August 2018).

[77] European Medicines Agency. EPAR summary for the public: Vfend. (2014). http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000387/WC500049751.pdf. (last access 06 August 2018).

[78] M. Mikulska, A. Novelli, F. Aversa, S. Cesaro, F.G. de Rosa, C. Girmenia, A. Micozzi, M. Sanguinetti, C. Viscoli. Voriconazole in clinical practice. J. Chemother., 24: 311–327 (2012).

[79] R.C. Rathbun, H.L. Hoffman. Review of the safety and efficacy of voriconazole. Expert Opin. Investig. Drugs, 11: 409–429 (2002).

[80] U. Theuretzbacher, F. Ihle, H. Derendorf. Pharmacokinetic/pharmacodynamic profile of voriconazole. Clin. Pharmacokinet., 45: 649–63 (2006).

[81] E.K. Manavathu, J.L. Cutright, P.H. Chandrasekar. Organism-dependent fungicidal activities of azoles. Antimicrob. Agents Chemother., 42: 3018–3021 (1998).

[82] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000387/WC500049756.pdf. (last access 06 August 2018).

[83] Pfizer. Vfend: Summary of product characteristics. (2017).

[84] M.A. Thiel, A.S. Zinkernagel, J. Burhenne, C. Kaufmann, W.E. Haefeli. Voriconazole concentration in human aqueous humor and plasma during topical or combined topical and systemic administration for fungal keratitis. Antimicrob. Agents Chemother., 51: 239–244 (2007).

[85] S.C. Heng, G.I. Snell, B. Levvey, D. Keating, G.P. Westall, T.J. Williams, H. Whitford, R.L. Nation,

M.A. Slavin, O. Morrissey, D.C.M. Kong. Relationship between trough plasma and epithelial lining fluid concentrations of voriconazole in lung transplant recipients. Antimicrob. Agents Chemother., 57: 4581–4583 (2013).

[86] European Medicines Agency. EPAR Scientific Discussion: Vfend. (2005). http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000387/WC500049753.pdf. (last access 06 August 2018).

[87] P.I. Siafaka, N.Ü. Okur, M. Mone, S. Giannakopoulou, S. Er, E. Pavlidou, E. Karavas, D.N. Bikiaris. Two different approaches for oral administration of voriconazole loaded formulations: Electrospun fibers versus β-cyclodextrin complexes. Int. J. Mol. Sci., 17: 1–15 (2016).

[88] I. Gueta, R. Loebstein, N. Markovits, Y. Kamari, H. Halkin, G. Livni, H. Yarden-Bilavsky. Voriconazole-induced QT prolongation among hemato-oncologic patients: clinical characteristics and risk factors. Eur. J. Clin. Pharmacol., 73: 1181–1185 (2017).

[89] M.J. Dolton, J.E. Ray, S.C.A. Chen, K. Ng, L.G. Pont, A.J. McLachlan. Multicenter study of voriconazole pharmacokinetics and therapeutic drug monitoring. Antimicrob. Agents Chemother., 56: 4793–4799 (2012).

[90] C. Kirbs. Development and application of in vitro and in vivo microdialysis methods contributing to biomarker profiling and characterisation of drug distribution processes. Freie Universitaet Berlin. (2015).

[91] S. Brunauer, H. Emmett, E. Teller. Adsorption of Gases in Multimolecular Layers. J. Am. Chem. Soc., 60: 309–319 (1938).

[92] E. Mutschler, G. Geisslinger, H.K. Kroemer, P. Ruth, M. Schäfer-Korting (eds.). Mutschler Arzneimittelwirkungen: Lehrbuch der Pharmakologie und Toxikologie. Wissenschaftliche Verlagsgesellschaft, Stuttgart, 9th ed. (2008).

[93] A. Field, J. Miles, Z. Field (eds.). Discovering statistics using R. SAGE Publications Ltd., London, 3th ed. (2014).

[94] F. Farowski, O.A. Cornely, J.J. Vehreschild, P. Hartmann, T. Bauer, A. Steinbach, M.J.G.T. Rüping, C. Müller. Quantitation of azoles and echinocandins in compartments of peripheral blood by liquid chromatography-tandem mass spectrometry. Antimicrob. Agents Chemother., 54: 1815–9 (2010).

[95] J. Martens-Lobenhoffer, V. Rupprecht, S.M. Bode-Böger. Determination of micafungin and anidulafungin in human plasma: UV- or mass spectrometric quantification? J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., 879: 2051–6 (2011).

[96] S.C. Heng, R.L. Nation, B. Levvey, G.I. Snell, M.A. Slavin, D.C.M. Kong. Quantification of voriconazole in human bronchoalveolar lavage fluid using high-performance liquid chromatography with fluorescence detection. J. Chromatogr. B Anal. Technol. Biomed. Life Sci., 913–914: 171–175 (2013).

[97] B. V Araujo, C.F. Silva, S.E. Haas, T. Dalla Costa. Microdialysis as a tool to determine free kidney levels of voriconazole in rodents: a model to study the technique feasibility for a moderately lipophilic drug. J. Pharm. Biomed. Anal., 47: 876–81 (2008).

[98] http://www.internetchemie.info/chemie-lexikon/daten/w/wasser-dampfdruck.php (last access 06 August 2018).

[99] Carl Roth GmbH + Co. KG. Sicherheitsdatenblatt: Acetonitril gemäß Verordnung (EG) Nr. 1907/2006 (REACH), geändert mit 2015/830/EU. (2016).

https://www.carlroth.com/downloads/sdb/de/A/SDB_AE00_DE_DE.pdf. (last access 06 August 2018).

[100] P. Kolbinger, M. Gruber, G. Roth, B.M. Graf, K.-P. Ittner. Filter Adsorption of Anidulafungin to a Polysulfone-Based Hemofilter During CVVHD In Vitro. Artif. Organs, 42: 200–207 (2017).

[101] C. Marchiori. Development of HPLC-UV methods for the determination of anidulafungin or caspofungin in plasma of ICU patients. UNIVERSITA' DEGLI STUDI DI PADOVA. (2013).

[102] Carl Roth GmbH + Co. KG. Sicherheitsdatenblatt: Methanol gemäß Verordnung (EG) Nr. 1907/2006 (REACH), geändert mit 2015/830/EU. (2015). https://www.carlroth.com/downloads/sdb/de/T/SDB_T193_CH_DE.pdf. (last access 06 August 2018).

[103] European Medicines Agency. EPAR Scientific discussion: Ecalta. (2007). http:// www.ema. europa.eu/ docs/en_GB/ document_ library /EPAR _-_Scientific_Discussion /human/ 000788/ WC500020676. pdf. (last access 06 August 2018).

[104] European Medicines Agency. Specifications and Control Tests on The Finished Product. : 83–94 (1992). http:// www.ema.europa.eu /docs/en_GB/document_library /Scientific_ guideline/ 2009/09/ WC500003368.pdf. (last access 06 August 2018).

[105] Merck. Cancidas: Summary of product characteristics. (2000). http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000379/WC500021033.pdf. (last access 06 August 2018).

[106] A. Pascual, C. Csajka, T. Buclin, S. Bolay, J. Bille, T. Calandra, O. Marchetti. Challenging recommended oral and intravenous voriconazole doses for improved efficacy and safety: Population pharmacokinetics-based analysis of adult patients with invasive fungal infections. Clin. Infect. Dis., 55: 381–390 (2012).

[107] Sigmar-Aldrich Co. Product Information: Human Serum Albumin. (2012).

[108] J.P. Galea, P.J. Tyrrell, H.P. Patel, A. Vail, A.T. King, S.J. Hopkins. Pitfalls in microdialysis methodology: an in vitro analysis of temperature, pressure and catheter use. Physiol. Meas., 35: N21-8 (2014).

[109] J. Chu, K. Hjort, A. Larsson, A.P. Dahlin. Impact of static pressure on transmembrane fluid exchange in high molecular weight cut off microdialysis. Biomed. Microdevices, 16: 301–10 (2014).

[110] X. Ao, X. Wang, M.R. Lennartz, D.J. Loegering, J.A. Stenken. Multiplexed cytokine detection in microliter microdialysis samples obtained from activated cultured macrophages. J. Pharm. Biomed. Anal., 40: 915–21 (2006).

[111] X. Ao, T.J. Sellati, J.A. Stenken. Enhanced microdialysis relative recovery of inflammatory cytokines using antibody-coated microspheres analyzed by flow cytometry. Anal. Chem., 76: 3777–84 (2004).

[112] X. Ao, J.A. Stenken. Microdialysis sampling of cytokines. Methods, 38: 331–41 (2006).

[113] A.J. Rosenbloom, D.M. Sipe, V.W. Weedn. Microdialysis of proteins: performance of the CMA/20 probe. J. Neurosci. Methods, 148: 147–53 (2005).

[114] M. May, S. Batkai, A. a Zoerner, D. Tsikas, J. Jordan, S. Engeli. Enhanced human tissue microdialysis using hydroxypropyl-ß-cyclodextrin as molecular carrier. PLoS One, 8: e60628 (2013).

[115] H. Rosdahl, U. Ungerstedt, J. Henriksson. Microdialysis in human skeletal muscle and adipose

tissue at low flow rates is possible if dextran-70 is added to prevent loss of perfusion fluid. Acta Physiol. Scand., 159: 261–2 (1997).

[116] D. Burau, P. Simon, L. Ehmann, D. Petroff, H. Wrigge, C. Kloft. Is relative recovery influenced by drug combination? 8th International Symposium on Microdialysis, Uppsala, Sweden, 25-27 May 2016. https:// reg.akademikonferens.se /app/Data/ProjectImages /6879/microdialysis 2016-abstracts.pdf, 42, (2016) P0222 (2015).

[117] C. Weiser, S.G. Wicha, M. Zeitlinger, C. Kloft. Microdialysis of highly adsorbing drugs: Evaluation of a probe coating approach exemplified by anidulafungin 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, Netherlands, 09-12 April 2016: EV0652 (2016).

[118] C. Weiser, M. Zeitlinger, C. Kloft. In vitro microdialysis of anidulafungin as prerequisite for an in vivo microdialysis study 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Copenhagen, Denmark, 25–28 April 2015: P0222 (2015).

[119] D. Kauzor, S. Schröpf, C. Kloft. In vitro characterisation of vancomycin as prerequisite for a microdialysis study in neonates 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Barcelona, Spain, 10–13 May 2014. Abstract book, 232 (2014).

[120] E. Lowdin, I. Odenholt, S. Bengtsson, O. Cars. Pharmacodynamic effects of sub-MICs of benzylpenicillin against Streptococcus pyogenes in a newly developed in vitro kinetic model. Antimicrob. Agents Chemother., 40: 2478–2482 (1996).

[121] J. Michael. Pharmacodynamic in vitro studies contributing to the rational use of linezolid in infections by vancomycin resistent Enterococcus faecium. Martin-Luther-Universitaet Halle-Wittenberg. (2011).

[122] K. Bartels, E. Goebgen, C. Kloft. Dynamic in vitro infection model enables mimicking of in vivo levofloxacin PK profiles showing eradication of E. coli infection. Annual Meeting of the Deutsche Pharmazeutische Gesellschaft (DPhG), Düsseldorf, Germany, 23-25 September 2015. www.dphg.de/fileadmin/downloads/DPhG2015_ConferenceBook_final.pdf, 184, (2015).

[123] I.K. Bartels. Validierung des dynamischen In-vitro -Infektionsmodells zur Bestimmung des pharmakodynamischen Effekts von Levofloxacin auf Escherichia coli. Freie Universitaet Berlin. (2015).

[124] W. Chen, H. Xie, F. Liang, D. Meng, J. Rui, X. Yin, T. Zhang, X. Xiao, S. Cai, X. Liu, Y. Li. Population Pharmacokinetics in China: The Dynamics of Intravenous Voriconazole in Critically Ill Patients with Pulmonary Disease. Biol. Pharm. Bull., 38: 996–1004 (2015).

[125] P. Liu, D.R. Mould. Population pharmacokinetic analysis of voriconazole and anidulafungin in adult patients with invasive aspergillosis. Antimicrob. Agents Chemother., 58: 4718–4726 (2014).

[126] M. Neely, T. Rushing, A. Kovacs, R. Jelliffe, J. Hoffman. Voriconazole Pharmacokinetics and Pharmacodynamics in Children. Clin Infect Dis., 27: 320–331 (2015).

[127] K. Han, B. Capitano, R. Bies, B.A. Potoski, S. Husain, S. Gilbert, D.L. Paterson, K. McCurry, R. Venkataramanan. Bioavailability and population pharmacokinetics of voriconazole in lung transplant recipients. Antimicrob. Agents Chemother., 54: 4424–4431 (2010).

[128] J.A. Dowell, W. Knebel, T. Ludden, M. Stogniew, D. Krause, T. Henkel. Population pharmacokinetic analysis of anidulafungin, an echinocandin antifungal. J. Clin. Pharmacol., 44: 590–8 (2004).

[129] A.H. Groll, D. Mickiene, R. Petraitiene, V. Petraitis, C.A. Lyman, J.S. Bacher, S.C. Piscitelli, T.J. Walsh. Pharmacokinetic and pharmacodynamic modeling of anidulafungin (LY303366): Reappraisal of its efficacy in neutropenic animal models of opportunistic mycoses using optimal plasma sampling. Antimicrob. Agents Chemother., 45: 2845–2855 (2001).

[130] Pfizer. Vfend: Summary of product characteristics. (2008).

[131] J. Seeger. Vergleich des pharmakodynamischen Effekts von Levofloxacin 750 mg qd und 500 mg bid auf resistente Escherichia coli im dynamischen In-vitro-Infektionsmodell. Freie Universitaet Berlin. (2017).

7 Publications

Abstracts

Oral presentations

I.K. Minichmayr, C. Weiser.
In vitro and *in silico* approaches to improve clinical drug monitoring and drug development.
Clinical Microdialysis Symposium Berlin-Buch, Berlin, Germany, 12 December 2014.

Poster presentations

C. Weiser, D. Kauzor; H. Brosig, F. Kees, M. Zeitlinger, C. Kloft.
HPLC method development and validation for the determination of anidulafungin in microdialysis samples of healthy volunteers and intensive care patients.
Annual Meeting of the Deutsche Pharmazeutische Gesellschaft (DPhG), Frankfurt, Germany, 24-26 September 2014. Abstractbook, 148-149, (2014).

C. Weiser, M. Zeitlinger, C. Kloft.
In vitro microdialysis of anidulafungin as prerequisite for an *in vivo* microdialysis study.
25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Copenhagen, Denmark, 25-28 April 2015.
www.escmid.org/escmid_library/online_lecture_library/material/?mid=29161, (2015).

C. Weiser, M. Zeitlinger, C. Kloft.
A new approach for *in vitro* microdialysis of anidulafungin.
Annual Meeting of the Deutsche Pharmazeutische Gesellschaft (DPhG), Düsseldorf, Germany, 23-25 September 2015.
www.dphg.de/fileadmin/downloads/DPhG2015_ConferenceBook_final.pdf, 141, (2015).

C. Weiser, S.G. Wicha, M. Zeitlinger, C. Kloft.
Microdialysis of highly adsorbing drugs: Evaluation of a probe coating approach exemplified by anidulafungin.
26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, INetherlands, 09-12 April 2016.
https://www.escmid.org/escmid_publications/escmid_elibrary/material/?mid=34684, (2016).

C. Weiser, T. Fuhrmann-Selter; C. Kloft.
Development of a dynamic *in vitro* microdialysis system.
8th International Symposium on Microdialysis, Uppsala, Sweden, 25-27 May 2016.
https://reg.akademikonferens.se/app/Data/ProjectImages/6879/microdialysis2016-abstracts.pdf, 41, (2016).

C. Weiser, M. Zeitlinger, C. Kloft.
Does pre-coating of probes enable *in vitro* and *in vivo* microdialysis investigation of anidulafungin?
Annual Meeting of the Deutsche Pharmazeutische Gesellschaft (DPhG), Munich, Germany, 04-07 October 2016.
www.dphg.de/fileadmin/downloads/DPhG2016_ConferenceBook_final.pdf, 120, (2016).

Further publications

C. Weiser, J. Kuß, C. Kloft.
Das leistet die Klinische Pharmazie. Umfrage zur Forschung in Klinischer Pharmazie von 2009 bis 2013.
Dtsch. Apoth. Ztg. 157: 1582-1585 (2017).

C. Weiser, J. Kuß, C. Kloft.
Wie viel Forschung leistet die Klinische Pharmazie.
Pharm. Ztg. 162: 2330-2334 (2017).

8 Appendix

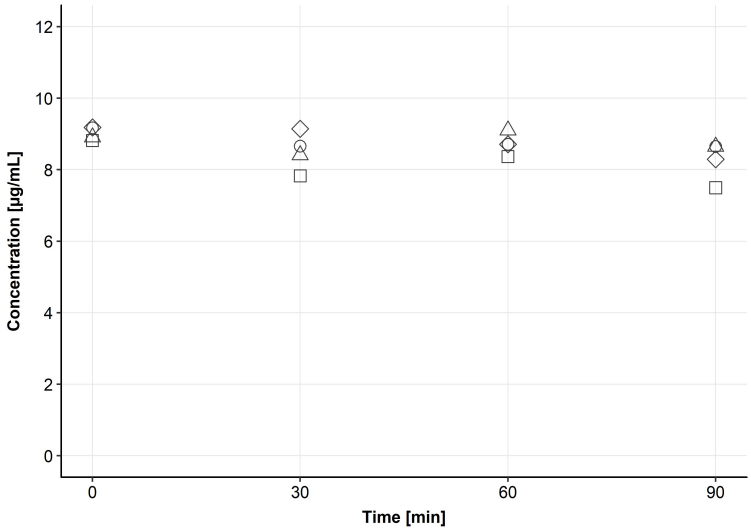

Fig 8-1: Anidulafungin concentrations in medium of CMA63® I (squares) and II (circles), and CMA71® I (triangles) and II (diamonds) catheters during 90 min investigated time.

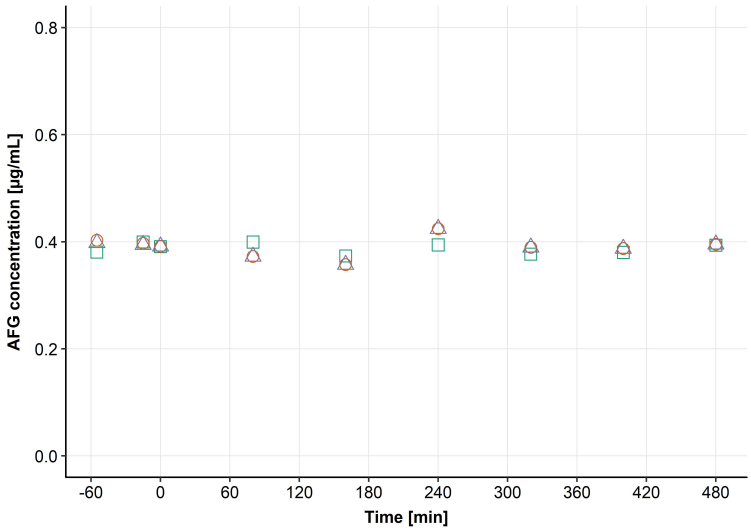

Fig 8-2: Anidulafungin (AFG) concentration in perfusate (n=9 per catheter) of recovery investigation from 1 µg/mL AFG medium and pre-coating with AFG during the investigated time: catheter 1 (blue circles), catheter 2 (green triangles) and catheter 3 (black triangles), taken in 80 min intervals.

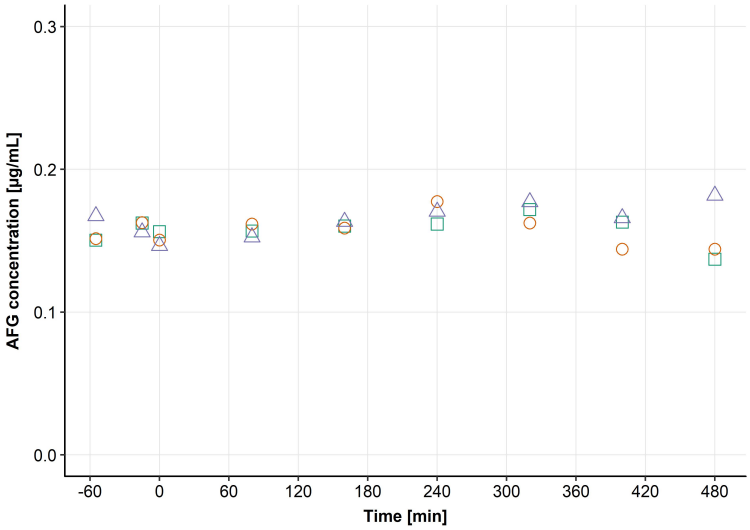

Fig 8-3: Anidulafungin (AFG) concentration in perfusate (from microsyringe) of recovery investigation from 8 µg/mL AFG medium and pre-coating with AFG during the recovery investigation of 480 min (8 h). Samples (n=9 per catheter) from perfusate of catheter 1 (green squares), catheter 2 (orange circles) and catheter 3 (purple triangles) were taken in 80 min intervals.

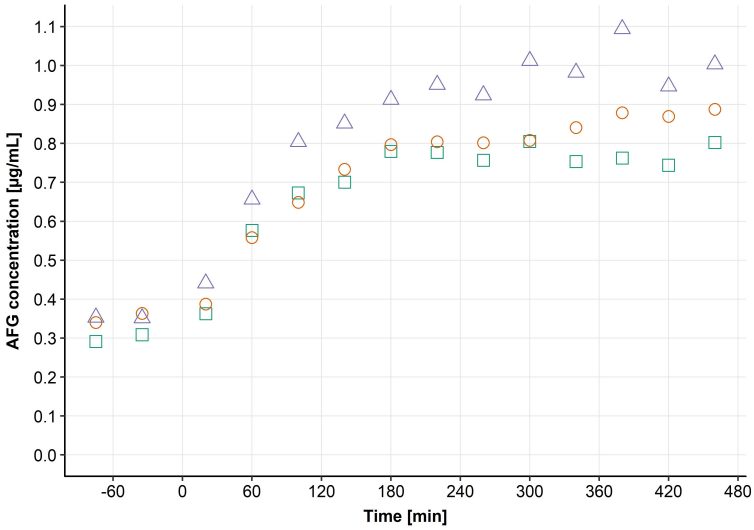

Fig 8-4: Observed Anidulafungin (AFG) concentration in microdialysate (n=14 per catheter) during pre-coating and the investigation with 1 µg/mL AFG in medium during the investigated time. Samples from catheter 1 (green squares), catheter 2 (orange circles) and catheter 3 (violet triangles) were taken in 40 min intervals.

Appendix

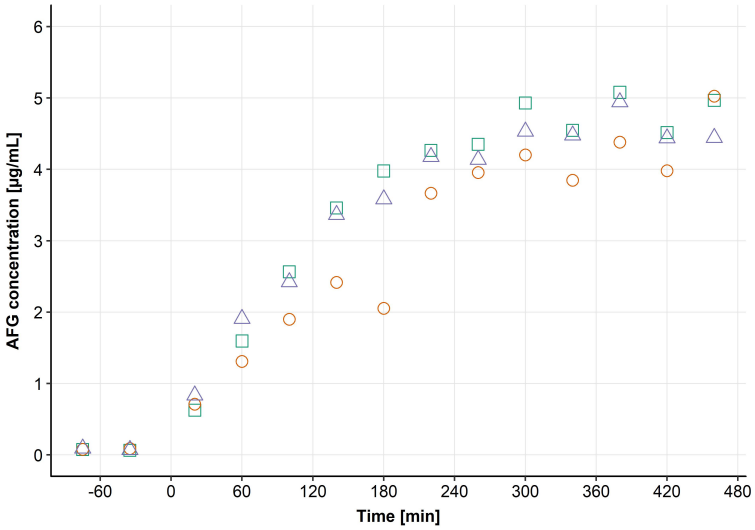

Fig 8-5: Observed Anidulafungin (AFG) concentration in µDialysate samples during pre-coating and the recovery investigation with 8 µg/mL AFG in medium for 480 min (8 h). Samples from catheter 1 (green squares), catheter 2 (orange circles) and catheter 3 (violet triangles) were taken in 40 min intervals.

Tab 8-1: Mean relative recovery (RR) of catheter 1, 2 and 3, standard deviation (SD) and coefficient of variation (CV) for microdialysis recovery investigations of different voriconazole concentrations (C_{VOR}: 0.5, 1.5, 3 and 4 µg/mL) and flow rates (FR: 1, 2 µL/min) (n=4 per catheter).

C_{VOR} [µg/mL]	FR [µL/min]	Mean RR, %	SD [%]	CV, %
0.5	**1**	85.9	6.48	7.54
0.5	**2**	89.3	3.43	3.84
1.5	**1**	87.3	3.46	3.96
1.5	**2**	87.5	3.84	4.39
3	**1**	86.2	3.47	4.03
3	**2**	85.3	4.34	5.09
4	**1**	88.9	2.44	2.75
4	**2**	85.9	2.82	3.28

Tab 8-2: Mean relative delivery (rD) of catheter 1, 2 and 3, standard deviation (SD) and coefficient of variation (CV) for microdialysis delivery investigations of different voriconazole concentrations (C_{VOR}: 0.5, 1.5, 3 and 4 µg/mL) and flow rates (FR: 1, 2 µL/min) (n=3-4 per catheter).

C_{VOR} [µg/mL]	FR [µL/min]	Mean rD, %	SD [%]	CV, %
0.5	**1**	77.9	2.56	3.28
0.5	**2**	78.0	2.87	3.68
1.5	**1**	91.2	1.30	1.42
1.5	**2**	90.4	2.69	2.97
3	**1**	96.1	1.51	1.57
3	**2**	95.2	1.02	1.08
4	**1**	94.2	7.04	7.48
4	**2**	95.5	1.60	1.67

Appendix

Tab 8-3: Individual mean relative delivery (rD) and corresponding coefficient of variation (CV) per catheter during retrodialysis of voriconazole (VOR) for perfusate (20 or 200 µg/mL) and medium (VOR-free, 1 or 10 µg/mL).

Catheter	C_{VOR} in perfusate	C_{VOR} in medium	Mean rD, % (n=3)	CV, % (n=3)
1	20	1	93.0	0.139
2	20	1	93.3	0.211
3	20	1	92.7	0.497
1	20	10	54.0	4.13
2	20	10	55.5	0.947
3	20	10	54.8	2.85
1	200	1	97.2	0.555
2	200	1	97.7	0.325
3	200	1	94.2	0.704
1	200	10	93.7	0.0972
2	200	10	92.7	0.750
3	200	10	91.2	0.978
1	20	-	95.0	0.535
2	20	-	97.1	0.247
3	20	-	94.0	0.615
1	200	-	95.8	0.895
2	200	-	97.4	0.239
3	200	-	95.0	0.389

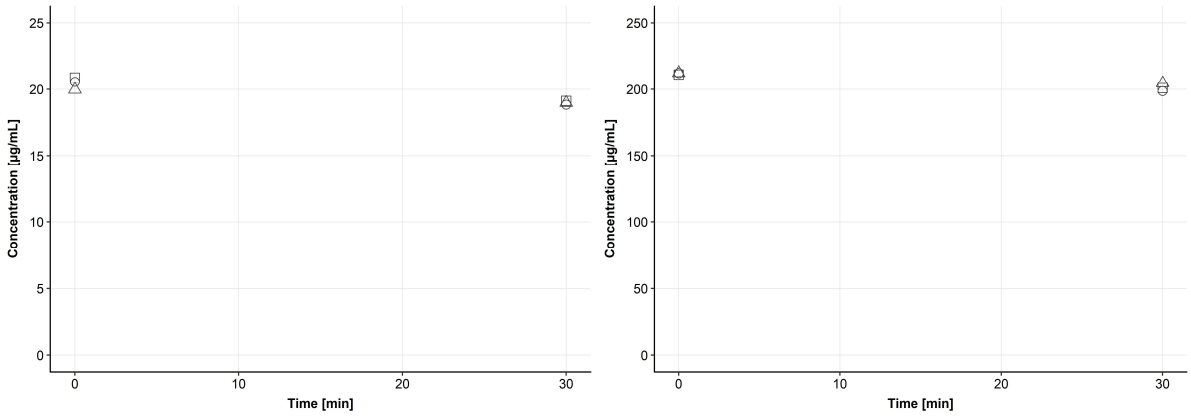

Fig 8-6: Voriconazole (VOR) concentration in perfusate during retrodialysis of microdialysis investigation in the dynamic *in vitro* microdialysis system with 20 µg/mL VOR (left (i)) and 200 µg/mL VOR (right (ii)) in perfusate. Catheter 1: squares; catheter 2: circles; catheter 3: triangles.

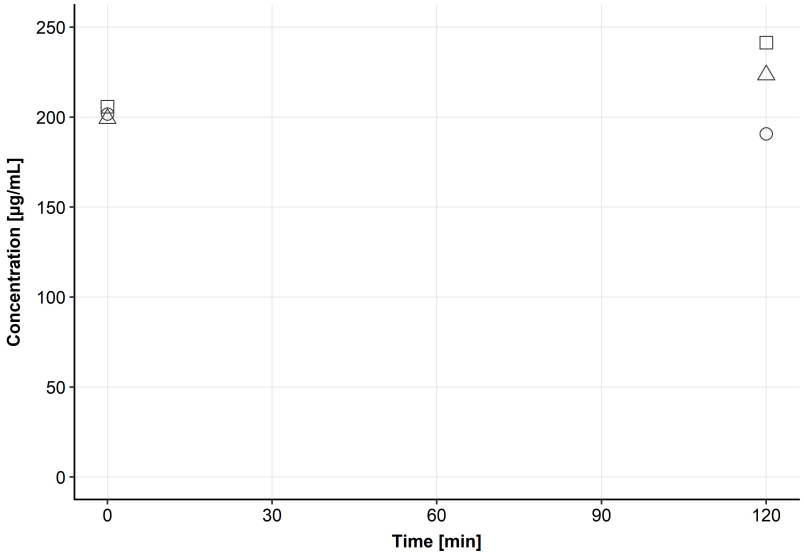

Fig 8-7: Anidulafungin (AFG) concentration in perfusate during retrodialysis from 0 to 120 min of microdialysis investigation in the dynamic *in vitro* microdialysis system with 200 µg/mL AFG in perfusate. Catheter 1: squares; catheter 2: circles; catheter 3: triangles.

Appendix

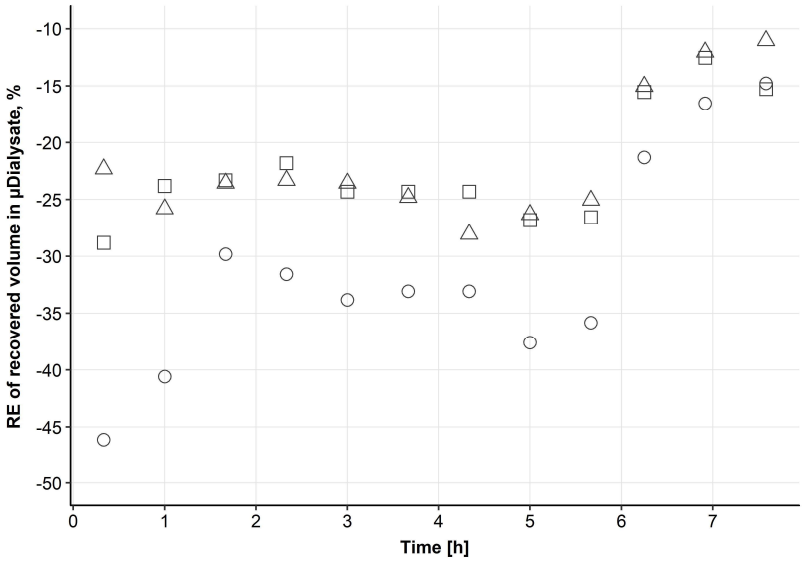

Fig 8-8: Accuracy (RE, %) of recovered to nominal volume in microdialysate samples of anidulafungin during recovery and retrodialysis investigation in the dIVMS. Catheter 1 (squares); catheter 2 (circles), catheter 3 (triangles).

9 Danksagung

Fünf Jahre ist es her, dass ich zum ersten Mal die Kelchstraße 31 von Innen gesehen habe. Seit dieser Zeit ist einiges passiert und hier möchte ich nun die Gelegenheit nutzen um den vielen Menschen Danke zu sagen, die mir in dieser Zeit begegnet sind.

Als allererstes gilt mein Dank natürlich meiner Doktormutter, **Frau Prof. Charlotte Kloft**. Ich danke Ihnen, dass Sie mich in Ihrem Arbeitskreis aufgenommen haben und für die Möglichkeit meine spannenden Projekte zu bearbeiten. Vor allem für die Freiheiten, die Sie mir gegeben haben. Nichts war von vorne herein festgesetzt, über alles konnte man disskutieren und sich am Ende auf die beste Lösung einigen. Auch ist die menschliche Seite nicht zu kurz gekommen und Sie hatten immer ein offenes Ohr für die schönen und auch weniger schönen Momente des Lebens. Danke für die vielen schönen Stunden.

Danke an meinen Kooperationspartner **Prof. Markus Zeitlinger**, ohne den es das Projekt „Anidulafungin“ nie gegeben hätte. Danke nicht nur für das herausfordernde Projekt, sondern auch für die, grade in der ersten Zeit der Doktorarbeit, aufbauenden Projekt-Telefonate und natürlich die einmalige Möglichkeit das AKH Wien zu besuchen!

Ein großer Dank gebührt Herrn **Prof. Kees** aus Regensburg, der mir gerade am Anfang der Doktorarbeit mit so unendlich viel Wissen zur Bioanalytik zur Seite stand. Da wurden so viele Emails geschrieben, Anrufe getätigt und ich durfte auf einen tollen Regensburg Besuch gehen, der bis obenhin voll war mit meinem Lieblingsthema Anidulafungin und wie man dieses am besten quantifiziert.

Bedanken möchte ich mich auch bei der lieben **Zoe**! Sie hat mich so lieb in Wien aufgenommen und hat dazu beigetragen, dass die Reise zu einem vollen Vergnügen wurde (unvergessen unser Ausflug zu Mary Poppins!) und ich natürlich mit ihr eine Leidensgenossin gefunden habe, da wir teilweise gleichzeitig mit Anidulafungin arbeiten durften.

Ohne meine Kollegen, die definitiv mehr als „nur“ Kollegen waren und sind, hätte rein gar nichts funktioniert! Ihr seid die Stütze für die Momente, in denen gar nichts mehr gegangen ist und mit denen ich auch während der eher grauen Momente aus vollem Herzen Lachen konnte. Danke, dass ihr mich regelmäßig im Mikrodialyse-Labor frequentiert habt und diesen zum „News und Gossip“ Ort gemacht habt: **Iris, Andrea, Johanna M., Johanna S., Eva, Daniela, Sebastian W., Sebastian F., Janin K., Martin K., Francis, Lisa, Ana-Marija, Franziska, Lena, Viki, Luisa, Niklas W., Niklas H., Saskia und Jane** ☺

Danksagung

Danke den ***In-vitro*-Freaks Eva, Johanna S.** und dem **Franck** für ihre Freakigkeit, die emotionalen Sofort-Hilfen im Labor und auch nach Feierabend!

Danke **Eva** für all die Stunden Reden und Zuhören über die Gott und die Welt. Vor allem für die Ratschläge und das gemeinsame austüfteln von Projekten und der Zukunft im Allgemeinen.

Danke **Johanna M.** für alle Gespräche, die Power-Point-Notfall-Sessions und all die schönen Stunden, die wir in Berlin, Frankfurt, Steinfurth und Göteborg verbracht haben! Und denk immer dran „Sie hatte nur noch Schuhe an!“

Danke an **Daniela**, die von mir besonders stark im ersten Jahr der Doktorarbeit frequentiert wurde und ich alle 15 min vor der Tür stand!! Danke für die guten Gespräche und die Geduld, die du mit mir hattest!

Danke **Iris** für deine Ausdauer mir immer Dinge bis ins letzte Detail erklären zu wollen, auch wenn ich schon längst mit dem Thema abgeschlossen habe, und vor allem Danke für die tolle Zeit beim Mikrodialyse Symposium in Berlin. Bestes Vortragsteam ever!!

Danke an die Girls von unten, **Andrea** und **Lena**, die mit mir IMMER Gossip und einen guten Schnack geteilt haben und soooo viel Zeit in meine R-Plots investiert haben wie kein anderer. Wahnsinn!!!

Danke **Johanna S.**, dass du mich mit den „Ladies“ bekannt gemacht hast und immer ein offenes Ohr sowie deine eigene Meinung hast! Sehr inspirierend!!

Ein gaaaanz großer Dank geht an meine Reviewer! Allen voran **Franziskarrrrr**, die mir wirklich sooooo viele Tips zu meiner englischen Ausdrucksweise gegeben hat, unglaublich… Die anderen fleißigen Bienchen, die meine Arbeit gelesen und kommentiert haben und ich ihnen sehr dafür danke waren **Johanna S., Claudia, Eva, Lisa, Daniela und Iris**.

Einen großen Dank möchte ich hier an **Claudia** richten, sie war nicht nur die Erste in der Kelchstraße, unter deren Fuchtel ich schaffen durfte, sie hat mich auch noch nach Dortelweil vermittelt, wo ich jetzt in ihrer ehemaligen Abteilung schaffen darf! Von dir hab ich den Großteil von dem, was ich über die Mikrodialyse weiß, gelernt. Danke dir!

Danke an die Menschen in der Kelchstraße, ohne die oft gar nichts mehr gehen würde:

Danke liebe **Tania** für ALLES. Stundenlange Probennahme um unsere Pumpenschläuche zu validieren: kein Problem! Zum wirklich 10. Mal den einen Anidulafungin-HPLC-Lauf pipettieren, weil es einfach nicht funktionieren will: kein Problem! Während ich noch im Urlaub verweile, mal eben den Prototyp des dynamischen Modells zusammenbasteln: wirklich überhaupt kein Probelm! Danke für deinen unbremsbaren Einsatz und deinen Tatendrang, der mich so oft mitgerissen hat. Danke auch an **Herrn**

Siebenbrodt für die vielen Gespräche, die gemeinsame Zeit im Praktikum und dass sie mich nach den Parties in der Kelchstraße immer mitgenommen haben und ich nicht Bahn fahren musste ☺ Danke an **Herrn Herrmann** für die tollen Reiseberichte, die Lust auf Urlaub gemacht haben und natürlich danke für die 1000 Abbildungen der Mikrodialyse, die Sie nicht nur am PC sondern auch fotographisch festgehalten haben!! Auch ein herzliches Danke an **Bea** und **Frau Böhnstedt**, die immer ein offenes Ohr für die großen und kleinen Dinge im Leben hatten und Mut zugesprochen haben, wenn es nötig war!

Danke an die vielen Wahlpflichtstudierenden, die sich mit dem dynamischen Mikrodialyse System (meine Backerbsen: **Michelle** und **Nicole**) sowie mit FoKP (**Janina, David, Johanna, Meike** und **Sophia**) auseinandergesetzt haben.

Danke an die Berliner Crew für all die Ablenkungen nach Feierabend und den Wochenenden: **Iris, Steffi, Marco und Marlene**! Wenn mal wirklich Stress war, habt ihr mich das in Null-Komma-Nix vergessen lassen!

Danke an meine liebsten Apothekerinnen Damen, **Boppöse** und **Frau Kolz**, dass ihr mich seit den ersten Monaten in Berlin und darüber hinaus begleitet habt, ihr mir immer beisteht und mich so gut ablenken könnte, dass ich nach 20 Sekunden schon alles um mich herum vergessen habe. Danke!

Danke meiner lieben Freundin **Nathalie**, die mit mir schon fürs Mathe-Abi gepaukt hat und daher wusste, dass ich während der Doktorarbeitszeit einen schlauen Ratgeber mit dem Titel „Write your dissertation in 15 min a day“ brauche (Ja, er war meine Bibel).

Danke möchte ich meiner Familie sagen, **Mama, Papa** und meiner **Siss Mel**. Ihr ertragt mich immer so wie ich bin, weist mich in meine Schranken und gebt mir meinen Raum, wenn es gefährlich wird ☺ Danke für alles, was ihr mir ermöglicht habt.

Danke an meine beste Freundin, mein **Stäbchen**, dass du immer riechst, wenn was nicht stimmt, meinen Geistes- und Gemütszustand immer auf dem Schirm hast („oh, oh! Hat jemand ein Snickers???“), mich wahrscheinlich schon von Kopf bis Fuß analysiert hast (ohne, dass ich was davon gemerkt habe) und dafür, dass ich dir jeden Mist, der grade in meinem Hirn vorgeht erzählen kann, was oft zu tiefgründigen mehrstündigen Telefonaten führt.

Danke an **Fabian**! Du warst noch vor der Kelchstraße da und bist es auch jetzt noch (Wow!). Dafür danke ich dir. Alle meine Launen hast du zuhause aufgefangen und hast dafür gesorgt, dass ich immer was zu essen bekomme. Danke, dass du immer darauf achtest, dass ich auch ja genug „schönes“ mache und auch sonst einfach immer da bist…