Innovative Approaches to Cardiovascular Drug Trials and Pharmacotherapy in Children

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I. Erklärung zur Dissertation

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf den Sergej Ramusovic

II. Danksagungen

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III. Zusammenfassung

Ziel der vorliegenden Arbeit ist es, durch mathematische Modellbildung und Simulation (M&S), eine hochspezifische Bestimmungsmethode für Arzneistoffe, sowie adaptierte Studiendesigns, Lösungsansätze für die Probleme bei Studien in der pädiatrischen Kardiologie zu bieten.

Im ersten Abschnitt der Arbeit wurde mittels Physiologie-basierter M&S eine neue Simulationsplattform konzipiert, welche die Effekte von Alsikiren, Losartan und Enalapril auf das Renin-Angiotensin-Aldosteron-System (RAAS) bezüglich der Plasmaspielgel von Angiotensin I und II und darüber hinaus die Pharmakokinetik (PK) von Enalapril vorhersagt. Außerdem ermöglichen die identifizierten Parameter eine Skalierung für Kinder, welche *Bridging* Studien ermöglicht. Durch diese kann das Problem kleiner Patientenzahlen in pädiatrischen Studien gelöst werden. *Bridging* Studien können Sicherheits- und Wirksamkeitsdaten von Erwachsenen auf Kinder übertragen und somit Daten für eine pädiatrische Arzneimittelzulassung generieren. Um dies zu ermöglichen, wurde außerdem eine HPLC tandem-MS Bestimmungsmethode für Enalapril entwickelt, welche aufgrund des geringen Probevolumens von nur 100 µL auch für die jüngsten Patienten geeignet ist. Ihre hohe Selektivität stellt sicher, dass die Arzneistoffbestimmung durch mögliche Komedikation nicht negativ beeinflusst wird.

Im zweiten Abschnitt wird eine Observationsstudie vorgestellt, in der spezifische und klinisch relevante Endpunkte für pädiatrische Studien untersucht wurden. Außerdem wurden bisher unbekannte Aspekte der Entwicklungspharmakologie von Amiodaron mittels M&S beleuchtet. Die engmaschige Überwachung von Herzfrequenz und Blutdruck während der intravenösen (IV) Amiodarongabe ermöglichte es, die klinische Hämodynamik dieser unter lebensbedrohlichen Herzrhythmusstörungen leidenden Kinder zu beschreiben – ein Ansatz, der für zukünftige klinische Studien in diesem Bereich unabdingbar ist. Mittels moderner M&S Verfahren konnte abschließend gezeigt werden, wie die Arzneistoffverteilung im Körper von Kindern das PK Verhalten von Amiodaron während der IV Therapie insbesondere bei den jüngsten Patienten dominiert.

IV. Summary

This thesis aims to propose novel ways to cope with challenges regarding trial design and data-analysis in the field of pediatric cardiology. It focusses on pediatric patients with cardiovascular disease by harnessing innovative modeling and simulation (M&S) tools, highly specific and sensitive drug analysis, and trial designs tailored for pediatric needs.

In the first part, a novel renin angiotensin aldosterone system (RAAS) platform was constructed by physiologically based (PB) modeling that predicts angiotensin I and II profiles in response to administration of aliskiren, losartan and enalapril as well as the pharmacokinetics (PK) of enalapril. The model identified parameters ready for scaling to children. This enables bridging studies that aim to overcome the obstacle of small patient numbers in pediatric trials. The bridging approach can link safety and efficacy data from children and adults to ultimately be submitted for regulatory drug approval. To achieve meaningful PK data even in the youngest patients, a HPLC tandem-MS assay for enalapril utilizing low sample volumes of only 100 µL was also developed. Its high selectivity ensured that no concomitant medication distorted the detection of the chemical entity.

The second part proposes an observational trial to gain specific and clinically relevant endpoints in children and to fill in important gaps of knowledge concerning the developmental pharmacology of amiodarone by advanced M&S techniques. Continuous monitoring and recording of blood pressure and heart rate during intravenous (IV) amiodarone administration allowed assessment of their changes by defining the hemodynamic behavior of pediatric patients with life-threatening tachycardia during IV therapy. Such approaches are mandatory for future pediatric trial designs. With advanced M&S, novel insights into the effect of ageing on the developmental pharmacology of amiodarone were additionally gained. It was possible to demonstrate how distribution dominates the drug's PK during the intravenous treatment period especially in the youngest patients.

V. Table of contents

VI. Figures

VII. Tables

VIII. Abbreviations

ACE	angiotensin converting enzyme
ACEI	angiotensin converting enzyme inhibitor
AHA	American Heart Association
ALT	alanine transaminase
ANOVA	analysis of variance
APE	absolute percentage error
AST	aspartate transaminase
AT1	angiotensin II receptor subtype 1
AUC	area under the curve
AUEC	area under the effect curve
AVSD	atrioventricular septal defect
BMI	body mass index
CDC	Centers for Disease Control and Prevention
CE	collision energy
CEP	cell entrance potential
CI	confidence interval
C_{max}	maximum concentration
C_{ss}	steady-state concentration
CXP	cell exit potential
CYP	cytochrome P450
d-TGA	d-transposition of the great arteries
DEA	desethylamiodarone
DCM	dilated cardiomyopathy
DP	declustering potential
DRI	direct renin inhibitor
ECG	electrocardiography
EFPIA	European Federation of Pharmaceutical Industries and Associations
ELISA	enzyme linked immunosorbent assay
EMA	European Medicines Agency
EP	entrance potential
ESI	electrospray ionization
FAT	focal atrial tachycardia
FDA	Food and Drug Administration
FP	focusing potential
γ-GT	gamma-glutamyl transaminase
geoSD	geometrical standard deviation
GET	gastric emptying time
HLHS	hypoplastic left heart syndrome
HOCM	hypertrophic cardiomyopathy
HPLC	high performance liquid chromatography
$IC50$	half maximal inhibitory concentration

ICRP	International Commission on Radiological Protection
IP	intestinal permeability
IS	internal standard
ITT	intestinal transit time
IV	intravenous
JET	junctional ectopic tachycardia
LLOQ	lower limit of quantification
logP	logarithmic partition coefficient
LSD	least significant difference
M&S	modeling and simulation
ME	matrix effect
MedAPE	median absolute percentage error
MedPE	median percentage error
MI	myocardial infarction
MRM	multiple reactions monitoring
MS	mass spectrometry
NHANES	National Health and Nutrition Examination Survey
PAH	pulmonary arterial hypertension
PBPK	physiologically based pharmacokinetic
PD	pharmacodynamic
PDE5	phosphodiesterase type 5
PE	process efficiency
PIP	pediatric investigation plan
PJRT	permanent junctional reciprocating tachycardia
PK	pharmacokinetic
PRA	plasma renin activity
PUMA	paediatric use marketing authorization
QC	quality control
QTc	heart rate corrected QT-interval
RAAS	renin angiotensin aldosterone system
RIA	radioimmunoassay
RSD	relative standard deviation
SD	standard deviation
SPE	solid phase extraction
SVT	supraventricular tachycardia
t_{max}	time of maximum concentration
TLC	thin layer chromatography
TOF	tetralogy of Fallot
ULOQ	upper limit of quantification
USP	United States Pharmacopeia
V_{max}	maximum reaction velocity
VSD	ventricular septal defect
VT	ventricular tachycardia
WPW	Wolf Parkinson White Syndrome

1. Introduction

1.1. General introduction

1.1.1. Cardiovascular drugs for children

Congenital heart defects are the most common congenital anomalies involving a single organ among children today (Schwedler, 2011). Obesity, which has been linked to cardiovascular diseases like hypertension in adults, is also increasing in incidence among children and contributes to the burden of cardiovascular disease in this age group (Sorof, 2002; Sorof, 2004). Consequently, there is a considerable therapeutic need to treat these conditions in the pediatric population. However, treatment is impeded by the marked lack of medications specifically licensed for this age group. Studies show that up to 90% of drug utilization is off-label or unlicensed for neonates (Choonara, 2004) and that rates of off-label use of medications in older children are also equally high (Lass, 2011; Lindkvist, 2011; Olsson, 2011; Kimland, 2012; Palčevski, 2012; Ribeiro, 2012). Rates of off-label prescribing, especially of medications used to treat cardiovascular diseases, have been documented to be high not only in hospitals but also in office-based settings (Radley, 2006; Hsien, 2008). This situation has been perceived as being dangerous for the highly vulnerable pediatric population. With safe and effective pediatric doses often being virtually unknown, children may be subjected to ineffectively low or potentially high drug exposures. Rates of adverse events have been shown to be high with off-label medication use and constitute an additional danger (Cuzzolin, 2006; Phan, 2010; Aagaard, 2011). It must also be noted that splitting tablets designed for adults may adversely affect dose conformity (Breitkreutz, 2007) and may destroy functional coatings designed for optimizing palatability, which is essential for pediatric drug adherence (Cram, 2009). A marked lack of drug formulations specifically tailored for pediatric use and the widespread off-label use of oral drug formulations originally designed for adults together result in suboptimal treatment of cardiovascular conditions among children and adolescents.

In 2007, the European Medicines Agency (EMA) implemented several mechanisms to increase the use of licensed drugs among children. These

mechanisms made it obligatory to formulate a pediatric investigation plan (PIP) to investigate and introduce drugs to treat diseases that could potentially occur among children. For drugs whose patents were yet to expire, a patent prolongation could now be obtained by conducting research on drug formulations or treatment indications for use in pediatric populations. However, patent protection has expired for many drugs in the realm of cardiovascular diseases. In such instances, the Pediatric-use Marketing Authorization (PUMA) provides an incentive to conduct pediatric research by extending patent protection of newly generated data on pediatric formulations and treatment indications of otherwise generic drugs (European Parliament and the Council, 2006). The first PUMA application was approved in 2011 for buccal midazolam (EFPIA, 2012), but the paucity of further approvals of PUMA applications demonstrates the low investment in this specific marketing authorization instrument. These facts collectively underline the urgent need for further academic research in the field of pediatric cardiovascular pharmacotherapy.

1.1.2.Clinical trials with children

Clinical studies in children impose a unique challenge (Abdel-Rahman, 2007). The small number of patients with cardiac conditions hampers trial recruitments. In addition, for the many drugs used off-label, practical experiences of clinicians have contributed to strong beliefs regarding drug effectiveness that are not substantiated by data obtained from clinical trials. This accumulated knowledge presents ethical challenges to the design of pediatric trials since clinicians may hesitate to use placebos given that their use could deny patients potentially effective treatment (Saul, 2005; Li, 2011). One other aspect of the challenges facing clinical studies in children is the lack of knowledge regarding the complex relationship between dose, exposure and effects. A large-scale trial on the use of carvedilol in pediatric chronic heart failure exemplifies this dilemma. Although carvedilol is routinely used in adult chronic heart failure (Hunt, 2009), its effectiveness in children has not been demonstrated. Therefore, Shaddy et al. conducted a placebo-controlled study of 161 children and adolescents to evaluate the effects of 2 doses of carvedilol on symptomatic systemic ventricular

systolic dysfunction (Shaddy, 2007). No significant differences between the treatment and the placebo groups could be demonstrated in this trial and the question of whether an age-appropriate dosing regimen could have yielded effective treatment remained unanswered. The authors speculated that this may have been because the study was underpowered to detect any differences. However, studies of carvedilol pharmacokinetics in children suggest that the trial doses chosen were probably too low to reach the drug exposure attained by dosing the drug based on the licensed adult regimen (Läer, 2002; Jadhav, 2010). Carvedilol is not currently indicated for pediatric chronic heart failure. Recent studies further exemplify challenges in conducting pediatric cardiology trials. A trial on the effects of enalapril in infants with single-ventricle heart physiology failed to demonstrate improvements in somatic growth, ventricular function, or heart failure severity (Hsu, 2010). Dosing in this study was also based on experience and the probability of the doses chosen being too low cannot be ruled out. Another trial aimed at investigating the effect of enalapril on mitral regurgitation, which is the most common indication for reoperation in children following repair of an atrioventricular septal defect, had to be prematurely cancelled during enrollment due to poor recruitment associated with a lack of clinical equipoise on the part of the recruiting clinicians (Li, 2011).

The challenges discussed above prompted the choice of pediatric cardiovascular conditions as the focus of this thesis. The objective was to evaluate innovative approaches to pediatric trial design that could address and overcome the problems discussed above. Enalapril and amiodarone were chosen as the drugs to be studied since they are widely used in the treatment of pediatric cardiovascular diseases. Their importance is evident from their inclusion in the EMA list for off-label drugs that warrant further research (EMA, 2012).

Enalapril is considered as a first line treatment for chronic heart failure in children by the European Medicines Agency Expert Group Meeting on Paediatric Heart Failure (EMA, 2010a). Its importance is further evident from the fact that it is among the 10 most commonly administered extemporaneous oral

preparations used in European hospitals (EMA, 2010b). The initial motivation for the research reported in this thesis was provided by the observation that the currently available pediatric PK data for enalapril was based on an outdated radioimmunoassay (RIA) whose specificity was likely to be adversely affected by cross reactions. This lack of specificity could lead to the overestimation of enalapril concentrations, call into question the data derived from plasma concentrations based on this assay, and even lead to incorrect drug dosing. From a broader perspective, there is currently no model available to effectively study the physiological effects of enalapril on the renin-angiotensin-aldosterone system (RAAS) and to quantitatively describe the effects of different drugs on the biomarkers of the RAAS. Development of such a model would permit the identification of the key parameters essential for scaling down the adult model for use in children and adolescents, thereby providing the first scientific basis for an understanding of the effects of enalapril on the pediatric RAAS.

Amiodarone is specifically recommended in the "Pediatric Advanced Life Support" guidelines of the American Heart Association (AHA) for use in children (AHA, 2005). However, clinical data as well as data on its developmental PK are lacking. The drug is not labeled for pediatric use but is nevertheless widely utilized in children and adolescents. It was therefore necessary to utilize approaches that could deal with situations wherein large randomized trials to derive clinical data would not be feasible. These approaches should also permit the generation of developmental PK data on amiodarone, which is complicated by its long half-life and pronounced lipophilicity (Latini, 1984; Freedman, 1991).

Discussed below are the specific motivations for the subparts of the research reported in this thesis:

- A physiologically based RAAS model
- Determination of enalapril and enalaprilat concentrations in human serum samples of small volume by high pressure liquid chromatography (HPLC) tandem-mass spectrometry (MS) for use in paediatric trials
- Observational study of intravenous (IV) amiodarone in children

1.2. Motivations for the subparts of the thesis

1.2.1. A physiologically based RAAS model

The renin-angiotensin-aldosterone system (RAAS) plays a central role in the homeostasis of blood pressure and is therefore a paramount target for the pharmacological treatment of cardiovascular diseases such as congestive heart failure and hypertension (Cagnoni, 2010 ;McMurray, 2011). Drugs targeting the various components of this system include the angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor subtype 1 (AT1) inhibitors and oral renin inhibitors, among others.

Modeling and simulation techniques are becoming increasingly utilized in cardiovascular research (Formaggia, 2009; Kimko, 2011; Grillo, 2012). Model development requires the incorporation of already existing data on parameters ranging from steady-state concentrations to enzyme kinetic constants. These data are derived from studies carried out in distinctly different experimental systems including in vitro, in vivo, and in silico systems. Until now, however, only a few RAAS models have been developed. Takahashi et al. developed a qualitative model to study the effect of ACE expression on blood pressure in mice (Takahashi, 2003). Hong et al. developed a compartmental, semi-mechanistic model describing the effect of aliskiren on RAAS biomarkers (Hong, 2008). Using a physiologically based modeling approach, Guillaud et al. constructed a model that described different aspects of the RAAS (Guillaud, 2010). However, the model could not be applied to predict drug effects since only one dose of the renin inhibitor aliskiren was used to evaluate the performance of the model. There was thus a significant need for developing a physiologically based model for the integration of PK data from different drugs administered in multiple clinically relevant doses. In addition, the pediatric scaling parameters for which ontogeny data had to be generated remained largely unknown.

1.2.2.A HPLC tandem-MS method for determination of enalapril and enalaprilat concentrations in pediatric trials

Enalapril (*N*-[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl-L-proline) is the prodrug for enalaprilat, an active metabolite acting as an ACE inhibitor (Vlasses, 1985). After oral absorption with a bioavailability of 59-73%, the drug is incompletely converted by hepatic esterases to enalaprilat (figure 1), and the plasma concentrations of enalapril and enalaprilat peak at one and four hours (Najib, 2003), respectively. Enalaprilat undergoes biphasic elimination with nearly 100% renal excretion (Vlasses, 1985). The elimination process is characterized by a rapid decline followed by a prolonged phase at concentrations below 8 ng/mL, which is due likely to the tight binding of the drug with ACE (Vlasses, 1985). Enalaprilat produces its biological action by decreasing the amount of angiotensin II which is generated from angiotensin I by the ACE (Atlas, 2007). Therefore, its main effect lies in lowering the blood pressure by lowering the peripheral vascular resistance (Atlas, 2007). However, changes in the renal blood flow and additional pleiotropic actions have also been postulated to contribute to its efficacy (Vlasses, 1985).

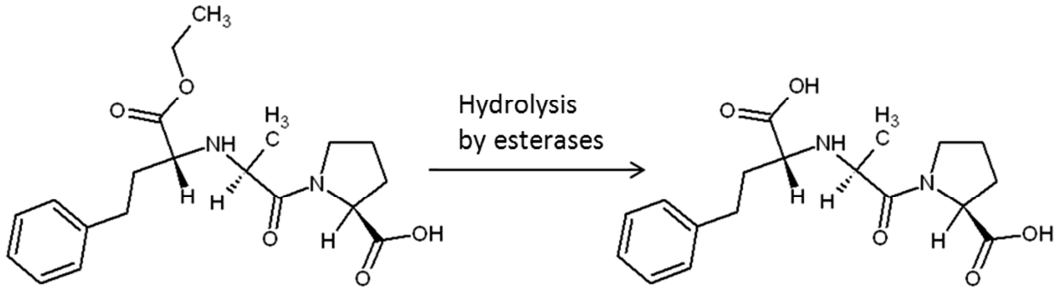

Figure 1. Hydrolysis of enalapril to the active metabolite enalaprilat primarily by hepatic esterases.

Although level A evidence supports the licensure of enalapril for the treatment of hypertension and chronic heart failure in adults (Rosendorff, 2007; Hunt, 2009), evidence supporting its use in pediatrics is sparse. Until now, only three trials have investigated the pediatric PK of enalapril and enalaprilat (Lloyd, 1989; Nakamura, 1994; Wells, 2001), quantifying the serum concentrations with a RIA (Hichens, 1981). However, as mentioned earlier, the RIA is likely to overestimate

enalapril and enalaprilat concentrations (figure 2), possibly due to cross reactions resulting from the low specificity of the antibody used. As a consequence, the antibody recognizes not only the drug but also other structures with similar 3-dimensional conformations, and the assay “sees” more analyte than is actually present in the sample – an effect well recognized for substances like aldosterone and estradiol among others (Santen, 2007; Hinchliffe, 2013; Hariharan, 1991; Strathmann, 2011). This casts doubt on the PK parameters derived in children using the RIA, especially with regard to data on the hydrolysis of enalapril to enalaprilat. Additionally, the RIA involves the time-consuming preparation of antibodies and the use of radioactive agents, making it cumbersome and potentially unsafe for routine application.

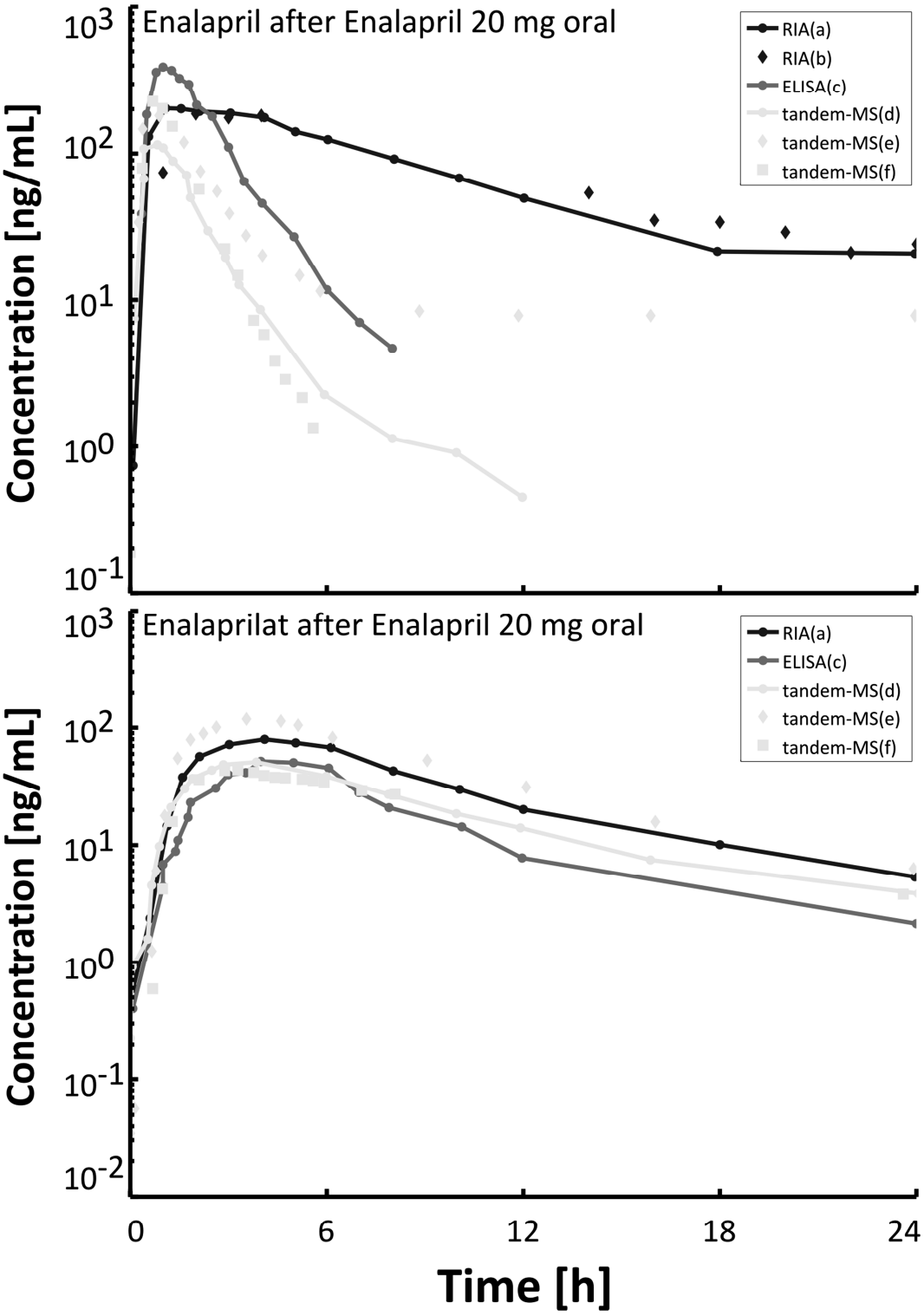

Figure 2. Comparison of different methods for determination of enalapril and enalaprilat concentrations, (a)= Ribeiro, 1996, (b)= Juillerat, 1990, (c)= Matalka, 2002, (d)= Najib, 2003, (e)= Ghosh, 2011, (f)= Ramusovic, 2012b. Despite the administration of equal enalapril doses (20 mg) and comparable test persons (age-matched healthy young male volunteers), pronounced differences especially in the elimination of enalapril can be observed. The RIA (black diamonds and solid black line with dots) seems to overestimate enalapril concentrations compared to ELISA and HPLC tandem-MS assays.

Multiple newer technologies have emerged for the determination of enalapril and enalaprilat concentrations in biological samples. These include enzyme kinetic assays (Thongnopnua, 2005) and gas chromatography tandem-MS analysis after derivatization (Shioya, 1992), but the HPLC tandem-MS appears to be the most suitable assay given its superior specificity–which is critical in patients receiving polypharmacy–and the fact that it constitutes a readily available platform. A MEDLINE search yields 7 publications on enalapril and enalaprilat determination using the HPLC tandem-MS assay (Lee, 2003; Najib, 2003; Gu, 2004; Lima, 2009; Lu, 2009; Gonzalez, 2010; Ghosh, 2011). The sample volumes used in these reports ranged between 200 and 500 µL. However, blood sample volumes available for pediatric studies are limited, especially in neonates, and the ability to conduct assays with reduced blood sample volumes is a critical prerequisite for modern pediatric trial designs, which incorporate extensive measurements of pharmacodynamic humoral biomarkers (Abdel-Rahman, 2007; Day, 2009). However, an assay that comprehensively addresses these needs for pediatric research is yet to be developed.

1.2.3.Observational study of intravenous amiodarone in children

Amiodarone ((2-butyl-1-benzofuran-3-yl)-[4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl]methanone) is an antiarrhythmic drug with unique pharmacodynamic and pharmacokinetic properties. Although it is generally categorized as a potassium channel blocker belonging to the Vaughan-Williams class III (Singla, 2012), a range of other pharmacodynamic effects are also associated with it. Amiodarone has beta receptor blocking properties and is also endowed with the ability to block calcium channels (Kodama, 1999). In addition, its interactions with thyroid hormone receptors are the subject of discussion (Kodama, 1999). The oral bioavailability of the drug is variable (22-86%) (Latini, 1984). Its pharmacokinetics reflects its pronounced lipophilicity. After administration, the drug exhibits a rapid and pronounced distribution into peripheral tissues (Latini, 1984), followed by a slow redistribution from these peripheral sites into the blood. This accounts for its long half-life of up to 77 days (Freedman, 1991). Amiodarone is oxidatively metabolized by the cytochromes, of

which cytochrome P450 3A4 (CYP 3A4) has the strongest contribution (figure 3) (Ohyama, 2000). Its most relevant metabolite is the pharmacologically active desethylamiodarone (DEA) (Nattel, 1986). In adults, amiodarone is licensed for the treatment of symptomatic tachycardias such as atrioventricular junctional tachycardia, supraventricular tachycardia, and atrial fibrillation (Ratiopharm GmBH, 2011). Since the drug exhibits potentially severe thyroid, pulmonary, and nerve toxicities, it is only indicated if other therapeutic alternatives cannot be tolerated by patients or were not sufficiently effective (Ratiopharm GmBH, 2011). Amiodarone is not licensed for pediatric applications in the US or Europe; however, its use is recommended in the "Advanced Life Support" Guidelines of the American Heart Association (AHA, 2005).

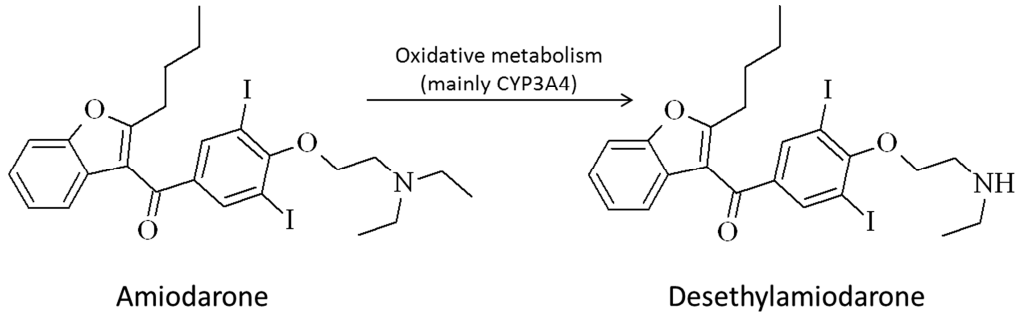

Figure 3. Amiodarone is metabolized to the active metabolite desethylamiodarone through oxidative metabolism, mainly by CYP3A4.

Although amiodarone is regarded as an effective treatment for severe pediatric arrhythmia, uncertainties persist about how safe it is for use in clinical practice (Saul, 2010; EMA (Paediatric Committee), 2012). Fears that bolus applications of amiodarone could cause hypotension, bradycardia, or hepatotoxicity, or result in excessive serum drug concentrations have prevented paediatricians from using optimal dosing regimens for this medication (eg, 5 mg/kg bolus combined with a maintenance infusion of 10 mg/kg/day), even though these regimens have yielded the highest success rates (82–94%) in multiple retrospective studies (Figa, 1994; Soult 1995; Perry, 1996; Burri, 2003; Laird, 2003). Chang et al. achieved success rates of only 34% when using lower bolus and maintenance doses of amiodarone (2.5 mg/kg and ~7 mg/kg/day), but reported that the lack

of a trained electrophysiologist may have dissuaded them from using optimal doses (Chang, 2010).

Some data is available on the efficacy, safety, and pharmacokinetics of intravenous amiodarone in paediatric intensive care patients who were treated with a loading dose of 5 mg/kg followed by a maintenance infusion of 10 mg/kg/day and achieved high rates of arrhythmia termination (Figa, 1994; Soult, 1995; Perry 1996; Burri 2003; Laird 2003). A prospective, randomized, double-blinded trial that evaluated loading and maintenance dose combinations of 1 mg/kg and 2 mg/kg/day, 5 mg/kg and 5 mg/kg/day, and 10 mg/kg and 10 mg/kg/day, respectively, achieved a lower maximum success rate of 80% at the end of the study (Saul, 2005). However, the study design did not accurately reflect clinical practice with respect to drug administration because the doses were applied as multiple boluses instead of being infused continuously, which could affect the serum concentrations of amiodarone. In addition, since IV amiodarone was administered in the intensive care setting, its half-life (6.9¬11.4 days) could only be determined during the trial in 3 of the 29 children in whom serum concentrations were evaluated (Saul, 2005). Further, PK observations only demonstrated a general trend towards decreasing amiodarone serum concentrations with increasing age. Moreover, comparisons with adult drug exposures are distinctly lacking. This has highlighted the need to increase confidence in the use of IV amiodarone in routine clinical practice and enhance knowledge of the developmental PK of amiodarone.

2. Aim of the thesis

The overall aim of the thesis was to develop and evaluate methods and assays for the optimal planning and conduct of pediatric clinical trials.

The first subproject (the RAAS model) was intended to demonstrate how physiologically based modeling could help with development of a modular systems biology platform that incorporated mechanistic insights and could have practical applications in pediatric clinical trials. Using this modeling approach, a comprehensive description of the RAAS and its modulation by diverse pharmacological interventions such as ACE- and renin inhibition or AT1 blockade may be achieved. Furthermore, the intent was to validate the RAAS model through rigorous evaluation of its performance at multiple levels of simulation, including the simulated administration of clinically reasonable doses of ACE and renin inhibitors as well as AT1 inhibitors. For pediatric applications, it was essential that the model incorporate interfaces for the integration of a physiologically based PK model and for mathematical fitting of pediatric data if such models were not readily available. Such a model would provide a solid foundation for the future development of physiologically based RAAS models for use in paediatric populations by facilitating the identification of relevant parameters that could be scaled down appropriately for use in pediatric clinical trials.

The second subproject consisted of the development and validation of a specific and rapid HPLC tandem-MS assay for the determination of enalapril and enalaprilat concentrations in low-volume clinical samples characteristically available for use in pediatric trials. The HPLC tandem-MS assay was preferred so as to generate valid pediatric PK data and permit its integration in the RAAS model described above.

The aim of the third subproject, a study of IV amiodarone in children with potentially life-threatening arrhythmias, was to perform a prospective observational clinical trial of IV amiodarone in children. This subproject was undertaken to enhance confidence in the use of amiodarone in children with potentially life-threatening arrhythmias by correlating amiodarone serum concentrations with efficacy and safety parameters including heart rate, blood pressure, QTc intervals, and laboratory values. Additionally this project aimed to demonstrate how new insights into the developmental pharmacology of amiodarone could be derived by comparison of pediatric data with that of a simulated adult population generated through compartmental modeling approaches.

3. Methods

3.1. General methods and their application within the framework of clinical trials for children

The following paragraph will provide a general overview of the methods applied to fulfill the aims of the three subprojects. Their detailed implementation will be discussed in section 3.2 to 3.4.

3.1.1. Modeling and simulation (M&S)

A model is a conceptual and simplified representation of a physical object or process. Its properties and behaviors are represented with a complexity based on the existing body of knowledge of those processes and the intent governing the construction of the model. Simulation refers to the process of applying a model within the context of a specific set of user-defined conditions ("inputs") to predict the behavior of the object or process. The term modeling and simulation (M&S) becomes idiomatic since this is often the final intent of a model. The impact of M&S is pronounced, with its applications spanning engineering (eg, airplane and car development), science (eg, climatic models or models for the movement of continents), and even reach as far as everyday life (eg, weather forecasting and "second-life" computer games).

Pharmaceutical sciences frequently use such modeling techniques, each of which serves different purposes. Organic chemistry uses molecular modeling techniques for virtual screening and lead structure optimization in drug development (Böhm, 2002). Drug formulation research sees the use of the Noyes-Whitney model to describe the dissolution behavior of solid drug particles (Dokoumetzidis, 2006).

In the design of clinical trials, modeling and simulation (M&S) may involve describing various aspects of PK and PD processes (Meibohm, 2005) as well as the simulation of complex clinical trials with sub-models for recruitment, patient attrition, and statistical power assessment for the modeled endpoints (Kimko, 2011). Such modeling approaches are being increasingly implemented following

their recognition by regulatory authorities in the USA (Gobburu, 2001) and in Europe (Manolis, 2009). Pharmacokinetic and PD modeling depends on the accessibility of data and involves the application of different methods to this end. Compartmental models represent an approach to describe the PK behavior of a drug in a human body (Gibaldi, 1982). Usually, a few compartments (mostly less than 5) are interconnected by rate constants and their values are estimated based on available data (figure 4, left model). In the presence of pediatric PK data, such compartmental models can be harnessed to predict drug concentrations reached under different dosing regimens to identify a regimen with the highest likelihood of therapeutically success. In the absence of pediatric data, it is possible to scale adult compartmental models for pediatric purposes by applying allometry. The principle of allometry assumes a correlation between size and function. A historical and still valid application of allometry is the finding that metabolic rate is proportional to body mass raised to the 3/4th power, first described by Max Kleiber in 1932 and given his name: "Kleiber's law" (1) (Kleiber, 1932).

$$q_0 \sim M^{3/4} \tag{1}$$

where q_0= metabolic rate and M= body weight

Based on body mass and a scaling exponent, parameters of adult PK models can also be scaled down to pediatric values since the same principles govern PK in humans of all ages. The value of the scaling exponent depends on the underlying constants. While the value equals 1 for volume dependent processes such as distribution, clearance dependent processes are scaled by an exponent of ¾ (Anderson, 2008). However, such scaling only reflects changes in size, while functional changes in organs (eg, enzyme maturation) are not adequately captured. This contributes to the decreased predictive performance of such models, especially in young age groups which naturally exhibit the lowest body weights (Anderson, 2011).

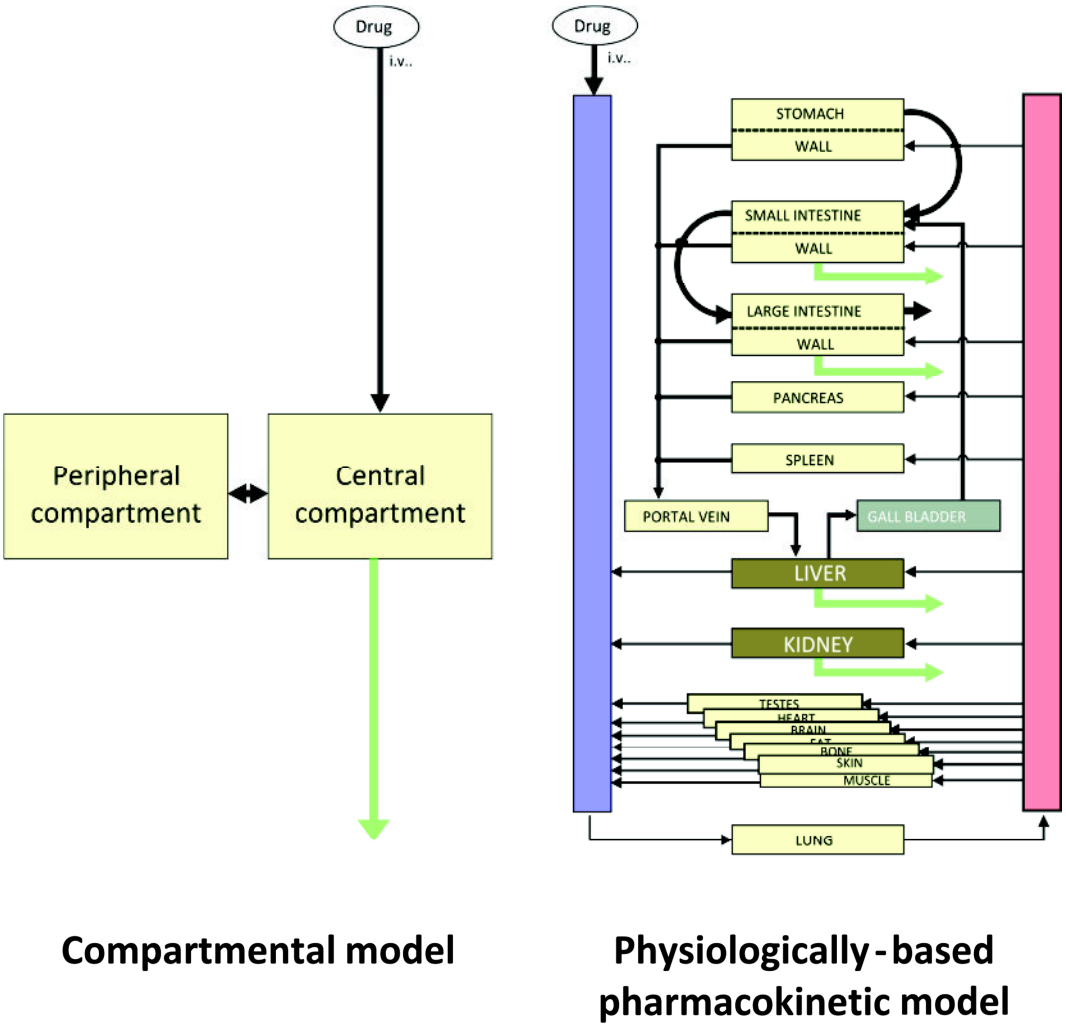

Figure 4. Schematic representations of a compartmental and a physiologically based PK model (modified after Willmann, 2003). Black arrows represent drug movements, while green arrows represent elimination pathways.

An additional strategy suitable for use in the absence of pediatric pharmacokinetic data is provided by physiologically based modeling methods, which offer mechanistic modeling approaches applicable to pediatric trial planning (Barrett, 2012). Mechanistic models incorporate physiological data on organ sizes, enzyme activities, and blood flows as the initial basis for a physiologically based adult model and then integrate this information with physicochemical drug properties (Willmann, 2003) (figure 4, right model). The pediatric model is derived by keeping the physicochemical drug data constant while the physiology data is scaled based on the known trajectories of physiological parameters and enzyme maturation during growth from children to adults (Edginton, 2006). With enzyme maturation data being applied, these

mechanistic models complement pediatric compartmental models scaled only by allometry that are lacking maturation trajectories of enzymes.

The potential value of a physiologically based PK modeling approach can be demonstrated from an example drawn from the literature on the phosphodiesterase type 5 (PDE5) inhibitor, sildenafil. This drug is used in the treatment of pediatric pulmonary hypertension. In a clinical trial whose objective was to evaluate the hemodynamic and corresponding PK responses to a single dose of oral sildenafil in children with pulmonary arterial hypertension (PAH), blood samples drawn 30 minutes after nasogastric administration showed the concentration of sildenafil to be below the limit of quantification in 42% of the patients (Apitz, 2010). However, physiologically based simulations of sildenafil PK in children demonstrated that the drug reached its maximum concentration 1.4 hours after oral administration (Hsien, 2010). Therefore, it is likely that a significant amount of drug had reached the venous blood stream after 30 minutes. Subsequent sampling through a trial protocol optimization based on physiologically based modeling may have yielded more informative results.

Although physiologically based modeling may also be harnessed for deriving pediatric PD models, this approach is applied to a lesser extent due to the inherently high complexity (eg, figure 5) and marked lack of preexisting adult physiologically based models. More sophisticated approaches are therefore required. For physiologically based PD models, it is often necessary to integrate the in vivo and in vitro data on human and drug properties (Eissing, 2011). One advantage of such an approach lies in the general character of the PD model, which can be utilized for the simulation of different drug applications since it incorporates the processes involved in the PD responses and their interactions with drugs in adults (Ramusovic, 2012a). Further, as described for the PK models, such PD models can be scaled for pediatric application following identification of the relevant parameters and their trajectories during growth from children to adults, and may therefore be harnessed for pediatric trial optimization.

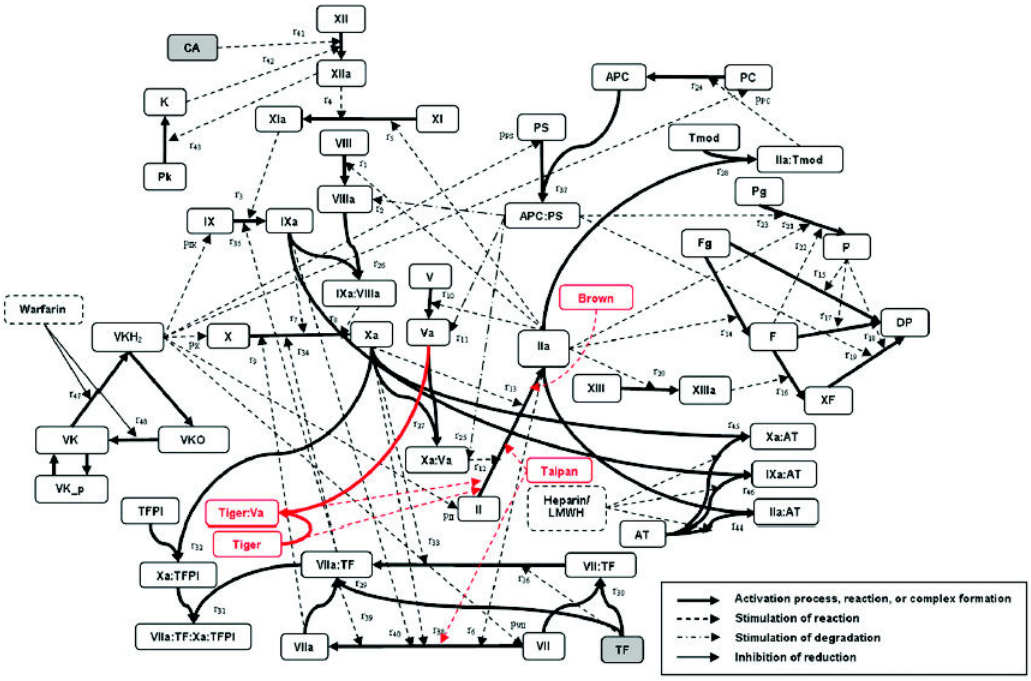

Figure 5. Schematic representation of a complex adult PD model for the blood coagulation cascade based on interaction and complex formation of the coagulation cascade factors. The specific targets of snake venoms (red lines and boxes) and drugs (heparins, solid black boxes; warfarin, dashed boxes) are depicted. From Gulati et al. (Gulati, 2013).

The principle of constructing pediatric models based on scaled adult data for generating an "in silico child" was the subject of a number of workshops with contributions from the Heinrich-Heine University in cooperation with the "HEXAL Initiative Kinderarzneimittel" (Läer, 2009). It is also being increasingly applied to the design of trials for pediatric investigation plans by companies, which gives them an advantage because it is now possible to simulate dosing regimens and optimize pediatric trial design in the absence of pediatric PK data (Manolis, 2011).

3.1.2.HPLC tandem-MS

HPLC tandem-MS permits the efficient quantification of drugs in different biological matrices by combining the chromatographic separation properties of an HPLC with the selectivity and detection abilities of a mass spectrometer. The analytical process for this method can be subdivided into three distinct steps:

1. Sample preparation

In this decisive step, the drug is separated from its biological matrix since it may adversely affect mass spectrometry detection due to a matrix effect that suppresses or enhances ionization of the analyte and thereby provides inconsistent results between samples with different matrices. This separation process commonly uses ion exchanger columns to withhold drugs in the column matrix and release them in a subsequent elution step, a technique referred to as "solid phase extraction" (SPE). Liquid-liquid extraction between aqueous and organic phases can also potentially be applied.

2. Chromatographic separation

Despite the high selectivity of the tandem-MS detection, chromatographic separation of the sample is crucial to further reduce the interaction of the drug with the remaining matrix components. Analytical C_{18}-reverse phase columns are routinely utilized in this process, but other configurations are also possible depending on the characteristic of the drug. Optimizing the mobile phase for a HPLC tandem-MS assay poses a significant challenge since standard buffers utilized in HPLC that incorporates ultraviolet or fluorescence detection (eg, phosphate buffer) are incompatible with the assay.

3. Tandem-mass spectrometric detection

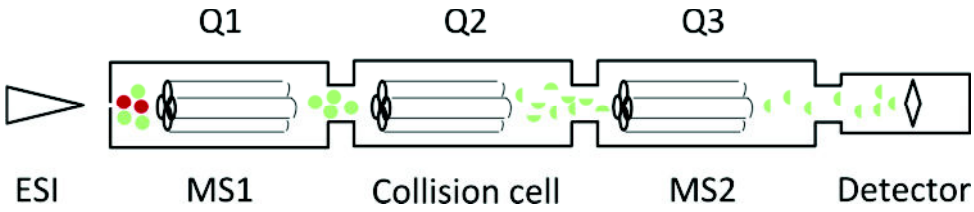

Figure 6. Schematic representation of a tandem mass spectrometer. ESI: electrospray ionization; Q: quadrupole; MS: mass spectrometer.

Selective detection with tandem-MS is based on the specific selection and fragmentation of ions. In mass spectrometers harnessing electrospray ionization (ESI), the processed sample is sprayed after preparation into the interface via an electrically-charged needle. A hot airstream then vaporizes the mobile phase and causes the sprayed droplets to shrink and the charges

to concentrate on the surface. With rising charge density, the droplet becomes unstable and finally explodes ("Coulomb explosion"), yielding free ions that are transferred into the mass spectrometer. Inside the mass spectrometer, quadrupole magnets (Q1-Q3) keep the ions in stable trajectories. In the first mass spectrometer (MS1), the mass of interest from all the incoming ions is selected and transferred to the collision cell where it is fragmented via collision with gas atoms (eg, nitrogen). One specific collision fragment of the former ion is then selected in the second mass spectrometer (MS2) (m/z transition) and finally detected. Due to its ability to monitor multiple fragmentations, the assay is referred to as multiple reactions monitoring (MRM) and proves useful for determining multiple drugs or metabolites in one sample. The dwell time of a MRM assay is the time during which data for a single data point is acquired and averaged. With increasing dwell times, the accuracy of a single data point increases at the expense of the total number of data points that can may be acquired for the peak of interest. Conversely, the more the number of reactions that are monitored in the MRM mode, the fewer the number of data points that may be gathered for an individual MRM transition. Consequently, dwell times need to be optimized while developing a MRM assay according to the number of MRM transitions and the total duration of the peak of interest resulting from the HPLC settings in order to generate a sufficient number of data points for the robust integration of each individual peak. In addition, the potential effects of crosstalk need to be ruled out when developing a MRM assay. Such effects may occur if fragments generated in the collision cell adversely affect the detection of the fragments finally aimed for. Furthermore, crosstalk can occur if two identical fragments from different parent compounds are monitored. Quantification errors may occur if insufficient time is allocated for evacuation of the collision cell when switching MS1 from the first to the second parent compound, causing the signal generated from the former to be accounted to ions of the latter (Vogeser, 2010).

The HPLC tandem-MS assay is highly suitable for use in pediatric clinical trials for two reasons. First, it can cope with the low blood volumes usually available in pediatric studies, especially in trials involving neonates (Fanaroff, 2002). Even if a greater volume of blood is available, the remaining amount may be used for quantification of other biological molecules, such as endogenous humoral biomarkers. Second, the high selectivity of the method may permit the efficient and accurate quantification of the drug of interest even in the presence of concomitant medications that are likely to be administered to children afflicted with life-threatening conditions who are cared for in an intensive care setting.

3.1.3.Observational trials/studies

A clinical trial is an experiment investigating the effect of exposure to “factors” on different health-related variables in humans. From an epidemiological perspective, these “factors” may constitute environmental risks while the pharmacological perspective may often focus on the effect of drug administration. Health-related variables can be derived from different sources and represent outcomes such as survival, myocardial infarction, blood pressure or adverse events as well as the quality of life measured by validated questionnaires. In addition, biochemical parameters such as drug concentrations and markers of biological function such as blood cortisol levels are also classified as health-related variables.

Depending on their design, clinical trials can be subdivided into two major groups: interventional and observational. Interventional trials are ideal for generating the high quality evidence necessary, for instance, to obtain drug approvals. These trials incorporate systematic and time-tested design elements to ensure the generation of data of the highest quality, such as:

1. Controls: One group of patients in the trial receives the investigational drug while another group receives an “active comparator” or “placebo” to help identify drug effects and differentiate drug effects from “placebo effects.” Definitions of placebo and active comparator are given below:

   a. Placebo: A sham formulation lacking the active drug. Although pharmacologically inactive itself, its application may trigger a physiologic response based on psychological expectations (the so called “placebo effect”).
   b. Active comparator: An approved and routinely available treatment for the disease of interest that is not the investigational drug. Comparing the investigational drug’s effects to those of active comparators can yield information on its efficacy vis-à-vis that of already marketed treatment options.
2. Randomization: The process of assignment of patients to investigational drug or active comparator/placebo group which is done by chance to prevent the occurrence of patient selection during assignment (“Bias”).
3. Blinding: Process whereby the patient (single-blind) or the patient and the treating physician (double-blind) remain unaware of the patient’s assignment to an investigational drug or active comparator/placebo group in order to avoid preconceptions regarding the effects of the drug on both sides.
4. Statistical trial planning: The number of patients to be recruited to answer the scientific question (the “statistical power”) is calculated in advance and trial enrollment performed accordingly.

However, the implementation of these design elements may pose significant financial, logistical, and ethical hurdles. Clinicians have gathered practical knowledge based on their individual experiences, especially in pediatrics. Therefore, they may refrain from participating in or helping with recruitment of patients for a placebo-controlled trial due to ethical concerns since one group of patients would be denied the putatively effective treatment (Saul, 2005). Statistical trial planning poses another problem owing to the lower patient numbers afflicted with pediatric cardiac disease.

Observational trials, by their very design, may be able to overcome some of these challenges since the choice of treatment for an individual patient does not

get influenced. The development of health-related variables is then recorded over the course of the treatment and analyzed. Compared to interventional trials, it may appear that the design elements ensuring data quality are lacking in observational trials since the effects of group selection, such as patients with more severe diseases being more likely to receive a specific drug, cannot be prevented and may only be accounted for at the level of data analysis. However, an observational trial may allow the gathering of scientific knowledge where other approaches are deemed inappropriate for whatever reason. Currently, the high rate of off-label use in pediatrics (Choonara, 2002; Lass, 2011; Lindkvist, 2011; Olsson, 2011; Kimland, 2012; Palčevski, 2012; Ribeiro, 2012) may be viewed as an ever-repeating therapeutic experiment with a patient number of 1 that does not facilitate the gathering of collective therapeutic knowledge (Ward, 2009). This inherently pediatric issue demands study designs that are free from the strict constraints of an interventional trial (Perry, 2012). Prospective observational trials may provide a framework to collate data from these individually treated patients without interfering with the clinical treatment routine. For adults, the successful implementation of such approaches has already been demonstrated in intensive care units (Sterba, 2008, Schmittinger, 2012). With adequate caution to account for the limitations of such observational studies, these frameworks may generate valuable data on pediatric treatments.

3.2. A physiologically based RAAS model

A literature search was first conducted to identify the relevant parameters of the RAAS. The model was then developed in several incremental steps. First, a basic RAAS model was developed into which equations for drug effects were included and missing parameters estimated to define steady-state conditions. Next, an integrated whole body physiologically based PK model for enalapril and enalaprilat was developed and coupled with the RAAS model. Finally, the simulation-based model evaluations were performed.

3.2.1. Pharmacodynamic RAAS model

Modeling was performed using the Mobi software package, which is a graphical and mathematical platform for modeling biological processes that accounts for mass-balance conservation and stoichiometry (Bayer Technology Service GmbH, Leverkusen, Germany, Version 2.3.5). Objects were implemented with different properties. “Species” described products or educts of reactions, or the substances that modified reactions. Reactions could be freely defined, for example, as rate laws or the Michealis–Menten reactions. The software also included a MATLAB™ toolbox for data-based parameter estimation and optimization (Bayer Technology Service GmbH, Leverkusen, Germany, Version 2.3). These procedures constituted a key element of integrative physiologically based modeling (Eissing, 2011), linking data values with unknown information and producing parameter value estimates that are consistent with the physiological framework.

The circulating RAAS is an endocrine proteolytic cascade. Triggered by decreasing blood pressure, sodium concentration, or systemic volume, the juxtaglomerular apparatus in the kidneys secretes renin which selectively cleaves the liver-derived, physiologically inactive glycoprotein angiotensinogen to angiotensin I (Atlas, 2007). In the plasma and the vascular beds of the lungs, angiotensin I is eventually converted by the ACE to the main effector of the RAAS–angiotensin II. The system is internally controlled by the negative feedback of angiotensin II that decreases renin levels with increasing angiotensin II levels. However, the more

complex aspects of the RAAS that involve local systems found in vascular endothelia involve the ACE2 enzyme, which cleaves angiotensin II to the peptide angiotensin 1-7 (Shahid, 2011) and its biological actions, which are the subject of current investigations. The proteolytic cascade leading to the formation of angiotensin II from angiotensin I and its precursor angiotensinogen have been modeled as follows: angiotensinogen was assumed to have a constant concentration in the plasma (as degradation by renin contributes only about 20% to its metabolic clearance (Hackenthal, 1987)). Angiotensinogen was modeled as being cleaved by renin into angiotensin I, which was the initial driving reaction of the whole cascade (2).

$$\frac{dc_{Angiotensin_1}}{dt} = \frac{\left(VMAX_{Renin} \times C_{Angiotensinogen} \times C_{Renin}\right)}{\left(K_{M_{Renin}} + C_{Angiotensinogen}\right)} \quad (2)$$

where $VMAX$= maximum reaction velocity; K_M= Michaelis-Menten constant; C= concentration

Then, angiotensin I was cleaved by ACE to angiotensin II (3).

$$\frac{dc_{Angiotensin_2}}{dt} = k_{Ang\_2\_gen} \times C_{Angiotensin_1} \quad (3)$$

where $k_{Ang\_2\_gen}$= rate constant for angiotensin 2 generation; C= concentration

The negative feedback effect of angiotensin II on renin secretion (Blair-West, 1971; Sancho, 1976; Schweda, 2012) was implemented in the first-order renin production step (4).

$$\frac{dc_{Renin}}{dt} = k_{Renin_{gen}} \times C_{Prorenin} \times \left(1 - \frac{C_{Angiotensin_2}}{\left(IC50_{Angiotensin_2} + C_{Angiotensin_2}\right)}\right) \quad (4)$$

where $k_{Renin_{gen}}$= rate constant for renin generation; $IC50$= half maximal inhibitory concentration; C = concentration

The degradations of angiotensin I and angiotensin II, as well as the renin concentrations were modeled following a first-order process defined by degradation constants and the respective substance or enzyme concentrations.

3.2.2.Drug effects

The effects of enalaprilat and benazepril (ACE inhibitors (ACEI)) and of the oral direct renin inhibitor (DRI) aliskiren were computed using a type I indirect response model (Jusko, 1994). The effects of aliskiren, which inhibits the generation of angiotensin I, were integrated into equation (2) to give equation (5.1). The effects of the ACEIs, leading to a suppression of the generation of angiotensin II, were integrated into equation (3) to give equation (5.2).

$$\frac{dc_{Angiotensin_x}}{dt} = (2) \text{ or } (3) \times \left(1 - \frac{C_{Inhibitor}{}^{Hill-Factor(Aliskiren\ or Enalaprilat)}}{\left(IC50_{Inhibitor}^{Hill-Factor} + C_{Inhibitor}{}^{Hill-Factor}\right)}\right) \qquad (5.1/5.2)$$

where (2) or (3)= equation (2) or (3); $IC50$= half maximal inhibitory concentration; C= concentration; for 5.1: x= 1 and $Inhibitor$= aliskiren; for 5.1: x= 2 and $Inhibitor$= enalaprilat

The effect of angiotensin receptor blockers was accounted for in the renin production step, attenuating the negative feedback of angiotensin II on renin production by competitively inhibiting the effect of angiotensin II on the angiotensin II receptor. The differential equation (4) was therefore modified to give equation (6).

$$\frac{dc_{Renin}}{dt} = k_{Renin_{gen}} \times C_{Prorenin} \times \left(1 - \frac{C_{Angiotensin_2}}{\left((IC50_{Angiotensin2} \times 1 + \frac{C_{Losartan}}{K_I Losartan}) + C_{Angiotensin_2}\right)}\right) \qquad (6)$$

where $k_{Renin_{gen}}$= rate constant for renin generation; C= concentration; $IC50$= half maximal inhibitory concentration; K_I= (competitive) inhibition constant

Figure 7 shows a schematic diagram of the final model. The plasma renin activity (PRA), which is a biochemical measurement rather than a physiological process, was not explicitly included in the RAAS model. However, its value reflected renin activity ex vivo, while angiotensin I concentration reflected renin activity in vivo (Chauveau, 1992). From trial data (Nussberger, 2002) it was possible to establish the following correlation between these values, which was confirmed by angiotensin I and the PRA data obtained on day 1 of that trial (R^2=0.9867) (7).

$$PRA = 0.083 \times C_{Angiotensin2} + 0.3828 \qquad (7)$$

where PRA= plasma renin activity; C= concentration

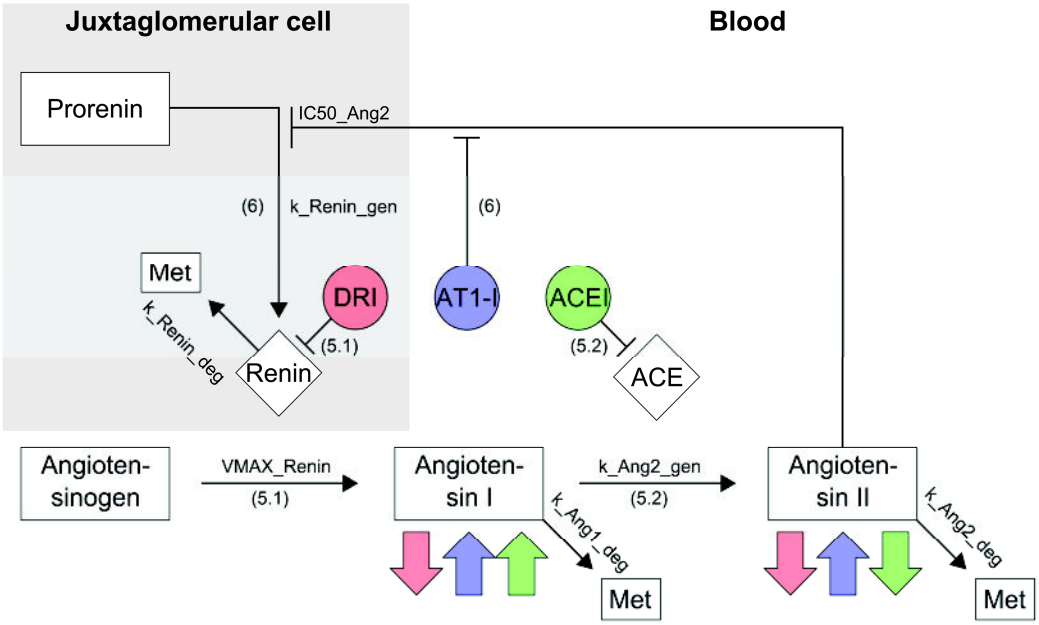

Figure 7. Schematic representation of the pharmacodynamic RAAS model.
The model includes the generation of angiotensin I, angiotensin II and renin and their respective degradation to metabolites (Met) in the circulating blood, and the negative feedback of angiotensin II on renin production within the juxtaglomerular cell compartment. The three drug classes, direct renin inhibitors (DRI: red), angiotensin II subtype 1 inhibitors (AT1-I: blue), and angiotensin converting enzyme inhibitors (ACEI: green) are depicted with their distinct modes of action and their effect on angiotensin I and II levels. The main model parameters are given and the governing equations are identified in brackets.

3.2.3.Parameter identification

Values in the published literature were used for model parameterization if available. Estimating missing parameters required the determination of steady-state values of the RAAS, for which a dataset was created containing the documented angiotensin I, angiotensin II, and renin concentrations obtained from the data of Juillerat et al. (Juillerat, 1990), Christen et al. (Christen 1991), and Nussberger et al. (Nussberger 2002). Mathematical fits of PK data from the respective trials were created and used as inputs for estimating basic RAAS parameters. A 20,000 minute break was inserted between drug administrations to allow the system to return to a steady-state after the weaning off of the PD effect of the respective drug. For enalapril, the HPLC tandem-MS PK measurements were available (Najib, 2003) and were superimposed on the angiotensin data since more indirect enalapril quantification methods had a tendency to overestimate enalapril and enalaprilat concentrations. In a final step, all the values for which estimates were required were applied to all data simultaneously in Mobi utilizing the “Monte Carlo” estimation routine followed by the estimation routine “fminsearch” with a root mean square error function. The resulting parameters used in the equations are listed in table 1.

3.2.4.Pharmacokinetic whole body physiologically based model development

The construction of models for the physiologically based integration of enalapril PK was approached as follows: First, models for enalaprilat and enalapril PK in adults were generated separately using PK-Sim, a software tool for whole body physiologically based modeling (Bayer Technology Service GmbH, Leverkusen, Germany, Version 4.2.4) (Willmann, 2003). This software includes validated physiological models to estimate substance-specific parameters for absorption and distribution, such as partitioning and permeability coefficients based on physicochemical compound properties (eg, membrane affinity, fraction unbound, solubility, and molecular weight). The program also integrates any interdependence of organ weight, blood flow, and intestinal parameters (gastrointestinal length, radius, gastrointestinal sections, and intestinal surface

areas) associated with the weight and height of the virtual individual, utilizing data from biometric databases such as the National Health and Nutrition Examination Survey (NHANES) (Centers for Disease Control and Prevention, 1997) or the International Commission on Radiological Protection (ICRP) (ICRP, 2002). Since PK-Sim is the graphical front-end of the Mobi platform, further refinements were made to the original PK-Sim model using Mobi. Two identical male individuals were created in PK-Sim, with demographic values taken from the ICRP 2002 population (ICRP, 2002). Age-, weight-, and height-related data were chosen to match the trial data from Najib et al. (Najib, 2003), and these parameters were used subsequently to develop the enalapril model (mean age: 23.25 ± 4.55 years; mean weight: 73.38 ± 9.39 kg; mean height: 175.96 ± 7.44 cm). These demographic parameters were sufficiently similar to the data from the enalaprilat trial (mean age: 25 ± 3 years and mean weight: 64 ± 10 kg in the "young population" described by Hockings et al.) (Hockings, 1986), such that the final models could be combined and interpreted as a single representative individual (age: 23.25 years, weight: 73.38 kg, and height: 175.96 cm). Finally, the enalaprilat and enalapril models were coupled in Mobi as described in section 3.2.6.

3.2.5.Enalaprilat model

A model for the effects of IV enalaprilat administration was set up using Pk-Sim. For the hydrophilic metabolite, the assigned LogP value was −1.7 (literature: −0.7 (National Center for Biotechnology Information, 2011c), −3 (Ranadive, 1992)), with an unbound fraction of 0.83 (literature: 0.84 (Sirianni, 1998)). At concentrations below 8 ng/mL, the rate-liming step of enalaprilat excretion has been documented to be its release from ACE, to which it is bound with high affinity (Ulm, 1982; Vlasses, 1985). To account for this effect, the model was exported to Mobi and a reversible binding process occurring in the venous plasma compartment was defined (8).

$$\frac{dc_{Enaat_{bound}}}{dt} = k_{Enaat_{bound}} \times C_{ACE} \times C_{Enaat_{unbound}} - k_{Enaat/ACE_{diss}} \times C_{Enaat_{bound}} \quad (8)$$

where C= concentration; $k_{Enaat_{bound}}$= rate constant for enalaprilat binding to ACE; $k_{Enaat/ACE_{diss}}$= rate constant for enalaprilat dissociation from ACE; ACE= angiotensin converting enzyme; $Enaat$= enalaprilat

Since the exact number of enalapril-binding sites was unknown (since these were present in both the circulating and tissue bound ACE, primarily in the lung and tissue beds (Ondetti, 1982)), the values of the species "ACE" and both binding constants were estimated from a dataset on enalapril IV administration (Hockings, 1986) and from oral enalapril data (Najib, 2003), with "ACE" being 9.00 mM, the binding constant of enalaprilat to ACE being 0.1 1/minute and the respective dissociation constant being 0.001 1/minute.

3.2.6.Coupled enalapril/enalaprilat model

The enalapril model was set up in Pk-Sim with a LogP value of 0.07 (Ranadive, 1992) and an unbound fraction of 0.55 (Sirianni, 1998). To perform further manipulations, the model was exported to Mobi. The gastrointestinal uptake was estimated using "intestinal permeability (IP)," "gastric emptying time" (GET), and the "intestinal transit time" (ITT) as variables. Intestinal permeability in the model was estimated to be 1.02 cm/second*10^{-03}, GET to be 6 minutes (PK-Sim default value: 30 minutes), and intestinal transit time was estimated to be 2 hours (PK-Sim default value: 3 hours). The Mobi models for enalaprilat and enalapril were then combined such that the decrease of enalapril served as the source for enalaprilat in the liver cell compartment. The specific hepatic conversion clearance was estimated based on enalaprilat concentrations after enalapril administration, with a value of 0.5 L/min/kg of the organ. The specific renal excretion clearance was set to 8 L/min/kg of the organ. The suitability of this approach has already been demonstrated for different transport and metabolism processes (Willmann, 2009; Kersting, 2012). Enalapril is converted to enalaprilat not only by the liver but also intra-renally (Lannoy, 1989; Lannoy, 1990). This was implemented in the model by the inclusion of an additional conversion clearance within the intracellular compartment of the kidneys. Data

on urinary excretion of enalapril, enalaprilat, and their combination (Rippley, 2000) were used to estimate clearance parameters, with a resulting specific intra-renal conversion clearance of $1.95*10^5$ L/min/kg of the organ, and an excretion clearance of 0.36 for intra-renal enalaprilat and 3.45 L/min/kg of the organ for enalapril.

3.2.7.Simulation-based model evaluations

Model evaluation was based on simulations. To investigate the impact of variability on the steady-state concentrations of angiotensin I and II, eight scenarios were established with a lognormal distribution on $VMAX_{Renin}$, $k_{Ang\_1\_deg}$, $k_{Ang\_2\_gen}$, $k_{Ang\_2\_deg}$, k_{Renin_gen}, k_{Renin_deg} and $IC50_{Ang\_2}$ (geoSD 1.4). Each scenario was simulated in 1000 replicates. A sensitivity analysis was performed for the PD RAAS model without the drug parameters but with a base model, and parameters were varied in ranges of 0.1-10 as the multiplication factor. Outcome parameters were the steady-state concentration (C_{ss}) of angiotensin II and area curves for angiotensin II suppression enalapril (AUEC) since they were judged to be the most relevant outcomes. Model development was performed on a desktop PC (2.93 GHz Intel Duo CPU, 4 GB RAM, 64 bit).

3.2.8.Statistical analysis

Statistical calculations were performed in MATLAB 2011a (Version 7.12.0.635, 32 bit; The Mathworks Inc., Natick, MA). Median percentage errors (MedPE) were calculated as the median of all individual percentage error values for all data points in one drug administration (9).

$$PE = \frac{(C_{pred} - C_{obs})}{C_{obs}} \times 100\% \qquad (9)$$

where PE= percentage error; C= concentration; $pred$= predicted; obs= observed

MedPE serve as a measure of accuracy with a value of 0% indicating the absence of a bias towards over- or under-prediction of angiotensin I and II concentrations obtained by simulation and compared to data. The median absolute percentage error (MedAPE) was calculated as the median of all absolute percentage error values for all data points in one-drug administration (10).

$$APE = \left|\frac{(C_{pred} - C_{obs})}{C_{obs}}\right| \times 100\% \quad (10)$$

where APE= absolute percentage error; C= concentration; $pred$= predicted; obs= observed

MedAPE serves to measure the precision of the model. A value of 0% indicates that predicted and observed values for angiotensin I and II concentrations are identical. MedPE and MedAPE were constructed with non-parametric 95% confidence intervals (CI) derived from 10,000 repetitions of random data sampling and recalculation (bootstrap). Area under the effect curves for the last data point ($AUEC_{last}$) were calculated as the integral of the angiotensin concentrations, applying the trapezoidal rule for simulated and observed concentrations from the first to the last data point of each drug administration. To calculate the integral of the steady-state concentrations, the $AUEC_{last\ (steady-state)}$ values were obtained by replacing the angiotensin concentrations obtained from data or simulations with the steady-state concentrations attained as outputs of the model. For comparison, the ratios of the observed (R_{obs}) or predicted drug effects (R_{pred}) and steady-state $AUEC_{last}$ were calculated (11).

$$R_{obs/pred} = \frac{AUEC_{last_{obs/pred}}}{AUEC_{last_{steady-state}}} \quad (11)$$

where R= ratio; $AUEC$= area under the effect curve; $pred$= predicted; obs= observed

The $AUEC_{last}$ values were a direct quantification of the exposure to angiotensins and could therefore serve to evaluate the effect of different drugs interfering with the RAAS. The $R_{obs/pred}$ values helped to compare simulations with the trial data. To compare steady-state concentrations and calculate the standard deviations, either the placebo data or data for the administration time 0 hours from the first day were extracted from Juillerat et al. (Juillerat, 1990), Christen et al. (Christen, 1991), Mazzolai et al. (Mazzolai, 1999), or Nussberger et al. (Nussberger, 2002). Steady-state concentrations were compared by applying a Kruskall–Wallis Test.

Table 1. Parameterization of the RAAS model.

Input parameter	Assigned Value	Literature value	Unit	Reference
Precursor molecules				
$C_{Angiotensinogen}$	1.00E+00	1.00	µM	Lynch, 1991
$C_{Prorenin}$	7.26E+01	-	µM	
General reaction parameters				
$VMAX_{Renin}$	1.02E+02	-	1/min	
$k_{Ang\_1\_deg}$	4.0E-01	0.98±0.06	1/min	Admiraal, 1990
$k_{Ang\_2\_gen}$	7.90E-01	-	1/min	
$k_{Ang\_2\_deg}$	1.47E+00	0.87;0.6±0.07;2.6±0.16	1/min	Donato, 1972; Al-Merani, 1978; Magness, 1994
k_{Renin_gen}	1.30E-09	-	1/min	
k_{Renin_deg}	3.00E-02	5.76E-03 - 5.73E-02	1/min	Schneider, 1969; Hiruma,1988
$IC50_{Ang\_2}$	6.59E-07	-	µM	
$Hill_{Ang\_2}$	1	-		
$K_{M\,Renin}$	1.35E+00	0.07-5.7	µM	Skinner, 1975; Streatfeild-James, 1998; Nguyen, 2003
Drug specific reaction parameters				
$IC50_{Enalaprilat}$	3.24E-03	1.2E-03-4.8E-03*; 1.26E-02 - 5.86E-02	µM	LeBlanc, 2006; National Center for Biotechnology Information, 2011c
$Hill_{Enalaprilat}$	1			
$IC50_{Benazepril}$	1.00E-02	5.18E-03 - 1.3E-02	µM	LeBlanc, 2006
$Hill_{Benazepril}$	1			
$IC50_{Aliskiren}$	1.64E-04	6.00E-04; 5.3E-04 - 6.00E-04*;0.33†	µM	Wood, 2003; Vaidyanathan, 2008; National Center for Biotechnology Information, 2011a
$Hill_{Aliskiren}$	0.35			
$K_I Losartan$	2.00E-02	2.00E-02; 3.00E-03 - 7.7E-02*	µM	Burnier, 2000; National Center for Biotechnology Information, 2011b

*pooled from in vitro data from human cell lines in different functional assays
†corrected for mean plasma protein binding of 50% (Wood, 2003)
C= concentration; $VMAX$= maximum reaction velocity; k= rate constant; Ang_1= angiotensin I; $Hill$= Hill factor for sigmoidal IMAX model; Ang_2= angiotensin II; gen= generation; deg= degeneration; $IC50$= half maximal inhibitory concentration; K_I= (competitive) inhibition constant

3.3. A HPLC tandem-MS method for determination of enalapril and enalaprilat concentrations in pediatric trials

For conversion of concentrations from ng/ml to µM multiply the former values by $2.66*10^{-3}$, $2.87*10^{-3}$ and $2.36*10^{-3}$ for enalapril, enalaprilat and benazepril, respectively. For conversion of concentrations from µg/ml to µM multiply the former values by 2.66, 2.87 and 2.36 for enalapril, enalaprilat and benazepril, respectively.

3.3.1. Chemicals and reagents

Enalapril maleate (≥ 98%, TLC) was obtained from Sigma Aldrich (München, Germany). Enalaprilat (USP standard) and benazepril hydrochloride (USP standard) were obtained from LCG-Standards (Luckenwalde, Germany). Methanol (HPLC grade), water (super gradient grade), and orthophosphoric acid (85%, p.a.) were purchased from VWR (Germany), while formic acid (98-100%, purity p.a.) and acetonitrile (gradient grade) were purchased from Sigma Aldrich (Seelze, Germany). Ammonium hydroxide solution (25%, p.a.) was purchased from Grüssing (Filsum, Germany) and normal serum was provided by employees of the Institute of Clinical Pharmacy and Pharmacotherapy (Düsseldorf, Germany).

3.3.2. Stock and working solutions, calibration standards, quality control samples, and stabilities

Stock solutions were prepared by dissolving 10 mg of accurately weighed enalapril, enalaprilat or the internal standard (IS) benazepril in 100 mL methanol. Working solutions were prepared by serial dilution of the stock solutions with water, leading to concentrations of 1 µg/mL for enalapril and enalaprilat and 1.66 µg/mL for IS (calculations based on free drug base). Calibration and quality control (QC) standards were prepared by spiking normal human serum with working solutions to yield a concentration of 200 ng/mL of enalapril and enalaprilat, followed by serial dilutions with blank serum to give concentrations of 100, 50, 25, 12.5, 6.25, 3.13, and 1.57 ng/mL. Stock solutions were stored at -20°C and equilibrated to room temperature prior to use, while working solutions

were freshly prepared for each of the daily measurements. The stability of stored stock solutions was assessed at a concentration of 100 ng/mL by comparing the peak areas of the working solutions made from stock solutions that had been stored for 3 and 6 months with working solutions made from freshly prepared stock solutions. Long-term stability was assessed by measuring QC standards of 200, 50, and 1.57 ng/mL with five standards for each concentration after one month of storage at -20°C. Freeze-thaw stability was investigated with QC standards after they had been subjected to three freeze (-20°C) and thaw (21°C) cycles. Short-term stability was assessed by preparing the QC standards 24 hours before extraction and storing them at room temperature while the post-preparative stability was assessed by extracting five QC standards at 200, 50, and 1.57 ng/mL and storing the extracted standards in the autosampler at room temperature for analysis 24 hours later. All concentrations for QC standards for stability assessment were finally determined with an 8-point calibration curve constructed using freshly prepared calibration standards.

3.3.3.Sample preparation

Negative pressure solid phase extraction (SPE) was performed using Plexa PCX Varian Bond Elut SPE cartridges (60 mg, 1 mL) (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Ten microliters of IS solution and 660 µL of a 2% solution of orthophosphoric acid in water were added to an aliquot of 100 µL serum and vortexed for 5 seconds. SPE columns were primed with 1 mL methanol followed by 1 mL water. Samples were extracted in the primed columns and subsequently washed using 500 µL of a 2% solution of formic acid in water followed by 500 µL of methanol/acetonitrile (1/1 (v/v)) solution. The SPE columns were finally eluted with 2 applications of 350 µL each of a mixture of methanol/acetonitrile (1/1 (v/v)) with 25% ammonium hydroxide in a 3/1 (v/v) ratio. The eluate was evaporated to dryness under a nitrogen stream and under steady shaking at 300 rounds per minute while being heated to 40°C. Evaporated samples were then reconstituted in 100 µL of the mobile phase.

3.3.4.Chromatographic and mass spectrometric conditions

The analyses were run on a modular HPLC system (Shimadzu Deutschland GmbH, Duisburg, Germany) assembled with a Shimadzu controller SCL10Avp, 2 separate Shimadzu pumps LC10Avp with a standard analytical mixing device for low- and high-pressure gradient configurations, a 3 channel prominence online degasser DGU 20A3, and a VWR/Hitachi Column Oven L-2300 (VWR International, Fontenay sous Bois, France). The HPLC system was serially connected to a triple quadrupole MS API 2000, while data was collected and analyzed using the Analyst 1.4.1® software package (Applied Biosystems/MDS SCIEX, Concord, Canada). A Luna® RP-C18(2) analytical column (50 mm×2 mm, 3 µm, 100 A, Phenomenex, Aschaffenburg, Germany) combined with a SecurityGuard® RP-C18 guard column (4 mm×2 mm, Phenomenex, Aschaffenburg, Germany) was used to perform chromatographic investigations. The run time was set to 5 minutes. During each run, the column was equilibrated to 30°C and 20 µL of reconstituted sample was injected. The mobile phase used for isocratic elution consisted of a mixture of methanol/water/formic acid (65/35/1 (v/v/v)) with a pH of 4.1. No splitting device was used to reduce the flow rate (0.4 mL/min), which resulted in a back-pressure of $190*10^5$ Pa. The tandem-MS was set to positive electrospray ionization and multiple reaction monitoring mode, monitoring the m/z transitions channels 377.3→234.2, 349.3→206.1 and 425.3→351.2 for enalapril, enalaprilat and IS, respectively (MS^2 spectrum, figure 8). Dwell times were set at 300 milliseconds (ms). The MS conditions and additional compound-specific MS parameters have been summarized in table 2.

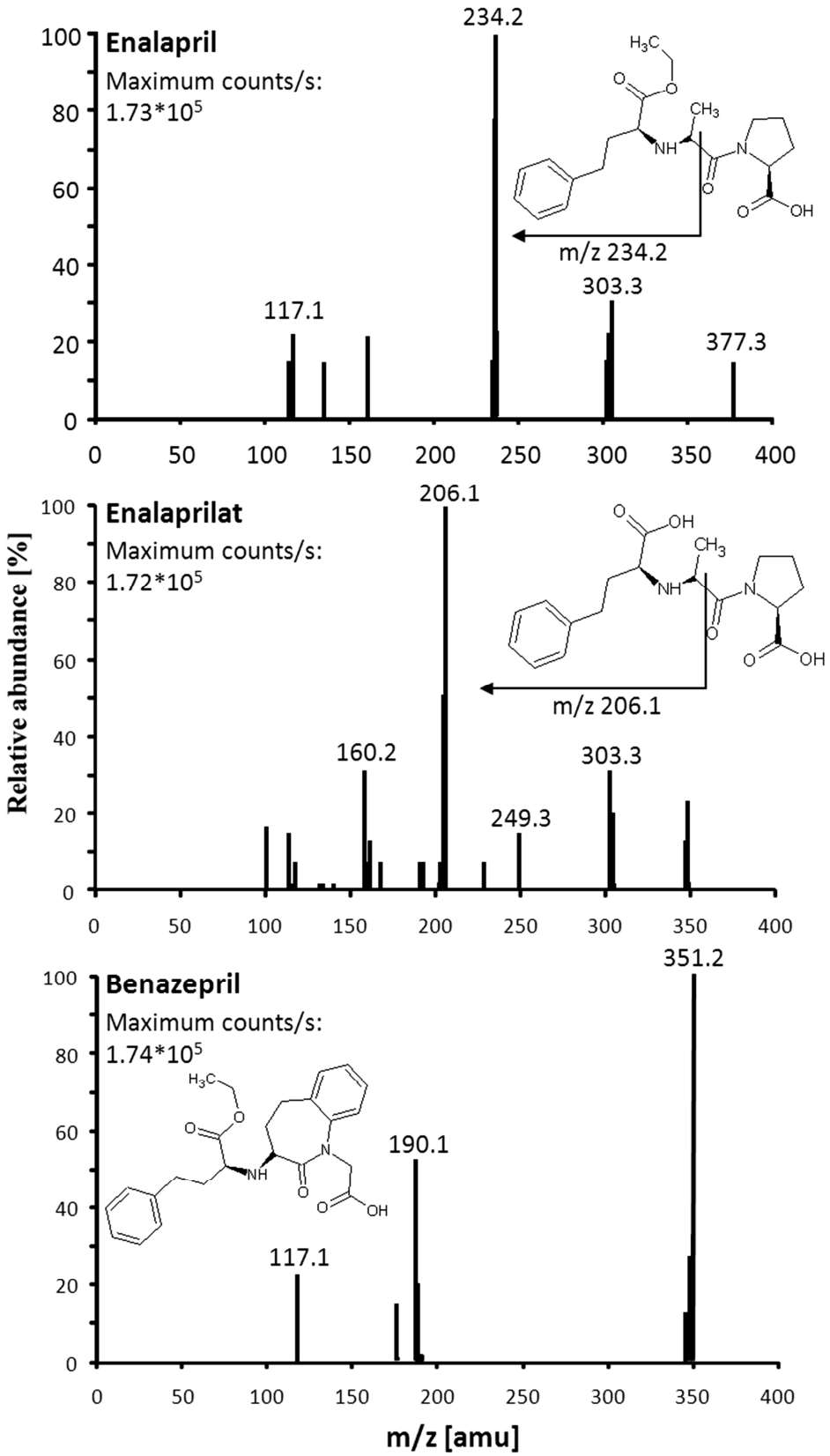

Figure 8. MS^2 product ion spectra $[M+H]^+$ for enalapril, enalaprilat, benazepril resulting from 3 cycles of multiple channel acquisition (300 ms each).

Table 2. Tandem-MS conditions and compound-specific parameters for enalapril and enalaprilat analysis.

MS conditions			
Mass spectrometer			API 2000
Interface			electrospray
Polarity			positive
Scan type			MRM[†]
Resolution (Q1, Q3)			unit[‡]
Curtain gas			10
Collision gas			6
IonSpray voltage			4000
Temperature			350
Ion source gas 1			30
Ion source gas 2			70
Solvent spilt ratio			none
Compound-specific parameters	**Enalapril**	**Enalaprilat**	**Benazepril**
Declustering potential (DP)	30	30	30
Focusing potential (FP)	360	360	370
Entrance potential (EP)	10.5	10	5
Cell entrance potential (CEP)	16	16	20
Collision energy (CE)	27	27	25
Cell exit potential (CXP)	12	18	10

[†]Multiple reactions monitoring [‡]0.6-0.8 atomic mass unit (amu) peak width at half peak height

3.3.5.Method validation

Precision, accuracy, linearity, matrix effects, recovery, selectivity, and stabilities of the tandem MS assay were assessed according to the US Food and Drug Administration (FDA) guidance for bioanalytical method validation (US Department of Health and Human Services, 2001). Validation runs were performed on three days with a new calibration curve for each day that was separately used to quantify the concentrations of enalapril and enalaprilat. Calibration curves were constructed by plotting the peak area ratios of enalapril and enalaprilat relative to the IS for eight concentrations ranging from 200 to 1.56 ng/mL. Linear regression was performed applying $1/x^2$ weighting for interpolation.

3.3.6.Recovery and matrix effect

Low recovery and the effect of the residual biological matrix can adversely affect HPLC tandem-MS measurements and result in inadequate process efficiency, impairing accuracy and the lower limit of quantification (LLOQ), especially if only small sample volumes are available. Therefore, the recovery and matrix effects were investigated at 5 different concentrations with 5 replicates each, covering the whole calibration range for enalapril, enalaprilat, and IS. Recovery (R) was evaluated comparing the peak areas of normal serum spiked with analyte prior to SPE extraction (A) with serum spiked after extraction (B) and calculating the ratio (12):

$$R(\%) = \frac{A}{B} * 100\% \quad (12)$$

where R= recovery; A= analyte spiked prior to extraction; B= analyte spiked after extraction

Matrix effects were assessed through the application of the post extraction addition technique (Taylor, 2005). The peak areas of measurement of the extracted serum reconstituted in analyte dissolved in mobile phase (C) was compared to the analyte in the same concentration dissolved in mobile phase only (D). The matrix effect (ME) was calculated as in equation 13:

$$ME(\%) = \left(\frac{C - D}{C}\right) * 100\% \quad (13)$$

where ME= matrix effect; C= extracted serum reconstituted with analyte dissolved in mobile phase; D= analyte dissolved in mobile phase

Process efficiency (PE) was defined as in equation 14 (Taylor, 2005):

$$PE(\%) = R(\%) * (\frac{100 - ME(\%)}{100}) \quad (14)$$

where PE= process efficiency; R= recovery ; ME= matrix effect

Process efficiency therefore constitutes a quantification of the combined efficiencies of the SPE related parameters recovery and matrix effect.

3.3.7.Selectivity

To investigate possible interactions with concomitant medications, selectivity was assessed by analyzing the sera of patients treated with sotalol, carvedilol, amiodarone and metoprolol, which are common concomitant medications in patients receiving enalapril. A cidofovir-containing sample (to investigate a highly hydrophilic drug) and a hemolyzed sample were also added to this list. These 6 samples were investigated for peaks that could potentially interfere with the method, with interfering peaks defined as those appearing 0.3 seconds before or after the analyte peak. Further, ion suppression or enhancement of these drugs was assessed by spiking the patient sera samples with enalapril and enalaprilat to yield a final concentration of 100 ng/mL and then extracting and measuring them and finally comparing the analyte peak areas to blank serum spiked with the same concentration of enalapril and enalaprilat. A deviation of more than 15% in peak areas was defined as ion suppression or enhancement. Crosstalk effects between the 3 MRM channels were investigated by injecting standard solutions at concentrations of 206, 236 and 201 ng/mL enalapril, enalaprilat or IS and monitoring the detector response of the respective remaining two channels.

3.3.8.Applicability

To demonstrate general applicability, the method was used to determine the human serum concentrations after oral administration of 20 mg of a commercially available enalapril maleate immediate release preparation (Enalapril Ratiopharm® 20 mg) in a healthy male volunteer aged 30 years. Blood samples were drawn every 30 minutes for 8 hours, with additional sampling in the absorption phase (0-45 minutes), around the time of the expected maximum concentration (time: t_{max}, concentration: C_{max}) of enalapril (after one hour), and enalaprilat (after four hours), and finally 24 h after oral administration of the drug. Blood samples were centrifuged at 3220*g for 20 minutes and the serum supernatant was frozen at -20°C until further analysis. The half-life ($t_{1/2}$), defined as the time period during which the serum concentration is halved, was

calculated by performing linear regression over the last 5 measureable concentration-time points to determine the elimination rate constant k_e, which was transformed to half-life using equation 15:

$$t_{1/2} = \frac{\ln(2)}{k_e} \tag{15}$$

where $t_{1/2}$ = half-life; k_e = rate constant for elimination

3.4. Observational study of intravenous amiodarone in children

3.4.1. Study protocol

The study was conducted at the pediatric intensive care unit (PICU) of the Georg-August University Hospital (Göttingen, Germany) in accordance with the Declaration of Helsinki (2008) and its subsequent amendments. It was approved by the local ethics committee (Proposal No. 4/4/10). Written consent was provided by parents, legal guardians, or when appropriate, patients.

3.4.2. Patients

Patients were eligible for inclusion if they required IV amiodarone for therapeutic purposes subsequent to episodes of incessant ventricular, supraventricular, or junctional ectopic arrhythmia (JET) with heart rates exceeding the 95th percentile for age, or if the episodes of tachycardia they encountered were associated with significant symptoms or that were judged to be life-threatening. The study was designed as an adjunct protocol to standard treatment as indicated by the respective attending physicians. Patients were excluded if amiodarone administration was contraindicated due to preexisting signs of pulmonary fibrosis, thyroid or hepatic dysfunction or other patient-specific contraindications, if body weight was below 2 kg, if there were other medical objections to drawing blood samples, or if informed consent was denied or withdrawn.

3.4.3.Amiodarone protocol, monitoring and objectives

Treatment consisted of a loading bolus of 5 mg/kg amiodarone for 30 minutes followed by an infusion of 10 mg/kg/day, during which heart rate, blood pressure, and electrocardiographic (ECG) changes were continuously monitored. Blood samples for amiodarone determination were drawn at 0, 15, 30, and 120 minutes and at 12 and 24 hours after the initiation of the amiodarone therapy. Blood samples at other time points were permitted if requested by the treating physician. Patients with an inadequate treatment response were permitted up to 2 additional loading boluses of 5 mg/kg for 30 minutes during the 10 mg/kg/day infusion.

The primary objective of the study was to measure serum amiodarone concentrations during IV therapy. Secondary objectives were to compare adult and pediatric amiodarone PK by using a model-based analysis, and to evaluate the efficacy and safety of amiodarone based on analysis of the heart rate, blood pressure, laboratory parameters, and QTc-interval.

3.4.4.Efficacy

Amiodarone therapy was considered successful if the issue of tachycardia was resolved and the basic rhythm was maintained. For patients with JET, success was defined as a reduction in the ventricular rate to less than 180 beats per minute and the heart rate to below 80% of the pre-amiodarone state.

3.4.5.Sample collection and amiodarone assay

A maximum of one mL blood per blood draw was collected and the serum frozen at −20°C until further analysis. Serum concentrations of amiodarone and its metabolite, DEA, were measured using a HPLC-UV standard kit (RECIPE Chemicals & Instruments GmbH, München, Germany) with a lower limit of quantification of 0.16 µM/L, accuracy of 95-105%, intra-assay precision of 2.1% and 2.5% for amiodarone and DEA, respectively, and an inter-assay precision of 4.0 and 4.2% for the 2 entities, respectively.

3.4.6.Pharmacokinetic assessment

The median peak concentrations of amiodarone were measured during the loading phase and the median concentrations of amiodarone during the maintenance phase were determined to have been reached 1.5 hours after the end of bolus. The metabolite DEA was evaluated for the time when it appeared first, and its serum concentrations at first and last quantification were measured. The area under the curve (AUC) of amiodarone was calculated by applying the trapezoidal rule from the first to the last serum concentration measured.

3.4.7.Adult compartmental model and comparison with pediatric data

A previously reported compartment model for intravenous amiodarone in adults (figure 9) (Vadiei, 1997) was implemented in MATLAB 2011a (Version 7.12.0.635, 32 bit; The Mathworks Inc., Natick, MA).

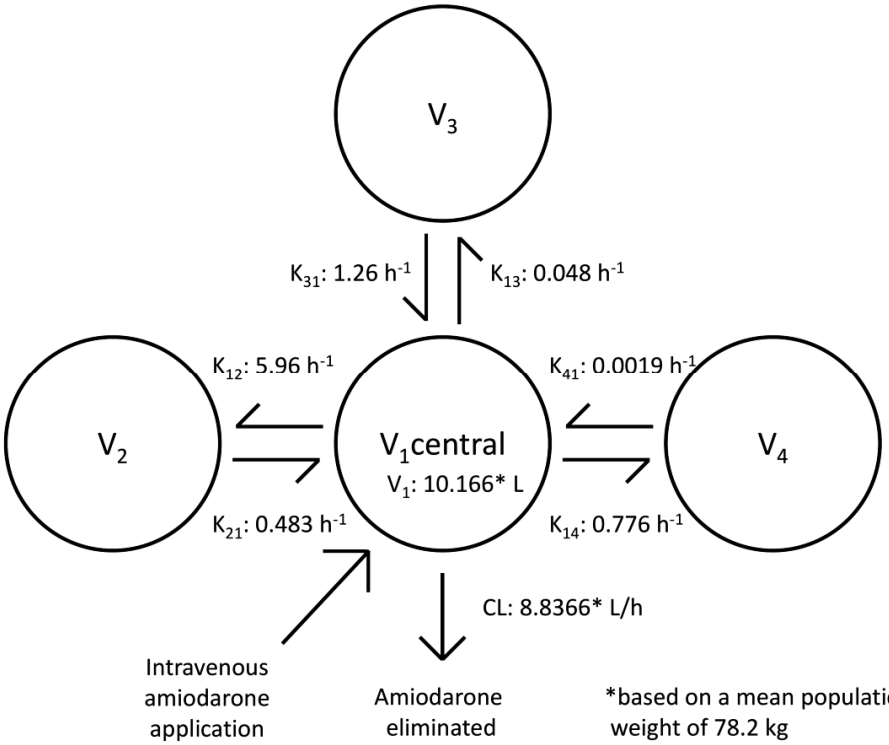

Figure 9. Compartmental model for amiodarone distribution in adults. Circles indicate distribution volumes (V) and arrows mass transports. K values represent rate constants normalized to the mean population weight.

Serum concentration-time profiles were simulated for an adult with body weight 78.2 kg (mean of the modeled population), using the weight-normalized dosing

regimen administered to study patients. Amiodarone has a long half-life due to its high lipophilicity and extensive tissue distribution (Freedman, 1991), and patients were therefore administered a short dosing regimen in the intensive care setting. Consequently, serum concentration and AUC were presumably driven mainly by volume of distribution, rather than by the clearance processes. In addition, CYP3A4 also has a lower activity in children below one year (Wildt, 1999) and thus was not expected to metabolize amiodarone extensively. This being the case, clearance probably affected dose- and time-related AUC changes even less in this cohort. We therefore used an allometric scaling exponent of 1 on the weight-normalized doses to reflect differences in volume of distribution between children and adults (Anderson, 2008). Any changes observed could then be attributed to developmental pharmacology. To detect such changes, amiodarone AUCs in adults were calculated based on simulated time points that matched those of study patients and were then compared to pediatric data.

3.4.8.Statistical evaluation

Statistical analysis was performed using Statistica 6.0 software (StatSoft, Tulsa, OK, USA). The AUC values were tested for normal distribution (Kolmogorov-Smirnov, Lilliefors test). Patient AUC values were compared with simulated adult values (Wilcoxon matched-pairs test). Correlation analysis (Pearson correlation) of patient data (age, weight, height, albumin, total plasma protein, AST, ALT, γ-GT, and relative AUC difference) was also performed. QTc intervals were assessed for normal distribution (Kolmogorov-Smirnov, Lilliefors test) and analyzed by using repeated measures analysis of variance (ANOVA) with one fixed factor (time) at three factor levels for a comparison of pre-, during- and post-therapy times. Differences of means were analyzed (Fishers Least Significant Difference (LSD) post-hoc test). Comparison of patients with phenobarbital comedication versus patients without phenobarbital medication was also performed (Wilcoxon rank-sum test). For all statistical analyses, $p<0.05$ was considered significant.

3.4.9.Heart rate and blood pressure assessment

Hypotension was defined as a decrease in systolic blood pressure of greater than 20 mm Hg or a decrease in diastolic blood pressure of greater than 10 mm Hg compared to baseline values, with potential clinical significance defined as hypotension commencing after amiodarone administration and lasting more than five minutes. Any decrease in heart rate below the 50^{th} and 2^{nd} age-adjusted percentiles was evaluated in accordance with Saul et al. (Saul, 2005) and Davignon et al. (Davignon, 1980), respectively, with the latter changes considered potentially clinically significant.

3.4.10.QTc intervals

QTc intervals were calculated through retrospective analyses of ECG strips. Pre- and post-therapy data included the time period between 4 weeks before and 4 weeks after the treatment, with the 'during-therapy' period ranging from the commencement of bolus until the end of continuous infusion of amiodarone. Heart rate correction of the QT intervals was performed using Bazett´s formula (16) (Bazett, 1920).

$$QTc = \frac{QT}{\sqrt{RR}} \tag{16}$$

where QTc= corrected QT interval; QT= QT interval; RR= interval from the onset of one QRS complex to the onset of the next QRS complex

3.4.11.Toxicity assessment and concomitant medication

Plasma creatinine was analyzed for toxicity when exceeding the age-adapted laboratory reference range during amiodarone therapy and was found to be within the reference range for up to 4 weeks before the initiation of therapy. Consistent with US FDA guidance, hepatic enzymes were analyzed if they exceeded the upper limits of the adult reference range (AST $\leq$45 U/L, ALT $\leq$49 U/L, γ-GT $\leq$38 U/L) by at least three times or were within the adult reference range prior to administration of amiodarone (U.S. Department of Health and Human Services, 2009). The median total plasma protein levels

during therapy were calculated based on patient records. Concomitant medications were documented and evaluated for their potential to alter serum concentrations of amiodarone.

4. Results

The first subproject demonstrated how a novel physiologically based model could predict the plasma concentrations of angiotensin I and angiotensin II in response to drug administration. Drug effects modeled included administration of different doses of aliskiren and losartan as well as the administration of benazepril and enalapril. The parameters of the first subproject allowed for pediatric scaling through identification of their trajectories during growth from children to adults. The model also demonstrated the effective integration of physiologically based PD with physiologically based PK at the final configuration, where it provided a comprehensive physiologically based description of enalapril PK.

The second subproject developed a HPLC tandem-MS method for the determination of enalapril and enalaprilat in human serum utilizing 100 µL of serum. Precision and accuracy of the assay were both consistent with FDA guidelines for bioanalytical method validation (US Department of Health and Human Services, 2001). Enalapril and enalaprilat were sufficiently stable under storage and assay conditions. The method was found to be suitable for pediatric studies, allowing them to potentially yield PK data for further use as input parameters for the RAAS model.

The third subproject conducted a prospective observational study of IV amiodarone in children which revealed that serum levels of the drug were within the therapeutic range reported for adults and that other safety parameters exhibited no significant changes. By simulating a virtual adult population treated with the drug, a lower amiodarone exposure was detected in the pediatric population, which could be attributed to the developmental changes in the protein binding characteristics of amiodarone.

4.1. A physiologically based RAAS Model

4.1.1. Simulation of steady-state conditions accounting for parameter variability

The simulated median steady-state concentrations of angiotensin I and II were consistent with values published in the literature (10.92 and 5.86 pM, figure 10). In the variability scenarios, the models did not differ in angiotensin I distribution but they did so in angiotensin II distribution (p=0.12 vs p=0.03). Variability of $k_{Ang\_1\_deg}$ or $k_{Ang\_2\_gen}$ had little impact on angiotensin II concentrations and caused its concentration to change less than did variations of the other aforementioned parameters. For angiotensin I and II concentrations, the median values of all variability scenarios were comparable to each other (10.67-10.92 pM for angiotensin I and 5.75-5.86 pM for angiotensin II). Comparison of the variability scenarios revealed that all resulting median concentrations were similar. Therefore, the scenario of variability on $VMAX_{Renin}$ was chosen for the numeric and graphic evaluation of the model.

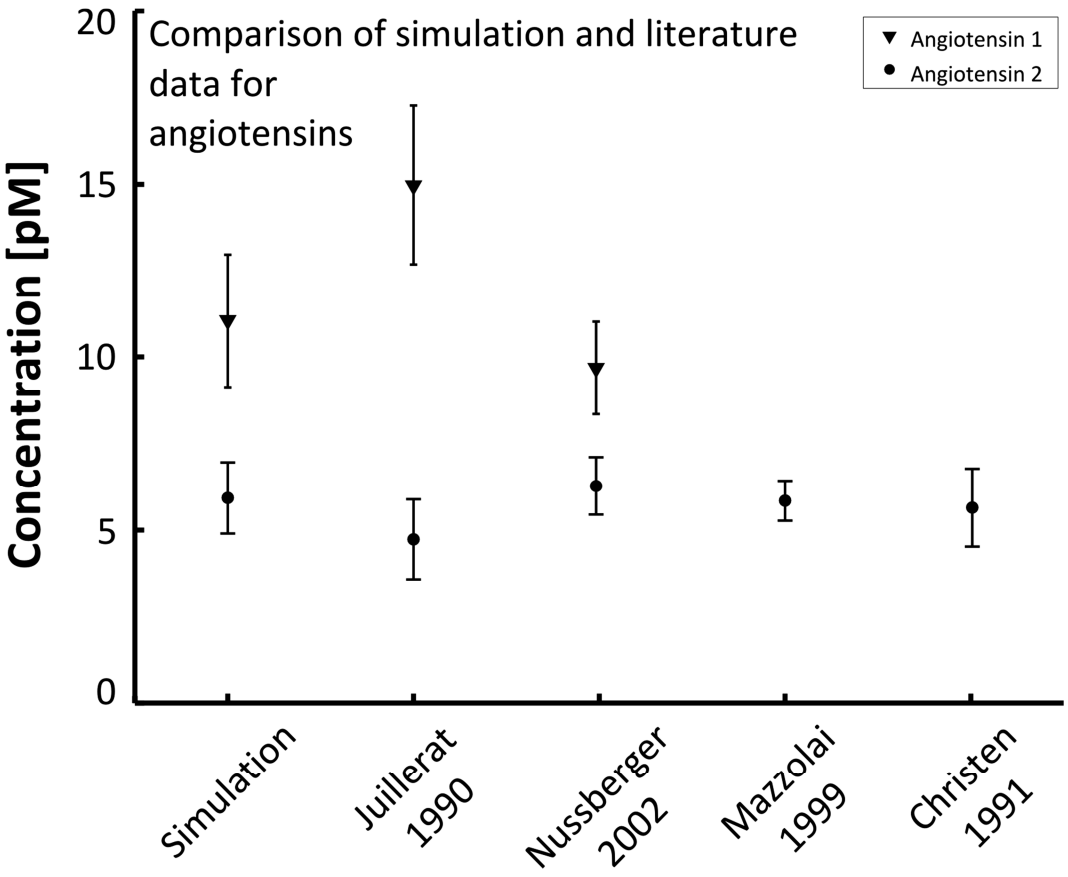

Figure 10. Comparison of simulated and previously published steady-state concentrations of angiotensin I and II. Data and standard deviations derived from Juillerat et al. (Juillerat, 1990), Christen et al. (Christen, 1991), Mazzolai et al. (Mazzolai, 1999), and Nussberger et al. (Nussberger, 2002).

4.1.2.General RAAS simulation results

Simulation of the effects of administration of the ACEIs enalapril and benazepril, the AT1 inhibitor losartan, and the oral DRI aliskiren on angiotensins I and II did not reveal significant over- or under-estimation by the physiologically based RAAS model (MedPE: angiotensin I: −9.97% (−19.2-3.26%); angiotensin II: −1.89% (−9.61-10.57%)). For all drug administrations, the median absolute errors were in an acceptable range (angiotensin I: below 50.5%, II: below 38.28%). All data on MedPEs, MedAPEs, and $AUEC_{last}$ have been summarized in table 3 and table 4.

Table 3. AUEC Ratios.

		$AUEC_{last}$ Angiotensin I ($pM*h*L^{-1}$)				$AUEC_{last}$ Angiotensin II ($pM*h*L^{-1}$)			
Drug	**Dose (mg)**	**Observed**	**R_{obs}**	**Predicted**	**R_{pred}**	**Observed**	**R_{obs}**	**Predicted**	**R_{pred}**
Enalapril PBPK	20	3.11E+03	4.75	2.76E+03	4.21	1.89E+02	0.54	2.06E+02	0.59
Enalapril fit	20	3.11E+03	4.75	2.95E+03	4.50	1.89E+02	0.54	1.92E+02	0.55
Benazepril	20	2.65E+03	4.05	3.21E+03	4.91	1.70E+02	0.48	2.19E+02	0.62
Benazepril	4X5	5.23E+03	7.99	3.87E+03	5.90	1.73E+02	0.49	1.58E+02	0.45
Aliskiren	640	1.28E+02	0.20	1.75E+02	0.27	8.27E+01	0.24	9.40E+01	0.27
Aliskiren	160	2.61E+02	0.40	2.29E+02	0.35	1.60E+02	0.45	1.23E+02	0.35
Aliskiren	80	1.96E+02	0.30	2.63E+02	0.40	1.18E+02	0.34	1.41E+02	0.40
Aliskiren	40	3.45E+02	0.53	3.48E+02	0.53	1.92E+02	0.55	1.87E+02	0.53
Losartan	40					7.02E+02	2.00	5.61E+02	1.59
Losartan	20					5.70E+02	1.62	4.67E+02	1.33

Observed and predicted area under the effect curves ($AUEC_{last}$) for all drug administrations for the scenario of variability on $VMAX_{Renin}$; PBPK physiologically based pharmacokinetics; $R_{obs/pred}$ observed or predicted/steady-state ratios. Steady-state AUEC values were obtained by replacing simulated data with simulated steady-state concentrations and calculating the respective $AUEC_{last}$: angiotensin I, II: 6.55E+02, 3.52E+02 $pM{\cdot}h{\cdot}L^{-1}$

Table 4. Numerical model evaluation.

		Angiotensin I		Angiotensin II	
Drug	**Dose (mg)**	**MedPE (%)**	**MedAPE (%)**	**MedPE (%)**	**MedAPE (%)**
Enalapril PBPK	20	-25.28 (-33.38-0.21)	30.92 (18.38-47.98)	0.34 (-20.42-31.14)	25.78 (16.27-30.01)
Enalapril fit	20	-14.68 (-29.12-4.68)	27.07 (13.24-46.69)	-0.46 (-18.08-18.08)	18.87 (14.49-31.06)
Benazepril	20	-9.18 (-14.50-23.15)	22.55 (13.02-36.48)	27.33 (-1.02-68.44)	34.90 (16.67-74.68)
Benazepril	4X5	-14.60 (-35.78-11.65)	25.38 (14.60-41.96)	4.44 (-45.18-19.72)	38.19 (15.80-47.45)
Aliskiren	640	50.58 (21.72-54.78)	50.58 (21.72-54.87)	13.84 (-16.03-15.30)	15.30 (13.84-34.17)
Aliskiren	160	-12.92 (-28.54-11.64)	18.76 (11.64-29.41)	-21.80 (-47.02- -8.45)	21.80 (8.45-47.02)
Aliskiren	80	16.30 (7.81-60.74)	16.31 (7.81-60.74)	-9.38 (-31.79-72.58)	31.79 (9.91-72.58)
Aliskiren	40	-20.46 (-25.40-42.72)	25.40 (20.46-42.72)	-35.32 (-38.28-42.95)	38.28 (35.52-42.95)
Losartan	40			-6.94 (-29.86-12.59)	23.90 (4.3-35.81)
Losartan	20			-6.22 (-31.85- -0.22)	6.22 (1.66-31.85)
ALL		-9.97 (-19.20-3.26)	14.50 (11.04-21.72)	-1.89 (-9.61-10.57)	19.66 (-14.77-28.12)

Median percentage error (MedPE) and median absolute percentage error (MedAPE) of simulated concentrations of RAA-biomarkers for all drug administrations. The data is presented as median (95%CI) for the scenario of variability on $VMAX_{Renin}$.

4.1.3.Simulation of the effect of enalapril and benazepril on angiotensin I and II

Administration of enalapril caused a drop in angiotensin II concentrations and a subsequent increase in angiotensin I, as is evident from the data and model predictions (figure 11 and figure 12). The effects of enalapril (20 mg) administration were computed in the model in two different ways: first by a mathematical fit of the enalapril PK data, and second by using the integrated physiologically based PK model.

Administration of enalapril leads to an increase in angiotensin I and a decrease in angiotensin II, with comparable results between the predictions from the physiologically based model (R_{pred} angiotensin I: 4.21, angiotensin II: 0.59) and the mathematical fit (R_{pred} angiotensin I: 4.5, angiotensin II: 0.55). Further, the median percentage errors for the physiologically based implementation (angiotensin I: −25.28%, II: 0.34%) were comparable to the mathematical fit (angiotensin I: −14.68%, II 0.46%). The trend shown by the data with regard to benazepril administration (20 mg and 4x5 mg) was captured by the model's prediction interval (figure 13 and figure 14). When comparing the simulated $AUEC_{last}$ values for the administration of benazepril at 4x5 mg and at 20 mg, the results for 4x5 mg displayed a pronounced increase in angiotensin I levels (5.9 vs 4.91-fold) and a stronger decrease in angiotensin II levels (0.45 vs 0.62-fold). For both enalapril and benazepril, a trend towards over-estimation of angiotensin I concentrations was apparent, while the initial reduction of angiotensin II concentrations appeared less steep in the data compared to the simulations.

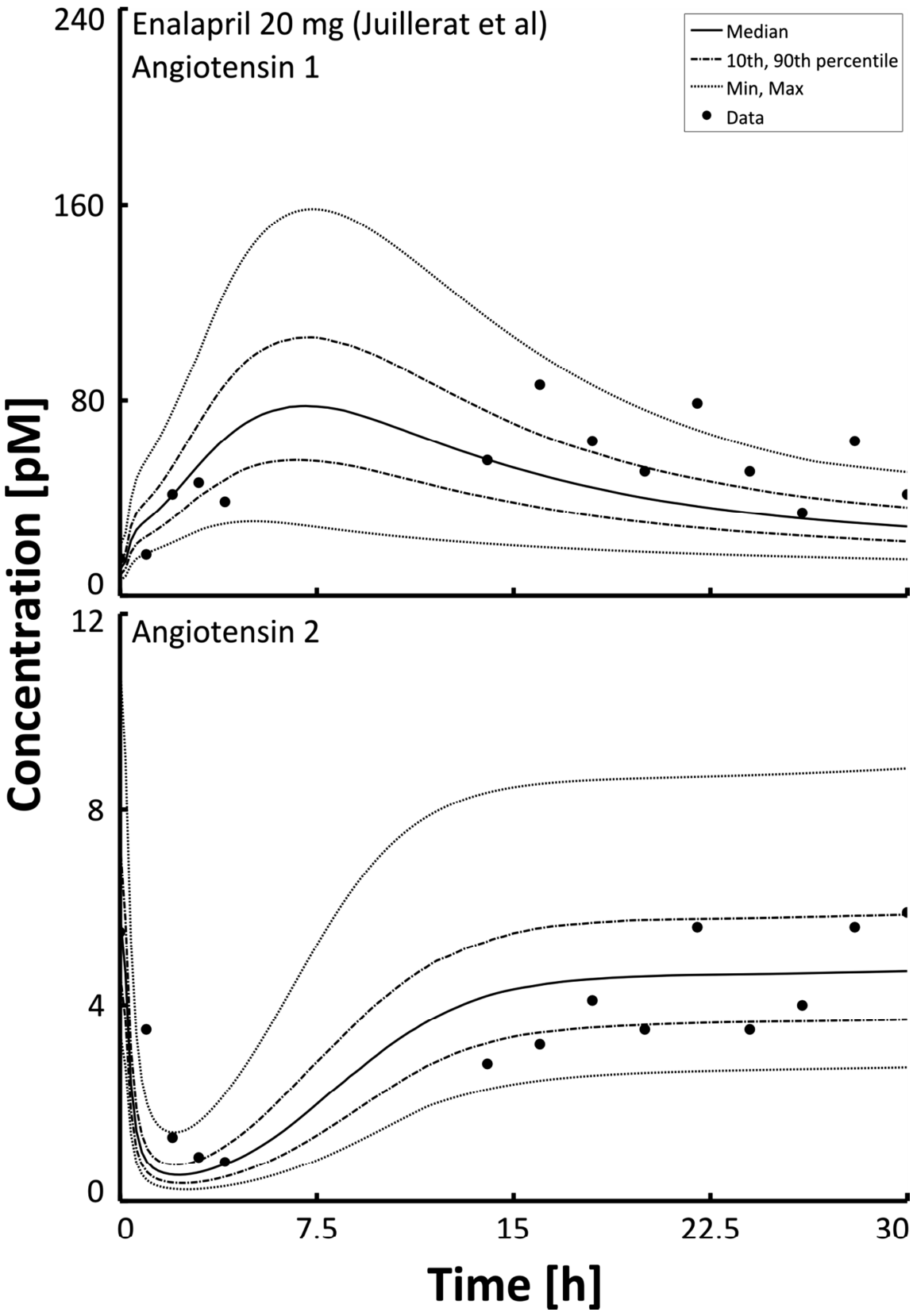

Figure 11. Effect of enalapril (20 mg) on angiotensin I and II levels. Dots depict literature data from Juillerat et al. (Juillerat, 1990). Lines show percentiles as well as minimum and maximum of the simulated data.

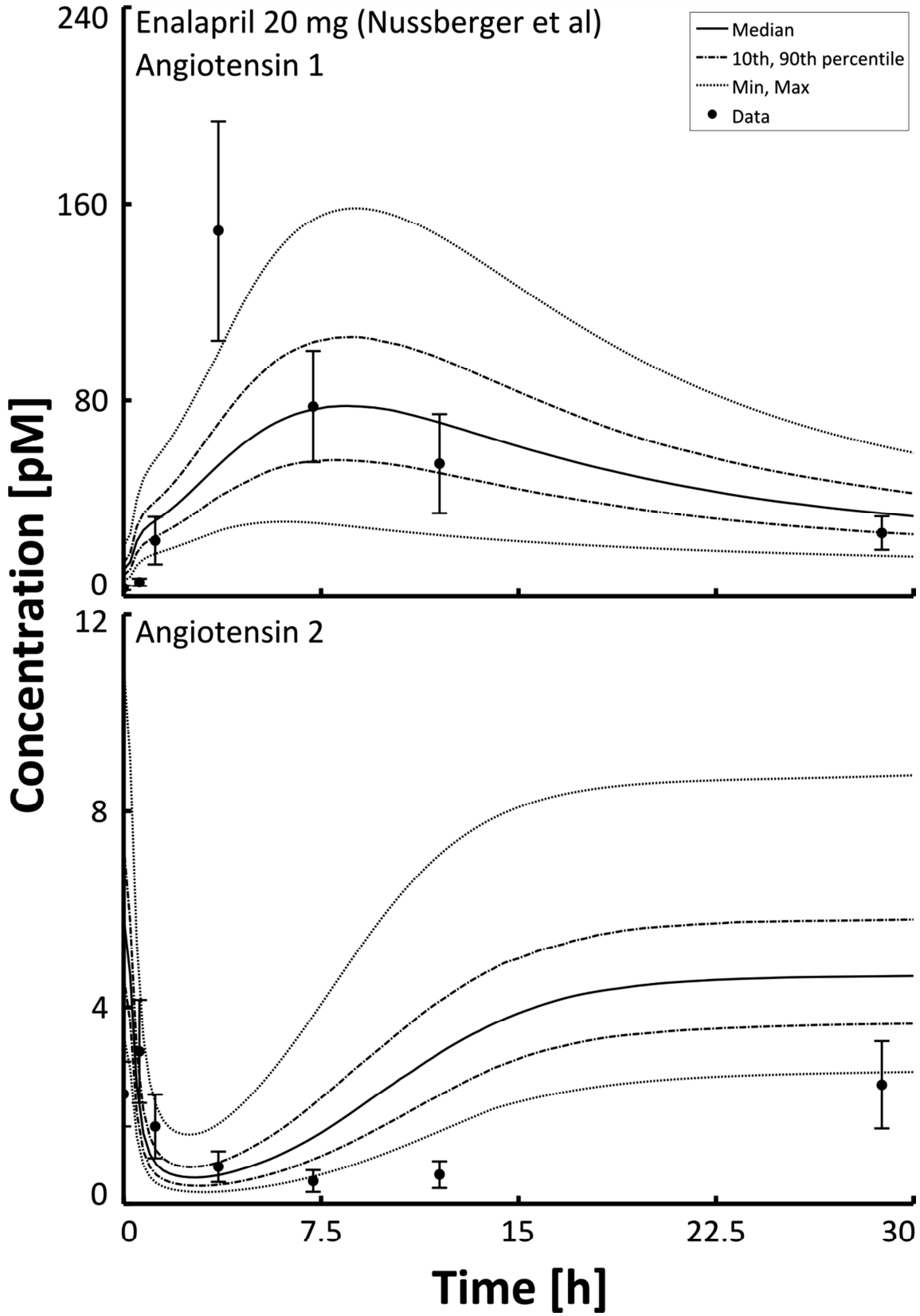

Figure 12. Effect of enalapril (20 mg) on angiotensin I and II levels. Dots depict literature data from Nussberger et al. (Nussberger, 2002) with standard deviations. Lines show percentiles as well as minimum and maximum of the simulated data.

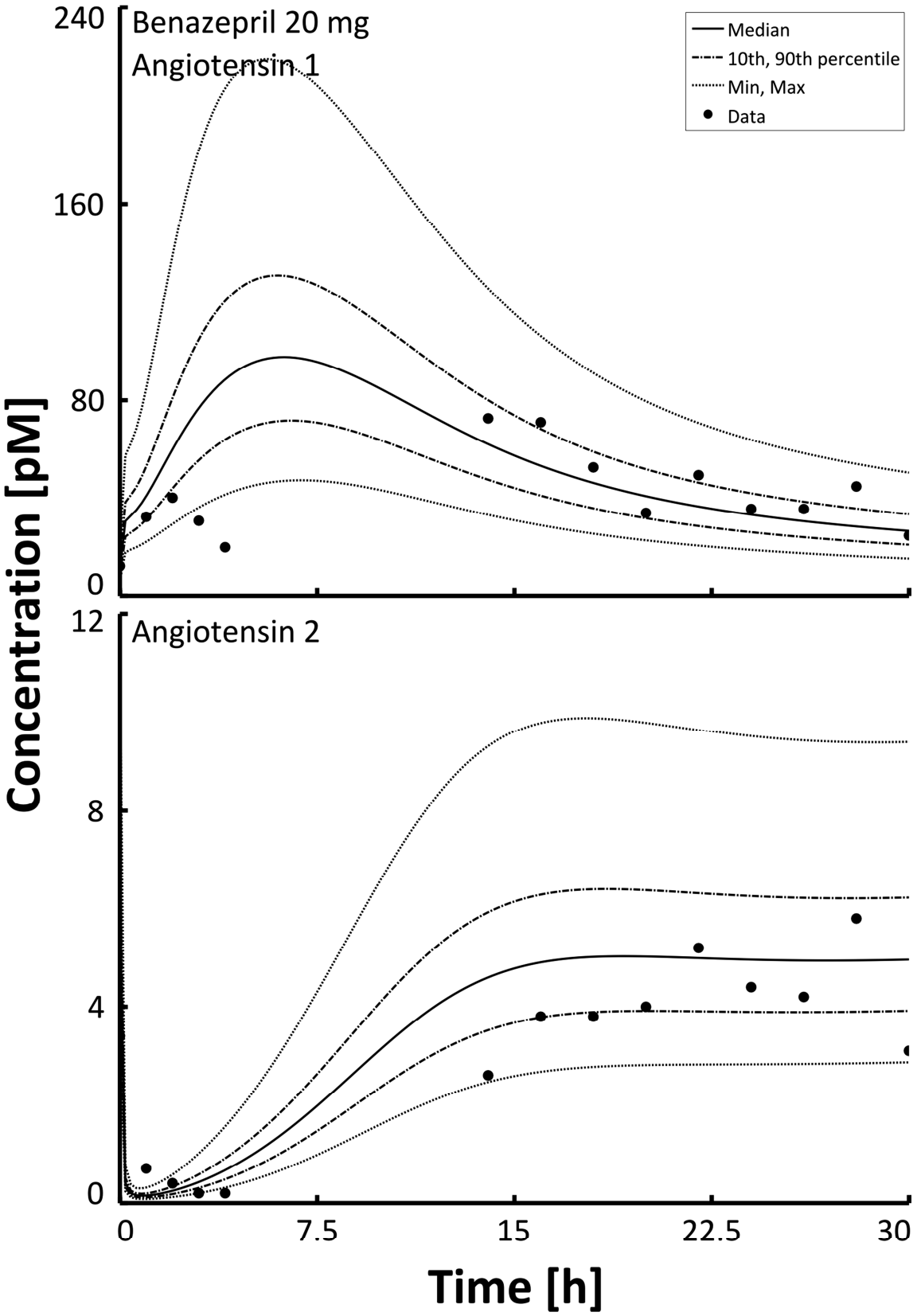

Figure 13. Effect of benazepril (20 mg) on angiotensin I and II levels. .Dots depict literature data from Juillerat et al. (Juillerat, 1990). Lines show percentiles as well as minimum and maximum of the simulated data.

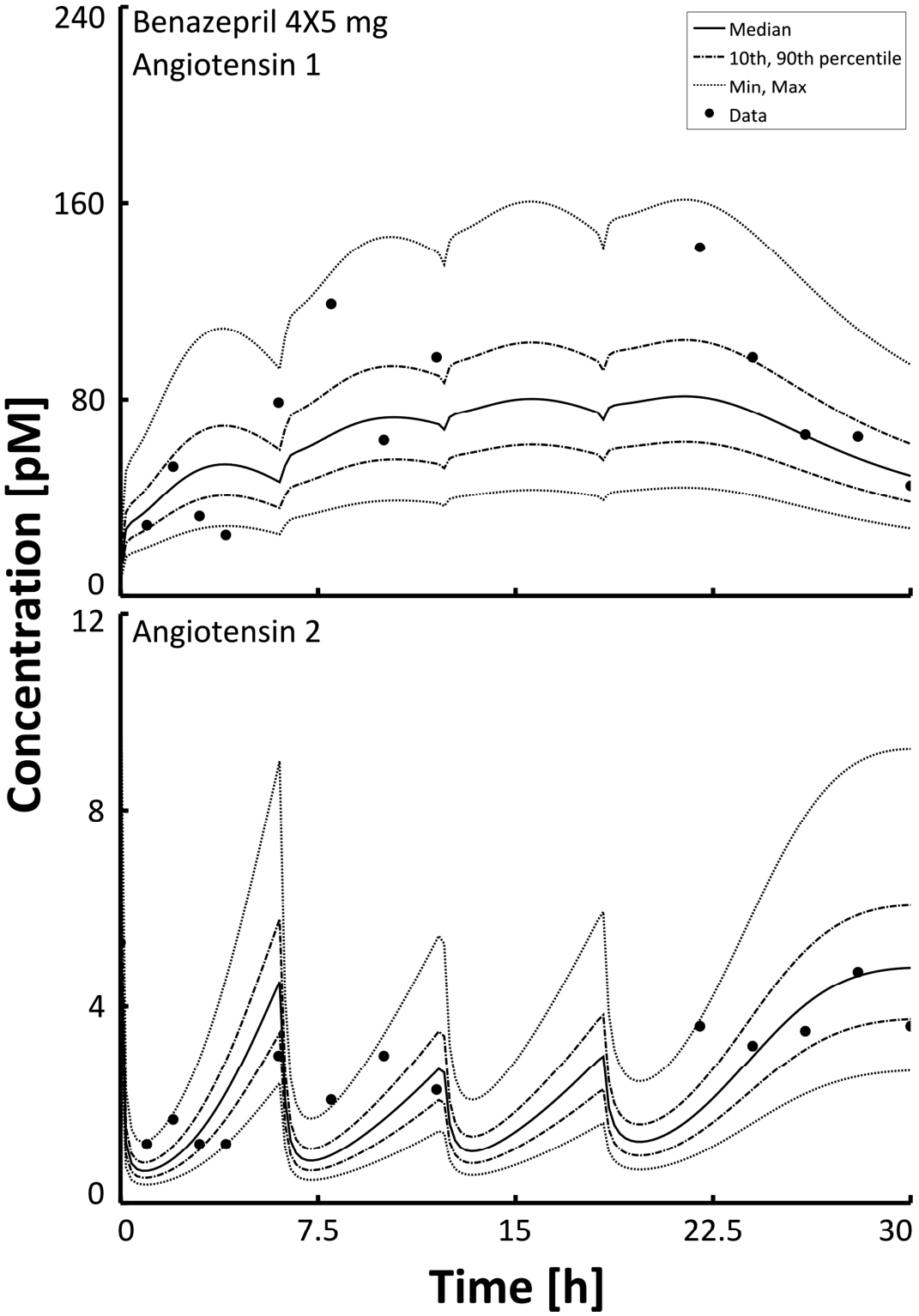

Figure 14. Effect of benazepril (4x5 mg) on angiotensin I and II levels. Dots depict literature data from Juillerat et al. (Juillerat, 1990). Lines show percentiles as well as minimum and maximum of the simulated data.

4.1.4.Simulation of the effect of aliskiren on angiotensin I and II

In general, the physiological response to administration of the direct renin inhibitor aliskiren manifests itself as a drop in the levels of angiotensin I and II. The model predicted this response over a 16-fold dosage range (640-40 mg) in a dose-dependent manner consistent with the data of Nussberger et al. (Nussberger, 2002). Although the response to the 40 mg dose exhibited a stronger deviation from the trial data than did the responses to the other doses, it remained within the prediction interval. In addition, the model visualized the undulation of angiotensin I and angiotensin II levels (figure 15 to figure 18). The dose-dependent and identical drop in angiotensin I and II (R_{pred}: 0.27-0.53 for 640-40 mg aliskiren) was confirmed by numerical calculations. Since angiotensin I concentrations and PRA can be converted by a linear equation, the model also predicted the PRA data (not shown) of Nussberger et al. (Nussberger, 2002).

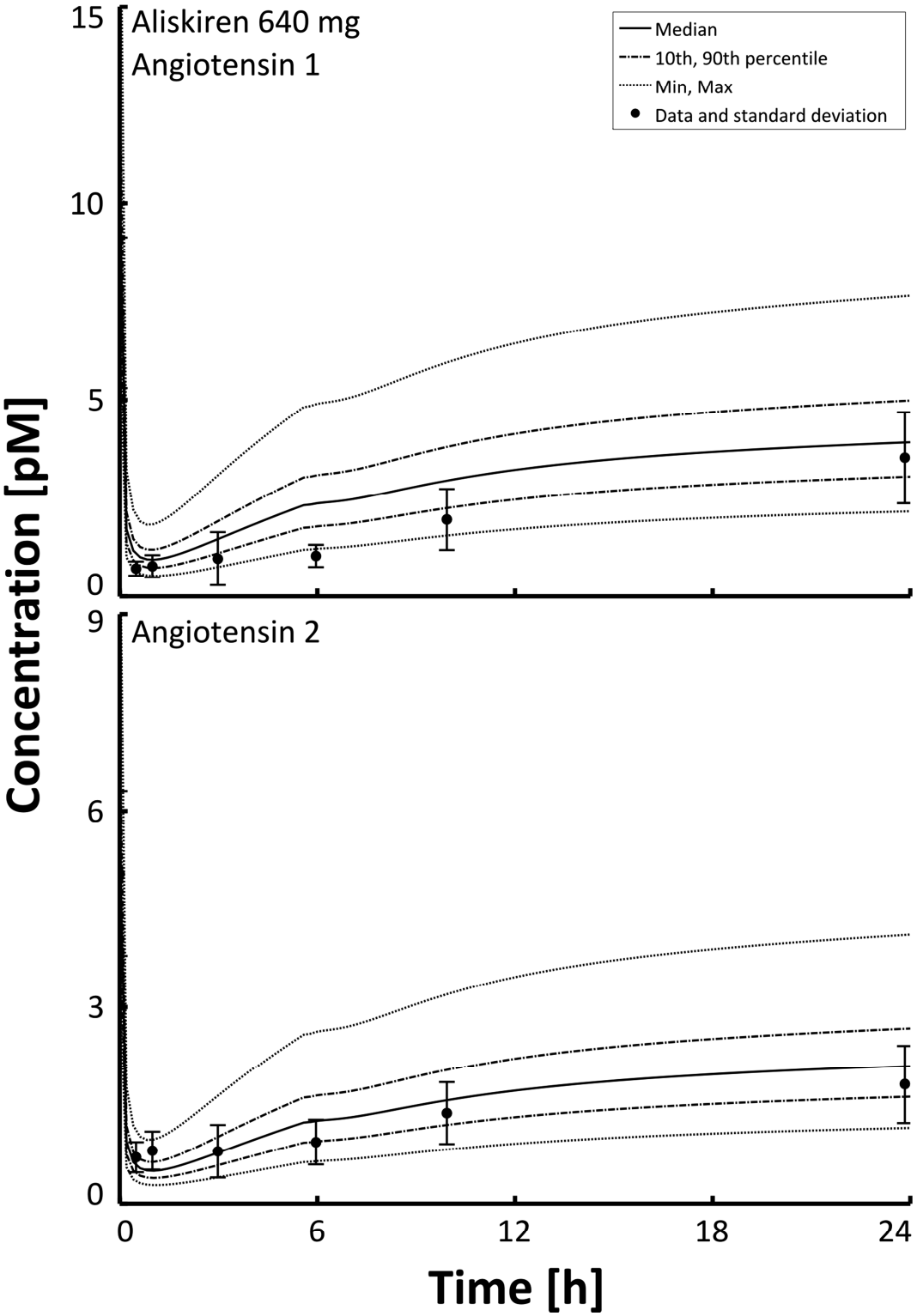

Figure 15. Effect of aliskiren (640 mg) on angiotensin I and II levels. Trial data (dots) with standard deviations are compared with the percentiles as well as minimum and maximum of the simulated data.

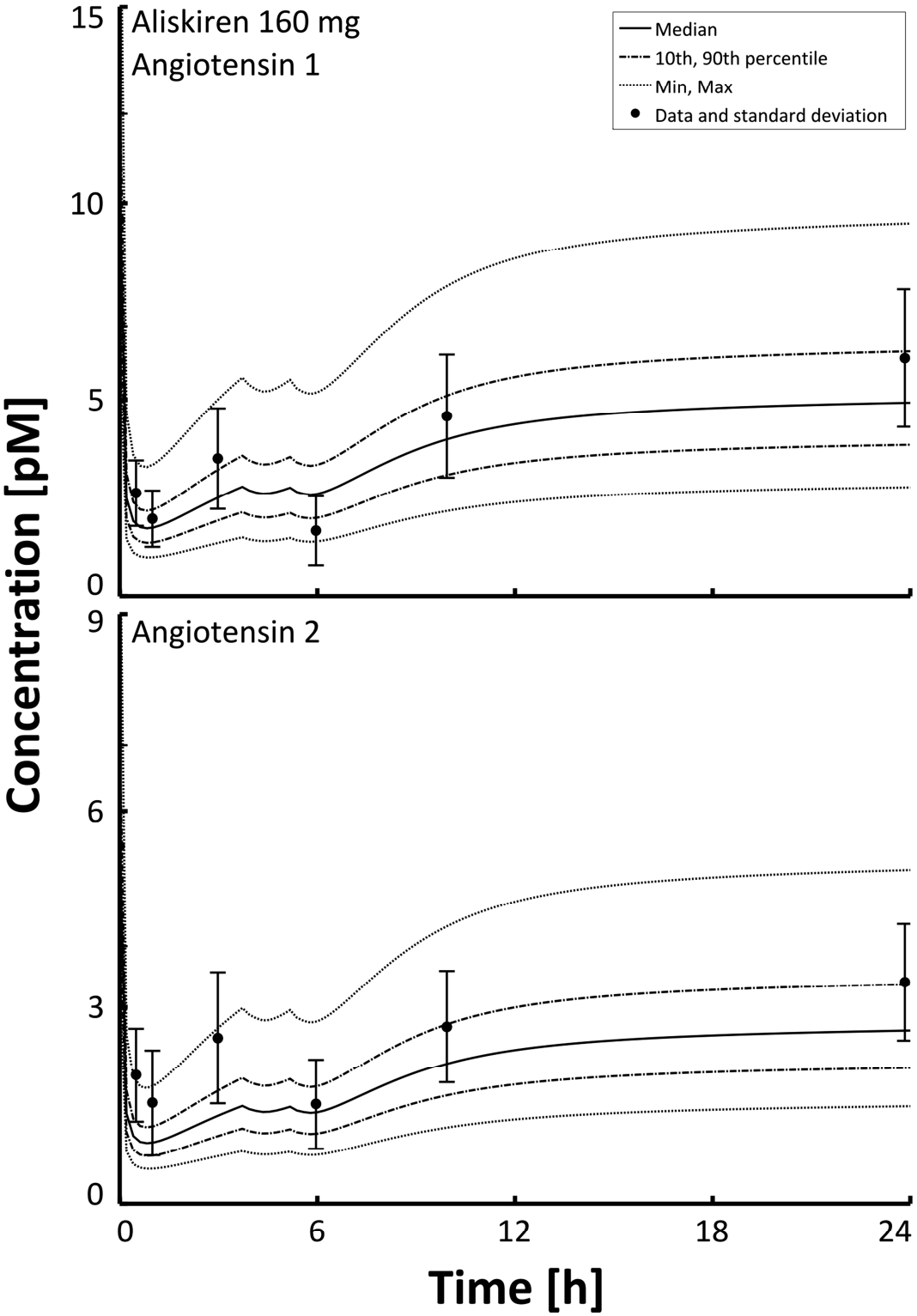

Figure 16. Effect of aliskiren (160 mg) on angiotensin I and II levels. Trial data (dots) with standard deviations are compared with the percentiles as well as minimum and maximum of the simulated data.

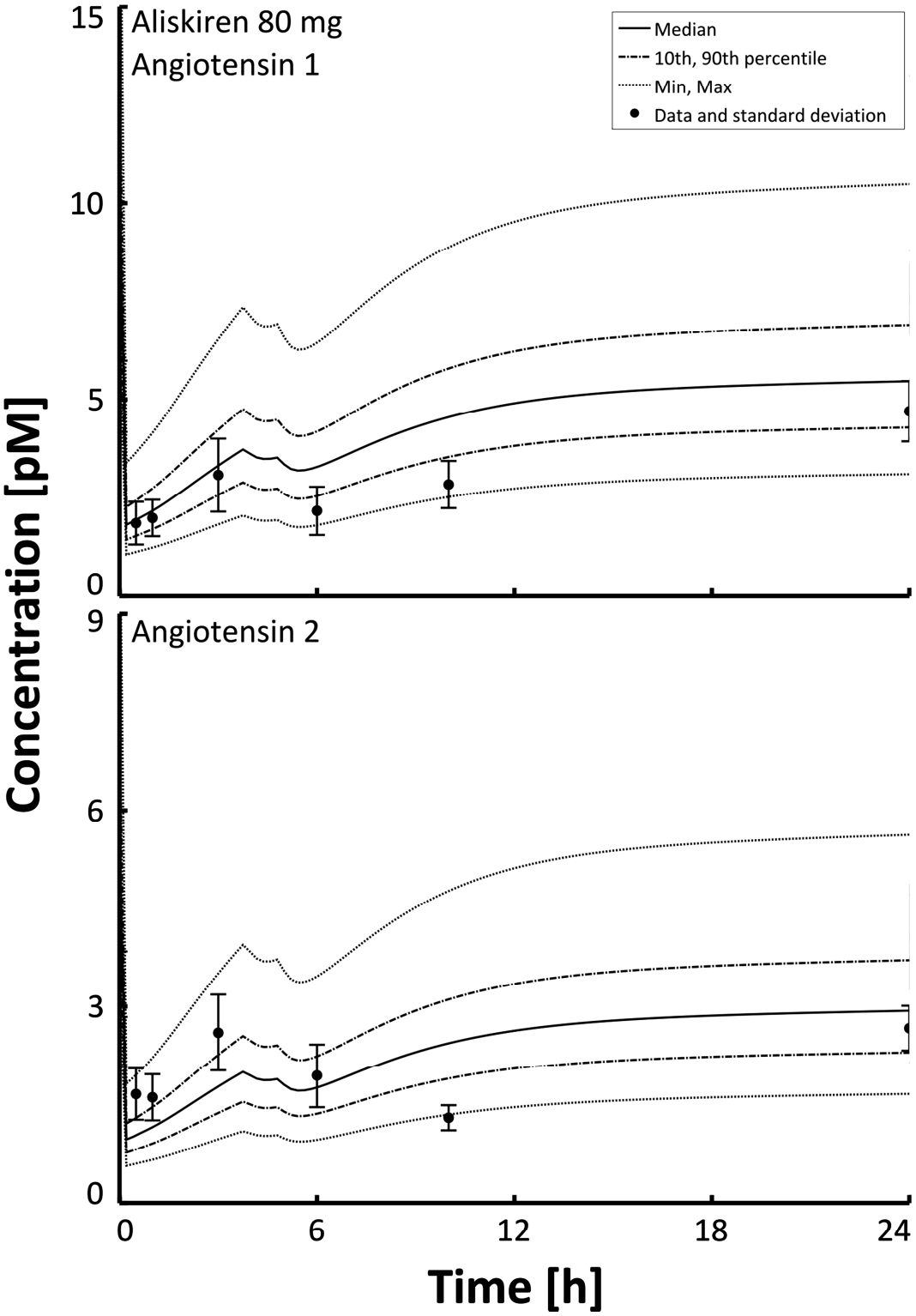

Figure 17. Effect of aliskiren (80 mg) on angiotensin I and II levels. Trial data (dots) with standard deviations are compared with the percentiles as well as minimum and maximum of the simulated data..

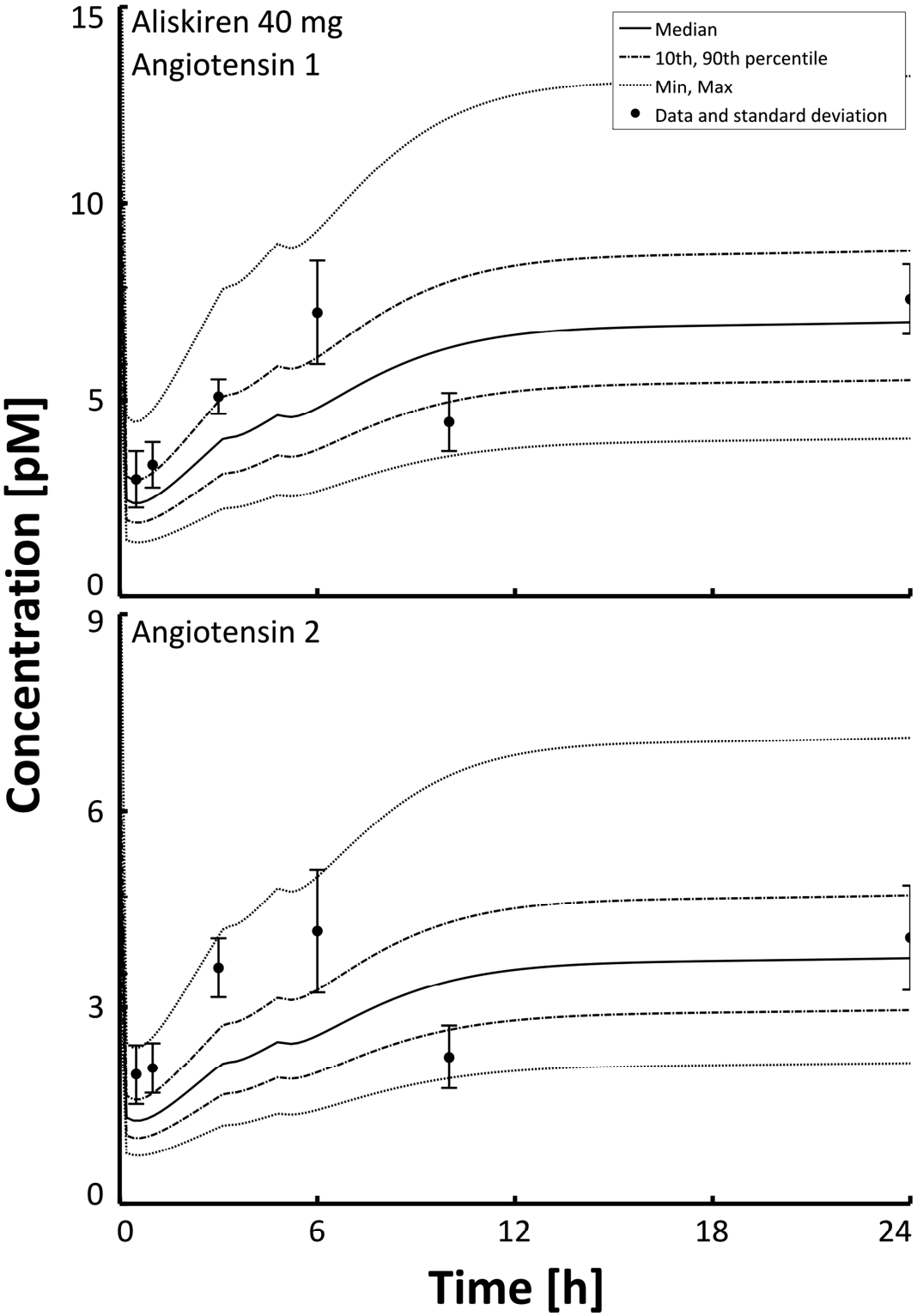

Figure 18. Effect of aliskiren (40 mg) on angiotensin I and II levels. Trial data (dots) with standard deviations are compared with the percentiles as well as minimum and maximum of the simulated data.

4.1.5.Simulation of the effects of losartan on angiotensin II

Angiotensin receptor blockers are assumed to interfere with the suppression of renin secretion by angiotensin II due to a blockade of renal AT1 receptors (Atlas, 2007). Levels of renin, angiotensin I, and angiotensin II rise as a consequence, a result that was supported by the simulation (figure 19). Plasma concentration data was only available for angiotensin II levels during oral administration of 20 and 40 mg of losartan, and this was predicted by the model with an acceptable deviation (20 and 40 mg: MedPE: −6.22% and −6.94 %, respectively, for the 2 doses; MedAPE: 6.22% and 23.9%, respectively). In addition, the model predicted a dose-dependent increase in angiotensin II $AUEC_{last}$ (20 mg R_{pred}: 1.33, 40 mg R_{pred}: 1.59), which was, to a greater extent, also present in the study data (20 mg R_{obs}: 1.62, 40 mg R_{obs}: 2.00).

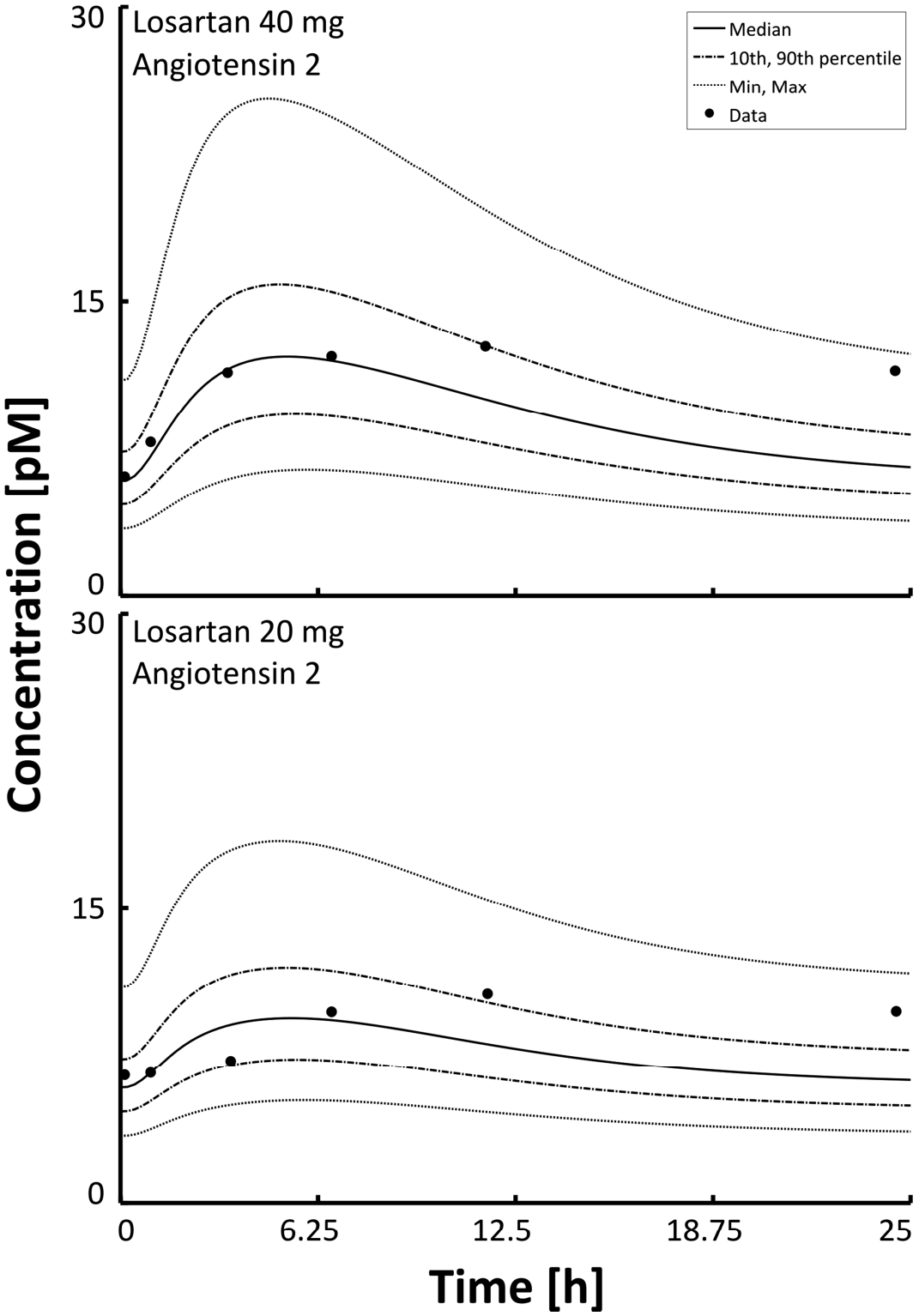

Figure 19. Effect of losartan (40 and 20 mg) on angiotensin I and II levels. Trial data (dots) are compared with the percentiles as well as minimum and maximum of the simulated data.

4.1.6.Sensitivity analysis for the RAAS model

To determine the sensitivity of the RAAS model, the relative change in steady-state concentrations and AUEC values for angiotensin II were quantified by multiplying the PD RAAS model parameters with 0.1–10 times their actual model value. Based on the maximum change obtained for angiotensin II steady-state levels and the AUEC, the parameters $k_{Ang_1_deg}$and $k_{Ang\_2\_gen}$ exhibited the lowest and equal sensitivity (C_{SS} angiotensin II: 0.47-1.2, AUEC angiotensin II: 0.38-1.06-fold change). $VMAX_{Renin}$, k_{Renin_deg}, $k_{Ang\_2\_deg}$, and $IC50_{Ang\_2}$ exhibited higher sensitivities (C_{SS} of angiotensin II: 0.22-, 3.28-, 3.28-, 0.33- to 2.38-, 0.43-, 0.42-, 2.56-fold, respectively; AUEC of angiotensin II: 0.33-, 2.25-, 3.02-, 0.31- to 2.38-, 0.43-, 0.42-, 2.56-fold, respectively).

4.1.7.Intravenous enalaprilat

The administration of 10 mg IV enalaprilat was simulated for the representative individual and compared to data in the published literature. The simulation followed the trend of the published data, but slightly over-estimated the tight-binding phase (figure 20). Based on MedPE and MedAPE values (table 5), it reflected better the data for the elderly population collated by Hockings et al. (Hockings, 1986). Urinary excretion 24h after enalaprilat administration was adequately captured (90% vs 72-99% (Vlasses 1985), figure 20).

4.1.8.Oral enalapril

The author achieved success in identifying a single parameter set for GET, gastrointestinal transit time, intestinal permeability, conversion clearance, and intrarenal enalapril conversion that integrated all data on plasma concentration-time curves of enalapril and enalaprilat, oral bioavailability of enalapril, and the renal excretion of enalapril and enalaprilat. For oral enalapril and the formation of enalaprilat, the resultant plots showed a good correlation between the simulated results and the data, with no significant bias (figure 21, table 5). The total fraction absorbed as a measure of oral bioavailability was comparable to values in the literature (63.1% vs 53-73% (Vlasses, 1985)), as was urinary excretion of enalapril and enalaprilat after 72h (23.9% and 35.5%, respectively,

vs 20.05±12.06% and 30.41±9.51% (Rippley, 2000)). While the urinary excretion kinetic for enalapril was consistent with the trial data, the model tended to overestimate the initial excretion for enalaprilat at the first three time points (figure 22).

Table 5. Numerical evaluation of enalapril and enalaprilat concentrations relative to data.

		Enalapril		**Enalaprilat**	
Drug	**Dose (mg)**	**MedPE (%)**	**MedAPE (%)**	**MedPE (%)**	**MedAPE (%)**
Enalaprilat IV	10				
"young"		23.39 (-11.62-90.36)	23.39 (12.51-90.39)		
"elderly"		-11.67 (-22.53-27.39)	22.53 (11.67-31.19)		
Enalapril (oral)	20	4.47 (-6.24-14.04)	13.97 (6.87-39.14)	5.78 (-21.32-25.19)	28.37 (20.85-76.14)

Data reported as median and 95% CI.

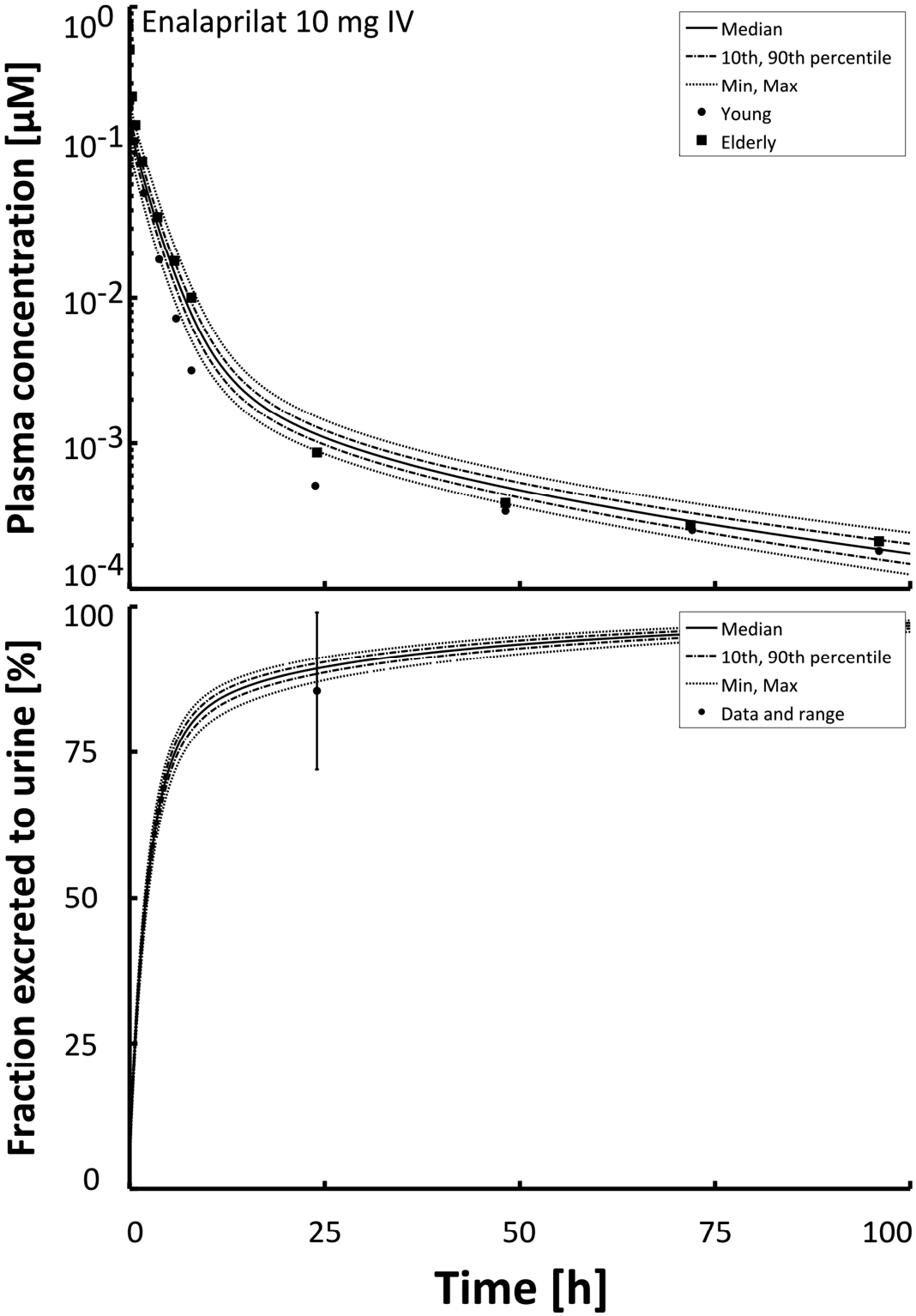

Figure 20. Relation of the PK of 10 mg IV enalaprilat to urinary excretion of enalaprilat. Simulation percentiles (lines) are shown in comparison to study data from young and elderly populations published by Hockings et al. (Hockings, 1986) (dots). The range is depicted for urinary excretion data (Vlasses, 1985).

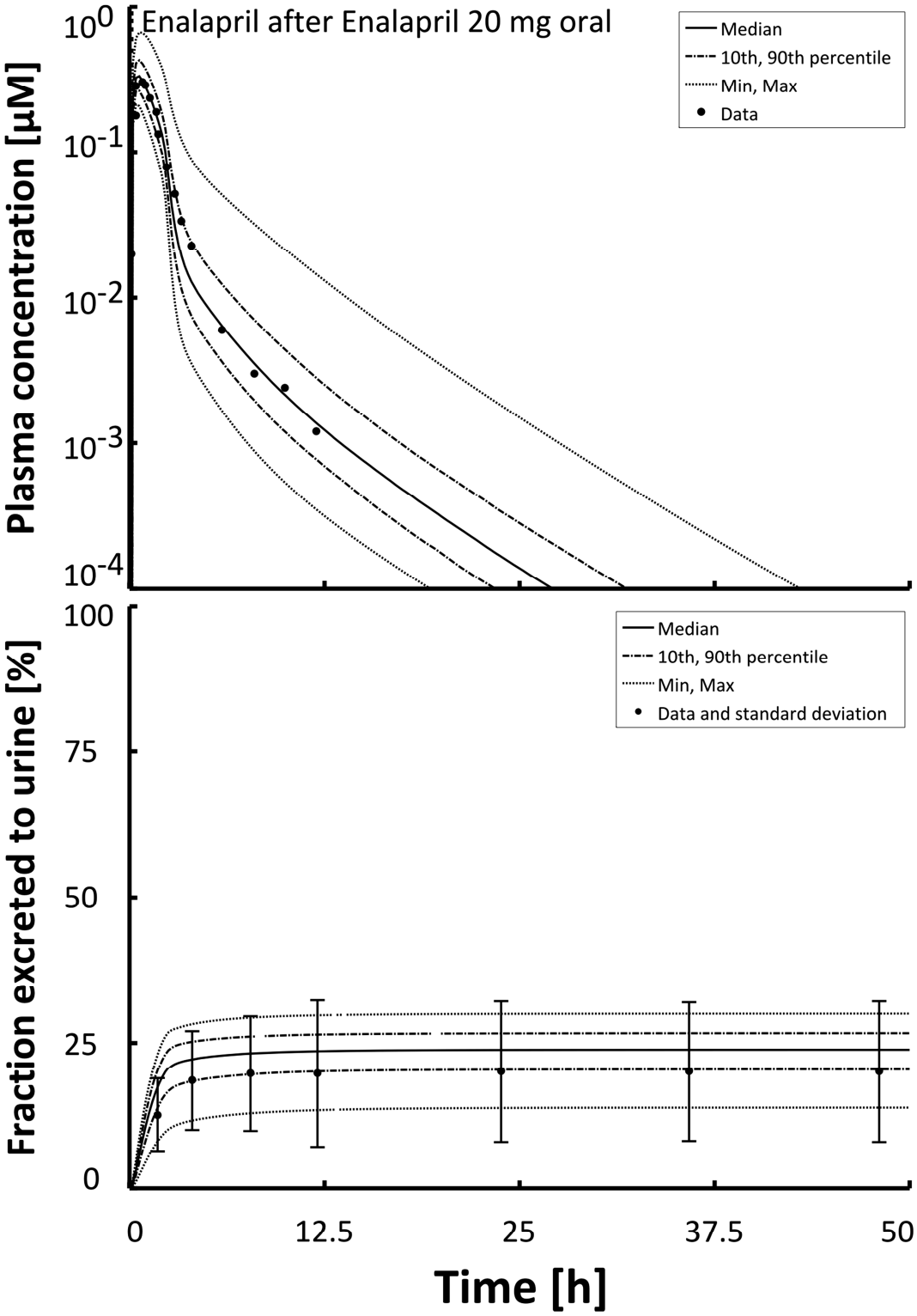

Figure 21. Relationship between the PK of enalapril (20 mg oral) and urinary excretion of enalapril. Simulation percentiles (lines) are shown in comparison with study data values (dots). Standard deviations are indicated for urinary excretion data (Rippley, 2000).

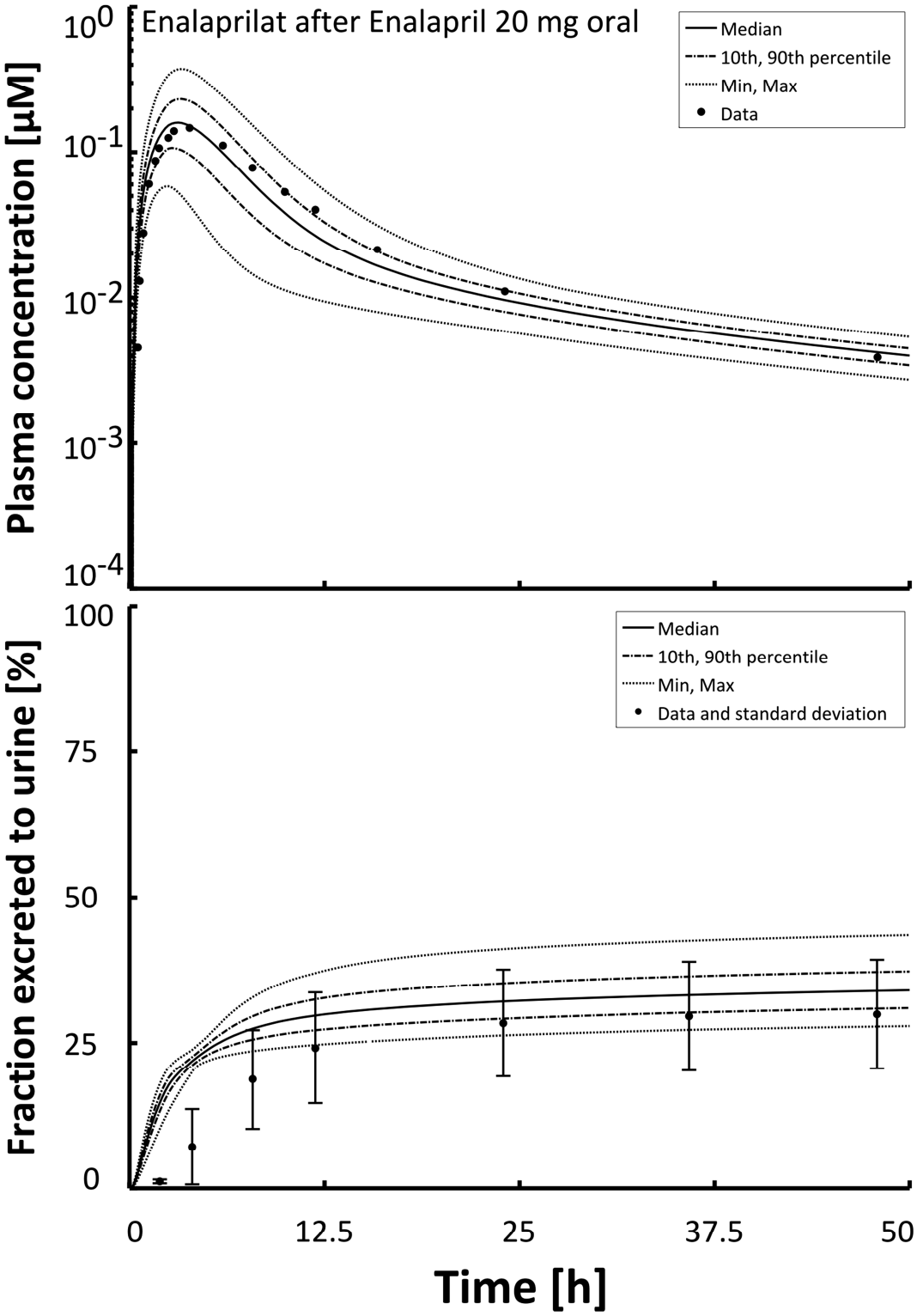

Figure 22. Relationship between the PK of intrahepatically and intrarenally converted enalaprilat (after oral administration of 20 mg enalapril) and urinary excretion of enalaprilat. Simulation percentiles (lines) are shown in comparison with study data values (dots). Standard deviations are indicated for urinary excretion data (Rippley, 2000).

4.2. A HPLC tandem-MS method for determination of enalapril and enalaprilat concentrations in pediatric trials

For conversion of concentrations from ng/ml to µM multiply the former values by $2.66*10^{-3}$, $2.87*10^{-3}$ and $2.36*10^{-3}$ for enalapril, enalaprilat and benazepril, respectively. For conversion of concentrations from µg/ml to µM multiply the former values by 2.66, 2.87 and 2.36 for enalapril, enalaprilat and benazepril, respectively.

4.2.1. Chromatography results, linearity, precision and accuracy

Retention time of the analytes and IS was less than one minute. The shape of all peaks was sufficiently symmetric and sharp to enable automated integration as shown for the upper and lower limits of quantification (ULOQ, LLOQ) (figure 23). The calibration curves were linear within the concentration ranges of 1.61-206 ng/mL (enalapril) and 1.84-236 ng/mL (enalaprilat). The respective calibration models of the analyte/IS ratios versus the nominal analyte concentrations for enalapril ($y=(1.74*10^{-3}\pm1.77*10^{-4})x+6.21*10^{-5}\pm2.16*10^{-5}$) and enalaprilat ($y=(4.41*10^{-4}\pm1.56*10^{-5})x+(-3.73*10^{-5})\pm3.04*10^{-6}$) exhibited a coefficient of determination >0.99. The method yielded acceptable results for precision and relative errors as a measure of accuracy (table 6). Precision was calculated using one-way ANOVA on the data measured with QC standards. For enalapril, intra-run precisions ranged between 3.46-5.6% and inter-run precisions ranged between 5.1-6.45%, while the relative error ranged between 1.35-5.68%. The corresponding results for enalaprilat were 5.11-5.86% for intra-run precision, 5.65-6.22% for inter-run precision, and 0.2-1.59% for the relative error. The standard deviations of the relative errors were below 7% for both analytes.

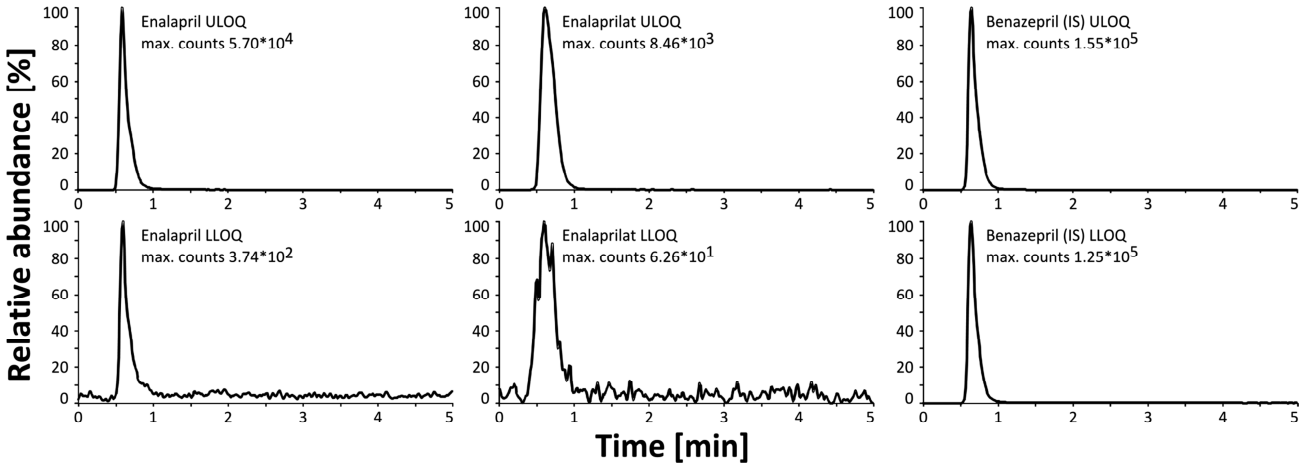

Figure 23. MRM chromatograms for enalapril, enalaprilat and IS from extracted samples at the lower (L) and upper (U) limits of quantification (LOQ).

Table 6. Precision and accuracy results for enalapril and enalaprilat.

Enalapril					
Concentration level	**Concentration added (ng/mL)**	**Concentration found (ng/mL)**	**Intra-run RSD (%)**	**Inter-run RSD (%)**	**Relative error (%)**
LLOQ	1.61	1.64±0.08	3.46	5.1	1.35±4.75
Medium	12.88	13.61±0.7	5.52	5.02	5.68±5.46
ULOQ	206	214.04±13.31	5.6	6.45	3.91±6.46
Enalaprilat					
Concentration level	**Concentration added (ng/mL)**	**Concentration found (ng/mL)**	**Intra-run RSD (%)**	**Inter-run RSD (%)**	**Relative error (%)**
LLOQ	1.84	1.88±0.11	5.86	6.22	0.2±0.78
Medium	14.75	14.79±0.86	5.77	5.86	0.29±5.85
ULOQ	236	237.31±13.06	5.11	5.65	0.56±5.53

Data presented as mean±standard deviation (SD) or relative standard deviation (RSD). ULOQ: upper limit of quantification; LLOQ: lower limit of quantification.

4.2.2.Recovery and matrix effects

Recoveries ranged from 75.92-88.69% for enalapril, from 76.13-92.62% for enalaprilat, and from 74.71-88.49% for IS, all with relative standard deviations (RSD) below 10% (table 7). The values were comparable with values in the literature (Lima, 2009; Gonzalez, 2010; Lu, 2009; Najib, 2003) and higher than those attained by liquid-liquid extraction (Gu, 2004). Matrix effects were pronounced in this setting, leading to an ion suppression of (−70.14)-(−76.87)% for enalapril, (−63.11)-(−78.45)% for enalaprilat and (−61.39)-(−69.82)% for IS. The matrix effect showed good reproducibility (RSD <4% for all measured standards). The process efficiencies for the SPE extraction procedure were between 16.4-33.41% for both analytes and for IS.

Table 7. Recovery, matrix effect and process efficiencies for enalapril, enalaprilat and benazepril.

Enalapril concentration (ng/mL)	**206**	**51.5**	**12.88**	**3.22**	**1.61**
Recovery mean ±SD (%)	75.92±4.86	83.11±8.59	72.6±6.74	83.95±8.13	88.69±6.26
Matrix effect mean±SD (%)	−71.68±1.45	−75.15±0.85	−76.87±1.13	−75.14±0.83	−70.14±1.64
Mean process efficiency (%)	21.5	20.65	16.79	20.86	26.48
Enalaprilat concentration (ng/mL)	**236**	**59**	**14.75**	**3.69**	**1.84**
Recovery mean ±SD (%)	92.62±4.48	87.19±8.63	76.13±6.54	79.62±8.78	90.59±7.67
Matrix effect mean±SD (%)	−74.41±1.53	−78.7±0.64	−78.45±1.45	−71.31±1.28	−63.11±1.18
Mean process efficiency (%)	23.7	18.57	16.4	22.84	33.41
Benazepril Concentration (ng/mL)	**187.3**	**4.83**	**11.71**	**2.93**	**1.46**
Recovery mean ±SD (%)	88.49±6.18	84.89±9.4	74.71±8.11	83.76±7.92	77.82±12.54
Matrix effect mean±SD (%)	−66.91±1.66	−70.68±1.2	−69.86±1.84	−67.18±3.46	−61.39±2.9
Mean process efficiency (%)	29.28	24.88	22.51	27.49	30.04

Data presented as mean or mean±standard deviation (SD)

4.2.3.Selectivity

No interacting peaks of concomitant drugs or hemolyzed blood were observed. The determined serum concentrations of spiked patient sera samples relative to the spiked blank sera were in the ranges of 98.3-109% for enalapril and 90.4-111% for enalaprilat, respectively, indicating that no significant ion suppression or enhancement occurred. Further, no crosstalk effect was detectable between the MRM channels.

4.2.4.Stabilities

Residual substance content of the stock solutions after 3 and 6 months of storage were calculated at 103.02±5.41% and 103.21±4.13% for enalapril, 97.09 ±0.5% and 90.18±2.14% for enalaprilat, and 96.46±2.84% and 98.30±2.76% for IS, respectively. The results of stability testing across all the steps of sample processing have been summarized in table 8. The acceptable stability pattern indicated that the method was applicable for routine analysis of pediatric blood samples.

Table 8. Analyte stabilities for enalapril and enalaprilat.

Storage Parameter Tested	**Concentration added (ng/mL)**		
	1.61	**51.5**	**206**
Enalapril	**Concentration recovered (ng/mL)**		
Long-term (−20°C for 30 days)	1.5±0.08	49.12±4.56	196.8±23.44
Short-term (21°C for 24h)	1.52±0.15	49.98±2.3	201±9.46
Post-preparative (21°C for 24h)	1.56±0.1	48.5±3.39	199±13.29
Three freeze-thaw cycles	1.6±0.1	49.5±3.22	203±10.54
	Concentration added (ng/mL)		
	1.84	**59**	**236**
Enalaprilat	**Concentration recovered (ng/mL)**		
Long-term (−20°C for 30 days)	1.88±0.18	60.92±3.87	246.2±10.21
Short-term (21°C for 24h)	1.88±0.09	57.48±2.56	249.2±11.17
Post-preparative (21°C for 24h)	1.83±0.16	60.08±4.99	244.6±31.29
Three freeze-thaw cycles	1.97±0.17	58.94±4.13	240±12.44

Data presented as mean±standard deviation (SD)

4.2.5.Application

The method was applied to determine the serum concentrations of enalapril and enalaprilat in a healthy male volunteer after oral administration of 20 mg enalapril maleate (figure 24). The main PK parameters were C_{max}: 226 ng/mL and 43.9 ng/mL, t_{max}: 0.68 h and 3.28 h, and elimination half-lives of 0.76 h and 5.56 h for enalapril and enalaprilat, respectively.

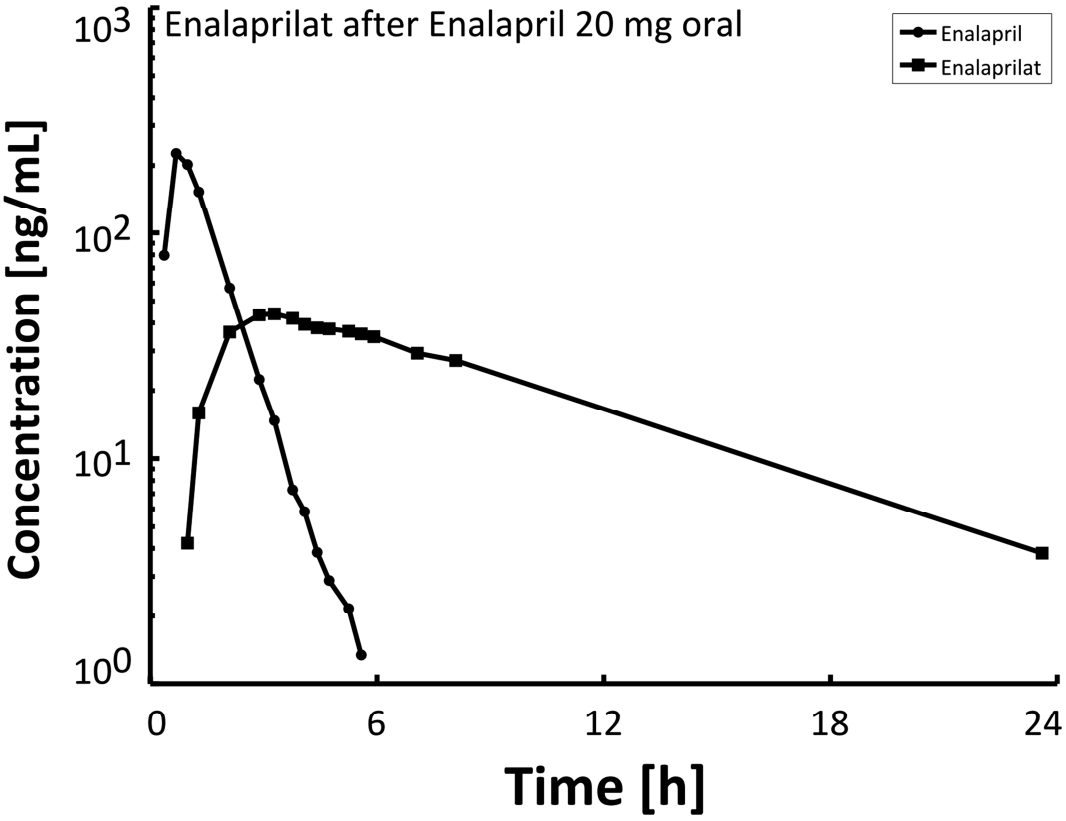

Figure 24. Serum concentration-time profile of a healthy male volunteer after oral administration of 20 mg enalapril maleate.

4.3. Observational study of intravenous amiodarone in children

4.3.1. Patient demographics

The cohort included 20 patients (median age, 0.23 years; range, 0.02–15). Postoperative or congenital JET and SVT were the most common tachyarrhythmias followed by ventricular, permanent junctional reciprocating and focal atrial tachycardia (table 9).

4.3.2. Amiodarone doses

Nine patients received one bolus, two received two boluses, and nine received three 5mg/kg boluses of amiodarone. On average, patients received a total of 221.9 mg amiodarone, and the median sampling period was 59.17 hours (table 9).

Table 9. Observational study of intravenous amiodarone in children: description of study cohort.

Patient No.	Age [years]	BMI Pct [%]	Duration of sampling [h]	Total amiodarone dose [mg]	Primary condition	Indication for amiodarone administration
1	15.04	<0.1	23.50	609.4	Muscular dystrophy type Duchenne, DCM	SVT
2	5.56	32.4	24.83	246.0	Ebstein's anomaly, WPW syndrome	SVT
3	3.75	69.0	26.00	956.3	s/p Ross procedure	JET
4	2.08	76.7	26.00	201.2	MI after Kawasaki fever	VT
5	0.49	<0.1	11.33	47.8	s/p (surgical) correction of pulmonary vein stenosis	SVT
6	0.40	1.9	58.33	263.3	s/p arterial switch for d-TGA	JET
7	0.33	0.4	24.83	70.5	HOCM, Noonan syndrome, s/p (surgical) myectomy	JET
8	0.33	0.6	111.25	370.0	Normal heart	JET
9	0.29	<0.1	10.16	64.7	s/p (surgical) VSD closure	JET
10	0.24	<0.1	244.00	326.3	s/p (surgical) AVSD correction	SVT
11	0.21	0.5	67.37	242.6	s/p (surgical) correction of TOF	JET
12	0.17	<0.1	72.00	122.5	Pulmonary vein stenosis	SVT
13	0.11	26.8	12.25	90.0	s/p (surgical) VSD closure	JET
14	0.08	28.8	136.00	424.4	Normal heart	PJRT
15	0.05	1.0	188.78	281.3	Normal heart	FAT
16	0.04	52.2	192.25	295.7	s/p Norwood I for HLHS	SVT
17	0.04	95.8	48.17	109.9	s/p (surgical) resection of myocardial rhabdomyoma	JET
18	0.02	5.4	104.50	146.3	s/p (surgical) AVSD correction	SVT
19	0.02	46.3	118.83	158.6	Normal heart, s/p asphyxia	VT
20	0.02	84.4	60.00	116.8	s/p (surgical) coarctectomy	SVT
Summary	0.23 (0.02-15.04)	-	59.17 (10.16-244.00)	221.9 (47.8-956.3)		

*age-adjusted percentile in brackets; summary data reported as median and range; AVSD atrioventricular septal defect; BMI body mass index; DCM dilated cardiomyopathy; d-TGA d-transposition of the great arteries; FAT focal atrial tachycardia; G gender; HLHS hypoplastic left heart syndrome; HOCM hypertrophic cardiomyopathy; JET junctional ectopic tachycardia; MI myocardial infarction; Pct percentile; PJRT permanent junctional reciprocating tachycardia; s/p status post; SVT supraventricular tachycardia; TOF tetralogy of Fallot; VSD ventricular septal defect; VT ventricular tachycardia; WPW Wolf Parkinson White syndrome

4.3.3.Analysis of amiodarone serum concentrations in blood samples

A median of 14 samples (range, 6–32) were drawn per patient. The highest absolute volume of blood drawn did not exceed 20 mL, which was substantially below the established upper limit for blood sample collection in children (5 mL/kg over several weeks) (Fanaroff, 2002).

During bolus administrations, marked increases in amiodarone levels were observed, followed by rapid decreases after the start of maintenance infusion (figure 25 to figure 29, table 10). The metabolite DEA was identified in 11 patients. The median interval between the start of the amiodarone therapy and the first appearance of DEA was 52.52 hours (range, 0.32–174 hours). The median DEA serum concentrations were 0.24 µM/L (range, 0.16–0.41 µM/L) at the time of first appearance and 0.24 µM/L (range, 0.19–0.62 µM/L) at final measurement. Patient 8 was excluded from this analysis because the DEA identified in the baseline sample was likely due to previous administration of amiodarone. Patients received a median of 9 (range, 2–13) concomitant drugs, of which phenobarbital could have lowered amiodarone serum concentrations by inducing the CYP3A4 enzyme that metabolizes amiodarone. However, this possibility can be discounted since the median maintenance phase serum concentrations did not differ significantly (p=0.24) between patients naïve to (n=13, median 1.68 µM/L range, 0.46–4.82 µM/L) or receiving (n=7, median 1.83 µM/L, range, 1.27–3.26 µM/L) phenobarbital.

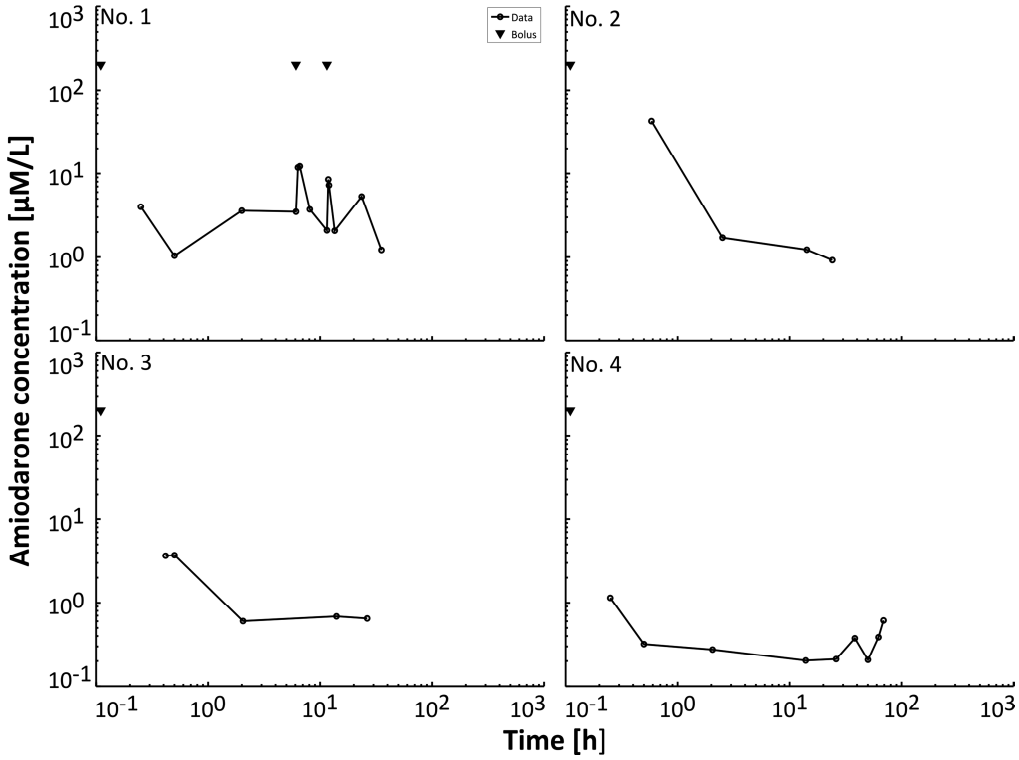

Figure 25. Serum amiodarone time profiles of study patients 1-4 (double logarithmic plots). Dots: measured values, triangles: timing of bolus applications.

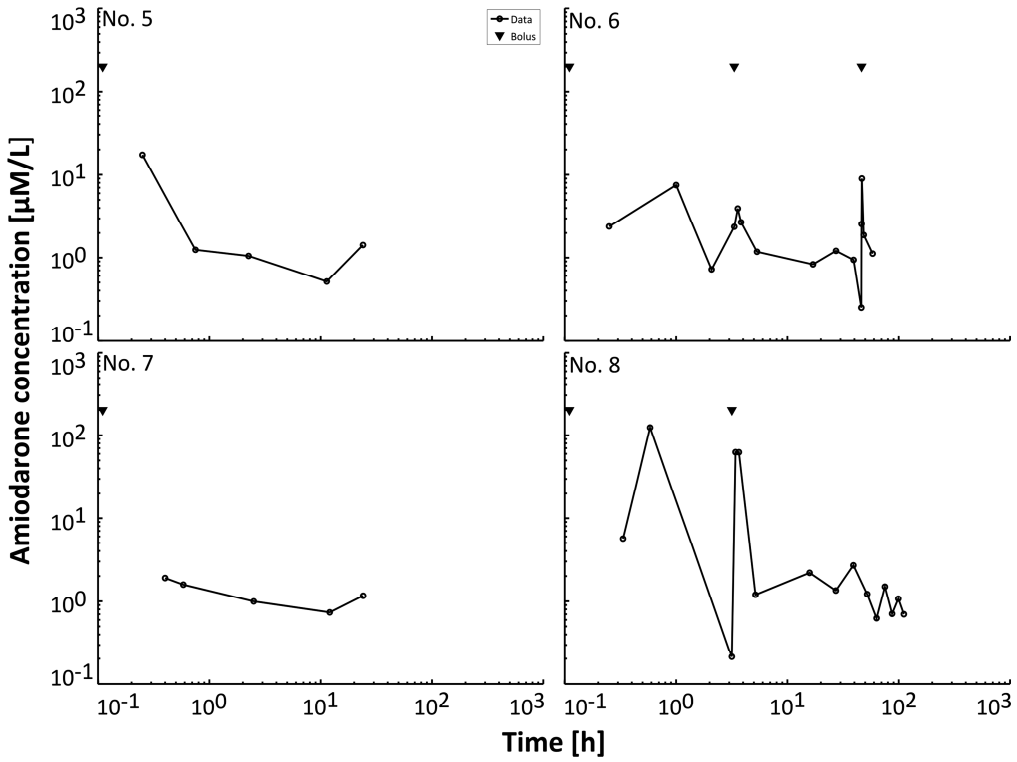

Figure 26. Serum amiodarone time profiles of study patients 5-8 (double logarithmic plots). Dots: measured values, triangles: timing of bolus applications.

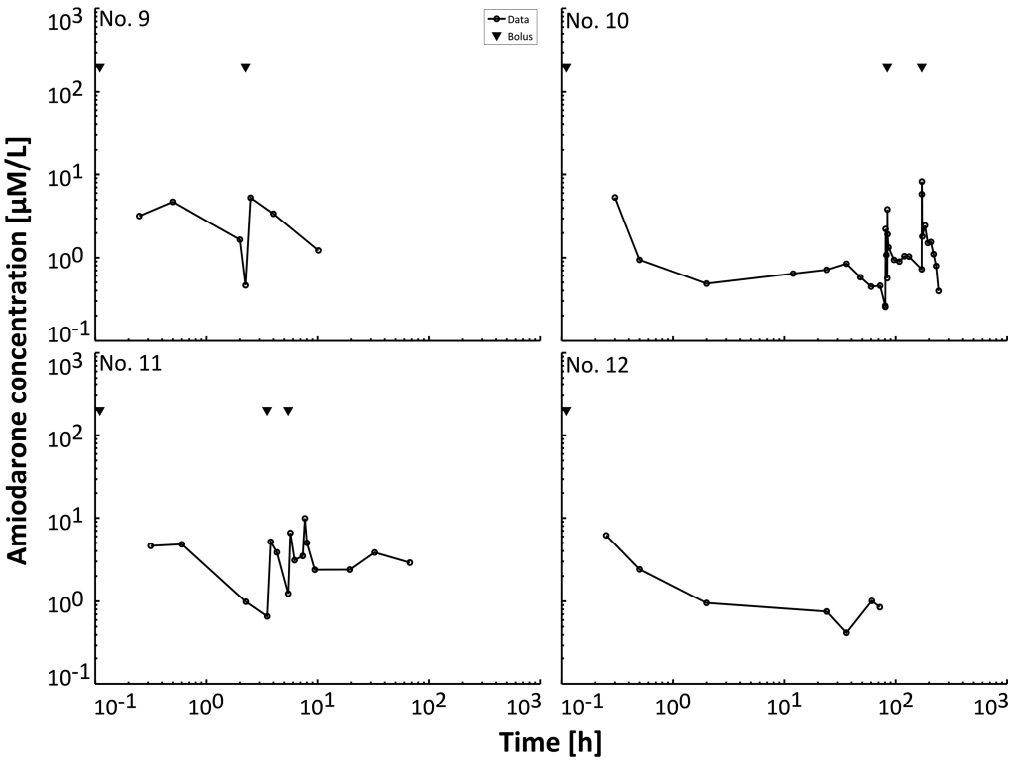

Figure 27. Serum amiodarone time profiles of study patients 9-12 (double logarithmic plots). Dots: measured values, triangles: timing of bolus applications.

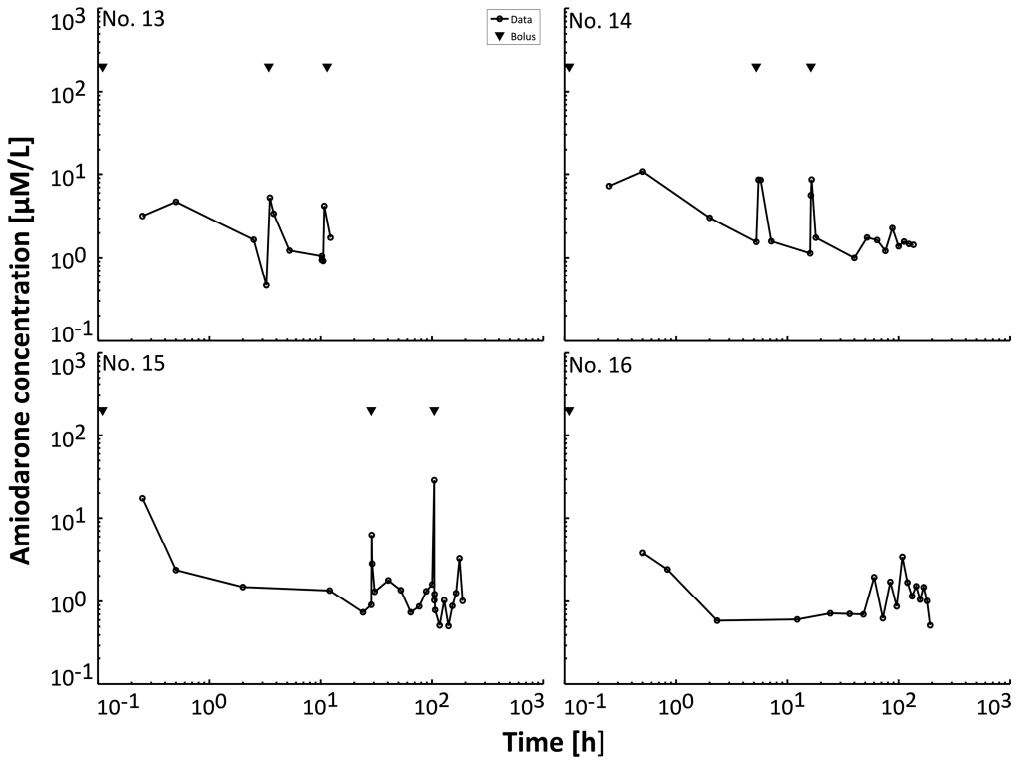

Figure 28. Serum amiodarone time profiles of study patients 13-16 (double logarithmic plots). Dots: measured values, triangles: timing of bolus applications.

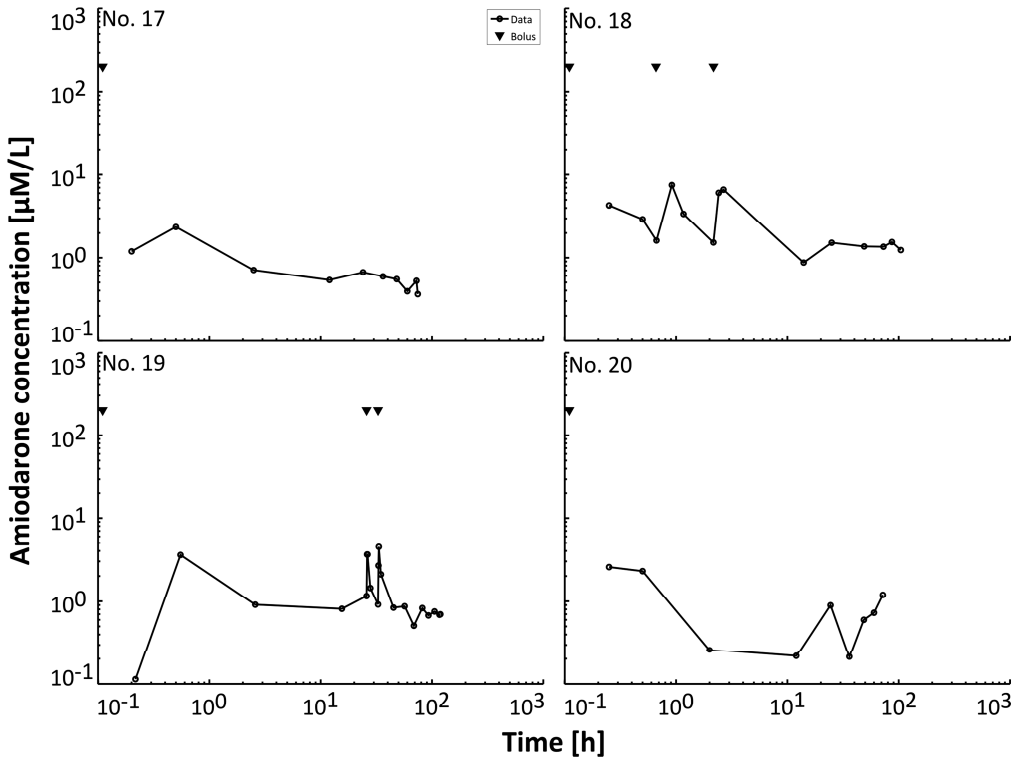

Figure 29. Serum amiodarone time profiles of study patients 17-20 (double logarithmic plots). Dots: measured values, triangles: timing of bolus applications.

Table 10. Amiodarone concentrations during bolus and maintenance phases of treatment.

Bolus and maintenance phase no.	Maximum concentration during bolus phase (µM/L)	Median concentration during maintenance phase (µM/L)
1	7.36 (1.78–191.63)	1.30 (0.31–8.01)
2	9.7 (5.61–97.16)	2.01 (0.89–5.78)
3	13.14 (6.54–45.36)	2.06 (0.62–8.23)

Concentration data reported as median and range.

4.3.4.Blood pressure and heart rate

During the 40 bolus applications, 15 episodes of systolic or diastolic hypotension were observed in 8 (20%) patients. Hypotension lasted longer than 5 minutes in 5 patients, while 1 of them had unstable low blood pressure prior to amiodarone administration. Accordingly, a potentially clinically significant hypotensive episode was noted during 4/40 (10%) boluses. No patients required termination of amiodarone, additional volume load, or adjustment of catecholamine administration to stabilize blood pressure during loading or maintenance administration.

Heart rates below the 50th percentile were noted in 21 boluses of 12 patients and below the 2nd age-adjusted percentiles in 2 boluses each in 1 patient, respectively (table 11). No cases of 2nd or 3rd degree AV blocks were observed.

Table 11. Blood pressure and heart rate data of study patients. All 20 patients received a first bolus, 11 received a second and 9 received a third bolus (all patients received maintenance infusions).

	Applied amiodarone boluses					
	1st bolus		2nd bolus		3rd bolus	
	No. patients (%)	Time (min)	No. patients (%)	Time (min)	No. patients (%)	Time (min)
Systolic BP >20mm/Hg less than baseline (any duration)	4 (20)	11 (1–30)	1 (9.1)	7	0	0
Systolic BP >20 mm/Hg less than baseline with duration >5 minutes	2 (10)	20 (10–30)	0 (0)	0	0	0
Diastolic BP >10mm/Hg less than baseline (any duration)	5 (25)	11 (1–30)	4 (36.4)	5 (2-9)	1 (11.1)	24
Diastolic BP >10mm/Hg less than baseline with duration >5 minutes	3 (15)	16 (6–39)	2 (18.2)	9 (8-9)	1 (11.1)	24
Heart rate less than *50th age-adjusted percentile*	11 (55)	16 (1–30)	6 (54.5)	15 (1–30)	4 (44.4)	13 (1–30)
2nd age-adjusted percentile	1 (5)	12	1 (9.1)	2.5	0	0

BP blood pressure; data are reported as number (%) of patients receiving bolus and maintenance doses.
Durations are expressed as median and range.

4.3.5.QTc intervals

Mean QTc intervals for pre-, during- and post-therapy periods were 443.2 ms (95% CI, 421.6–464.8 ms), 458.4 ms (95% CI, 444.0–472.8 ms), and 467.25 ms (95% CI, 449.7–484.8 ms), respectively. QTc during therapy was not prolonged compared to pre-therapy values (mean difference, −15.2 ms, 95% CI, 34.1–3.7 ms, p=0.11). Pre- versus post-therapy QTc intervals showed a significant mean difference of 24.05 ms (95% CI, −42.9 – −5.2 ms, p=0.01), but there was no significant difference between during-therapy and post-therapy intervals (mean difference, 8.9 ms, 95% CI, −27.7–10.0 ms, p=0.36).

4.3.6.Laboratory assessment

The mean observational period for laboratory parameters was 6 days (range, 2–11 days). Plasma creatinine increases of more than twice the pre-amiodarone values were not observed. The highest creatinine value (146.77 µM/L) occurred in a patient (No. 20) whose creatinine levels had been increasing before amiodarone was administered. Two instances of elevated AST and ALT values and 5 cases of elevated γ-GT levels were identified, but these increases were ≤3-fold higher than baseline values. The median total plasma protein was 50.3 g/L (range, 41–63.0 g/L).

4.3.7.Efficacy

Successful resolution of tachycardia or sufficient suppression of heart rate during junctional ectopic tachycardia was achieved in 18/20 (90%) patients.

4.3.8.Comparison of patient PK data with adult simulations and correlation analysis

Simulated adult patients exhibited higher AUCs than their pediatric counterparts (figure 30 and figure 31).

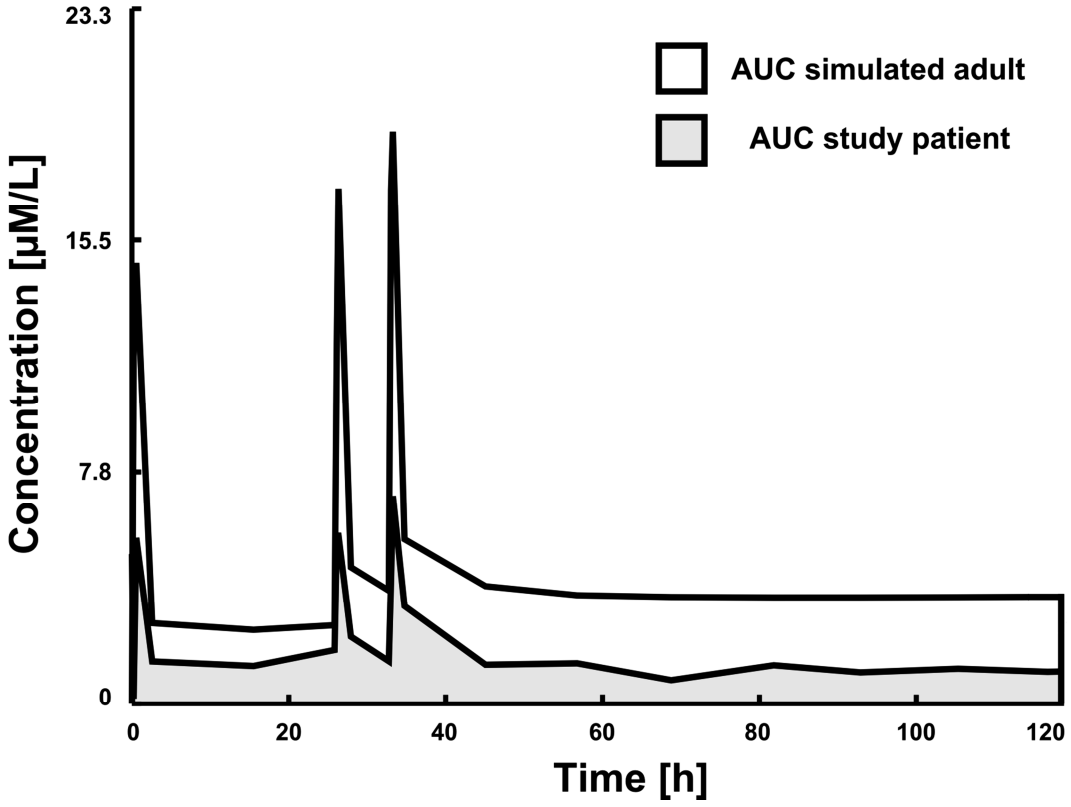

Figure 30. Representative profiles and corresponding AUCs for amiodarone in a study patient (grey) and a simulated adult (white) receiving the same weight-normalized dosing regimen.

As expected from pediatric growth trajectories, age correlated significantly with height ($r=0.95$, $p<0.001$), weight ($r=0.88$, $p<0.001$), and total plasma protein concentration ($r=0.47$, $p<0.04$) values. Further, gender significantly correlated with height ($r= -0.46$, $p<0.04$) and weight ($r= -0.46$, $p<0.04$). Due to collinearity, total plasma protein concentration also correlated with height ($r= 0.57$, $p<0.01$), weight ($r= 0.55$, $p<0.02$), and albumin concentration ($r= 0.47$, $p<0.04$). A correlation also existed between AST and ALT liver enzyme values ($r= 0.59$, $p<0.04$). The relative difference between simulated adult and pediatric AUCs for amiodarone was correlated with age ($r= 0.56$, $p<0.02$), height ($r= 0.59$, $p<0.01$), weight ($r= 0.57$, $p<0.01$), and total plasma protein ($r= 0.74$, $p<0.001$; figure 32).

Visual inspection demonstrated the correlation of relative AUC difference and total plasma protein to be most relevant.

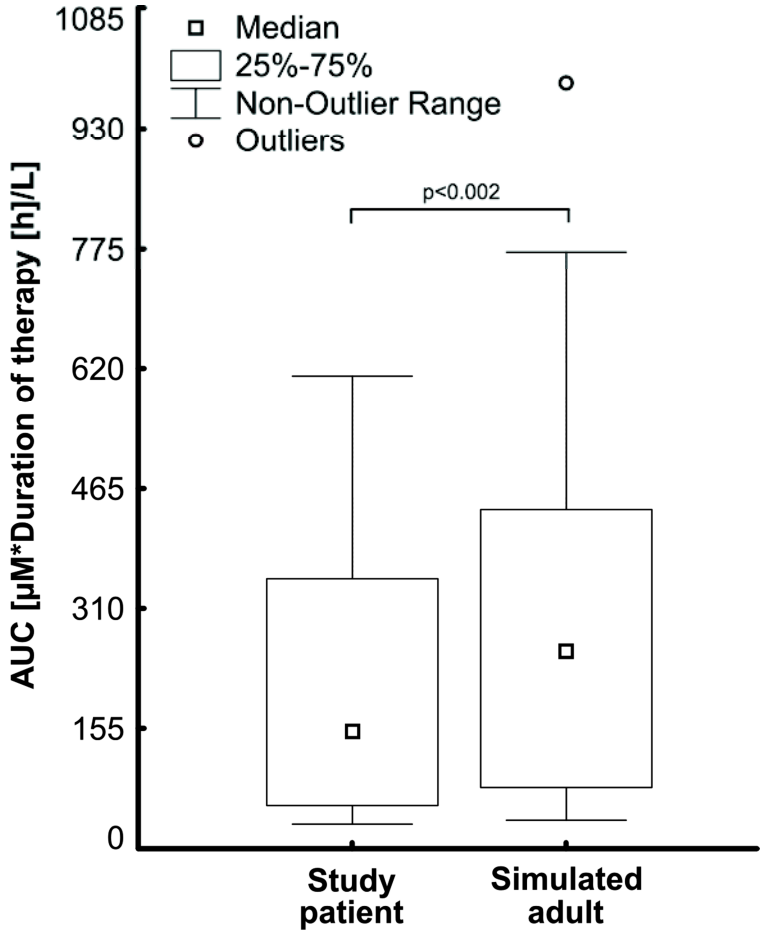

Figure 31. Boxplots depicting the difference between study patients and corresponding simulated adult profiles (p <0.002).

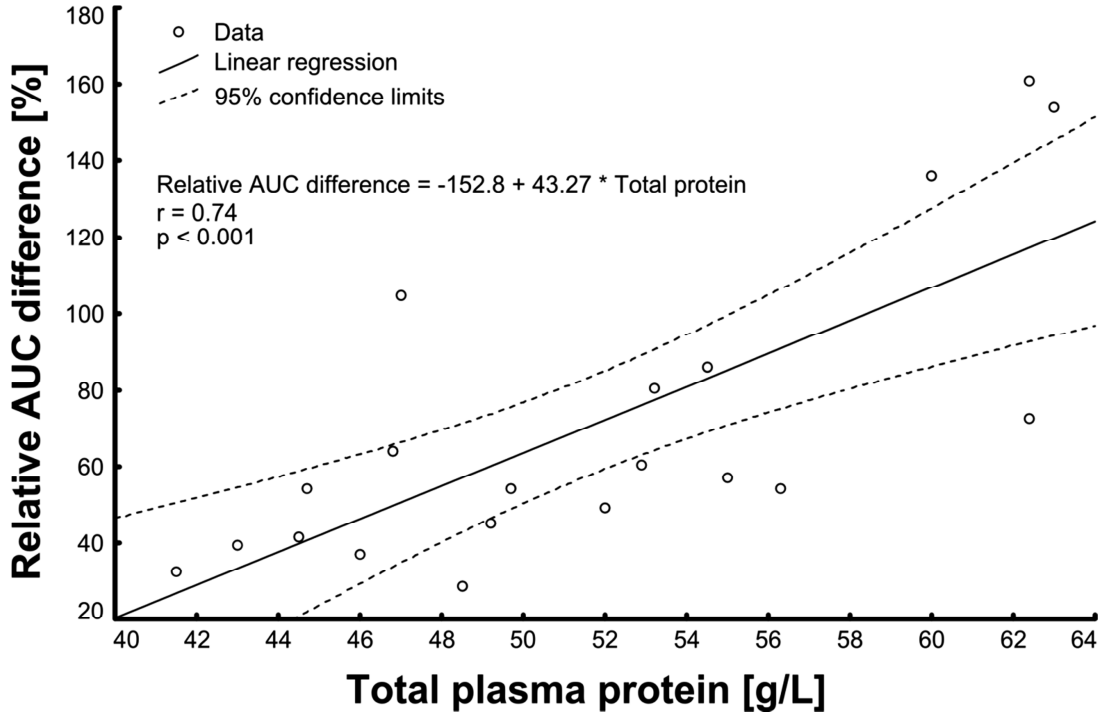

Figure 32. Correlation of relative difference in AUC and total plasma protein.

5. Discussion

In this work, the author has attempted to demonstrate the concepts of modeling and simulation, application of HPLC tandem-MS analysis, and prospective observational study designs as potential tools for improving pediatric clinical investigations.

Modeling and simulation were implemented from both PK and PD perspectives. Focusing on the PK of amiodarone, it was possible to simulate an adult population utilizing a compartmental model and to harness this for model simulations to derive insights on the developmental pharmacology of the drug by comparison with data obtained in a pediatric observational study. Since a real adult cohort for data comparison was lacking, the M&S approach was substantially employed for data analysis and interpretation. From the PD perspective, a physiologically based model mapping the response of the RAAS biomarkers angiotensin I and II following drug exposure was constructed by incorporating in vitro and in vivo data. Further, enhancing this physiologically based PD RAAS model with a physiologically based model for enalapril and enalaprilat PK demonstrated the successful integration of both approaches. Pediatric scaling may be achieved by substituting the adult values for parameters such as angiotensin I and II generation and degradation with pediatric values that take into consideration pediatric growth trajectories, or by utilizing the model to estimate these parameter values based on pediatric angiotensin I and II data after drug exposure. The success of such an approach for physiologically based PK models has already been demonstrated (Edginton, 2006).

HPLC tandem-MS analytical procedures seamlessly integrate into this concept by providing the methodology to quantify enalapril and enalaprilat serum concentrations in small-volume samples from pediatric patients. Such data may then directly serve as inputs into the RAAS model, adding to the potential for pediatric simulations.

How a prospective observational study can be harnessed to generate data that explore safety and efficacy, and provide insights into the developmental pharmacology for amiodarone has been demonstrated here. This is of special relevance to life-threatening pediatric arrhythmias since the observational design offered the opportunity to collate these data without having to devise a placebo-controlled trial design accompanied with tight ethical constraints. The results of this study underline the suitability of the pediatric applied dosing-regimen and may thereby improve clinical certainty in the administration of IV amiodarone for the treatment of children with severe tachycardia.

5.1. A physiologically based RAAS Model

Mathematical models can be used to quantitatively describe the current understanding of physiological systems and their response to drug exposure. Further, physiologically based modeling provides a framework that can integrate diverse sources of data. A novel physiologically based model for the plasma concentrations of angiotensin I and angiotensin II in response to drug administration is presented here. Drug effects modeled include different doses of aliskiren and losartan as well as the administration of benazepril and enalapril. To demonstrate the effective integration of physiologically based PD with a physiologically based PK model, the final configuration also included a physiologically based description of enalapril pharmacokinetics.

Currently, the inhibition of renin represents the most upstream intervention in the RAA cascade. The model described here correctly reflects the effect of renin inhibition in the clinically relevant doses of 40-640 mg of aliskiren and performs comparably to other existing models. The data set generated by Nussberger et al. (Nussberger, 2002) used in this modeling study was also utilized by Hong et al. (Hong, 2008) for the development of a semi-mechanistic model, and parts of the data were used by Guillaud et al. (Guillaud, 2010) for a physiologically based model. Based on a visual comparison of the plasma concentration-time plots, this model predicts plasma concentrations of angiotensin I and angiotensin II as well as the model by Hong et al., including the undulation of concentration in response to aliskiren. Hong et al. interpret this undulation as a circadian rhythm for renin and integrate this into their model. However, their compartmental model of aliskiren does not reflect the double-peaked shape of the aliskiren plasma concentration time curves found in the data set of Nussberger et al. The model presented herein yields an exact representation of aliskiren PK, including the double-peaks, as obtained by mathematical fits. This model therefore describes angiotensin I and II plasma concentrations in response to aliskiren treatment sufficiently, without the need for inclusion of circadian rhythm parameters. For pharmacotherapeutic evaluation of their model, Guillaud et al. used the data pertaining to an aliskiren dose of 640 mg from the study by

Nussberger et al., yielding a normalized root mean square deviation of 34% for angiotensin I and 81% for angiotensin II. For comparison, predictions of the same data by the model described here produce an error of 22.71% for angiotensin I, and 17.4% for angiotensin II.

Inhibition of ACE, the central enzyme of the RAA cascade, results in decreased plasma concentrations of angiotensin II and an increase in angiotensin I concentrations. In general, this model reflects this effect for the administration of 20 and 4x5 mg of the ACEI benazepril and for 20 mg of enalapril sampled over 30 hours, but with some deviations. Mechanistically, the more pronounced rise in the angiotensin I concentration for both drugs may have been caused by a stronger renin induction than predicted by the model, which can be attributed to a more attenuated angiotensin II inhibition or a decreased degradation of angiotensin I. Possible explanations for the slower initial drop in angiotensin II obtained by these simulations compared to the published data include a delayed onset of ACE inhibition caused by diffusion and signal transduction processes, or an over-estimation of angiotensin II degradation. However, in the absence of any published evidence for such mechanisms, these parameters were not considered in the model. Additional error may have been introduced by differences between the study populations of the two source studies for PK data, Juillerat et al. (Juillerat, 1990) and Najib et al. (Najib, 2003). However, this would have only affected enalapril simulations as PK and dynamic information for benazepril originated from the same trial. Since the simulation data deviated from the trial data in both simulations, a major effect of the differences in the study population seemed quite unlikely. Although angiotensin profiles exhibit visual deviation from the data, this does not lead to a statistically significant bias in MedPE-values or produce pronounced differences in simulated versus data-derived $AUEC_{last}$ values. Therefore, it may be concluded that the model has the potential to adequately describe the effect of ACE inhibition on angiotensin I and II.

Blocking of the AT1 receptor represents the furthest downstream pharmacological intervention in the RAAS, causing increases in angiotensin I and II concentrations that have both been adequately predicted by the model. The simulated increase in angiotensin II concentration-time integrals caused by the AT1 receptor blocker losartan is comparable to observed data, with 79.5% and 82.1% of $AUEC_{last}$ values for the administration of 40 mg and 20 mg, respectively. However, data generated by Christen et al. (Christen, 1991) indicates a prolonged action of losartan on angiotensins and is therefore contradictory to simulations that exhibit a more rapid return to steady-state values. This prolonged action may be attributed to the effect of EXP3174, an active metabolite of losartan (Burnier, 2000), which also blocks AT1 in vivo. However, as the PK data used for modeling did not take the effect of EXP3174 into consideration, this model could only reflect the effect of losartan. With the incorporation of such data, the mathematical fit of losartan PK could be replaced by a full physiologically based model accounting for the conversion of losartan to EXP3174, similar to the modeling of enalapril and its metabolite, which would yield a better description of the drug's effect on the RAAS.

In its final configuration, this physiologically based model links enalapril PK to patient demographics such as age and weight, urinary excretion, or hepatic function. Binding parameters for enalaprilat were estimated to simulate IV administration with this physiologically based PK model. The resulting simulations reflect data from the elderly population in the Hockings et al. study better than those from the younger population (Hockings, 1986). In contrast, simulation of oral enalapril did not produce any age-related differences and closely captured the PK of both enalapril and enalaprilat. The initial over-estimation of simulated urinary excretion of enalaprilat after oral enalapril could have been caused by specific binding processes in the kidney that decreased the speed of enalaprilat excretion. However, there is no published data to support this hypothesis. Not all parameters can be described by published values for oral uptake modeling, such that the model also includes estimated values that deviate from actual data. In the model described here, intestinal permeability

was set at 1.02*cm/s*10^{-3}, which differed substantially from published data, which report it to be 2.5-3.5 cm/s*10^{-6} (Morrison, 1996). PK-Sim default values for gastric emptying time and gastrointestinal transit time also required adaptation. In general, defining appropriate parameters for oral uptake in physiologically based modeling proved challenging, and this is illustrated extensively through several data sets. Generic physiologically based modeling of plasma concentration-time profiles for a broad range of drugs yielded medium to high quality simulations for only 23% of substances administered orally, compared to 69% for the IV route (Poulin, 2011). Controversial data that support (Friedman, 1989) or contradict (Morrison, 1996) a functional role for peptide transporters in the oral uptake of enalapril further complicate oral uptake modeling. Considering these challenges, the above mentioned deviations are acceptable since the final parameters chosen for oral uptake allowed for the adequate description of the available data on plasma concentration-time profiles, urinary excretion of enalapril and enalaprilat, and the oral bioavailability of enalapril. This could be important to describe the PK behavior of enalapril in very old, young, and renally or hepatically impaired patient populations.

To describe the diverse mechanisms by which drugs interact with the RAAS in a physiologically based model, it was possible to devise a single set of parameters either from published data or through estimation procedures that closely reflected human physiology. The Michaelis-Menten constant for renin, the angiotensinogen concentration, the half-lives of renin and angiotensin II, and, for the study of drug effects, the $IC50$ values of enalapril and benazepril, the K_I of losartan, and the $IC50$ of aliskiren were either derived from human, animal, or in vitro studies. The degradation of angiotensin I was estimated at 40% of the value published in the literature, but this had little impact as the sensitivity analysis revealed this parameter to be the least sensitive. For several processes of the RAAS and the enalapril PK model, the parameter values were not available from published data. However, based on insights into the underlying biology (such as for angiotensin II generation) or on qualitative knowledge (exemplified by the renal conversion of enalapril to enalaprilat) (Lannoy, 1989; Lannoy, 1990),

plausible estimates for these parameters could be obtained. To obtain these estimates, all available plasma concentrations of angiotensin I and II and all mathematical fits of the different drugs were used within a single step. It may therefore be hypothesized that these estimates are less biased than those obtained by estimation procedures based on sparser data.

In summary, it may be concluded that a physiologically based model for the interaction of the RAAS biomarkers angiotensin I and II with enalapril, benazepril, aliskiren, and losartan that allows for an adequate description of the RAAS response after single administration of the drugs was successfully constructed and validated. The availability of additional data would permit a revision of its modular structure to incorporate long-term drug application. The model would then have to additionally cover “angiotensin escape,” which could lead to attenuated blood pressure reduction during long-term ACE inhibition (Meiracker, 1992), possibly caused by angiotensin II production due to the enzyme chymase (Lorenz, 2010). Additionally, more complex aspects of the RAAS model could include angiotensin converting enzyme 2, angiotensin II cleavage products such as angiotensin 1-7 and their biological actions, RAAS in local tissues, and blood pressure or cardiac hypertrophy.

5.2. A HPLC tandem-MS method for determination of enalapril and enalaprilat concentrations in pediatric trials

Pediatric research demands tailored approaches at all levels. Although methods for the determination of enalapril and enalaprilat via HPLC-tandem MS have already been reported, most of them appear unsuitable for pediatric applications due to their sample volume requirements, which range from 200 µL up to 500 µL (Lee, 2003; Najib, 2003; Gu, 2004; Lima, 2009; Lu, 2009; Gonzalez, 2010; Ghosh, 2011). This volume was reduced to 100 µL in the experiments that were part of this study, thus allowing for more samples to be taken at different time points or for more parameters to be investigated per sample from each individual patient, thereby maximizing the information that can possibly be gathered in a pediatric trial. Precision and accuracy of the assay were consistent with FDA guidance for bioanalytical method validation (US Department of Health and Human Services, 2001). Further, enalapril and enalaprilat were sufficiently stable under storage and assay conditions.

The only reported clinical trial investigating pediatric PK of enalaprilat (Wells, 2001) found the mean concentrations to range from 2 and 25 ng/mL. These concentrations were covered by the linear range of the assay presented here, thereby underlining its applicability for pediatric research. In contrast to the assay utilized by Wells et al., which may lack specificity because it used radiolabeled polyclonal antibodies, the assay presented here used selective mass spectrometry detection and was therefore thought to permit more precise quantification of the concentrations of enalapril and its metabolite, thereby adding to the existing knowledge on the PK of enalapril in pediatric populations. Use of this assay also eliminates safety concerns since it does not call for the use of radiolabeled reagents, a feature that also makes it possible for the assay to be used extensively without the need for specialized facilities or safety oversight.

The selectivity of the HPLC tandem-MS assay in this setting was demonstrated by the observation that the addition of potentially interfering drugs did not result in

ion suppression or enhancement effects. Polypharmacy with beta blockers and antiarrhythmics is common in patients receiving enalapril for chronic heart failure. The results obtained in this study helped exclude the possibility of interference by the drugs frequently administered concomitantly in these patients (eg, sotalol, carvedilol, metoprolol, bisoprolol, and amiodarone). Further, neither the very hydrophilic drug cidofovir nor the use of a hemolyzed sample adversely affected the assay.

In conclusion, the HPLC tandem-MS assay presented here is able to efficiently and accurately determine the concentrations of enalapril and enalaprilat in 100 µL of human serum and complies with the FDA guidance for bioanalytical method validation (US Department of Health and Human Services, 2001).

5.3. Observational study of intravenous amiodarone in children

Prospective observational studies and trials represent an innovative approach that may help overcome some of the hurdles associated with interventional trials, especially when applied to pediatric populations. In the study reported here, serum levels of amiodarone were within the therapeutic range reported for adults, and other safety parameters exhibited no significant changes. A lower amiodarone exposure was detected in the pediatric population compared to adults receiving the same weight-normalized dose, which might have been related to age-related changes in the protein binding characteristics of amiodarone. The data presented here helped increase confidence regarding the use of IV amiodarone in clinical practice and provided additional knowledge on the developmental PK of the drug.

Maintenance amiodarone concentrations (1.3–2.06 µM/L) were within range of those reported for adults treated for atrial fibrillation (1.77±1.5 µM/L) (Galve, 1996) or for sustained ventricular tachyarrhythmia (2.53 (0.77-5.89) µM/L; modified from Horowitz et al.) (Horowitz, 1985). During bolus application, maximum serum concentrations of amiodarone remained below 31 µM/L in the majority of study patients. This result was similar to data in adults from a study by Shiga et al., who reported mean amiodarone serum concentrations of 21.17±5.29 µM/L following the administration of a 5 mg/kg bolus over 15 minutes (Shiga, 2010). However, 3 study patients showed markedly high serum concentrations of up to 191.63 µM/L. Since none of these patients experienced adverse events such as hypotension, bradycardia, or increases in hepatic enzymes, it was considered likely that these samples had been inadvertently contaminated with amiodarone solution when blood was drawn near the IV line.

In this study, the active metabolite DEA appeared later during treatment at sub therapeutic serum concentrations and did not seem to contribute to the overall effect of IV amiodarone in the pediatric population. This result contrasts with the

far higher DEA concentrations reported from studies of long-term oral amiodarone therapy (Kannan, 1987).

Efficacy was achieved in 90% of the patients in the study cohort. This finding is consistent with previously reported trials of the same dosing regimen (Figa, 1994; Soult, 1995; Perry, 1996; Burri, 2003; Laird, 2003), and is higher than the 34% efficacy reported by Chang et al. (Chang, 2010) and the maximum efficacy of 80% reported by Saul et al. (Saul, 2005). The lower amiodarone loading dose (2.5 mg/kg) and maintenance dose (7 mg/kg/day) utilized by Chang et al. could explain the lesser efficacy observed in their study. The decreased efficacy found by Saul et al. could also result from differences in dosing regimens—during the maintenance phase in this study, the drug was administered as bolus applications instead of as a continuous infusion. Serum amiodarone concentrations in the study by Sault et al. were below 1.55 µM/L in all patients, and therefore lower than those measured during the second and third maintenance infusion phases in the patients participating in our study.

The use of IV amiodarone was safe in this cohort as no patient had severe adverse outcomes requiring clinical intervention. The overall hypotension rate of 10% during bolus application in our prospective study resembled rates of 5–13.6% reported in the prospective trial of Saul et al. and was comparable to rates of 0–10% reported in a number of retrospective studies (Figa, 1994; Soult, 1995; Perry, 1996; Burri, 2003; Laird, 2003). Using the same definition as Saul et al., 52.5% of study patients exhibited bradycardia, as against 30% observed by Saul et al. (Saul, 2005). Further, only 5% of the patients had heart rates less than the 2nd age-adjusted percentile, which is within the range reported by Saul et al. (with significant bradycardia being 5-9.1%) and consistent with the 0-10% range described in several retrospective studies (Figa, 1994; Soult, 1995; Perry, 1996; Burri, 2003; Laird, 2003). The hemodynamic safety of the study dosing regimen has also been demonstrated by Haas et al. (Haas, 2008).

No proarrhythmic effect of amiodarone was observed. The median pre- versus post-therapy QTc prolongation was 5.4%, which was less than the 12.12% reported for adults receiving the same IV maintenance dose (Saksena, 1984). Furthermore, changes in laboratory parameters did not indicate toxicity, as there was no case in which plasma creatinine, AST, ALT or γ-GT values increased >3-fold during therapy compared to baseline values.

The model-based analysis detected lower amiodarone exposure in the pediatric patient cohort than in the virtual adult population. This could be attributed to developmental pharmacological changes in protein binding by amiodarone and distribution rather than to changes in clearance. Although increased drug clearance could cause this difference, amiodarone was primarily metabolized by CYP3A4, whose activity was 50% lower in infants below one year of age as has been demonstrated for midazolam, which is also metabolized by CYP3A4 (Wildt, 1999). Clearance also was unlikely to explain lower patient exposure because amiodarone is highly lipophilic (Latini, 1984), resulting in a prolonged distribution phase of up to 231 days (three amiodarone half-lives (Freedman, 1991)) which markedly exceeds the median treatment period of ~60 hours, because of which no steady-state could be attained. During the steady-state, drug concentration in the bloodstream is mainly driven by elimination from the body, but before the steady-state is reached, it is mainly driven by distribution. Pediatric and adult doses are interconverted by allometric scaling by considering the ratio of adult to pediatric weight, and this factor is increased to the power of the allometric scaling exponent. During the steady-state, when clearance dominates drug elimination, this exponent is 0.75, but before the steady-state, this exponent is 1 (Anderson, 2008), as was the case for our study cohort. However, this interconversion only accounts for size differences and not for developmental changes in amiodarone PK. The hypothesis that clearance is subordinate in pediatric IV short-term amiodarone PK is also supported by similar maintenance concentrations observed for study patients, regardless of phenobarbital administration. Phenobarbital potently induces CYP3A4, and animal experiments show that if clearance had predominated, lower concentrations should have

been observed (Fruncillo, 1985). In contrast, this was not demonstrated for patients treated with this drug. Instead, lower pediatric amiodarone exposure may be attributed to increased tissue distribution as a result of lipophilicity and decreased plasma protein binding, which indicates developmental changes in amiodarone PK. Decreased plasma protein binding in neonates and infants has been demonstrated for ampicillin, phenobarbital, disopyramide and other drugs (Ehrnebo, 1971; Wallace, 1976; McNamara, 2002). Further, in this study, AUCs correlate with plasma protein concentrations. These relative differences also correlate with age, height, and weight, suggesting a developmental effect on amiodarone PK as depicted in the schematic drawing in figure 33. Such an effect is also hypothesized by Saul et al. based on observations of serum amiodarone concentrations that vary by age (Saul, 2005). In this pediatric setting, simulating a virtual adult population offers a valuable avenue to further investigate the possible developmental aspects of amiodarone PK in the absence of an adult comparison group.

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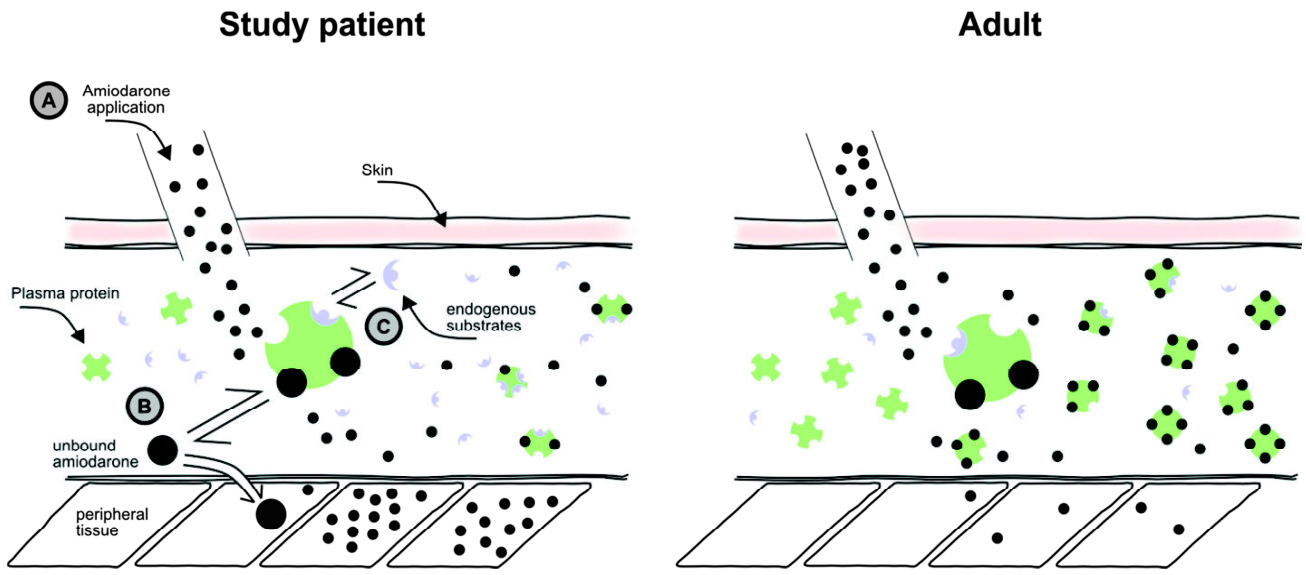

Figure 33. Graph depicting mechanisms of protein binding and distribution of amiodarone into peripheral tissues as a potential cause for lower AUC values in the study compared to adult patients. A) Amiodarone is applied intravenously. B) Only unbound amiodarone can distribute into peripheral tissues; alternatively, it can reversibly be bound to plasma proteins such as albumin, preventing tissue distribution. C) Different endogenous substrates also bind to plasma proteins, hampering the binding of amiodarone by reducing the number of available binding sites. Lower plasma protein concentrations and higher numbers of endogenous substances (Kearns, 2003) in study patients facilitated peripheral tissue distribution, which decreased the AUCs in comparison to adults.

Limitations of this interpretation may result from differences in fluid management, possibly influencing protein levels. A developmental increase in protein levels, however, is well documented in the pediatric population (Kearns, 2003). Physiologically higher levels of endogenous substances like bilirubin are found in children potentially displacing drugs from protein binding (Kearns, 2003). However, no evidence for the relevance of this effect for amiodarone is available in the literature.

In summary, data generated by this study may increase confidence in the clinical use of amiodarone and encourage providers to use an adequate, safe, and effective IV dosing regimen to treat severe pediatric arrhythmia in routine clinical practice. Harnessing a model-based analysis enables us to hypothesize the potential developmental impact of plasma protein binding on the PK of amiodarone.

6. Conclusion and perspectives

In the first part of the thesis, the strategy of physiologically based modeling allowed for the construction of a novel integrated RAAS platform predicting angiotensin I and II profiles in response to drug applications. For research on antihypertensive drugs or drugs for use in congestive heart failure in pediatric patients, such a model may, in the first place, direct investigational attempts by facilitating the study of the growth trajectories of the model parameters, which is essential for pediatric scaling. This may be done by in vitro determination of values for parameters such as angiotensin I and II generation or degradation. On the other hand, the model may also be utilized for estimation of these parameters based on pediatric in vivo data on angiotensin I and II levels following drug administration. This final in silico framework of a model that encompasses the whole spectrum of RAAS responses following drug exposure spanning neonates, children, adolescents and adults provides a major step towards bridging from adult to pediatric drug safety and efficacy aiming at approval by the regulatory authorities. In pediatric cardiology, large-scale trials for drug approvals in the area of off-patent drugs are currently not realistic facing the constraints of small patient numbers available, leading to long lasting multi-center recruitment and consequently to enormous costs associated. Bridging may overcome this obstacle and, in fact, currently represent the only feasible way of drug approval for children currently receiving off-patent medication for cardiovascular disease. The attempt to enhance treatment options for the severe condition of pediatric dilated cardiomyopathy with oral enalapril can visualize this approach. In a bridging study for enalapril in pediatric dilated cardiomyopathy, based on the knowledge of a similar pathophysiology in adults and children, an attempt can be made to link safety and efficacy data from adults to children if drug exposure is comparable. The RAAS model can exactly serve that purpose: not only are physiologically-based descriptions of drug pharmacokinetics integrated to predict drug exposures, but also the response of angiotensin I and II to drug administrations can be predicted. Furthermore, the same processes may also be described backwards from adults to neonates yielding the extent of drug responses through the spectrum of human ageing.

The small number of patients realistically enrolled on a pediatric trial even with the constraints regarding blood sampling can now serve the purpose to inform the model predictions. Then, if similar weight-normalized drug exposures, accompanied with similar pharmacodynamic response are attained, bridging based on this holistic pharmacokinetic and – dynamic model along with the small size pediatric trial can be regarded as successful. For this approach, the RAAS model would also need the pediatric serum concentration-time profiles of enalapril and enalaprilat as additional input parameters and as confirmatory data. The presented HPLC tandem-MS assay is ideally poised for this research attempt, since it is precisely developed to serve the needs of pediatric investigations. The low sample volume required (100 µL) permits its application throughout all pediatric age groups from adolescents down to neonates. Also, due to the low volume demand, less blood is needed for a given number of samples or vice versa, more samples may be taken to enrich PK data while keeping the total blood volume constant. For more informative trial designs not solely focussing on PK, the saved blood volume may also be harnessed to determine humoral pharmacodynamic biomarkers, such as angiotensin I and II levels, which are crucial for pediatric bridging. The high selectivity of the HPLC tandem-MS assay ensures that the determination is not adversely affected by concomitant medications likely to be encountered in severely diseased children.

The second part of the thesis demonstrated observational trials as an innovative methodology to gain clinical practice efficacy and safety data of intravenous amiodarone in the treatment of life-threatening pediatric tachycardia. The observational design simplified the integration of the study into clinical routine without compromising patient treatment. The study was readily accepted in the pediatric intensive care unit since its protocol did not include “blinding” or placebo controls which may have been problematic due to a marked lack of equipoise among the physicians in charge. Continuous monitoring and recording of blood pressure and heart rates during intravenous amiodarone administration allowed further assessment of their changes during therapy. Although rates of bradycardia or hypertension resembled those reported as adverse events in

previous studies, in this study neither bradycardia nor hypertension warranted any clinical intervention. It may be assumed that the clinical effect of those outcomes may have been overrated by previous studies that did not implement such close prospective monitoring. Therefore, a broad implementation of such observational strategies may serve the purpose of defining a specifically pediatric spectrum of outcomes, and would inform future pediatric trial design by providing sensitive trial endpoints. The treatment of adult tachycardia with intravenous amiodarone differs from the pediatric strategy in terms of the timing of amiodarone bolus administrations. As a consequence, an adult cohort receiving comparable amiodarone doses cannot be identified, and pediatric drug exposure cannot be related to adults to gain data on the developmental pharmacology of amiodarone. The simulation of a virtual adult population in the absence of a real one therefore provided a valuable strategy to gain novel insights into the developmental pharmacology of amiodarone and specifically into the importance of plasma protein binding for serum concentrations of the drug. Although amiodarone is known to extensively bind to plasma proteins, this model-based analysis is the first investigation that demonstrates the impact of aging on this process.

All elements discussed in the present work point towards the same direction: facilitating advances in knowledge and patient care despite the restrictions faced by pediatric research. Data on the off-label use of drugs in pediatric populations are perpetually generated, but also frequently lost, since methodologies to prospectively collate and analyze these pieces of information are lacking. However, the broad implementation of observational studies may challenge this current situation. Not only may efficacy and safety data be collected, but also the development of a set of appropriate pediatric drug analyses may enable the gathering of pediatric PK data to an extent yet to be reached. The parallel implementation of modeling and simulation would then help both the analysis of the data gained from the observational frameworks, and the design of pediatric trials, which could subsequently make use of this knowledge for rational choices regarding drug doses and sampling times for drugs and biomarker assessment.

As a conclusion, harnessing these innovative approaches to cardiovascular drug trials and pharmacotherapy in children may finally lead to improved therapeutic outcomes in this vulnerable patient population.

7. References

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8. Curriculum vitae

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EDUCATION

04/2007 - present - **PhD in Clinical Pharmacy**
Department of Clinical Pharmacy and Pharmacotherapy, Heinrich-Heine-University Düsseldorf, Germany
Focus of research: Modeling and simulation of enalapril and amiodarone for pediatric applications; Physiology-based modeling of RAA-system biomarkers; Development of HPLC tandem-MS methods for pediatric trials

02/2007 **Degree in Pharmacy and Certification as Registered Pharmacist in Germany**

10/2001 - 11/2006 - **Study of Pharmacy**
Heinrich-Heine-University Düsseldorf, Germany
Including one-year of research and work experience in the United States and in Germany

RESEARCH EXPERIENCE

04/2006 - 10/2006 - **Academic research placement**
Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, USA
Focus of research: Development and physicochemical characterization of novel polymerizable surfactants with pH-sensitive amphiphilicity and cell membrane disruption for efficient siRNA delivery

08/2004 – 09/2004 – **Pharmaceutical industry work placement**
Computational Chemistry and Biology, BASF AG, Ludwigshafen, Germany
Work included introduction into different molecular modeling techniques

WORK EXPERIENCE

02/2007 – present **Work at pharmacy (supervising pharmacist)**
Finken-Apotheke, Duisburg, Germany
Regular work as locum pharmacist on weekends and holidays; responsible for the operation of the pharmacy

10/2005 – 04/2006 **Work as pre-registration pharmacist at pharmacy**
Apotheke am Kirchplatz, Düsseldorf, Germany

01/2002– 10/2004 **Work as nurse-assistant**
Malteser Hospital, Duisburg, Germany
Nurse-assistant during night-shifts on weekends; responsible for all aspects of patient care under supervision of nurse in charge

PEER-REVIEWED PUBLICATIONS

1. **Ramusovic S**, Paul T, Lagler F, Meibohm B, Läer S. Intensified pharmacokinetic and safety monitoring of a pediatric cohort receiving intravenous amiodarone – the SIMON trial. *Circ Arrhythm Electrophysiol* (under review)
2. **Ramusovic S**, Läer S. An integrated physiology-based model for the interaction of RAA system biomarkers with drugs. *J Cardiovasc Pharmacol*. 2012;60(5):417-428. doi: 10.1097/FJC.0b013e3182676f06.
3. Burckhardt BB, **Ramusovic S**, Tins J, Läer S. Determination of aliskiren in human serum quantities by HPLC-tandem mass spectrometry appropriate for pediatric trials. *Biomed Chromatogr*. 2012. doi: 10.1002/bmc.2815.
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5. Wang XL, **Ramusovic S,** Nguyen T, Lu ZR. Novel polymerizable surfactants with pH-sensitive amphiphilicity and cell membrane disruption for efficient siRNA delivery. *Bioconj Chem.* 2007;18(6): 2169-2177.

PUBLICATIONS WITHOUT PEER REVIEW

1. **Ramusovic S,** Läer S, Breitkreutz J, Bosentan in der Pädiatrie. Die ideale Arzneiform in der klinischen Studie (Bosentan in pediatrics. The ideal dosage form in a clinical trial). *Pharmazie in unserer Zeit*. 2010;39(6):454-458.
2. **Ramusovic S.,** Läer S. Quo vadis, Rimonabant?. Arzneiverordnung in der Praxis 2008;35:129-130.

ORAL PRESENTATIONS AND POSTERS

1. **Ramusovic S.** A systems biology approach for a better use of intravenous amiodarone in children – the SIMON trial. 7th Seminar on "Pharmacokinetics & Pharmacodynamics in Pediatrics", Genua; 2011 (oral presentation).
2. **Ramusovic S**, Frobel A-K, Läer S. Physiology Based Modeling to Support Pediatric Cardiovascular Drug Research - Potential Applications Using Amiodarone and Enalapril as Examples. American Heart Association, Scientific Sessions 2010, Chicago; Circulation 122, A19468; 2010 (oral presentation).
3. **Ramusovic S,** Intravenöse Anwendung von Amiodaron auf der Intensivstation – Chancen und Risiken – Pharmakologische Aspekte (Intravenous amiodarone in the ICU – Chances and risks – Pharmacological aspects). 41st Annual Congress of the German Society for Paediatric Cardiology; October 6, 2009; Weimar (oral presentation).
4. **Ramusovic S,** Willmann S, Läer S. A physiology based model for the Renin-Angiotensin Aldosterone-system. 12th Biannual Congress of the European Society for Developmental Perinatal and Pediatric Pharmacology; June 17-20, 2009; Chamonix (oral presentation).
5. **Ramusovic S,** Willmann S, Läer S. A physiologically based pharmacodynamic model of the Renin-Angiotensin-Aldosterone-System. Annual Meeting of the Population Approach Group Europe (PAGE); 2009; St. Petersburg (poster).
6. **Ramusovic S,** Vogt W, Läer S. Simulation of a virtual paediatric population as a basis for an amiodarone dosing regimen. Cardiology in the Young 18, Supplement 1, 40;2008; (poster).
7. **Ramusovic S,** Vogt W, Frobel A-K, Läer S. Physiologically based modeling for the development of a paediatric dose regimen for oral long-term therapy with amiodarone. In: Proceedings of the 11th ESDP Congress on Safe and Effective Medicines for Children, Rotterdam, pp.90; 2008; (poster).
8. **Ramusovic S,** Vogt W, Frobel A-K, Läer S. Simulation of a virtual paediatric population as a basis for an amiodarone dosing regimen for children. Annual Meeting of the Association for European Pediatric Cardiology (AEPC), Venice; 2008; (poster).