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Child-appropriate analytical technologies as key elements for pharmacokinetic and pharmacodynamic investigations of drugs acting on the renin-angiotensin-aldosterone system

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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 keinen erfolglosen Promotionsversuch unternommen.

Düsseldorf, Björn B. Burckhardt

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III. Zusammenfassung

Mittels der vorliegenden Arbeit wird ein umfassender und regulatorisch konformer Lösungsverbund von analytischen Kleinstvolumina-Applikationen vorgestellt, der die Konzentrationsbestimmung von kardiovaskulären Arzneistoffen über die Zeit wie auch deren Effekte auf das Renin-Angiotensin-Aldosteron-System (RAA-System) in pädiatrischen klinischen Studien ermöglicht. Die so etablierte bioanalytische Plattform stellt einen vielversprechenden Ansatz zur Erhebung dichter Daten in pharmakokinetischen (PK) wie auch pharmakodynamischen (PD) Studien in Kindern jeglichen Alters dar.

Hierzu wurden für die Arzneistoffe Aliskiren und Enalapril wie dessen aktiven Metaboliten Enalaprilat hoch sensitive HPLC-MS/MS Bestimmungsmethoden aus nur 50 µL bzw. 100 µL Serum, Urin und Speichel entwickelt. Neben der Arzneimittelkonzentrationsbestimmung im Serum, wurde die nicht-invasive Bestimmung in Speichel als Alternativansatz untersucht. Des Weiteren wurden die Methoden zur Erfassung der Pharmakokinetik auf einen Probendurchsatz skaliert, welcher den aktuellen Anforderungen an analytische Verfahren im Rahmen von klinischen Studien gerecht wird.

Zur Untersuchung der Effekte einer Pharmakotherapie auf das RAA-System sind immunologische Methoden genutzt worden. Diese ermöglichen die umfassende Bestimmung von Änderungen innerhalb dieses physiologischen Regelmechanismus u.a. durch die Parameter Renin, Angiotensin I, Angiotensin II und die Plasma Renin Aktivität in insgesamt nur 1,1 mL Plasma pro Abnahmezeitpunkt. Die Kombination der PK- und PD-Methoden ermöglicht die Untersuchung der Dosis-Wirkungsbeziehung in Kindern.

Die durchgeführte PK/PD-Studie an gesunden Erwachsenen diente der Verifizierung der Kleinstvolumina-Methoden für zukünftige klinische Studien in Kindern und Jugendlichen. Die angewendete engmaschige Probenentnahme, erlaubte die präzise Beschreibung eines Doppelpeak-Phänomens bei der Absorption von Aliskiren. Die durchgeführten Untersuchungen zur nicht-invasiven Quantifizierung der Arzneimittel im Speichel zeigten für die untersuchten Arzneistoffe keine ausreichende Übereinstimmung zu Konzentrations-Zeit-Profilen in Serum, um zur Substitution geeignet zu erscheinen.

Abschließend wurde ein den „Gute Klinische Laborpraxis“ (GCLP)-Richtlinien konformes Qualitätssystem für das Labor etabliert. Durch die erfolgreiche Durchführung einer Phase I Studie im Rahmen eines pädiatrischen Arzneistoffentwicklungsprogrammes (gefördert durch das FP7 Programm; EU Grant Agreement 602295) konnte die Applikabilität der bioanalytischen Plattform sowie der GCLP-konformen Prozesse gezeigt werden. Die erhobenen Daten liegen in einem pädiatrischen Investigationsplans dem *Paediatric Committee* der Europäischen Arzneimittelbehörde vor (EMA Verfahrensnr.: EMEA-001706-PIP).

IV. Summary

A comprehensive and regulatory-compliant bioanalytical platform is presented that enables the reliable and systematic determination of drugs acting on the RAA system and their pharmacodynamic effects in low-volume samples. This tailored bioanalytical platform empowers sophisticated studies in children across all age groups and helps to gain reliable pharmacokinetic (PK) and pharmacodynamic (PD) knowledge of drugs acting on the RAA system.

Developed bioanalytical HPLC-MS/MS assays enabled the precise quantification of aliskiren, enalapril and its active metabolite enalaprilat in only 50 µL or 100 µL serum, urine and saliva. Beside the current gold standard of drug determination in serum, saliva was investigated as non-invasive alternative to substitute invasive PK sampling. All assays were scaled up to high-throughput approaches meeting current expectations for a clinical setting on sample throughput and corresponding timely duration.

The bioanalytical quantification of pharmacodynamic changes in the RAA system was conducted through immunological assays. Using the parameters renin, angiotensin I, angiotensin II and plasma renin activity, amongst others, the changes in the endocrine system can be sophisticatedly evaluated in a total plasma volume of 1.1 mL per sampling point. Combining both, the PK and the PD assays, a bioanalytical platform was formed which enable to elucidate the relationship between dose-exposure and effect in children.

The platform was developed to address existing limitations in paediatric studies and to improve the availability of information for current pharmacotherapy in children. However, as there is often only one single chance to conduct a clinical trial in children, all assays were validated according to international bioanalytical validation guidelines and its applicability was proven by a proof-of-concept study in healthy volunteers prior the platform will be used in paediatric drug development. Owing to the very dense sampling a double-peak phenomenon in the absorption of aliskiren could be identified. Saliva seemed not suitable to substitute invasive sampling for the investigated drug entities.

Lastly, a “Good Clinical Laboratory Practice”-compliant quality system was enrolled at the bioanalytical laboratory. Imbedding the “ready-to-use” bioanalytical platform into a regulatory-compliant quality environment, the platform performed successfully as part of a Phase I study of a paediatric drug development program (funded by FP7 programme; EU Grant Agreement 602295). Finally, the obtained bioanalytical results could be included into regulatory documents such as Paediatric Investigation Plan (PIP), which was submitted to the Paediatric Committee at the European Medicines Agency (EMA Procedure Number: EMEA-001706-PIP).

V. Table of Contents

I. Erklärung zur Dissertation III

II. Danksagungen IV

III. Zusammenfassung V

IV. Summary VI

V. Table of Contents VII

VI. Figures X

VII. Tables XII

VIII. Abbreviations XIII

1. Introduction 1

1.1 Current developments in paediatric research 1

1.2 Approaches to optimise paediatric study planning and conduction including their limitations 3

1.3 Blood sample volumes in paediatric research and impact on analytics 7

1.4 Renin-angiotensin-aldosterone system 8

1.5 Heart failure and hypertension in children 11

1.6 Aliskiren 14

1.7 Enalapril 16

2. Aim of the thesis 19

3. Methods 21

3.1 Sample purification 21

3.1.1 Solid-phase extraction (SPE) 21

3.2 Chromatographic separation and detection 23

3.2.1 High performance liquid chromatography (HPLC) 23

3.2.2 Mass spectrometry 24

3.3 Immunoassay 26

3.4 Bioanalytics for determination of the drug concentration in different biological fluids 27

3.4.1 Serum 27

3.4.2 Urine 47

3.4.3 Saliva 56

3.5 Bioanalytics for determination of hormones and proteins allowing for the assessment of pharmacodynamics within the RAA system 62
3.5.1 Determination of angiotensin I and plasma renin activity 65
3.5.2 Determination of prorenin 66
3.5.3 Determination of angiotensin II 67
3.5.4 Determination of renin 68
3.5.5 Calculation of angiotensin converting enzyme activity 69
3.5.6 Validation of the immunological assays 69
3.5.7 Applicability of all assays used for determination of pharmacodynamics 69
3.6 Proof-of-concept study 70
3.6.1 Study design and participants 70
3.6.2 Sampling schedule of the drug levels 71
3.6.3 Sampling schedule of the RAA system parameters 72
3.6.4 Determination of drug concentrations for pharmacokinetic evaluation 74
3.6.5 Determination of RAA system parameters 74
3.6.6 Calculation of pharmakokinetic parameters 75
3.6.7 Safety assessments 75
3.7 Establishment of a “Good Clinical Laboratory Practice”-compliant quality system for bioavailability studies in support of marketing authorisation by EMA 76
4. Results 80
4.1 Bioanalytics for determination of drug concentration in different biological fluids 84
4.1.1 Serum 84
4.1.2 Urine 102
4.1.3 Saliva 117
4.2 Bioanalytics for determination of hormones and proteins allowing for the assessment of pharmacodynamics within the RAA system 125
4.3 Proof-of-concept study 127
4.3.1 Study population 127
4.3.2 Determination of drug concentration for pharmacokinetic determination 129
4.3.3 Changes in humoral parameters 138
4.3.4 Change in humoral parameters after administration of enalapril/enalaprilat 138
4.3.5 Safety assessment 142
4.4 Establishment of a “Good Clinical Laboratory Practice”-compliant quality system for bioavailability studies in support of marketing authorisation by EMA 143
5. Discussion 148

5.1 Bioanalytics for pharmacokinetic determination 150
5.1.1 Methods for determination of drug concentrations in serum 150
5.1.2 Method for determination of drug concentrations in urine 152
5.1.3 Method for pharmacokinetic determination in saliva 154
5.2 Bioanalytics for determination of hormones and proteins allowing for the assessment of pharmacodynamics within the RAA system 156
5.3 Proof-of-concept study 158
5.4 Establishment of a Good Clinical Laboratory Practice-compliant quality system for bioavailability studies in support of marketing authorisation by EMA 166
6. Conclusion and perspective 168
7. References 172
8. Appendix 184
9. Curriculum vitae 200

VI. Figures

Figure 1 ***Scheme of the RAA system*** *11*
Figure 2 ***Chemical structure of aliskiren*** *14*
Figure 3 ***Chemical structure of enalaprilat (left) and enalapril (right).*** *17*
Figure 4 ***Capture of enalaprilat by solid-phase extraction*** *22*
Figure 5 ***Overview of adjustable gases, potentials and further parameters as well as their locations in the mass spectrometer (AB Sciex API 2000).*** *26*
Figure 6 ***Typical electrospray mass spectra of aliskiren and benazepril*** *32*
Figure 7 ***Obtained electrospray mass spectrum of enalapril*** *33*
Figure 8 ***Obtained electrospray mass spectrum with modified fragmentation pathways of enalaprilat*** *34*
Figure 9 ***Excerpt of the technical drawing of the heatable water bath*** *45*
Figure 10 ***Scale-up process and optimisations steps.*** *46*
Figure 11 ***Electrospray ionisation mass spectra of all three analytes of interest and the internal standard*** *49*
Figure 12 ***Intended GCLP setting at the bioanalytical laboratory*** *79*
Figure 13 ***Comparison of required time for bioanalysis*** *80*
Figure 14 ***Achieved optimisations in required sample volume for determination of aliskiren and its pharmacodynamics in different paediatric age groups*** *82*
Figure 15 Examplary ***Multiple Reaction Monitoring (MRM) chromatograms of aliskiren.*** *85*
Figure 16 Exemplary ***Multiple Reaction Monitoring (MRM) chromatograms of enalapril and enalaprilat*** *86*
Figure 17 ***Typical chromatogram obtained by the enalapril/enalaprilat serum method*** *94*
Figure 18 ***Serum concentration-time profile of a healthy male volunteer after oral administration of 300 mg aliskiren hemifumarate*** *95*
Figure 19 ***Different kinds of oral administrations of aliskiren hemifumarate*** *96*
Figure 20 ***Comparison of the two developed assays determining enalapril and enalaprilat in serum*** ... *97*
*Figure 21 A****ccuracy results of 22 calibration curves of enalapril and enalaprilat*** *98*
Figure 22 ***Determined split peak in serum*** *99*
Figure 23 ***Obtained total ion counts*** *101*
Figure 24 ***Typical chromatogram of all analytes and the internal standard using the urine method.*** .. *102*
Figure 25 ***MRM chromatograms of analytes and internal standard.*** *103*

Figure 26 ***Comparison of linear and quadratic regression of the urinary method*** *105*
Figure 27 ***Comparison of one-step and two-step SPE extraction*** *108*
Figure 28 ***Comparison of unprocessed vs processed urinary samples*** *110*
Figure 29 ***Obtained excreted drug amount-time profiles of aliskiren and enalapril***..... *115*
Figure 30 ***Accuracy results of 7 calibration curves of enalapril and enalaprilat in urine*** *116*
Figure 31 ***Typical chromatogram of aliskiren and the internal standard***..... *117*
Figure 32 ***MRM chromatogram of analyte and internal standard*** *118*
Figure 33 ***Obtained concentration-time profiles in serum and saliva***..... *124*
Figure 34 ***Consort diagram*** *127*
Figure 35 ***Obtained urine profiles of aliskiren***..... *129*
Figure 36 ***Individual serum concentration-time profiles of 300 mg aliskiren*** *130*
Figure 37 ***Comparison of aliskiren concentrations in serum and saliva***..... *132*
Figure 38 ***Obtained urine profiles of enalapril and enalaprilat***..... *134*
Figure 39 ***Scatter plots of measured enalapril and enalaprilat concentrations in serum and saliva*** *135*
Figure 40 ***Comparison of enalapril and enalaprilat concentrations in serum and saliva***..... *137*
Figure 41 ***Change in ACE activity***..... *139*
Figure 42 ***Obtained mean concentration-time profile after administration of 300 mg aliskiren hemifumarate***..... *141*
Figure 43 ***Excerpt of the SOP-list of the bioanalytical laboratory***..... *144*
Figure 44 ***Final GCLP setting implemented*** *147*
Figure 45 ***Detailed overview of the successfully conducted steps to establish and prove the low-volume bioanalytical platform***..... *169*

VII. Tables

Table 1 ***Volume limits for the blood sampling in young children.*** *7*

Table 2 ***Adjustable parameters and gases at the mass spectrometer.*** *25*

Table 3 ***Compound dependent as well as source and gas parameters of enalapril/enalaprilat*** *31*

Table 4 ***Summarised details of the in-house validation plan*** *for bioanalytical PK methods* *38*

Table 5 ***Detailed MS conditions and compound-specific parameters for all analytes of interest*** *50*

Table 6 ***Established in-house validation plan*** *for PD methods* *64*

Table 7 ***Precision and accuracy of aliskiren quantification*** *by the HPLC-MS/MS serum method.* *87*

Table 8 ***Precision and accuracy of enalapril/enalaprilat quantification*** *by the HPLC-MS/MS serum method* *87*

Table 9 ***Recovery, matrix effect and process efficiency of aliskiren in serum*** *88*

Table 10 ***Recovery, matrix effect and process efficiency of enalapril/enalaprilat in serum.*** *89*

Table 11 ***Relative matrix effect of the enalapril/enalaprilat serum method.*** *89*

Table 12 ***Stability results of aliskiren in serum.*** *91*

Table 13 ***Stability results of enalapril and enalaprilat in serum*** *92*

Table 14 ***Accuracy and precision of all three analytes of interest of the urinary method*** *106*

Table 15 ***IS-normalised relative matrix effect in urine samples of sixteen subjects*** *109*

Table 16 ***Recovery, matrix effect and process efficiency of the urinary method*** *111*

Table 17 ***Stability results (a) of all analytes of interest of the urinary method*** *113*

Table 18 ***Stability results (b) of all analytes of interest of the urinary method*** *113*

Table 19 ***Accuracy and precision of aliskiren determination in saliva*** *119*

Table 20 ***Internal standard normalised relative matrix effect of the salivary method*** *119*

Table 21 ***Recovery, matrix effect and process efficiency of aliskiren and benazepril (IS)*** *120*

Table 22 ***Stability results of aliskiren in saliva.*** *121*

Table 23 ***Main pharmacokinetic parameters of aliskiren in serum and saliva*** *123*

Table 24 ***Baseline characteristics and pharmacokinetics of aliskiren as well as enalapril and enalaprilat*** *128*

Table 25 ***Comparative dataset of pharmacokinetics in saliva and serum of aliskiren, enalapril and enalaprilat*** *131*

Table 26 ***Dataset of pharmacodynamics in all healthy volunteers.*** *140*

Table 27 ***Change in blood pressure*** *142*

VIII. Abbreviations

ACE	Angiotensin converting enzyme	ELISA	Enzyme linked immunosorbent assay
ACEI	Angiotensin converting enzyme inhibitor	EMA	European Medicines Agency
A*e*	Amount excreted into urine	EP	Entrance potential
Ang I	Angiotensin I	ESI	Electrospray ionisation
Ang II	Angiotensin II	eV	Electronvolt
ANOVA	Analysis of variance	FDA	US Food and Drug Administration
API	Active pharmaceutical ingredient	FIA	Flow injection analysis
ARB	Angiotensin receptor blocker	FP	Focusing potential
AUC	Area under the concentration-time curve	FP7	EU's Seventh Framework Programme for Research
BCS	Biopharmaceutics classification system	fu	Fraction unbound
CAD	Collisionally associated dissociation	FIA	Flow injection analysis
CE	Cell entrance	g	Earth's gravitational acceleration
CEM	Channel electron multiplier	GCP	Good Clinical Practice
CHMP	Committee for medicinal products for human use	GCLP	Good Clinical Laboratory Practice
CL	Clearance	GLP	Good Laboratory Practice
CLIA	Chemiluminescentsassay	GMP	Good Manufacturing Practice
C_{max}	Maximum concentration	GS	Gas
Conc	Concentration	h	Hour
cps	Counts per second	HPLC	High performance liquid chromatography
CRS	Chemical reference standard	(HP)LC-MS/MS	High performance liquid chromatography-tandem mass spectrometer
CUR	Curtain gas	IC 50	Half maximal inhibitory concentration
CV	Coefficient of variation	IS	Internal standard
CXP	Cell exit potential	IS-ME	Internal standard normalised matrix effect
DBS	Dried blood spot	ISV	Ionspray voltage
DF	Deflector	k*a*	Absorption constant
DM	Diabetes mellitus	k*e*	Elimination constant
DRI	Direct renin inhibitor	kV	Kilovolt
EC	European Commission	LENA	Labelling enalapril from neonates up to adolescents
EDTA	Ethylenediaminetetraacetic acid	LLE	Liquid-liquid extraction
EIA	Enzyme immunoassay		

LLOQ	Lower limit of quantification	s/n	Signal to noise ratio
MAX	Mixed mode anion exchanger	SD	Standard deviation
MCX	Mixed mode cation exchanger	SPE	Solid-phase extraction
ME	Matrix effect	$t_{½}$	Half-life
min	Minute	t_{max}	Time point of maximal concentration
mM	Millimolar	TLC	Thin layer chromatography
MRM	Multiple reaction monitoring	TMB	Tetramethylbenzidine
MS	Mass spectrometer	ULOQ	Upper limit of quantification
ms	Millisecond	Q	Quadruple
Na	Not applicable	QC	Quality control
nm	Nanometre	RAA	Renin-angiotensin-aldosterone
pa	Per analysis	RE	Recovery
PBPK	Physiology-based pharmacokinetics	RI	Renal impaired
PBS	Phosphate buffered saline	RIA	Radioimmunoassay
PD	Pharmacodynamics	RIN	Renal infection
PE	Process efficiency	rpm	Revolutions per minute
PIP	Paediatric investigation plan	US	United States
PK	Pharmacokinetics	USP	United States Pharmacopoeia
pk*a*	Negative decimal logarithm of acid dissociation constant	UV	ultraviolet
PPT	Protein precipitation	v/v	Volume/volume
PRA	Plasma renin activity	Vd	Volume of distribution
PUMA	Paediatric use marketing authorisation	Vol	Volume
RR	Riva-Rochi	WAX	Weak anion exchanger
RSD	Relative standard deviation	WHO	World Health Organisation
		y	Year

1. Introduction

1.1 Current developments in paediatric research

In January 2007 the European Paediatric Regulation (EC 1901/2006) came into force with the aim to ensure high-quality medicine in children and to improve the availability of information on its use in children (Paediatric regulation 2007). This marked an important milestone in a paradigm change from the thought of guarding children against clinical research to protect them through clinical research.

However, many drugs which are effectively used to treat serious diseases in adults are not sufficiently investigated in children although they are often extensively used in them. Consequently, labelled medicines for children and adolescents are still mostly lacking. The incentives established in the regulation, such as prolongation of supplementary protection certificate of approved drugs by 6 months or the option to gain Paediatric Use Marketing Authorisations (PUMA) with 10 years of data protection for drugs whose intellectual properties rights have already expired do not lead to the hoped increase in research in the paediatric population. Until now only one drug (midazolam) received a PUMA in 2011. A second positive recommendation for a PUMA was given by the Committee for Medicinal Products for Human Use (CHMP) in 2014 (Propranolol). The European Community tries hard to eliminate those drawbacks and has initiated a funding for off-patent drugs by 7th framework programme with e.g. the aim of increasing research activity for well-established drugs not only by pharmaceutical industry but also by academia and by other organisations. Several programmes are underway aspiring the generation of data for having medicinal products suitable for children. One project is called LENA (Labelling Enalapril from Neonates up to Adolescents).

With regard to the "Successes of the Paediatric Regulation after 5 years-"report of the European Medicines Agency (EMA), around 350-400 paediatric clinical trials were conducted per year that account for a proportion of 9 % of all clinical trials in 2012 (European Medicines Agency 2013c). At a glance, this positive trend is clouded by the following: First, only 63 % of all submitted Paediatric Investigations Plans (PIP) have been completed so far (European Medicines Agency 2013b). Second, recently conducted clinical trials in children did not show the effectiveness of the investigated drugs being established in paediatric therapy for long time. The reason for the failed outcomes are diverse and current attempts to justify this are based on e.g. assumptions that the study might be underpowered to detect any difference (Shaddy et al. 2007). The study by Shaddy *et al.* required 26 centres and nearly five years to enrol 161 children and adolescents, exemplifying the difficulties faced in paediatric trials. Third, there is a correlation

between the amount of paediatric data and the age with increasing amount of data pertaining to children above 12 years and older (Samiee-Zafarghandy et al. 2014). Despite the initiatives of the U.S. Food and Drug Administration (FDA) and EMA, data of newly approved drugs for neonates and toddlers are still very rare. Comparing the availability of paediatric data of drugs at time point of initial approval by the FDA, Samiee-Zafarghandy *et al.* found no significant increase since regulations came into force (Samiee-Zafarghandy et al. 2014). Compiling these findings it becomes obvious that the lack of information on safety and efficacy is continued also in newly approved drugs. Although legislative efforts have been undertaken to overcome these obstacles, current findings suggest that – apart from any economic driven reason – especially more child-appropriate tools for study planning, conduction and evaluation are required to empower sophisticated investigations across all age groups and to achieve the aim of comprehensive knowledge and better medicine in children.

Within clinical routine the current drawback on the availability of labelled paediatric drugs entail a broad off-label or unlicensed use. Being defined as the use of a drug that is not included in the approved labelling or did not gain marketing authorisation for the e.g. age group at all, respectively. The younger the children, the higher the rate of off-label use is. This results in a particular risk for neonates, infants and children younger than 2 years (Shah et al. 2007) exemplified by approximately 90 % off-label use in the subpopulation of neonates (Committee for Medicinal Products for Human Use, Kumar et al. 2008). Nevertheless, older children are likewise affected (Olsson et al. 2011, Ribeiro et al. 2013, Lass et al. 2011). A conducted survey in all 27 EU member states found 45 to 60 % of all medicines applied in children were used off-label (European Medicines Agency 2010a). The off-label or unlicensed use results not only in physicians bearing the responsibility and the corresponding legal risk, but furthermore emphasises the current lack of comprehensive pharmacokinetic and pharmacodynamic knowledge in this vulnerable population. Ultimately, this situation puts the less investigated paediatric age groups in jeopardy as efficacy and drug safety are insufficiently investigated. In particular, if the underlying physiological mechanisms and developmental changes are insufficiently evaluated, the risk of unexpected adverse drug reactions or inefficient therapy are more likely (Laeer et al. 2002).

1.2 Approaches to optimise paediatric study planning and conduction including their limitations

During the last years several methodologies, techniques and approaches were established to optimise paediatric clinical studies and add knowledge to this field, although data sources are restricted.

An innovative and acknowledged methodology to support and optimise paediatric clinical trials is the so called modeling and simulation approach by utilising specialised mathematic software. Currently two different model approaches can be applied. While data extraction in compartmental models is limited, physiology-based models enable the simulation of in-silico children, taking anatomical and physiological development into account. This allows for cross age scaling and seems more suitable for paediatric research especially as the models are able to predict the dose optimal sampling points if age-depended pharmacokinetics is conceivable.

Prior to this thesis physiology-based models had been successfully developed for cardiovascular drugs (Ramusovic 2013, Khalil and Läer 2014). But their validation is challenging, as valuable pharmacokinetic and pharmacodynamic datasets in children after administration of cardiovascular drugs like enalapril are rare. Furthermore, the spare data was obtained by different authors and often indicates a lack of uniformity. Reasons for the latter are diverse and based on facts like: e.g. different assays were applied to obtain the data; sampling times varied between the undertaken studies, baseline characteristics of the investigated population were different etc. This ends up in highly variable datasets that might not represent a drug's pharmacokinetics and pharmacodynamics precisely.

Moreover, the physiology-based simulations also take developmental changes into account and allow the prediction of pharmacodynamics. Since the physiological mechanisms in children are not as well investigated and understood as in adults, the validity of such predictive tools is limited until appropriate analytical investigations allow the systematic evaluation of those body's own mechanisms/systems (e.g. RAA system) also in children. Thus, before such powerful tools should be applied in study planning for regulatory purposes and to generate reliable datasets, they require sophisticated validation for the intended scope. In the best case, such validation is performed – regardless whether for pharmacokinetic or pharmacodynamic models – by using comprehensive paediatric clinical datasets – which are currently often lacking.

If there is a high demand in obtaining dense data while ethical burdens hinder to conduct the necessary investigations in a single subject, it is tenable to gain the required data out of a group instead of a single subject. In particular for clinical studies in vulnerable patient populations, the spare blood sampling approach has therefore been introduced. The notion of the spare blood sampling approach is to collect only a few samples per individual and each individual contributes to a complete e.g. concentration-time curve. This approach precludes an intensive and physically strenuous sampling of single person and adds potential to meet ethical considerations and guideline recommendations. Therefore, it is a meaningful optimisation in current paediatric research.

Nevertheless, a huge limitation for drug development is that sparse sampling is not useful in drugs known to show high variability (e.g. aliskiren) (Rebello et al. 2011c). Furthermore, if only few subjects per age group can contribute to a profile, the estimated exposure per time point might be worse. The same applies to the estimates of standard error or confidence interval unless more subjects have been investigated at the same sampling points. To obtain dense and reliable data especially for drugs with high inter-individual variability the connection of sparse blood sampling and population pharmacokinetics as is common in clinical pharmacology is not advisable.

Irrespective of this, the question must be posed whether more appropriate analytical approaches can additionally overcome current limitations in the vulnerable group and even allow for dense datasets in each individual of this population. Proper assays would circumvent limitations faced in enrolment of children as fewer children might be required (Shaddy et al. 2007). If comprehensive sampling in few children is empowered, the spare sampling approach can be refrained from, which substantially reduces the required number of study participants. This in turn reduces costs and accelerates the time required for the completion of study. Therefore, reliable low-volume assays are urgently required to allow these comprehensive studies also in very young children and to obtain the required observed data intended e.g. for the validation of the paediatric models.

With regard to bioanalytical assays, many endeavours have been made to develop sampling techniques and methods reducing the required blood volume. A sound approach to adopting current analytical methods for paediatric research is by tailoring the required sample volume for reliable determination to few microliters instead of millilitres. Utilising mass spectrometric detection, which is characterised by its superior selectivity and its high sensitivity, has resulted in the drive to exploit this potential in combination with low-volume assays for cardiovascular drugs (Ramusovic et al. 2012, Burckhardt et al. 2013). As the sampling volume can significantly be reduced and the blood withdrawal undertaken with an indwelling

cannula, this analytical method is favoured by competent authorities (U.S. Food and Drug Administration 1998). Ethical principles on drawn blood volume are followed, while pain and distress are reduced to a minimum. Nevertheless, it is a major analytical challenge to establish precise low-volume methods encompassing a huge calibration range in biological matrix-rich fluids.

Another meaningful approach is blood collection by capillary. It has increased in importance since high resolution mass spectrometry has gained a well-established detection standard and thus enables the use of low blood volumes for reliable determination. Nevertheless, it is necessary to prick the subject per each single sampling point, resulting in more stress than using an indwelling cannula. The technique also asks for highly trained staff to obtain accurate results, as the capillary blood collection is more challenging compared to blood collection utilising an indwelling cannula. Consequently, this technique appears inappropriate for systematic drug investigations which are highly required if first pharmacokinetic profiles of a drug are generated in a poorly investigated population like children.

A related approach also requiring minimum blood volumes (~5-100 µL) is the collection of dried blood spots. The blood is "drawn" by pricking the heel or the fingertip and depositing the blood drop on specially prepared Guthrie cards. Dried blood spots have been used for decades in particular in genetic sampling or newborn screening, but has been enhanced for therapeutic drug monitoring during recent years. Owing to the minimal required volume and its ease in storage and shipment it has advantages when compared to low-volume assays. However, there are also ongoing concerns - particularly in neonates and toddlers as in these age groups the haematocrit varies and therefore alters the distribution of the blood on the card (O'Broin 1993, O'Broin et al. 1995, Adam et al. 2000, Denniff and Spooner 2010, Mei et al. 2010). Since the drops need to be punched out of the card prior to analysis this effect influences the drug content per punch amongst different sources. Furthermore, a high assay sensitivity (drug concentrations below 1 ng/mL) will be hard to establish with this technique. Additionally, it bears the discomfort of regular needle pricks during the blood collection process in a trial. In general, it is an advanced technique, but the current limitations in very young children indicate that dried blood spots need further optimisations before this technique should be applied in systematic clinical paediatric trials. Moreover, FDA has not officially weighed in on its use for new drug applications.

By contrast, competent authorities mention non-invasive sampling as an alternative approach (Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC 2008, U.S. Food and Drug Administration 1998). Biological fluids like urine and saliva can substitute invasive sampling if a correlation of drug levels in these fluids compared to blood were shown. While urine samples are usually collected at a collection interval of 1-4 hours, saliva sampling facilitates a denser sampling which is favoured to obtain more meaningful pharmacokinetic data. Moreover, compared to blood samples, saliva collection is painless, less stressful, non-invasive and consequently less frightening for paediatric patients. Complications of infection and thrombosis caused by venous puncture can be excluded. The high sensitivity of a LC-MS/MS method coupled with the non-invasive and painless sample collection predestines this analytical approach for children. Physicochemical properties of the drug investigated – in particular the unbounded fraction of the drug in blood and its pk_a – determine whether a drug sufficiently penetrates into saliva. Nevertheless, this non-invasive sampling presents a promising methodology in paediatric research if the drug properties allow for sufficient detection in the corresponding fluid. However, one should bear in mind that for determination of pharmacodynamic changes in plasma parameter or hormones, salivary sampling is not constructive and cannot substitute invasive sampling.

The ethical aspects, the recruitment and the diversity of children regarding ontogeny highlight some challenges in paediatric study planning and conduction. Undertaken optimisations support sophisticated planning, elaborateness and analytical methodology with a focus on paediatric specific characteristics and facilitate addressing the aims of the European Paediatric Regulation effectually. Nevertheless, considering ethical burdens on blood volume, physiology-based models or the spares blood sampling approach, all these processes and requirements necessitate appropriate low-volume analytical methods to develop full functionality or achieve their aims. In particular rich sampling that is mandatory for the intended systematic investigation of pharmacokinetics and pharmacodynamics within this thesis make blood collection techniques by capillary withdrawal or dried blood spots appear inappropriate.

1.3 Blood sample volumes in paediatric research and impact on analytics

Especially in paediatric research, ethical as well as analytical burdens are high. Paediatric clinical trials on pharmacokinetics, efficacy and safety should be performed by preventing distress, fear and pain or even minimise the risks as far as possible (Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC 2008).

One additional challenge is the restricted volume of blood that can be maximally drawn in children without endangering them. The total blood volume is related to a person's body weight. In children this volume is around 75-85 mL/kg body weight (Pearson 2003). While the FDA has no specific guideline on the volume that can be drawn in paediatric patients and endorses keeping the cumulative blood loss to a minimum, EMA recommends a maximum clinical trial-related blood loss of 3 % of the total blood volume during a period of four weeks. It is additionally stated that the loss should not exceed 1 % at any single time (Committee for Medicinal Products for Human Use, Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC 2008). This accounts for about 2.4 mL blood per kilogram body weight and is comparable to the statement of the US Department of Health and Human Services which claimed 3 mL/kg restricted to a maximum of 50 mL of total blood volume (Office for Human Research Protections (OHRP)).

Age	Sex	Body weight [kg] with 50th percentile	Whole blood volume [mL]	Max. blood volume recommended to draw acc. to EMA recommendation [mL]
Birth	♂	3.4	272	8.2
	♀	3.3	264	7.9
9 months	♂	9.3	744	22.3
	♀	8.7	696	20.9
36 months	♂	13.8	1104	33.1
	♀	13.4	1072	32.2

*Table 1 Example ethical **volume limits for the blood sampling in young children.** Values for body weight were obtained from WHO percentiles; EMA recommendations according to Ad-hoc group for the development of implementing guidelines for Directive 2001/20/EC 2008.*

Moreover, the bulletin of WHO based on a review by Howie mentions a recommended volume limit of 1-5 % in children given by several ethic committees worldwide (Howie 2011). However, in sick children the maximum amount should be restricted to 3 mL/kg. Current norm body weights listed by the agencies represent only the mean; taking the 3-fold standard deviation of a 28 days old boy the body weight is in the range of 2.5 to 5.8 kg. This resulting in a maximum blood volume to be drawn of about 6 to 14 mL. The aforementioned safe limits of total blood volume include both clinical care and paediatric research.

This makes it obvious that assays broadly applied in drug research in adults are inappropriate for paediatric investigation and low-volume assays are highly required for sophisticated and systematic investigation. Especially if combined investigations of pharmacokinetics and pharmacodynamics are intended, which marks a reasonable approach to investigate drug exposure and pharmacodynamic effect in parallel. Such low-volume assays will also contribute to better research in adults, particularly in multiple drug studies with a cross-over design as in those investigations even high blood volumes of adults are required. An ethical, safe and reasonable approach might be to develop child-appropriate assays and to demonstrate their usefulness while applying them to healthy volunteers before use in children.

1.4 Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone (RAA) system plays a key role in the homeostasis of blood pressure by controlling blood volume and systematic vascular resistance. Moreover, the system is important in regulation of electrolyte and water in the body. The RAA system moderates vasoconstriction, myocyte hypertrophy, and myocardial fibrosis, an effect that has translated into clinically meaningful treatment approaches. The blockade of the RAA system was shown to result in reduced blood pressure but also in improved fibrosis stages (Tyralla et al. 2011). Consequently, it is a paramount target for several drug classes that are used to effectively treat elevated blood pressure as well as correlated cardiac diseases (e.g. heart failure) in humans. With regard to the international guideline on heart failure by the European Society of Cardiology (McMurray et al. 2012), three out of four drug classes used as first line therapy (symptomatic systolic heart failure) target an enzyme, receptor or structure involved in the RAA system. Within this thesis, the newest approved drug class of direct renin inhibitors and the well-established drug class of angiotensin converting enzyme inhibitors (ACEI) were investigated. While the sole orally available direct renin inhibitor (DRI) aliskiren blocks at the first and time rating step of the cascade, the ACEI (e.g. enalapril) are known to inhibit the conversion of angiotensin I to the vasoconstrictor angiotensin II.

The enzyme renin is an aspartic protease that cleaves the inactive angiotensinogen to yield angiotensin I. While it is known that renin is primarily synthesised by kidney, other organs like the uterus, adrenal glands or saliva glands or central nervous system (DiBona 2000) also contribute to its generation and secretion. The renin secretion by the juxtaglomerular apparatus in the kidney is thereby affected by perfusion pressure in the kidney controlled by baro receptors, sodium concentration detected in the macula densa or triggers of the autonomic nervous system. Angiotensin I is converted to angiotensin II, a strong vasoconstrictor, by the angiotensin converting enzyme in plasma and the lungs. The vasoconstriction is caused by the interaction of angiotensin II with the angiotensin I receptor. The water- and sodium

retention is affected by the liberation of aldosterone, initiated by the interaction of angiotensin II in the adrenal glands. Finally, the RAA system is internally regulated by a negative feedback that controls the renin level. The effects caused by AT_1-receptor activation can be partly masked by natriuretic peptides. The latter represents antagonistic properties compared to the vasoconstriction, profibrotic and prohypertrophic effects of angiotensin II. Natriuretic peptides (atrial natriuretic peptide, B-type natriuretic peptide) show vasodilatory, antifibrotic, antihypertrophic effects, and natriuetic effects (Lueder et al. 2013) (Figure 1).

Although multiple target structures of the RAA system have been inhibited by drugs developed in the last three decades, the system is still of high interest for pharmaceutical drug development. A new drug class might be established soon that highlights the importance of this system in current drug research on heart failure. Very recently, dual blocking by angiotensin-neprilysin inhibition has been found to be a new target in controlling the RAA system especially in patients with heart failure. Neprilysin is responsible for the degradation of natriuretic peptides and in turn is inhibited by angiotensin II (Figure 1). The published promising results of the study in New England Journal of Medicine by Mc Murray et al. investigated the influence on survival after administration of this novel drug class compared to enalapril (ACEI) (McMurray et al. 2014).

The above-mentioned mechanism of action of the RAA system, its effects and adaptations as well as the effects of the pharmacological interventions is based mainly on experiences in adults. In children this data is much more fragmentary. It has been acknowledged that the renin-angiotensin-aldosterone system is particularly active in the newborns and young infants if compared to adults (Van Acker et al. 1979, Fiselier et al. 1983). This inverse relationship between age and measurable components in children has been shown to depend more on age than on e.g. sodium intake. Such a finding, being contrary to facts in adult research, gives insight in a different activation of the system in this vulnerable population especially after birth and in the growing child. While in adults the RAA system mainly maintains the blood pressure homeostasis, in the paediatric organism the system is additionally involved in ontogeny of e.g. the myocardium. This implies that on the current level of knowledge paediatric pharmacotherapy with drugs acting on the RAA system is challenging, as it is ambiguous whether and how strong an interaction with the system is beneficial and at which stage the natural development of organs is seriously affected.

Also, the risk of severe side effects is hard to estimate. Recent outcome of a review on nephrotoxicity of ACEI in neonates with cardiac diseases highlighted the risk of renal failure in this age group (Lindle et al. 2014). Premature neonates have been reported to have a greater likelihood of experiencing ACEI-related

renal failure (55 %) than term newborns (23 %, p > 0.001)(Lindle et al. 2014), highlighting once again that the poorest investigated age groups are the most severely affected ones.

Broughton Pipkin *et al.* showed that mean baseline levels of angiotensin II in children (n=63 [2 months to 12 years]) were approximately double of the values in adults (n=25 [18-34 years]) (37.8 ± 3.7 vs 18.4 ± 3.1 pg/mL) (Broughton Pipkin et al. 1981). Broughton Pipkin *et al.* detected within the paediatric group also a decline of angiotensin II concentrations with age. The blood withdrawal was performed only once per subject which might be based on the high required blood volume of 10 mL per determination. For children in the age of 3 months to 15 years a decrease of plasma renin activity (PRA) was described by van Acker *et al.* (Van Acker et al. 1979). Of high importance are their findings on the amplitude of variations after stimulation of RAA system parameters with higher variability in younger children. It indicates how sensitively the RAA system reacts to external triggers especially in the youngest age group. Furthermore, it emphasises the importance of accurate and precise investigations of pharmacokinetics and pharmacodynamics for a safe pharmacotherapy in all age groups. The latter can only be established if extensive comprehensive knowledge is gained in this area. A comprehensive description of concentration or activity changes of PRA, angiotensin I and II, aldosterone and renin over time, owing to pharmacotherapy by e.g. ACEI in children, is not available until the present.

Understanding the role of all components involved in the RAA system, it might allow not “only” to establish a safer pharmacotherapy but also to rank the available drugs according to their effect on treating the disease and support in the ontogeny. Meaning, if e.g. high PRA levels would be important for the development of the myocardium, it might be more useful to use ACEI that increase the PRA level and lower the blood pressure instead of applying a DRI that reduces PRA levels in parallel to the blood pressure. For clarification of such hypotheses, sophisticated paediatric studies investigating pharmacodynamic changes in children are of high interest.

With focus on the drug interventions in children only sparse data of the pharmacodynamics on the RAA system of aliskiren and enalapril are available. The rare data sets were obtained in hypertensive children while data on children with heart failure are lacking. Sullivan et al. (Sullivan et al. 2013) determined only PRA by utilising 2 mL blood per sampling point in a recently conducted paediatric trial while for adult trials the same amount of blood was reported. In further adult studies 7-9 mL blood were required to determine

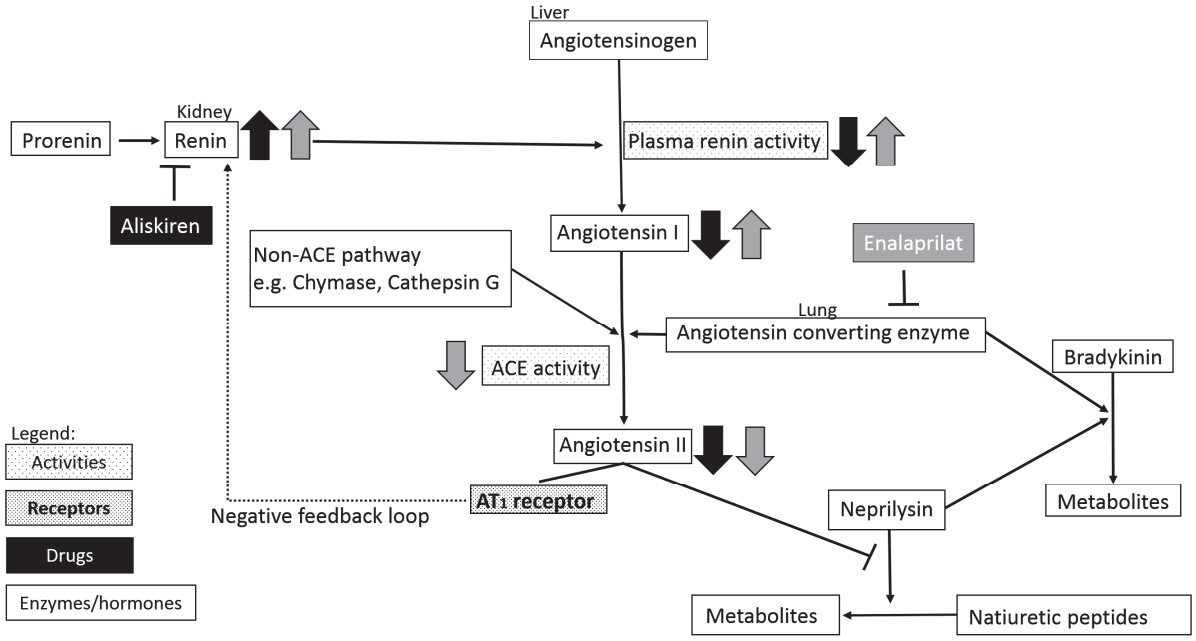

Figure 1 ***Scheme of the RAA system*** *with aliskiren and enalapril as well as their effects on the system (arrows). Based on its route of action, aliskiren causes an increase of renin and decreases plasma renin activity (PRA), angiotensin I and II. By contrast, enalaprilat increases renin, PRA, and angiotensin I, but effects a drop in ace activity and angiotensin II. Additionally, the neprilysin pathway is included being of high interest in current research for new drug entities for treating heart failure.*

angiotensin I, angiotensin II and further RAA biomarkers (Ménard et al. 1995, Nussberger et al. 2002) which would be ethically questionable to draw in neonates and toddlers for one sample. Even commercial assays with low sample requirements mostly need 6 mL blood to determine angiotensin I, angiotensin II, PRA, renin and prorenin. This again clearly indicates the need for child-appropriate analytical approaches that empowers the evaluation of pharmacokinetics combined with pharmacodynamics of cardiovascular drugs used to treat heart failure or hypertension across all age groups.

1.5 Heart failure and hypertension in children

Heart failure describes a clinical condition of under-supply of the metabolic demands in tissue caused by insufficient cardiac output. In contrast to adult in which heart failure is mainly caused by coronary heart disease or myocardial infarct, in the paediatric population congenital heart disease (CHD) and cardiomyopathy are the main reasons for the manifestation of heart failure. CHD occurs in almost 1 % of live births in the United States of America (Hoffman and Kaplan 2002). Although it is known that the incidence of heart failure is large in children with congenital malformations (Kay et al. 2001), the overall incidence and prevalence of the disease in the paediatric population is unknown. There are currently two

European studies available that give insight in the fraction of heart failure amongst all cardiac admissions. Both reported rates varied between 10 – 33 % (Sommers et al. 2005, Massin et al. 2008). The rate of heart failure is even higher amongst children with cardiomyopathies and was identified to range between 65 – 80 % and represents between 5 – 19 % of all heart failure cases. Although the causes of heart failure in adults and children differ highly, the mortality rate between both groups is nearly comparable (7.9 vs 7.5 %)(Webster et al. 2006).

In children with congenital heart failure, the diseases' drawback of under-supplying vital components to the tissue is less distinct in the foetus than after birth. In the womb the foetal heart works in parallel and therefore allows the compensation of impairments of one ventricle. Days after birth specific anatomic structures like the ductus arteriosus botalli are re-built and result in a series pump function. This often marks the point of impaired supply of the demands in tissue and causes an activation of the central nervous system and renin-angiotensin-aldosterone system. While initially the body compensates for the under-supply by physiological reactions like increased heart rate, the persistent stress is maladaptive and contributes to a progression of hypertrophy and dysfunction. This process – also known as remodelling – worsens the heart failure by hypertrophy, dilatation, loss of myocytes, and increased interstitial fibrosis (Eichhorn and Bristow 1996, Sutton and Sharpe 2000). Angiotensin converting enzyme inhibitors have been found to be twice beneficial for patients with heart failure. First, the ACEIs reduce the blood pressure and in turn the afterload which disburden the myocardium. Second, ACEIs are either able to reduce the progression of remodelling or even enable a so-called reverse remodelling (Greenberg et al. 1995). The latter results in a push-back of the disease's progression as long as the syndrome is not end-stage. It was shown that the cardiac remodelling is attenuated after administration of drugs that interact with the RAA system (Nakamura et al. 2013). This interference emphasises the important role of the RAA system in cardiovascular regulations and its effect on the progression of heart failure but also indicates the role of pharmacotherapy in slowing down or stopping this progression.

Whether these benefits translate fully to children is an important question. While Mori *et al.* found a beneficial effect in paediatric patients with valvular regurgitation who have been treated with ACEI (Mori et al. 2000), Hsu *et al.* evaluated no beneficial effect on growth, ventricular function, or heart failure severity in children with single ventricle (Hsu et al. 2010). These contradictory findings, combined with reported cases of renal failure under ACEI therapy (Leversha et al. 1994, Schilder and van den Anker 1995, Dutta and Narang 2003, Hsu et al. 2010), reinforce the establishment of appropriate analytical tools

enabling comprehensive paediatric studies, contributing to the efficacious and safe treatment of children with cardiac diseases.

Drugs acting on the RAA system are likewise used to treat hypertension. According to the European Society of Hypertension, elevated blood pressure (BP) is more common in children than previously assumed (Lurbe et al. 2009, Berendes et al. 2013, Chirita-Emandi et al. 2013). The incidence of high blood pressure in the paediatric population is increasing due to overweight and obesity (Hedley et al. 2004, Obarzanek et al. 2010, Onis et al. 2010, Benmohammed et al. 2011, Chirita-Emandi et al. 2013, Rascher et al. 2013). McNiece *et al.* who investigated pre-hypertension and hypertension in adolescents, reported a prevalence of more than 30 % in boys and between 23 – 30 % in girls (McNiece et al. 2007). In particular regarding the given long-term complications of uncontrolled blood pressure and tracking of elevated blood pressure from childhood to adults, an early implementation of suitable therapy and management of children with elevated values has significant clinical and financial implications.

Antihypertensive drugs classes highly recommended in treatment of hypertension of adults are also considered useful in children although available data is limited (Lurbe et al. 2009). A review (Simonetti et al. 2007) of 27 paediatric trials investigated angiotensin converting enzyme inhibitors (ACEI), angiotensin receptor blockers and calcium antagonists with regard to their blood pressure lowering efficiency in children. Most of the studies included were limited to certain age groups without any reasonable up-titration of the dose. This might explain why the sole non-renal-excreted ACEI fosinopril failed to establish a dose-response effect. Such study outcomes suggest that there is still a lack of knowledge on drug absorption, distribution, metabolism and excretion along with information on maturation (ontogeny, anatomical and physiological development etc.) in children and adolescents.

These facts, amongst others, represent motives why the direct renin inhibitor aliskiren and the angiotensin converting enzyme inhibitor enalapril were selected for further research within this thesis. Both investigated drugs are approved for the treatment of hypertension in adults, but available pharmacokinetic and pharmacodynamic data in children is either missing, limited or available data is not reliable owing to the limitations of the utilised analytical method. Regarding heart failure, no comprehensive data in children is available for both drugs yet.

1.6 Aliskiren

The pharmaceutical industry has been working on direct renin inhibitors for more than 20 years. Many drug entities did not achieve marketability due to low oral bioavailability (Stanton 2003a). Aliskiren (Rasilez®, Tekturna®), is the first orally available direct renin inhibitor shown to lower blood pressure in humans (Weir et al. 2007). Representing a new class of cardio-vascular drugs approved for treatment of essential hypertension in adults, it was approved by EMA and FDA in 2007. Since then further combination products utilising hydrochlorothiazide, valsartan and amlodipine were registered whereof - besides the mono product - only the combination of aliskiren/hydrochlorothiazide is still available on the market. Others were withdrawn due to safety issues concerning hypotension and hyperkalaemia. The occurrence of such severe side effects indicates that although novel drugs being intensively studied in adults prior to be marketed, their detailed mechanisms of action are still not fully understood. Moreover, it emphasizes how much more important it is to carefully and sophisticatedly study each drug in children, in whom much less is known about drug distribution, metabolism and interaction.

Pharmacokinetics in adults

Aliskiren is rapidly absorbed with the maximum plasma concentrations reached within 1-3 hours after oral administration. The relative bioavailability after administration of 75 mg orally is 2.6 % (Azizi et al. 2006). Based on the high specific binding on the target structure with a 50 % inhibition concentration (IC_{50}) of 0.6 nmol/L (Wood et al. 2003) and its long elimination half-life, the low bioavailability is sufficient to block renin for days. Published clinical data in adults shows a high inter-individual variability of aliskiren pharmacokinetics exemplified by the C_{max} value after oral application in healthy volunteers ranging from 34 to 871 ng/mL after single administration of 300 mg aliskiren (Rebello et al. 2011c).

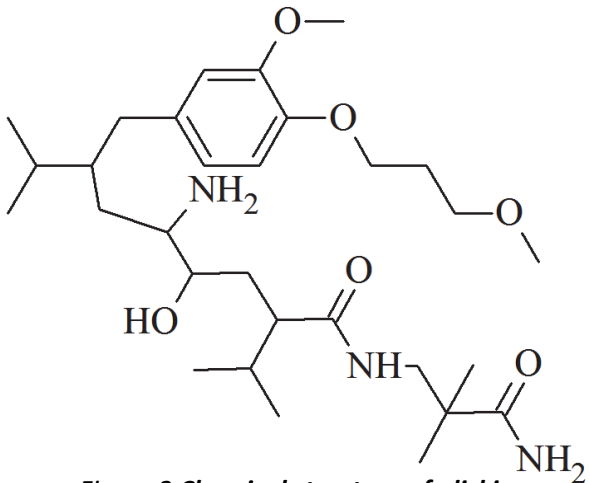

Figure 2 ***Chemical structure of aliskiren***

The drug substance shows non-linear dose proportionality in AUC and C_{max}; however this is considered not to be clinically relevant owing to the high reported variability of aliskiren (Vaidyanathan 2005).

Pharmacodynamics in adults

Aliskiren directly inhibits human renin, the central enzyme of the RAA system which is essential for the regulation of blood pressure. Its antihypertensive potency is reported to be comparable to angiotensin I receptor blockers (ARB) (Gradman et al. 2005, Stanton et al. 2003b). One main difference compared to widely used ACEI and ARB is the decrease in plasma renin activity (PRA) after administration of aliskiren. This effect is being discussed to be an advantage as high PRA levels are associated with an increased risk of myocardial infarction (Alderman et al. 1997). Confirmatory long-time data is currently not available.

Available data in children

With regard to children, by end of 2010 two publications became available (Flynn 2010, Kelland et al. 2010) reporting first experiences with aliskiren in children. In the 14 case reports presented, aliskiren (37.5 – 300 mg per day) was used as add-on therapy if the common treatment was not successfully in lowering the blood pressure in children. Half of them showed adverse events like hyperkalaemia or hypotension which indicates the urgency to investigate the detailed mechanism of action and making the current off-label application of aliskiren safer.

Recently, the first open-labelled paediatric study became available evaluating children and adolescents aged 6 to 17 years. The study investigated two doses and their effect. For pharmacokinetic determination one millilitre blood was required per sample (Sullivan et al. 2013). Due to the high intra- and inter-individual variability of aliskiren sparse sampling with population pharmacokinetic analysis was not recommended to gather first in child pharmacokinetic data (Vaidyanathan et al. 2008b). One millilitre of blood for one sample, however, appears inappropriate for systematic investigations, especially in younger children. With regard to information given by the originator, preclinical data in very young animals revealed unexpectedly high drug concentrations that might led to the decision to exclude children younger than 6 years from the above-mentioned study. To avoid danger to this age group by off-label use, valuable studies with appropriate analytical methods covering all age groups are necessary.

Published high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methods for determination of aliskiren required between 200 µL (Rebello et al. 2011a) and 700 µL (Tapaninen et al. 2011a) of serum or plasma. The corresponding lowest quantitation limit was 0.25 ng/mL by applying 700 µL plasma (Tapaninen et al. 2011a). A child-appropriate assay utilising a small amount of plasma or

serum combined with a low quantitation limit for paediatric research was not established at the time of literature search. In general, pharmacokinetic data in paediatric patients is rarely available yet.

Data on excretion in paediatric patients are lacking. Neither the percentage distribution of aliskiren to the different excretion pathways (renal, biliary etc.) nor the influence of simultaneous drug administration was reported. Since safety concerns have been raised on the combined blocking of the RAA system (Harel et al. 2012, Maggioni et al. 2013) it is necessary to also determine the concentrations of the applied drugs in urine. This allows further elucidation of the mechanisms of the potential risks triggered by the combination of the drugs. A conducted literature search revealed no method simultaneously quantifying aliskiren and enalapril in urine.

Based on the physicochemical properties of aliskiren, a high penetration rate from serum to saliva can be assumed which predestines it for non-invasive sampling. Aliskiren is classified as BCS class 3 drug (high solubility, low permeability) with a pk_a value of 9.45. The reported fraction unbound in blood of 47-51 % (Waldmeier et al. 2007) should also enable a sufficient penetration from blood into saliva. Although the saliva collection has several advantages for paediatric research, the determination of salivary aliskiren concentrations had not been reported yet.

1.7 Enalapril

The second investigated drug acting on the RAA system is enalapril, an angiotensin converting enzyme inhibitor (ACEI) which has been established in the therapy of hypertension and heart failure in adults for over 20 years. The introduction into therapy marked a landmark as the drug class improved the prognosis and for the first time showed a significant effect on mortality [Consensus-, SOLVD- & V-Heft III study (Consensus Trial study group 1987, SOLVD 1991)]. ACEI are nowadays recommended for patients with heart failure and low ejection fraction to reduce morbidity and mortality (Level A evidence) (Yancy et al. 2013). With regard to the ESH guideline 2013 ACEI are additionally endorsed to treat hypertensive patients especially with diabetes mellitus or renal dysfunction (Mancia et al. 2013).

Pharmacokinetics in adults

Enalaprilat, the active metabolite of enalapril, has a very low bioavailability owing to its high polarity and permeability properties (Biollaz et al. 1982). Therefore it is orally administered by its prodrug enalapril that is reported to have a bioavailability of about 60 % (Hexal AG 2011) but is not highly active in the pharmacodynamic sense. While enalapril is rapidly absorbed within one hour after oral administration (Ulm et al. 1982, Swanson et al. 1984), the bioactivation by hepatic carboxyesterases (CES 1) is responsible

for the "delayed" t_{max} of enalaprilat at approximately 4 hours postdose (Sweet et al. 1981). The prodrug and the active metabolite are both primarily excreted via the kidney. The area under the concentration time curve of the drug do not differ significantly between fed and fasted conditions accounting for no food effect (Swanson et al. 1984).

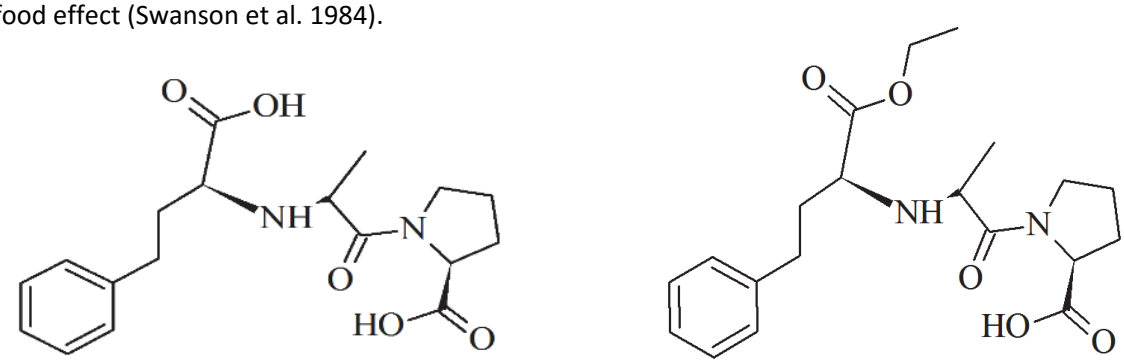

Figure 3 ***Chemical structure of enalaprilat (left) and enalapril (right).***

Pharmacodynamics in adults

By blocking the angiotensin converting enzyme, enalapril inhibits the generation of the vasoconstrictor angiotensin II. Therefore, the peripheral resistance is decreased, accounting for the blood pressure lowering effect of the drug. Moreover, enalapril causes an increase in plasma renin activity and renin concentration. By blocking the angiotensin-converting enzyme the metabolism of bradykinin is also interrupted, which causes dry cough in many patients treated with enalapril (> 1 per 10 treated persons).

Available data in children

Competent authorities and the world health organisation (WHO) are highly interested in data regarding ACE inhibitors in children. With regard to the "Priority list of needs in pediatric therapeutics", which is developed by the National Institutes of Health in consultation with FDA, angiotensin converting enzyme inhibitors should be investigated for paediatric hypertension with priority (National Institutes of Health 2012). In addition, WHO mentioned the ACEI enalapril in the present "List of essential medicines for children" which was issued in April 2013 (World Health organisation 2013) and EMA's current "draft inventory of paediatric therapeutic needs" (European Medicines Agency 2013a) recommends investigating ACEI extensively for paediatric patients. Based on EMA's revised priority list for studies into off-patent paediatric medicinal products in 2012, the last call of Seventh Framework Programme (FP7) by European Commission has led to a project investigating enalapril from neonates up to adolescents. The so named LENA project (funded by FP7 programme; EU Grant Agreement 602295) started by end of 2013 and will access to assays being presented in this thesis.

Reliable data on pharmacokinetics of enalapril in paediatric patients is currently questionable. Until now enalapril has been investigated in three paediatric pharmacokinetic trials, which revealed paediatric data sets in 40 hypertensive children aged 2 months to 16 years and 22 patients (neonates to children) with congenital heart failure (Lloyd et al. 1989, Nakamura et al. 1994, Wells et al. 2001). However, in all studies the drug concentrations were determined by a radioimmunoassay (Hichens 1981). As shown by Ramusovic (Ramusovic 2013) this analytical technique tends to overestimate the serum concentrations especially of the active metabolite enalaprilat. This might be due to cross reactions and a lack of selectivity but ends up in non-reliable datasets. Consequently, the paediatric clinical data displays differences in results. Wells *et al.* (Wells et al. 2001) showed similar pharmacokinetic results to those reported for healthy adults in literature, while Nakamura *et al.* found increased half-lives of enalapril and enalaprilat in children (Nakamura et al. 1994). The investigated paediatric population differed between both studies, indicating how variable this paediatric population is within itself and how important comprehensive research across all age groups is to improve the rare knowledge about the pharmacokinetics of enalapril and enalaprilat. Nevertheless, enalapril is already recommended as first line therapy in children with heart failure and hypertension in children ≥ 20 kg (European Medicines Agency 2010b, Kantor et al. 2013).

To reflect the current state-of-the-art and to obtain robust data, determination by HPLC-MS/MS appears to be the most suitable assay given its superior selectivity. During recent years effort was made to develop appropriate HPLC-MS/MS assays for the reliable determination of enalapril and enalaprilat in serum (Najib et al. 2003, Gu et al. 2004, Lima et al. 2009, Lu et al. 2009, Gonzalez et al. 2010, Gosh et al. 2012, Ramusovic et al. 2012,). Unfortunately, none of them has been developed as a low-volume assay for high-throughput.

As mentioned previously, it is also of high importance to study the routes of drug elimination in children. With regard to adults, enalapril and enalaprilat are nearly completely excreted via kidney, this elimination pathway is therefore also of particular interest in children and adolescents. Furthermore, Lannoy *et al.* (Lannoy et al. 1989) reported a conversion of enalapril to enalaprilat not only by the liver but also intra-renally. This conversion has not been reported to be investigated in children yet. Neither the existence nor the amount are studied in children and present an important step towards building up comprehensive knowledge on the mechanism of action in paediatric patients. As introduced earlier, while saliva collection has several advantages for paediatric research, the appropriateness for pharmacokinetic determination should be evaluated. Salivary concentrations of enalapril and enalaprilat have neither been reported in adults nor in children yet.

2. Aim of the thesis

The aim of the thesis was to develop and evaluate a tailored comprehensive bioanalytical setting for pharmacokinetic as well as pharmacodynamic investigations of drugs acting on the RAA system in children. This setting needs to be designed to allow the systematic investigation of drugs acting on the RAA system in neonates up to adolescents. Without these low-volume assays current obstacles in paediatric trials cannot be overcome and consequently no comprehensive and meaningful data can be obtained in this underserved population. Such a novel bioanalytical platform can actuate the currently underpowered research in all paediatric age groups and improve the availability of information in paediatric patients. The overall aim was graded into the following subprojects:

First, with the development and validation of low-volume HPLC-tandem mass spectrometric methods a high-throughput determination of aliskiren, enalapril and enalaprilat concentrations in serum and urine was intended to be established. The chosen analytical technique represents the current state-of-the-art in clinical analytics appropriate for bioanalytical drug level measurements and is commonly used in pharmaceutical research. This approach was preferred due to its high selectivity to obtain accurate and precise pharmacokinetic datasets as well as its ability to cope with low sample volumes being common in paediatric research. These low-volume assays would support future paediatric investigation of drug absorption, distribution, metabolism and its renal elimination.

Second, novel salivary assays were developed and validated with the aim of checking on the ability of drug level determinations of aliskiren, enalapril and enalaprilat by non-invasive sampling. These innovative assays shall prove if the investigated drugs penetrate sufficiently into this biological fluid and therefore allow for a less painful and less stressful collection and determination of pharmacokinetic data compared to serum or plasma. The combination of a selective HPLC-MS/MS method and non-invasive sampling predestines this analytical approach for paediatric research if the drug properties permit sufficient penetration into the oral fluid. Additionally, this approach allows the generation of first pharmacological knowledge of the drug distribution in the salivary compartment.

Third, this part of the thesis was aimed at evaluating pharmacodynamic assays for paediatric research and establishing a comprehensive platform covering the main parameters of the RAA system. These parameters (angiotensin I, angiotensin II, ace activity, plasma renin activity, renin, and prorenin) allow for the comprehensive pharmacodynamic investigation of the target system of aliskiren, enalapril and its active metabolite enalaprilat. At present, comprehensive and systematic investigation of the

pharmacodynamics on the RAA system in the underserved paediatric population is not possible. Such a comprehensive approach would exclude possible bias due to different sample handling, different determination methods or assays etc. that negatively effect the strength of generated data.

Fourth, by the conduction of a proof-of-concept study, a confirmatory step was established to demonstrate the usefulness of the tailored setting. The aim of this subproject was to check the applicability of the tailored bioanalytical setting in healthy volunteers investigating serum, urine and saliva after oral administration of aliskiren hemifumarate or enalapril maleate. This subproject allowed to obtain pharmacokinetic and pharmacodynamic data in a comprehensive way which is useful to prove the ability of the low-volume assays in generating reliable data. Moreover, the data is urgently required to empower the validation of developed models and simulations.

The final step within this thesis was intended to create a “ready-to-use” platform by imbedding the low-volume bioanalytical assays after proving their applicability into a first-time quality control setting that complies with current regulatory requirements for clinical studies. The Good Clinical Laboratory Practice (GCLP) appeared most appropriate to meet current regulatory requirements when the bioanalytical platform is implemented into a clinical study for gaining marketing authorisation. Consequently, GCLP was intended to be established at the bioanalytical laboratory of the Institute of Clinical Pharmacy and Pharmacotherapy.

Ultimately, the setting aimed to be used in a Phase I study of the LENA project to evaluate the pharmacokinetic behaviour of a new child-appropriate dosage form. The generated pharmacokinetic data within this study required highest quality and integrity as it was intended to be incorporated in the Paediatric Investigation Plan. The latter undergoes evaluation by the Paediatric Committee at the European Medicines Agency.

3. Methods

3.1 Sample purification

Biological fluids like plasma, serum, urine and saliva present a varying composition of e.g. lipids, proteins, electrolytes, cells, co-eluting metabolites, impurities, degradation products etc. All of these components might interfere with the analyte of interest during detection and restrain its precise quantification by e.g. signal suppression. Furthermore, the matrix contributes clearly to a reduced lifetime of the analytical equipment (e.g. HPLC column). To reduce this interference several approaches had been developed in the past. The most often used are protein precipitation (PPT), liquid/liquid extraction (LLE) and solid-phase extraction (SPE). PPT is a fast and simple approach, but works best only in protein rich matrices such as whole blood, plasma or serum. Nevertheless, PPT does not remove matrix interferences others than proteins and does not concentrate the sample. Moreover, the effectiveness is often limited as the precipitation is not complete or the loss ratio of the analytes of interest is high. It is a useful and fast technique to optimise lifetime of the equipment but does not increase the analytical sensitivity as required for low-volume assays. LLE allows the separation of analytes of interest from proteins and other hydrophilic components, but if emulsions are formed the separation of the organic solvent later on is difficult and might result in incomplete and varied analyte diffusion. At time of method development, in particular for high-throughput analytics LLE is not first choice as the extraction can currently not be performed automatically. Recently, a new emulsion-free 96-well plate approach was announced for LLE, which needs to be evaluated on its applicability in future trials. Consequently, SPE was chosen owing its superior purification properties and flexibility in extraction protocol coping with diversity of analytes, purifications solvents and biological fluids.

3.1.1 Solid-phase extraction (SPE)

Solid-phase extraction is considered the current “gold-standard” in sample preparation of biological fluids, owing to its potential of high purification levels on many compositions like proteins, electrolytes, lipids etc. This technology is a chromatographic approach by using silica or polymeric sorbent material to chemically purify the biological fluid. Applying different pH values and solvents, the compounds of the sample matrix can be fractionated according to their pk_a values (acid dissociation constant) and hydrophilic/lipophilic properties before the final eluate is submitted to analytical testing. The selection of e.g. solvents and the corresponding application follow the same mind-set known from liquid chromatography. In general, a valuable separation protocol should be based on the drug’s polarity and its

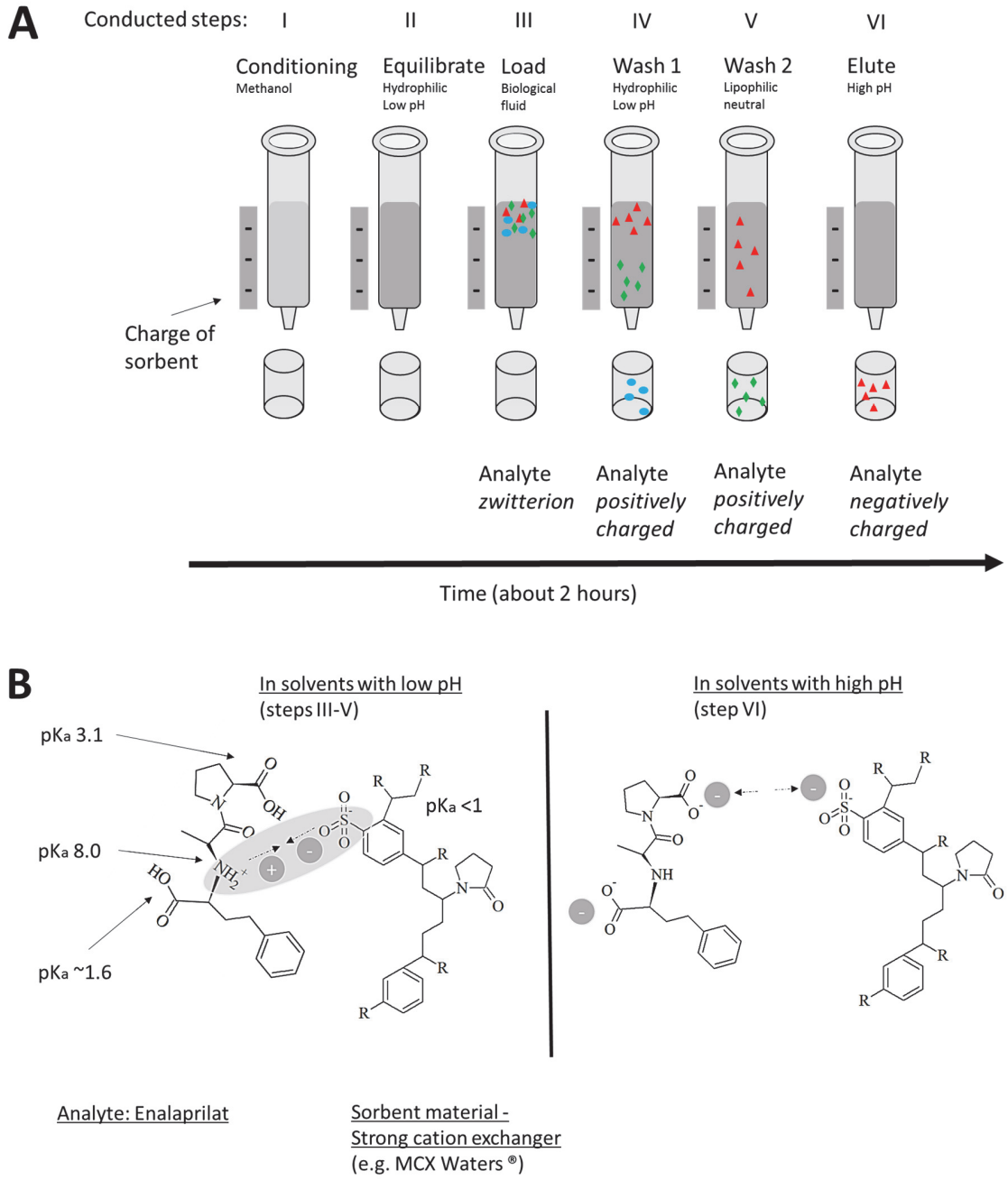

Figure 4 ***Capture of enalaprilat by solid-phase extraction*** *(simplified scheme). In the upper graph (A) the process of a solid-phase extraction over time is sketched. Blue cycles define matrix compounds like proteins, green diamond indicate other potential drugs in the biological fluid and red triangles show the analyte(s) of interest. In the lower graph (B) the ion interaction between analyte and sorbent material is illustrated. While the sulfonic acid of the sorbent material is charged at all time, the charge of the analyte of interest can be influenced by the solvents used. On the left side, the interaction during the extraction step III-V is depicted, while the interaction during the elution (step VI) is shown on the right side. Apart from the shown ion interaction with the permanent negatively charged sulfonic acid of the polymeric sorbent material, additional interaction is induced by van-der-Waals and hydrogen bonds.*

pk_a. This allows components to interact with or elute from the sorbent material. Nevertheless, challenges are raised by e.g. simultaneously eluting compounds with contrary properties (e.g. enalaprilat [calculated $logD_{(pH\ 6.5)}$ -8.3] is much more hydrophilic than its ester prodrug enalapril [calculated $logD_{(pH\ 6.5)}$ -1.0] (Rautio 2011), but refraining from other interfering matrix compounds like salts, proteins, phospholipids and other endogenous compounds in parallel. As shown in a simplistic and exemplified way in Figure 4, enalaprilat is loaded as zwitterion together with other compounds and matrix into the cartridges and separated by additional wash steps. Using solvents with neutral or low pH values, enalaprilat is charged positively at the nitrogen and interacts by van-der-Waals, hydrogen bond and ion interaction with the permanent negatively charged sulfonic acid of the polymeric sorbent material. Not until the solvent pH is changed to higher values (at least 2 pH ranges above the pk*a* of the basic structure = 99 % deprotonated) enalaprilat is released and can be eluted. Amongst many benefits, this technology is the most cost- and laboratory-intensive technique of the approaches presented here, as each drug requires development and optimisation based on an individual protocol. This is especially the case if sensitive and insusceptible methods are required.

The solid-phase extraction is the sole technique that cleans up, concentrates and produces a final sample ready for further analysis. First, it allows the matrix to be highly purified, which results in a more robust method especially if the matrix varies between different individuals. Furthermore, the purification improves the baseline, leading to a better accuracy of the analytical results. Second, by removing the interferences it enables the reduction of ion suppression or enhancement in the MS detection. Better signals permit building up more sensitive methods. Third, owing to its separation approach being comparable to the HPLC, it facilitates the fractionation of the sample matrix and permits the classification of the compound for more effective analysis. It is therefore the most superior of the three preparation techniques discussed and applied in the following assays developments.

3.2 Chromatographic separation and detection

3.2.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography is a well-established and fast chromatographic separation technique. Apart from being the “classical” approach separating substances of interest and making them available for single quantification, such as for the determination of content per tablet, HPLC is additionally implemented in bioanalytical chromatography as a further step to reduce the interferences of the analytes

of interest and the remaining matrix. This has the potential to significantly lower the effect on ion suppression or enhancement of the analyte of interest during mass spectrometric detection (Chambers et al. 2007). Other than in widely used LC-UV, common puffers (e.g. phosphate puffer) are inappropriate for LC-MS. Additionally, inorganic acids like hydrochloric acid as well as trifluoracetic acid or triethylamine are not recommended as they are either non-volatile or cause high background signals making the selection of a proper mobile phase important and challenging. Based on the opposed operation principles of HPLC and MS, common flow rates of 1 mL/min used for e.g. LC-UV result in poor ionisation performances of the MS. This is grounded in the fact that a flow rate of 1 mL/min of an aqueous solution generates 1200 mL/min of gas volume which influences the spray and may also negatively effects the high vacuum performance of the device. Commonly used flow rates compatible with the mass spectrometer (if no splitting device is incorporated) vary between 0.3 – 0.5 mL/min.

3.2.2 Mass spectrometry

In bioanalytical settings intended for pharmacokinetic analysis not only in healthy but also diseased volunteers, it is hard to conceive which concomitant drug will be administered to a patient in future. Thus, it is advisable to establish an analytical method that is known to be very selective owing its detection method. With regard to its superior selectivity and sensitivity, the detection by masses utilising a mass spectrometer is preferred in bioanalytical analysis. Due to its susceptibility to a possible matrix effect, full efficiency is gained only by either the elimination or separation of interfering matrix. Within this thesis, the mass spectrometric analysis was performed by ESI (electrospray ionisation)-tandem mass spectrometer with an electron multiplier detector. The following account is therefore restricted to this technique.

Nowadays the ESI-MS is a common and widely used technique that serves as a mild ionisation technique useful for peptides, proteins and thermally labile pharmaceuticals. However, for the latter the adjustment in the ESI-source is a tightrope walk as higher temperatures allow for good desolvation and evaporation results and consequently result in better sensitivity but may destroy the analyte. Additionally, ESI is an ion source known to be more susceptible for matrix effect than others (Dams et al. 2003). However, the ion source acts as the link between HPLC and mass spectrometer. The arriving mobile phase from HPLC is nebulised in the ion source into droplets by gases. Owing to pH value of the mobile phase, additives and the charged capillary in the ion source most substances get charged in the droplet. The impact of temperature and the gas let the droplet shrink. By increasing charge density, the positively charged substances orient themselves at the surface of the droplet. With rising charge density, the droplet

Ion source	Mass analyser		Detector
	Interface	Quadrupol rail	
Flow rate	Declustering potential (DP)	Stubbies	Channel electron multiplier (CEM)
Position of spray cone	Focusing potential (FP)	Quad 1 (IE 1)	Deflector (DF)
Curtain gas (CUR)	Skimmer	Cell entrance potential (CEP)	
Ionspray voltage (ISV)	Entrance potential (EP)	Quad 2 (CE) A	
Nebuliser gas (GS 1)	Interquad lens (IQ 1)	Cell exit potential (CXP)	
		Quad 3	

becomes unstable and finally explodes ("Coloumb explosion"). This marks the point at which free ions are formed that can pass the interface of the mass spectrometer.

Table 2 ***Adjustable parameters and gases at the mass spectrometer.***

After ions have been emitted by the ion source the ions are analysed by their mass-to-charge ratio (m/z) in the mass analyser. The tandem mass spectrometer offers the option to highly selectively scan for the analytes of interest by using quadrupole mass analysers in series. Running the device in the MRM (Multiple Reaction Monitoring) mode means that in the first quadruple (Q1) the parent ion is filtered. By passing the collision cell (Q2) the ion is then processed and fragmented into specific product ions and after selecting specific m/z transitions of the fragments in Q3 the corresponding ions are detected by their mass-to-charge ratios. In our setting a continuous electron multiplier (CEM) was used as detector that is characterised by a high selectivity and good accuracy and therefore useful for wide calibration ranges or low analyte amount in the sample, as it is the case in low-volume assays.

Although LC-MS/MS is predestined for a precise determination in low sample volumes, it requires an exceptional method development as e.g. only for MS method development about 20 parameters (potentials, gases etc.) should be adopted to warrant a stable, sensitive and accurate determination. The so called "flow-injection analysis" (FIA) is a useful tool to optimise exactly these compound-dependent as well as source and gas parameters (Table 2). Figure 5 gives insight to the number of potentials and gases that need adaptation especially if not only a robust but also sensitive method needs to be established.

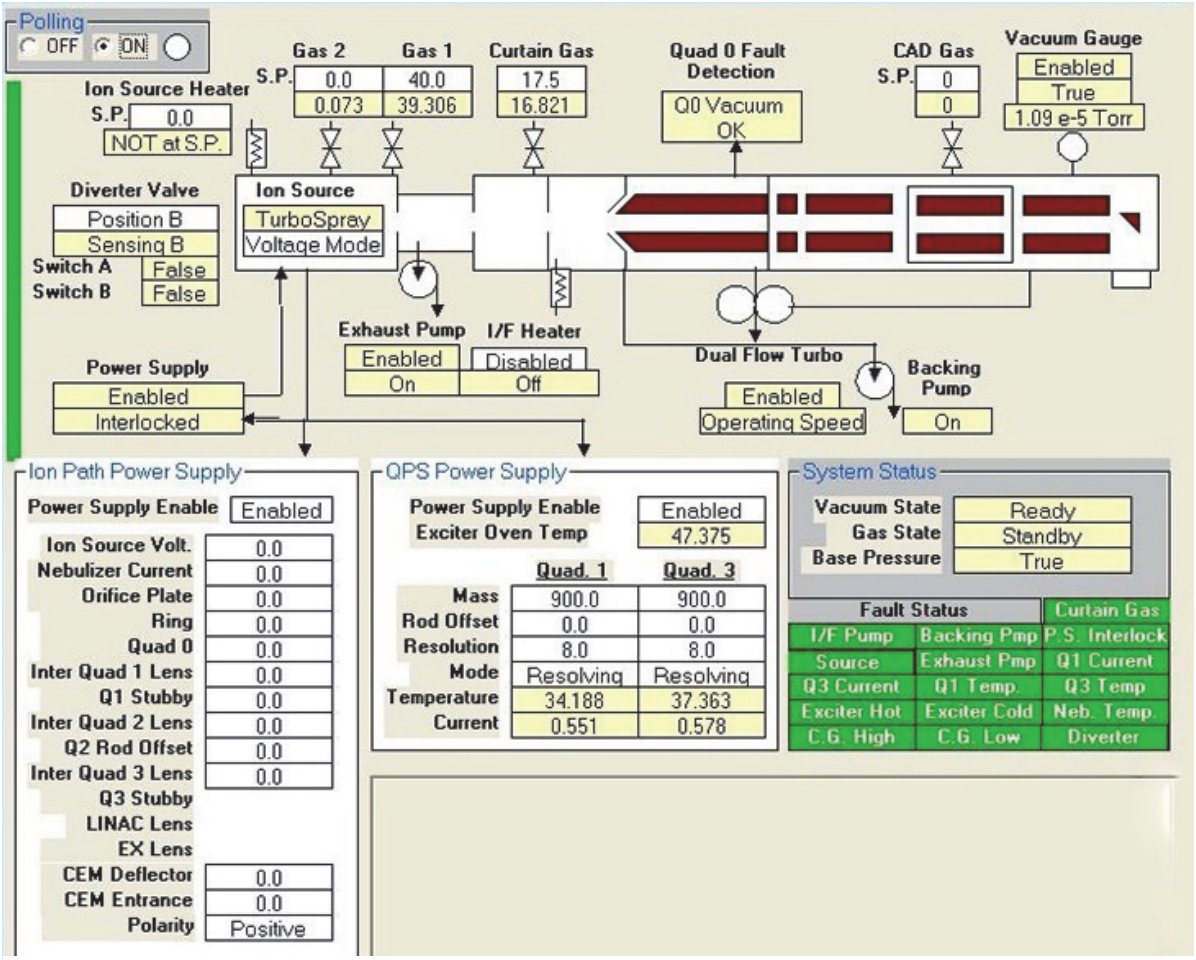

Figure 5 ***Overview of adjustable gases, potentials and further parameters as well as their locations in the mass spectrometer (AB Sciex API 2000).***

3.3 Immunoassay

A further commonly used bioanalytical detection approach is the quantification by immunoassays. This diagnostic method covers several techniques such as enzyme linked immunosorbent assay (ELISA), radio immunoassay (RIA) or chemiluminescence immunoassay (CLIA). In contrast to the aforementioned mass spectrometric detection that is often used to quantify drug concentration in biological fluids and is harnessed to pharmacokinetic determinations, immunoassays are mostly applied in detection and quantification of proteins. This fact makes the latter essential as laboratory assays for evaluation of pharmacodynamics. These assays are charaterised by a specific determination owing the immunological reaction of antibodies. This specificity creates the conceivable approach of low-volume assays that are mandatory for paediatric research. The possibility of a cost-efficient, high-throughput approach with a moderate amount of equipment is of further advantage for bioanalytical detection of toxins, hormones or enzyme activities.

3.4 Bioanalytics for determination of the drug concentration in different biological fluids

3.4.1 Serum

The biological fluid of choice to measure drug concentrations and to evaluate corresponding pharmacokinetic parameters is still plasma or serum. The approach to establish robust, fast, precise but also sensitive low-volume methods with detection limits in pg/mL magnitudes was only achieved by a much elaborated method development. The following paragraph provides detailed information on the development, scale-up and validation of such low-volume assays.

3.4.1.1 Determination of aliskiren and enalapril in serum by HPLC-MS/MS method

The methods for determination of aliskiren as well as enalapril and its active metabolite enalapril in serum were developed as two independent methods. However, both methods were established as high throughput assays with the aim of enabling the quantification of at least 600-800 low-volume samples per week.

Chemicals and materials

The active pharmaceutical ingredient (API) aliskiren hemifumarate (≥ 98 %, potentiometry & HPLC) was supplied by MSN Laboratories (Hyderabad, India). Enalapril maleate CRS and enalaprilat dihydrate CRS (both European Pharmacopoeia Reference Standards) were purchased at the European Directorate for the Quality of Medicine & Healthcare (Strasbourg, France). The internal standard benazepril hydrochloride (≥ 98 %, HPLC) and ethyl acetate (100 % p.a.) were obtained from Sigma-Aldrich (Seelze, Germany). Methanol (HiPerSolv® Chromanorm® HPLC grade), water (super gradient grade), hydrochloric acid (AnalaR Normapur), acetone (AnalaR Normapur) and ammonia solution (AnalaR Normapur) were purchased from VWR (Germany). Alternative supplier of methanol (HPLC grade) was Fisher Scientific (Loughborough, United Kingdom). Orthophosphoric acid (85 % p.a.) and formic acid (98-100 % p.a.) were delivered by AppliChem (Gatersleben, Germany). Ammonium formiate (99 %, HPLC-grade) was obtained from Fluka (Seelze, Germany). Blank human serum was provided by employees of the Institute of Clinical Pharmacy and Pharmacotherapy (Düsseldorf, Germany). Oasis® MCX cartridges (30 mg), Oasis® MCX 96-well plates (30 mg), Oasis® MAX 96-well plates (10 mg), XSelect™ CSH C18 3.5 µm columns (3.0 mm x 150 mm), XSelect™ CSH C18 3.5 µm guard columns (3.0 mm x 20 mm) and XBridge® BEH C18 3.5 µm columns (3.0 mm x 150 mm) were obtained from Waters (Eschborn, Germany).

Preparation of standards and quality control samples

Stock solution of aliskiren was prepared by dissolving approximately 12 mg of accurately weighed drug substance in 100 mL water, while the stock solution of enalapril, enalaprilat and the internal standard (IS) benazepril were obtained in the same manner to yield 0.10 mg/mL in methanol. These stock solutions were diluted with water to obtain working solutions with 10 µg/mL enalapril and enalaprilat as well as 166 ng/mL benazepril. All above-mentioned calculations were based on the free drug base. As for all analytes deuterated internal standards were not commercially available, benazepril was chosen as IS. It is structurally related to the analytes of interest, shows comparable stability, and behaves similarly during the analytical determination due to the comparable physicochemical properties.

The highest calibration standard was obtained by spiking serum with stock solution or working solution, respectively, to yield the upper concentration level of 1200 ng/mL of aliskiren, 400 ng/mL enalapril, and 360 ng/mL enalaprilat (Upper limit of quantification [ULOQ]). Using serial dilution of the latter with blank serum resulted in the following nominal concentrations: 600, 300, 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17, 0.59, 0.29 and 0.146 ng/mL. Regarding enalapril and enalaprilat, the following concentration levels were additionally gained by applying the same technique: 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.57, 0.79, and 0.395 ng/mL enalapril and 180, 90, 45, 22.5, 11.25, 5.63, 2.81, 1.41, 0.70, and 0.35 ng/mL enalaprilat. Quality control (QC) samples were independently prepared in the same way resulting in a calibration range of 0.15 - 1200 ng/mL aliskiren or 0.395 – 400 ng/mL enalapril and 0.35 – 360 ng/mL enalaprilat. However, a dilution step (please refer to the following section “sample preparation”) of extracted sample/standard containing enalapril/enalaprilat with mobile phase was responsible for a final analytical calibration range of 0.2 – 200 ng/mL enalapril and 0.18 – 180 ng/mL enalaprilat [expressed as detection range of the mass spectrometer]. All below listed concentration levels of QC samples etc. within the validation of enalapril/enalaprilat refer to this analytical detection range.

All of the aforementioned dilutions were prepared freshly, while the stock solutions of the aliskiren and IS were stored at 7 °C and the ones for enalapril plus enalaprilat were stored at -20 °C for a maximum period of 6 months. The stock solutions were equilibrated to room temperature prior to use.

Sample preparation

Aliskiren

To a volume of 100 µL serum sample or QC sample additionally 500 µL phosphoric acid (2 %, v/v) and 10 µL IS working solution (166 ng/mL) were pipetted. The IS was added to compensate for variations during

sample extraction and to adjust for remaining matrix effects. The prepared samples were vortexed for five seconds and centrifuged at 16100 g at 4 °C for 10 minutes. The centrifuged samples were purified by solid-phase extraction (SPE) utilising a strong cation exchanger on sulfonic acid base (Oasis MCX) (30 mg, 1 mL). Differently to the generic SPE protocol the sorbent was preconditioned with 1.0 mL ammonia solution in methanol (3 %, v/v) followed by a conditioning step with 1.0 mL methanol and finally equilibrated with 2 % formic acid. The acidified samples were transferred into the primed cartridges, eluted and the sorbent subsequently washed with 2.0 mL of formic acid (2 %, v/v) followed by 2.0 mL of methanol. The analytes were released from the sorbent by elution with three times 0.5 mL and one time 0.3 mL of ammonia solution in methanol (10 %, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C shaking at 550 rpm on a Thermomixer comfort (Eppendorf, Hamburg, Germany) and finally the residue reconstituted with 100 µL mobile phase.

Enalapril

Prior to sample preparation 50 µL serum were mixed with 5 µL benazepril working solution (IS) and 1100 µL water. The samples were extracted with Oasis® MAX solid-phase extraction cartridges (10 mg, 1 mL) – a mixed mode, reverse-phase, strong anion exchanger. The MAX 96-well plates chosen allowed for high sample throughput and were primed with 1 mL of formic acid in methanol (2 %, v/v) followed by an equilibration step with 1 mL water. After the sample mixture was load into the cartridges and passed, the sorbent of the cartridges was washed by 1.0 mL of water, 1.0 mL methanol-acetone mixture (60:40, v/v), 1.0 mL of ethyl acetate and 500 µL methanol. Finally, the analytes were eluted from the cartridges with once 0.4 mL of formic acid in methanol (2 %, v/v). The eluate was evaporated to dryness under a gentle stream of compressed air while shaking at 550 rpm at 40 °C. The residue was reconstituted with 100 µL of methanol and water (40:60, v/v). This final step made for a dilution of the sample/standard concentration in the ratio of 1:2.

Chromatographic and mass spectrometer conditions

The utilised modular HPLC system (Shimadzu Deutschland GmbH, Duisburg, Germany) consisted of a controller SCL-10Avp, two separate pumps LC-10ADvp, three channel online degasser DGU-20A^3 prominence, autosampler SIL-10ADvp and a column oven (L-2300, VWR/Hitachi). Chromatographic separation of aliskiren was carried out on a XSelect^{TM} CSH C18 column (3.0 mm x 150 mm, 3.5 µm) protected by a corresponding XSelect^{TM} CSH C18 guard column (3.0 mm x 20 mm, 3.5 µm). Compounds were separated with a mobile phase consisting of a mixture of methanol and water acidified with formic acid (74.998:24.998:0.005, v/v/v) by applying isocratic elution at 0.4 mL/min. Column temperature was maintained at 40 °C and run time was set to 5 minutes. For determination 20 µL of the reconstituted sample solution were used per analysis.

For the separation of enalapril and enalaprilat an XBridge® BEH C18 3.5 µm columns (3.0 mm x 150 mm) was used. After injection of 10 µL sample solution (Methanol/water 40:60, v/v) the samples were separated under gradient conditions within 6 minutes run time utilising a methanol/water mixture (40:60, v/v) buffered by formic acid (1 %, v/v) and ammonium formiate (2 mM). The applied gradient started with 40 % of methanol, and increased stepwise after 0.5 minutes to 60 % and after 1 minute to 80 %. Between 1 and 4 minutes the amount of methanol was continuously increased to 95 % and reduced at 4.5 minutes to 40 % of methanol again. It stayed at this level till the end of the run time. After an equilibration time of 3 minutes the next samples were injected. The flow rate was 0.4 mL/min and the column temperature was maintained at 50 °C which resulted in a moderate back-pressure of 125 bar.

Triple-quadrupole tandem mass spectrometric detection was performed on an Applied Biosystems SCIEX API 2000 (Applied Biosystems/MDS SCIEX, Concord, Canada) with an electrospray ionisation (ESI) interface running in positive ionisation mode. The ion transitions were mass-to-charge ratio (m/z) 552.2 to 436.2 m/z for aliskiren and 425.3 to 351.2 m/z for benazepril (Figure 6). Both dwell times were set to 300 milliseconds (ms). Regarding enalapril (ENA) and enalaprilat (ENAAT) detection, the device screened the transitions channels 377.2 to 234.2 m/z (ENA), 349.1 to 206.1 m/z (ENAAT) and 425.3 to 351.2 m/z for benazepril in multiple reaction monitoring (MRM) mode (Figure 7 and 8). All dwell times were set to 250 ms. Detailed MS conditions for aliskiren were as follows: Curtain gas and collision gas were adjusted to 21 and 5 a.u. (arbitrary units), respectively. Nitrogen was employed as collision gas. Ion source gas 1 and ion source gas 2 were set to 26 and 70 a.u., while the ESI source was held at 500 °C and the ion spray voltage was fixed at 5.5 kV. The compound-specific parameters declustering potential, focusing potential, entrance potential and cell entrance potential were adjusted to 97, 360, 11 and 23 eV, respectively. The

optimal voltages of the parameters collision energy and cell exit potential were 24 and 11 eV, respectively. Regarding enalapril and enalaprilat, the corresponding source and compound dependent parameters are listed in Table 3. While the collected data of aliskiren was analysed using Analyst 1.4.1, the updated software version 1.5.1 was applied for enalapril/enalaprilat determination (Applied Biosystems/MDS SCIEX, Concord, Canada). The data processing was done with IntelliQuan® as integration algorithm without smoothing for both methods.

Source parameters and gases			
Curtain gas	18		
Collision gas	5		
Ionspray voltage	1.5 kV		
Temperature	350 °C		
Ion source gas 1	30		
Ion source gas 1	90		
Compound dependent parameters			
	Enalapril [eV]	**Enalaprilat [eV]**	**Benazepril [eV]**
Declustering potential	56	56	26
Focusing potential	300	370	150
Entrance potential	11.5	11.5	12
Cell entrance potential	16	14	13
Collision energy	27	25	28
Cell exit potential	10	10	8

Table 3 ***Compound dependent as well as source and gas parameters of the enalapril/enalaprilat method in serum.***

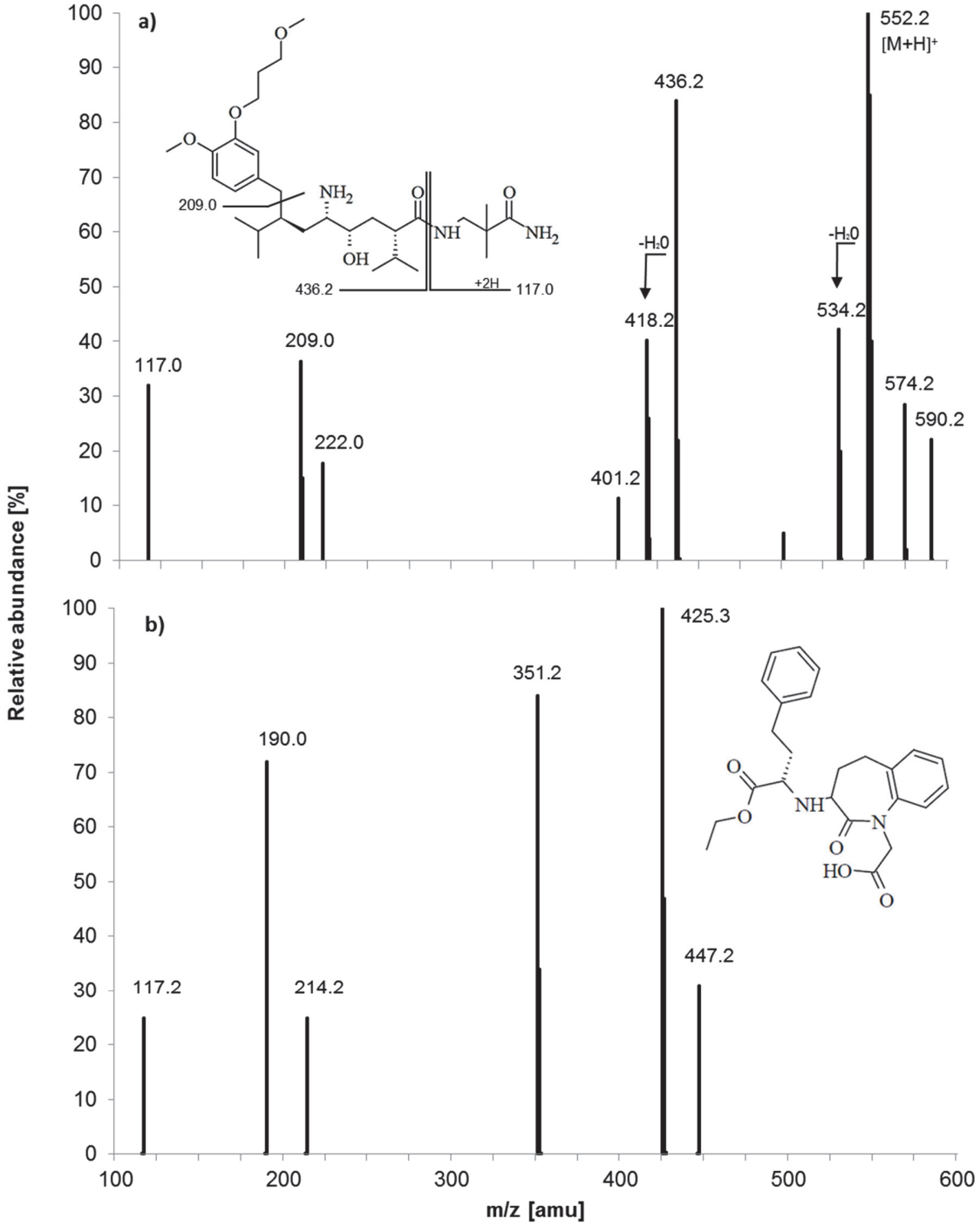

Figure 6 ***Typical electrospray mass spectra of aliskiren*** *(a)* ***and benazepril*** *(b). Both spectra were obtained by electrospray ionisation carried out in positive ion mode by Q1 scan (100 – 600 m/z). Possible fragmentations of aliskiren are additionally indicated by means of the enclosed chemical structure. Each fragment is adumbrated in the structure by a separation line and the corresponding fragment mass. $[M+H]^+$: molecular weight of the substance plus one proton, H_2O: water.*

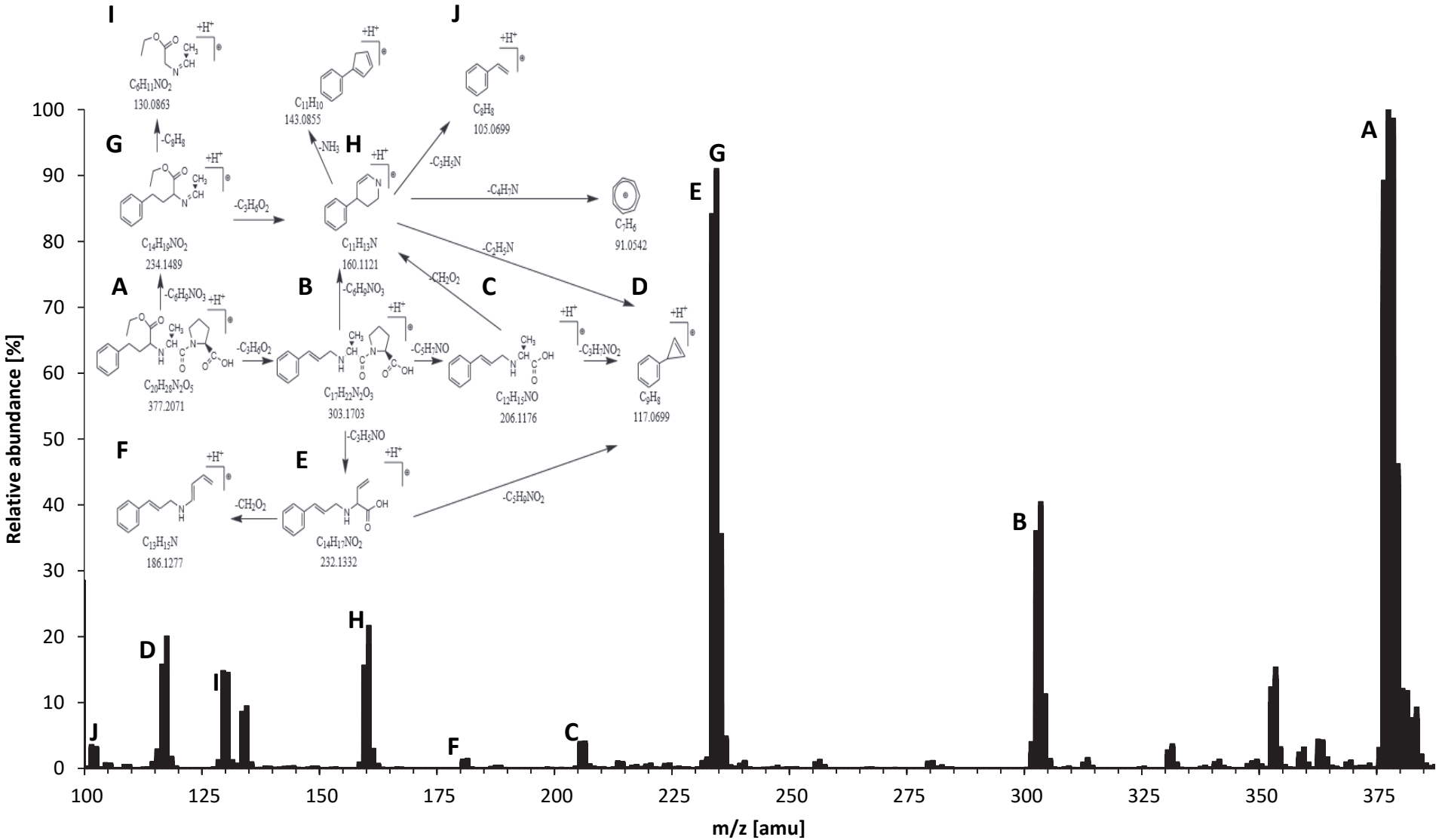

Figure 7 ***Obtained electrospray mass spectrum of enalapril*** *with modified possible fragmentation pathways of enalapril (suggested by Feng et al. PMo-004 IMSC 2012) Q1 scan was performed with electrospray ionisation in positive ion mode. Scan range was 100 – 400 m/z. Each fragment is identified by a capital letter (starting with "A" for the parent compound) in the chromatogram and next to the corresponding chemical structure (upper left corner). Arrows illustrate the possible fragmentation pathway beginning with the parent compound A (377.2071 m/z).*

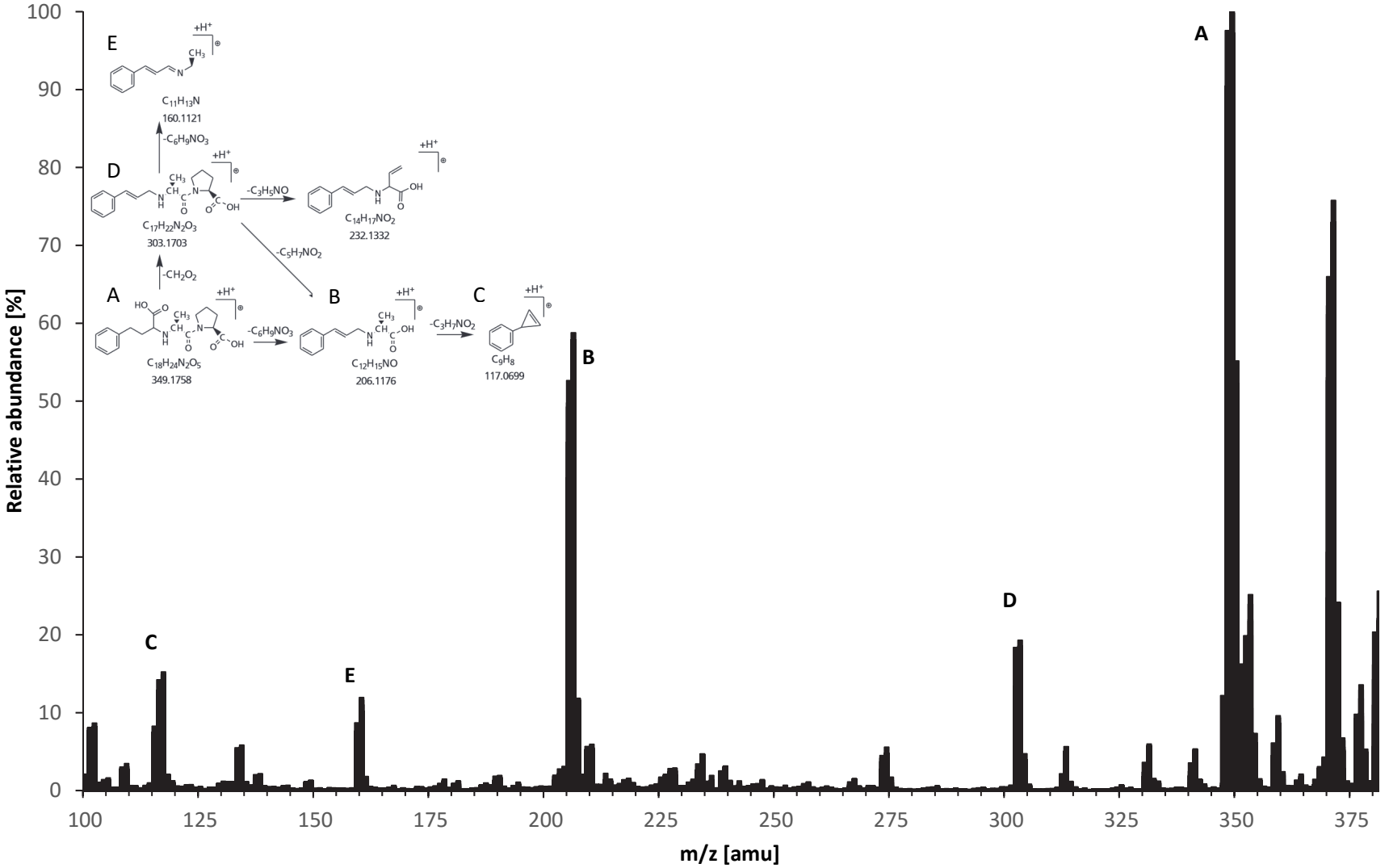

Figure 8 ***Obtained electrospray mass spectrum with modified fragmentation pathways of enalaprilat*** *(suggested by Feng et al. PMo-004 IMSC 2012). Q1 scan was performed with electrospray ionisation in positive ion mode. Scan range 100 – 400 m/z. Each fragment is identified by a capital letter (starting with "A" for the parent compound) in the chromatogram and next to the corresponding chemical structure (upper left corner). Arrows illustrate the possible fragmentation pathway starting from the parent compound A (349.1758 m/z).*

Bioanalytical method validation

A compiled in-house validation plan was established based on current FDA guidance (2001) and EMA guidelines (2012) on bioanalytical method validation (European Medicines Agency 2012b, Food and Drug Administration 2001). A specific ICH guideline on bioanalytical method development was not applicable and consequently ICH guidelines were omitted from the compilation of the in-house validation plan. Summarised details on the in-house validation requirements, which were equal or even stricter than the strictest criteria of international guidelines, are outlined exemplarily for aliskiren in Table 4. Slight deviations to the validation plan illustrated in Table 4 arised for enalapril. For example, the determinations of accuracy and precision for enalapril and enalaprilat were determined using four different concentration levels instead of five levels. Details are given in the following sections. Both the aliskiren and the enalapril/enalaprilat method were independently and fully validated following the in-house validation plan and comply with international bioanalytical guidelines. Each validation run was based on a freshly prepared calibration curve.

Selectivity and cross-talk

Selectivity is the assay's ability to distinguish between different compounds in the biological sample. In this context, differentiation is made between interference caused by matrix and by other drug substances. Regarding the latter, it is hard to conceive which further medication a patient will receive; consequently it is difficult to validate the assays for all future "cross-reaction-scenarios". Additionally, aliskiren is a new drug in treatment of cardio-vascular diseases that is likely to be used in combination if previous treatment is insufficient. Applying the tandem MS in multiple reaction monitoring (MRM) mode clearly simplifies this challenge owing its superior selectivity by screening for parent compound and its fragments. To demonstrate the ability of the assay to differentiate and quantify the analyte in the presence of co-medications, several different drugs in serum of different sources were checked for peaks that could potentially interfere with the analyte peak or the one of the IS. An interaction was defined as a signal in the range of 0.3 minutes before or after the retention time of the analyte or IS and the potential to induce ion suppression or enhancement. A deviation of known concentration of more than minus 15 % was classified as ion suppression and of more than 15 % as enhancement. Drugs investigated in the aliskiren method were: Acetylsalicylic acid, ibuprofen, amiodarone, sotalol, bisoprolol, metoprolol, cavediol, enalapril, enalaprilat, ramipril, ramiprilat, cidofovir, cyclosporine and heparin. The enalapril/enalaprilat method was assessed on interfering peaks by checking serum samples with acetylsalicylic acid, aliskiren, ramipril, ramiprilate, candesartan, atenolol, metoprolol, bisoprolol, pantoprazole, pravastatin and L-

thyroxine. Furthermore, in line with FDA and EMA guidelines (European Medicines Agency 2012b, Food and Drug Administration 2001), six (for aliskiren) or seven (for enalapril/enalaprilat), individual sources of drug-free human serum from healthy subjects were processed and analysed for any interference of endogenous matrix compounds. Haemolysed and hyperlipidaemic samples were likewise checked. Finally, cross-talk of the assays was determined by monitoring the response of the analyte channel in the presence of a serum sample only spiked with internal standard on the one hand and the response of the internal standard channel in the presence of serum with the analyte(s), on the other hand. Zero samples were inspected within daily sets of the calibration standards.

Linearity and Lower Limit of Quantification (LLOQ)

When linearity of an analytical methods is given within a defined range, the obtained signals are directly proportional to a drug concentration. The lower limit of quantification determines the lowest drug amount in an individual analytical method that can be quantified within the pre-defined limits for accuracy and precision.

Calibration curves were obtained by measuring freshly spiked human serum samples with aliskiren at 14 concentration levels or by 11 concentration levels of human serum samples spiked with enalapril and enalaprilat, respectively. These levels as well as blank and zero samples were determined in triplicate. The calibration range of the analytes of interest were set according to published drug levels in humans under various conditions (fed, fasted, age, gender, drug interaction etc.) empowering the measurement across all reported drug levels by only one analytical method with a wide calibration range. The concentration of added internal standard benazepril was chosen on the notion of allowing for a slope of the calibration curve that enables a good distinction at all analyte concentration levels. Linearity of each curve was evaluated by plotting the concentration ratio of the analyte to IS versus peak area of the analyte to IS. While for aliskiren a weighted linear regression of 1/x was applied, the regression of enalapril and enalaprilat was weighted by $1/x^2$. The suitability of the calibration curve was assessed by the mean value of accuracy and precision per concentration level which had to be within 15 % of nominal concentration except LLOQ for which the deviation must not exceed 20 %. Moreover, a correlation coefficient of ≥ 0.99 was expected to be sufficient to prove the goodness of the regression. Additionally, a signal-to-noise ratio of at least 5:1 compared to the blank sample was required for the LLOQ. Determination of LLOQ concentration was performed on six (for enalapril: seven) different individual sera to verify the assay's reproducibility under changing conditions at this extremum.

Accuracy and Precision

While accuracy is described by the deviation of the determined analyte concentration from the nominal concentration and presented as a percentage; precision defined the closeness of repeated individual measures of an analyte concentration. For determination of accuracy of the aliskiren method, quality control samples at 0.146, 1.17, 18.8, 150 and 1200 ng/mL aliskiren were evaluated by five-fold replicate analysis on four different runs on four different days. QC samples of enalapril and enalaprilat were determined at 0.2, 3.13, 25 and 200 ng/mL enalapril, as well as 0.18, 2.81, 22.5 and 180 ng/mL enalaprilat, respectively, and were evaluated by five-fold measurements (independently processed) at each concentration level. Inter-run accuracy and precision of enalapril and enalaprilat were investigated by five runs on five days. Based on the requirements of EMA and FDA (European Medicines Agency 2012b, U.S. Food and Drug Administration 2001), the mean value per concentration of within-run and between-run accuracy must be within 15 % of the nominal concentration except for the LLOQ at which the deviation of 20 % is acceptable. The same applies to the investigation of precision which must be demonstrated at the above-mentioned concentrations by five samples per concentration level and the mean value must not exceed 20 % for the LLOQ and 15 % for all other concentration levels, respectively. The deviation in precision was calculated using one-way ANOVA and was evaluated for intra-runs and inter-runs which also cover variability in batches of reagents, equipment and two different analysts.

Calculation of repeatability (intra-run precision):

$$CV\ [\%] = \frac{\sqrt{s^2}}{\bar{X}} * 100 = \frac{\sqrt{MS_{wg}}}{\bar{X}} * 100 \quad (1)$$

where CV = coefficient of variation; $\bar{X}$ = mean; s^2 = repetition variance; MS_{wg} = mean squares error within group

Calculation of time-different intermediate precision (inter-run precision):

$$s_t^2 = \frac{MS_{bg} - MS_{wg}}{n} \quad (2)$$

where s^2_t = variance between days; MS_{wg} = mean squares error within group; MS_{bg} = mean squares error between group; n = amount of repetitions per day

$$CV\ [\%] = \frac{\sqrt{s^2 + s_t^2}}{\bar{X}} * 100 \quad (3)$$

where CV = coefficient of variation; $\bar{X}$ = mean; s^2 = repetition variance; s^2_t = variance between days

Additionally, the precision of the system (LC-MS) was evaluated by replicate injection.

Validation parameter		Strictest guideline requirement according to FDA and/or EMA	Corresp. guideline*	In-house validation plan for aliskiren serum method development
Selectivity	*Interference of matrix*	6 different sources of blank matrix; criterion: response ≤ 20 % LLOQ/≤ 5 % IS	EMA	6 different sources of blank matrix; criterion: response ≤ 20 % LLOQ/≤ 5 % IS
	Interference of substances	Investigation of potential interfering drugs/ metabolites/ degradation products etc.	FDA + EMA	14 potential co-administered drugs in different human sources; criterion: no peak within ± 0.3 min of retention time of analyte + IS
	Cross-talk	--	--	Cross-talk between analyte and IS channel, criterion: no response
Linearity		At least 6 concentrations per calibration curve; at least 3 calibration curves; accuracy & precision ≤± 15 % (≤± 20 % at LLOQ)	EMA	14 concentration levels per calibration curve; 17 calibration curves in total; accuracy & precision ≤± 15 % (≤± 20 % at LLOQ)
Limit of quantification		S:N ≥ 5:1; accuracy & precision ≤ 20 %	FDA + EMA	S:N ≥ 5:1; accuracy & precision ≤ 20 %; 6 different human sources
Accuracy	*Within-run*	Four concentration levels; n=5 per concentration level; max. ± 15 % (± 20 % at LLOQ) deviation	EMA	Five concentration levels; n=5 per concentration level; max. ± 15 % (± 20 % at LLOQ) of nominal concentration
	Between-run	3 runs on two days; four concentration levels; n=5 per conc. level; Mean: ≤± 15 % (≤± 20 % at LLOQ)	EMA	4 runs on 4 days; 5 concentration levels; n=5 per concentration level; Mean: ≤± 15 % (≤± 20 % at LLOQ) of nominal concentration
Precision	*Within-run*	Four concentration levels; n=5 per conc. level; CV: ≤ 15 % (≤ 20 % at LLOQ)	EMA	Five concentration levels; n=5 per conc. level; ≤ 15 % (≤ 20 % at LLOQ)
	Between-run	3 runs on two days; four concentration levels; n=5 per conc. level; CV: ≤ 15 % (≤ 20 % at LLOQ)	EMA	4 runs on 4 days; 5 concentration levels; n=5 per concentration level; ≤ 15 % (≤ 20 % at LLOQ)
Matrix effect		6 lots of matrix; 2 concentrations (low and high); CV ≤ 15 %	EMA	*Absolute effect*: 5 concentrations over whole range; n=5 per level *Relative effect*: 6 lots of matrix; 3 concentrations (n=5); CV: ≤ 15 %
Recovery		Determination at 3 concentration levels	FDA	Determination at 5 concentration levels; n=5 per level
Stability		Freeze-thaw/ short-term/ long-term/ stock-solution and post-preparation stability	FDA + EMA	Freeze-thaw/ short-term/ long-term/ stock-solution and post-preparation stability
Dilution integrity		n=5 per concentration; criterion: accuracy & precision ≤± 15 %	EMA	n=5 per concentration level; criterion: accuracy & precision ≤± 15 %
Carry over		Blank samples after a high concentration sample; criterion: response ≤ 20 % of LLOQ/ ≤ 5 % of IS	EMA	Blank after one and after 20 samples of high concentration and ULOQ; criterion: response ≤ 20 % of LLOQ/ ≤ 5 % of IS

IS= internal standard CV= coefficient of variation S:N= signal-to-noise ratio ANOVA= analysis of variance

Table 4 ***Summarised details of the in-house validation plan*** *based on the strictest criteria of EMA (European Medicines Agency 2012b) and FDA bioanalytical guidelines (Food and Drug Administration 2001). Exemplified by the aliskiren method.*

Matrix effect and recovery

Even though LC-MS/MS is a highly sensitive and selective analytical technique for quantification of bioanalytical samples it is susceptible to the effects of residual biological matrix. Combined with the risk of low recovery in solid-phase extraction, the analytical method might not be applicable especially for small sample volumes because these effects united tighten the calibration range by increasing the LLOQ. Thus, recovery and absolute matrix effect were investigated at five concentration levels in the range of the whole calibration curve for aliskiren and at four concentration levels for enalapril and enalaprilat, respectively. Both, recovery and absolute matrix effect were determined by five-fold independent sample preparation and corresponding analysis per concentration level. Investigation concerning aliskiren was conducted at the LLOQ (0.146 ng/mL), low (1.17 ng/mL), medium (18.8 ng/mL), high concentration (150 ng/mL) and the ULOQ (1200 ng/mL). Owing to the smaller calibration range of the enalapril/enalaprilat method, the matrix effect was studied at four concentration points (LLOQ, low, medium and ULOQ). The matrix effect and the recovery of the internal standard was determined at the applied concentration. With regard to results of *Chambers et al.* (Chambers et al. 2007), low pH value of mobile phase in combination with polymeric mixed-mode exchange SPE was chosen for purifying the biological fluid and lowering the matrix effect (strong cation exchanger for aliskiren and strong anion exchanger for enalapril/enalaprilat). For determination of the absolute matrix effect, blank processed serum after solid-phase extraction was spiked with analyte in reconstitution solution and the obtained signal compared to the ones of analyte in mobile phase only. According to EMA guideline (European Medicines Agency 2012b) additionally serum of six different sources was explored at low, medium and high aliskiren concentration levels as well as at low and high enalapril/enalaprilat concentrations levels (seven different sources) by triplicate measurement each to determine the relative matrix effect. The relative matrix effect is considered more relevant for biological fluids and resulting methods than the absolute effect owing to the possible variability in the matrix composition of different sources (Matuszewski et al. 2003). Investigations on the influence of haemolysed blood samples were also performed at 0.146, 18.8 and 1200 ng/mL aliskiren as well as 200 ng/mL enalapril and 180 ng/mL enalaprilat. Calculation of the matrix effect was conducted by calculating the ratio of the peak area of extracted human serum post spiked with analyte ($Area_x$) to the peak area of the analyte in the same concentration dissolved in mobile phase ($Area_y$). The absolute matrix effect must be evaluated, but is not limited to a certain range. Using the post-extraction addition technique (Taylor et al. 2005) the matrix effect was calculated as follows:

$$ME\ [\%] = \left(\frac{Area_X - Area_Y}{Area_X} \right) * 100 \quad (4)$$

where ME= matrix effect; Area x= detected peak area of extracted human serum post spiked with analyte; Area y= detected peak area of dissolved analyte in mobile phase

The IS-normalised relative matrix effect (IS-ME) was calculated according to EMA with acceptance criteria expressed as coefficient of variation (CV) per concentration level of maximum 15 % of the inter-subject deviations (European Medicines Agency 2012b). The calculation of the latter was done by evaluation of the individual IS-normalised matrix effect being defined as the matrix factor of the analyte divided by the matrix factor of the IS. The matrix factor represents the ratio of the peak area in the presence and the absence of the matrix of the corresponding substance. The CV of the IS-normalised matrix effects of all six or seven, respectively, subjects were used to assess the relative ME.

$$IS - ME\ [\%] = \left(\frac{\text{Peak area of analyte}_{\text{Presence of matrix}} / \text{Peak area of analyte}_{\text{Absence of matrix}}}{\text{Peak area of IS}_{\text{Presence of matrix}} / \text{Peak area of IS}_{\text{Absence of matrix}}} \right) * 100 \quad (5)$$

where IS-ME= internal standard normalised matrix effect

The ratio of peak area of serum spiked with analyte prior to solid-phase extraction ($Area_A$) with the peak area of blank serum spiked with analyte after the extraction ($Area_B$) yielded the absolute recovery of the assay. Recovery was determined at five (aliskiren) and four (enalapril/enalaprilat) concentration levels with five replicates per level. Calculation was performed as follows:

$$RE\ [\%] = \left(\frac{Area_A}{Area_B} \right) * 100 \quad (6)$$

where RE= recovery; Area A= peak area of serum spiked with analyte prior to extraction; Area B= peak area of blank serum spiked with analyte after the extraction

To calculate the process efficiency of the solid-phase extraction the following equation by *Taylor et al.* (Taylor et al. 2005) was used:

$$PE\ [\%] = RE\ [\%] * \frac{(100 - ME\ [\%])}{100} \quad (7)$$

where PE= process efficiency; RE= recovery; ME= absolute matrix effect

Stability

The effect of freezing and thawing on the concentrations of aliskiren, enalapril and enalaprilat was evaluated utilising QC samples, which were subjected to four freeze (-20 °C) and thaw (21 °C) cycles before analysis. Stability of stock solution was investigated by comparing the peak area of freshly prepared stock solution to the peak areas of three and six months aged solutions stored at 7 °C (aliskiren) or -20 °C (enalapril and enalaprilat). For determination of short-term stability, the peak areas of QC samples stored unprocessed for 24 h at room temperature were compared to the peak areas of freshly prepared QC samples at 0.146, 1.17, 18.8, 150 and 600 ng/mL aliskiren and at 0.195, 3.13, 25, 200 ng/mL enalapril and 0.175, 2.81, 22.5, 180 ng/mL enalaprilat. Long-term stability of aliskiren in serum was studied at 0.146, 1.17, 18.8, 150 and 1200 ng/mL after one and three months at -20 °C and -80 °C. Enalapril and enalaprilat were checked regarding long-term stability after 54 days after being frozen at -80 °C at above-listed enalapril and enalaprilat concentration levels. Furthermore, post-preparation stability (autosampler stability) was investigated by comparing the measured concentrations stored in the autosampler at room temperature for 24 h to the nominal concentrations. Regarding enalapril and enalaprilat, the drug stability in the autosampler was evaluated after 48 h. Processed dry extracts of aliskiren, as well as of enalapril and enalaprilat (post-extraction), were additionally stored at -20 °C for 24 h in the freezer to evaluate stability of the extract after the samples were dried. In all cases, the analytes of interest were considered to be stable as long as degradation of concentration was $\leq$ 15 % of the nominal concentration and $\leq$ 20 % at the LLOQ. These limits represent acceptable thresholds for accuracy and precision of bioanalytical methods according to international guidelines.

Dilution integrity and carry over

It cannot be ruled out that serum samples with concentrations higher than the calibration range need to be measured as aliskiren shows high variability in C_{max} for example (Vaidyanathan et al. 2007b). Also for enalapril, few concentrations have been reported that are not covered by the current calibration range (Arafat et al. 2005). To prove if concentrations exceeding the upper limit of quantification can still be measured accurately after dilution, the dilution integrity was checked by spiking human blank serum with concentrations above the ULOQ and serially diluted with blank serum to yield exactly the ULOQ. Additionally, this investigation allows checking for possible hook-effects at high concentrations, occurring in samples with saturable binding sites of reagents or biological compounds. Higher concentrations of drugs might cause unproportionally higher or even lower signals compared to the lower concentrations detected and consequently lead to false concentrations than the actual level presented in the sample. Although this

effect is known to occur mainly in immunological tests it was not reported to be examined for aliskiren and was therefore evaluated as it could not be ruled out in principle. Accuracy and precision were judged suitable if the mean percentage-deviation was below 15 % (expressed as CV (%) for precision and percentages (%) of nominal concentration for accuracy). With regard to EMA guidelines (European Medicines Agency 2012b), carry over was assessed by injecting a sample of mobile phase right after a serum sample with aliskiren concentration at 125 ng/mL and 1200 ng/mL. Enalapril and enalaprilat were investigated at 200 ng/mL and 180ng/mL, respectively. Carry over was obviated if not more than 20 % of the peak area of the analytes of interest at the LLOQ and 5 % of the IS were detected.

Robustness and method development

Investigations on the robustness of the developed aliskiren assay were performed to prove the appropriateness of the assay under slightly changing conditions. Following parameters were studied with main focus on resulting peak areas: Temperature of column oven between 20 – 50 °C, pH value of mobile phase was varied between 0 to 1 %, flow rate of mobile phase was changed by $\pm$ 10 % and percentage of organic solvent in mobile phase ranged from 72.5 – 77.5 %. Moreover, batch-to-batch consistency of utilised XSelect^{TM} HPLC-columns (lot 106312361, 108313471 and 108320131) was studied. Additionally, compound specific parameters of the mass spectrometer were analysed by flow injection analysis. Curtain gas was optimised in the range of 10 – 35 a.u. (arbitrary units), collision gas was varied from 3 to 7 a.u., ion source gas 1 was investigated between 0 – 50 a.u. and ion source gas 2 between 55 – 70 a.u. Furthermore, resulting peak areas and intensities by temperature changes of the ion source between 300 – 500 °C and changes in ion spray voltage of 4.5 – 5.5 kV were investigated. Prior to method validation, sophisticated method development regarding solid-phase extraction, high performance liquid chromatography and mass spectrometry were undertaken. At the time point of the aliskiren method development, only methods supported by the originator were published. Thus, reported data was limited and consequently extensive development was necessary. In brief, five different HPLC columns were investigated on appropriate peak separation, shape, signal intensity and retention time. Due to the lack of deuterated internal standards, two structurally related substances were investigated (Acebutolol vs benazepril). 20 mass spectrometer parameters of the API 2000 were identified and optimised by flow injection analysis. Finally, the sample purification was developed by investigation of different approaches on their effectiveness (LLE, PPT and SPE), whereof SPE performed best regarding purification and recovery of analyte. The procedure was than optimised with regard to washing steps and elution of the aliskiren.

With focus on method development for enalapril and enalaprilat, in particular the solid-phase extraction was optimised with effort on highest possible matrix effect reduction and speed of extraction cycles as well as liquid chromatographic separation to support reducing the matrix effect by SPE. Therefore, sample preparation was evaluated by different acids (formic acid, phosphoric acid and hydrochloric acid) and water, as well as its best suitable mixing ratio of acid and sample volume. Different reverse-phase sorbents were evaluated on their suitability for sample purification. Additionally, the wash and elution steps were investigated on matrix effect reduction and analyte breakthrough in hydrophilic, lipophilic, organic as well as inorganic solutions at different pH values. Moreover, after selection of the best elution solution, rated on the lowest eluted matrix amount, an investigation on the required volume was conducted. In parallel, three different types of HPLC columns were investigated on their separation capacity of analytes and matrix compounds. Likewise, as for aliskiren, the flow injection analysis on source and gas parameters for enalapril and enalaprilat was done in the following ranges: the curtain gas was adjusted between 10 and 30 a.u., ion source gas 1 tuned in the range of 20-50 a.u. and ion source gas 2 was optimized between 50 and 90a.u. The collision gas was adopted for best performance between 4 -7 a.u., while temperature of the ion source was varied between 150 and 550 °C. Finally, the oven temperature of the HPLC system was varied from 25 – 50 °C by checking on the change in intensity, retention time and peak shape.

Application

Due to the absence of detailed individual concentration-time profiles, especially the developed aliskiren assay was proved to demonstrate its applicability. Additionally, the effect of aliskiren administration under fasted and fed conditions, as well as the effect of different dosing (1 x 300 mg vs 2 x 150 mg Rasilez® [each administered as single dose]) were evaluated. Dense sampling was applied to generate as valuable pharmacokinetic data as possible allowing the obtaining of single individual concentration-time profiles. Samples drawn in healthy volunteers within 72 hours after administration of 20 mg enalapril (EnaHEXAL®) were additionally analysed. Blood samples were centrifuged at 3220 g for at least 20 minutes and the corresponding serum was frozen at -20 °C or below until analysis. Half-life was calculated by performing a regression over the last four measured concentration-time points to determine the elimination rate constant k_e, which was transformed to half-life by using equation 5:

$$t_{½} = \frac{\ln 2}{ke} \tag{8}$$

where $t_{½}$ = elimination half-life; ke= elimination rate constant

The AUC was calculated by applying the trapezoidal rule with infinity extrapolation. With regard to guidelines, the maximum acceptable percentage of the extrapolated AUC must be less than 20 % of the total AUC.

$$AUC_{0-t\ last} = \sum_{i=0}^{n-1} ((c_{i+1} + c_i)/2) * (t_{i+1} - t_i) \quad (9)$$

$$AUC_{0-\infty} = AUC_{0-t\ last} + \frac{c_{t\ last}}{k_e} \quad (10)$$

where ke= elimination rate constant; t= time; c=concentration; AUC= area under the curve; c_t last= concentration of the last sampling point

Within this thesis two enalapril/enalaprilat assays were developed. The first assay [former assay] was used to determine the drug concentrations in the proof-of-concept study (section 3.4). The assay's applicability was shown prior to its application. The second assay was applied for the Phase I study within the LENA project. The new assay is charaterised by a broader calibration range, a lower required sample volume and a higher degree of sample purification. The comparability of both assays was checked by determination of 1 hour postdose samples of all volunteers of the proof-of-concept study with both assays. The statistical evaluation was performed by a paired t-test.

3.4.1.2 Scale up of the extraction process

To meet current demands in sample through-put within a clinical study, the scale-up to off-line positive pressure extraction was conducted. For reproducible and high-quality solid-phase extraction (SPE) with a high run-to-run consistency as desired for clinical studies, the switch from single cartridges by vacuum extraction to 96-well positive pressure extraction was conducted. This scaling presented the highest possible off-line scaling for the used SPE material. SPE-formats with higher amounts of SPE cavities per plate were commercially not available. The conventional vacuum manifold had the disadvantage of irreproducible analyte recoveries due to variable processing times in the columns. This happened if some columns run dry, which allowed a free flow path of vacuum and negatively affected the solvent flow in all other cavities. But even improperly closed ports or any leakage also effected the run-to-run precision. For highest possible reproducibility during extraction, the controlled and appropriate flow rate is much more essential than applying either vacuum or positive pressure. However, the positive pressure manifold had the advantage of being equipped with a monitor to check for the flow rate of the liquid. Especially the sample load, washing- and elution step are known to be most sensitive and critical regarding the flow rate. The exact adjustment of the flow rate of the liquids is important to generate a well-balanced setting of high-

reproducibility, best extraction speed, and duration of sample extraction because for critical steps the flow can be reduced to enable best possible interaction conditions while it is increased during insensitive steps.

The solid-phase extraction was performed as described above by utilising a 96-well Positive-Pressure Manifold (Waters, Germany). The transfer from vacuum extraction to positive pressure did not only enable a semi-automated extraction but also to tremendously increase the sample amount up to about thousand samples which can be purified per week by one laboratory technician. Since appropriate equipment for rapid and continuous drying was not commercial available, required equipment for the drying process was self-developed to suit best to the laboratory pre-conditions and needs (Figure 9). Especially equipment for the drying process (e.g. heatable water bath for 96 deep-well plates and special drying top frame) as well as a 2 ml deep-well changer for the Shimadzu SIL10 ADvp which enabled the direct injection of the samples out of the deep-well into the HPLC-system, were developed. Figure 10 is enclosed illustrating the scale-up from single cartridges using vacuum technique to the positive pressure extraction in 96-well-formate.

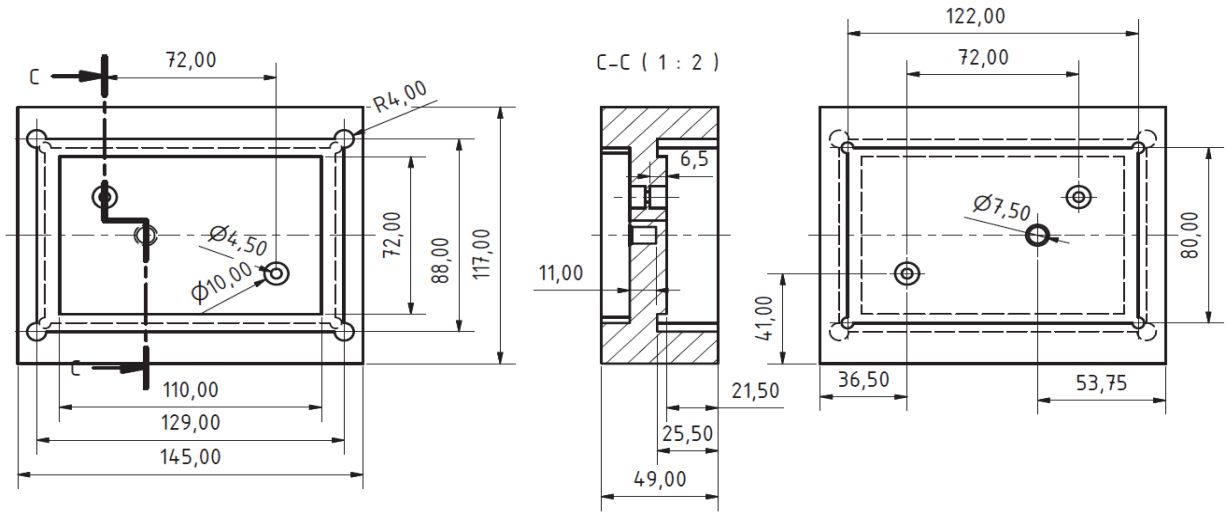

Figure 9 ***Excerpt of the technical drawing of the heatable water bath*** *used in the high throughput approach.*

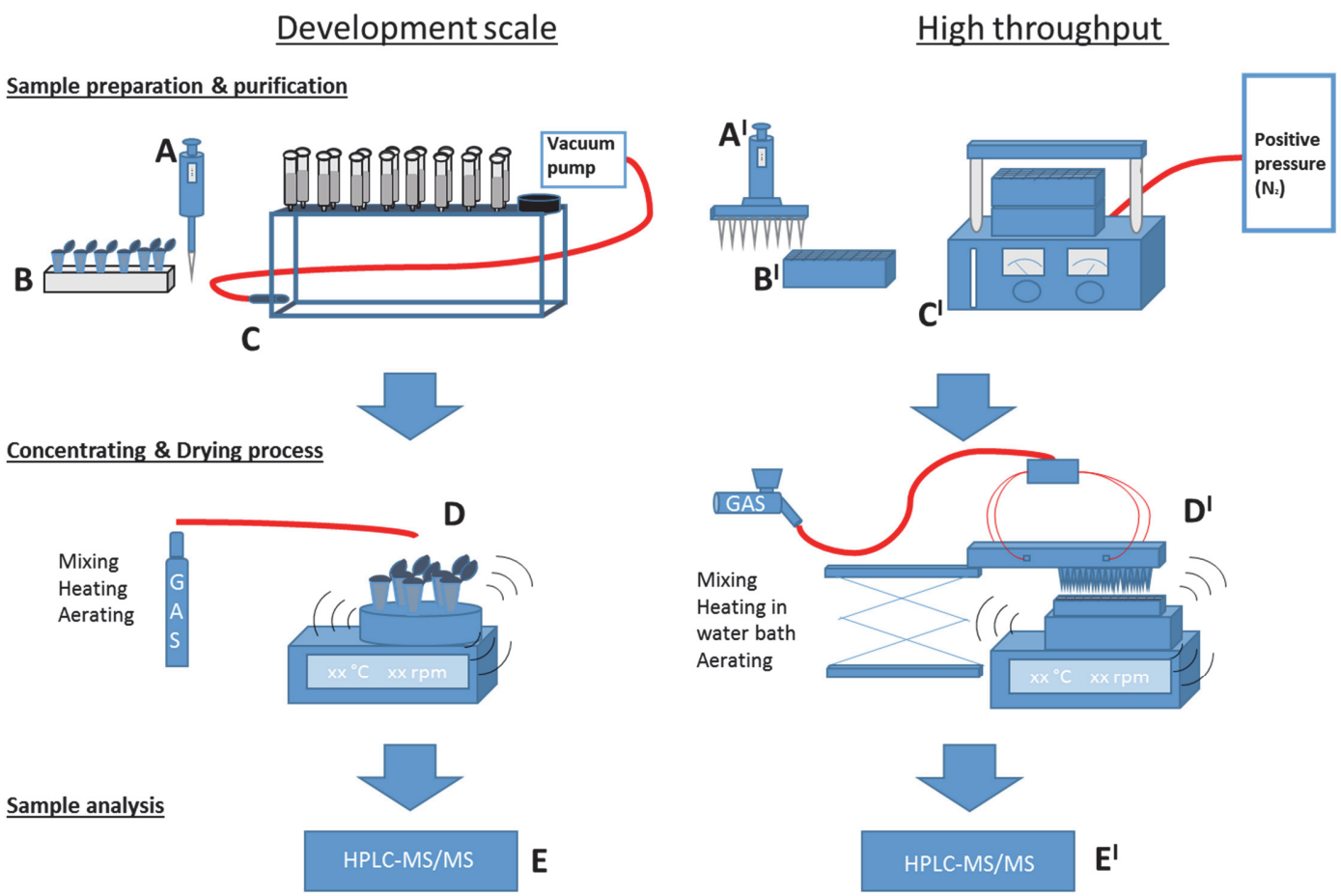

Figure 10 ***Scale-up process and optimisations steps*** *to establish a high through-put approach of all bioanalytical assays utilizing solid-phase extraction. On the left side the development scale of sample preparation and purification is illustrated while on the right side the scale-up is shown.*

Established advance=

A: Single channel pipette (manual)	*→ A'*	*Multi-channel pipette (electronic);*
B: Single micro tubes	*→ B'*	*96-deep-well approach;*
C: Vacuum manifold using single cartridges	*→ C'*	*Positive pressure manifold using 96-well plates;*
D: Gas bottle (nitrogen) and Thermomixer with thermoblock (24 positions)	*→ D'*	*Nitrogen / compressed air generator and modified; Thermomixer with headable temperature-controlled water bath and a self-developed special drying top frame;*
E: Sample injection by autosampler using HPLC vials	*→ E'*	*The whole 96 deep-well plate can optional be used directly in the autosampler to inject the samples without decanting*

3.4.2 Urine

In addition to the determination of drug concentration in serum, it is important to determine its excretion pathways in order to fully describe its pharmacokinetics. Even if drugs are substantially studied in healthy volunteers in whom pharmacokinetic trials are commonly conducted, other subpopulations are equally meaningful. In particular body weight/composition, excretory and metabolic functions and pathophysiological features may alter dose-concentration relationships. On the one hand, renal excretion of aliskiren in children and adolescents has not been reported yet. On the other hand, enalapril, which is extensively eliminated by the kidney, might vary more in children with congenital heart failure or patients suffering from renal failure than in healthy volunteers who receive the same drug dosage. Therefore, a reliable assay for evaluation of urinary concentrations of aliskiren and enalapril is of relevance in comprehensive pharmacokinetic investigations and is presented hereinafter.

3.4.2.1 Simultaneous determination of aliskiren, enalapril and enalaprilat in urine

The drug aliskiren, the prodrug enalapril and its active metabolite were all determined by one method simultaneously quantifying the drug levels in urine. The method was developed as single cartridge approach and then scaled to meet current demands on high sample through-put by HPLC-tandem MS (Figure 10).

Chemicals and materials

Aliskiren hemifumarate (≥ 98 %, potentiometry & HPLC) was obtained from MSN Laboratories (Hyderabad, India). Enalapril maleate (≥ 98 %, TLC) and the internal standard benazepril hydrochloride (≥ 98 %, HPLC) were purchased from Sigma-Aldrich (Seelze, Germany). Enalaprilat was USP standard and received from LCG-Standards (Luckenwalde, Germany). Methanol (HiPerSolv® Chromanorm® HPLC grade), water (super gradient grade) and ammonia solution (≥ 25 % p.a.) were supplied by VWR (Germany). Formic acid (98 - 100 % p.a.) and acetonitrile (HPLC grade) were delivered by AppliChem (Gatersleben, Germany). Human urine was provided by employees of the Institute of Clinical Pharmacy and Pharmacotherapy (Düsseldorf, Germany) and their relatives. Oasis® MCX and WAX cartridges (each 30 mg, 1 mL), XSelect™ CSH C18 3.5µm columns (3.0 mm x 150 mm) and XSelect™ CSH C18 3.5 µm guard columns (3.0 mm x 20 mm) were purchased from Waters (Eschborn, Germany).

Preparation of standards and quality control samples

The stock solutions of aliskiren (0.12 mg/mL), enalapril (0.10 mg/mL), enalaprilat (0.10 mg/mL) and the internal standard (IS) benazepril were prepared comparably to the procedure described in section 3.2.1. By contrast to serum, working solutions of enalapril and enalaprilat were not prepared to avoid a falsification

by a high degree of sample dilution during spiking of the high concentration levels being necessary for the urine method.

Serial dilution of the upper concentration limit of 9600 ng/mL of aliskiren with blank urine yielded the following nominal calibration standards: 4800, 2400, 1200, 600, 300, 150, 75, 37.5, 18.8 and 9.4 ng/mL. The same procedure was applied to dilute enalapril and enalaprilat and to prepare the Quality Control (QC) samples leading to concentration levels of 12000, 6000, 3000, 1500, 750, 375, 187.5, 93.8, 46.9, 23.4 and 11.6 ng/mL of enalapril and of 9000, 4500, 2250, 1125, 562.5, 281.3, 140.6, 70.3, 35.2 ,17.6 and 8.8 ng/mL of enalaprilat. All of these dilutions were prepared freshly while the stock solutions of the analytes of interest and IS were stored at 7 °C for a maximum period of 6 months and equilibrated to room temperature prior to use.

Sample preparation

Urine is known to be a highly inter- and intra-variable saline mixture which is prone to causing matrix effect. Therefore, special focus was on the sample purification to yield highly cleaned samples in which neither degradation- and excretion products nor other residuals still interfere. Sample preparation took place without any dilution of the urine samples. 100 µL of the urine were mixed with 10 µL IS (166 ng/mL) and 490 µL formic acid (2 %, v/v) before the mixture was subjected to solid-phase extraction. By applying a two-step clean-up phase using solid-phase extraction consisting of a weak anion exchanger (WAX) followed by a strong mixed mode cation exchanger (MCX) the samples were purified. The weak anion exchanger was conditioned with 1 mL of formic acid in methanol (2 %, v/v) followed by an equilibration step with 1 mL of formic acid in water (2 %, v/v) . The previously centrifuged samples were added to the cartridges (30 mg, 1 mL) und slowly dropped through. To remove interferences two washing steps were performed by first using 500 µL of formic acid (2 %, v/v) and secondly 450 µL of an acid mixture (2 %) of methanol/water (10:90, v/v). The analytes were eluted afterwards with two times 350 µL each of acidified methanol (2 formic acid, v/v). During the second clean-up phase the centrifuged samples obtained from the WAX elution were further on extracted with Oasis® MCX solid-phase extraction (SPE) cartridges (30 mg, 1 mL). The MCX sorbent was preconditioned with 1 mL ammonia solution in a mixture of methanol/acetonitrile (10:45:45, v/v/v) followed by a conditioning step with 1 mL methanol and finally equilibrated with 2 % formic acid (v/v). The methanolic samples were mixed with 700 µL of formic acid (2 %, v/v) and afterwards transferred into the primed cartridges, eluted and the sorbent subsequently washed with 1 mL of 2 % formic acid (v/v) followed by 1 mL methanol/water (80:20, v/v). The analytes were released from the sorbent by elution with three times 0.5 mL and one time 0.4 mL of ammonia solution in a mixture of

methanol/acetonitrile (10:45:45, v/v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C, shaking at 550 rpm and finally the residue reconstituted with 100 µL of acidified (0.1 % formic acid) methanol/water (20:80, v/v).

Chromatographic and mass spectrometer conditions

The utilised modular HPLC system (Shimadzu Deutschland GmbH, Duisburg, Germany) is the same as for serum determination and is described in detail in section 3.2.1.1. Liquid chromatographic separation was performed utilising a XSelect™ CSH C18 column (3.0 mm x 150 mm, 3.5 µm) protected by a corresponding XSelect™ CSH C18 guard column (3.0 mm x 20 mm, 3.5 µm). The total run time was set to 7.5 minutes with a mobile phase gradient of methanol (A) and water (B) to separate the compounds. Both solvents were acidified with formic acid prior to use (0.1 %, v/v) and gradient was as follows: Starting with 30 % of A, after 0.2 min 50 % A, at 6 min 80 % A, at 6.3 min 100 % A, decreased to 50 % A at 7 min and staying at 50 % for the rest of the run time. The total run time was chosen on the fact that fast gradient LC promotes matrix effects which were not favoured (Chambers et al. 2007). The column was re-equilibrated for 3 min with the initial mobile phase. The flow rate was 0.4 mL/min and column temperature was maintained at 40 °C. 2 µL of the reconstituted sample solution were used for determination.

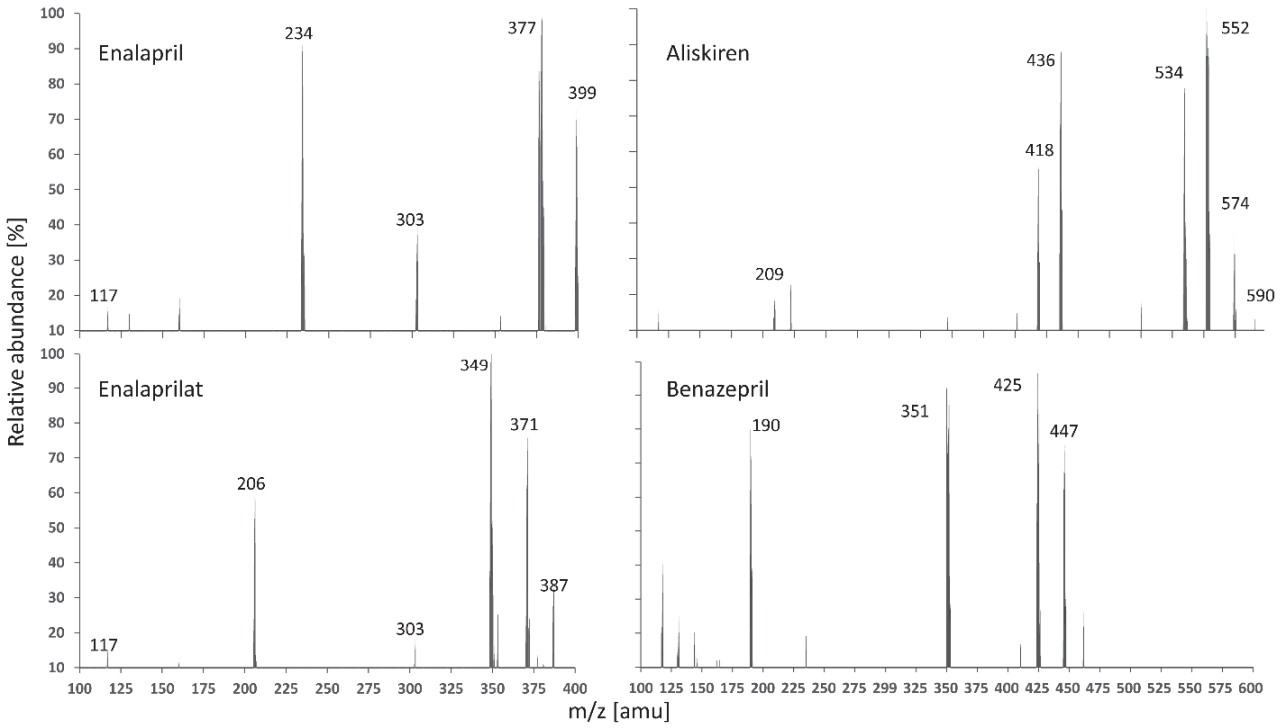

Figure 11 ***Electrospray ionisation mass spectra of all three analytes of interest*** *and the internal standard.*

Mass spectra were obtained by an Applied Biosystems SCIEX API 2000 (Applied Biosystems/MDS SCIEX, Concord, Canada) with an electrospray ionisation (ESI) interface operated in positive ionisation mode. The ion transitions were mass-to-charge ratio (m/z) 552.2 to 436.2 m/z for aliskiren, 377.2 to 234.2 m/z for enalapril, 349.1 to 206.1 m/z for enalaprilat and 425.3 to 351.2 m/z for benazepril. All dwell times were set to 150 milliseconds. Detailed mass spectrometric and compound dependent conditions are compiled in Table *5*. The data acquisition and processing was carried out with Analyst 1.4.1 (Applied Biosystems/MDS SCIEX, Concord, Canada) with IntelliQuan® as integration algorithm without smoothing.

Mass spectrometer-conditions		
Mass spectrometer		API 2000
Interface		electrospray
Polarity		positive
Scan type		MRM[b]
Resolution	Q1	unit[a]
	Q3	unit[a]
Curtain gas		15
Collision gas		5
IonSpray voltage		5.5 kV
Temperature		550 °C
Ion source gas 1		26
Ion source gas 2		80
Solvent split ratio		none

Compound-dependent parameters				
	Aliskiren [eV]	**Enalapril [eV]**	**Enalaprilat [eV]**	**Benazepril (IS) [eV]**
Declustering potential	97	95	101	97
Focusing potential	360	380	380	370
Entrance potential	11	11	11	9
Cell entrance potential	23	8	8	17
Collision energy	24	36	36	30
Cell exit potential	11	11	14	10

Table 5 ***Detailed MS conditions and compound-specific parameters for all analytes of interest*** *and the internal standard benazepril for the urine method ([a] 0.7 ± 0.1 amu peak width at half peak maximum; [b] Multiple Reaction Monitoring).*

Bioanalytical method validation

The urine method was fully validated as a quantitative confirmatory method in terms of linearity, specificity, accuracy, precision, recovery, matrix effect and stability. Each validation run was based on a freshly prepared calibration curve consisting of a set of spiked standard samples of eleven concentrations over the concentration range. All validation parameters were evaluated using the in-house validation plan introduced in section 3.2.1.

Selectivity and cross-talk

Sufficient treatment of cardiovascular diseases by reaching e.g. the recommended target blood pressure is often gained by multiple drug administration only. Aliskiren and enalapril are both drugs approved for the treatment of hypertension and might therefore be combined with other drugs for appropriate treatment of the corresponding diseases. The selectivity of the developed assay was investigated by proving the lack of interaction caused by other co-medications. In this context, human urine samples of several patients were analysed for any peak in the transitions of aliskiren, enalapril and enalaprilat. In total 24 drugs, their corresponding active metabolites or degradation products which are typically administered to cardiovascular and diabetes mellitus patients were evaluated. No drugs were spiked to urine samples; all drugs were administered routinely to the patients and their urine collected. Drugs investigated: aspirin, ibuprofen, captopril, ramipril, ramiprilat, hydrochlorothiazide, omeprazole, candesartan, simvastatin, pravastatin, bisoprolol, amlodipine, thyroxin, mirtazepine, levetiracetam, baclofen, metformin, methionine, levodopa, entacapone, carbidopa and sumatriptan. Furthermore, fourteen individual sources of human urine were processed and analysed for any interference of endogenous matrix compounds with the corresponding analyte channels. Although not required by the bioanalytical guidelines, cross-talk of the assay was determined. The response of one analyte channel in the presence of a urine sample only spiked with internal standard and the other corresponding analytes as well as the response of the internal standard channel in the presence of urine spiked separately with all three analytes of interest, respectively, were examined. The response in the corresponding transitions of urine samples in absence and presence of the analytes of interest were checked for potentially interfering peaks. A deviation of known analyte concentration of more than minus 15 % was classified as ion suppression and of more than 15 % as enhancement if a signal in the range of 0.3 minutes before or after the retention time of the analytes or IS was detected.

Linearity and Lower Limit of Quantification (LLOQ)

A set of eleven non-zero concentration levels as well as blank and zero samples were used to establish a calibration curves utilising freshly spiked human urine samples. Linearity of each curve was evaluated by plotting the concentration ratio of the analyte to IS versus peak area of the analyte to IS. Mean value of accuracy and precision per concentration level had to be within 15 % of nominal concentration. At the lower limit of quantification (LLOQ) these criteria were defined as not more than 20 % for precision, accuracy within ±20 %, and S/N $\geq$ 5. Moreover, fourteen different individual urine samples were evaluated at the LLOQ to verify the assay's reproducibility at the concentration level that might vary highest.

Accuracy and Precision

Quality control samples at 9.4, 75, 600 and 9600 ng/mL of aliskiren, 11.6, 93.5, 750 and 12000 ng/mL of enalapril, as well as 8.8, 70.5, 565 and 9000 ng/mL of enalaprilat, were determined for accuracy and precision. Each concentration level was evaluated by fivefold replicate analysis on four different runs on four different days. Intra- and inter-runs were assessed for accuracy and precision of the method, as described in section 3.2. Mean values per concentration were defined suitable if the nominal concentration did not deviate more than 15 % and the mean values of the LLOQ were within 20 % (European Medicines Agency 2012b, Food and Drug Administration 2001). Statistical comparisons of precision were again made using one-way analysis of variance. The analysis was performed for intra-runs and inter-runs, which also cover variability in lots of reagents, equipment and two different analysts.

Matrix effect and recovery

Urine represents an especially highly complex matrix. Although it mainly consists of water the composition of the urine depends on diet influences, age, sex, diseases etc. and differs highly between inter- and also intra-subjects if compared to blood (Georgi and Boos 2006). This saline mixture contains a huge and very variable mixture of degradation products, products of metabolism, electrolytes, proteins, steroids etc. The circadian variability in kidney function (flow in urine, pH value, glomerular filtration rate, excretion of electrolytes etc.) is the highest amongst all organs, resulting in a permanently changing composition of the excreted fluid. For example, it is known that pH of urine varies between 4.5 and 7.5 under forced conditions and contrasts to the narrow and highly controlled range of about pH 7.4 of arterial plasma (Rowland and Tozer 1995). For method development, this variability makes it difficult to build up a method insensitive particularly to relative matrix effect. Matrix effects might be controlled by dilution of the sample matrix, purification of the sample matrix by e.g. solid-phase extraction or corresponding chromatographic conditions. Dilution of the samples was excluded to avoid imprecisions in measured concentrations owing

to dilution steps and rounding errors caused. To bring the effect to a minimum without diluting the sample, on the one hand chromatographic conditions were chosen to elute all analytes of interest after 4.5 minutes as many matrix components occur early in the chromatogram and on the other hand by utilising solid polymeric mixed-mode exchanger (Chambers et al. 2007). Owing the absence of commercially available deuterated internal standards for all compounds, benazepril was chosen. This asks for an even higher degree of required sample purification to avoid any falsification as the retention time of the internal standard and the analytes of interest differ. Therefore, the corresponding effect by co-detected matrix on the signals of the substances and IS might also influence differently, which is especially challenging for the relative matrix effect.

Recovery and absolute matrix effect were investigated at four concentrations in the range of the whole calibration curve (LLOQ, low, medium and high concentration [ULOQ]) by five-fold measurement per concentration level for aliskiren, enalapril, enalaprilat and at the concentration used in the internal standard. Additionally, urine from sixteen different sources was explored separately at a low and high concentration level by triplicate measurement each to determine the relative matrix effect. This allowed the evaluation of the method's efficiency in purifying the variable urine samples from different sources and is consequently considered more relevant for biological fluids than the absolute effect (Matuszewski et al. 2003). The urine sources were not pooled. The chosen urine sources represent children, adults and elderly as well as both genders, renally impaired and diabetes patients. Furthermore, urine samples drawn at different circadian time points were investigated. Calculation of the absolute matrix effect (ME) was conducted by calculating the ratio of the peak area of extracted urine post spiked with analyte ($Area_x$) to the peak area of the analyte in the same concentration dissolved in mobile phase ($Area_y$). The equation for calculation of the absolute matrix effect is given in equation *(4)*. The IS-normalised relative ME (IS-ME) was calculated according to EMA at the LLOQ and ULOQ with an accepted maximum intersubject deviation per concentration level of maximum 15 % (CV) (European Medicines Agency 2012b). The individual IS-normalised ME was calculated according to equation (5). The CV of the IS-normalised matrix effects of all sixteen subjects were used to assess the relative ME. Absolute recovery (RE) was determined at four concentrations with five replicates per level, by calculating the ratio of the peak area of urine spiked with analyte prior to solid-phase extraction ($Area_A$) to the peak area of blank urine spiked with analyte after the extraction ($Area_B$). Calculation was performed according to equation *(6)*. The process efficiency (PE) of the solid-phase extraction arose from the combined efficiencies of recovery and matrix effect. Its calculation is described in equation *(7)*.

Stability

Stability investigations of stock solutions of all analytes of interest in either water (aliskiren) or methanol (enalapril/enalaprilat/benazepril) were covered by investigations for the serum method. Short-term stability of the urine samples was determined by comparing the peak areas of QC samples of four concentration levels stored unprocessed for 24 h at room temperature with a freshly prepared calibration curve. Additionally, processed short-term storage at 7 °C for a period of 24 h was evaluated. The effect of long-time storage on spiked urine samples was studied at four concentration levels after one month at -20 °C. Freeze and thaw stability of aliskiren, enalapril and enalaprilat was assessed utilising QC samples, which were subjected to three freeze (-20 °C) and thaw (21 °C) cycles before analysis. Furthermore, by comparing the measured concentrations stored in the autosampler at room temperature for 48 h to the nominal concentrations, the post-preparation stability (autosampler stability) was investigated. Processed dry extracts (post-extraction) were additionally stored at -20 °C for 24 h in the freezer to evaluate stability of the extract after the samples were evaporated to dryness. With further regard to practicability aspects, stability of the eluate obtained by the first SPE (WAX only) was stored for 24 h in the fridge (7 °C) prior to being purified with the second SPE the following day (discontinuation stability). In all cases, all analytes were considered to be stable as long as degradation of concentration was $\leq$ 15 % of the nominal concentration and $\leq$ 20 % at the LLOQ.

Carry over

Carry over was accessed similarly to the serum methods by injecting a sample of mobile phase right after a urine sample at the ULOQ. This procedure is in accordance with the current EMA guideline (European Medicines Agency 2012b). Carry over was obviated if not more than 20 % of the peak area of the LLOQ and 5 % of the IS were detected.

Application

To prove the applicability of the developed assay, the method was used to obtain excreted drug amount-time profile of aliskiren, enalapril and enalaprilat in human urine. The local ethics committee approved the clinical investigation of these drugs in human urine and all volunteers gave written informed consent. After single oral administration of 300 mg of aliskiren hemifumarate (Rasilez ®) to a healthy 30 year old male volunteer, spontaneous urine samples were collected for the following 192 h after administration. Additionally, urine was collected for 72 h after a 20 mg single oral dose of enalapril maleate (EnaHEXAL ®) to a healthy 26 year old female volunteer. Each above-mentioned time frame represents approximately five reported elimination half-lives of the corresponding drug and therefore corresponds per definition to

full drug elimination from the body. Blood samples were collected by indwelling catheter into monovettes® over the same time frame and analysed according to the previously developed serum methods for aliskiren and enalapril (Burckhardt et al. 2013, Ramusovic et al. 2012). The urine samples were frozen at -20 °C until analysis. Each urine sample was analysed on its own without pooling. Enalapril and enalaprilat, respectively, are excreted renally unchanged. Additionally, Waldmeier *et al.* reported a sum of 1.4 % of metabolized aliskiren in all excreta only (Waldmeier et al. 2007). A metabolite balance in urine was conducted and revealed that only trace amounts of metabolites were detected in urine. The low amount in urine and limitations in the commercial availability of the metabolites as pure substance led to the decision to evaluate only the parent substance quantitatively. The obtained profiles were assessed on amount excreted to the urine (A*e*) within 5 elimination half-lives and renal clearance (CL_R). Both were determined according to the following equations:

$$Ae = \sum_{i=1}^{n} (Conc_i * Vol_i) \quad (11)$$

where Ae= amount of unchanged drug excreted into urine; Conc= determined concentration of drug in the specific urine sample; Vol= corresponding total urinary volume of this specific sample

$$CL_R = \frac{Ae_{0-t}}{AUC_{0-t}} \quad (12)$$

where CL_R= renal clearance; $Ae_{0\text{-}t}$= amount of unchanged drug excreted into urine in the period of 0 to t; $AUC_{0\text{-}t}$= area under the curve of drug in serum

3.4.3 Saliva

For pharmacokinetic determination serum and plasma still represent the matrices of choice in adults, but for vulnerable populations like children, non-invasive sampling of matrices like saliva are more suitable if a blood to saliva correlation can be established (U.S. Food and Drug Administration 1998). Whether a drug penetrates into saliva and consequently can be determined in the oral fluid depends substantially on its physicochemical properties. The reported properties of aliskiren theoretically account for 50 % penetration of aliskiren from blood into saliva which suggests the ability of the drug to be determined non-invasively in saliva. Whereas enalapril and enalaprilat notionally do not penetrate highly into saliva based on their unbounded fraction and pk_a values. Both drugs were investigated on their drug concentrations in saliva by HPLC-MS/MS, but only the aliskiren method had been fully validated according the bioanalytical guidelines until now.

3.4.3.1 Determination of aliskiren and enalapril in saliva by HPLC-MS/MS method

Two separate methods were developed to determine on the one hand aliskiren in saliva and on the other hand enalapril and enalaprilat in saliva. In the following sections the analytical development and its validation as well as the salivary sampling of this non-invasive and therefore highly child-appropriate technique is described.

Chemicals and materials

Aliskiren hemifumarate (≥ 98 %, potentiometry & HPLC) was delivered by MSN Laboratories (Hyderabad, India). Enalaprilat was provided by LCG-Standards (Luckenwalde, Germany) as USP standard. Enalapril maleate (>98 %, TLC) and the internal standard benazepril hydrochloride (≥ 98 %, HPLC) were purchased from Sigma-Aldrich (Seelze, Germany). Methanol (HiPerSolv® Chromanorm® HPLC grade), acetonitrile (HiPerSolv® Chromanorm® HPLC grade) and ammonia solution (≥ 25 % p.a.) were obtained by VWR (Germany). Water (HPLC grade) was purchased from Fisher scientific (Loughborough, United Kingdom). Formic acid (98-100 % p.a.) was supplied by AppliChem (Gatersleben, Germany). Saliva was provided by employees of the Institute of Clinical Pharmacy and Pharmacotherapy (Düsseldorf, Germany). Oasis® MCX cartridges (30 mg, 1 mL), XSelect™ CSH C18 3.5 µm columns (3.0 mm x 150 mm) and XSelect™ CSH C18 3.5 µm guard columns (3.0 mm x 20 mm) were purchased from Waters (Eschborn, Germany).

Preparation of standards and quality control samples

Blank saliva was spiked with stock solution of aliskiren in water (120 µg/mL), and was serially diluted to yield the concentration range of 0.586 – 1200 ng/mL. Regarding enalapril and enalaprilat a concentration

range of 0.1 – 50 ng/mL was pipetted by spiking 1 mL blank saliva with 50 µL enalapril and enalaprilat (working solution of 1 µg/mL each in methanol), respectively. All other concentrations followed from serial dilution. The internal standard was prepared by dissolving approximately 10 mg of accurately weighted substance in 100 mL methanol followed by further dilution step with water to yield 166 ng/mL benazepril. Quality control (QC) samples were independently prepared in the same manner as described above. All dilutions were prepared freshly while the stock solutions of the aliskiren, enalapril, enalaprilat and IS were stored at 7 °C for a maximum period of 6 months and equilibrated to room temperature prior to use.

Sample collection and preparation

Although the amount of proteins in saliva is low compared to blood, this hypotonic biological fluid is also characterised by significant inter- and intra-subject variability (Thomas et al. 2009) and the challenge is especially brought by mucins and, in turn, the high viscosity. To overcome possible effects and interferences by the viscosity of the fluid, samples were collected by commercial available saliva collection aid (Sarstedt, Nuembrecht, Germany) and were directly centrifuged prior to a freezing cycle. For sample preparation 100 µL and 500 µL, respectively, of undiluted fluid containing either aliskiren or enalapril and enalaprilat was mixed with 10 µL IS (166 ng/mL) and 490 µL formic acid (2 %, v/v) before the mixture was subjected to solid-phase extraction (Oasis® MCX). The cartridges (30 mg, 1 mL) were preconditioned with 1 mL of a mixture of ammonia solution-methanol-acetonitrile (10:45:45, v/v/v) followed by a conditioning step with 1 mL methanol and finally equilibrated with 2 % formic acid (v/v). Afterwards, the samples were transferred into the primed cartridges, eluted and the sorbent subsequently washed with 1 mL of formic acid (2 %, v/v) followed by a second wash step utilising 1 mL methanol-acetonitrile (50:50, v/v). For the sample preparation of enalapril and enalaprilat the elution force of the second wash step was reduced with regard to the analytes of interest by using methanol-water (80:20, v/v) instead of methanol-acetonitrile (50:50, v/v). The analytes were released from the sorbent by elution with three times 0.5 mL and one time 0.4 mL of a mixture of ammonia solution-methanol-acetonitrile (10:45:45, v/v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C shaking at 550 rpm and finally the residue reconstituted with 100 µL of acidified methanol/water (20:80, v/v).

Chromatographic and mass spectrometer conditions

With the same HPLC system (Shimadzu Deutschland GmbH, Duisburg, Germany) as described previously for the serum and urine method (section 3.2.1.) and a XSelect™ CSH C18 column (3.0 mm x 150 mm, 3.5 µm) protected by a corresponding XSelect™ CSH C18 guard column (3.0 mm x 20 mm, 3.5 µm) chromatographic separation of the analytes was achieved within 7.5 minutes run time. The flow rate was 0.4 mL/min and

column temperature was maintained at 40 °C while gradient of acidified methanol (A) and acidified water (B) (each with 0.1 % formic acid, v/v) was applied to separate the compounds. Gradient was as follows: Starting with 30 % of A, after 0.2 min 50 % A, at 6 min 80 % A, at 6.3 min 100 % A, decreased to 50 % A at 7 min and staying at 50 % for the rest of the run time. The column was re-equilibrated for 3 min with the initial mobile phase. 20 µL of the reconstituted sample solution were used for determination.

Mass-spectrometric detection of the analytes was carried out on an Applied Biosystems SCIEX API 2000 (Applied Biosystems/MDS SCIEX, Concord, Canada). Operating in positive ionisation mode with electrospray ionisation (ESI) the mass-to-charge ratio (m/z) 552.2 to 436.2 m/z for aliskiren and 425.3 to 351.2 m/z for benazepril were monitored. All dwell times were set to 150 milliseconds. The scanned transitions of enalapril and enalaprilat were mass-to-charge ratio 377.2 to 243.2 m/z and 349.1 to 206.1 m/z respectively. Applying Analyst 1.4.1 (Applied Biosystems/MDS SCIEX, Concord, Canada) with IntelliQuan® as integration algorithm without smoothing, the data acquisition and processing was carried out.

Bioanalytical method validation

The aliskiren method was fully validated according to the previously reported in-house validation plan in Table 4. The method developed for enalapril and enalaprilat has not been validated according to the international guidelines yet.

Selectivity and cross-talk

To prove the lack of any interaction caused by co-medications, the following drugs were investigated for any peak in the transitions of aliskiren and benazepril: Aspirin, ramipril, ramiprilat, clopidogrel, simvastatin and levetiracetam. The selectivity of the established saliva method was investigated on saliva samples of volunteers treated with typical co-medications routinely and were not spiked. This procedure constitutes correct saliva compositions and penetration ratios of the drugs investigated. Signals in the range of 0.5 min before and after the retention time of the analyte and IS were classified as interference and were subdivided into ion suppression or ion enhancement if the interference alters the analyte or IS peak area by more than + 15 % or – 15 %, respectively. Additionally, six different sources of blank saliva were analysed for any interference of endogenous matrix compounds with the corresponding analyte channels.

Linearity and Lower Limit of Quantification (LLOQ)

Linearity of the saliva method was determined in the range of 0.586 - 1200 ng/mL by twelve non-zero concentration levels. Linearity of each curve was evaluated by plotting the concentration ratio of the analyte to IS versus peak area of the analyte to IS. At the lower limit of quantification (LLOQ) of 0.586 ng/mL

precision and accuracy were defined acceptable within ± 20 % with a S/N ratio of at least five times when the signal of the analyte is compared with the blank sample. As effects of background or matrix might lead to the highest variability at the lowest concentration level it was evaluated in six different sources to check for the assay's reproducibility.

Accuracy and Precision

Quality control samples at 0.586 (LLOQ), 4.69 (low concentration), 75 (medium concentration) and 1200 (ULOQ) ng/mL of aliskiren were determined for accuracy and precision. Each concentration level was evaluated by five-fold replicate analysis on three different runs at three different days. For accuracy and precision of the method, intra- and inter-runs were evaluated with an acceptance range of mean values per concentration of not more than 15 % (20 % at the LLOQ) compared to nominal concentration. Calculation of precision was made using one-way analysis of variance as previously described in section 3.2. The analysis was performed for intra-runs and inter-runs which also cover variability in lots of reagents, equipment and two different analysts.

Matrix effect and recovery

The oral fluid mainly consists of water, electrolytes and proteins. Nevertheless, it is known that this hypotonic biological matrix shows significant intra- and inter-individual variability (Thomas et al. 2009). As the secretion activity of the three pairs of major glands underlies a circadian rhythm and the saliva excreted by each gland differs in its viscosity, it is obvious that the matrix is changing during day and night. Furthermore, salivary flow and pH vary, which results in changing electrolyte composition and concentration in saliva especially under resting or stimulated conditions. Beside age and diet even diseases like diabetes mellitus, kidney dysfunction or cystic fibrosis additionally influence the matrix. The combined power of solid-phase extraction and chromatographic separation by HPLC was used to minimise the matrix effect and reduce the interference during the ionisation process in mass spectrometer.

Recovery and absolute matrix effect were investigated at three concentrations covering the whole calibration curve (LLOQ, medium and ULOQ) by five-fold measurement per concentration level for aliskiren and at the concentration used of the internal standard. Calculation of the absolute matrix effect, the absolute recovery and the corresponding process efficiency was conducted as described in section 3.2.1 and according to equations *(4)*, *(6)*, and *(7)*, respectively.

Additionally, saliva of six different sources was explored at a low and high concentration level by triplicate measurement each to determine the IS-normalised relative matrix effect. The previously described

procedure for data analysis were executed with the presented limits for the relative matrix effect. Consequently, the inter-subject deviations expressed as coefficient of variation (CV) per concentration level of maximum 15 % were considered acceptable (European Medicines Agency 2012b). The calculation of the latter was done on the basis of equation (5) determining the individual matrix factor of analyte and internal standard.

Stability

Stability investigation of stock solution of aliskiren was done for serum. Since the concentration, the solvent and storage conditions were unchanged, no further investigation was necessary (Burckhardt et al. 2013). Short-term and long-term stability tests in saliva were performed on four concentration levels after 24 h at room temperature and 1 month at -20 °C, respectively. Furthermore, the stability of the reconstituted solution at room temperature for 24 h (autosampler stability) and processed dry extracts (post-extraction) were investigated as previously described for serum and urine. In all cases, the drug substances were considered to be stable as long as degradation of concentration was ≤ 15 % of the nominal concentration and ≤ 20 % at the LLOQ.

Application

The quantitative as well as qualitative penetration of the drug in saliva was studied to evaluate the pharmacokinetic behaviour in this fluid. Based on theoretical assumptions of the drug properties of aliskiren, nearly 50 % of the measured serum concentration should also be detectable in saliva. This amount was calculated by applying the following equation:

$$\frac{1+10^{pH\ saliva-pka}}{1+10^{pH\ plasma-pka}} * \frac{fu,plasma}{fu,saliva} \qquad (13)$$

where fu= fraction unbound

To investigate the penetration into the saliva after a single oral administration of 300 mg aliskiren hemifumarate (Rasilez®), serum concentrations were compared with the saliva concentrations in healthy volunteers. The study was approved by the local ethics committee and all participants gave written informed consent before participation. Saliva samples and serum samples were collected for the first 8 hours every 20 minutes and then every 24 hours up to five elimination half-lives of aliskiren (192 h) following oral administration to compare pharmacokinetic behaviour of the drug in both fluids. After administration of the tablet and eating, respectively, a glass of 250 mL water was given to assure that no contamination by any tablet residual, liberated drug substance in the mouth or food led to misdetection.

Saliva samples were collected following each blood sample draw. The participant was asked to swallow one minute before saliva collection to avoid any dilution etc. of the sample. Collection itself took place with saliva swabs without any stimulation like citric acid. The investigation was conducted with two commercial saliva collection devices to determine the best applicable system. Whatman™ OmniSwaps™ (GE Healthcare, Germany) and Salivetten® from Sarstedt (Nuembrecht, Germany) were tested.

The collected sample was stored on ice before centrifugation. The centrifuged samples were frozen at -20 °C within 20 minutes after collection until analysis. The serum method which was utilised to determine aliskiren in serum was the one described within this thesis. To compare saliva and blood concentrations the saliva/serum ratio of aliskiren was calculated by dividing the calculated area under the curves (AUC) of saliva to the AUC of serum. AUC was calculated by trapezoidal method. Furthermore, pharmacokinetic parameters like C_{max}, t_{max} and k*e* were evaluated in both biological fluids utilising a non-compartmental model (WinNonLin, Phoenix 6.3.0.395). For comparison of drug levels between serum and saliva only, the absorption rate constants were calculated for both peaks of the aliskiren double peak by the method of residuals.

3.5 Bioanalytics for determination of hormones and proteins allowing for the assessment of pharmacodynamics within the RAA system

This section serves to provide information on the utilised bioanalytical assays for evaluation of pharmacodynamics. The sophisticated sample collection for this evaluation is highly important for valuable results and stands in close connection to the here presented assays; it is described in detail in section 3.4.

Prior to analysis of pharmacodynamic parameters, available commercial assays were screened for their suitability for investigations in paediatric patients by assessing the assay's detection range, sensitivity, selectivity and the required blood volume. If necessary, the chosen assays were modified to fit to the user's claim on enclosed concentration levels or required sample volume. The included assays covered the main parameters of the RAA system and enabled the investigation of their changes which facilitated the comprehensive description of the pharmacodynamics of aliskiren as well as enalapril and enalaprilat.

All assays listed below were validated by the manufacturer before marketing. The obtained data was made accessible in the form of a corresponding data sheet entailed for details in the appendices (Appendix 9, Appendix 10, Appendix 11, and Appendix 12). With regard to the EMA guideline on bioanalytical method validation, a partial validation is considered suitable if e.g. the bioanalytical method is transferred to another laboratory or the sample volume is limited (European Medicines Agency 2012b). Thus, all assays were checked for their suitability and validity through partial validation during their implementation. An in-house validation plan for all assays, that determine the pharmacodynamic parameters, was established on the EMA guideline for ligand binding assays and immunoassays (European Medicines Agency 2012b). Details are arranged in Table 6. Accordingly, the smallest partial validation is the check on the intra-day precision and accuracy. However, for all below mentioned assays at the minimum intra- plus inter-day precision and accuracy were determined prior to their establishment in the laboratory utilising quality controls of precisely known concentrations. According to the EMA guideline on bioanalytical method validation for ligand binding assays and immunoassays, the within-run precision and accuracy should not exceed 20 % (25 % at the LLOQ and ULOQ) within a calibration curve of at least 6 calibration levels. Precision and accuracy of all parameters were determined by quality control samples of known concentration in at least seven independent assay runs. Additionally, the total error must be within 30 % (40 % at the LLOQ and ULOQ). The challenging check on recovery was not covered by above cited guideline. In contrast to the outlined procedure for the assays determining drug concentrations in biological fluids, it is not sufficient to just spike blank plasma with a known concentration of hormones and convert back the determined concentration to evaluate the recovery. As blank plasma samples of any human contain

e.g. angiotensin II in an unknown concentration, a further step is required to determine the concentration of the hormone in blank prior to being spiked.

For angiotensin II, an individual protocol for the solid-phase extraction was developed. Using the latter, the recovery of the solid-phase extraction was evaluated according to the following equation:

$$Recovery\ [\%] = \left(\frac{\frac{Conc_{SS}}{Sample\ volume_{SS}}}{\frac{Conc_{US} + Conc_{KC}}{Total\ sample\ volume\ of\ US + KC}} \right) * 100 \quad (14)$$

where $Conc_{SS}$ = determined concentration of spiked plasma sample; $Conc_{US}$ = determined concentration of unspiked plasma sample; $Conc_{KC}$ = Concentration of spiked quality control with known concentration to US

No international bioanalytical guidelines recommend a limit or threshold for recovery. However, the recovery pertains to the extraction efficiency of an analytical method and its extent should be known and consistent.

In general, obtained values were statistically exploited using the mean, standard deviation, coefficient of variation and the root mean square. The latter is used for frequent analysis in clinical chemistry in accordance with the German Medical association guideline on quality assurance in medical laboratories (Bundesärztekammer 2013) applicable to medical laboratories.

Validation parameter for immunoassays according to EMA guideline	Full validation	Partial validation *Based on the change intended a partial validation can range from as little as the evaluation of the within-run precision and accuracy to almost full validation.*
		Example In-house validation plan for all hormones and proteins acting on the RAA system
Selectivity	10 different sources of blank matrix (if possible matrix from diseased population), spiked at/near the LLOQ; criterion: accuracy ≤ 20 % (25 % at LLOQ) of at least 80 % of the evaluated matrix	Not performed; no change in sample matrix; kit has been approved for human serum/plasma
Specificity	Requires investigation with "related molecules" or drugs into drug/hormone-native matrix; Determination on the LLOQ and ULOQ, criterion: accuracy within 25 % of nominal concentration	Not performed; samples investigated were from healthy subjects
Linearity	At least 6 concentrations per calibration curve; at the minimum in duplicates; anchor points outside the calibration range are allowed; 6 independent runs; accuracy ≤ ± 20 % (≤ ± 25 % at LLOQ and ULOQ)	Validated due to extension of calibration range; criterion: at least 7 independent runs; accuracy ≤ ± 20 % of the samples with known concentration
Accuracy and precision	Five concentration levels; max. ± 20 % (± 25 % at LLOQ and ULOQ) deviation / same for within- and between runs / total error within 30 % (40 % at the LLOQ and ULOQ)	Validated due to extension of calibration range; within- and between run accuracy and precision ≤ ± 20 % (± 25 % at LLOQ and ULOQ) at the investigated levels
Matrix selection	If extraction is needed; calibration curve may be prepared in surrogate matrix while QC samples should be in actual sample matrix. Criterion: accuracy ≤ ± 20 %	Not performed separately; no unlabeled matrix purification done
Dilution linearity	Concentration levels above the ULOQ needs to be investigated; criterion after dilution with blank matrix: accuracy & precision ≤ ± 20 %	Not performed; calibration range was only extended at the lower end
Parallelism	Check between calibration standard curve and serially diluted samples (at least three concentration steps). Criterion: precision within 30 %	Not performed
Stability	Freeze-thaw/ short-term/ long-term/ criterion: 20 % of nominal concentration	Not performed; samples were stored as recommended by manufacturer
Minimal required dilution	Check for minimum required dilution steps in buffer to optimise accuracy and precision	Not performed; accuracy and precision were conform
Carry over	Should be evaluated in robotic liquid handling by a blank sample following a sample with high concentration	Not performed; no robotic liquid handling

Table 6 ***Established in-house validation plan*** *for pharmacodynamic assays with justification of chosen validation parameters for partial validation. The in-house validation plan bases on recommendations by the European Medicines Agency bioanalytical guideline (European Medicines Agency 2012b).*

3.5.1 Determination of angiotensin I and plasma renin activity

Although blood volumes required for reliable determination of angiotensin I by commercial assays range from 20 – 500 µL, an assay (75 µL) with a broad range, instead of the lowest possible volume, was chosen. Moreover, a radioimmunoassay was selected as it is accompanied by high sensitivity. This property allows the assay to be applied to all age groups because PRA varies significantly between adults and children (Dillon and Ryness 1975). Angiotensin I and plasma renin activity were both determined with a commercial Iodine^{125} (I^{125}) radioimmunoassay (RIA) (Immunotech, Prague, Czech Republic) with CE certification.

Chemicals and materials

Tubes with anti-angiotensin I antibodies (polyclonal), marked anti-angiotensin I antibody solution, ready for use standards of angiotensin I in bovine serum, wash buffer, and a bottle of enzyme inhibitor were all obtained from Beckman Coulter GmbH (Krefeld, Germany). Water was prepared freshly each day through an ion exchanger at the medical laboratory Dr. Spranger (Ingolstadt, Germany) and human serum was provided by employees of the Institute of Clinical Pharmacy and Pharmacotherapy (Düsseldorf, Germany).

Sample preparation

The calibration standards were determined utilising ready for use calibration standards verified according to RP 86/536. Enzyme inhibitor and plasma samples as well as calibration standards were evenly mixed and divided into two aliquots stored in micro tubes. One was kept at 4 °C in an ice bath, while the second one was placed in a water bath at 37 °C in a circulating water stream. After incubation for 3 hours and rapid cool-down to 4 °C, the samples (75 µL) were transferred into the coated anti-angiotensin I antibody tubes. Right after adding the tracer, all tubes were shaken at room temperature for 2 hours. Prior to detection in the gamma counter, the samples were extracted by suction and washed with 2 mL buffer.

Analytical determination

The per sample concentration of angiotensin I was determined by a gamma counter (Berthold, Multi Crystal Counter LB 2104) with a measuring time of 1 minute. Concentrations of angiotensin I at both temperatures allow the evaluation of the plasma renin activity (PRA). Within each determination a freshly prepared calibration curve was determined. Angiotensin I concentrations were revealed by plotting the calibration concentration versus the normalised percentage of bound angiotensin I expressed as B/B_0 (%).PRA was calculated according to equation (*15*):

$$PRA\ [ng/mL/h] = \frac{(Concentration\ Ang\ I\ 37°C - Concentration\ Ang\ I\ 4°C) * 2}{Hours\ of\ incubation} \qquad (15)$$

where Ang I= angiotensin I; PRA= plasma renin activity

3.5.2 Determination of prorenin

An enzyme linked immunosorbent assay (ELISA) was used to analyse prorenin in 100 µL plasma within a calibration range of 0.02 – 10 ng/mL. This applied assay required the lowest volume amongst those evaluated, detected prorenin directly, and had a broad calibration range (Molecular innovations, Novi, USA).

Chemicals and materials

Tris(hydroxymethyl)aminomethane (p.a.), bovine serum albumin fraction V (≥ 98 %), and sodium chloride (p.a.) were provided by Sigma-Aldrich (Seelze, Germany). Hydrochloric acid (p.a.) was obtained from VWR (Germany). Water was prepared fresh by ion exchanger at the medical laboratory Dr. Spranger (Ingolstadt, Germany). All further required reagents of the kit were purchased from Loxo (Dossenheim, Germany). The kit contained a 96-well microtiter plate coated with anti-human prorenin antibody, wash buffer, lyophilised human prorenin, tetramethylbenzidine (TMB) substrate solution as well as anti-human prorenin primary antibody and anti-house horseradish peroxidase antibody.

Sample preparation

The calibration standard with 20 ng prorenin was reconstituted by 1.0 mL of deionised water. All other concentrations were obtained by serial dilution of the upper concentration level. As the highest concentration level was omitted, a range of 0.02 – 10 ng/mL of prorenin was established. After addition of 100 µL standard or sample to each well, the microtiter plates were slightly agitated for 30 minutes at 500 rpm. The wells were washed three times with wash buffer and dried prior to adding the primary antibody to each well, then incubated for 30 minutes followed by a washing step. All wash steps were conducted utilising an automatic microtiter plate washer (Tecan Deutschland GmbH, Crailsheim). Under gentle shaking at 500 rpm the second antibody was added and unbounded composites were washed prior to activating the colour reaction through a 100 µL TMB substrate solution. The TMB substrate solution stayed for some minutes in the wells until the solution turned blue and was finally stopped by 50 µL hydrochloric acid (1 N).

Analytical determination

All wells were measured utilising a 96-microplate Sunrise reader (Tecan Deutschland GmbH, Crailsheim, Germany) at 450 nm. Magellan (version 7.1) was applied as quantification software. Zero samples were subtracted from standards and samples.

3.5.3 Determination of angiotensin II

Nussberger *et al.* point out that direct angiotensin II measurements are technically difficult and complex to conduct (Nussberger et al. 2007), appear inappropriate for clinical routine and alternatively suggest PRA determination as an easier approach. However, for lack of valuable angiotensin II levels in paediatric pharmacotherapy of aliskiren and enalapril and since angiotensin II is the main effector in the RAA system which is mandatory for understanding the regulation of the RAA system in children, assays and kits of several manufactures/distributers (Loxo, IBL, AssayPro, SPIbio) were evaluated. Investigated commercial ELISAs were all inappropriate for precisely determing low angiotensin II concentrations (~1 pg/mL) in low volumes. Owing to the expected decline in angiotensin II concentration after administration of aliskiren or enalapril, the LLOQ was set as low as possible. Therefore, the assay was optimised by combining SPE (in-house) and a modified ELISA. The assay required 500 µL plasma, whereas common ELISAs required about one to two millilitres. The purification was performed on Oasis® HLB 96-well plates, which allowed angiotensin II concentrations to be reliably determined with a calibration range of 1 – 125 pg/mL.

Chemicals and Materials

Methanol (HiPerSolv® Chromanorm® HPLC grade) and water (super gradient grade) were obtained from VWR (Germany). Formic acid was supplied by AppliChem (Gatersleben, Germany) while trifluoroacetic acid (p.a.) was delivered by Sigma-Aldrich (Seelze, Germany). For the ELISA, the required EIA buffer, angiotensin II standard (calibrated with the standard WHO 86/538), anti-angiotensin II IgG tracer, glutaraldehyde, borane trimethylamine, dithionitrobenzoic acid (ellman's reagent), tween 20 and wash buffer were purchased from IBL (Hamburg, Germany). All aforementioned reagents were enclosed in one commercial ELISA reagent kit. Plasma was provided by employees of the Institute of Clinical Pharmacy and Pharmacotherapy. Oasis® HLB 96-well plates (30 mg, 1 mL) were obtained from Waters (Eschborn, Germany).

Sample preparation

The developed sample preparation was as follows: First, the samples were mixed in an equal amount of formic acid (2 %, v/v) and stored at 7 °C until the solid-phase extraction was arranged. Then, the 96-well plates were conditioned with 1 mL methanol and 1 mL formic acid (2 %, v/v). Afterwards, the cooled samples were loaded into the primed cavities of the 96-well plate and slowly dropped through. The wash step was conducted by applying a mixture of acetonitrile and 0.1 % trifluoroacetic acid in water (10:90, v/v). Subsequently, the analyte was released from the sorbent material through a mixture of acetonitrile and water (80:20, v/v) and evaporated to dryness under a gentle stream of nitrogen in a tempered water

bath (40 °C). The evaporated samples were constituted in 125 µL EIA. 100 µL were transferred into a well and incubated for 1 hour at room temperature. Subsequently, 50 µL glutaraldehyde followed by borane-trimethylamine were added and incubated for 5 minutes each. After washing the wells five times, the anti-angiotensin II antibody was added and the wells were stored over night at 4 °C. The next day, the wells were washed again and 200 µL Ellman's reagent was added to start the colour reaction. The wells were gently shaken in the dark until a yellowish colour was identified.

Analytical determination

Finally, the angiotensin II concentrations were determined with the help of a 96-microplate Sunrise reader (Tecan Deutschland GmbH, Crailsheim, Germany) at 405 nm using Magellan (version 7.1) for detection and data analysis.

3.5.4 Determination of renin

The immunological test on renin concentration in human plasma was conducted by a CE certified chemiluminescence assay (CLIA) utilising monoclonal antibody and allowing for high-throughput.

Chemicals and materials

Deionised water was freshly prepared at the medical laboratory Dr. Spranger (Ingolstadt, Germany). The suspension of PBS puffer, bovine serum albumin and magnetic particles coated with anti-renin/prorenin monoclonal antibody, the starter solution with colouring agent as well as the conjugate solution containing PBS buffer bovine serum albumin and monoclonal anti-renin antibody were all obtained from Diasorin (Saluggia, Italy).

Sample preparation

The chosen CLIA was fully automatically through a robotic system and required no manual sample preparation. The device incubated the plasma samples with the magnetic particle suspension, followed by a wash step prior to another incubation with the monoclonal anti-renin antibody. After a further washing step and the addition of the starter solution the procedure was finalised.

Analytical determination

Using the Liason® analyser the renin concentration in plasma was detected by chemiluminescence immunoassay. The linear calibration range was 2 – 500 µIU/mL.

3.5.5 Calculation of angiotensin converting enzyme activity

The ACE activity was accessed by determing the ratio of plasma angiotensin II to angiotensin I concentration in plasma. The change in activity was a percentage and calculated according to the following equation:

$$Change\ in\ ACE\ activity\ [\%] = \left(100 \Big/ \left(\frac{Angiotensin\ II\ predose\ [\frac{pg}{mL}]}{Angtiotensin\ I\ predose\ [\frac{pg}{mL}]}\right)\right) * \left(\frac{Angiotensin\ II\ t\ [\frac{pg}{mL}]}{Angiotensin\ I\ t\ [\frac{pg}{mL}]}\right) - 100 \qquad (16)$$

where Angiotensin II t= Angiotensin II concentration at time point t; angiotensin I t= Angiotensin I concentration at time point t

3.5.6 Validation of the immunological assays

As mentioned in section 3.3, the assays were partially validated at least regarding accuracy and precision. Limits for within-run and between-run precision and accuracy were defined as ± 20 % (± 25 % at the LLOQ and ULOQ). These requirements are in accordance with the limits given by EMA for bioanalytical method validation of ligand binding assays and immunoassays. Please see section 3.3 for further details.

3.5.7 Applicability of all assays used for determination of pharmacodynamics

The applicability of the pharmacodynamic assays were evaluated in the proof-of-concept study introduced in the following chapter (section 3.4). Furthermore, modalities on the sensitive sample collection are also described in this chapter.

3.6 Proof-of-concept study

This study was designed to prove the applicability of the developed bioanalytical low-volume platform in the frame work of a clinical trial. The bioanalytical platform defines itself in a body of all LC-MS/MS assays for determination of drug concentrations and the established immunological tests. The platform was established for determination of aliskiren, enalapril and enalaprilat concentrations in serum, urine and saliva as well as for the evaluation of hormone and protein concentrations within the RAA system.

3.6.1 Study design and participants

3.6.1.1 Study protocol

This study was conducted at the Department of Clinical Pharmacy and Pharmacotherapy, Heinrich-Heine-University (Düsseldorf, Germany), with approval from the university ethics committee (Proposals no. 3809 and 3930 [including all amendments]) and following the Declaration of Helsinki and Good Clinical Practice recommendations. All subjects provided written, informed consent before participation. Volunteers were enrolled into two investigation groups. One group received aliskiren hemifumarate, whereas enalapril maleate was administered to volunteers in the second group.

3.6.1.2 Study population

Healthy volunteers were eligible for inclusion if they were in an age range of 18 to 35 years. Both genders were accepted for enrolment if no permanent medication was taken (except oral contraception), if their body weight was above 50 kg, if no organ disease had been formerly diagnosed and if no other reason for any other medical intervention existed. Pregnant women were excluded as well as subjects with history of angioedema, urticaria and/or low blood pressure values (<90/60 mmHg). Healthy subject who became aware of developing an allergy after administration of both drugs were also not included. If the informed consent was denied or withdrawn during the study, the healthy volunteer was excluded.

3.6.1.3 Study procedure

The study was designed as an open-label, single-sequence, single-dose study in healthy subjects. After collection of a predose sample in the morning of the first investigation day, each participant received 1 tablet of 300-mg aliskiren hemifumarate (Rasilez, Novartis Europharm Limited, Frimley, UK) or 1 tablet of 20-mg enalapril maleate (EnaHEXAL, HEXAL, Holzkirchen, Germany). The participants fasted at least 10 hours before drug administration. The study medication was administered with 250 mL of water. No further fluid intake was allowed for 30 minutes before and 1 hour after dosing with enalapril or four hours after administration of aliskiren. The first meal was served four hours postdose. The participants went

home 8 hours postdose and returned every 24 hours until the end of the study. Intake of xanthine-containing food or beverages and alcohol were not permitted for at least 48 hours after drug administration.

On day 1 of the study, an indwelling cannula was inserted into a peripheral arm vein for blood sampling. On the following investigation days, blood samples were taken by direct venipuncture. All participants rested in a supine position for at least 30 minutes before sampling to determine humoral parameters.

3.6.2 Sampling schedule of the drug levels

Serum

Blood samples were collected predose followed by sample withdrawals every ten minutes for the next three hours. This was continued every twenty minutes over the next three hours followed by sampling every 30 minutes until eight hours postdose. Further samples were taken 24, 48 and 72 hours postdose. Volunteers in the aliskiren group provided additional samples 96, 120, 144, 168 and 192 hours postdose. Marked, multiple-peak phenomena had previously been noted in patients given aliskiren; hence, an intensive sampling strategy was used to make a precise determination of the multiple-peak behavior per individual. This was important because individual concentration-time profiles showing this phenomenon are lacking. To describe the pharmacokinetic parameters (e.g., T_{max}) as exactly as possible, the frequent sampling was also used for enalapril/enalaprilat.

Despite the dense sampling schedule, a total blood volume of only approximately 105 mL per volunteer was taken for full pharmacokinetic determination in serum. This is less than one quarter of a usual blood donation volume and, consequently, was ethically acceptable in adults. This dense sampling allowed to obtain substantially more than the recommended 15 sampling points during the time of drug exposure. Drawn samples were kept at room temperature until centrifuged at 2000 x g for 10 minutes at 4 °C, and then the supernatant was frozen at -80 °C until analysis. For the determination of aliskiren as well as of enalapril and enalaprilat, 100 µL serum was required per sampling point.

Urine

Urine samples were collected predose. Samples were collected for up to 192 hours thereafter in the aliskiren group and up to 72 hours in the enalapril group. Every spontaneous urination was collected and analyzed as a discrete sample without pooling. Although a correction for creatinine concentration is sometimes used to minimize error in urinary concentrations because of the variability in fluid composition, this study did not require the correction because the excreted amount of the drug was calculated by multiplying the urine volume by the urinary drug concentration.

Saliva

Saliva was examined as an alternative fluid for obtaining drug concentrations and, in turn, pharmacokinetic determination, which might constitute an advantage in pediatric research. Although some authors collect saliva from adults by having them spit the oral fluid into collection bowls (Cawello *et al.*), Hiremath et al. concluded that collection of saliva by oral swabs offers methodological advantages over passive drool in the pediatric population. Preliminary investigations were performed with 2 commercially available devices. One swab was primarily designed to collect buccal cell samples for DNA testing (Omniswab, GE Healthcare, Germany), and the second was a chewing swab on a cotton base (Salivette, Sarstedt, Nuembrecht, Germany). The latter was selected for the proof-of-concept study because these swabs are routinely used to determine concentrations in saliva (e.g., cortisol) and have already been used in pediatric research (Davis *et al.*). The saliva samples were taken without any stimulation every twenty minutes for the first eight hours after drug administration and then every 24 hours. Immediately after blood collection, the participant was asked to chew slightly on the swab for 1 minute. The swab was stored on ice until centrifuged at 1000 x *g* for 2 minutes at 4°C, then frozen at −20°C or below until analysis.

3.6.3 Sampling schedule of the RAA system parameters

All humoral parameters were determined predose and again at 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hours postdose. On subsequent days, samples were taken every 24 hours. In total, a minimum blood volume of 2.1 mL was required for analysis of all RAA system parameters per timepoint. Although blood withdrawal was performed with substantial excess, allowing for replicate determinations, overall, only 250 mL blood were collected for determination of the pharmacokinetics of drugs and humoral parameters. This is equal to about one-half of the usual blood-donation volume. Collection tubes intended for angiotensin I and II determination were equipped with an inhibitor cocktail consisting of 0.44 mM *o*-phenanthroline, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.12 mM pepstatin A, and 1 mM *p*-hydroxy-mercuric benzoic acid (absent in angiotensin I tubes). This inhibition cocktail was selected based on results from Kohara *et al.* (Kohara et al. 1991) who investigated the effect of additives on angiotensin I and angiotensin II. The extremely instable angiotensin II with its half-life of 30-60 seconds required intense inhibition of degradation to facilitate valuable evaluations of the angiotensin II concentrations. The metabolism of both peptides after sampling was significantly reduced by the above-mentioned inhibitor cocktail. In addition, the pharmacodynamics were characterized by systolic and diastolic BP and heart rate. The corresponding RAA system is illustrated in Figure 1.

Prorenin and Renin

No chemical addition or inhibitors were spiked to the blood tubes to keep the pH neutral. This assured that many undesired activations like the one by aspartyl proteases at low pH were minimised, as described by Schalekamp *et al.* (Schalekamp et al. 2008). To prevent renin cryoactivation, blood samples for renin and prorenin were collected in EDTA tubes and immediately centrifuged at 2000 x *g* for 10 minutes at room temperature. The supernatant was snap-frozen before being stored at −80°C until assayed. Before the analysis of prorenin by enzyme-linked immunosorbent assay (ELISA), the plasma samples (100 µL) were rapidly thawed. Renin required a minimum of 250 µL of plasma for determination with a chemiluminescent immunoassay based on monoclonal antibodies.

Plasma renin activity

Plasma renin activity was determined using angiotensin I antibodies and circulating immunoreactive angiotensin I concentrations because that approach was expected to be more precise (Nussberger et al. 2007). The chosen ^{125}I radioimmunoassay (RIA) has a wide angiotensin I calibration range. This property enables the application even in children who have up to 15-fold greater PRA than adults have (Dillon and Ryness 1975). Samples were drawn in cooled EDTA tubes without inhibitor. The tubes were then centrifuged at 2000 x *g* for 10 minutes at 4°C and subsequently snap-frozen and stored at −80°C.

Angiotensin I and Angiotensin II

Cooled and inhibitor-spiked EDTA tubes were used to collect samples for angiotensin I and II determination. During blood withdrawal, the tubes were kept on ice and were immediately centrifuged at 2000 x *g* for 10 min at 4°C. Obtained plasma was snap-frozen and stored at −80°C until analysis; 100 µL of plasma was used to obtain the angiotensin I concentration with a commercial ^{125}I RIA, whereas 500 µL was necessary for the angiotensin II measurement using a modified ELISA. During the entire analysis, all samples were kept between 2°C and 8°C, unless otherwise described.

3.6.4 Determination of drug concentrations for pharmacokinetic evaluation

Serum, saliva and urine concentrations of aliskiren, enalapril and enalaprilat

In serum the concentration of the direct renin inhibitor aliskiren as well as the angiotensin converting enzyme enalapril and enalaprilat were determined with the two low-volume assays introduced in section 3.2.1. At the time of sample analysis, the required serum volume for determination of enalapril and enalaprilat was 100 µL and had been optimised to 50 µL later on. The urine concentrations of all three analytes of interest were determined by the simultaneous analysis introduced in section 3.2.2. The salivary assays presented in section 3.2.3 were used for aliskiren, enalapril and enalaprilat. All analytical settings were scaled for high sample throughput through at least 96-well-plate measures. In brief, sample purification of serum and saliva was done through solid-phase extraction (SPE) utilising Oasis® MCX (Waters, Eschborn, Germany). SPE of urinary samples was performed with a combination of Oasis® WAX and MCX cartridges. High performance liquid chromatography was carried out using an XSelect™ CSH C18 columns (Waters, Eschborn, Germany) followed by MS/MS detection with an API 2000 (AB Sciex, Concord, Canada). Concerning aliskiren the calibration range in the serum was 0.15 (LLOQ) – 1200 ng/mL, in urine it ranged from 9.4 (LLOQ) to 9600 ng/mL and the salivary assay encompassed a range of 0.59 (LLOQ) – 1200 ng/mL. With regard to enalapril and enalaprilat the quantification limit was 0.78 ng/mL and 0.70 ng/mL respectively in serum as well as 0.1 ng/mL in saliva. The calibration curve in urine covered a range of 11.6 (LLOQ) – 12000 ng/mL of enalapril and 8.8 (LLOQ) – 9000 ng/mL of enalaprilat respectively.

3.6.5 Determination of RAA system parameters

Plasma assays for angiotensin I, plasma renin activity, angiotensin II, prorenin and renin

All five pharmacodynamic assays were determined in plasma applying either RIA, CLIA or ELISA. The applied assays are described in detail in section 3.3. In total, the developed assays required only 1.05 mL of plasma per sample point to evaluate the concentration levels and enzyme activities of all five parameters. Not less than 96-well approaches were established for all of them to assure a high-throughput and to establish the low-volume assays in a routine setting. All immunological assays were successfully validated prior their implementation in the study. In summary, the assays allowed for the reliable determination of angiotensin I in a calibration range of 0.2 – 30 ng/mL while angiotensin II can be determined in 500 µL in the range of 1 – 125 pg/mL. Plasma renin activity was determined indirectly by applying angiotensin I with a LLOQ of 0.2 ng/mL angiotensin I. The ELISA of prorenin determination covers a calibration range of 0.02 – 10 ng/mL and the established renin assay evaluates the renin levels reliably between 1.96 to 500 µIU/mL.

3.6.6 Calculation of pharmakokinetic parameters

The pharmacokinetics of the compounds in serum were characterized by maximum serum concentration (C_{max}), time to maximum concentration (T_{max}), area under the curve from zero to timepoint *t* (AUC_{0-t}), AUC from zero to infinity ($AUC_{0-\infty}$), half-life (t½), elimination rate constant (k_e), and oral clearance of a drug (CL_F). The AUC was calculated by applying the trapezoidal rule with infinity extrapolation. The oral clearance was determined by dividing the administered dose by the $AUC_{0-\infty}$. The dose was adopted for enalaprilat according to the molar equivalent of enalapril maleate. Furthermore, the conversion ratio of enalapril and enalaprilat was calculated using the ratio of the $AUC_{0-\infty}$ of enalaprilat to the $AUC_{0-\infty}$ of enalapril. All of the above parameters were calculated according to equations (8), (9) and (10). The apparent volume of distribution (Vd_F) was determined by a non-compartmental analysis as follows: $Vd_F = CL_F/k_e$, whereas CL_F was defined as Dose/$AUC_{0-\infty}$. In urine, the unchanged amount of the compounds excreted into this fluid (A_e) and the renal clearance (CL_R) were evaluated according to equation (11) and (12). Saliva concentrations were examined for C_{max}, T_{max}, AUC_{0-t}, and the corresponding ratio between serum and saliva. Pharmacokinetic parameters were determined by non-compartmental analysis using a Phoenix 6.3.0.395 (Certara, St. Louis, MO). For humoral parameters, C_{max} and T_{max} were analysed. Because the primary objective of this study was not to provide new insights into the pharmacokinetics of the 3 drugs in adults, but rather to ensure the appropriateness of the low-volume analytic method and to assess the agreement of the results with those reported in the literature, a non-compartmental analysis, rather than a compartmental analysis, was used.

3.6.7 Safety assessments

The patient's health status was evaluated in personal interviews at least three days before as well as on day 1 of the study. Based on the interview, only subjects who were 18 years and older with a body weight greater than 50 kilograms and with no known organ diseases were allowed to participate. In addition, subjects with angioedema, urticaria, known allergies, or low blood pressure values in the past (<90/60 mmHg) were excluded for safety reasons. The hypotensive value limit was defined according to the recommendations of the National Heart, Lung and Blood Institute (National institutes of health 2010). Before drug administration on day 1, all female subjects were asked to peform a pregnancy test. For the duration of the study, blood pressure (BP) and heart rate measurements were conducted regularly. BP was measured after at least 5 minutes of rest in a supine position using an automatic upper arm oscillometric blood pressure monitor (Omron M5, Omron, Mannheim, Germany). Adverse events were monitored, their duration was recorded, and, if necessary, any concomitant drug given was documented.

3.7 Establishment of a "Good Clinical Laboratory Practice"-compliant quality system for bioavailability studies in support of marketing authorisation by EMA

There are several Good Practice guidelines, e.g. Good Manufacturing Practice (GMP), Good Laboratory Practice (GLP), Good Clinical Practice (GCP), established to support high-quality work e.g. within the pharmaceutical and medical environment. GxP is a commonly used term for the aforementioned guidelines. No GxP-standard was established in the laboratory at the beginning of this thesis. As a consequent and final step within this thesis the "Good Clinical Laboratory Practice" ("GCLP") was intended to be implemented for the bioanalytical laboratory of the Institute of Clinical Pharmacy and Pharmacotherapy.

GCLP is applied in this thesis to address regulatory expectations within the framework of Good Clinical Practice guidelines for laboratories performing the analysis of clinical trial samples, in particular for bioanalysis of samples. It should be noted here, that within the European regulatory environment the term of "GCLP" is not used officially (e.g. in regulations by EMA). The "GCLP" is an accepted European guidance and describes the combination of the ethical and scientific standards within Good Clinical Practice (GCP) on design, conduct and reporting of a laboratory study for analysis of clinical trial samples including bioanalysis. It resembles the Principles of Good Laboratory Practice (GLP) but considers the special aspects of GCP related to the rights, safety and well-being of the trial subjects. The latest official European document dealing with laboratories that conduct the analysis of clinical trial samples was a reflection paper which entered into force in 2012 (European Medicines Agency 2012a). "GCLP" can be used to establish the harmonised quality mind-set for organisations and individuals that undertake analysis of samples from clinical studies. Therefore, it appeared appropriate to imbed the novel low-volume bioanalytical methods introduced within this thesis into a quality system compliant with the current regulatory environment.

In doing so, a "ready-to-use" platform determining appropriately results for clinical trials can be established. The high quality standard is closely tied to the notion not only to develop high quality assays useful for research in children and to enable the highly required investigations in this vulnerable population but also to accomplish these investigations in direct accordance to the strict regulatory requirements. Consequently, by applying GCLP to the novel methods described within this thesis, the reliability and integrity of the analytical data can be assured and this marks an important step to support regulatory submissions to competent authorities for marketing authorisation.

The bioanalytical part of bioequivalence trials (Phase I) should be performed in accordance with the principles of Good Laboratory Practice (GLP). However, as human bioanalytical studies do not fall inside the scope of GLP (being confirmed by the responsible supervisory authority of North Rhine-Westphalia), the sites conducting the studies as well as the corresponding bioanalysis are not required to be monitored as part of a national GLP compliance program. Therefore, the GxP standard considered most appropriate for the studies within the LENA-project was Good Clinical Laboratory Practice.

The GCLP setting was required to be implemented prior to the first clinical study. An evaluation was conducted to identify the areas that mandatorily be embraced for the high quality standard. The intended setting is illustrated in Figure 12. The following elements are subject to GCLP at the Institute and necessitate coverage: (1) the physical facility, (2) its personnel and their safety, (3) the equipment and its qualification for use ("fit for purpose"), (4) the methods and procedures in the bioanalytical laboratory, (5) documented procedure for collection, transportation and receipt of specimens, (6) suitable quality control and (7) the recording and archiving system at the Institute. Furthermore, the corresponding roles, their responsibility, training needs, process flow and reporting mechanisms at the laboratory were defined and assigned. An internal and external quality control needed to be implemented. In this framework, Standard Operating Procedures (SOPs), corresponding form sheets, working instructions, and general quality documents were mandatory to ensure that the purpose and objectives of GCLP were satisfied. The complex procedures within the LENA specific studies necessitated for detailed descriptions. This was addressed by unambiguous manuals instead of working instructions. These manuals were meant of particular importance to ensure a suitable and accurate exchange of data and information at defined interfaces. Namely the interfaces to the study site where the volunteers were enrolled and treated as well as to the statistical institute were covered. Manuals on optimal sampling, storage and shipment of biological samples were initiated and the involved Phase-I unit was trained accordingly.

This was of special relevance as the clinical site was not familiar with the consumables/ sampling devices selected for blood sampling. A system was chosen that allows for collection of blood either through vacuum technique or by aspiration. While the vacuum collection is common in adult studies, there is often a need for soft collection through aspiration in children. Additionally, the blood collection system had been used prior to this to collect blood for the method validations of the corresponding serum methods. The reason to use the validated equipment in research and clinical studies was to apply the same level of quality procedures from research to the Phase I study of LENA and also in future paediatric studies within

the LENA project. The required materials and devices for blood withdrawal were selected, purchased and distributed to the clinical site by the laboratory.

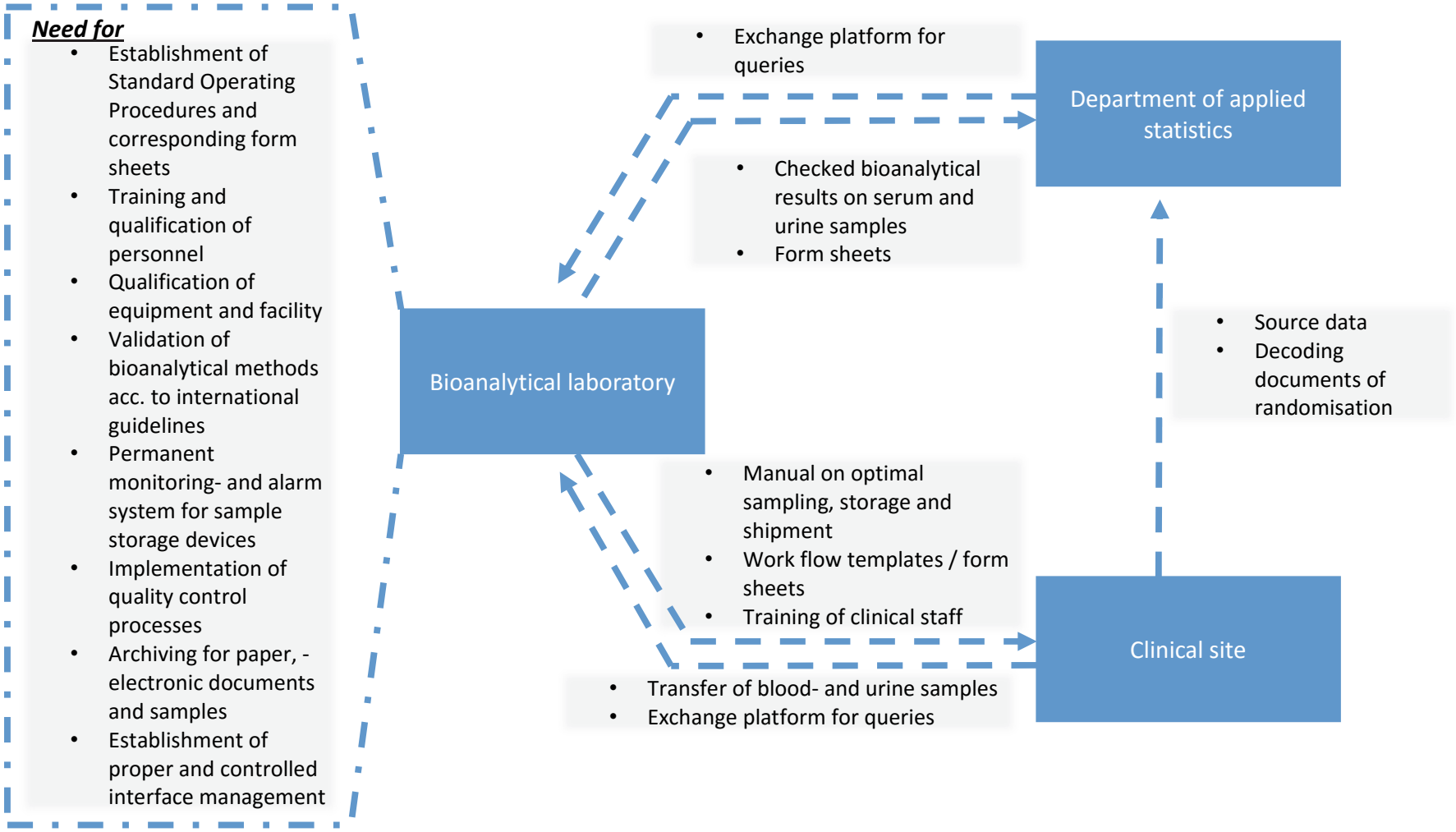

Figure 12 ***Intended GCLP setting at the bioanalytical laboratory*** *including interface management and data exchange according to the "GCLP" standard. Dashed lines and arrows indicate the mandatory processes and documents that needed to be established to meet the requirements of GCLP. The bioanalytical laboratory is responsible for performing all measurements according to this standard. The mandatory systems and processes that needs to be followed to ensure the reliability of produced data are listed on the left-hand side. Add-on responsibility of the laboratory is on appropriate training of related sites involved in the bioanalytical processes and to enable a proper exchange of material, samples and documents. This is an important indicator of patient safety within clinical trials.*

4. Results

In total, five low-volume bioanalytical HPLC-MS/MS methods for the determination of aliskiren and enalapril/enalaprilat were successfully developed. Moreover, the change of pharmacodynamic parameters after administration of aliskiren and enalapril can be evaluated by additional five low-volume assays based on immunological techniques. All of them were successfully applied in the conducted proof-of-concept study in healthy volunteers. The developed tailored bioanalytical platform that enables the description of pharmacokinetics and pharmacodynamics only requires a total blood volume of at most 2.3 mL per sampling point.

The assays utilised for evaluation of aliskiren and enalapril concentrations in serum were successfully validated according to international bioanalytical method validation guidelines and additionally scaled up for high sample throughput. This setting enabled a 6-fold higher sample throughput than in former established methods (Figure 13). For the scaling process, the required technical equipment especially for the drying process is mostly based on own developments.

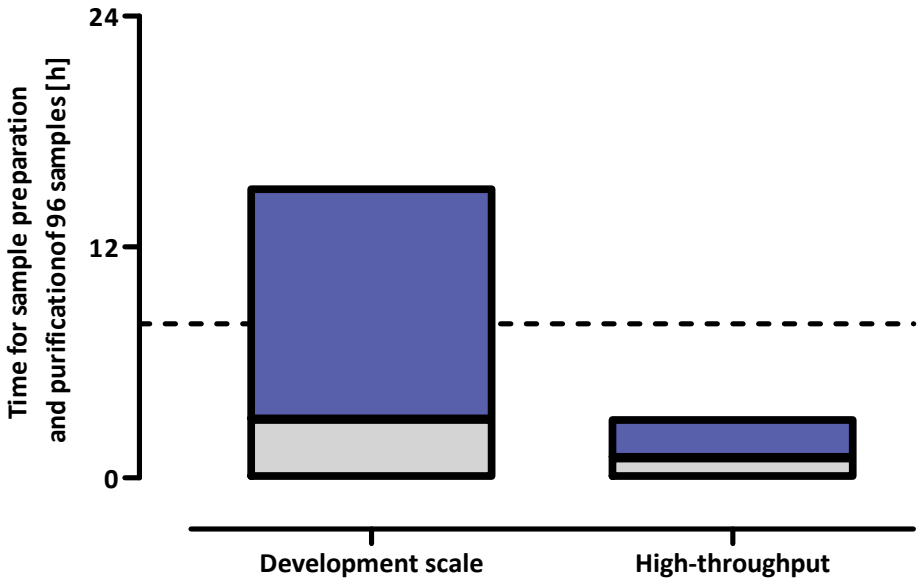

Figure 13 ***Comparison of required time for bioanalysis*** *between development scale by applying single cartridges and the high-throughput approach with 96 well-plates. The calculation is based on a sample amount of 96 samples. The blue areas mark the required time for sample purification by solid-phase extraction and the grey areas identify the time frame required for sample preparation. The dashed line represents one full working day (8 hours). By applying the high-throughput approach, the sample preparation and purification is finalised within 2 hours while the same amount of samples is impossible to purify within one working day by one lab technician using the previous development scale.*

The following graph gives insight into the undertaken analytical optimisation by comparing the required blood volumes in the first recently conducted paediatric clinical trial in aliskiren (Sullivan et al. 2013) with the newly established low-volume platform. According to the EMA guideline the maximum clinical trial-related blood loss should not exceed 3 % of the total blood volume during a period of four weeks and should not exceed 1 % at any single time. Consequently, both scenarios were given by the Figure 14. First, for four exemplarily chosen young children the maximum blood loss according to EMA recommendations with in four weeks as well as at a single time was shown. Second, the sample volumes required in the first paediatric study for aliskiren (children with 6 years and older) act as reference and were enclosed into the graph. The volumes were calculated for a minimum sampling setting of one sample for PK and one for PD as well as a full PK and PD profile (five PK samples and five PD samples). The same was done for the novel bioanalytical platform.

It became obvious that with the new platform, single PK sampling is possible even in pre-term born babies. The full PK and PD profile can be obtained in newborns with 4 kg body weight and higher without infringing ethical considerations. For children with less body weight than 4 kg only some of the five pharmacodynamic parameters can be determine if necessary. However, it is possible to also generate data is this age group now and collect also highly required information in this vulnerable population. By contrast, utilising former assays (e.g. Sullivan *et al*. 2013) the full PK and PD profile is only conceivable in children with 8 kg body weight and higher. In children in whom Sullivan et al. would be able to only draw one PK and one PD sample, the novel bioanalytical platform enables the collection of a full PK and PD profile. The full profile could consists of e.g. five determinations of drug concentration and five pharmacodynamic parameters.

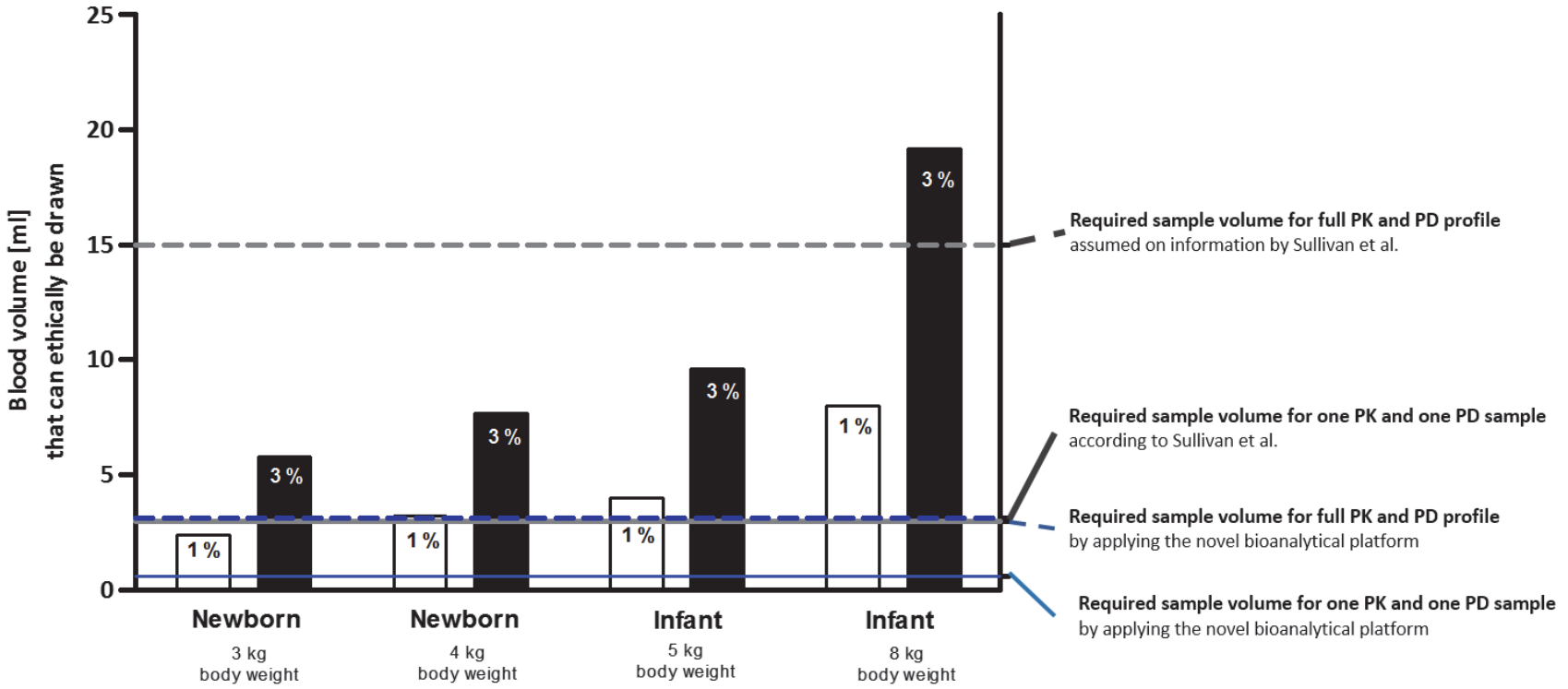

Figure 14 ***Achieved optimisations in required sample volume for determination of aliskiren and its pharmacodynamics in different paediatric age groups.*** *Four different young children were exemplarily shown on the x-axis. Based on each body weight, the maximum volume that should be drawn at any single day (white bars, 1 % of total blood volume) and within four weeks (black bars, 3 % of total blood volume) are illustrated. The blue line (——) indicates the required sample volume for reliable determination for the minimum set of one PK and one PD sample, while the dashed line (------)marks the required sample volume for full PK and PD profile using the novel bioanalytical platform. A full PK and PD profile is meant as five samples for determination of aliskiren and five pharmacodynamic samples. The grey line (——) marks the required volume reported by Sullivan et al. who conducted recently the first paediatric study using aliskiren in children with 6 years and older. He was therefore chosen as reference. The corresponding grey dashed line (------) shows the assumed amount for a full PK and PD sampling. Since it was ethically not possible to conduct these comprehensive PD studies in children by now, the required blood volume is assumed on current publications only. Blood volumes mandatory for clinical routine are not covered and require additional attention. According to information given by the Central Institute of Clinical Chemistry and Laboratory Medicine at the Universitätsklinikum Düsseldorf about 1.2 mL are required for clinical routine per sampling point.*

Finally, the tailored bioanalytical setting was embedded into the new established GCLP environment. This junction of precise low-volume bioanalytical assays, the successful scale-up for high sample through-put, the conformity to international bioanalytical guidelines and the international quality system (GCLP) enables the novel “ready-to-use” platform for paediatric trials. This platform meets current standards for clinical trials and was successfully used in parts within the Phase I-study of the LENA-project.

Detailed results on the single steps are provided in the following paragraphs.

4.1 Bioanalytics for determination of drug concentration in different biological fluids

4.1.1 Serum

4.1.1.1 Determination of aliskiren and enalapril in serum by HPLC-MS/MS method

The established in-house validation plan consisted of 14 validation parameters. The EMA guideline was strictest in 9 parameters whereas FDA guidance requirements were incorporated into 3 parameters equal to EMA recommendations. The validation parameter "recovery" was established based on FDA guidance as it contained stricter requirements than EMA. The parameter "cross-talk" was an in-house parameter which is not listed in both international guidelines. For practical reasons, some parameters were more comprehensive than the strictest parameter mentioned in both guidelines. Details are summarised in Table 4.

For the aliskiren method in serum, the retention time was about 2.7 minutes for aliskiren and 2.9 minutes for the IS benazepril. In the enalapril method, enalapril peaked at 4.9 minutes, the retention time of enalaprilat was 4.4 minutes and that of benazepril 5.1 minutes. Good results in terms of symmetric peak shape, resolution and retention time of all analytes of interest were obtained allowing for an automated integration.

Selectivity and cross-talk

No additional peaks above the guideline limits within $\pm$ 0.3 minutes of the retention time of aliskiren nor of the ones of enalapril, enalaprilat and benazepril were observed in the evaluated serum samples spiked with co-administered drugs. Regarding aliskiren, additional six different sources of human sera were analysed as blank and after spiking the sera with analyte at the LLOQ for any interference. No interferences in blank samples of the different sources were detected and the signal-to-noise ratios of spiked to blank samples of all sources were above 5:1. The latter also applied to the seven investigated sources during the validation of the enalapril/enalaprilat method. Typical chromatograms of blank human serum and QC sample at LLOQ as well as ULOQ of aliskiren are presented in Figure 15. Figure 16 shows the chromatograms at the same above-mentioned concentration levels for enalapril and enalaprilat. No effects of cross-talk were observed for all analytes of interest. Haemolysed blood as well as hyperlipidaemia blood samples had no detectable influence on the specific MS-channels of aliskiren, enalapril, enalaprilat and IS, respectively.

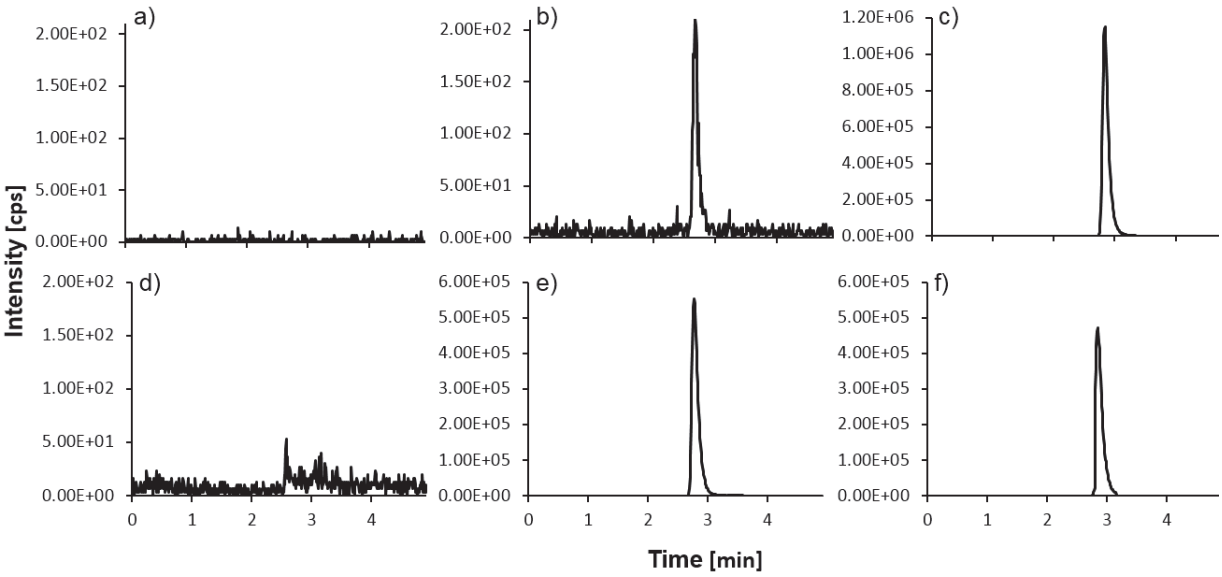

Figure 15 ***Multiple Reaction Monitoring (MRM) chromatograms of aliskiren*** *(upper row) and benazepril (lower row) at three concentration levels in serum. Blank samples of aliskiren (a), at the lower limit of quantification (b) and at the upper limit of quantification (c) are compiled with the chromatograms of benazepril for blank (d), at the lower limit of quantification (e), and at the upper limit of quantification (f).*

Linearity and Lower Limit of Quantification (LLOQ)

Aliskiren

Over the complete concentration range of 0.146 - 1200 ng/mL the investigated serum standard samples of aliskiren prepared with benazepril as internal standard show a linear relationship. The mean linear regression for the conducted calibration curves (n=17) was

$y = (0.01598 \pm 3.4*10^{-3}) x + (0.001234 \pm 8.1*10^{-4})$ with a coefficient of determination > 0.998. The lower limit of quantification for aliskiren was 0.146 ng/mL aliskiren in serum with provided values of precision and accuracy within the range of 20 %. The signal-to-noise ratios of all LLOQ samples were above 5:1. All results comply with EMA and FDA guidelines (European Medicines Agency 2012b, Food and Drug Administration 2001).

Enalapril/enalaprilat

Similar results were gained with the enalapril method within the linear calibration range of 0.2 – 200 ng/mL enalapril and 0.18 – 180 ng/mL enalaprilat, respectively. Best fit of the regression curve was gained by $1/x^2$ weighting for both analytes. The concentrations of both analytes at the LLOQ were within the guideline

limits regarding precision, accuracy and the signal-to-noise ratio. The mean linear regression based on six individual calibration curves each and the corresponding linear equations were:

$$y = (0.0438 \pm 0.0020)\, x + (0.0026 \pm 0.0024) \qquad \text{Enalapril}$$

$$y = (0.0055 \pm 0.0016)\, x + (0.0005 \pm 0.0001) \qquad \text{Enalaprilat}$$

with a correlation coefficient of at least 0.996 for enalapril and 0.997 for enalaprilat.

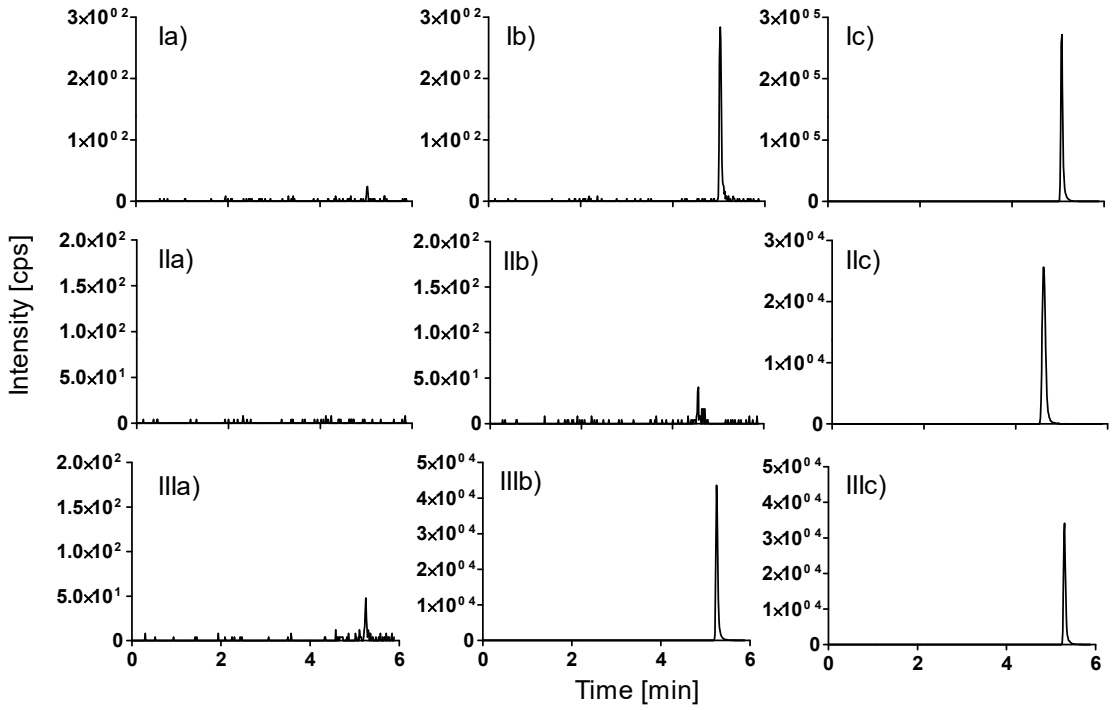

*Figure 16 Exemplary **Multiple Reaction Monitoring (MRM) chromatograms of enalapril (I), enalaprilat** (II) and benazepril (III) at blank (a), LLOQ (b) and ULOQ (c) utilising the serum method for determination of enalapril and enalaprilat concentrations.*

Accuracy and precision

Aliskiren

Regarding the direct renin inhibitor, five concentration levels covering the whole concentration range with 4 runs on 4 different days were investigated on accuracy and precision. All obtained values were within the predefined criteria according to the EMA and FDA guidelines (European Medicines Agency 2012b, Food and Drug Administration 2001). Obtained precision values by one-way ANOVA were between 0.4 – 7.2 % for intra-run and 0.6 – 12.9 % for inter-run precision. Precision and accuracy results are summarised in Table 7.

Concentration spiked [ng/mL]		Concentration found [ng/mL]	Intra-run R.S.D. [%]	Inter-run R.S.D. [%]	Relative error [%]
0.146	(LLOQ)	0.149 ± 0.01	7.01	12.93	1.75 ± 6.9
1.17		1.17 ± 0.07	4.45	8.19	0.0 ± 5.1
18.8		18.65 ± 0.37	0.40	0.58	- 0.8 ±1.6
150		152.7 ± 4.5	1.71	3.73	1.8 ± 2.4
1200	(ULOQ)	1189.1 ± 21.5	2.90	3.34	- 0.9 ± 1.5

Table 7 ***Precision and accuracy of aliskiren quantification*** *by the HPLC-MS/MS serum method – Data compiled as mean ± standard deviation (S.D.) or relative standard deviation (R.S.D.).*

Enalapril/enalaprilat

Intra-run and inter-run accuracy and precision for enalapril and enalaprilat, respectively, was determined on five days by investigating five different runs. Obtained results by one-way ANOVA of the intra-run precision ranged from 2.2 to 5.0 % for enalapril and from 4.9 to 18.0 % for enalaprilat. These precision results all conform to the current bioanalytical guidelines. Mean accuracy results of the quality control samples were likewise within the guideline requirements of ± 15 % at all concentration levels except the LLOQ (± 20 %). Further details are listed in Table 8.

	Concentration spiked [ng/mL]		Concentration found [ng/mL]	Intra-run R.S.D. [%]	Inter-run R.S.D. [%]	Relative error [%]
Enalapril	0.195	(LLOQ)	0.197 ± 0.02	5.0	10.8	1.1 ± 10.6
	3.125		3.06 ± 0.19	3.5	6.6	-2.0 ± 6.1
	25		25.8 ± 0.92	2.2	3.8	3.2 ± 3.7
	200	(ULOQ)	197.5 ± 5.73	2.3	3.0	-1.3 ± 2.9
Enalaprilat	0.175	(LLOQ)	0.177 ± 0.03	18.0	18.6	1.4 ± 14.0
	2.81		2.7 ± 0.17	7.0	11.9	-3.7 ± 5.9
	22.5		23.4 ± 1.3	6.5	12.0	3.8 ± 1.3
	180	(ULOQ)	186 ± 7.6	4.9	10.9	3.3 ± 4.2

Table 8 ***Precision and accuracy of enalapril/enalaprilat quantification*** *by the HPLC-MS/MS serum method – Data compiled as mean ± standard deviation (S.D.) or relative standard deviation (R.S.D.).*

Matrix effect and recovery

Aliskiren

At all concentration levels of aliskiren, the matrix led to ionisation suppression. The evaluated concentration levels regarding matrix effect were 0.146, 1.17, 18.8, 150 and 1200 ng/mL aliskiren. Absolute mean matrix effect values vary between -8.35 ± 7.1 % to -23.1 ± 4.4 %. Information on matrix effect is available in Table 9. At low (0.5 ng/mL), middle (150 ng/mL) and high (1200 ng/mL) concentration level the CV (%) of six sources of serum within a concentration level was highest at 12.5 % and within the guideline limits for relative matrix effect. The matrix effect of benazepril was calculated to be -8.9 ± 4.1 % based on nine measurements and also indicates ion suppression. This accounts also for the investigated haemolysed samples. The recoveries of aliskiren ranged from 89.2 to 98.2 %. Process efficiencies of the solid-phase extraction led to values between 72.6 to 81.8 %. Recovery of the internal standard was 95.3 ± 3.6 %. Details are compiled in Table 9.

Concentration [ng/mL]	0.146	1.17	18.8	150	1200
Absolute matrix effect ± S.D. [%]	-14.7 ± 10.5	-8.35 ± 7.1	-17.41 ± 1.4	-21.43 ± 8.6	-23.11 ± 4.4
Mean recovery ± S.D. [%]	92.9 ± 4.2	89.2 ± 3.6	91.5 ± 1.2	92.38 ± 0.6	98.16 ± 2.0
Mean process efficiency [%]	79.24	81.75	75.57	72.58	75.48

Table 9 ***Recovery, matrix effect and process efficiency of aliskiren in serum*** *– Data compiled as mean or mean ± standard deviation (S.D.).*

Enalapril/enalaprilat

The effect of the matrix on the determination of enalapril and enalaprilat was evaluated at the LLOQ, one low concentration, one middle concentration and at the ULOQ. By combining SPE and chromatographic separation, the matrix effect was observably reduced in this setting, leading to an ion suppression of -9.9 to -24.6 % for enalapril and of -7.2 ± 2.8 % for the internal standard benazepril. The sample matrix has no or a slight ion enhancing effect on detection of enalaprilat. It ranged between -1.6 to 9.5 %. All analytes were fully recovered from the sorbent of the mixed-mode anion exchanger, resulting in a process efficiency of 66 to 93 % for enalapril, 95 – 118 % for enalaprilat and 71 % for benazepril (IS) (Table 10).

	Concentration [ng/mL]	Absolute matrix effect ± S.D. [%]	Mean recovery ± S.D. [%]	Mean process efficiency [%]
Enalapril	0.195	-14.4 ± 16.0	77.1 ± 0.7	66.0
	3.125	-9.9 ± 2.9	103.5 ± 3.7	93.3
	25	-24.6 ± 7.0	102.9 ± 6.3	77.6
	200	-21.2 ± 5.3	92.0 ± 6.9	72.5
Enalaprilat	0.175	-1.6 ± 13.0	99.9 ± 7.8	98.3
	2.81	0.2 + 4.2	118.3 ± 4.6	117.8
	22.5	9.5 ± 4.1	102.4 ± 4.7	112.1
	180	2.1 ± 3.4	92.9 ± 9.2	94.9
Benazepril	25	-7.2 ± 2.8	76.9 ± 2.3	71.3

Table 10 ***Recovery, matrix effect and process efficiency of enalapril/enalaprilat in serum*** *– Data compiled as mean or mean ± standard deviation (S.D.).*

The relative matrix effect in enalapril samples at a low concentration (0.39 ng/mL [enalapril] and 0.35 ng/mL [enalaprilat], respectively) was 5.49 % (CV) for enalapril and 12.56 % for enalaprilat. At the ULOQ, a coefficient of variation of 1.87 % for enalapril and 8.96 % for enalaprilat were evaluated for all seven different human sources. Details are arranged in Table 11.

		Enalapril		Enalaprilat	
Concentration / Donor		0.39 ng/ml	200 ng/ml	0.35 ng/ml	180 ng/ml
Healthy adults 29 – 86 years old	♂ Donor 1	106.7	96.8	162.5	135.9
	♂ Donor 2	99.1	95.7	122.1	108.2
	♂ Donor 3	96.6	96.6	120.9	119.4
	♀ Donor 4	111.1	97.6	132.1	126.6
	♀ Donor 5	107.2	97.8	121.0	114.5
	♀ Donor 6	98.8	94.5	123.1	112.1
	♂ Donor 7	98.4	92.8	114.4	106.7
Mean value of normalised relative ME ± S.D. [%]		102.6 ± 5.6	96.0 ± 1.8	128.0 ± 16.1	117.6 ± 10.5
CV [%] within donors		5.49	1.87	12.56	8.96

Table 11 ***Relative matrix effect of the enalapril/enalaprilat serum method –*** *S.D.: standard deviation; CV: coefficient of variation.*

Stability

Peak areas of stock solution of aliskiren after three and six months of storage at 7 °C deviated by 7.0 ± 1.8 % and 0.4 ± 0.9 % compared to fresh stock solution and confirmed a stable solution. Measured peak areas of benazepril of 96.46 ± 2.8 % and 98.3 ± 2.8 % also indicated a stable stock solution of the internal standard under these conditions. The obtained results of the post-preparation solution stored in HPLC-vials at room temperature for 24 h demonstrated sufficient stability. Post-extraction samples frozen at -20 °C, freeze-thaw stability evaluated for four cycles of freeze and thaw as well as samples at -20 °C and -80 °C stored over a period of one and three months were stable since none of the investigated stability parameters were out of specification and proved the suitability for routine analysis. Details are summarised in Table 12.

	Concentration spiked [ng/mL]					
	0.146	1.17	18.8	150	600	1200
	Concentration found [ng/mL]					
Short-term (21 °C)	0.150 ± 0.004 (+2.7)*	1.36 ± 0.02 (+14.5)*	21.6 ± 0.5 (+14.8)*	171 ± 2.8 (+14.0)*	649 ± 8.0 (+8.2)*	---
1 month long-term (-20 °C)	0.121 ± 0.002 (-17.1)*	1.21 ± 0.03 (+3.4)*	18.3 ± 0.6 (-2.7)*	139 ± 3.2 (-7.3)*	---	1107 ± 37 (-7.8)*
1 month long-term (-80 °C)	0.121 ± 0.002 (-17.1)*	1.25 ± 0.05 (+6.8)*	16.7 ± 0.2 (-11.2)*	128 ± 4.9 (-14.6)*	---	1045 ± 17 (-12.9)*
3 months long-term (-20 °C)	0.136 ± 0.014 (-6.8)*	1.14 ± 0.03 (-2.6)*	18.2 ± 0.6 (-3.2)*	142 ± 4.6 (-5.3)*	---	1057 ± 33 (-11.9)*
3 months long-term (-80 °C)	0.157 ± 0.013 (+7.5)*	1.33 ± 0.03 (+13.7)*	20.6 ± 0.8 (+9.6)*	160 ± 3.5 (+6.7)*	---	1157 ± 43 (-3.6)*
Post-preparative (21 °C)	0.138 ± 0.01 (-5.5)*	1.22 ± 0.04 (+4.3)*	20.9 ± 0.1 (+11.2)*	159 ± 3.4 (+6.0)*	---	1190 ± 29 (-0.8)*
Post-extraction (-20 °C)	0.147 ± 0.01 (+0.7)*	1.29 ± 0.03 (+10.3)*	21.4 ± 0.4 (+13.8)*	163 ± 1.3 (+8.7)*	---	1246 ± 9.4 (+3.8)*
Freeze-thaw (-20/21 °C)	0.119 ± 0.008 (-18.5)*	1.03 ± 0.02 (-12.0)*	18.1 ± 0.5 (-3.7)*	142 ± 1.5 (-5.3)*	---	1032 ± 7.9 (-14.0)*

Table 12 ***Stability results of aliskiren in serum*** *– Data compiled as mean concentration ± standard deviation (S.D.); *Percentage deviation compared to nominal concentration. All results were within the required accuracy of ±15 % of nominal concentration (±20 % at the LLOQ) and confirm the stability.*

The stability of enalapril and enalaprilat under the investigated conditions was well within the international guideline limits for bioanalytical methods. Enalapril and enalaprilat was stable in serum for 24 h if stored at room temperature (25 °C). The investigation on frozen unprocessed serum samples showed results within the accuracy and precision limits of bioanalytical methods and confirmed also the stability. Furthermore, a new storage condition of extracted dried eluate in deep-well plates at -20 °C for 48 h was successfully amended to the validation plan and prove the drug's stability at this condition. Details for all concentration levels as well as conducted stability investigations and their conditions are listed below.

	Nominal conc. [ng/mL]	Short-term stability (25 °C for 24 h)	Long-term stability (-80 °C for 54 days)	Autosampler stability (25 °C for 24 h)	Freeze-thaw stability (-20 °C/21 °C; 4 times)	Extraction stability (-20 °C for 24 h)
Enalapril	0.195	0.187 ± 0.012 (-4.0)*	0.196 ± 0.009 (+0.6)*	0.221 ± 0.026 (+13.5)*	0.218 ± 0.009 (+11.7)*	0.217 ± 0.02 (+11.1)
	3.13	2.84 ± 0.06 (-9.2)*	3.14 ± 0.03 (+0.5)*	3.13 ± 0.02 (+0.3)*	3.30 ± 0.008 (+5.6)*	2.94 ± 0.06 (-6.0)
	25	23.4 ± 0.8 (-6.3)*	25.4 ± 0.1 (+1.5)*	25.6 ± 0.2 (+2.3)*	26.1 ± 0.2 (+4.6)*	23.7 ± 0.2 (-5.3)
	200	187 ± 4.4 (-6.5)*	189 ± 2.7 (-5.4)*	201 ± 1.8 (+0.7)*	202 ± 0.5 (+1.0)*	198 ± 2 (-1.0)
Enalaprilat	0.175	0.198 ± 0.02 (+13)*	0.194 ± 0.06 (+10.7)*	0.171 ± 0.023 (-2.35)*	0.156 ± 0.023 (-10.8)*	0.196 ± 0.002 (+11.8)
	2.81	2.74 ± 0.12 (-2.7)*	2.78 ± 0.15 (-1.0)*	2.81 ± 0.04 (±0.0)*	2.95 ± 0.09 (+5.0)*	2.94 ± 0.15 (+4.8)
	22.5	21.1 ± 0.4 (-6.4)*	23.1 ± 0.2 (+2.6)*	22.27 ± 0.6 (+1.0)*	24.6 ± 0.4 (+9.3)*	22.7 ± 0.2 (+1.0)
	180	176 ± 0.6 (-2.4)*	173 ± 0.8 (-3.6)*	185 ± 2.8 (+2.5)*	201.7 ± 14.3 (+12.0)*	190 ± 3 (+5.6)

Table 13 ***Stability results of enalapril and enalaprilat in serum*** *– Data compiled as mean concentration ± standard deviation (S.D.); *Percentage deviation compared to nominal concentration; conc: concentration.*

Dilution integrity and carry over

For aliskiren, spiked blank human serum concentrations at 2400 ng/mL were serial diluted to the ULOQ and showed accuracy and precision well within the accepted deviation range of ± 15 %. Five determinations were performed with mean accuracy of 98.5 ± 1.9 %. Dilution integrity for enalapril and enalaprilat was checked by diluting serum samples containing 400 ng/mL enalapril and 360 ng/mL

enalaprilat with blank serum to the ULOQ. The determined accuracy was 86.9 ± 2.0 % for enalapril and 89.2 ± 0.9 % for enalaprilat (n=3). Carry over into the blank sample following a single injection of 125 ng/mL or 1200 ng/mL (ULOQ) aliskiren or 200ng/mL enalapril and 180 ng/mL enalaprilat, respectively, was not detected. Nevertheless, transitions into the followed blank sample were observed after 20 injections of aliskiren at 1200 ng/mL. Consequently, after five samples with unknown or high concentrations one blank sample was injected to avoid residual possibility of carry over.

Robustness

Undertaken investigations on method robustness are exemplified described for aliskiren in serum. The amount of formic acid in mobile phase was varied from 0 to 1 %. No acidified mobile phase led to any evaluable results because either no peak was detected or peak shape was not integrable. By increasing the amount of formic acid higher than 0.005 %, the obtained peak areas decreased by up to 54 % reaching a minimum at 1 % formic acid in mobile phase. A change in temperature of column oven in the range of 20 to 50 °C caused an increase of peak areas with higher temperatures of up to 44 % with a lower slope for temperatures above 40 °C. The change in the peak areas between the final method temperature of 40 °C compared to 50 °C was 8 %. Overall, the retention time shifted 0.4 minutes from 20 °C to 50 °C. The obtained peaks were of good shape as the full widths at half maximum were ≤ 0.13 and were automatically integrated by IntelliQuan®. Furthermore, the flow rate of the mobile phase was modified ± 10 % which changed the peak area by less than 0.8 %, whereas retention time decreased with higher flow rates by 0.5 minutes. Variations of organic fraction in mobile phase between 72.5 to 77.5 % changed the peak area by less than 5 %. During the full validation, 4 different HPLC columns were investigated which represent 3 different batches to prove batch-to-batch and intra-batch consistency. Aliskiren standard at about 120 ng/mL (n=5 per batch) showed peak areas with a maximum of 4.7 % variability over all column lots. Retention time varied by 0.13 minutes and intensity by a maximum of 6.1 %. Optimised flow-dependent MS parameters (gases, temperature and ionspray voltage) evaluated by flow-injection analysis are summarised under section 3.4. *Chromatographic and mass spectrometer conditions*.

Although many efforts were undertaken to purify the serum containing enalapril, enalaprilat and benazepril as much as possible by solid-phase extraction, the different physicochemical properties of enalaprilat and enalapril limit the separation of matrix and analyte to a certain amount. A more intensive washing step did not allow for the collection of all analytes within one fraction and was therefore neglected. Enalaprilat is much more hydrophilic (calculated log D -8) compared to enalapril (calculated log D -1). Therefore, chromatographic separation was of utmost importance to additionally support reducing

the matrix effect. The separation of enalaprilat and matrix was difficult because the retention times of many matrix residuals and enalaprilat were highly comparable even with different gradients and HPLC columns. In Figure 17 the chromatographic separation with the most appropriate gradient is shown. The gradient facilitates the separation of matrix and analytes with retention times close to each other to enable the use of only one internal standard. If the time difference between the analytes and the IS is large, it is more likely that residuals which influence the compounds of interest and elute early are different from the residuals that interfere with the internal standard and consequently lead to different behaviour that alters the quantification. Apart from the difficult separation of residual matrix and enalaprilat, the hydrophilic enalaprilat showed a split peaking at its retention time by applying solid-phase extraction by MCX material. This could only be erased by change of the SPE phase from cation to anion exchanger. Further details are arranged in the next section “Challenges during method development”.

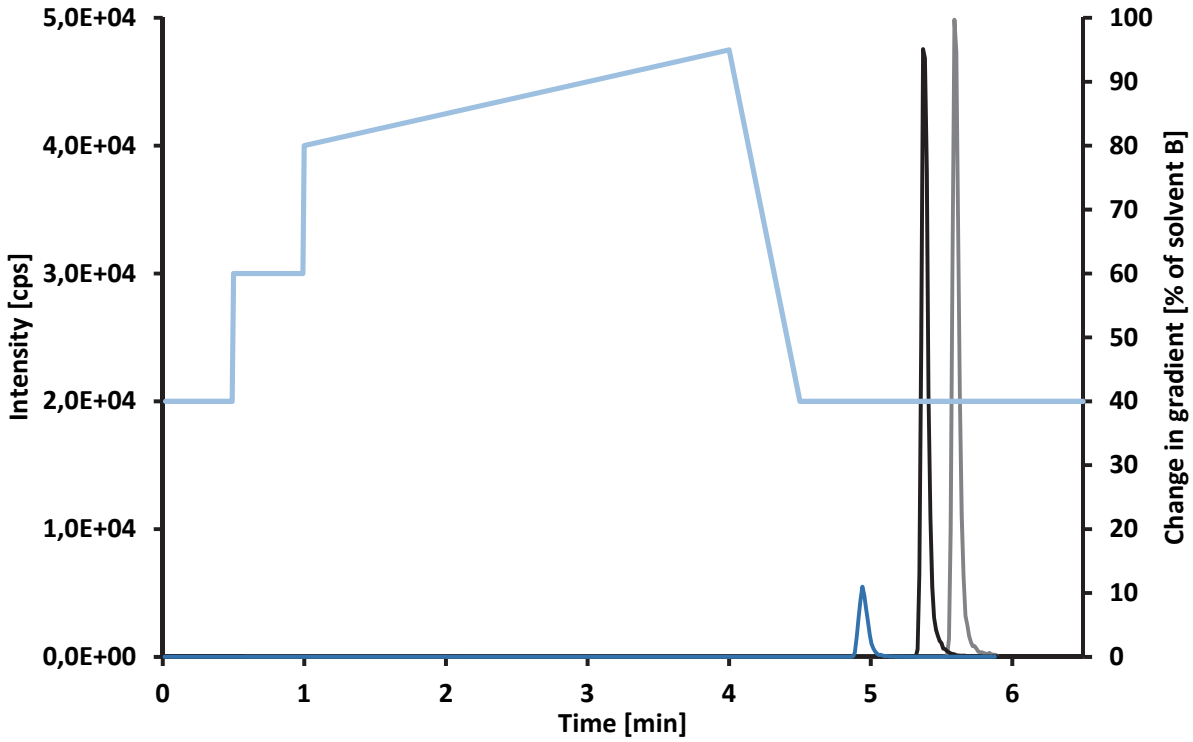

Figure 17 ***Typical chromatogram obtained by the enalapril/enalaprilat serum method****. Enalaprilat is illustrated by a blue line, enalapril is shown with a black line and the internal standard by the grey line. The corresponding intensities of all analytes are given on the primary y-axis. The trend of the applied gradient is shown as a line representing the percentage of solvent B (methanol).*

Application

The method was applied to determine the serum concentrations of aliskiren in a healthy male volunteer after single oral administration of 300 mg aliskiren. The concentration-time profile was obtained 2 hours after a light breakfast (two slices of bread, marmalade and 250 mL milk). The main pharmacokinetic parameters were C_{max}: 246 ng/mL, t_{max}: 4 h, AUC: 1589 ng*h/mL and an elimination half-life of 41 h. A comparison to mean concentration-time profiles published by the originator is arranged in Figure 18.

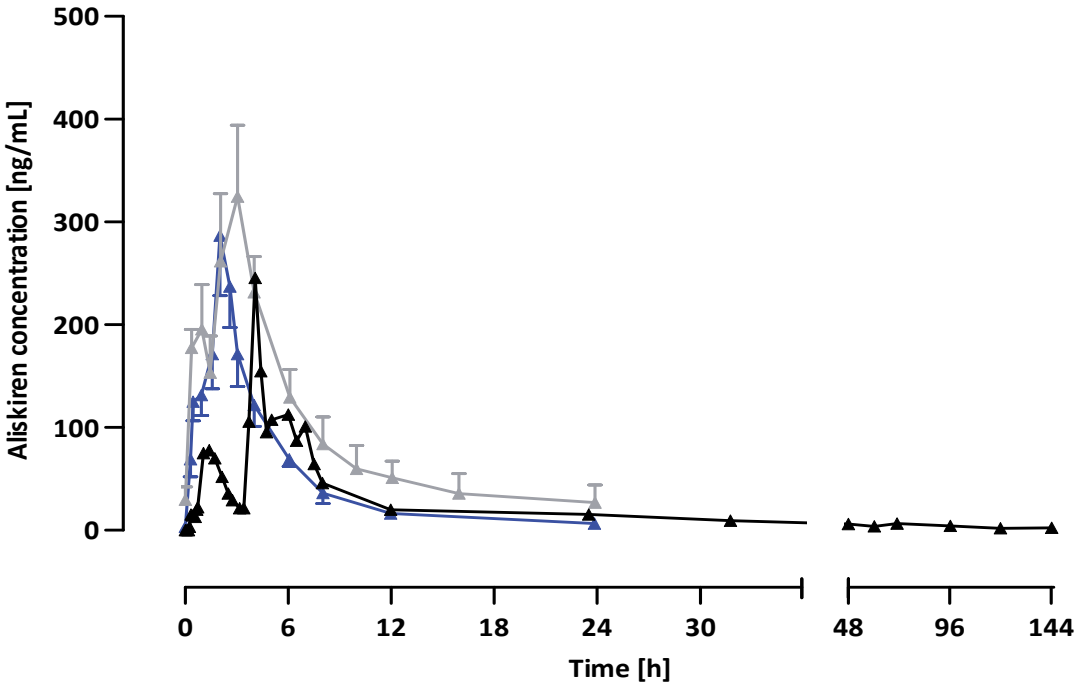

Figure 18 ***Serum concentration-time profile of a healthy male volunteer after oral administration of 300 mg aliskiren hemifumarate*** *(black line, triangles). Drug was administered 2 hours after a light breakfast (two slices of bread, marmalade and 250 mL milk). Grey triangles represent mean concentration-time points of 28 young subjects (Vaidyanathan et al. 2008c), while blue triangles represent mean concentration-time points of 17 subjects (Vaidyanathan et al. 2007b).*

Moreover, the double peak phenomenon was investigated by different administrations of aliskiren hemifumarate. The male volunteer (29 years, 63 kg) took 2 tablets à 150 mg (at once) 2 hours after a light meal, 2 tablets á 150 mg aliskiren hemifumarate under fasting conditions and finally, 1 tablet á 300 mg of the drug also under fasting conditions. Between each dosing a wash-out period of at least three months were included. The obtained concentration-time profiles all revealed double peaks to various extents. Both administrations under fasting conditions showed t_{max} after 2 and 2.6 hours, while the t_{max} of the administration after the light breakfast was after 4 hours. The determined AUC_{0-t} ranged from 1244 (2 x

150, fasten) to 5448 ng*h/mL (1 x 300 mg, fasten). The corresponding concentration-time profiles are available in Figure 19.

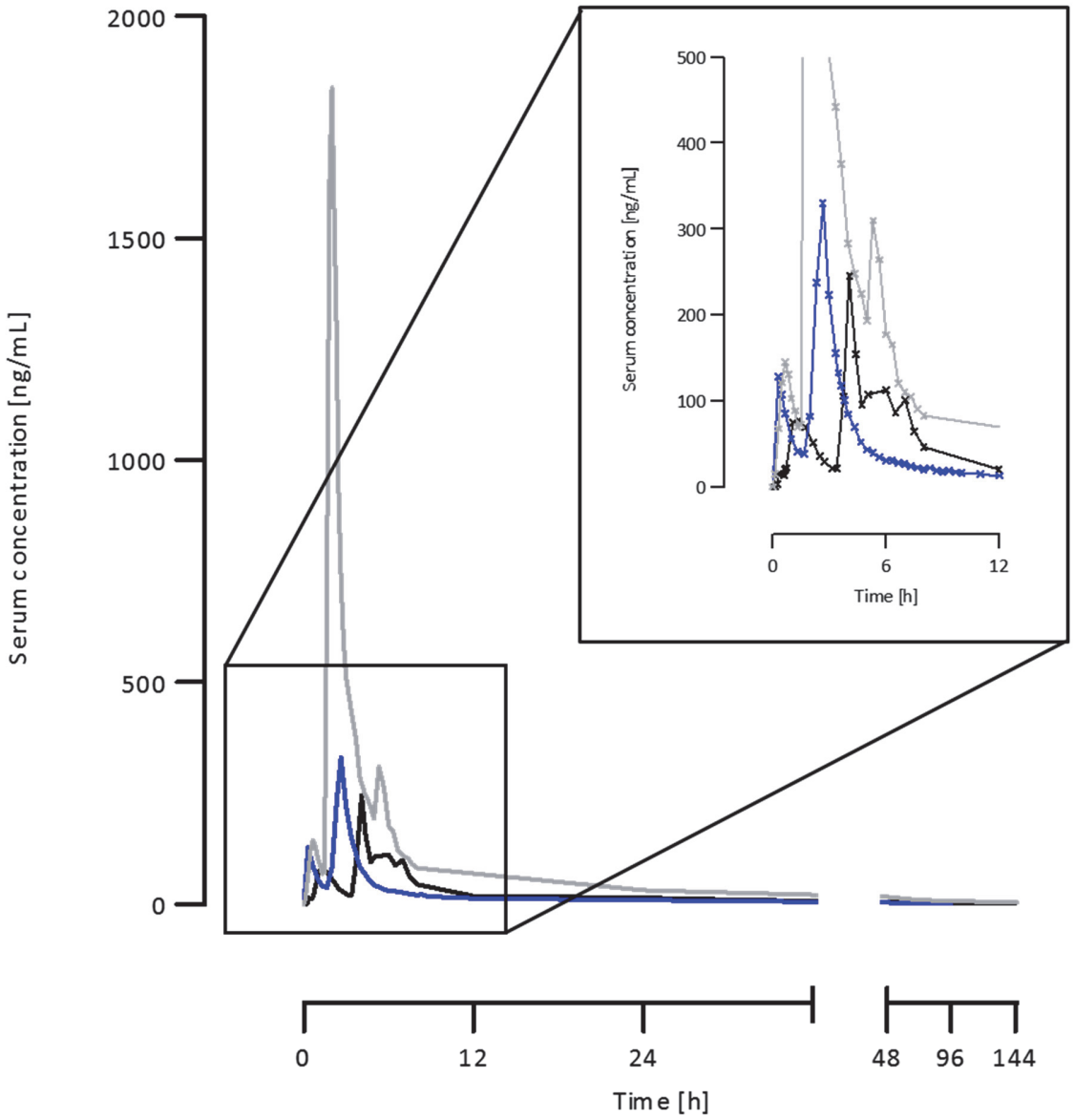

Figure 19 ***Different kinds of oral administrations of aliskiren hemifumarate*** *to a healthy male subject. The black line shows the profile after administration of 2 x 150 mg aliskiren at once after a light breakfast, the blue line represents the profile after administration of 2 x 150 mg aliskiren at once under fasting conditions and the grey line illustrates the one-time administration of 300 mg aliskiren under fasting conditions. To better identify the double peaks occurring after all kinds of administration and the high variability, an inlet was arranged for the first 12 hours after oral administration.*

Regarding enalapril and enalaprilat, two assays were successfully developed within this thesis. To evaluate whether results obtained by both enalapril/enalaprilat assays were comparable, serum samples at the one hour postdose time point after administration of 20 mg enalapril maleate [EnaHexal®] to seven healthy volunteers were measured by both assays. The obtained drug concentrations showed no significant difference for enalapril ($p = 0.30$) according to the performed paired t test (two-tailed p-value). The comparison for enalaprilat revealed a significant difference ($p = 0.02$) which is not remarkable as the new assay is characterised by a lower LLOQ and a more sophisticated sample extraction yielding higher purified samples and less ion suppression. In total, this accounts for a higher sensitivity of the method and allows for a more precise differentiation in the lower range of the calibration curve as this area is commonly effected strongest by ion suppression. The level of significance was α=0.05. Figure 20 shows the direct comparison of the concentrations detected in each sample using both assays.

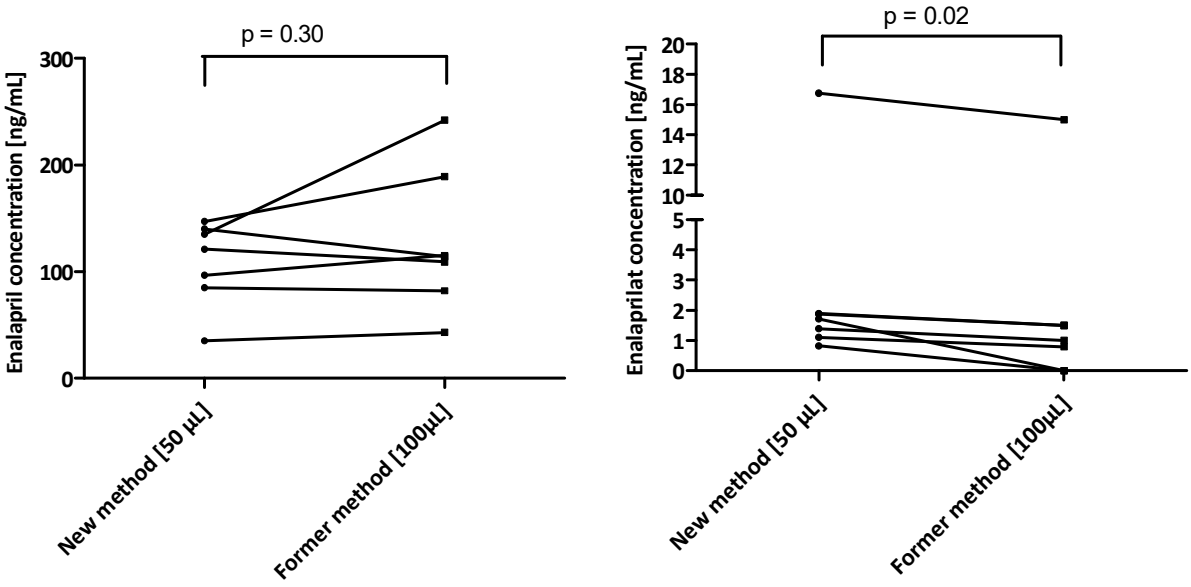

Figure 20 ***Comparison of the two developed assays determining enalapril and enalaprilat in serum****. Both assays were developed at different stages of the thesis and were applied in different studies. The "former method" was used for the proof-of-concept study, while the "new method" was applied in the Phase I study. To evaluate whether results of both methods were comparable, the comparison with statistical analysis utilising a paired t test was conducted. All seven samples were withdrawn from different subjects one hour after administration of 20 mg enalapril maleate and frozen at -80 °C until analysed (n=1 determination per sampling point).*

A proof of applicability of the latest enalapril/enalaprilat method is given using the results of 22 calibration curves which had been used to analyse the serum samples of the bioavailability study of the LENA-project. The enclosed Figure 21 shows the accuracies at all eleven concentration points per calibration curve of enalapril and enalaprilat. Outliers in the calibration curve were rejected prior the final calibration curve was established and the analysis of the analytical run performed. However, all calibration curves conformed to the EMA bioanalytical guideline which stated at least 75 % of all calibration standards (minimum six levels per calibration curve) must comply with the requirements of accuracy (± 15 %; ± 20 % at the LLOQ). During bioanalysis a maximum of one calibration standard needed to be excluded per calibration curve accounting for a maximum of 9 % rejection rate.

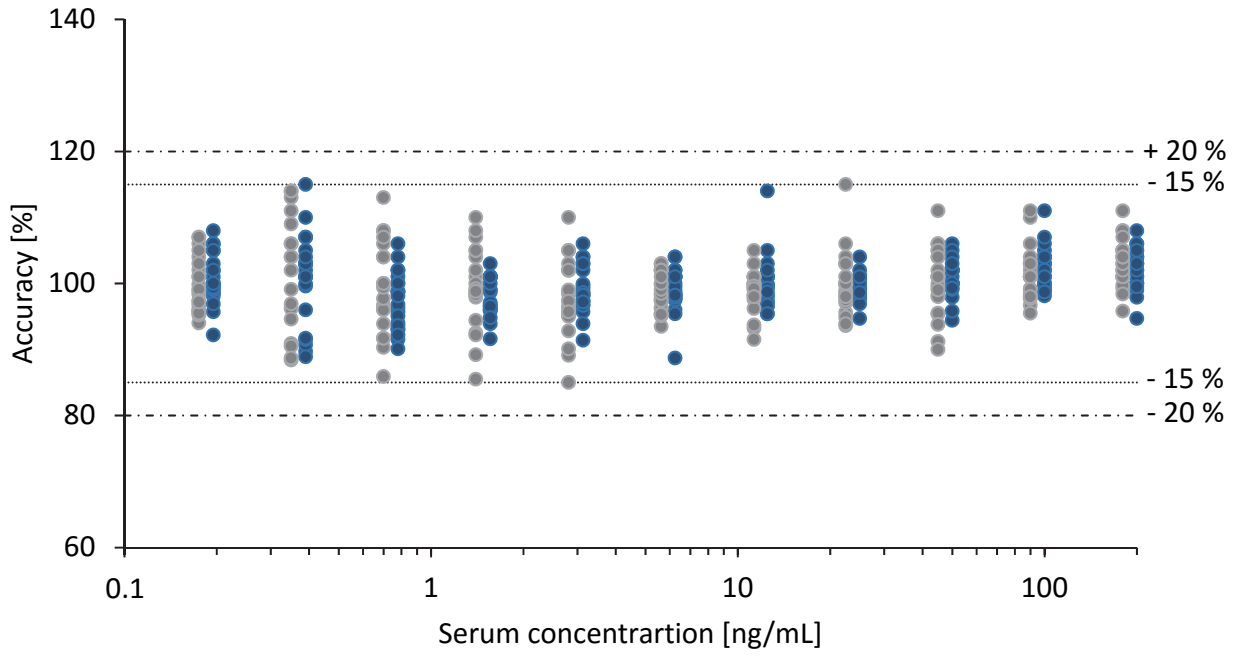

*Figure 21 The plot shows the **accuracy results of 22 calibration curves** (each covering 11 concentration levels per drug substance) of enalapril (blue) and enalaprilat (grey). Indicated are additionally the accuracy thresholds (dashed lines) according to FDA and EMA bioanalytical guidelines for all concentrations levels (±15 %) and the LLOQ (±20 %).*

4.1.1.2 Challenges during method development

This section is intended to present some exemplary challenges during method development prior to the validation steps. Method development of the bioanalytical method for enalapril and enalaprilat was exemplarily chosen.

A peak with the identical transition as enalaprilat with different retention time occurred in the chromatogram (split peak phenomenon) and it was not possible to erase the peak either by thought-out SPE or by any number of different LC gradients (Figure 22). Although the second peak does not significantly influence the performance during method validation, this phenomenon carried the risk of less robustness. This is mostly like to occur if automated integration of the chromatogram is preferred. By modification of the acidified mobile phase with ammonium formiate a stable buffer system was established, but it did not influence the occurrence of the split peak. The checks for contamination in mobile phase, autosampler or solutions for SPE were additionally all negative.

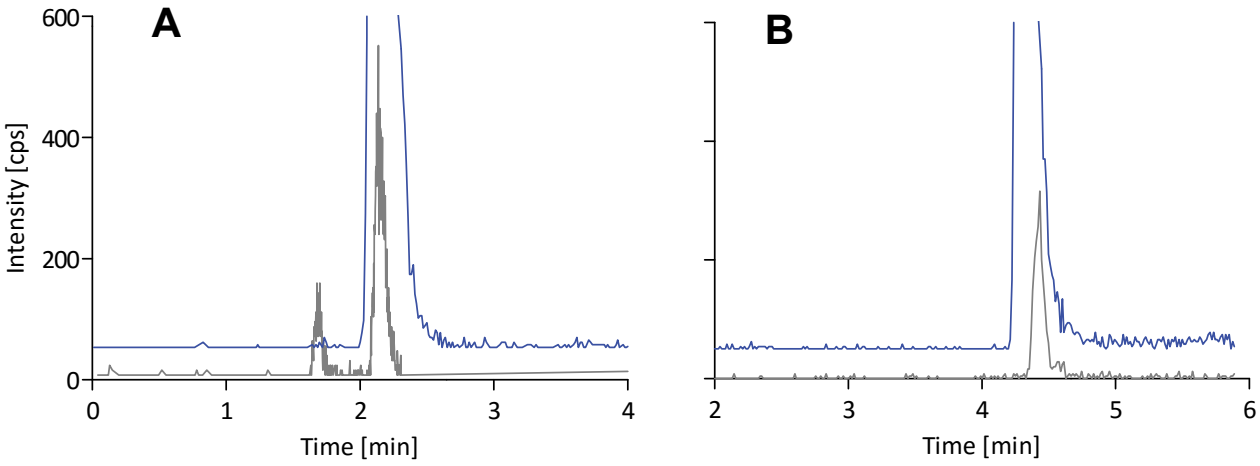

Figure 22 ***Determined split peak in serum*** *with the transition 349.1->206.1 m/z during method development. The split peak - shown in graph A - was measured on several HPLC-columns after SPE purification by Oasis MCX. In grey, the ion count of a low enalaprilat concentration in serum is shown that clearly identifies the split peak. As reference, enalaprilat solved in mobile phase is presented by the blue line (same scaling) without any split peak [base line is nudged to prevent overlap]. Graph B represents the final method without a split peak for enalaprilat. In grey, the ion count of the purified serum sample is shown, while the blue line illustrates again the analyte solved in mobile phase.*

After also scanning for the second most intense transition of enalaprilat (please see Figure 8), clarity was brought to the question as the peak did not belong to enalaprilat. As a quantification with two transitions would result in a higher LLOQ and a smaller calibration range, the sorbent material of the SPE was changed

from cation exchanger to strong anion exchanger and a totally new protocol was developed. This brought success to the method, as the compound with the same transition as enalaprilat (349.3->206.1 m/z) could be detached by SPE and consequently the split peak was removed. This allowed to scan with one transition for enalaprilat which results in a broad calibration range with the low LLOQ of 175 pg/mL. Even though MCX sorbent material was used for sample purification of urine and saliva, this double peak was not previously detectable. Reasons for this might be grounded in the different LLOQ, the amount of sample volume used and the applied gradient during chromatography, but most conceivable is the different composition of the various biological fluids themselves. However, it expounds how diverse the several biological fluids are and emphasises the effort required in method development to reduce the relative matrix effect.

The effectiveness of sample purification by solid-phase extraction was monitored by Q1 scans within the range of 100 – 800 amu. As indicated in the section “Robustness and method development” the degree of the purification of the serum samples containing enalapril, enalaprilat and benazepril depends on the amount and kind of sample preparation solution, wash- and elution solutions, injection volume and the solid-phase extraction phase itself.

With regard to the mass spectrometer it was denoted by flow injection analysis that higher ion spray voltages lead to higher intensities. However, during validation it became obvious that the mass spectrometer was not able to produce robust results with this setting and therefore the high intensity gained was discarded in favour of achieving a more robust method. All other optimal values for the compound-dependent parameters were confirmed during the validation. The attached total ion current (TIC) obtained by flow injection analysis are attached in Figure 23.

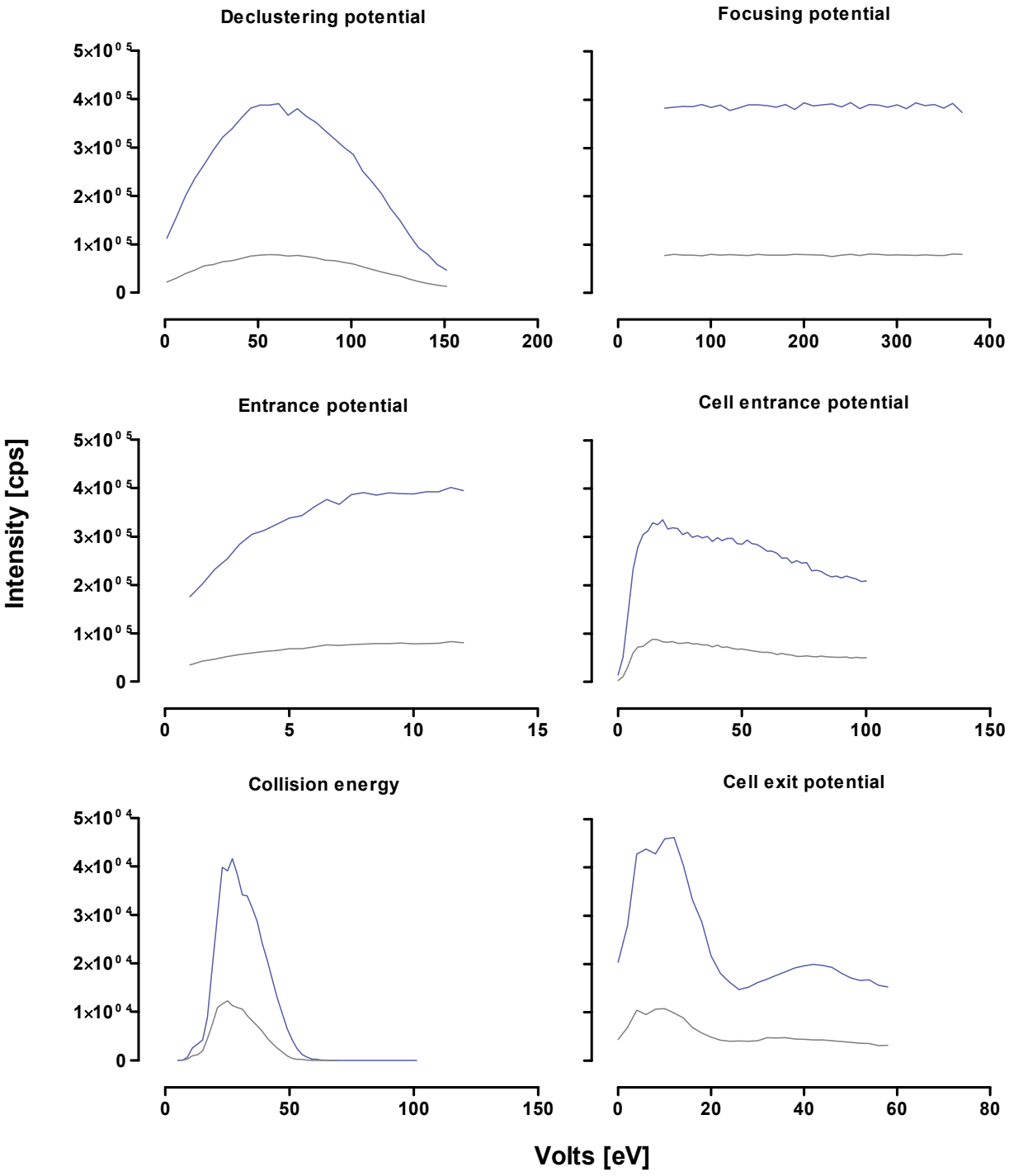

Figure 23 ***Obtained total ion counts*** *(TIC) of compound dependent parameters by flow injection analysis (FIA) of enalapril and enalaprilat. The blue line indicates the intensity of enalapril (377.2->234.2 m/z) and the grey line determines the intensity of enalaprilat (349.1->206.1 m/z) for parameter. Intensity is given as counts per second.*

4.1.2 Urine

4.1.2.1 Simultaneous determination of aliskiren, enalapril and enalaprilat in urine

The method validation was successfully executed according to the in-house validation plan presented in Table 4.

All analytes of interest were baseline separated using HPLC. The retention times were 6.8 minutes for aliskiren, 5.3 minutes for enalapril, 4.6 minutes for enalaprilat and 6.5 minutes for the IS benazepril. The peak shapes were sufficiently symmetric enabling an automated integration. A representative chromatogram is shown in Figure 24.

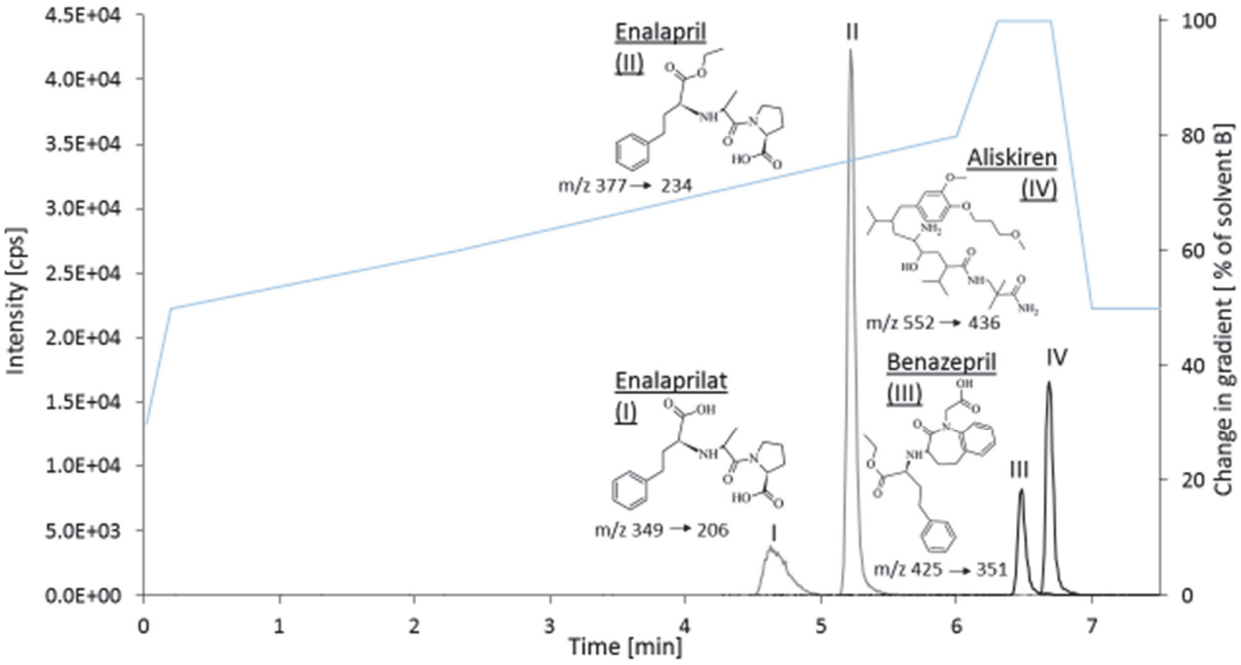

Figure 24 ***Typical chromatogram of all analytes and the internal standard using the urine method.*** *Additionally, chemical structures and the corresponding transitions of the analytes are indicated. The trend of the applied gradient is shown as a line representing the percentage of solvent B (methanol). Enalaprilat is indicated as (I), enalapril shown as (II), aliskiren as (IV) and benazepril as (III).*

Selectivity and cross-talk

The investigation of urine samples containing co-administered drugs proved the selectivity of the developed MS-method. No peaks within ± 0.3 minutes of the retention time of all three analytes were detected in the evaluated urine samples. Additionally, no effect of cross-talk was observed. No interferences in blank samples of all fourteen investigated sources were found and the signal-to-noise

ratios of spiked to blank samples of the different sources were above 5:1. Moreover, investigated urine samples of renally impaired and diabetes patients had no detectable influences on the specific MS-channels of aliskiren, enalapril, enalaprilat, and IS, respectively. Typical multiple reaction monitoring (MRM) chromatograms of blank human urine and QC samples at LLOQ as well as ULOQ are presented in Figure 25.

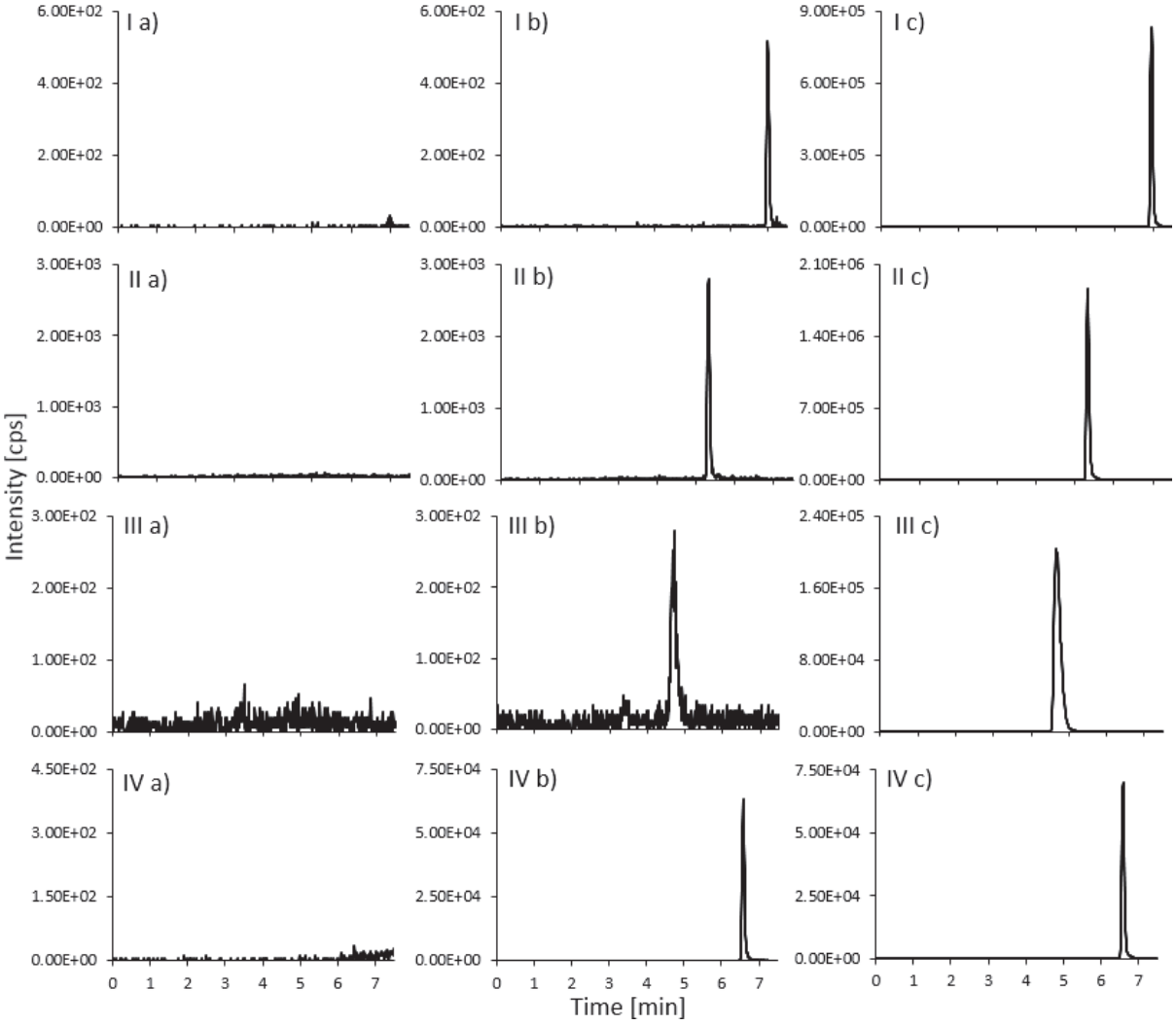

Figure 25 ***MRM chromatograms of analytes and internal standard*** *obtained with the urine method- Blank samples of all analytes of interest (a), at the lower limit of quantification (b) and at the upper limit of quantification (c) are compiled with chromatograms of the internal standard. Aliskiren is illustrated as (I), enalapril as (II), enalaprilat as (III) and benazepril is shown as (IV).*

Calibration curve and Lower Limit of Quantification (LLOQ)

Calibration curves were generated over the range of 9.4 – 9600 ng/mL of aliskiren, 11.6 – 12000 ng/mL of enalapril and 8.8 – 9000 ng/mL of enalaprilat respectively. Best fittings of the calibration curves of all three analytes were gained by quadratic squared regression ($1/x^2$). The accurateness of the calibration curve model was proven by a coefficient of determination (r^2) greater than 0.993 (aliskiren), 0.998 (enalapril) and 0.997 (enalaprilat). The mean regressions for the conducted calibration curves (n=10) were

$y= (2.45*10^{-8} \pm 9.4*10^{-9}) x^2 + (1.09*10^{-3} \pm 9.1*10^{-5}) x + (-3.21*10^{-3} \pm 8.0*10^{-4})$ Aliskiren

$y= (-7.78*10^{-8} \pm 2.1*10^{-8}) x^2 + (3.4*10^{-3} \pm 4.5*10^{-4}) x + (-7.51*10^{-4} \pm 2.8*10^{-3})$ Enalapril

$y= (-1.36*10^{-8} \pm 8.4*10^{-9}) x^2 + (1.05*10^{-3} \pm 1.7*10^{-4}) x + (-1.33*10^{-3} \pm 1.5*10^{-3})$ Enalaprilat.

Additionally, IS-normalised peak areas of the analytes of interest were checked for significant influence through concentration levels utilising ANOVA (SPSS® 22). The calculated coefficient of determination of the quadratic regression model was higher than 0.999 for all analysed drugs (p-value < 0.05). With a linear regression model the r^2 was only 0.988. Figure 26 visualises the different fit of the regression on lower concentration levels.

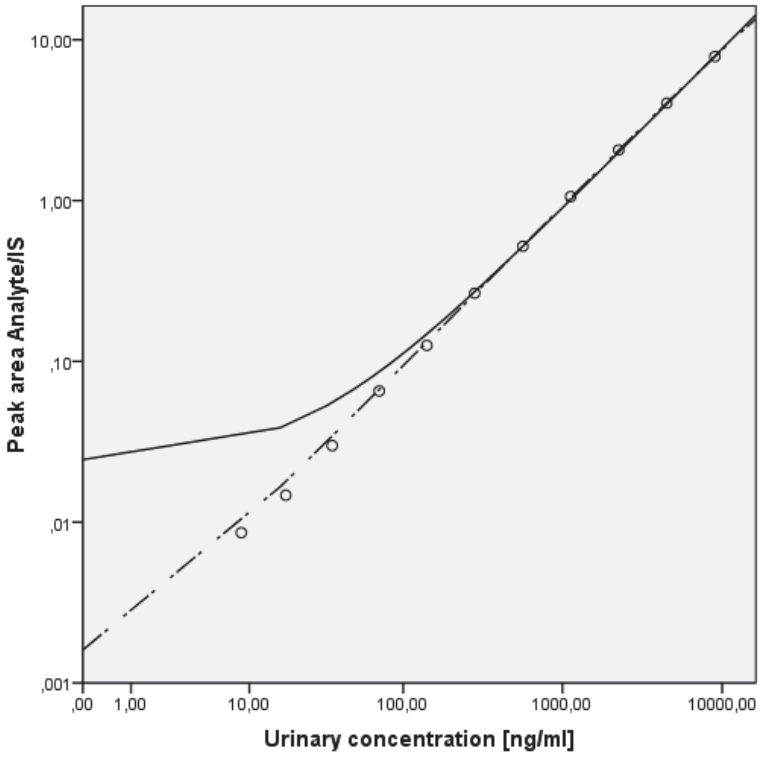

Figure 26 ***Comparison of linear and quadratic regression of the urinary method*** *exemplified for enalaprilat (semi logarithmic plot). Cycles represent observed concentrations; dashed line: weighted quadratic squared regression; black line: weighted linear regression.*

The lower limits of quantification of aliskiren, enalapril and enalaprilat in urine were 9.4, 11.6 and 8.8 ng/mL with values of precision and accuracy within the range of 20 %. The signal-to-noise ratios of all LLOQ samples were above 5:1.

Accuracy and precision

Inter- and intra-day accuracy and precision were investigated on four concentration levels covering the whole concentration range in four runs on four different days. The accuracy of each concentration level was determined by five independent quality control samples. All obtained accuracy values fulfilled the current international guideline requirements (European Medicines Agency 2012b, Food and Drug Administration 2001). Utilising one-way ANOVA, the obtained precision values of aliskiren were 3.2 – 5.8 % for intra-run and 6.1 – 10.3 % for inter-run precision. For enalapril, the precision was 2.4 – 6.1 % (inter) and 3.9 – 7.9 % (intra) and for the active metabolite enalaprilat between 3.1 – 9.4 % (inter) and 4.7 – 12.7 % (intra). Precision and accuracy results are summarised in Table 14.

	Concentration spiked [ng/mL]		Concentration found [ng/mL]	Intra-run R.S.D. [%]	Inter-run R.S.D. [%]	Relative error [%]
Aliskiren	9.4	(LLOQ)	10.1 ± 0.3	5.75	6.10	7.0 ± 3.1
	75		69.6 ± 3.6	4.34	7.10	- 7.2 ± 4.8
	600		646.9 ± 49.4	3.15	7.90	7.8 ± 8.2
	9600	(ULOQ)	9461.0 ± 775	4.60	10.32	-1.5 ± 8.6
Enalapril	11.6	(LLOQ)	11.6 ± 0.6	5.44	7.93	0.0 ± 5.4
	93.5		94.4 ± 1.7	3.72	3.89	1.0 ± 1.8
	750		734.9 ± 33.6	2.37	3.90	- 2.0 ± 4.5
	12000	(ULOQ)	12521.0 ± 396	6.08	6.55	4.3 ± 3.3
Enalaprilat	8.8	(LLOQ)	8.8 ± 0.7	9.38	12.66	0.4 ± 8.2
	70.5		70.5 ± 2.5	4.53	5.78	0.0 ± 3.6
	565		575.1 ± 23.6	3.08	5.49	1.8 ± 4.2
	9000	(ULOQ)	8935.3 ± 259	3.60	4.66	- 0.7 ± 2.9

Table 14 ***Accuracy and precision of all three analytes of interest of the urinary method*** *– Data compiled as mean ± standard deviation (S.D.) or relative standard deviation (R.S.D.). Reported results base on n=5 determinations of individual prepared samples per concentration level.*

Matrix effect and recovery

The absolute matrix effect was investigated at four concentration levels over the whole calibration range of aliskiren, enalapril and enalaprilat whereby the matrix led to ionisation suppression. The two-step SPE enabled a marked reduction of the absolute mean matrix effect in urine samples which was calculated to be between -1.3 to -11.5 % for aliskiren, -1.9 to -4.9 % for enalapril, and -0.8 to -3.9 % for enalaprilat. The matrix effect of benazepril was calculated to be -6.56 $\pm$ 2.5 % (mean $\pm$ SD) based on five measurements and also indicated ion suppression. Further information on absolute matrix effect is available in Table 16. Based on current state of the art and with regard to the fact that the urine method will be applied to a broad spectrum of patients with different diseases and of different age (e.g. within the LENA project), the relative matrix effect was studied intensively on sixteen different sources at the upper and lower LOQ. The investigated patients represent an age range of 2 – 90 years, covering both genders, different diets, drug intake and diseases known to influence the urine composition like diabetes or renal impairment. The intersubject comparison of alteration or interference caused by the presence of other compounds in urine was calculated by the coefficient of variation (CV).

During method development the effect of a two-step solid-phase extraction on the relative matrix effect was evaluated. Nine different sources were extracted by one-step (MCX) or two-step (WAX + MCX) extraction and the obtained internal standard normalised relative matrix effects for aliskiren, enalapril and enalaprilat determined. The conducted statistical analysis by Mann-Whitney-test indicated a significant difference ($p < 0.05$) for enalapril and enalaprilat. The use of the two-step extraction showed reduced matrix effects and indicated a higher degree of sample purification. The result for aliskiren was not significant ($p = 0.06$). In summary, the results suggest a better purification by the two-step solid-phase extraction if compared to the single purification procedures. Details are arranged in Figure 27.

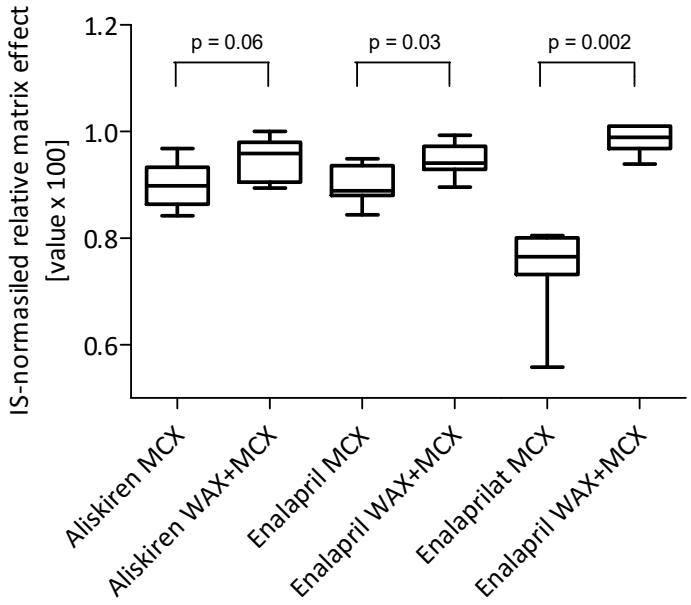

Figure 27 ***Comparison of one-step and two-step SPE extraction*** *on the internal standard normalised relative matrix effect of aliskiren, enalapril and enalaprilat in urine. The solid-phase extraction by Oasis® MCX is compared with the two step extraction using Oasis® WAX+MCX. Each box plots indicate the median, 25th, 75th percentile plus minimum and maximum values as whiskers. N=9 measurements per boxplots. Statistical analysis is done by Mann-Whitney-test (two-tailed p-value). MCX: mixed-mode cation exchanger; WAX: weak anion exchanger; IS: internal standard.*

By applying the final two-step extraction the following results were obtained: At the LLOQ the CV was 5.70 %, 4.04 % and 6.62 % for aliskiren, enalapril and enalaprilat, respectively. A CV of 1.73 % for aliskiren, 1.26 % for enalapril and 1.25 % for enalaprilat was calculated at the ULOQ and was therefore well within the EMA requirements of 15 % (European Medicines Agency 2012b). Details on the IS-normalised relative matrix effect are compiled in Table 15. The encountered high variability of urine compositions as well as the marked drop in total ion current between unprocessed and processed blank urine samples by the established purification process can be seen in Figure 28. Irrelevant substances, metabolites, residuals and other interfering urine ingredients were either excluded by the effective purification or do not affect the determination of all three analytes of interest based on the chosen chromatographic conditions.

				Aliskiren		Enalapril		Enalaprilat	
Donor			Concentration	9.4 ng/mL	9600 ng/mL	11.6 ng/mL	12000 ng/mL	8.8 ng/mL	9000 ng/mL
Healthy adults	26 – 60 years old	♀	Donor 1	na	101.6	na	100.6	na	101.2
		♂	Donor 2	na	101.2	na	102.1	na	103.2
		♂	Donor 3	107.2	100.6	105.6	100.8	108.5	103.1
		♂	Donor 4	103.7	101.3	108.1	102.8	110.5	103.0
		♂	Donor 5	99.2	101.5	98.4	100.1	99.5	102.3
		♀	Donor 6	107.3	102.3	110.7	103.2	124.8	102.7
		♀	Donor 7	117.3	102.3	112.8	102.1	116.5	100.1
Elderly	DM	♂	Donor 8	106.7	103.6	98.3	101.0	111.7	100.6
	H	♀	Donor 9	106.9	105.0	106.0	103.1	94.4	103.1
	RI	♀	Donor 10	106.2	102.5	100.2	101.7	105.5	102.0
Children/adolescents	6 y	♂	Donor 11	109.2	101.4	103.2	101.0	106.7	103.1
	4 y	♂	Donor 12	103.7	100.3	102.7	101.1	109.1	102.8
	6 y	♀	Donor 13	109.2	100.4	102.9	101.9	102.1	104.5
	9 y	♂	Donor 14	107.0	97.0	100.5	103.8	104.8	104.4
	12 y	♂	Donor 15	105.3	102.3	103.2	104.6	103.5	104.9
	2 y, RIN	♂	Donor 16	88.7	104.1	106.8	103.9	110.2	104.5
Mean value of normalised relative ME ± S.D. [%]				105.5 ± 6.0	101.7 ± 1.8	104.3 ± 4.2	102.0 ± 1.3	107.7 ± 7.1	102.9 ± 1.3
CV (%) within donors				5.70	1.73	4.04	1.26	6.62	1.25

Table 15 ***IS-normalised relative matrix effect in urine samples of sixteen subjects*** *– Abbreviations: ME, matrix effect; CV, coefficient of variation; S.D., standard deviation; DM, diabetes mellitus; H, healthy; RI, renally impaired; RIN, renal infection; y, years; na, not applicable*

Recoveries of the first SPE only (WAX) and of the combined SPE (WAX + MCX) was determined. After applying the anion exchanger WAX (First SPE step only), the recoveries of aliskiren ranged from 74.4 – 88.0 %, 96.5 – 103.8 % for enalapril and 81.3 – 94.0 % for enalaprilat. The recoveries of aliskiren ranged for the combined SPE from 63.5 to 87.4 %. For enalapril and enalaprilat, the range was between 91.7 to 108.8 % and 66.7 to 91.0 % respectively. Process efficiencies of the solid-phase extraction led to values between 56.3 to 85.4 % (aliskiren), 87.2 to 106.8 % (enalapril) and 64.1 to 90.2 % (enalaprilat). Recovery of the internal standard was 89.1 ± 2.9 %. Details are summarised in Table 16.

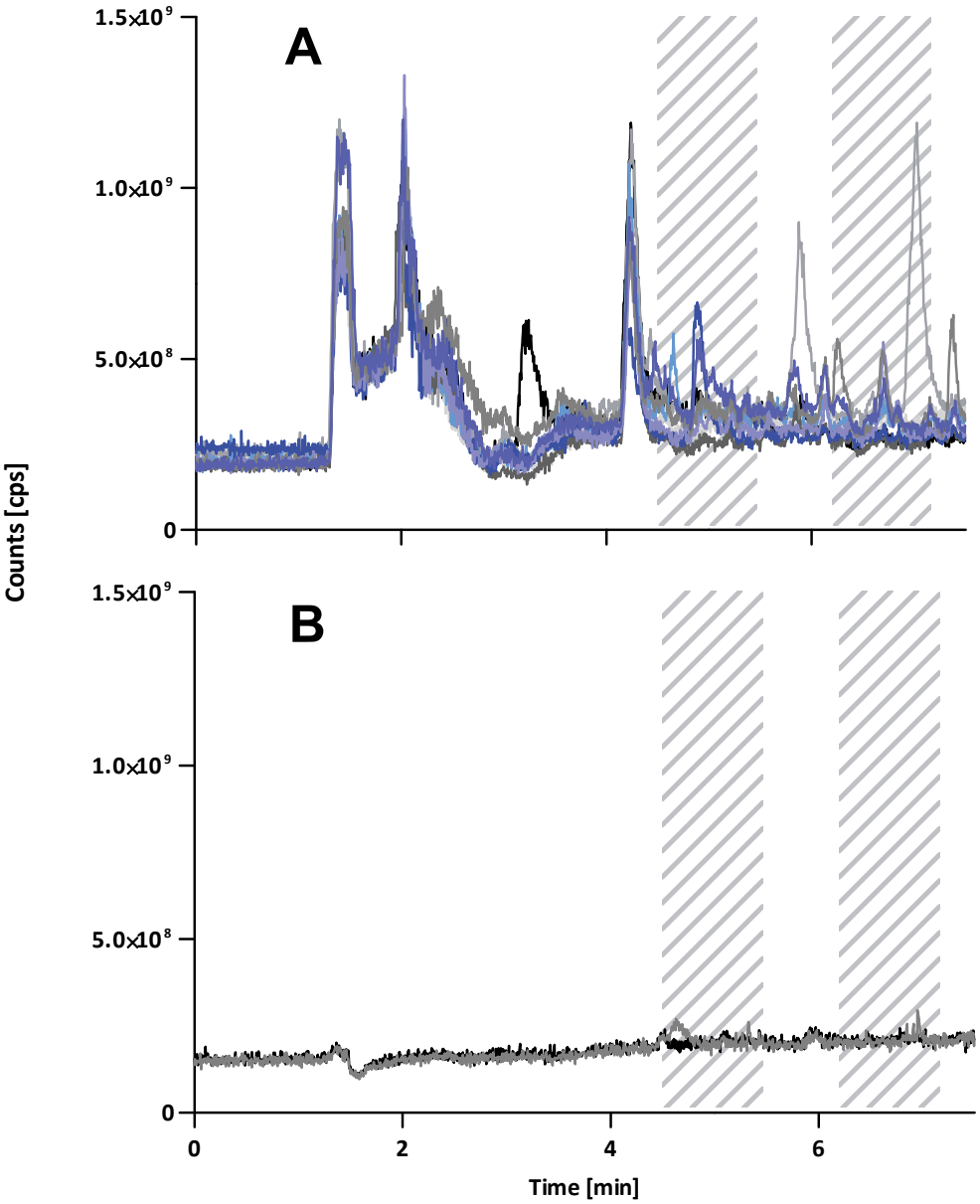

Figure 28 ***Comparison of unprocessed vs processed urinary samples****. Graph A: Q1 scans of 12 different unprocessed urine samples obtained with the API 2000 within the range of 100 – 800 m/z to illustrate the variable matrix amongst the sources. Graph B shows exemplary processed urine samples. In both graphs the retention times of aliskiren, enalapril, enalaprilat and benazepril are indicated by grey patterned fills. Matrix which peaks at this time interferes with the analytes and is responsible for ion suppression. By comparing graph A and B, the gained reduction of matrix by the two-step solid-phase extraction gets visible.*

	Aliskiren				Enalapril				Enalaprilat			
Concentration [ng/mL]	9.4	75	600	9600	11.6	93.5	750	12000	8.8	70.5	565	9000
Mean recovery of first SPE-step only ± S.D. [%]	74.4 ± 5.5	76.6 ± 2.1	81.7 ± 0.2	88.0 ± 0.2	98.3 ± 0.0	99.1 ± 0.7	96.5 ± 1.0	103.8 ± 4.4	94.0 ± 2.4	82.3 ± 1.3	81.3 ± 0.9	92.4 ± 2.8
Mean recovery ± S.D. [%]	63.5 ± 4.1	79.2 ± 1.9	87.4 ± 5.6	71.4 ± 3.2	91.7 ± 9.4	92.8 ± 1.8	92.6 ± 2.2	108.8 ± 1.5	69.2 ± 2.9	73.8 ± 0.0	66.7 ± 5.9	91.0 ± 0.3
Absolute matrix effect ± S.D. [%]	-11.5 ± 7.0	-4.8 ± 6.2	-2.3 ± 5.1	-1.3 ± 0.9	-4.9 ± 2.6	-2.9 ± 2.8	-3.8 ± 6.0	-1.9 ± 0.9	- 2.0 ± 1.8	-1.2 ± 5.7	-3.9 ± 7.3	-0.8 ± 1.2
Mean process efficiency [%]	56.3	75.4	85.4	70.5	87.2	90.1	89.0	106.8	67.8	72.9	64.1	90.2

Table 16 ***Recovery, matrix effect and process efficiency of the urinary method*** *– Data compiled as mean or mean ± standard deviation (S.D.). N=5 determinations per concentration level.*

Stability

Stock solutions of aliskiren, enalapril, enalaprilat and benazepril stored in the fridge were stable for six months. Results of the latter are given in section 4.1.1 (aliskiren) and by Ramusovic *et al.* (enalapril/enalaprilat) (Ramusovic et al. 2012). Peak areas of the analytes at five different concentration levels stored for one month at -20 °C were comparable to those obtained from the freshly prepared calibration curve. Also stored at -20 °C and without any outlier were the post-extraction stability as well as freeze and thaw stability. Results of the discontinuation and post-preparative stability indicated stable quality control samples if compared to freshly prepared calibration curves at four concentration levels over the whole calibration range. Mean accuracies of the processed short-term stability were 106-112 % for aliskiren, 93-101 % for enalapril and 95-100 % for enalaprilat confirming these conditions as stable. Additionally, the successfully conducted 'unprocessed short-term' stability proved the suitability for routine analysis. Details are summarised in Table 17 and Table 18.

	Aliskiren				Enalapril				Enalaprilat			
	Concentration spiked [ng/mL]											
	9.4	75	600	9600	11.6	93.5	750	12000	8.8	70.5	565	9000
	Mean concentration found [ng/mL] (n=3)*											
Short-term unprocessed (21 °C)	10.0 (3.2)	64.1 (0.4)	555 (1.3)	8730 (0.4)	10.0 (1.0)	87.4 (0.7)	653 (0.9)	10533 (0.5)	8.8 (7.1)	68.0 (0.5)	514 (1.3)	8403 (0.4)
Short-term processed (7 °C)	10.0 (4.3)	83.6 (1.4)	674 (1.3)	9744 (1.5)	11.7 (3.6)	88.8 (1.6)	694 (2.1)	11540 (3.8)	8.8 (7.1)	68.3 (2.7)	547 (2.9)	8534 (2.9)
Discontinuation stability (7 °C)	9.5 (7.2)	66.5 (2.3)	622 (2.1)	10067 (0.5)	9.5 (2.0)	79.8 (0.3)	666 (1.2)	11333 (1.7)	8.2 (4.2)	62.6 (2.2)	535 (1.2)	9010 (1.7)
Post-preparative (21 °C)	8.5 (3.5)	67.0 (3.4)	601 (1.5)	8524 (4.3)	9.9 (4.3)	89.0 (1.1)	734 (2.4)	12800 (6.5)	8.4 (2.1)	64.5 (4.4)	555 (4.2)	9124 (5.2)

Table 17 ***Stability results (a) of all analytes of interest of the urinary method*** *- * values are presented as mean concentration with the corresponding CV [%] in brackets. All results were within the required accuracy of ±15 % of the nominal concentration (±20 % at the LLOQ). N=3 determinations per concentration level.*

	Aliskiren					Enalapril					Enalaprilat				
	Concentration spiked [ng/mL]														
	9.4	37.5	150	1200	9600	11.6	46.5	186	1500	12000	8.8	35.3	141	1130	9000
	Mean concentration found [ng/mL] (n=3)*														
Post-extraction (-20 °C)	11.1 (4.9)	32.2 (3.2)	147 (1.8)	1270 (2.1)	9960 (1.3)	12.0 (3.3)	49.3 (1.7)	193 (1.8)	1520 (0.9)	12600 (1.1)	8.9 (4.4)	35.3 (3.5)	148 (1.4)	1180 (2.0)	9460 (1.3)
1 month long-term (-20 °C)	9.9 (3.9)	32.6 (1.9)	141 (3.8)	1217 (0.8)	9090 (0.3)	11.0 (4.9)	42.3 (2.7)	167 (2.2)	1303 (1.4)	11733 (1.5)	8.6 (5.9)	32.4 (0.9)	134 (2.8)	1083 (2.0)	7800 (1.0)
Freeze-Thaw (-20 °C/ 20 °C)	10.7 (5.2)	34.6 (6.9)	154 (5.3)	1357 (5.4)	10300 (0.8)	11.7 (0.4)	48.7 (4.2)	190 (1.1)	1460 (0.0)	12433 (4.3)	9.0 (9.2)	39.2 (5.8)	145 (2.1)	1113 (1.1)	9138 (2.3)

Table 18 ***Stability results (b) of all analytes of interest of the urinary method*** *- * values are presented as mean concentration with the corresponding CV [%] in brackets. All results were within the required accuracy of ±15 % of the nominal concentration (±20 % at the LLOQ). N=3 determinations per concentration level.*

Carry over

No carry over into a sample with pure mobile phase right after a sample at the ULOQ was detected by injection of 2 µL of the sample.

Application

The method was applied to obtain the urine concentration-time as well as the excreted amount-time profiles of aliskiren hemifumarate (300 mg) and enalapril maleate (20 mg) in healthy male and female volunteers after single oral administration. The main evaluated pharmacokinetic parameters were (1) amount excreted into the urine (A*e*) within 5 elimination half-lives and (2) renal clearance (CL_R). For aliskiren, Ae was 3.57 mg within 192 h. Renal clearance was calculated as excreted amount into urine divided by the serum area under the curve and was 1.41 $L*h^{-1}$. Regarding enalapril and enalaprilat, the amount excreted into the urine was 3.84 mg and 5.44 mg, respectively, within 72 h. It results in a calculated renal clearance of 10.31 $L*h^{-1}$ and 8.68 $L*h^{-1}$ respectively. Corresponding urine profiles and the comparison to obtained serum concentrations are compiled in Figure 29.

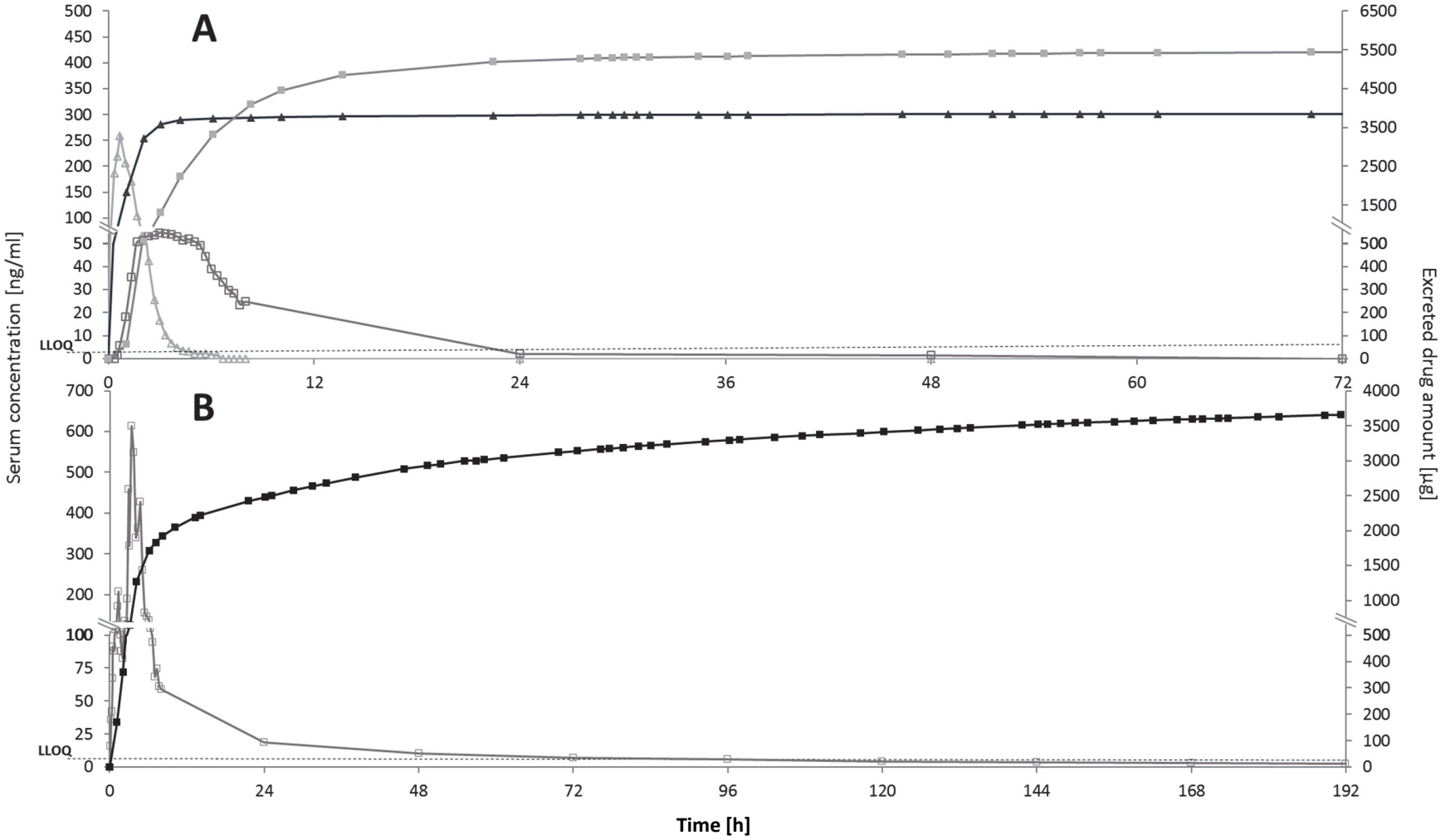

Figure 29 ***Obtained excreted drug amount-time profiles of aliskiren and enalapril*** *- Excreted amount-time profiles of enalapril (Δ) and enalaprilat (□) (20 mg, orally administered) [A] as well as aliskiren (□) (300 mg, orally administered) [B] of healthy volunteers (n=1 each). Solid symbols represent the cumulated amount of the drug in urine and unfilled ones show the obtained serum concentrations for each drug. LLOQ: lower limit of quantification of serum method.*

Drug concentrations in urine were also investigated within the Phase I study of LENA. As indicated above, no details can be given regarding the results of the study since it falls under the property rights of the LENA consortium. However, the obtained calibration curves during the bioanalysis proved the applicability of the developed assay also for the high throughput approach. The enclosed Figure 30 shows the accuracy at all eleven concentration points per calibration curve of enalapril and enalaprilat. Obtained outliers were rejected according to guideline requirements and the calibration curve was evaluated without them (including regression analysis) prior to conduction of sample evaluation. The maximum amount of outliers (maximum 2 outliers per calibration curve ≙ < 25 % of all calibration standards per calibration curve) as well as the determined accuracy values comply with EMA and FDA guidelines.

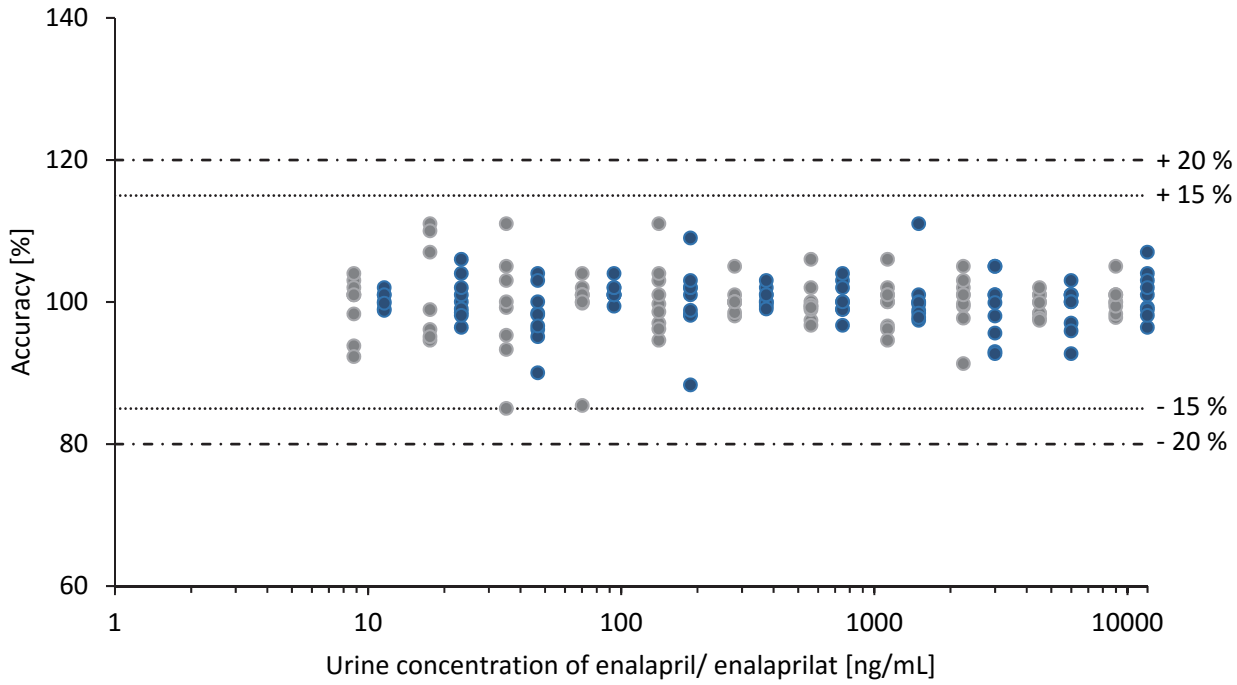

*Figure 30 The plot shows the **accuracy results of 7 calibration curves** (each covering 11 concentration levels per drug substance) of enalapril (blue) and enalaprilat (grey) obtained during analysis of urinary samples of the Phase I-study of LENA. In addition, the accuracy thresholds according to FDA and EMA bioanalytical guideline for all concentrations levels (±15 %) and the LLOQ (±20 %) are also indicated.*

4.1.3 Saliva

4.1.3.1 Determination of aliskiren and enalapril in saliva by HPLC-MS/MS

Aliskiren and benazepril were baseline separated by the chosen chromatographic conditions. The retention times were 6.8 minutes for aliskiren and 6.5 minutes for the IS benazepril. The salivary method for enalapril and enalaprilat obtained retention times of 5.1 minutes for enalapril and 4.6 minutes for enalaprilat, again utilising benazepril as the internal standard. Good results in terms of symmetric peak shape, resolution and retention time were obtained in both methods allowing for an automated integration. A representative chromatogram of the salivary method of aliskiren is shown in Figure 31.

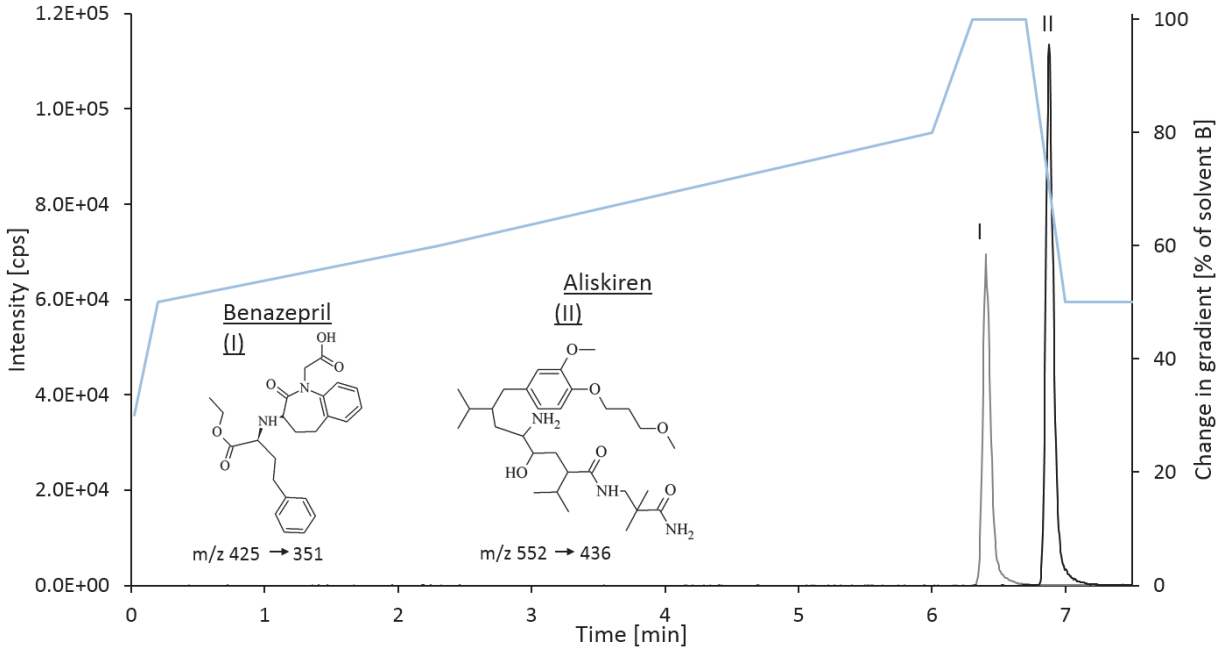

Figure 31 ***Typical chromatogram of aliskiren and the internal standard*** *determined by the salivary LC-MS/MS method. In addition, chemical structures and the corresponding transitions of the analytes are indicated next to the peak. The trend of the applied gradient is shown as a line representing the percentage of solvent B (methanol).*

In the following sections, only validation results of the aliskiren method are summarised because the salivary method for enalapril and enalaprilat was fully developed but not yet validated.

Selectivity and cross-talk

Typical multiple reaction monitoring (MRM) chromatograms of blank saliva and QC sample spiked with aliskiren and benazepril are compiled in Figure 32 and proved the selectivity of the developed method. Additionally, no effects of cross-talk and no interferences in blank samples in all six sources were observed.

No peaks within ± 0.5 minutes of the retention times of the analytes were detected in the evaluated saliva samples with concomitant drugs.

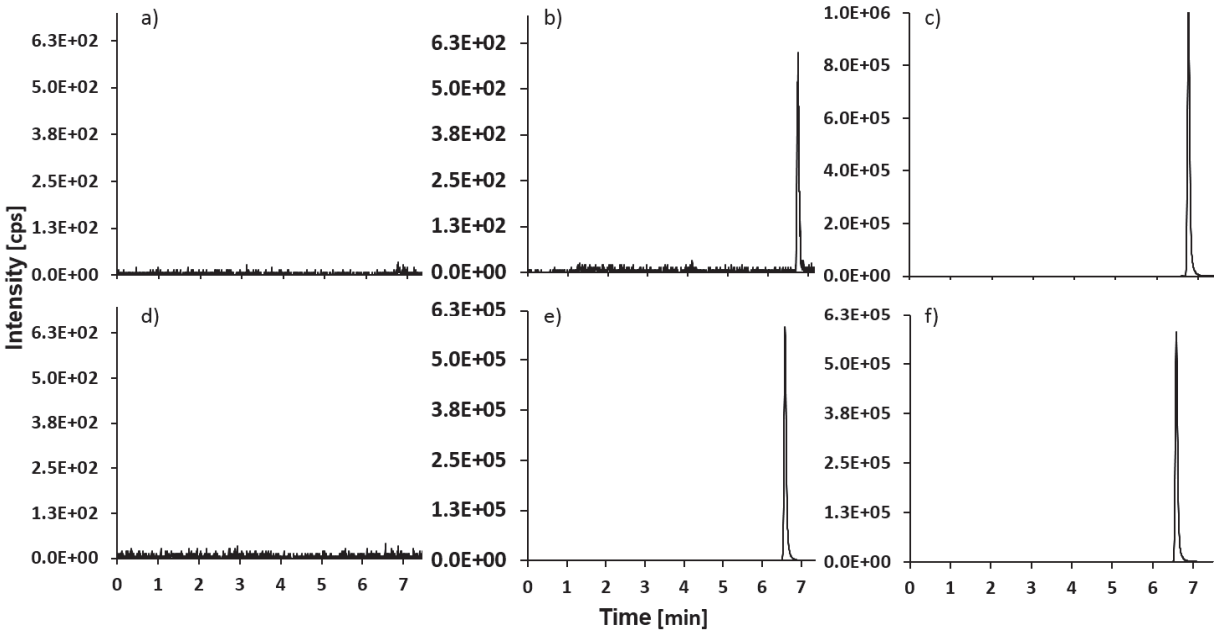

Figure 32 ***MRM chromatogram of analyte and internal standard*** *of purified salivary samples – MRM chromatograms of aliskiren of a blank sample a), at the lower limit of quantification b) and at the upper limit of quantification c) are compiled with chromatograms of the internal standard d)-f). All chromatograms were obtained through the salivary method.*

Linearity and Lower Limit of Quantification (LLOQ)

Calibration curves were constructed with twelve standards within the range of 0.586 – 1200 ng/mL of aliskiren. Best fitting of the curves was obtained by linear regression with $1/x^2$ weighting. The obtained coefficient of determination (r^2) was greater than 0.99 with a mean regression of

$y = (1.68*10^{-3} \pm 5.74*10^{-4})\ x + (-8.58*10^{-5} \pm 2.51*10^{-4})$ obtained by 11 measured calibration curves. The mean values of the LLOQ of aliskiren at 0.586 ng/mL of all sources were within the guideline limits of 20 % for accuracy and precision. The signal-to-noise ratios of all LLOQ samples were above 5:1.

Accuracy and precision

Inter- and intra-day accuracy and precision were investigated over four concentration levels covering the whole concentration range in three runs on three different days. All obtained accuracy values fulfilled the current international guideline requirements (European Medicines Agency 2012b, Food and Drug Administration 2001). Utilising one-way ANOVA, the obtained precision values of aliskiren were 3.8 – 8.1 % for intra-run and 3.4 – 8.9 % for inter-run precision. Precision and accuracy results are summarised in Table 19.

Concentration spiked [ng/mL]		Concentration found [ng/mL]	Intra-run R.S.D. [%]	Inter-run R.S.D. [%]	Relative error [%]
0.586	(LLOQ)	0.56 ± 0.04	6.50	6.66	-4.4 ± 6.9
4.69		4.23 ± 0.19	4.00	4.76	- 9.8 ± 4.1
75		74.0 ± 6.21	8.08	8.89	- 1.3 ± 8.3
1200	(ULOQ)	1338 ± 45.81	3.83	3.43	11.5 ± 3.8

Table 19 ***Accuracy and precision of aliskiren determination in saliva*** *– Data compiled as mean ± standard deviation (S.D.) or relative standard deviation (R.S.D.) n=5 per concentration level.*

Matrix effect and recovery

Ion suppression caused by the residual matrix was detected for both, aliskiren and benazepril (IS). The absolute matrix effect at four concentration levels ranged between -2.9 to -11.6 % for aliskiren. The matrix effect of benazepril was calculated to be -4.9 ± 2.2 % based on five measurements. Further information on absolute matrix effect is available in Table 21. The relative matrix effect was determined by investigating six different sources of human saliva spiked with aliskiren at the LLOQ and ULOQ.

			Aliskiren	
		Concentration	0.586 ng/mL	1200 ng/mL
Donor				
Healthy adults 25 – 46 years old	♀	Donor 1	116.4	92.6
	♂	Donor 2	93.4	96.4
	♂	Donor 3	94.8	93.5
	♂	Donor 4	99.0	93.0
	♂	Donor 5	97.8	91.5
	♀	Donor 6	110.0	92.8
Mean value of normalised relative ME ± S.D.			101.9 ± 9.2	93.3 ± 1.5
CV (%) within donors			9.0	1.63

Table 20 ***Internal standard normalised relative matrix effect of the salivary method*** *for aliskiren – Abbreviations: ME: matrix effect; CV: coefficient of variation; S.D.: standard deviation*

The inter-subject comparison of alteration or interference caused by the presence of other compounds in saliva was calculated using the coefficient of variation (CV). At the LLOQ the CV was 9.0 % while a CV of 1.63 % was calculated for aliskiren at the ULOQ and therefore well within the EMA requirements of 15 %. Details on the IS-normalised relative matrix effect are compiled in Table 20. The established effective SPE purification in combination with the chromatographic separation reduced any interaction by irrelevant substances, metabolites, residuals and other interfering ingredients in saliva to a minimum which did not affect the determination by LC-MS/MS.

The recovery of aliskiren ranged for the conducted SPE from 56.2 to 70.1 %. Process efficiencies of the solid-phase extraction led to values between 54.5 to 62.0 %. Recovery of the internal standard was 79.9 ± 1.8 %. Details are summarised in Table 21.

	Aliskiren			Benazepril
Concentration [ng/mL]	0.586	37.5	1200	
Mean recovery ± S.D. [%]	60.2 ± 6.4	56.2 ± 7.0	70.1 ± 5.0	79.9 ± 1.8
Absolute matrix effect ± S.D. [%]	-2.9 ± 1.5	-2.9 ± 1.2	-11.6 ± 3.8	-4.9 ± 2.2
Mean process efficiency [%]	58.4	54.5	62.0	76.0

Table 21 ***Recovery, matrix effect and process efficiency of aliskiren and benazepril (IS)*** *- Data compiled as mean or mean ± standard deviation (S.D.). N=5 determinations per concentration level.*

Stability

Stock solutions of aliskiren and benazepril stored in the fridge were stable for six months as shown in section 4.1.1. Comparable stability results were obtained for quality control samples stored for one month at -20 °C at five concentration levels against a freshly prepared calibration curve. Furthermore, dry samples stored at -20 °C as well as samples stored in the autosampler (post-preparative stability) were stable within the investigated period of 24 hours. Stability of the saliva samples kept at room temperature for 24 h were successfully assessed against a freshly prepared calibration curve. Details are summarised in Table 22.

	Aliskiren			
	Concentration spiked [ng/mL]			
	0.586	9.4	150	1200
	Mean concentration found [ng/mL] (n=3)*			
Short-term (21 °C)	0.683 ± 0.04	8.8 ± 0.2	133 ± 3.9	1101 ± 97
1 month long-term (-20 °C)	0.525 ± 0.02	8.7 ± 0.3	139 ± 3.9	1049 ± 45
Post-extraction (-20 °C)	0.645 ± 0.03	8.8 ± 0.5	143 ± 11	1193 ± 33
Post-preparative (21 °C)	0.584 ± 0.03	8.8 ± 0.1	169.5 ± 2	1290 ± 10

Table 22 ***Stability results of aliskiren in saliva*** *– * values are presented as mean concentration with the corresponding standard deviation. All results were within the required accuracy of ± 15 % of nominal concentration (±20 % at the LLOQ).*

Application

The serum and saliva concentrations were determined in three healthy volunteers after oral administration of 300 mg aliskiren (Rasilez®). For pharmacokinetic assessment, C_{max}, t_{max}. $AUC_{0\text{-}4.9h}$, $AUC_{0\text{-}192h}$, $ke_{0\text{-}5h}$, $ke_{0\text{-}192h}$ and corresponding $t_{½}$ were compared in both fluids.

First, with regard to the huge difference in concentration levels between both fluids the detected C_{max} distinguished highly (e.g. C_{max}: 21 ng/mL in saliva vs 1840 ng/mL in serum). The C_{max} in saliva was about 0.5 - 1.2 % of the one in serum. Due to the high difference in C_{max} of both fluids, the saliva swabs used for the collection were eluated belatedly by a mixture of ammonium in acetonitrile/methanol to ensure that the drug did not stick in the swab. No additional drug substance was obtained by this approach which denotes that the low concentrations are based in the drug and its distribution.

Second, as a result of the latter, the calculated AUC between serum and saliva was also highly different. The observed ratio in $AUC_{(0\text{-}4.9h)}$ of saliva and serum varied between 0.8 – 1 %. The $AUC_{(0\text{-}192h)}$ in saliva was between 8 to 19 % of the one in serum. Third, obtained t_{max} values in saliva and serum were similar. The known high inter-individual variability of C_{max} and AUC was detected in both fluids. The shapes of saliva concentration-time profiles were comparable to the ones obtained in serum within the first five hours only. The correlation between aliskiren concentration in saliva and serum was analysed in the time frame up to five hours and 0 to 192 h after administration. Within the first five hours a good correlation of r=0.94 was gained while for the whole observation period a coefficient of determination of only 0.38 was calculated.

Moreover, the elimination rate constants of 0-4.9 h and 0-192 h were evaluated in both fluids by non-compartmental analysis. When comparing the ratio of the obtained k*e* between 0-4.9 h it only differed

between 0.84 – 1.03 and consequently supported the above-mentioned assumption that the pharmacokinetic behaviour of aliskiren is similar in both fluids within the first hours. The ratio in k*e* between saliva and serum for the time frame of 0-192 h ranged from 0.37 – 1.89. The calculated $t_{½}$ confirmed the findings.

Furthermore, the absorption rate in both fluids was estimated to add further credibility to the undertaken distinction of the profiles rather than sole visual inspection. Nevertheless, the reported k*a* values were for the comparison of both fluids only. The ratio in k*a* values for the double peak differed between 0.72 and 1.12 and confirmed the comparability within the first hours after administration. Corresponding concentration-time profiles in saliva and serum are compiled in Figure 33. Evaluated pharmacokinetic parameters in serum and saliva are summarised in Table 23.

The investigations on the most suitable salivary sample collection system showed, that the Salivettes® from Sarstedt were more convenient for the volunteers. It was easier to untrained persons to chew the cotton of the Salivettes ® and collect the required amount of saliva per sampling point than to wipe or skim an appropriate amount (> 200 µL) of the saliva by the Omniswaps®. The evaluation was done in 7 different persons.

Pharmacokinetic Parameter	Serum	Saliva	Ratio
Volunteer no.1			
C_{max}	1840 ng/mL	21.25 ng/mL	0.012
t_{max}	2 h	2 h	1
$ke_{0\text{-}4.9\ h}$	0.8500 h^{-1}	1.2191 h^{-1}	1.43
$ke_{0\text{-}192\ h}$	0.0107 h^{-1}	0.0080 h^{-1}	0.75
ka_{1}	5.8335 h^{-1}	6.1452 h^{-1}	1.05
ka_{2}	5.028 h^{-1}	5.6151 h^{-1}	1.12
$AUC_{0\text{-}4.9\ h}$	2297 ng*h/mL	23 ng*h/mL	0.01
$AUC_{0\text{-}192\ h}$	5448 ng*h/mL	968 ng*h/mL	0.18
Volunteer no.2			
C_{max}	564 ng/mL	2.9 ng/mL	0.005
t_{max}	2.33 h	2.68 h	1.15
$ke_{0\text{-}4.9\ h}$	1.3911 h^{-1}	1.2912 h^{-1}	0.93
$ke_{0\text{-}192\ h}$	0.0055 h^{-1}	0.0090 h^{-1}	1.64
ka_{1}	3.9144 h^{-1}	3.5223 h^{-1}	0.90
ka_{2}	4.9093 h^{-1}	3.5372 h^{-1}	0.72
$AUC_{0\text{-}4.9\ h}$	723 ng*h/mL	6 ng*h/mL	0.008
$AUC_{0\text{-}192\ h}$	1286 ng*h/mL	118 ng*h/mL	0.09
Volunteer no.3			
C_{max}	1290 ng/mL	10.8 ng/mL	0.008
t_{max}	4 h	4 h	1
$ke_{0\text{-}4.9\ h}$	2.6742 h^{-1}	2.2499 h^{-1}	0.84
$ke_{0\text{-}192\ h}$	0.0111 h^{-1}	0.0150 h^{-1}	1.35
ka_{1}	1.7323 h^{-1}	1.8283 h^{-1}	1.06
ka_{2}	3.0857 h^{-1}	2.6099 h^{-1}	0.85
$AUC_{0\text{-}4.9\ h}$	1428 ng*h/mL	14 ng*h/mL	0.01
$AUC_{0\text{-}192\ h}$	3409 ng*h/mL	687 ng*h/mL	0.20

Table 23 ***Main pharmacokinetic parameters of aliskiren in serum and saliva*** *after single oral administration of 300 mg aliskiren to three healthy volunteers – C_{max}: maximum concentration; t_{max}: time point of maximum concentration; ke: elimination rate constant; ka 1: absorption rate constant of the first peak; ka 2: absorption rate constant of the second peak; AUC: area under the concentration-time curve.*

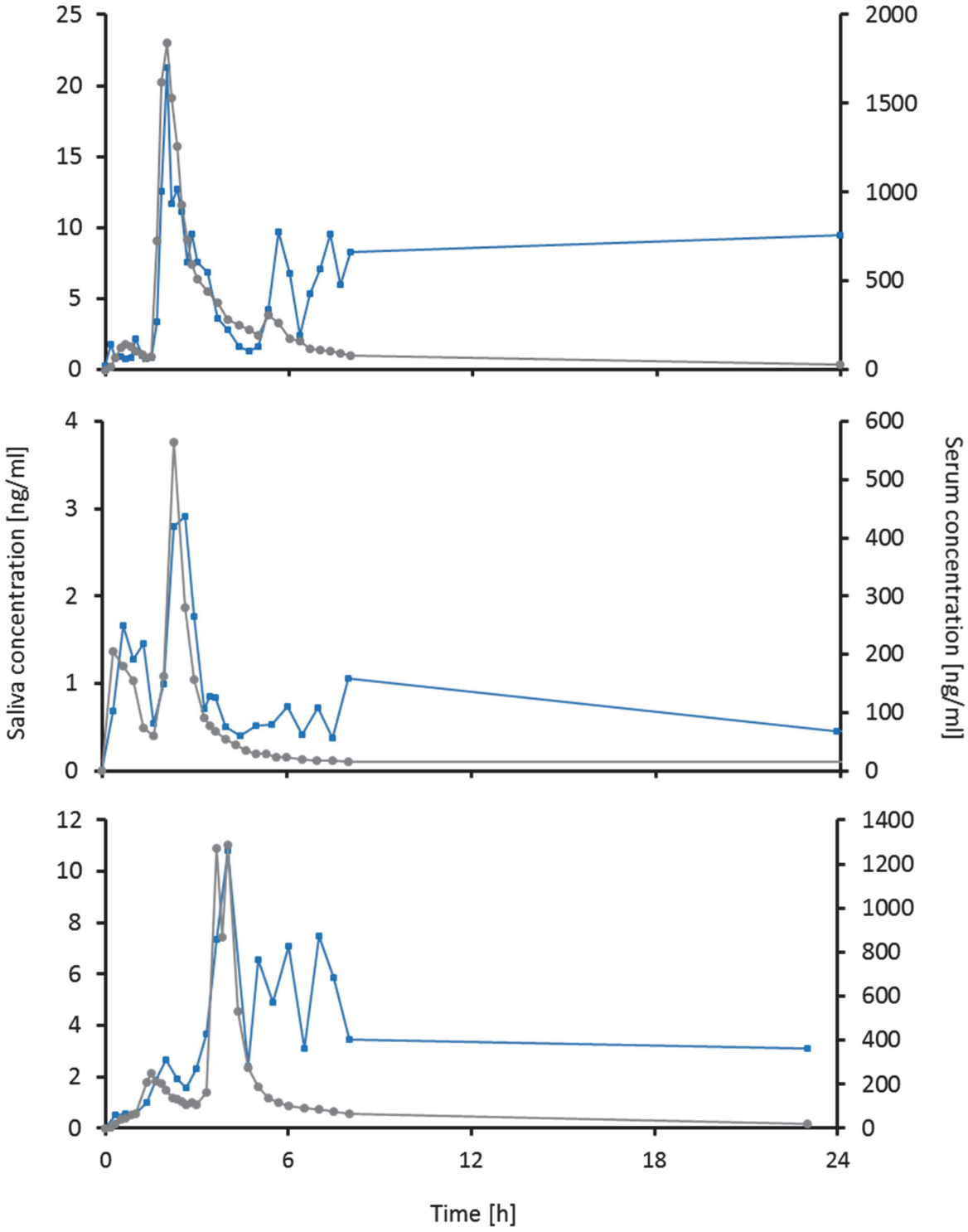

Figure 33 ***Obtained concentration-time profiles in serum and saliva*** *– The graph shows the three single concentration-time profiles in serum and saliva within the first 24 hours after administration of 300 mg aliskiren hemifumarate to three healthy volunteers. Solid grey circles represent the concentration in serum and solid blue squares mark the observed saliva concentration.*

4.2 Bioanalytics for determination of hormones and proteins allowing for the assessment of pharmacodynamics within the RAA system

The chosen immunological assays for pharmacodynamic evaluation were partially validated. All applied assays were at least cross-validated on the assays' accuracy and precision after establishment at the laboratory. With regard to the EMA guideline for ligand binding assays and immunoassays (European Medicines Agency 2012b), a deviation of at the most ± 20 % of the nominal concentration or mean for the estimation of precision and accuracy respectively prove the assay's conformity to the guideline. The LLOQ and the ULOQ of the assay must be within 25 % of the nominal concentration. The total error should be less than 30 %. Details on full validation of the assays are summarised in the manufacturer's data sheets enclosed in the appendix.

Angiotensin I and plasma renin activity

Angiotensin I and plasma renin activity were both determined by a commercial ^{125}I radioimmunoassay (RIA) (Immunotech, Prague, Czech Republic). The assay's intra-day precision was determined in 10 runs on one day while inter-day precision was evaluated on 10 different days. Obtained coefficients of variation (CV) were 10.2 and 8.2 % respectively, determined by a provided calibration control standard of known concentration. The root mean squares were 11.8 and 9.0 % respectively. The mean intra-day accuracy was 6.0 ± 10.8 % and the inter-day accuracy was 4.1 ± 8.3 %. The assay complies with current EMA requirements on ligand binding assays. In addition, the manufacturer's reported validation results are attached in Appendix 9.

Prorenin

An enzyme linked immunosorbent assay (ELISA) was used to analyse prorenin in 100 µL plasma within a calibration range of 0.02 – 10 ng/mL. The inter-day precision was evaluated on 9 different days with a CV of 5.2 %. Regarding the intra-day precision, the determined coefficient of variation was 8.2 %, having been determined through 10 different runs. The mean intra-day accuracy of the prorenin assay was 2.5 ± 8.4 % while the inter-day accuracy was -5.4 ± 5.0 %. The assay complies with current EMA requirements on ligand binding assays. Appendix 11 lists the complementary validation results of the assay manufacturer.

Angiotensin II

The in-house optimised assay consisting of a combined SPE plus modified ELISA to determine angiotensin II in 500 µL plasma was investigated for its precision by four runs on one day. Furthermore, inter-day run precision was evaluated through 7 different runs on four different days. The assay revealed a mean intra-

day accuracy of -7.9 ± 2.4 % and a CV for precision of 2.6 %. Regarding the inter-day determination, the mean accuracy was -4.5 ± 6.4 %. The coefficient of variation of the inter-day precision was 6.7 %. Determined root mean squares were 8.1 and 7.4 % respectively. Additionally, the recovery of the solid-phase extraction was determined to be 84 % (n=3). Calculation was done according to equation (15). The assay complies with current EMA requirements on ligand binding assays. The manufacturer's information on validation of the unmodified ELISA kit is attached in Appendix 10.

Renin

The calibration range of renin ranged from 2.53 to 500 µIU/mL with an LLOQ of 2.53 µIU/mL. The intra-day precision was evaluated by 10 runs on one day with a CV of 2.5 % while the inter-day precision was determined by 10 different runs on 10 different days. The ascertained coefficient of variation was 3.9 %. The corresponding mean inter-day accuracy was 10.0 ± 4.3 %. The intra-day accuracy was determined at two different concentration levels (25.1 and 98.9 µIU/mL) with 11 % and 9 % deviation respectively, compared to the nominal concentration and was therefore each within the given guideline limits. The assay complies with current EMA requirements on ligand binding assays. Additional validation results from the manufacturer are available in Appendix 12.

Angiotensin converting enzyme activity

It was calculated using the ratio of angiotensin I to angiotensin II concentrations and was not analytically determined.

4.3 Proof-of-concept study

4.3.1 Study population

All 22 volunteers enrolled were white and healthy. The pupolation includes a subpopulation of six subjects (5 males, 1 female) of Arabic descent. A group of 13 volunteers (6 males, 7 females), aged 19 to 31 years, were enrolled to the aliskiren group, and six men and three women (aged 19 to 30 years) were enrolled to the enalapril group. Baseline characteristics for all participants are shown in Table 24.

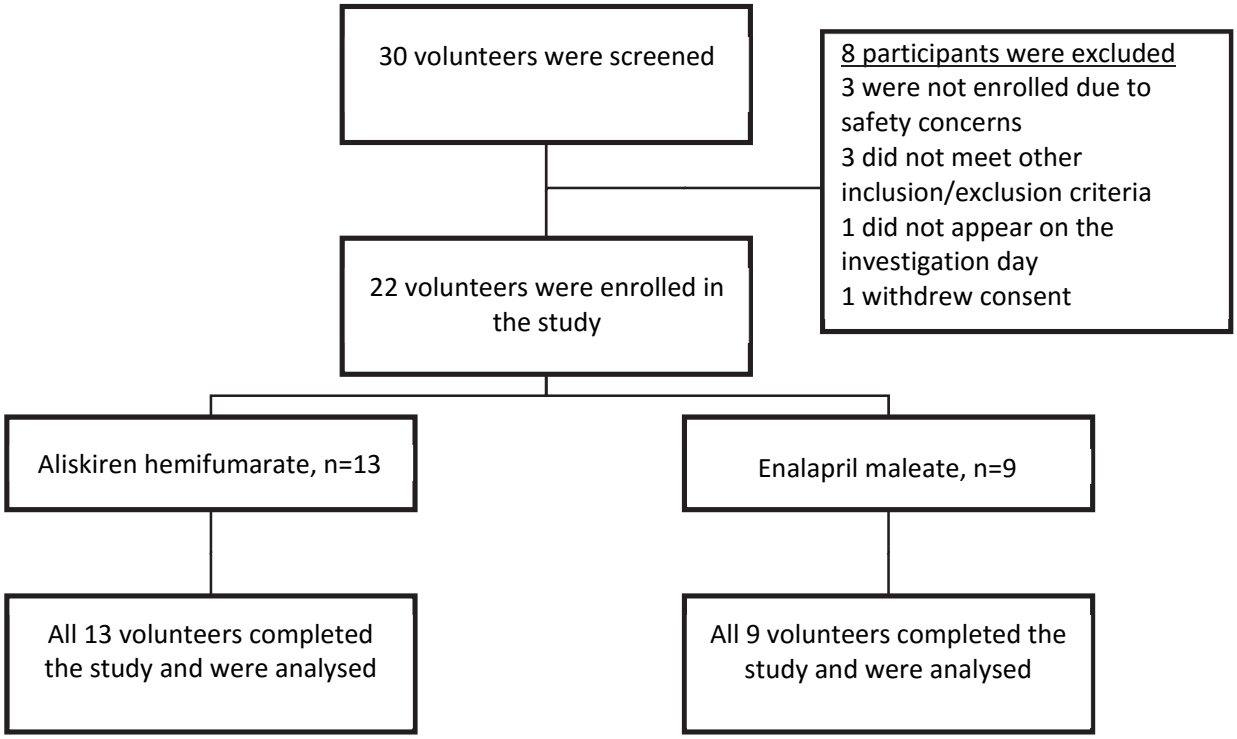

Figure 34 ***Consort diagram*** *of the conducted proof-of-concept study.*

	Aliskiren group (n=13)	Enalapril group (n=9)
Age [years]	26 ± 3	23 ± 4
Male sex	6 (46%)	6 (67%)
Caucasians [%]	100	100
Height [cm]	176 ± 8	181 ± 10
Bodyweight [kg]	76 ± 19	70 ± 13
BMI [kg/m²]	24.3 ± 4.7	21.2 ± 2.4

Pharmacokinetic parameters	Aliskiren[a]	Enalapril	Enalaprilat
c_{max} 1 [ng/mL]	161.5 ± 135.5	159.7 ± 67.8	52.5 ± 25.3
c_{max} 2 [ng/mL]	502.5 ± 503.1	-	-
t_{max} 1 [h]	0.67 (0.35 – 1.5)	1 (0.7 – 1.0)	4 (2.8 – 4.4)
t_{max} 2 [h]	2.67 (0.8 – 4.3)	-	-
AUC_{0-t} [ng*h/mL]	1946.2 ± 1285.3	230.2 ± 78.2	491.4 ± 148.0
$AUC_{0-\infty}$ [ng*h/mL]	2076.1 ± 1340.9	231.6 ± 78.3	513.5 ± 145.9
$t_{½}$ [h]	75.6 ± 20.0	0.9 ± 0.3	10.5 ± 11.3
k_e [1/h]	0.0097 ± 0.002	0.8050 ± 0.247	0.0968 ± 0.036
CL/F [L/h]	191.5 ± 97.2	97.5 ± 40.3	29.7 ± 7.8
Vd/F [L]	21368.3 ± 12694.2	134.0 ± 71.9	502.1 ± 672.9
A*e 24h* [mg]	1.51 ± 1.0	-	-
A*e last* [mg]	2.15 ± 1.36	3.41 ± 1.08	3.78 ± 1.63
CL_R [L/h]	1.18 ± 0.43	15.1 ± 3.5	7.5 ± 2.1

Table 24 ***Baseline characteristics and pharmacokinetics of aliskiren as well as enalapril and enalaprilat*** *in the proof-of-concept study– All values are reported as mean ± standard deviation except t_{max} (median and range).* [a] *Aliskiren showed biphasic absorption and both C_{max} and t_{max} values are presented. BMI, body mass index; c_{max}, maximum serum concentration; t_{max}, time point of maximum concentration; AUC, area under the serum concentration-time curve; $t_{½}$, terminal half-life; k_e, elimination rate constant; CL/F, oral clearance; Ae, amount excreted into urine within 24 h and full observation period; CL_R, renal clearance.*

4.3.2 Determination of drug concentration for pharmacokinetic determination

Aliskiren, enalapril and enalaprilat were investigated in serum, urine and saliva by highly dense sampling of the drug concentrations in the corresponding biological fluids for five elimination half-lives of the drugs. The presented low-volume assays were applied for all aforementioned drugs by using the high-throughput approach.

4.3.2.1 Aliskiren in serum and urine

Eleven out of 13 subjects had double peaks of aliskiren in serum. The extent and time point of the occurrence of the double peak differed among subjects. However, the first peak occurred with a median t_{max} at 0.7 hours and reached lower maximum concentrations compared to the second peak. The t_{max} of the second peak ranged between 0.8 and 4.3 hours, with the median at 2.7 hours. Individual concentration-time profiles are arranged in Figure 36. The mean $AUC_{0-\infty}$ of aliskiren was 2076.1 ± 1340.9 ng*h/mL with a C_{max} of 502.5 ± 503.1 ng/mL (mean ± S.D.; n=13). The mean terminal half-life of aliskiren was 75.6 hours. The total amount recovered in urine was 0.7 ± 0.4 % (mean ± S.D.; n=13) of the administered dose. The mean calculated CL_R was 1.18 L. Corresponding urinary profiles are arranged in Figure 35. Obtained pharmacokinetic parameters in serum and urine are listed in Table 24.

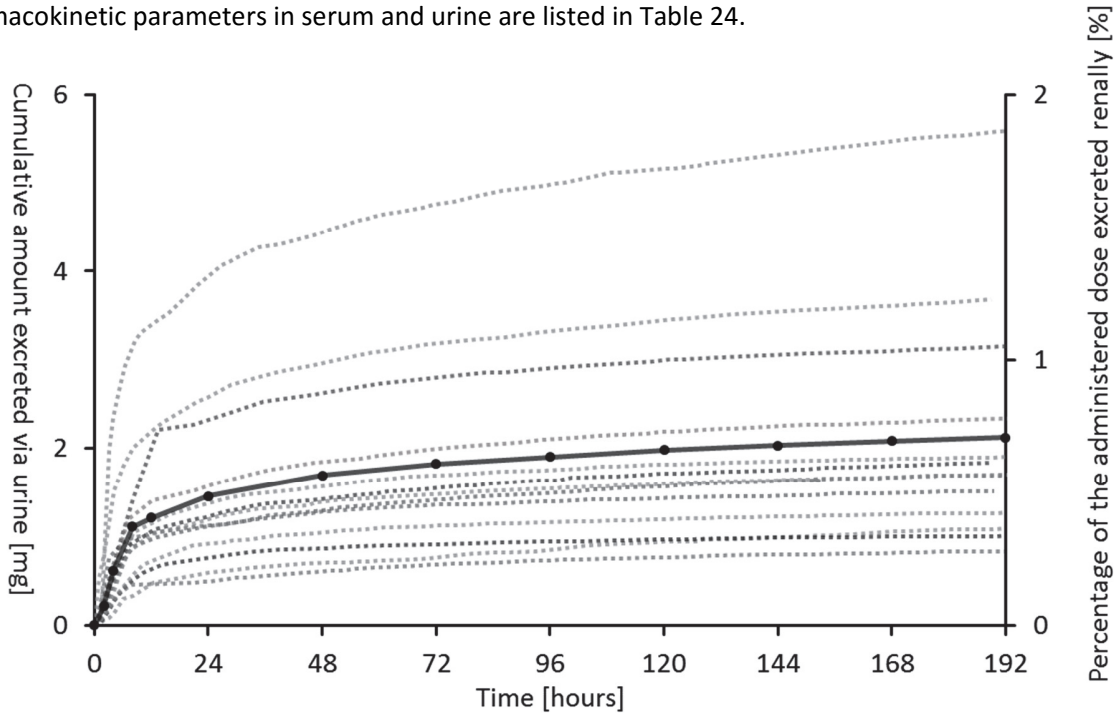

Figure 35 ***Obtained urine profiles of aliskiren*** *within the proof-of-concept study – Dotted lines represent the individual cumulative amount (left y-axis) excreted into urine after administration of 1 x 300 mg aliskiren hemifumarate to healthy volunteers. Additionally, the black line shows the mean percentage of excreted urinary aliskiren (right y-axis) n=13.*

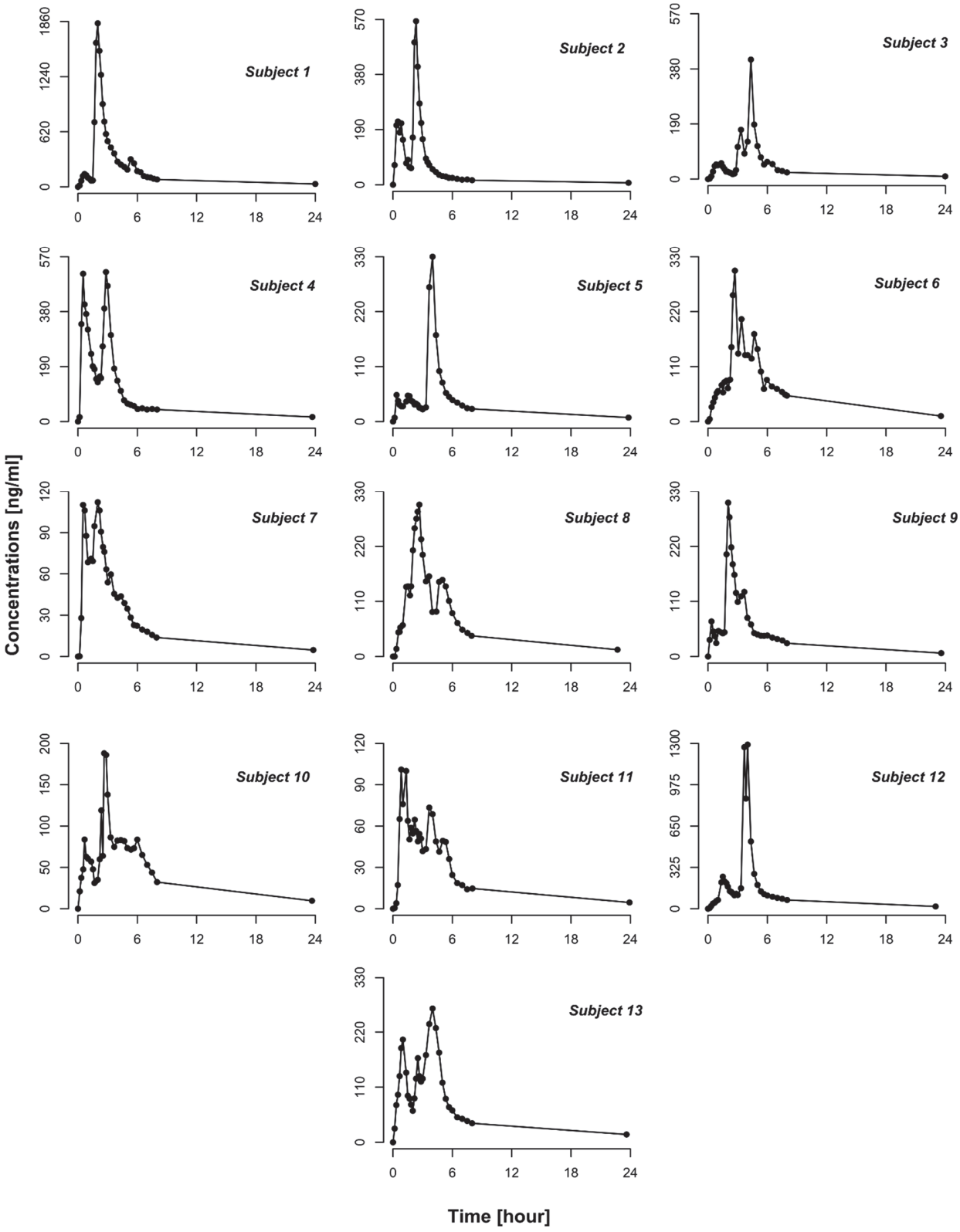

Figure 36 ***Individual serum concentration-time profiles of 300 mg aliskiren*** *in healthy volunteers (19-31 years).*

4.3.2.2 Aliskiren in saliva

The concentration-time profiles of aliskiren in saliva showed two different distribution phases over time with a comparable profile shape up to 5 hours, whereas it changed substantially at later timepoints when compared with the serum concentration. The obtained mean $C_{max0\text{-}4.9h}$ of aliskiren in saliva was 7.1 ± 8.4 ng/mL while $C_{max0\text{-}192h}$ was 7.3 ± 7.1 ng/mL. The $AUC_{0\text{-}4.9h}$ was 11.0 ± 10.5 ng*h/mL and changed to 352.2 ± 367.4 ng*h/mL from 0 to 192 h. The observed t_{max} in the investigation period up to 5 hours was 2.7 h, whereas it changed to 6 hours when the entire study period (192 h) was considered. The saliva-to-serum ratio of k*e* ranged between 0.84 to 1.31 for 0 to 4.9 h and clearly altered for 0 to 192 h (0.2 – 4.4). If compared with serum, the mean C_{max} in saliva - up to the first five hours after administration - was 1.4 %, and the area under the curve reflected 1.5 % of the serum concentration. In contrast, the $AUC_{0\text{-}192h}$ was 18.2 % of that seen in serum. Comparative individual concentration-time profiles of aliskiren in serum and saliva are presented in Figure 37, and a comparative dataset is compiled in Table 25.

	Serum	Saliva
	Aliskiren (n=13)	
$C_{max0\text{-}4.9h}$ [ng/mL]	502.5 ± 503.1	7.1 ± 8.4
$C_{max0\text{-}192h}$ [ng/mL]	502.5 ± 503.1	7.3 ± 7.1
$t_{max0\text{-}4.9h}$ [h]	2.67	2.7
$t_{max0\text{-}192h}$ [h]	2.67	6
$AUC_{0\text{-}4.9h}$ [ng*h/mL]	743.7 ± 574.7	11.0 ± 10.5
$AUC_{0\text{-}192h}$ [ng*h/mL]	1946.2 ± 1285.3	353.2 ± 367.4
	Enalapril (n=5)	
$C_{max0\text{-}72h}$ [ng/mL]	151.8 ± 61.2	5.5 ± 6.1
$t_{max0\text{-}72h}$ [h]	1	1
$AUC_{0\text{-}72h}$ [ng*h/mL]	219.5 ± 46.9	6.6 ± 6.5
	Enalaprilat (n=5)	
$C_{max0\text{-}72h}$ [ng/mL]	53.4 ± 31.1	0.3 ± 0.1
$t_{max0\text{-}72h}$ [h]	4.3	4
$AUC_{0\text{-}72h}$ [ng*h/mL]	514.5 ± 163.7	2.34 ± 1.9

Table 25 ***Comparative dataset of pharmacokinetics in saliva and serum of aliskiren, enalapril and enalaprilat*** *– All values represent mean ± standard deviation except t_{max} which is listed as median.*

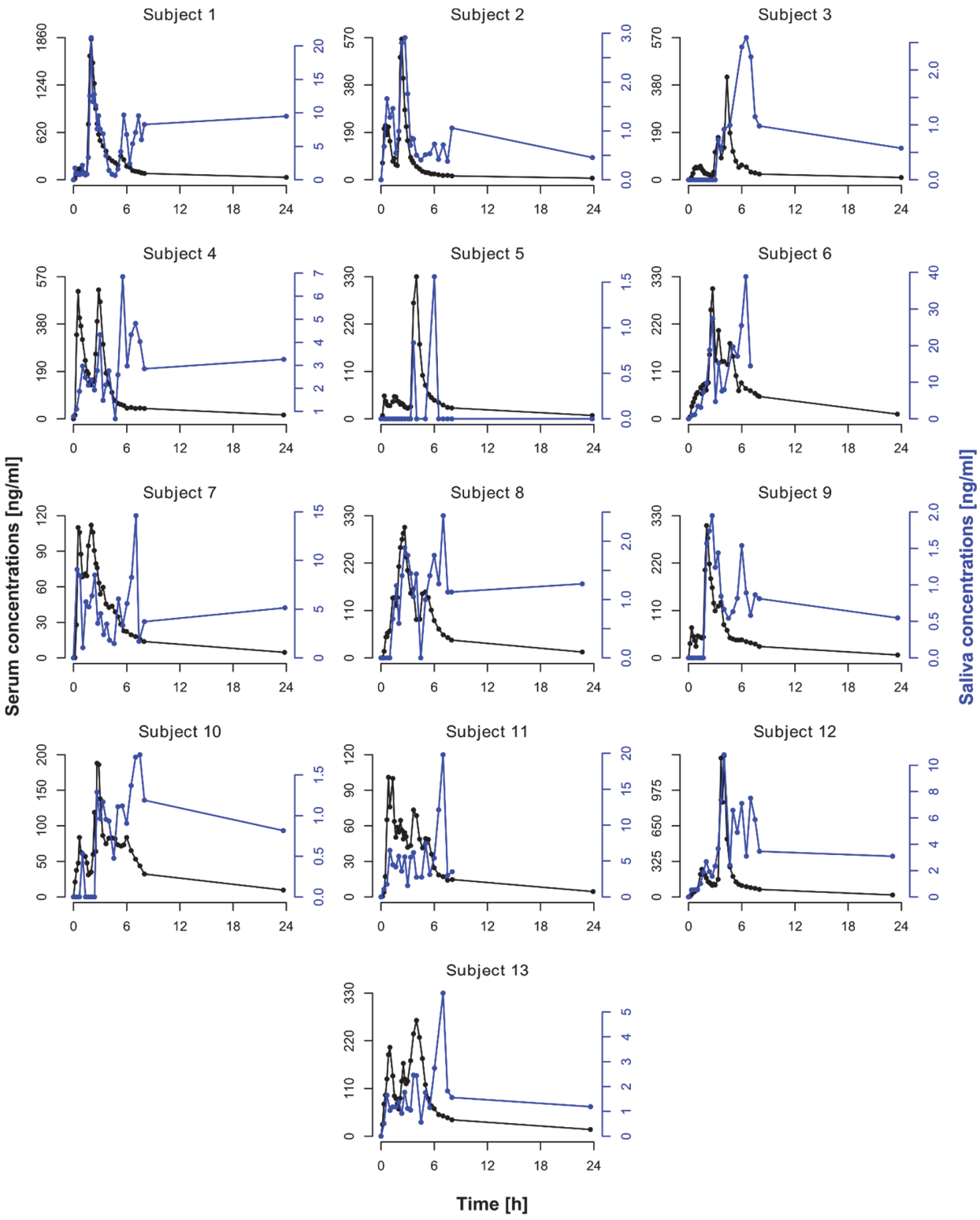

Figure 37 ***Comparison of aliskiren concentrations in serum and saliva*** *after oral administration of 300 mg aliskiren hemifumarate in 13 healthy volunteers for the first 24 h. Black line: serum concentration of aliskiren [primary y-axis], blue line: saliva concentration of aliskiren [secondary y-axis]*

4.3.2.3 Enalapril and enalaprilat in serum and urine

After administration of 20 mg enalapril maleate, the prodrug was rapidly absorbed (mean t_{max}: 0.9 h) with maximum concentration of 159.7 ± 67.8 ng/mL (mean ± S.D.; n=9). The $AUC_{0-\infty}$ was 231.6 ng*h/mL, therefore, it was about half that of enalaprilat. The calculated mean elimination half-life of enalapril was 0.9 h. In total, 17 % of enalapril was found in urine after 72 h, with a renal clearance of 15.1 ± 3.5 L/h (mean ± S.D.; n=9). The active metabolite enalaprilat reached a mean C_{max} of 52.5 ± 25.3 ng/mL (mean ± S.D.; n=9) after 4 hours. The observed t_{max} ranged between 2.8 – 4.4 hours. The mean elimination half-life was 11 hours. The mean AUC_{0-72h} was 491.4 ± 148.0 ng*h/mL (mean ± S.D.; n=9). The converting rate of enalapril and Enalaprilat, calculated by the corresponding $AUC_{0-\infty}$, ranged from 1.66 to 4.92, with the median of 2.0. The amount of excreted enalaprilat was about 27 % of the dose administered. The calculated renal clearance of enalaprilat was 7.5 ± 2.1 L/h (mean ± S.D.; n=9). Figure 38 shows the urinary excretion amount of enalapril and enalaprilat. Obtained pharmacokinetic parameters in serum and urine are listed in Table 24.

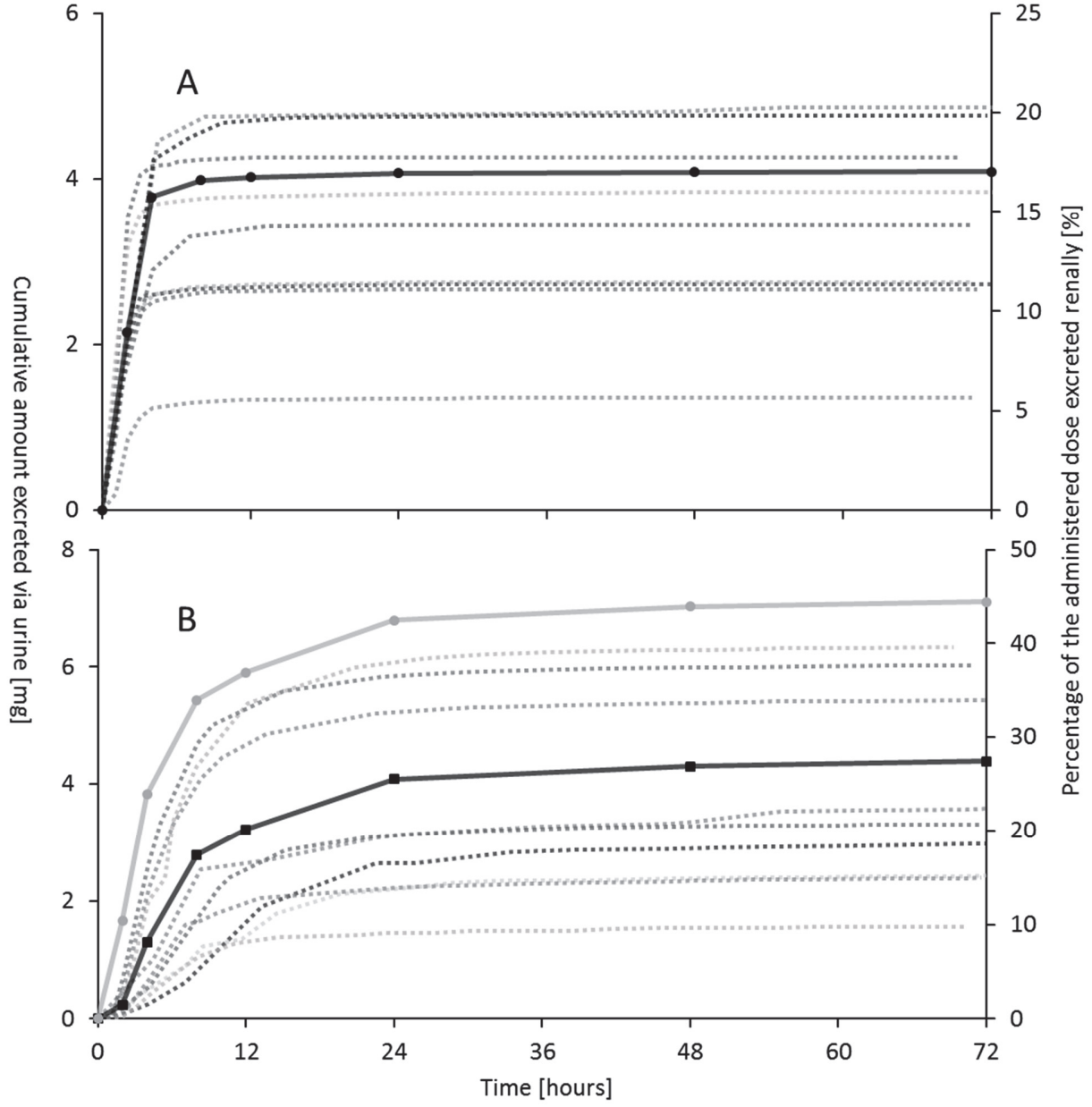

Figure 38 ***Obtained urine profiles of enalapril and enalaprilat*** *– In dotted lines the individual cumulative amount excreted into urine after administration of 1 x 20 mg enalapril maleate to healthy volunteers are presented. Additionally, the black lines represent the percentage of excreted enalapril (A) and of enalaprilat (B). The grey line in B shows the total combined excreted amount of prodrug and active metabolite. n=9.*

4.3.2.4 Enalapril and enalaprilat in saliva

The correlations between serum and saliva concentrations of enalapril and enalaprilat were investigated in seven volunteers. In five of seven volunteers, salivary concentration-time profiles were obtained. One subject was excluded because the mouth was not washed after drug administration and thus detected

salivary drug concentrations represented a combination of residual of the tablet itself and its penetration into saliva. The second subject developed gum bleeding, and the concentrations obtained cannot, therefore, safely be accounted for saliva only. The evaluable concentration-time profiles revealed low concentrations of enalapril and in particular very low concentrations of enalaprilat in saliva. For enalapril, the C_{max} in saliva was 5.5 ± 6.1 ng/mL and varied between 1 to 14 % of that found in serum. The median t_{max} was at one hour in both fluids. The $AUC_{0\text{-}72h}$ of enalapril was 6.6 ± 6.5 ng*h/mL, and the individual ratios of $AUC_{0\text{-}72h}$ of saliva/serum ranged between 1 to 10 %. The determined $AUC_{0\text{-}72\,h}$ of enalaprilat was 2.34 ± 1.9 ng*h/mL, with a mean maximum concentration of 0.3 ± 0.1 ng/mL at 4 hours (t_{max}). Although visual inspection confirmed a comparable profile shape of the prodrug enalapril in serum and saliva, that cannot be claimed for the active metabolite enalaprilat. The penetration was less than the one of that for enalapril and occasionally below the lower limit of quantification. The evaluated correlation between saliva and serum concentrations was not good because the shape of the concentration time profiles and the corresponding elimination rates were different in both fluids. The corresponding comparative individual profiles in serum and saliva are compiled in Figure 40 and further comparative pharmacokinetic parameters are arranged in Table 25. A comparison between the two determination methods for the drug concentration in serum and saliva is arranged in Figure 39 indicating the determined difference between the concentration levels of enalapril and enalaprilat in serum and saliva.

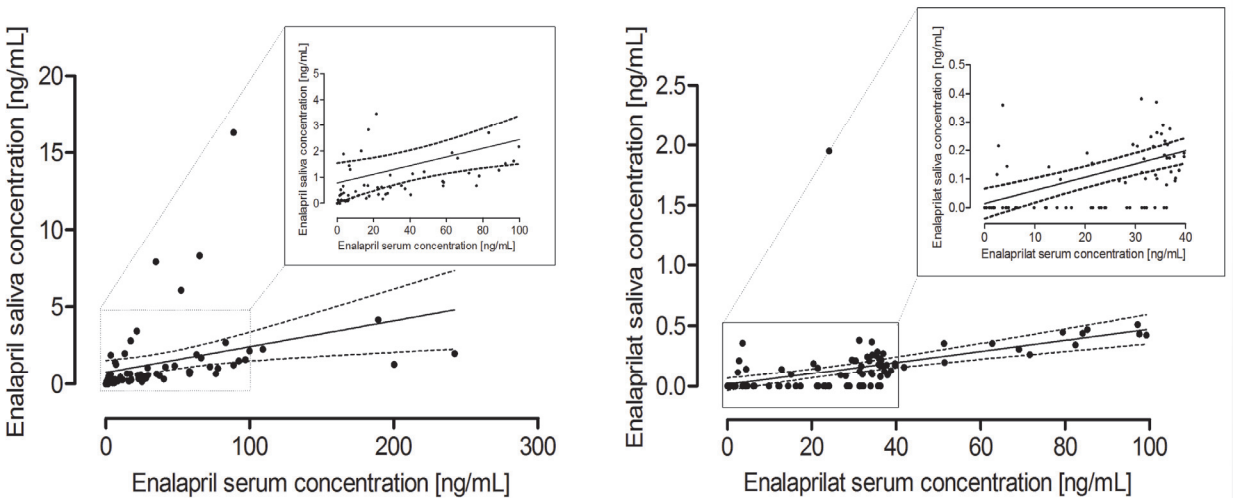

Figure 39 ***Scatter plots of measured enalapril and enalaprilat concentrations in serum and saliva.*** *The closeness of agreement of the two different pharmacokinetic determination methods in serum and saliva were assess by a scatter plot. The left graph illustrates the measured concentrations of enalapril in saliva and serum, while the right graph shows the measured concentrations of enalaprilat (data obtained in five healthy volunteers). The solid lines indicate the regression lines. The 95th confidence intervals are shown by dashed lines.*

The correlation, determined by Spearman correlation, was $r_s = 0.74$ for enalapril and $r_s = 0.71$ for enalaprilat. In total, the visual description, the comparison of the pharmacokinetic parameters in both fluids and the evaluation by the scatter plots confirmed all that determination of both drugs in saliva are not appropriate to substitute the determination of the drug concentrations in serum.

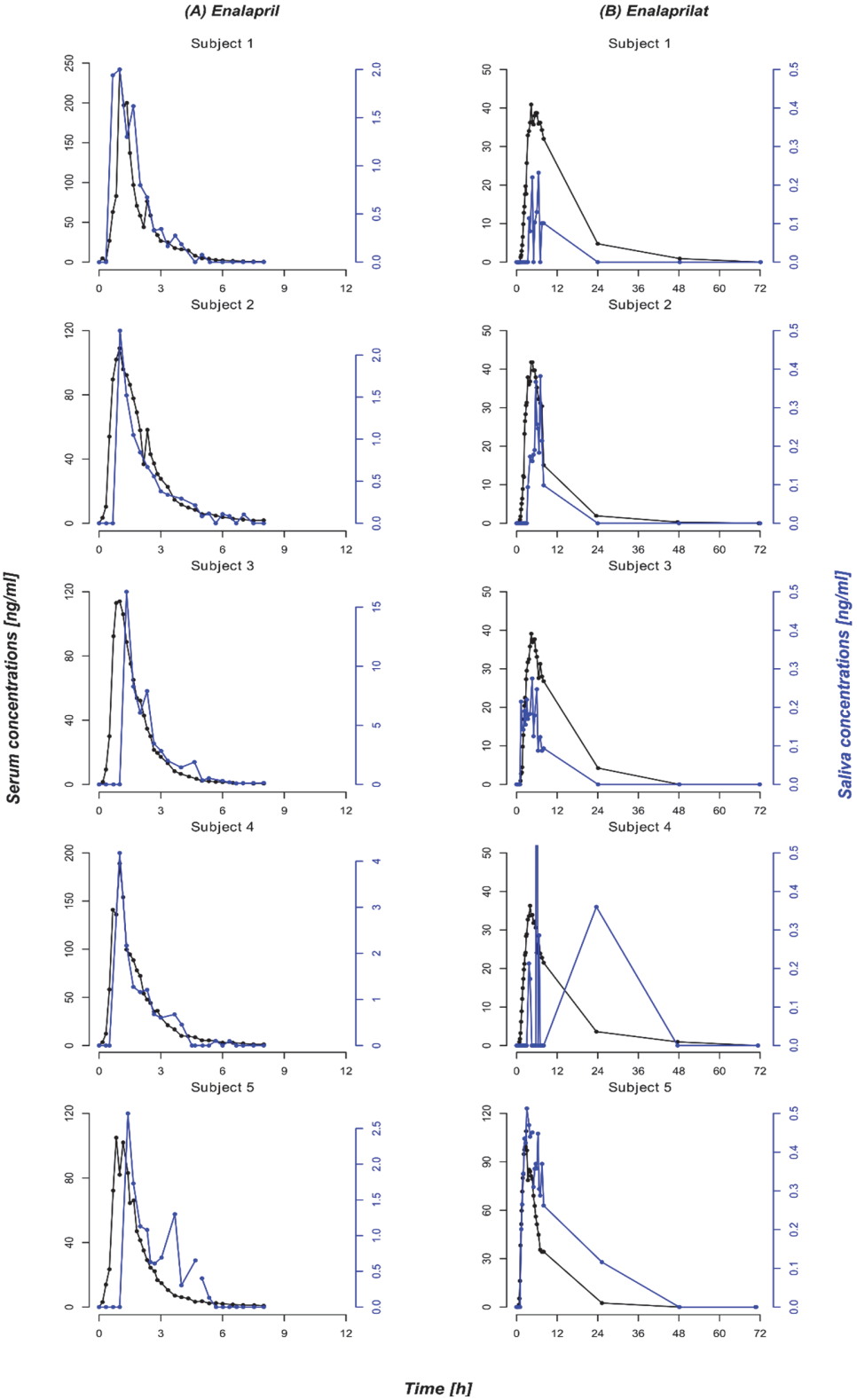

Figure 40 ***Comparison of enalapril and enalaprilat concentrations in serum and saliva*** *after oral administration of 1 x 20 mg tablet (EnaHEXAL®). The blue line describes the saliva concentration-time curve [secondary y-axis], while the black line determines the serum concentrations [primary y-axis].*

4.3.3 Changes in humoral parameters

4.3.3.1 Change in humoral parameters after administration of aliskiren

Based on published results in adults, and as shown in Figure 1, aliskiren directly blocks the enzyme renin and subsequently affects the plasma renin activity, which was confirmed by the study results. Mean PRA was maximally reduced 34-fold with a t_{max} of 1 hour. Moreover, the plasma renin activity did not return to the predose values within the observation time. As a result of aliskiren administration, the RAA system biomarkers angiotensin I and angiotensin II were expected to be influenced. However, no noticeable change in angiotensin I was observed. All mean predose values were already borderline with or at the LLOQ, and subsequently, no drop in angiotensin I concentrations was seen as a result of aliskiren administration. Because a more-sensitive assay requiring the same small amount of blood was not available, a re-analysis could not be performed. In contrast, the optimised assay was able to measure concentrations of angiotensin II at all time points. The angiotensin II concentrations evaluated had already decreased 30 minutes after administration of aliskiren and reached their minimum around 2 hours postdose. After 48 h, at least the predose values had again been reached, whereas, in the meantime, there was a mean reduction of 70 % from the baseline observed. Renin concentration increased approximately 19-fold compared with initial values, with a t_{max} of 5 hours. On day 9, the renin in the aliskiren group was still about 4.7-fold higher than it was for the predose value. Renin concentration was high variable, asindicated by the standard deviation of 434 µIU/mL for aliskiren at the C_{max}. Prorenin was only determined in participants taking aliskiren hemifumarate. The mean predose value for prorenin was 0.71 ng/mL and varied between 0.21 and 1.83 ng/mL. The mean predose prorenin concentrations were about 45-fold higher than the measured renin concentrations. Within the first eight hours, the concentrations started to increase slightly and reached their maximum mean concentration 48 h after dosing aliskiren (156 % of mean predose value).

4.3.4 Change in humoral parameters after administration of enalapril/enalaprilat

Enalaprilat, the active metabolite of enalapril, binds competitively to the angiotensin converting enzyme and blocks the conversion of angiotensin I to angiotensin II. The angiotensin I concentration rose with enalaprilat and reached maximum concentrations in all nine subjects in 5 to 7 hours after oral administration of the prodrug enalapril. The mean increase was 3.7-fold when compared with baseline concentrations. In contrast, angiotensin II levels were reduced and reached a maximum reduction between 3 to 7 hours postdose. The mean inhibition was 64 % of the predose value, showing a broad range of 30 to 95 % reduction. When comparing aliskiren and enalapril, the inhibition of angiotensin II synthesis was reduced for 24-48 hours with Aliskiren, whereas with enalapril treatment, the value was reduced for 8 hours

only. The angiotensin converting enzyme activity was specifically inhibited by enalaprilat and was, therefore, calculated in volunteers taking enalapril only. The mean activity was maximum reduced by 76 % between 3 – 8 hours postdose. The median t_{max} was 6 hours. Within 24 – 48 hours postdose, almost all patients had returned to at least predose values. The ACE activity was calculated based on the obtained angiotensin I and angiotensin II concentrations. Figure 41 illustrates the determined change from baseline over time.

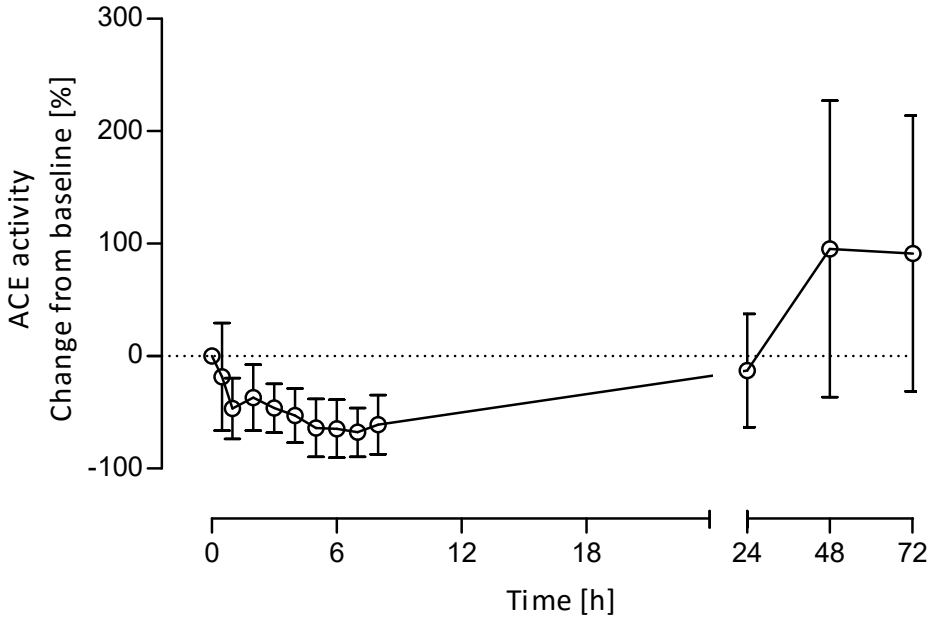

Figure 41 ***Change in ACE activity*** *after single administration of 20 mg enalapril maleate. Presented are the mean ± S.D. Each measurement point is described by n=9 determinations.*

Determined renin concentrations increased with enalapril approximately 16-fold from initial values, with a t_{max} of about 6 hours after enalapril administration. The concentration slowly went back to a 2.7-fold increase by day 3 of the study. The variability in renin concentration is indicated by a standard deviation of 89 µIU/mL (464 µIU/mL if the volunteer with the short-term hypotensive values is included) at the C_{max}. With enalapril, the plasma renin activity was nearly unchanged for the first three hours after dosing. The activity started to increase 3 hours after administration of enalapril and reached a maximum concentration 5 hours postdose. The mean activity was increased by up to 19-fold compared with the mean predose value.

Figure 42 represents the mean pharmacokinetics and pharmacodynamics of aliskiren and enalapril/enalaprilat while Table 26 lists the corresponding pharmacodynamics results. Individual pharmacokinetic and pharmacodynamic profiles of all involved volunteers are attached in Appendix 1-Appendix 8.

	Renin		Plasma renin activity		Angiotensin I		Angiotensin II	
	C_{max} [µIU/mL]	t_{max} [h]	Activity [ng/mL/h]	t_{max} [h]	Max change [%]	t_{max} [h]	Max change from baseline [%]	t_{max} [h]
Aliskiren (n=13)								
Mean	272	5[a]	0.01	1	[b]		- 70	2
Range	(48 – 1694)	(3 – 7)	0.01	(0.5 – 6)			(0 – (-98))	(0.5 – 4)
Enalapril/Enalaprilat (n=9)								
Mean	128 [280[c]]	6[a]	9 [14[c]]	5[a]	263 [396[c]]	6[a]	- 64	4.5[a]
Range	(50 – 300) [1499[d]]	(5 – 6)	(2 – 17) [51[d]]	(5 – 6)	(151 – 400) [1460[d]]	(5 – 7)	((-30) – (-95))	(3 – 7)

Table 26 ***Dataset of pharmacodynamics in all healthy volunteers*** *– [a] t_{max} values were reported as median, [b] mean predose value was already at the LLOQ. A reliable change could not be determined, [c] obtained mean value if subject with hypotensive blood pressure values is included and not marked as outlier, [d] obtained upper limit of range if subject with hypotensive blood pressure values is included and not marked as outlier.*

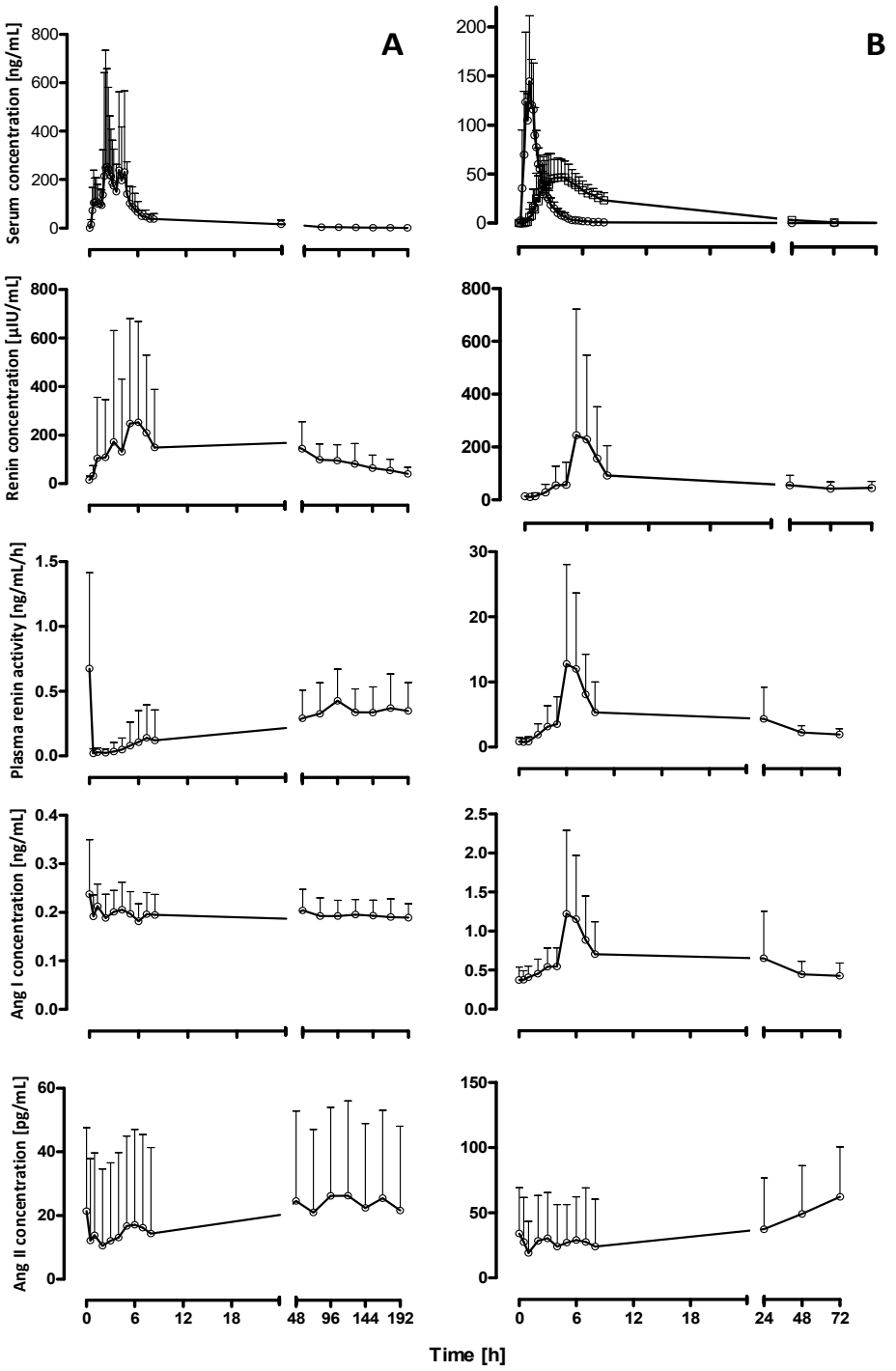

Figure 42 ***Obtained mean concentration-time profile after administration of 300 mg aliskiren hemifumarate*** *to 13 healthy volunteers (A) and mean concentration-time profile after administration of 20 mg enalapril maleate to 9 healthy volunteers (B). Followed by pharmacodynamics of aliskiren (left column) and enalapril/enalaprilat (right column). All profiles are represented by mean ± standard deviation. Error bars are only one-sided owing to a clear-cut arrangement.*

4.3.5 Safety assessment

Both drugs were generally well tolerated. Three adverse events of dizziness were reported by the subjects taking 20 mg enalapril maleate. Subjects who received 300 mg aliskiren hemifumarate also reported three adverse events: two complained about headaches, one about dizziness. All adverse events were mild or moderate and discontinued within 12 hours after the first incidence. No notable change in the blood pressure values were detected in the healthy volunteers of either group. The ratio of the mean systolic blood pressure value of the whole observation period to the predose value was 0.95 with aliskiren and 0.99 with enalapril. The ratios of diastolic blood pressure also remained nearly unchanged. Details are summarised in Table 27. Nevertheless, one subject taking enalapril experienced approximately 30-minutes of hypotensive BP values of 80/44 mm Hg RR. The timing of the hypotensive values occurred around the t_{max} of the active metabolite Enalaprilat; however, the volunteer was clinically asymptomatic.

	Aliskiren group (n=13)	Enalapril group (n=9)
Systolic blood pressure [mmHg]		
Baseline	125 ± 14	109 ± 11
Average 0-24 h	118 ± 12	107 ± 10
Average 0-end	118 ± 12	107 ± 10
Ratio (average 0-24 h/baseline)	0.95 ± 0.05	0.98 ± 0.04
Ratio (average 0-end/baseline)	0.95 ± 0.04	0.99 ± 0.04
Minimum value	107 ± 11	97 ± 11
Diastolic blood pressure [mmHg]		
Baseline	70 ± 6	67 ± 6
Average 0-24 h	65 ± 7	64 ± 6
Average 0-end	66 ± 7	64 ± 6
Ratio (average 0-24 h/baseline)	0.93 ± 0.07	0.95 ± 0.04
Ratio (average 0-end/baseline)	0.95 ± 0.06	0.96 ± 0.04
Minimum value	55 ± 7	54 ± 7
Heart rate [1/minute]		
Baseline	59 ± 10	65 ± 10
Average 0-24 h	62 ± 9	69 ± 9
Average 0-end	63 ± 10	69 ± 9
Ratio (average 0-24 h/baseline)	1.06 ± 0.07	1.07 ± 0.1
Ratio (average 0-end/baseline)	1.07 ± 0.07	1.07 ± 0.1
Maximum heart rate	77 ± 13	85 ± 15

Table 27 ***Change in blood pressure*** *– Data is compiled as mean ± standard deviation; baseline: blood pressure value before administration of drug*

4.4 Establishment of a "Good Clinical Laboratory Practice"-compliant quality system for bioavailability studies in support of marketing authorisation by EMA

GCLP compliant procedures were successfully implemented in the bioanalytical laboratory prior to the Phase I study of the LENA project. Requirements related to the appropriateness of the facility and equipment as well as to qualification of staff defined by GCLP were fulfilled. For the laboratory rooms in which the bioanalytical analysis and the creation and use of reagents were performed even stricter requirements in accordance with GLP were followed. All roles have been defined and trainings undertaken according to the requirements of the guideline. SOPs were created and approved for use that cover the areas of the bioanalytical techniques applied, reagents and materials, as well as equipment including service, maintenance and calibration. In addition, SOPs for quality control, procedures to assure patient safety and blinding, sample shipment and storage, recording, archiving, disposal and cleaning etc. were newly implemented. Since safety of the personnel has been dealt with in internal university instructions no additional work instructions or SOPs on this topic were set in force. The procedure on data privacy of patient's personal information followed international requirements regarding recording, handling and storage of samples at the bioanalytical laboratory (ICH E6 (R1) §2.11) (ICH 1996). In total, 51 SOPs, manuals and form sheets entered into force prior to their contribution to the Phase I study of LENA. An excerpt of the current SOP list is attached as Figure 43.

The organisational chart of the LENA project is appended (Appendix 14) to provide an overview about the involved partners and interfaces which must be addressed by the GCLP-compliant quality system. Involved staff at the Institute of Clinical Pharmacy and Pharmacotherapy as well as physicians, nurses and laboratory assistants at the Phase I centre in Leuven (Belgium) were trained in the relevant procedures (sampling, storage, shipment, sample preparation, analysis etc.) and its documentation. The monitoring of proper documentation and compliant bioanalytical procedures at the Institute was secured through an internal and an independent external quality control. This two-fold quality control was established to provide a suitable control at the bioanalytical laboratory because the internal quality control was performed only with limited work experience. This was complemented with a well reputable external one (Dr. Klinkner und Partner, Germany). Additionally, all equipment used in the bioanalysis had been qualified for installation (IQ) and for its purpose by documented operation qualification (OQ). Prior to each analytical run, a daily performance qualification (PQ) was successfully performed. These qualifications verified the appropriate performance of the established analytical equipment at the Institute.

Owner	Classification/ Categor	Type	Version	Effective date [YYYYMMDD]	Valid	Title in local language	Title in English
UDUS	BA	S	01	08.05.2014	yes	Ena-Probenvorbereitung	Ena-Sample preparation
UDUS	BA	S	01	08.05.2014	yes	HPLC-MS	HPLC-MS
UDUS	BA	S	01	08.05.2014	yes	Spülmaschine	Washer
UDUS	BA	S	01	08.05.2014	yes	Lagerung	Storage
UDUS	BA	S	01	08.05.2014	yes	MS-Reinigung	MS-Cleaning
UDUS	BA	S	01	08.05.2014	yes	MS-Kalibrierung	MS-Calibration
UDUS	BA	S	01	08.05.2014	yes	SOPs	SOPs
UDUS	BA	S	01	08.05.2014	yes	Mitarbeiter	Staff
UDUS	BA	S	01	08.05.2014	yes	Rohdaten und Protokollierung	Raw-data and recording
UDUS	BA	S	01	08.05.2014	yes	GCLP-Archiv	GCLP-Archive
UDUS	BA	S	01	08.05.2014	yes	Prüfpläne	Study Protocol
UDUS	BA	S	01	08.05.2014	yes	Abschlussberichte	Final Report
UDUS	BA	F	03	11.09.2014	yes	Lösungen Serum	Solutions Serum
UDUS	BA	F	03	11.09.2014	yes	Kalibrierreihe Serum	Calibration series Serum
UDUS	BA	F	03	11.09.2014	yes	IS-Lösungen Serum	IS-Solutions Serum
UDUS	BA	F	03	11.09.2014	yes	Festphasenextraktion Serum	Solid Phase Extraction Serum
UDUS	BA	F	03	03.07.2014	yes	Eindampfen	Extract to dryness
UDUS	BA	F	04	03.07.2014	yes	HPLC-Vials	HPLC-Vials
UDUS	BA	F	01	03.07.2014	yes	SpühImaschienen-Reinigung	Washer-Cleaning
UDUS	BA	F	01	03.07.2014	yes	SpühImaschienen-Bedienung	Washer-instructions
UDUS	BA	F	01	03.07.2014	yes	MS-Reinigung	MS-Cleaning
UDUS	BA	F	02	10.09.2014	yes	Abwicklung Probenannahme	Sample receipt
UDUS	BA	F	02	10.09.2014	yes	Vorbereitung Porbenaufbereitung	Arrangements for Sample preparation
UDUS	BA	F	02	10.09.2014	yes	Umgang mit Proben(WP 07)	Sample handling
UDUS	BA	F	02	10.09.2014	yes	Dokumentation Sekretariat (WP 07)	Office Documentation (WP 07)
UDUS	BA	F	02	10.09.2014	yes	Vernichtung aufgearbeiteter Proben in HPLC-Vials	Destruction of examinded Samples
UDUS	BA	F	02	09.09.2014	yes	Einsicht Archivordner	Archive Access
UDUS	BA	F	01	09.09.2014	yes	Schlüsselübergabe Archiv	Key handover protocoll
UDUS	BA	S	01	27.06.2014	yes	Validierung des Enalapril-, Enalaprilat Assays in Serum	Validation of the Enalapril-, Enalaprilat Assays in Serum
UDUS	BA	F	01	09.09.2014	yes	Zu-/Abgängen von LENA-Archivdokumenten	Archive additions and disposals
UDUS	BA	F	01	10.09.2014	yes	Kontrollbesuch	Compliance visit
UDUS	BA	F	02	10.09.2014	yes	Zugang Tiefkühltruhe	Access freezer
UDUS	BA	F	01	11.09.2014	yes	Lösungen Urin	Solutions Urine
UDUS	BA	F	01	11.09.2014	yes	Kalibrierreihe Urin	Calibration series Urine

Figure 43 ***Excerpt of the SOP-list of the bioanalytical laboratory*** *of the Institute of Clinical Pharmacy and Pharmacotherapy.*

Care was taken that newly purchased equipment and software even complied with the FDA rule 21 CFR part 11 (“electronic records, electronic signatures”). The storage conditions of freezers and fridges at -80 °C, -20 °C and 4 °C were permanently monitored and all devices were connected to an alarm system. This enabled a proper storage of the samples prior to analysis and also long-term storage in terms of archiving the specimens. All data recorded, including failure analysis and records, were also stored compliant with recommendations of the guidance in a paper archive. Access to the documents is restricted to the responsible archivist only and follows the strict procedure of documentation in accordance with GCLP. Data submitted electronically from one site to another was characterised with an MD5 checksum to warrant data integrity.

Based on the successfully enrolled GCLP standard at the Institute, the bioanalytical platform was embedded into current regulatory requirements for clinical studies. During the bioanalytical method validation for sample analysis, positive and negative quality controls were included in the measurements to assure an appropriate determination. According to the established manual, samples of the Phase I study were checked for physical integrity, completeness, identity, storage and transport conditions to verify that a smooth and non-dissipative exchange of specimens and documents was performed.

For the Phase I study of the LENA project, 1562 serum samples and 586 urine samples were analysed within 26 days. During the run-time of the study, three sample shipments from the clinical site to the bioanalytical laboratory were successfully conducted. Revealed protocol deviations such as insufficient labelling, duplicates etc. were addressed by notes-to-file. If possible, the deficits were corrected till the next sample shipment took place.

The overall result after the final bioanalysis for pharmacokinetics, the successful conduct of the Phase I study, reporting of the results, treatment of special incidences etc. proved the reliability, quality and integrity of the work and the “ready-to-use” bioanalysis platform. Identified queries, notes-to-file etc. were able to be addressed properly and were answered within predefined limits confirming the suitability of the processes established following GCLP requirements. The interfaces in particular had been classified as critical prior to the Phase I study, but the aforementioned procedures which all deal with the different interfaces in the entire bioanalytical processes confirmed the suitable integration of participating facilities and institutions. A frequently performed exchange of experiences between the partners was an extraordinary tool to further increase compliance and subsequently the quality of data generated within this setting. Due to the supply of the clinical site with devices and material for blood withdrawal - apart from the “ready-to-use” platform - a full bioanalytical concept in support of a Phase I study was established.

Details on processes and outcomes of the Phase I study within the GCLP frame work are the intellectual property of the EU-funded LENA project; it is not permissible to report them. The final GCLP setting implemented for the Phase I study is shown in Figure 44.

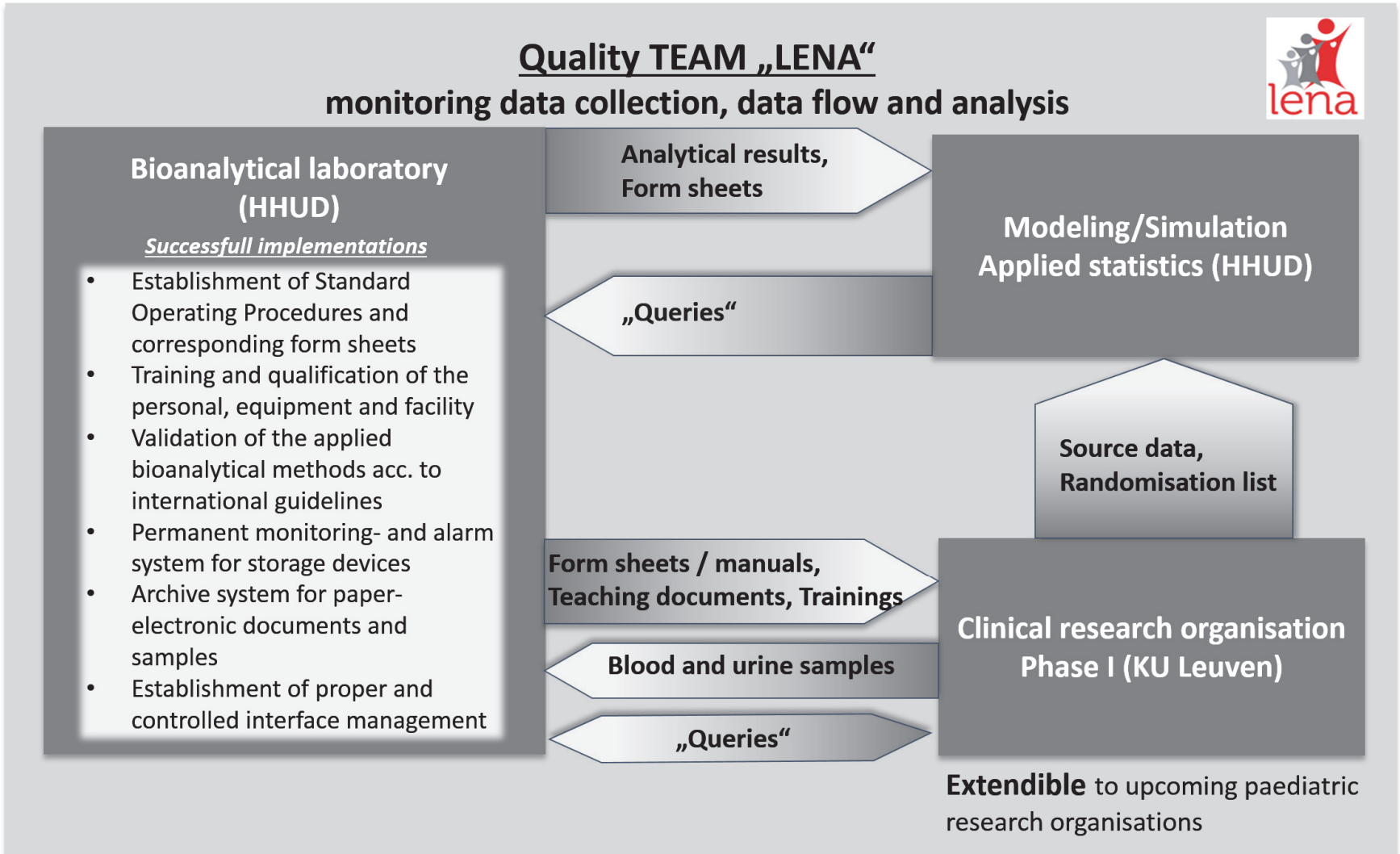

Figure 44 ***Final GCLP setting implemented*** *at the Institute of Clinical Pharmacy and Pharmacotherapy. In addition, the involved partners/institutes as well as the interface processes are illustrated. The study at the clinical site was performed according to GCP. All obtained samples were collected and treated according to the manuals and shipped to the bioanalytical laboratory. All processes at the laboratory were conducted in line with GCLP and the data is submitted for statistical analysis. The collection, preparation, acquisition and analysis of samples, generated data and exchange of documents were covered by established GCLP-compliant procedures.*

5. Discussion

Within this thesis a comprehensive bioanalytical platform is presented that enables the reliable and systematic determination of drugs acting on the RAA system and their pharmacodynamic effects in children. This tailored bioanalytical platform empowers sophisticated studies in children across all age groups and helps to gain reliable pharmacokinetic and pharmacodynamic knowledge of drugs that act on the RAA system. This will enable the relationship between dose-exposure and effect in paediatric patients to be elucidated. The platform was developed to address existing limitations in paediatric studies and to improve the availability of information for current pharmacotherapy in children. All assays were developed and validated as high through-put approaches according to international bioanalytical validation guidelines and its applicability was proven by a proof-of-concept study in healthy volunteers. Combined with the newly established GCLP quality standard, a "ready-to-use" bioanalytical platform for clinical studies compliant to regulatory requirements was implemented and successfully performed at the bioanalytical laboratory. Finally, the obtained bioanalytical results were included into the Paediatric Investigation Plan which was submitted to the Paediatric Committee at the European Medicines Agency (EMA Procedure Number: EMEA-001706-PIP).

The bioanalytical platform comes along with a clear reduction of required blood volume per patient. Compared with recently published literature by Sullivan *et al.* (Sullivan et al. 2013), the volume for determination of pharmacokinetics and pharmacodynamics of aliskiren in the same setting in children (Setting: 6 pharmacokinetic determinations+ one pharmacodynamic parameter with 3 determinations) was able to be decrease by 86 %. This allows for the investigation even in neonates and infants. Even if the pharmacodynamic changes in the RAA system were be comprehensively described by five instead of only one parameter (which until now has not been done in children), the developed platform, by gaining much more pharmacodynamic data, still requires only 62 % of the blood needed by the published approach above. It was shown that the low-volume assays additionally are of high use for denser sampling without infringing on ethical considerations. It made detailed concentration-time profiles of e.g. aliskiren accessible and explicitly described a biphasic absorption of the drug.

Apart from invasive methods, non-invasive methods were also elucidated by investigating urine and saliva to evaluate their ability to optimise current clinical settings in paediatric research. The penetration of aliskiren and enalapril had not been investigated prior to this thesis although non-invasive sampling is recommended by the competent authorities. Unfortunately, the saliva sampling of aliskiren or enalapril

did not appear useful for substituting invasive sampling in the context of pharmacokinetic evaluations. Additionally, one should bear in mind that for pharmacodynamic evaluations invasive methods are still inevitable. However, the obtained data gave insights into a potential complex mechanism of distribution of aliskiren.

Furthermore, the determination of both drugs in urine allow to also monitor the drug excretion and its amount by the renal pathway. This data in children is until now completely missing for aliskiren while the rare available data on enalapril and enalaprilat are based on non-robust results. Consequently, results in such a more comprehensive data set might lead to a safer pharmacotherapy and increase knowledge.

By developing pharmacodynamic assays besides the pharmacokinetic ones, a seamless approach has been successfully established that analyses in parallel the enzyme activities or peptide levels of the main parameters in the renin-angiotensin-aldosterone system and permits to study drug exposure and its effect.

To prove the applicability and reliability of this low-volume setting a proof-of-concept study in healthy volunteers was successfully conducted. This data, in turn, might then directly serve as input into modeling and simulation, enabling for system validation and improving future paediatric study planning. By adding to the potential for paediatric simulations, studies bridging from adults to the paediatric population or between different paediatric age groups might be simplified. Consequently, not only can the knowledge of drug safety in paediatric patients be improved through the facilitated data collection, but current study planning and conduction can also be highly improved, resulting in a reduction of required study subjects, amount of necessary studies and decreased study costs and recruitment times.

The final and logical step was the successful implementation of GCLP at the bioanalytical laboratory. The conducting of the pharmacokinetic assays according to this international acknowledged quality setting assured the reliability and integrity of the generated data sets. The validated assays, the trained staff and standardised procedures and work flows within the laboratory as well as for associated partners (clinical sites etc.) makes the developed bioanalytical platform “ready-to-use” for further clinical studies in compliance with international regulatory expectations. Since the laboratory took over responsibility for selection, purchasing, labelling, packaging, distribution etc. of all consumables required for bioanalytical sampling, the bioanalytical laboratory of the Institute of Clinical Pharmacy and Pharmacotherapy became an important partner for the Clinical Research Organisation (CRO) within the conducted Phase I study. The applicability and functionality of the bioanalytical laboratory to conduct the analysis and to act as a reliable partner for the CRO and statistical institute were assessed in the Phase I study and confirmed.

5.1 Bioanalytics for pharmacokinetic determination

Five bioanalytical methods for pharmacokinetic determination in serum, urine and saliva have been successfully developed, whereby four of them have been validated according to the strictest criteria of FDA and EMA bioanalytical method validation guidelines (European Medicines Agency 2012b, Food and Drug Administration 2001).

5.1.1 Methods for determination of drug concentrations in serum

Aliskiren

The developed HPLC-ESI-MS/MS method allows for a fast and reliable determination of serum concentrations of aliskiren requiring only 100 µL of human serum with the widest linear calibration range from 0.146 – 1200 ng/mL and the lowest LLOQ of 0.146 ng/mL reported at the date of publishing in 2012.

The reported assay can be applied to paediatric patients due to the small serum amount (100 µL) required and its high sensitivity. In 2012, the lowest LLOQ of published HPLC-MS/MS methods was 0.25 ng/mL utilising 700 µL (Tapaninen et al. 2011a) for determination which was successfully halved to 0.146 ng/mL through the current method requiring a sample volume of only 100 µL serum. This sensitive method requiring small serum quantities is potentially suitable for highly accurate and precise determination of serum concentrations of aliskiren in term-born or premature babies, assessed by intra- and inter-run determination of 0.4 % to 7.0 % and 0.6 % to 12.9 %, respectively, with a bias of -0.9 % to 1.8 %.

Interferences by serum components on co-administered drugs did not affect the quantitative determination of aliskiren. No relative matrix effect obtained at three concentration levels in six different human sources was detected. The applied SPE was successfully optimised to the drug properties attaining better sample clean-up and leading to high recovery values of 89 % to 98 % which is more effective compared to other extraction results by Tapaninen *et al.* of more than 80 % (Tapaninen et al. 2010a). Low pH value combined with polymeric mixed-mode strong cation exchange SPE revealed mean absolute matrix effects of -8.35 % to -23.1 % indicating ion suppression over the whole calibration range. The obtained matrix effects and the recovery of the current assay resulted in a high process efficiency of more than 72 %. These results in combination with the successfully examined selectivity by fourteen different drugs proved the ability of the reported assay to discriminate against matrix, background and other chemical species.

The range of the current assay (0.146 ng/mL to 1200 ng/mL) sufficiently enables the quantification of the high inter-subject variability of aliskiren already demonstrated in adults and elderly people. After

administration of 300 mg aliskiren to healthy volunteers, mean maximum plasma concentrations of 374.5 ± 290.8 ng/mL were measured (Vaidyanathan et al. 2007b). Moreover, in elderly people the same dose results in concentrations of 604.1 ± 605.4 ng/mL (Vaidyanathan et al. 2007b). Furthermore, increased maximum plasma concentrations due to drug-drug interactions of up to 81 %, (Vaidyanathan et al. 2008a, Rebello et al. 2011a, Rebello et al. 2011b) nutritional effects reducing its concentration by up to 80 % (Product information aliskiren 2012, Tapaninen et al. 2011a) and influences of diseases like renal impairment revealing mean C_{max} values of 545.7 ± 430.2 ng/mL (Vaidyanathan et al. 2007a) are all likewise covered by the available assay ensuring its broad applicability.

The applicability of the assay for routine analysis was confirmed as all conducted stability investigations were within the required accuracy and precision limits of ± 15 % (± 20 % at LLOQ) compared to nominal concentration. Furthermore, Sullivan *et al.* (Sullivan et al. 2013) investigated two different doses (2 mg/kg body weight and 6 mg/kg body weight) of aliskiren in children of 6 years of age and older. The aliskiren concentration ranged between 2 and 485 ng/mL aliskiren. These values are well within the concentration range of the implemented aliskiren assay.

Finally, it was shown that biphasic absorption of aliskiren is neither correlated to the dosing of 300 mg once or 2 x 150 mg (at once) nor by a light breakfast. In all cases the double peak phenomenon was visible.

Enalapril/enalaprilat

Enalapril and enalaprilat were both determined by an LC-MS/MS method. The conducted literature search let on about apparent differences between published results on drug concentration and correlated pharmacokinetic parameters of enalapril and enalaprilat. This seems to be related to the different analytical methods which were applied (radio immunoassay (RIA), enzyme immunoassay or LC-MS methods). While pharmacokinetics determined by RIA overestimates the drug levels of enalapril and enalaprilat, the specific and more sensitive approaches of liquid chromatography/ tandem mass spectrometry methods appear more precise (Ramusovic 2013).

Unfortunately, all available paediatric datasets available in literature are based on RIA and explain why these datasets are graded as unreliable. This drawback can now be appropriately addressed by the novel child-appropriate LC-MS/MS assay utilising 50 µL serum only. This volume is the lowest required so far for reliable high-throughput determination of enalapril and enalaprilat concentrations in serum and, in turn, enables high-value concentration-time profiles with 12-15 sampling points even in neonates. The required volume of 750 µL for this tremendous dense sampling in a child represents one sixth of the maximum

volume that should ethically be drawn with in four weeks in neonates with only 2 kg (!) body weight. The established calibration range of the successfully validated LC-MS/MS assay covers reported peak concentrations but also adequately addresses the known target mediated drug disposition of the drugs through a low limit of quantification (175 pg/mL enalaprilat and 195 pg/mL enalapril). The only published clinical study evaluating the pharmacokinetics of enalapril and enalaprilat in children reported mean concentrations between 2 to 25 ng/mL (Wells et al. 2001). These concentrations were fully covered by the here presented assay and confirm its applicability for paediatric research.

The assay's intra- and inter-run precision was 2.2 to 5.0 % and 3.0 to 10.8 % for enalapril and 4.9 to 18.0 % and 10.9 to 18.6 % for enalaprilat. The relative error was at the most 3.3 % for enalapril and -4.6 % for enalaprilat. This novel high-throughput assay will be applied in a paediatric clinical trial covering neonates up to adolescents (LENA) and will for the first time make systematic pharmacokinetic data in paediatric patients of all age groups with heart failure available. Current contradictory results (Ferguson et al. 1982, Ulm et al. 1982, Nakamura et al. 1994) on pharmacokinetic parameters like half-life, AUC etc. in children with heart failure emphasise the need for the assay to clarify this antagonism. By applying the assay within the LENA project, this research work might lead to more reliable data for a safer treatment of this vulnerable population.

5.1.2 Method for determination of drug concentrations in urine

A novel LC-ESI-MS/MS method was successfully developed and validated which simultaneously precisely quantifies the sole orally available direct renin inhibitor aliskiren and the angiotensin converting enzyme blocker enalapril as well as its active metabolite enalaprilat in undiluted urine. This LC-MS/MS method allows to reliably quantify all three analytes of interest utilising 100 µL of undiluted urine by encompassing a wide calibration range of 9.4 – 9600 ng/mL of aliskiren, 11.6 – 12000 ng/mL of enalapril and 8.8 – 9000 ng/mL of enalaprilat using quadratic squared regression with $1/x^2$ weighting. The broad ranges enable a precise detection of the concentration in urine without diluting the sample even if samples are highly concentrated by the kidney due to e.g. low fluid intake of the patients during the day. The known high inter- and intra-individual variability in urine composition and the resulting variable matrix effect have been effectively reduced to a minimum. The variability amongst all investigated sixteen individuals concerning relative matrix effects range between 1.25 to 6.62 % (CV) for all three analytes confirming the high purification of the highly complex sample matrix. This allows for the possibility to apply this assay to healthy and impaired patients whose diseases influence the renal excretion and subsequently change the matrix.

The developed method reports on the simultaneous urinary analysis of aliskiren and enalapril/enalaprilat utilizing LC-MS/MS as the detection method. There are, however, already reports on individual assays on the analysis of urinary aliskiren and on urinary enalapril/enalaprilat. For aliskiren, an LC-MS/MS assay was developed (Waldmeier et al. 2007), for enalapril/enalaprilat a radio immunoassay (Rippley et al. 2000) was used. Compared with the individual assays reported by Waldmeier and Rippley, the here developed method has several advantages. First, the run time of the here developed assay is substantially lower (7.5 minutes in this assay compared to 80 minutes in the one reported by Waldmeier *et al.*). Second, urinary enalapril in this developed assay will generate data which is more specific than data generated so far, since Rippley *et al.*´s urinary enalapril/enalaprilat concentrations are based on an unspecific radio immunoassay with a polyclonal antibody. Third, the upper limit of quantification in this developed assay is the highest reported so far allowing for a broad applicability even to highly concentrated urine samples and, thus, is less sensitive to the highly variable renal capacity of concentrate urine. Fourth, comprehensive analysis of matrix effects caused by the known highly variable composition of urine samples were intensively evaluated which were not reported to be assessed by Rippley *et al.* or Waldmeier *et al.* Thus, this manuscript provides a suitable analytical method for the determination of all compounds in a routine setting and gives detailed data about urinary excreted amount-time profiles of aliskiren and enalapril/enalaprilat over five elimination half-lives of each drug.

This urinary assay was mainly developed to investigate the excreted amount of these drugs to gain more knowledge about the excretion pathway especially in rarely investigated populations like children. Age is related to kidney maturation which accounts for drug elimination; especially for enalapril and enalaprilat. This represents an important aspect to be investigated for a safe pharmacotherapy in children.

Even though urine data for aliskiren is only rarely available, most authors give information on 150 mg instead of 300 mg aliskiren which makes a comparison difficult as non-linear pharmacokinetic behavior of the drug is known (CHMP Product information aliskiren 2012). Nevertheless, calculated parameters reported by Vaidyanathan *et al.* (Vaidyanathan et al. 2007a) who administered 300 mg aliskiren to an adult population mentioned a renal clearance of 1.4 L/h for the healthy volunteers which is identical to the value obtained within this investigation. Furthermore, the amount excreted to the urine is about 0.6 % within 24 h of the administered dose, which was likewise confirmed by 0.8 %. Regarding enalapril and enalaprilat, renal excretion rates of 19.86 and 30.2 %, respectively, had been reported after administering a 10 mg dose (Rippley et al. 2000) which was also confirmed through this investigation with 19.19 % for enalapril

and 27.18 % for enalaprilat for the given dose. By comparing these findings to the rare data available in literature, the suitability and reliability of the developed method was demonstrated.

Since there is still an ongoing discussion among experts about the benefit to risk ratio of combined use, this analytical method enables a detailed analysis of pharmacokinetics regarding renal excretion especially of high risk patients. In particular, by combining this assay with small quantity methods in the serum of all three analytes of interest (introduced in section 3.2.1), extensive knowledge of the pharmacokinetic behaviour in poorly investigated populations like paediatric patients can be gained.

5.1.3 Method for pharmacokinetic determination in saliva

An innovative approach was followed through the development of LC-ESI-MS/MS methods that quantifies the sole orally available direct renin inhibitor aliskiren and enalapril/ enalaprilat for the first time in saliva.

It is known that saliva varies highly if compared to serum as both the pH value and the composition (proteins, electrolytes, water concentration etc.) show high intra- and interindividual variability. The composition of children with e.g. Down syndrome, who are known to often have congenital heart failures and therefore regularly require cardiovascular medication, show different salivary compositions than children or adults (Siqueira et al. 2007). In order to apply the salivary method to all age groups which might require cardiovascular medication like aliskiren, the matrix effect had only been minimised through solid-phase extraction in combination with gradient elution of the LC-method.

By applying 100 µL saliva, this LC-MS/MS method allows to obtain reliable pharmacokinetic data within the concentration range of 0.586 – 1200 ng/mL of aliskiren. This range was established on assumptions based on measurable serum concentrations, pH, pka and free fraction of aliskiren and its solubility. The reported plasma protein binding of aliskiren (Waldmeier et al. 2007) coupled with the pka of about 9.45 suggests a theoretical penetration of about 50 % of aliskiren into saliva. In contrast to this calculation, only low levels of the direct renin inhibitor were detected in saliva after administration of 300 mg aliskiren to the three participants.

The C_{max} value in saliva was about 1 % of that in serum. No additional drug substance was eluted by belated elution of the saliva swabs, which denotes that the reason for the low penetration lies in the substance itself and its distribution. As a consequence, the areas under the curves differ in the same manner. The ratio of area under the concentration-time curves$_{(0\text{-}4.9h)}$ in saliva and serum calculated by trapezoidal method varies between 0.008 and 0.01. For the whole observation period the ratio of AUC was 0.09 to 0.20. The determined t_{max} was similar in both fluids. Since only the free fraction can penetrate into saliva

a probable reason of the low findings might be that the reported protein binding of approximately 50 % in humans is too low. In marmosets the binding was 92 % (European Medicines Agency 2007).

When compared to serum in the first five hours, the saliva sampling described the pharmacokinetics of aliskiren in the central circulation with a good correlation between both fluids (r = 0.92). For longer postdose sampling (> six hours), this good correlation was not confirmed through this trial as the saliva concentration did not decrease similar to the serum concentrations. The comparison of the absorption rates of both peaks in serum and saliva varied from 0.72 to 1.12 and confirmed the similar behaviour of aliskiren in both fluids. The arbitrary visual distinction which was supported by the results of the non-compartmental model indicated slightly more comparable ratios in $ke_{0\text{-}4.9h}$ than in $ke_{0\text{-}192h}$ with less variability in the results obtained from the three volunteers. Taking the high volume of distribution of aliskiren (135 Litres) into account, this might indicate an additional liberation of the drug from other compartments like the surrounding tissue instead of sole penetration by the saliva glands at later observation time points. The liberation of the drug from the surrounding tissue especially changes its concentration in biological fluids that are of low-volume. This might explain why the liberation from the surrounding tissue results in detectable fluctuations in saliva concentration and was not detected in blood/serum before.

Moreover, the amount of drug which penetrates into saliva correlates with the salivary pH value and saliva flow which can both change after a meal. Since the penetration rate shifted approximately from 5 hours after drug administration until end of the investigation period, this shift cannot be justified by changes due to ingestion as these should only be of short-term. The observed shift in the penetration ratio of all three patients nearly five hours after administration cannot be fully substantiated by what is currently published in literature and the reasons of such a shift can for now only be assumed.

Regarding enalapril the correlation through visual inspection between measured salivary and serum concentrations were good. However, the salivary concentrations of enalaprilat did not alter comparable to the serum concentrations when measured by an API 2000. The fact that determined concentrations of the active metabolite in saliva and serum cannot be correlated explains the decision to refrain from further investigations on both compounds within this thesis.

5.2 Bioanalytics for determination of hormones and proteins allowing for the assessment of pharmacodynamics within the RAA system

The developed low-volume assays for pharmacodynamic analysis of the renin-angiotensin-aldosterone system were successfully established for angiotensin I, angiotensin II, plasma renin activity, renin and prorenin. The reduced volume required for the determination of all five parameters in plasma (1.05 mL for all parameters per sampling point) enables the investigation of the pharmacodynamics in neonates and toddlers. This total volume is comparable to the commonly drawn volumes for only one parameter within the recently conducted paediatric studies. As a consequence, the low-volume assays enable either a much denser sampling of one pharmacodynamic parameter or allow for a more comprehensive sampling of the whole RAA system without infringing on ethical considerations for the withdrawable blood volume in children. Given the maximum drawable blood volume in neonates with 3 months of age for clinical care and paediatric research (~8 mL plasma), the developed assays facilitate obtaining a complete PD profile of the RAA system with 4-5 sampling points per patient plus a corresponding sampling for pharmacokinetic determination. Besides the focus on paediatric patients, the chosen calibration range of the applied assays was broad enough to cover blood levels in adults and in children. For instance, the mean angiotensin II levels in adults reported by Shimamoto *et al.* (Shimamoto et al. 1984) after overnight fasting were 12 ± 2.1 pg/mL (mean $\pm$ SE) while Miyawaki *et al.* investigated angiotensin II concentrations in the early neonatal period. They concluded that children with low birth weights of 1500 g have significant higher levels of angiotensin II than children with normal birth weights (>2500 g) [19 vs 76 pg/mL] (Miyawaki et al. 2006). Levels of angiotensin II are additionally elevated in patients with e.g. heart failure owing to the activation of the RAA system. However, published results were covered by the developed angiotensin II assay. Plasma renin activity (PRA) is known to vary highly between adults and children. Dillon mentioned 15-fold higher values for children (Dillon and Ryness 1975). Therefore, a selective RIA assay was established which indirectly measured PRA utilising angiotensin I. The assay offered the flexibility to adjust the incubation time, the latter being an important parameter for creating reliable results since in the indirect method, the amount of angiotensin I available per sample is limited. By adjusting the time, it can be ensured that a sufficient product (Ang I) for conversion is available at all time and that therefore the plasma renin activity can be precisely determined.

By now the little which is known in paediatric pharmacotherapy about drugs acting on the RAA system is based on many different studies and assays. In the past, this obstacle resulted in situations of contradictory results and complicated interpretation. The here developed platform enables the comprehensive

investigation of all parameters within one trial, even in neonates, owing the low-volume it requires for full pharmacodynamic sampling and evaluation. With regard to paediatric pharmacotherapy, this novel platform consequently excludes possible bias due to the different handling of samples, the different determination methods or assays, the different investigated populations etc. and facilitates a more reliable insight into pharmacodynamic processes.

Although several optimisation approaches were investigated during the development of the methods, two main aspects were kept unchanged to avoid full validation. First, antibodies were always kept in excess as the blood volume was only reduced and not increased. Second, the volume in a cavity during the measurement step was left unchanged to always assure the same known path length in the cavity. This is of high importance for precise determination by e.g. photometric detection as the sample concentration can be deduced from the known path length and attenuation coefficient (Lambert beer law). Given that partial validation is expected sufficient if the analytical method is transferred from one laboratory to another, or if only a limited volume of blood is available, all methods were successfully evaluated regarding accuracy and precision prior to the implementation of the assays.

5.3 Proof-of-concept study

In summary, the proof-of-concept study confirmed the applicability of the child-appropriate assays for determining phramacokinetics of Aliskiren, enalapril, and Enalaprilat and the corresponding changes in the humoral parameters in the RAA system. These assays reliably quantified blood, urine, and saliva concentrations of both drugs and the corresponding humoral parameters over the course of 5 elimination half-lives.

Tailoring drug assays to determine drug concentrations and biomarkers of the RAA system in small-volume samples fully succeeded with the required blood volumes. Despite the dense sampling (in total, 40 pharmacokinetic sampling points for one pharmacokinetic profile), the tailored assays applied here allowed for the determination of drug concentrations and the subsequent changes in humoral parameters in only 45 mL blood per subject. Although the drug concentrations were determined in a maximum of 100 µL serum per sampling point, all five humoral parameters of the RAA system were analysed in 2.1 mL blood. Compared to Sullivan *et al.* (Sullivan et al. 2013) who investigated only one pharmacodynamics parameter in children and required 2 mL of blood, this optimisation in sample volume by developing the low-volume asssays should be a huge benefit in paediatric pharmacotherapy. By applying this bioanalytical platform, evaluation of pharmacokinetics and pharmacodynamics is even possible in neonates and infants. This novel option facilitates obtaining sufficient data points per child and circumvents the population pharmacokinetic approach with its possible imprecisions. The results obtained by the low-volume assays proved their reliability by comparing them with values in the literature. This legitimizes the chosen bioanalytic settings to be applied for pediatric populations and provides systematic data sets for enalapril/enalaprilat. For aliskiren, all bioanalytic assays were suitable for a precise determination, except the angiotensin I assay. Because of the low angiotensin I baseline in the aliskiren group, the expected decrease in concentration was not detectable. Consequently, its suitability for determining those decreases are not yet possible, and further optimisation is required for future investigations.

Additionally, this comprehensive pharmacokinetic and pharmacodynamic dataset allows to validate physiological-based computer models that are of increasing interest for an optimal planning and conduction of paediatric clinical trials. The modelling approach is highly recommended by EMA and FDA for sophisticated paediatric research. The combination of both tools enable the future conduct of high quality clinical trials in the paediatric population with a maximum of gained knowledge by requiring the

minimum of blood volume. Such an acknowledged approach is highly useful to gain paediatric use marketing authorization in future.

The findings regarding the pharmacokinetics of aliskiren in adults were in line with those in literature and reflected high inter-individual variability in serum concentration and a low urinary excretion. Aliskiren displayed a biphasic absorption behaviour in varying degree among all healthy volunteers, that could be precisely described by applying the dense sampling used in this study. Currently, mean concentration-time profiles for Aliskiren are primarily published, which only vaguely suggest this behavior. The reason for this double-peak phenomenon can only be assumed because it has not been previously reported in detail. Since data on the novel direct renin inhibitor aliskiren is limited, a literature search was performed to check for similar behaviour in other drugs. A comparable behaviour with a first small peak followed by a second, much larger peak can be seen with phenazopyridine (Shang et al. 2005), but the reason has not been reported either. In literature, double peaks are generally correlated with e.g. enterohepatic recirculation, change in salt, change in bile solubility, with the formulation, with p-glycoprotein etc. For aliskiren, a change in salt, an additional absorption site along the gastrointestinal tract, or precipitation are likely but needs to be investigated further.

The mean variability in AUC of 65 % in this study is in line with data reported by Vaidyanathan *et al.* (Vaidyanathan et al. 2008b) who claimed a range between 40 to 70 %. Because of the confirmed high intra- and inter-individual variability, population pharmacokinetics and spare sampling are not advisable for aliskiren. Moreover, the elimination half-life obtained by this study differed from those compared in previous publications. In studies with an investigation period of 96 hours, a half-life of 40 hours was claimed (Vaidyanathan et al. 2008b), but this study, with its investigation period of 192 h, revealed a mean elimination half-life of 75.6 hours (median, 65.9 hours). The long sampling period fulfilled three aspects: first, the sampling period covered nearly the whole elimination phase being per definition 5 drug half-lives after its administration. Unfortunately, not exact 5 elimination half-lifes were covered as the the investigation period of the study was designed on elimination half-lifes published in literature before. Since in the here presented study a longer half-life was determined than aforementioned it got obvious that not the full elimination phase was covered; second, the sampling period enabled an exact determination of the elimination constant as it reflects the “pure” elimination and no other process like absorption or distribution; third, it promoted the investigation of target mediated drug disposition of aliskiren because this phenomenon is best detectable in the late elimination phase (Gossas et al. 2012).

These results should be considered during the planning and conduction of future studies; in particular, the calculation of the washout period in crossover studies should be made carefully because aliskiren elimination is much slower than previously assumed. It was possible to determine drug concentrations even three weeks after a single oral administration. The other pharmacokinetic parameters, such as C_{max} and T_{max} as determined by the low-volume LC-MS/MS assay, were generally comparable to those found by Vaidyanathan et al (Vaidyanathan et al. 2008b). Likewise, the amount recovered in urine (0.7 %) was comparable with Waldmeier *et al.*'s data (Waldmeier et al. 2007) who found 0.6 % aliskiren and reported the main excretion pathway to be faecal. The mean CL_R was comparable to that reported by Vaidyanathan *et al.* (Vaidyanathan et al. 2007a). As mentioned, weeks after administration of Aliskiren, drug concentrations were still detectable in concentrated urine samples, which accounts for a more prolonged elimination phase than previously assumed.

Pharmacokinetic simulations for aliskiren are available using input data reported by Nussberger *et al.* (Ramusovic and Laeer 2012). As illustrated above, these reported pharmacokinetic results appear imprecise as they seem to underestimate the aliskiren concentrations. Therefore, these simulations require revalidation based on current published individual data such as the data published here to allow for reliable predictions in the future. This is of especial importance if the model is to be scaled to the paediatric age groups as the goodness of model is mandatory for reliable predictions and for a proper treatment of this vulnerable population.

For enalapril, successful implementation of assays to determine serum and urinary concentrations were demonstrated. Mean C_{max} values of 160 ng/mL were obtained for the prodrug enalapril, as well as 53 ng/mL for the active metabolite Enalaprilat, which are comparable to those of Najib *et al.,* who reported 124 ng/mL and 54 ng/mL also using LC-MS/MS (Naijb et al. 2003). While enalapril was rapidly absorbed within one hour after oral administration, the bioactivation by hepatic esterases is responsible for the "delayed" t_{max} of enalaprilat at about 4 hours postdose (Sweet et al. 1981). Determined elimination half-lives of enalapril (0.9 h) and enalaprilat (10 h) were consistent with Najib *et al.* The bioavailability of enalapril is about 50 %, which is primarily excreted renally. This study found a mean total renal excretion of 44 % of the administered dose, which differs slightly from data represented by Rippley *et al.,* who found 50 %, and Noormohamad *et al.,* who reported 51 % (Noormohamad et al. 1990, Rippley et al. 2000). This difference might be explained by the variability in bioavailability of 21% to 61%, which, consequently, affects the amount excreted into urine (Noormohamad et al. 1990). The amount of enalaprilat in urine was 27 %, which is comparable with other reported values of 30 – 36 % (Kelly et al. 1986, Noorhohamad

et al. 1990, Rippley et al. 2000). The conversion rate of enalapril and enalaprilat (61 %) was within the ratios determined by Till *et al.* (67 %) (Till et al. 1984) and Rippley *et al.* (60 %) (Rippley et al. 2000). Observed CL_R of enalapril (15 L/h) and enalaprilat (8 L/h) were also in line with the literature (Ulm et al. 1982, Till et al. 1984). All findings confirmed the suitability of the preparation of urinary samples, avoided extensive degradation, and prove the applicability of the applied low-volume assay. However, it became obvious that the chosen serum sampling points of the study did not sufficiently cover the late phase of the drug elimination phase, especially of enalaprilat, in order to conduct precise pharmacokinetic determinations such as evaluation of the elimination constant. For further planning it is therefore important to establish additional sampling points over the time period of 24 to 72 hours. In addition, this incident exemplified the importance of modeling in paediatric studies of enalapril as it is known that sample points require adaptations to cover the whole drug exposition in different age groups in children (Willmann 2009) and the modeling and simulation approach allows to predict optimal sampling point and enables optimal data density per individual patient.

To the best of our knowledge, salivary concentrations of aliskiren have not previously been determined. However, the replacement of invasive pharmacokinetic sampling using saliva seems to be inappropriate for the drugs investigated. The evaluation of samples by validated assays did not result in saliva drug profiles comparable to profiles obtained in serum, which prohibits a meaningful interpretation of the data. The aliskiren concentrations obtained were substantially less than the expected 50 % penetration from serum to saliva, as calculated based on physicochemical properties. The penetration ratio was derived from the total drug exposure as calculated through AUC range from between 0.5 – 8 %. There was a change in the penetration at five hours, which persisted for the next days. The shift in penetration rate was observed in nearly all volunteers taking aliskiren and was evaluated by a posteriori analysis. The subsetting for 0 to 5 hour and 0 to 192 hours was performed after visual inspection of the data and was strengthened by the estimations of pharmacokinetic parameters using a non-compartmental analysis. The persistence points towards additional drug penetration from other compartments as the cause of the change because ingestion would have resulted in a temporary change only. However, a similar finding has not been reported in literature, and the reason for such a shift requires further research.

Regarding enalapril and enalaprilat, the expected low penetration rates, based on theoretical assumptions about the unbound fraction in blood and the pk_a values, were confirmed. Nevertheless, although salivary enalapril lconcentrations are comparable to concentrations in serum, the pharmacokinetics of the active metabolite enalaprilat was not covered sufficiently by saliva when compared to serum. The reason for the

poorer penetration of enalaprilat into saliva might be due to the drug's ability to form a zwitterion, which could contribute to insufficient penetration from serum into saliva. Based on these findings we cannot recommend using saliva as a biological fluid to determine the pharmacokinetics of the two drugs.

Both drugs showed marked and sustained inhibition of the RAA system in healthy volunteers. The measured changes in all humoral parameters were in temporal concordance with serum drug concentrations. They confirmed the current knowledge about the RAA system, with the exception of angiotensin I in the aliskiren group, because of the low predose values. Levels of the latter fall below the quantification limit and hinder a meaningful profile. Although the first parameters changed within 0.5 to 1 hour after administration of aliskiren, change took about 3-4 hours in the enalapril group because of the bioactivation of enalapril to enalaprilat. Although the determined t_{max} of aliskiren in this study was about 2.7 hours after administration, most humoral parameters were altered earlier. Aliskiren showed a double peak phenomenon, which suggests that the first peak of the determined double peak was sufficient to intensively interact with the RAA system. This first peak appeared between 0.3 – 1.5 hours after oral administration. Additionally, this assumption is supported by the aliskiren concentrations of the first peak (162 ng/mL), which exceed the IC_{50} of renin (0.6 nmol/L) (Wood et al. 2003). Even on day 9 of the investigation, the evaluated mean drug concentration in serum was 1.2 ng/mL and, therefore, still accounted for at least 50 % inhibition of the target structure.

The detected rise in renin concentration after oral administration of aliskiren and enalapril was comparable to the observations of Nussberger *et al.* (Nussberger et al. 2002). They mentioned an up to 20-fold dose-dependent increase after administration of 640 mg aliskiren, whereas this study showed a 19-fold rise in renin following a 300 mg aliskiren hemifumarate administration (maximum approved dose). Furthermore, after the administration of 20 mg enalapril, a 16-fold up-regulation of renin was confirmed through the child-appropriate assay. The time to reach the highest mean renin concentration under aliskiren treatment differed between Nussberger *et al.* and this study (10 vs 5 hours). The difference might be explained by the much denser sampling in this study, which allowed a more precise determination of the t_{max}. Furthermore, by comparing the results obtained by Nussberger *et al.* with those of the current study, the data from Nussberger *et al.* showed a C_{max} of renin with enalapril after 3 hours, whereas, with Aliskiren, it was reached 10 hours postdose. With regard to the known pharmacokinetic parameters of both compounds, an earlier C_{max} with aliskiren and a later one with enalapril treatment would be more rational. This study found the C_{max} of renin after administration of the compounds at about the same time

postdose (5 hours aliskiren vs 6 hours enalapril). The variability in C_{max} of the renin was equally high. Finally, an additional benefit for future paediatric studies is the low required volume of only 250 µL plasma.

The alteration of PRA with enalapril/enalaprilat and aliskiren, respectively, was comparable to that found by Nussberger *et al.* (Nussberger et al. 2002). Nevertheless, an appreciable improvement was gained by the reduction in required blood volume for a reliable determination. Although Sullivan *et al.* required 2 mL of blood per sampling point (Sullivan et al. 2013), only 400 µL of blood was necessary for reliable determination of PRA in children in this study.

The low-volume assays developed reliably determined angiotensin II concentrations. Because available commercial assays to determine angiotensin II were less sensitive, a combination of high sample purification and sample concentration by solid-phase extraction and an ELISA with monoclonal antibodies were established to measure the low concentrations adequately. This enabled detection in 500 µL plasma, which is a reduction of up to 75 % compared with the required volumes of kits available on the market. The decreased angiotensin II concentrations with enalapril were comparable to the ones reported by Brunner *et al.* (Brunner et al. 1981). This study found a decline of 64 %, whereas Brunner mentioned 58 %. The slight deviation in angiotensin II concentrations is best explained by the different analytic methods and the high variability observed. The reduction in angiotensin II results with aliskiren of 70 % were comparable to those found by Nussberger *et al.,* who claimed a 70 % decline with 160 mg aliskiren.

In the course of the later phase of the study for some pharmacodynamic values, higher/lower values were measured than the selected C_{max} or C_{min} concentrations for the evaluation of the volunteers' data. If such a value occurred at time points $\geq$ 24 hours and could be classified as an outlier because the value followed no trend, it was excluded from the evaluation. Especially the facts that two different blood collection systems (indwelling catheter on the first investigation day and direct venepuncture on the following days) and that the volunteers did not stay overnight at the institute but instead each day went to the sample collection in the morning might indicate reasons for such deviations. Variability in the day-to-day heart rate of day 2 till end of study additionally supports this assumption if the rate is compared with the values of day 1. However, those findings show how important a sophisticated study planning and conduction are for the generation of reliable datasets.

A further observation being connected to the above-mentioned finding relates to the change in variability of some humoral parameters if the first 24 hours were compared to the late phase of the study (24 -192 h). The encountered increase in variability (expressed as standard deviation) for late time points might

also be explained by the different blood collection system. The time-point 24 h (first time when volunteers is pricked with the butterfly by a different person than on day 1) was often characterised by a different alteration in humoral parameters than expected. Especially if the volunteer required a repeated prick with the butterfly to successfully draw the blood, a change in the humoral parameters is most likely owing to the stressful situation. Is was noticed that this unexpected alteration per individual subject was less or not detectable for very late time-points which might account for an adaptation of the volunteer to the situation and supports the assumption.

The conducted proof-of-concept study did not only verify the applicability of the low-volume assays, but also provided a dense and comprehensive dataset of healthy volunteers. This comprehensive dataset of drug concentrations and their effect on humoal parameters allow to validate physiological-based computer models for pharmacokinetics and pharmacodynamics. Those models are of increasing interest for an optimal planning and conduction of paediatric clinical trials. The modelling approach is highly recommended by EMA and FDA for sophisticated paediatric research (Food and Drug Administration 2012, European Medicines Agency 2012c). The combination of both tools enables the future conducting of high quality clinical trials in the paediatric population with a maximum of gained knowledge whilst requiring the minimum blood volume. Such an acknowledged approach is highly useful gaining paediatric use marketing authorisation in the future. The usefulness of the dense sampling in this proof-of-concept study was shown by the fact that the first available model for aliskiren based on concentration levels reported by Nussberger *et al.* (Nussberger 2002) which deviate significantly to the drug concentrations found through this study. Since recent publications on pharmacokinetics of aliskiren revealed comparable parameters to those determined within the proof-of-concept study, this suggests that the presented individual concentration levels are more meaningful. And it seems that the concentration levels reported by Nussberger in 2002 underestimate the “real” levels. As no further individual concentration-time profiles of aliskiren are currently available, the obtained dataset should support the further development and improvement of existing models through more reliable datasets. It enables more precise predictions and contributes to a better study planning.

The study was not designed to directly compare the direct renin inhibitor aliskiren and the ACE inhibitor enalapril instead of investigating the applicability of the low-volume assays. Additionally, an explorative pharmacological approach was not at all intended to be established with the proof-of-concept study. This justifies the single sequence study design instead of a cross-over design. Additionally, the concept avoids any cross-reaction or bias especially on the pharmacodynamic side because investigations prior to this

study revealed that aliskiren can be detected in urine weeks after the drug was administered (Nussberger et al. 2002). This suggests that its effect might also persist and would influence the subsequent investigations.

All patients were monitored by regular measurements of patients' blood pressure and heart rate during their stay at the institute. On the one hand, the collected data was used to evaluate the safety of all patients. Only one person with a body weight of 52 kg once showed short term reduction at the t_{max} of enalaprilat. Nevertheless, the volunteer was asymptomatic. On the other hand, the data was used to investigate the pharmacodynamic effect of both drugs on blood pressure and heart rate. The collected data indicated that in healthy volunteers, the drug intake of an antihypertensive drug has no significant influence on the parameters over time. A comparison of predose, 24 h and end-of-study values did not indicate a reduction in systolic and diastolic blood pressure or heart rate. The latter showed slight increases in the time frame of 4 – 8 hours postdose. The effect cannot be clearly assigned to ingestion activity or drug effect as both, the t_{max} of the compound as well as time point of meal, coincide at about 4 hours after drug intake. The findings for blood pressure argue for potent counter regulation of the healthy organism by other pathways such as the autonomic nervous system. Data from literature confirm this finding (Stangier et al. 2000, Nussberger 2002).

5.4 Establishment of a Good Clinical Laboratory Practice-compliant quality system for bioavailability studies in support of marketing authorisation by EMA

The successful implementation of the GCLP-compliant processes was a key success factor for establishing the “ready-to-use” platform. The covered elements included the facilities, staff, validated assays according to current international guidelines, reporting and archiving procedures encompassing storage of paper- and electronic data as well as specimens. Embedding the validated bioanalytical assays into this quality system accounted for reliable and accurate test data within clinical studies but also contributed to auditable, consistent and reproducible data. Since the conducted Phase I study within the LENA project is to be followed by several other paediatric studies, the established quality standard warrants a maximum standardisation within the project. To date, the required assays for determination of pharmacokinetics of enalapril/enalaprilat are fully established, however, the corresponding bioanalytical assays for pharmacodynamic evaluation are to follow. This will be necessary to empower the full potential of the “ready-to-use” platform for prospective paediatric studies. It was not implemented at the Institute of Clinical Pharmacy and Pharmacotherapy yet, because the medical laboratory Dr. Spranger, at which the pharmacodynamic assays were conducted, was DIN EN ISO 15189 (Medical laboratories - Particular requirements for quality and competence) and DIN EN ISO/IEC 17025 (General requirements for the competence of testing and calibration laboratories) certified (both valid till 31.12.2014). This was regarded sufficient for the proof-of-concept study, but requires optimisation for the upcoming paediatric studies of the LENA project.

All bioanalytical analyses utilised for the Phase I study of LENA were conducted successfully according to GCLP. Good Clinical Laboratory Practice is an accepted European guidance, but the term is not officially implemented yet. This explains why GCLP is not yet designated to be audited by a regulating authority as is the case with e.g. GCP or GLP. An external audit that confirm the compliance to GCLP has not yet been performed at the Institute but is scheduled for the next year. However, the future audits of the laboratory will monitor the compliance and determine if further improvements are necessary in adherence to the GCLP mind-set. Outcomes of the collaboration between the bioanalytical lab, the clinical site for the bioavailability study and the Institute for applied statistics showed up with a smooth work flow of the corresponding working packages by utilising the applicable SOPs and working instructions. Working processes at the interfaces were characterised by a seamless data transfer accompanied by properly addressed exchange of confirmations, feedbacks or queries. The quality and condition of the provided serum and urine samples, the exchange process for notes-to-file and exchange of experiences to increase

the quality already during the run-time of the Phase I study itself were proof that the important topics were properly addressed in the manuals for sampling, storage and shipment.

Since the laboratory of the Institute of Clinical Pharmacy and Pharmacotherapy provided not only a “ready-to-use” platform by validated bioanalytical assays and processes compliant to GCLP but also supply the involved clinical sites with required devices and materials for blood sampling, storage and shipment, a sophisticated bioanalytical concept in support of Phase I studies was implemented and in addition successfully proven.

6. Conclusion and perspective

In final summary, this thesis presents a novel comprehensive analytical approach of child-appropriate assays enabling the determination of the pharmacokinetics and pharmacodynamics of drugs acting on the RAA system in all paediatric age groups (Figure 45). Owing to the low sample volume required, it also enables the vitally necessary systematic determination of drug concentrations and their effect in neonates and infants. This bioanalytical platform overcomes current ethical, analytical and regulatory obstacles in paediatric research and it primarily facilitates the evaluation of drug exposure, safety and efficacy of widely used e.g. off-label drugs in this vulnerable population and contributes to the availability of information on these drugs. In total, five bioanalytical HPLC-tandem mass spectrometric methods were developed investigating the direct renin inhibitor aliskiren as well as the angiotensin converting enzyme inhibitor enalapril and its active metabolite enalaprilat in serum, urine and saliva. The drive to analyse microliter sampling volumes while obtaining more information per sample was successfully challenged by the ultrasensitivity of the combined SPE and LC-MS/MS bioanalytical methods. Beyond these pharmacokinetic assays, efforts were successfully undertaken to investigate the pharmacodynamics on the renin-angiotensin-aldosterone system by five immunological low-volume assays. This holistic bioanalytical setting is ideally suited for conducting comprehensive clinical studies in poorly investigated populations such as neonates. Against the backdrop of the newly implemented GCLP setting, the bioanalytical setting can be appropriately used in clinical studies by meeting regulatory demands.

The developed low-volume mass spectrometric methods (50-100 µL per sample) ensure the crucial determination even in presence of other concomitant drugs and amongst different sources of biological fluids. The low volume not only enables the determination of drug concentrations in the poorly investigated young age groups, but in addition, it allows for the generation of enriched paediatric pharmacokinetic data in children through denser blood sampling by keeping the overall required blood volume constant. This intensive sampling might give insight into different drug absorption, distribution, metabolism and elimination of drugs if compared with adult data due to e.g. maturational processes. If fewer blood samples are needed, the saved blood volume may also be used to investigate systematically humoral pharmacodynamic markers such as renin, plasma renin activity or angiotensin II which are mandatory to ensure a safe and efficacious drug application. All pharmacodynamic parameters captured by this bioanalytical platform can be determined in a blood volume of as little as 2.1 mL. The conducted scale-up empowers the applicability also in complex and comprehensive paediatric studies. Apart from the

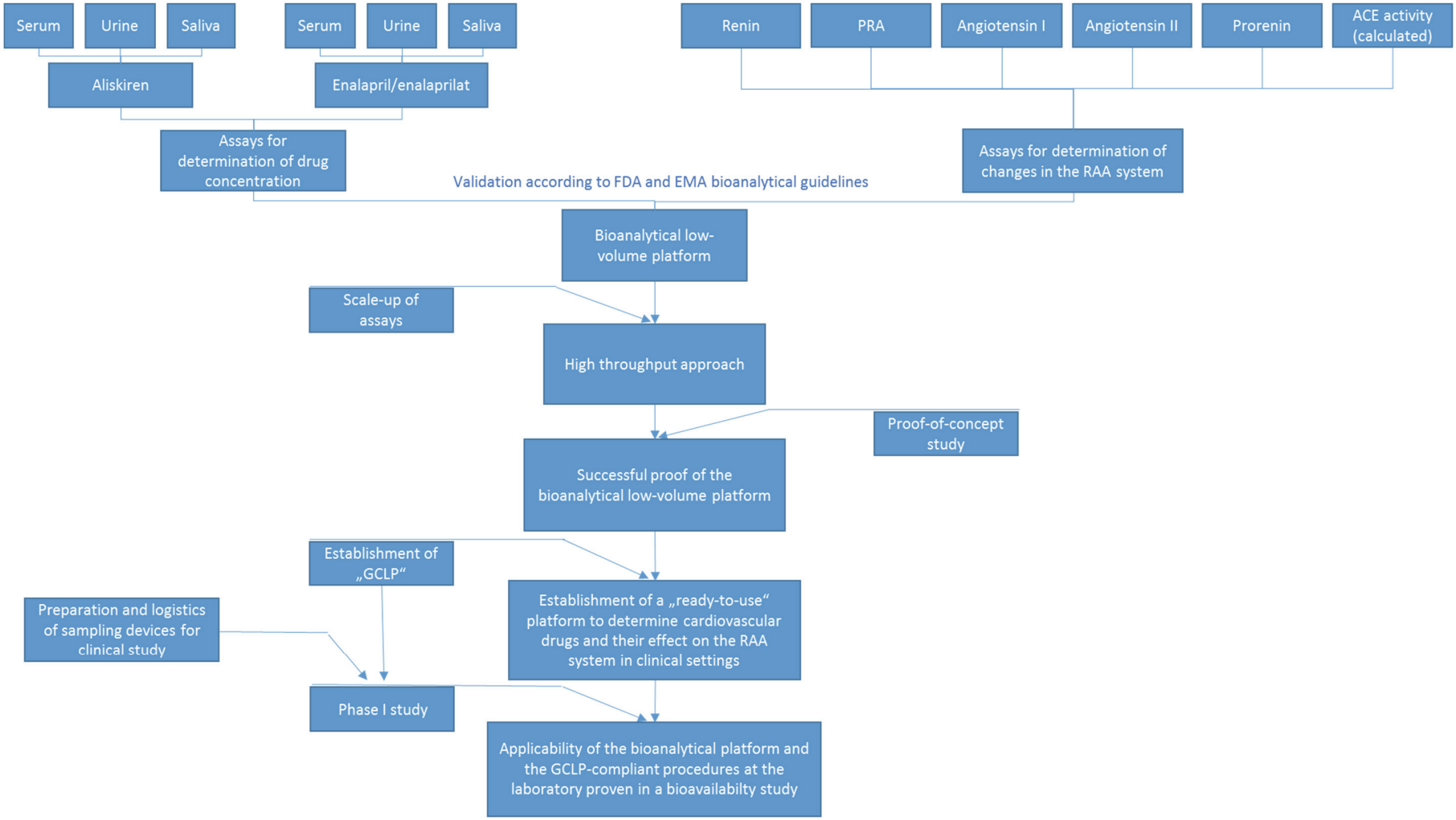

Figure 45 ***Detailed overview of the successfully conducted steps to establish and prove the low-volume bioanalytical platform*** *useful to determine cardiovascular drug concentrations in serum, urine and saliva plus to evaluate the effects on the renin-angiotensin-aldosterone system. Within this thesis a "ready-to-use" bioanalytical platform was installed which is GCLP-compliant and meets current regulatory requirements for sample analysis within a clinical study.*

high sample throughput approach (100-200 samples per day) a further advantage is – based on the microliter approach per marker - the possibility to deal with blood collections by indwelling catheters or if necessary even with reduced blood volume by capillary blood withdrawal. Combining these properties, it offers a high flexibility for paediatric studies.

Furthermore, such an analytical platform is essential for the validation of models and simulations which are promising tools in paediatric study planning and conduction. As the platform encompasses the whole spectrum of RAA system responses following drug exposure across all age groups, it is not only an important step to generate reliable paediatric data on drug concentrations in serum and urine but also to make valuable and comprehensive pharmacodynamic data sets in all paediatric age groups available.

The conducted open-label clinical study in healthy volunteers verified the suitability and applicability of all developed low-volume assays prior to their use in vulnerable populations. Utilising these tailored assays, the pharmacokinetics of enalapril and aliskiren were reliably evaluated by dense sampling of serum and urine over 192 hours. Due to the use of the dense sampling scheme for aliskiren, a detailed description of its biphasic absorption was obtained, the reasons behind are currently being discussed. Due to these findings of the biphasic absorption behaviour of aliskiren, population pharmacokinetic analyses with spare sampling as suggested by EMA and FDA for paediatric studies are not advisable in paediatric patients. Furthermore, the pharmacodynamics in the RAA system after single dose of both drugs were fully investigated and the applied assays enable a comprehensive determination in children. Consequently, these positive findings in blood and urine of the proof-of-concept study empower the established low-volume assays to be applied in paediatric research and their application should increase the knowledge of drug exposure and its effects in children. The child size assays allow for the conduction of paediatric clinical trials in line with regulatory guidelines as well as ethical principles and significantly supports to gain paediatric use marketing authorizations.

In addition, following FDA recommendations to elucidate non-invasive methodologies in the determination of pharmacokinetics in children, a novel and innovative approach of investigating salivary drug concentrations of aliskiren, enalapril and enalaprilat was established and described within this thesis. These investigations in healthy volunteers gave insight into a complex mechanism of the distribution of aliskiren in humans. This notion is based on the different time-dependent penetration rates of the drug entity in both biological fluids. However, it was shown that the salivary sampling and the evaluations of salivary aliskiren, enalapril and enalaprilat concentration do not appear suitable to substitute serum sampling in order to obtain valuable pharmacokinetic data.

Figure 46 illustrates an overview of all established steps to install a GCLP-compliant bioanalytical platform at the Institute of Clinical Pharmacy and Pharmacotherapy.

Owing to the proven suitability of this bioanalytical platform, the setting will be applied in the LENA research project. Whereas in the past, determining pharmacokinetics and pharmacodynamics on paediatric patients was complex and resulted in only few investigations, the bioanalytical platform might result in a more sophisticated investigation especially in large paediatric clinical studies such as in the LENA-project. The idea behind LENA is to address current shortcomings of enalapril in children and to conduct meaningful research in the aim of establishing basis for future paediatric use marketing authorisation (PUMA). The gathering of new information through the planned studies across all paediatric age groups is only feasible due to the developed bioanalytical platform. For the first time, LENA makes sophisticated paediatric pharmacokinetic as well as pharmacodynamic datasets of enalapril and enalaprilat in children with heart failure available. The combination of validated physiology-based modeling, the low-volume bioanalytical platform and a child-appropriate solid dosage form within this project may facilitate overcoming current obstacles in paediatric clinical studies. It empowers the generation of urgently required paediatric datasets in neonates up to adolescents in due consideration of regulatory quality requirements (GxP) and contributes to a safer treatment of the youngest patients amongst us.

7. References

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8. Appendix

Appendix 1 Individual profiles of healthy volunteers 1-3 after administration of 20 mg enalapril maleate 185

Appendix 2 Individual profiles of healthy volunteers 4-6 after administration of 20 mg enalapril maleate 186

Appendix 3 Individual profiles of healthy volunteers 7-9 after administration of 20 mg enalapril maleate 187

Appendix 4 Individual profiles of healthy volunteers 002-004 after administration of 300 mg aliskiren hemifumarate..... 188

Appendix 5 Individual profiles of healthy volunteers 005-007 after administration of 300 mg aliskiren hemifumarate..... 189

Appendix 6 Individual profiles of healthy volunteers 008-010 after administration of 300 mg aliskiren hemifumarate..... 190

Appendix 7 Individual profiles of healthy volunteers 011 - 013 after administration of 300 mg aliskiren hemifumarate..... 191

Appendix 8 Individual profiles of healthy volunteer 014 after administration of 300 mg aliskiren hemifumarate..... 192

Appendix 9 Available validation data of the RIA for angiotensin I and PRA determination given by the kit distributer. 193

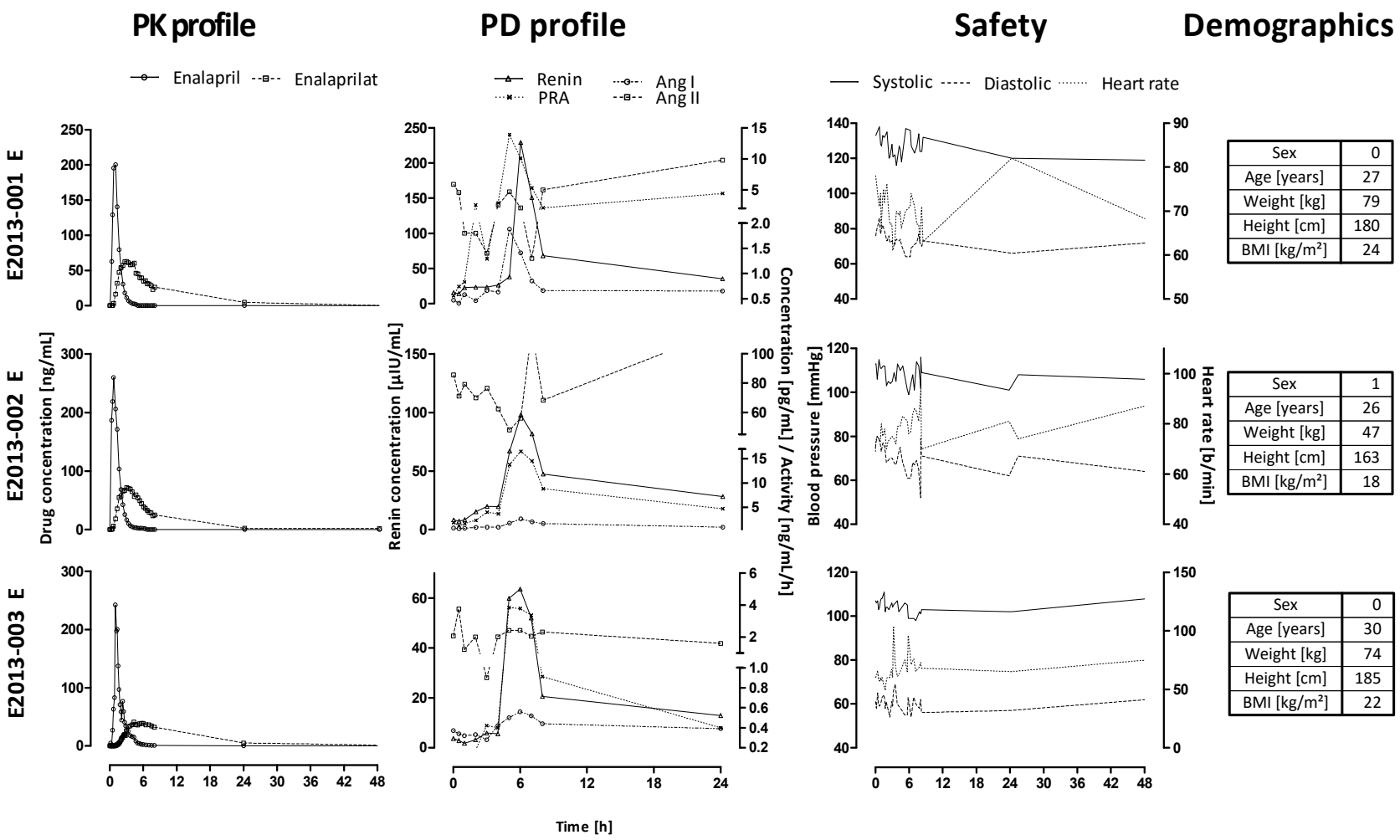
Appendix 10 Available validation data of the unmodified ELISA for angiotensin II determination given by the kit manufacturer 194

Appendix 11 Available validation data of the ELISA for prorenin determination given by the kit manufacturer..... 195

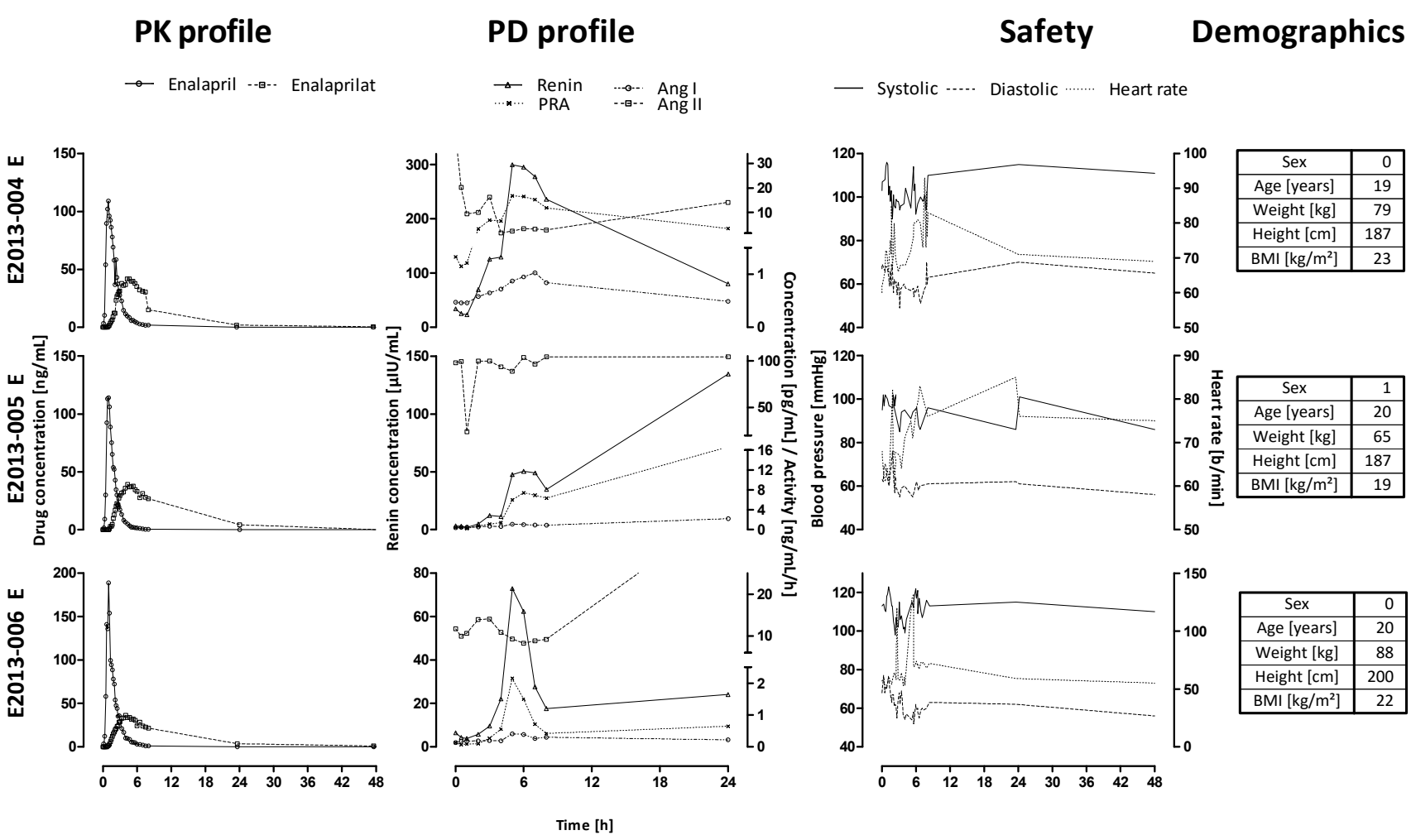
Appendix 12 Available validation data of the CLIA for renin determination given by the kit manufacturer 197

Appendix 13 Excerpt of the project plan describing the schedule issues and milestones within the LENA project. 198

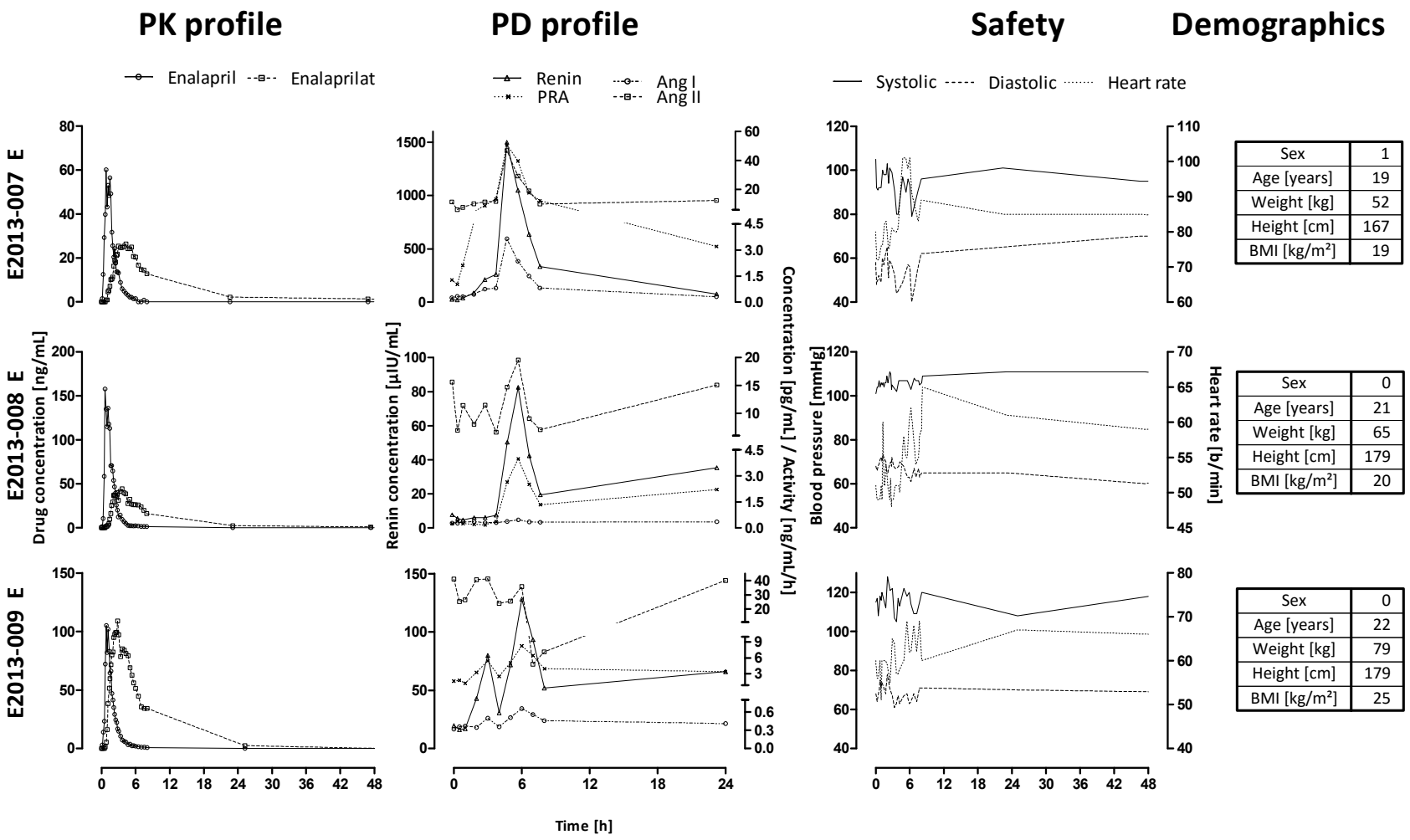
Appendix 14 Organisational chart of the LENA project (version 03, 20th August 2014)..... 199



Appendix 1 Individual profiles of healthy volunteers 1-3 after administration of 20 mg enalapril maleate



Appendix 2 Individual profiles of healthy volunteers 4-6 after administration of 20 mg enalapril maleate

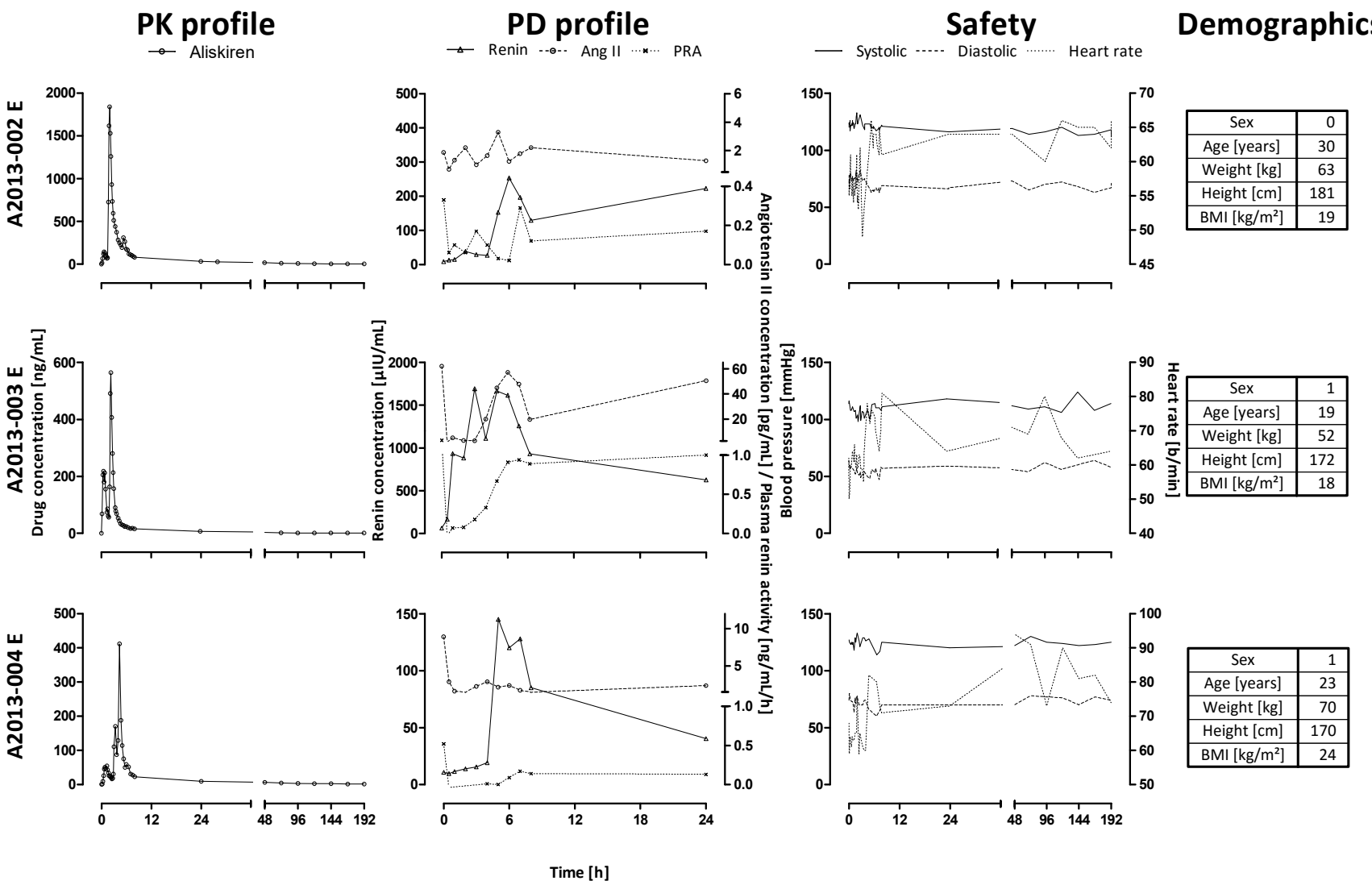


Sex	1
Age [years]	19
Weight [kg]	52
Height [cm]	167
BMI [kg/m²]	19

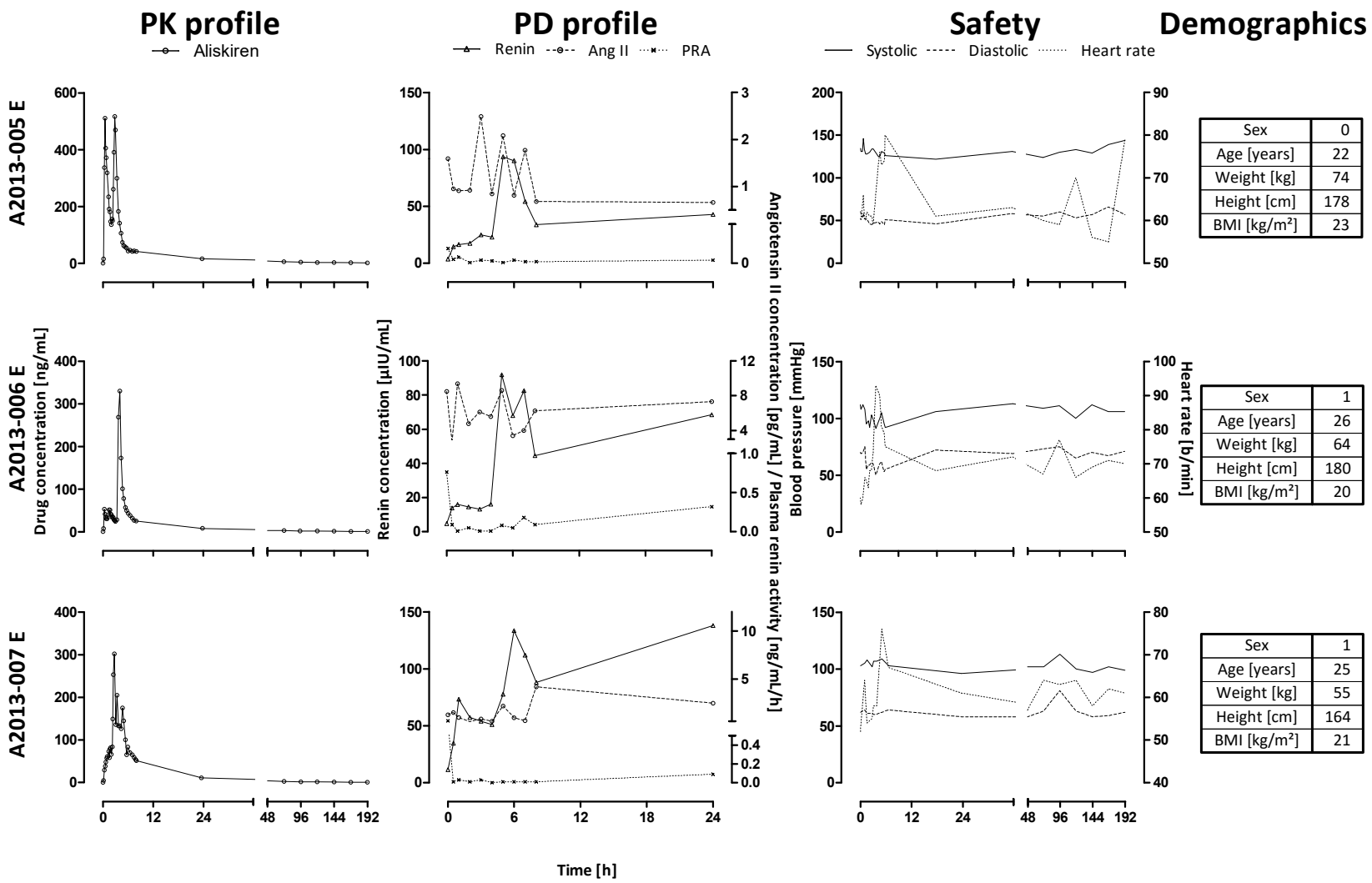
Sex	0
Age [years]	21
Weight [kg]	65
Height [cm]	179
BMI [kg/m²]	20

Sex	0
Age [years]	22
Weight [kg]	79
Height [cm]	179
BMI [kg/m²]	25

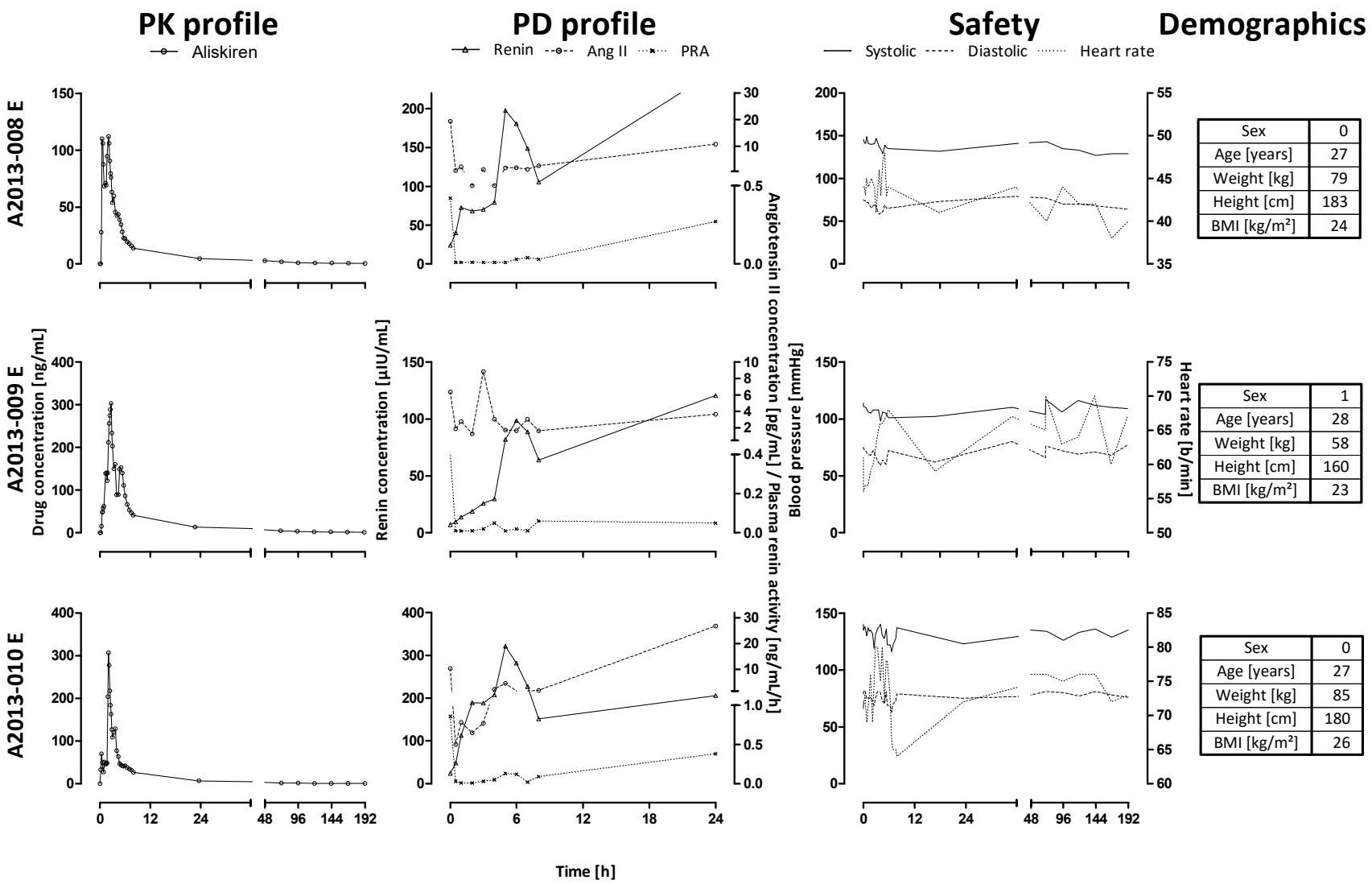
Appendix 3 Individual profiles of healthy volunteers 7-9 after administration of 20 mg enalapril maleate



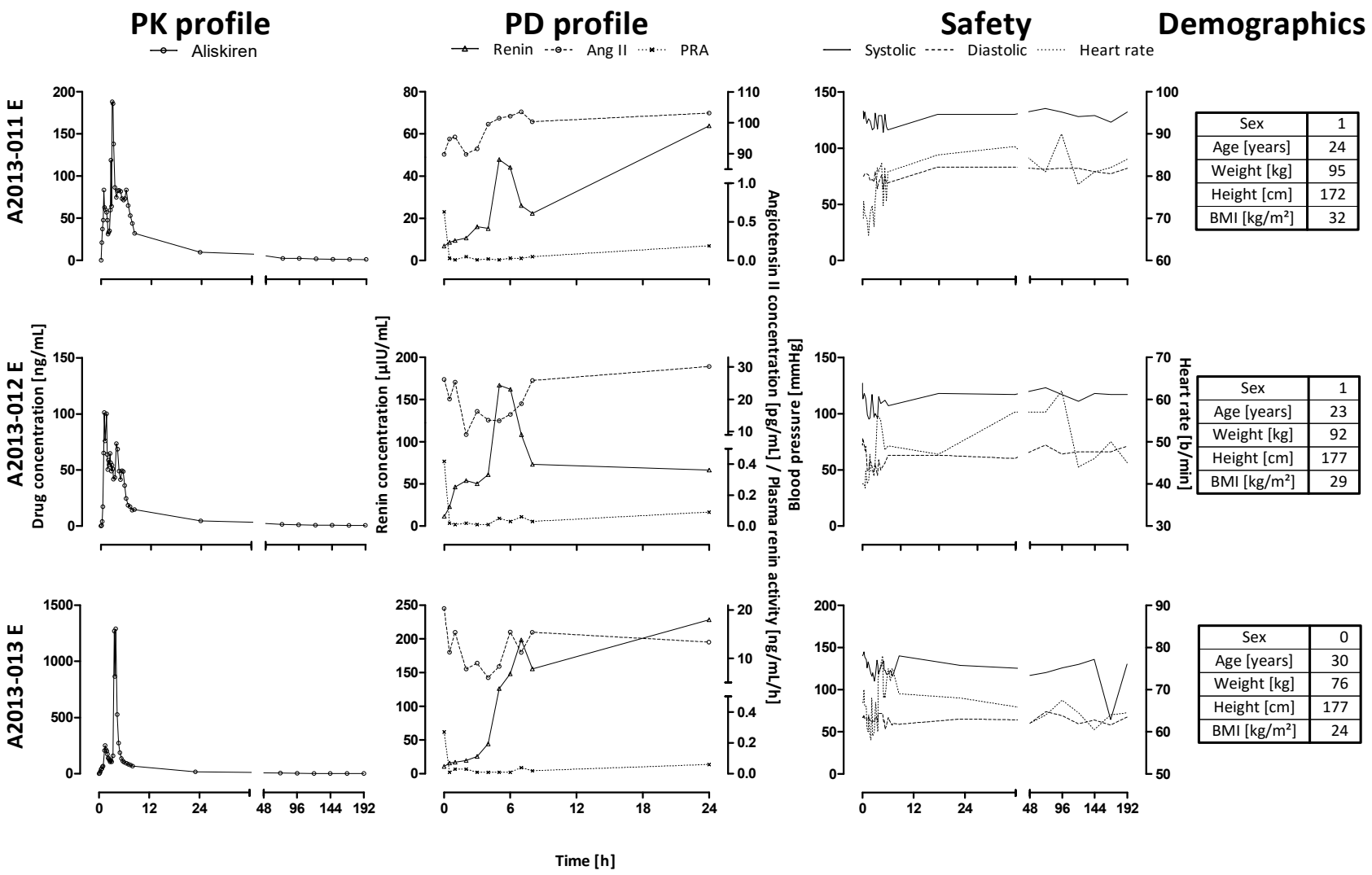
Appendix 4 Individual profiles of healthy volunteers 002-004 after administration of 300 mg aliskiren hemifumarate



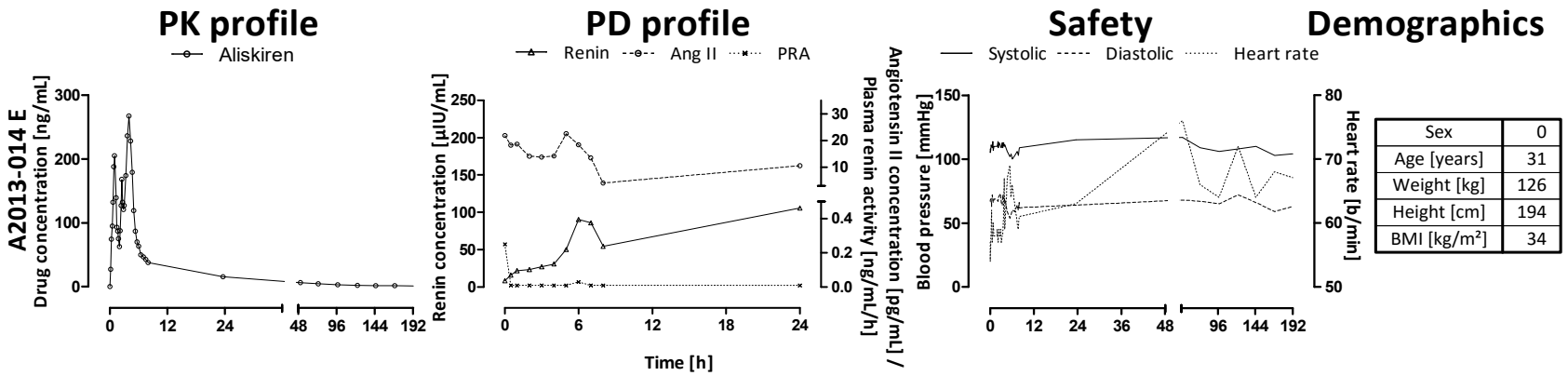
Appendix 5 Individual profiles of healthy volunteers 005-007 after administration of 300 mg aliskiren hemifumarate



Appendix 6 Individual profiles of healthy volunteers 008-010 after administration of 300 mg aliskiren hemifumarate



Appendix 7 Individual profiles of healthy volunteers 011 - 013 after administration of 300 mg aliskiren hemifumarate



Appendix 8 Individual profiles of healthy volunteer 014 after administration of 300 mg aliskiren hemifumarate

BECKMAN COULTER

APPENDIX

IVD

Angiotensin I RIA KIT

REF IM3518

RADIOIMMUNOASSAY OF ANGIOTENSIN I FOR THE IN VITRO DETERMINATION OF PLASMA RENIN ACTIVITY (PRA) IN HUMAN PLASMA

CE

Representative data are provided for illustration only. Performance obtained in individual laboratories may vary.

A. Accuracy

A.1 Dependence on time of enzymatic incubation

No dependence of PRA on time was observed.

Time, minutes		60	120	180
PRA ng/mL/hour	Sample A	0.30	0.32	0.33
	Sample B	0.56	0.48	0.53
	Sample C	1.04	1.15	1.19
	Sample D	1.64	1.56	1.65
	Sample E	5.12	5.19	4.93

A.2 Dilution test

Three plasma samples were diluted with the zero calibrator and assayed according to the procedure of the kit.

Sample	Dilution	Theoretical conc. (ng/mL)	Observed conc. (ng/mL)	Recovery (%)
A	undiluted	-	2.45	-
	1/2	1.23	1.21	98.8
	1/4	0.61	0.56	91.4
	1/8	0.31	0.27	88.2
B	undiluted	-	3.05	-
	1/2	1.53	1.46	95.7
	1/4	0.76	0.64	83.9
	1/8	0.38	0.34	89.2
C	undiluted	-	9.21	-
	1/2	4.61	4.30	93.4
	1/4	2.30	1.82	79.0
	1/8	1.15	0.90	78.2
	1/16	0.58	0.45	78.2

A.3 Recovery test

Plasma samples were spiked with known quantities of angiotensin I and assayed according to the procedure of the kit.

Initial conc. (ng/mL)	Added angiotensin (ng/mL)	Observed conc. (ng/mL)	Observed addition (ng/mL)	Recovery (%)
0.88	1.01	1.89	2.09	111
	1.30	2.18	2.68	123
	1.93	2.81	3.20	114
1.39	1.01	2.40	2.64	110
	1.30	2.69	2.98	111
	1.93	3.32	3.59	108
1.54	1.01	2.54	2.84	112
	1.30	2.84	3.31	117
	1.93	3.46	4.03	116
2.60	1.01	2.54	2.84	112
	1.30	2.84	3.31	117
	1.93	3.46	4.03	116
2.62	1.01	3.62	3.75	104
	1.30	3.92	4.19	107
	1.93	4.55	4.82	106

B. Precision

B.1 Intra-assay

Samples	1	2
Number of determinations	25	25
PRA, ng/mL/hour	1.49	2.94
CV (%)	11.25	11.25

B.2 Inter-assay

After generation of Angiotensin I, samples were determined in duplicates in 10 different series according to the procedure of the kit. Plasma renin activity was obtained and used for the calculation of inter-assay precision.

Samples	A	B	C	D	E
Number of determinations	10	10	10	10	10
PRA, ng/mL/hour	0.69	3.37	7.08	15.1	24.2
CV (%)	20.9	9.57	8.72	9.74	11.8

C. Specificity

	Cross reactivity (%)
Angiotensin I	100
Angiotensin II	ND
Angiotensin III	ND
Tetradecapeptide	ND
Angiotensinogen	ND

D. ^{125}I Characteristics

$T_{1/2}$ (^{125}I) = 1443 h = 60.14 d

^{125}I	E (MeV)	%
γ	0.035	
X	0.027	114
	0.032	25

PI-3518-2012-10-24

Appendix 9 Available validation data of the RIA for angiotensin I and PRA determination given by the kit distributer.

Explore our innovative technologies for your research

ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunoassay of Angiotensin II has been fully validated for its use in plasma after extraction.

☞ The Minimum Detectable Concentration (MDC) of Angiotensin II corresponding to the NSB average plus three standard deviations (n = 8) is: 1 pg/mL.

☞ Quality Ccontrol (QC) samples intra & inter-assay variation: established by measuring each QC five times per assay and in six different assays (i.e. 30 assays per QC):

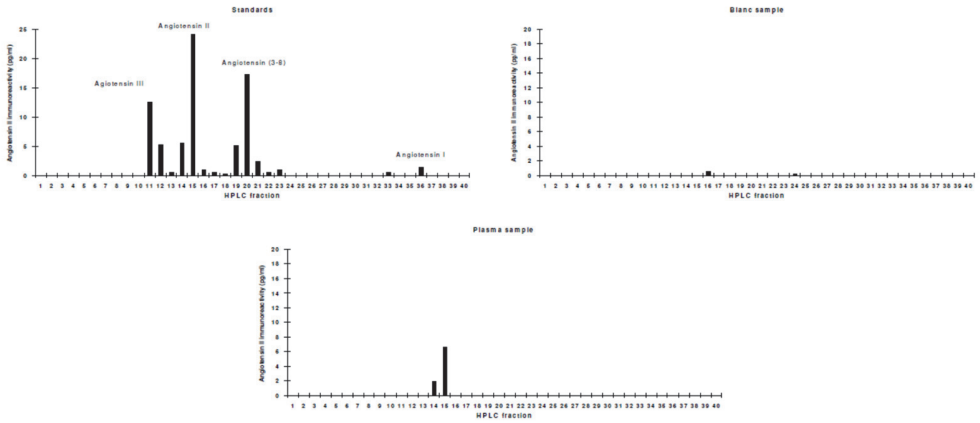
pg/mL	Intra-assay coefficient of variation	Inter-assay coefficient of variation
100	7 %	7 %
20	2 %	5 %
5	6 %	10 %
2	10 %	15 %

☞ Limit of quantification: 2 pg/mL

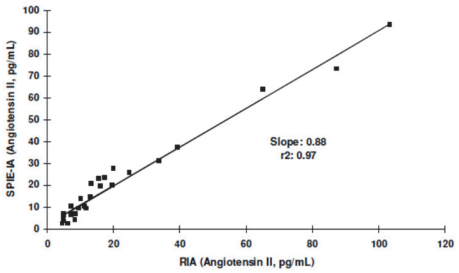
☞ Cross-reactivity:

Angiotensin II:	100 %
Angiotensin I:	4 %
Angiotensin III:	36 %
Angiotensin 3-8:	33 %
Angiotensin 1-7:	<0.01 %

☞ Specificity: comparison of HPLC profiles of Angiotensin standards, a blank sample and a plasma sample.



☞ Comparison with RIA on 29 samples:



Appendix 10 Available validation data of the unmodified ELISA for angiotensin II determination given by the kit manufacturer

Intra-assay Precision: Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Sample	1	2	3
n	20	20	20
Mean (ng/ml)	0.345	0.464	6.22
Standard Deviation	0.020	0.017	0.237
CV (%)	5.74	3.61	3.81

Inter-assay Precision: Three samples of known concentration were tested in ten independent assays to assess inter-assay precision.

Sample	1	2	3
n	10	10	10
Mean (ng/ml)	0.342	0.439	6.16
Standard Deviation	0.045	0.056	0.353
CV (%)	13.2	12.6	5.72

Recovery: The recovery of antigen spiked to levels throughout the range of the assay in depleted plasma was evaluated.

Sample	1	2	3	4
n	4	4	4	4
Mean (ng/ml)	0.205	0.747	2.02	4.94
Average % Recovery	102	93	101	103
Range	86-122%	90-97%	95-106%	99-108%

Linearity: To assess the linearity of the assay, samples containing and/or spiked with high concentrations of antigen were serially diluted to produce samples with values within the dynamic range of the assay.

Sample	1:2	1:4	1:8	1:16
n	4	4	4	4
Average % of Expected	96	102	107	119
Range	93-100%	98-110%	99-121%	104-145%

Specificity: This assay recognizes recombinant and natural human prorenin. The factors listed below were prepared at 10 ng/ml in depleted plasma and assayed for cross-reactivity. No significant cross-reactivity was observed.

Recombinant human renin
Recombinant mouse prorenin
Recombinant rat prorenin

Sensitivity: The minimum detectable dose (MDD) was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates (range OD_{450}: 0.066-0.074) and calculating the corresponding concentration. The MDD was 0.013 ng/ml.

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1. Schalekamp MADH, *et al.*: J Hypertens., 2008,

Appendix 11 Available validation data of the ELISA for prorenin determination given by the kit manufacturer

15. SPECIFIC PERFORMANCE CHARACTERISTICS

15.1. Analytical specificity

Analytical specificity may be defined as the ability of the assay to accurately detect specific analyte in the presence of potentially interfering factors in the sample matrix (e.g., haemolysis, lipaemia, bilirubinaemia).

Interference. Controlled studies of potentially interfering substances or conditions showed that the assay performance was not affected by concentrations of bilirubin up to 20 mg/dL, haemoglobin up to 500 mg/dL or triglycerides up to 3000 mg/dL.

Cross-reactions. The presence of the following potentially cross-reactive molecules in the assay showed the interference illustrated in the table below. The test was performed in accordance with the guidelines of Clinical and Laboratory Standards Institute (CLSI, USA), Document No. EP07-A2.

Compound	Spiked amount, µg/mL	% Cross-reactivity
$Beta_2$-microglobulin	50	– 7.0
Cathepsin D	1.5	– 6.9
Trypsin	1.6	– 4.2
Plasmin	100	0.8

In the absence of a standard prorenin preparation, cross-reactivity by prorenin was evaluated using two commercial preparations of recombinant prorenin and of recombinant renin. Two sets of five solutions with the same concentrations of the two molecules were prepared and the resulting samples (range: 0.78-12.5 ng/mL) were tested by direct renin immunoassays claiming no cross-reactivity by prorenin. The ratio of the signal emitted by each prorenin solution to the signal emitted by the corresponding renin solution is reported in the following table.

Immunoassay	CLIA direct renin test	IRMA direct renin test	Liaison Direct Renin
Prorenin-to-renin mean ratio	0.16	0.56	0.26

15.2. Precision with LIAISON® Analyzer

Different samples, containing different concentrations of specific analyte, were assayed to estimate repeatability and reproducibility of the assay (i.e., within- and between-assay variability). The results refer to the groups of samples investigated and are not guaranteed specifications, as differences may exist between laboratories and locations.

Repeatability. Twenty replicates were performed in the same run to evaluate in-house repeatability.

Repeatability	A	B	C	D	Control 1	Control 2
Number of determinations	20	20	20	20	20	20
Mean (µIU/mL)	15.1	33.8	82.2	258.0	27.1	99.0
Standard deviation (µIU/mL)	0.6	0.9	1.7	3.1	1.5	2.3
Coefficient of variation (%)	3.7	2.8	2.0	1.2	5.6	2.4
Min value (µIU/mL)	13.4	32.4	78.5	252.8	24.1	95.3
Max value (µIU/mL)	15.7	35.9	84.6	264.4	29.9	102.5

Reproducibility. Twenty replicates were performed in different days (one or two runs per day) with two different lots of integral per site to evaluate reproducibility. The tests were performed in two sites, in house (site 1) and in an independent laboratory (site 2) using the same instruments.

Reproducibility - Site 1	E	A	B	C	D	Control 1	Control 2
LOT No. 02							
Number of determinations	20	20	20	20	20	20	20
Mean (µIU/mL)	5.1	13.2	34.1	82.4	260.3	27.2	103.2
Standard deviation (µIU/mL)	0.5	1.6	1.9	4.3	12.3	1.9	4.4
Coefficient of variation (%)	10.0	12.4	5.7	5.2	4.7	6.9	4.2
Min value (µIU/mL)	4.1	11.4	30.8	74.3	241.6	24.3	93.7
Max value (µIU/mL)	5.9	17.2	38.4	88.7	278.8	30.4	111.0
LOT No. 03							
Number of determinations	20	20	20	20	20	20	20
Mean (µIU/mL)	5.3	13.1	34.7	83.5	266.7	27.3	102.9
Standard deviation (µIU/mL)	0.5	1.7	1.8	2.8	7.8	1.8	3.3
Coefficient of variation (%)	9.5	12.8	5.3	3.4	2.9	6.6	3.2
Min value (µIU/mL)	4.4	10.9	31.8	77.4	254.9	24.3	93.9
Max value (µIU/mL)	6.9	17.7	40.3	88.4	282.7	31.7	107.2
Inter-lot coefficient of variation (%)	2.7	0.6	1.2	0.9	1.7	0.4	0.2

Reproducibility - Site 2	A	B	C	D	Control 1	Control 2
LOT No. 01						
Number of determinations	20	20	20	20	20	20
Mean (µIU/mL)	18.8	38.4	79.1	228.6	27.5	97.2
Standard deviation (µIU/mL)	3.1	2.8	5.9	22.4	1.9	9.5
Coefficient of variation (%)	16.5	7.4	7.5	9.8	7.0	9.8
Min value (µIU/mL)	15.8	34.2	61.1	188.6	24.2	83.0
Max value (µIU/mL)	26.4	44.1	88.9	270.0	32.0	116.5
LOT No. 03						
Number of determinations	20	20	20	20	20	20
Mean (µIU/mL)	15.8	34.8	85.4	260.8	26.8	103.2
Standard deviation (µIU/mL)	2.7	2.1	5.2	26.5	2.8	7.5
Coefficient of variation (%)	17.1	6.1	6.1	10.2	10.6	7.3
Min value (µIU/mL)	12.9	32.3	75.2	214.5	22.1	91.0
Max value (µIU/mL)	22.7	38.4	93.7	306.2	31.7	116.3
Inter-lot coefficient of variation (%)	12.2	7.0	5.4	9.3	1.9	4.2

15.4. Linearity by dilution test

Two plasma samples with high renin levels were tested as such and after serially diluting with a renin-free plasma. Mea-sured versus expected renin levels were analyzed by linear regression. The correlation coefficients (r) ranged from 0.999 to 1.000.

Dilution	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery	Dilution	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery
neat	–	333.4	–	neat	–	> 500.0	–
1:2	166.7	190.5	114.3	1:2	–	396.5	–
1:4	83.4	91.7	110.1	1:4	198.3	200.0	100.9
1:8	41.7	47.3	113.5	1:8	99.1	103.1	104.0
1:16	20.8	24.7	118.6	1:16	49.6	51.3	103.6
1:32	10.4	12.1	116.3	1:32	24.8	26.2	105.8

Two additional plasma samples with renin levels above the assay range were tested as such and after serially diluting with LIAISON® Endocrinology Diluent. Measured versus expected renin levels were analyzed by linear regression. The correlation coefficients (r) were 1.000.

Dilution	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery	Dilution	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery
neat	–	> 500.0	–	neat	–	> 500.0	–
1:2	–	365.8	–	1:2	–	> 500.0	–
1:4	182.9	192.9	105.5	1:4	–	397.3	–
1:8	91.5	98.2	107.4	1:8	198.7	209.5	105.5
1:16	45.7	53.6	117.2	1:16	99.3	109.3	110.0
1:32	22.9	25.5	111.5	1:32	49.7	54.9	110.5

15.5. Trueness by recovery test

Two sets formed of a high- and a low- to normal-renin sample (samples X and Y in set 1, and samples W and Z in set 2) were mixed in 1:5, 1:2, 1:1, 2:1 and 5:1 ratios and assayed. Percent recoveries were determined from results of undiluted samples. Measured versus expected renin concentrations were analyzed by linear regression. The correlation coefficients (r) ranged from 0.996 to 1.000.

Set 1	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery	Set 2	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery
X neat	–	5.3	–	W neat	–	23.9	–
5:1	25.8	24.5	95.0	5:1	65.4	62.3	95.2
2:1	46.3	49.4	106.6	2:1	106.9	103.4	96.7
1:1	66.9	69.3	103.6	1:1	148.4	148.4	100.0
1:2	87.4	92.1	105.4	1:2	189.9	186.6	98.2
1:5	107.9	108.0	110.1	1:5	231.4	230.0	99.4
Y neat	–	128.4	–	Z neat	–	272.9	–

15.6. High-dose hook effect

The high-dose hook effect (HDH) was determined by addition of recombinant renin to a human plasma pool up to a maximum of 150,000 µIU/mL.

Whenever samples containing extremely high analyte concentrations are tested, the high-dose hook effect can mimic concentrations lower than real. Analysis of high-dose hook effect was evaluated by testing one high-concentration renin-spiked sample. The sample resulted in a calculated concentration value above the assay range, indicating no sample misclassification.

15.7. Analytical and functional sensitivity

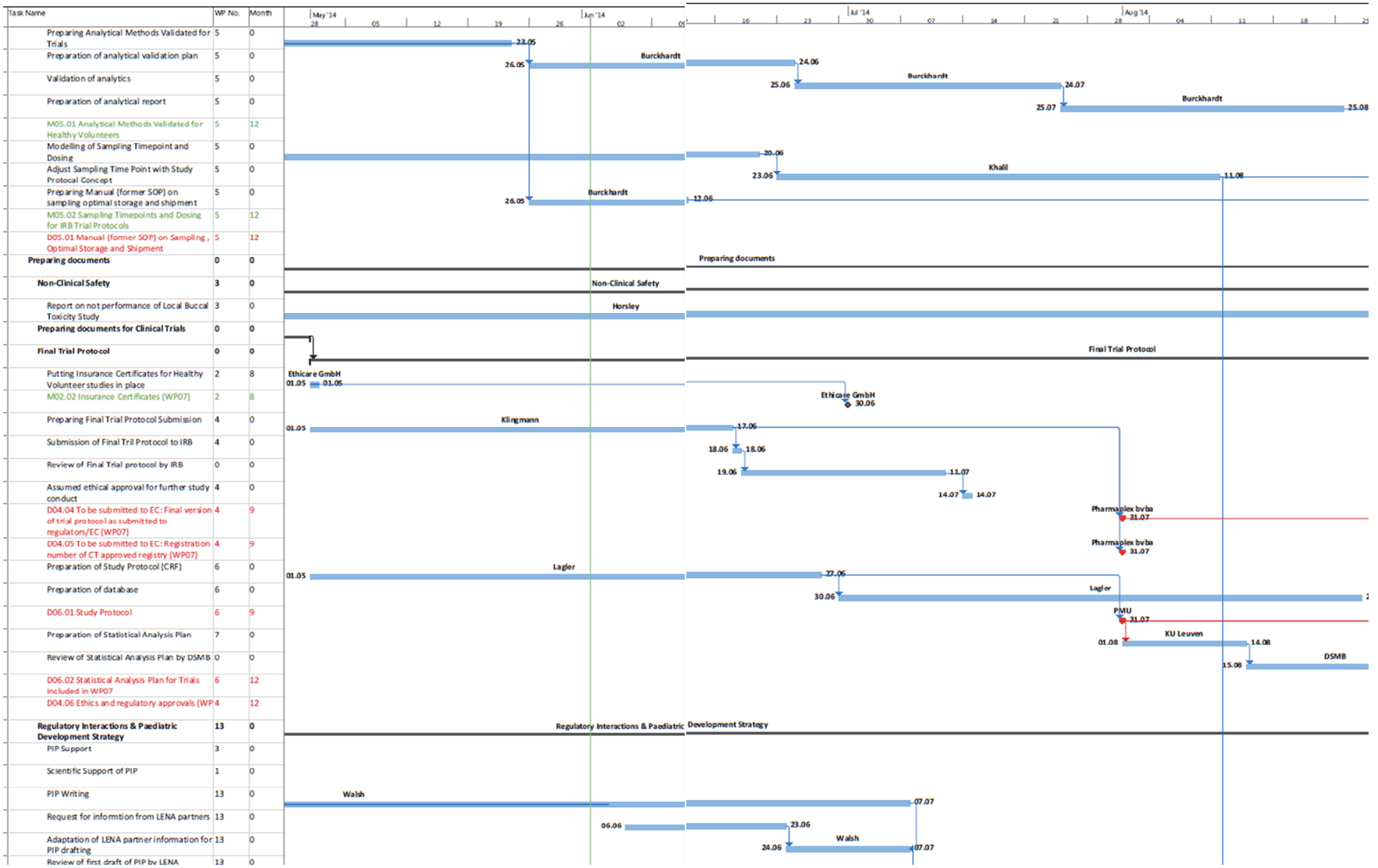
Analytical sensitivity (detection limit) is defined as the minimum detectable dose that can be distinguished from zero.

Analytical sensitivity, calculated in accordance with the guidelines of Clinical and Laboratory Standards Institute (CLSI, USA), Document No. EP17-A, ranges from 0.52 µIU/mL to 0.97 µIU/mL (as assessed by several assay runs, kit lots and instruments).

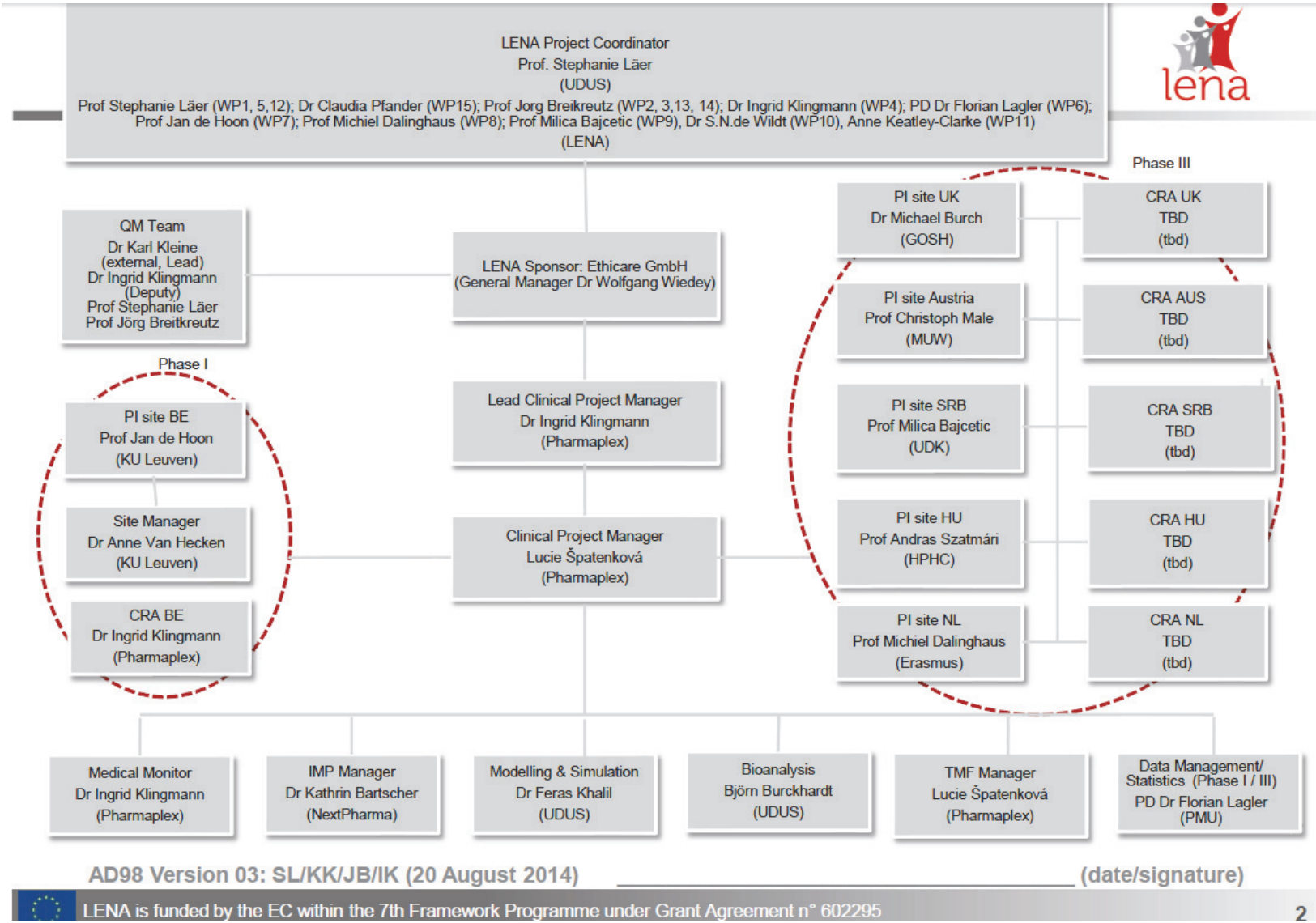
Analytical sensitivity, defined as the minimum detectable dose that can be distinguished from zero by two standard deviations (that is, two standard deviations above zero), ranges from 0.13 µIU/mL to 0.53 µIU/mL (as assessed by several assay runs, kit lots and instruments).

Functional sensitivity, defined as the concentration at which the between-assay coefficient of variation (CV) exceeds 20%, ranges from 1.60 µIU/mL to 1.96 µIU/mL (as assessed by several assay runs, kit lots and instruments).

Appendix 12 Available validation data of the CLIA for renin determination given by the kit manufacturer



Appendix 13 Excerpt of the project plan describing the schedule issues and milestones within the LENA project. In the upper area of the project plan required bioanalytical topics were illustrated. All were finalised earlier than the planned delivery date.



Appendix 14 Organisational chart of the LENA project (version 03, 20th August 2014)

9. Curriculum vitae

Personal information	
First name / Last name	**Björn Bengt Burckhardt**
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Professional experience	
Nov 2010 - today	**Heinrich-Heine-Universität Düsseldorf, Institut für Klinische Pharmazie und Pharmakotherapie**
Nov 2010 – today	PhD student / Scientific co-worker
Nov 2007 – Oct 2010	**Sandoz-Hexal (a company of Novartis), Holzkirchen/Kundl, Germany/Austria**
Jul 2008 – Oct 2010	Regulatory affairs manager
Nov 2007 – Apr 2008	Pharmazeut im Praktikum (pre-registration pharmacist)
May 2007 – Oct 2007	**Kranich Apotheke, Neukirchen-Vluyn, Germany**
	Pharmazeut im Praktikum (pre-registration pharmacist)
Professional / school education	
	Apothekerkammer Nordrhein, Düsseldorf
Jun 2008	**Certification as registered pharmacist in Germany**
Jun 2008	**Pharmazeut (pre-registration pharmacist)**
Degree	3. Staatsexamen (final exam)
	Heinrich-Heine-Universität Düsseldorf
April 2007	**Student (subject pharmacy)**
Degree	2. Staatsexamen (second exam)
	Student (subject pharmacy)
Degree	1. Staatsexamen (first exam)
Jun 2002	**Julius-Stursberg-Gymnasium, Neukirchen-Vluyn, Germany**
Degree	Allgemeine Hochschulreife (Abitur)

Parts of this thesis have already been published in peer-reviewed international journals or at conferences:

PEER-REVIEWED PUBLICATIONS

Burckhardt BB, Läer S. Sample preparation and extraction in low-volume samples suitable for paediatric clinical studies – challenges, advances and experiences from a bioanalytical HPLC-MS/MS method validation using enalapril, enalaprilat and benazepril, *International Journal of Analytical Chemistry* 2015, doi: 10.1155/2015/796249.

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***Burckhardt BB**, Ramusovic S, Tins J, Läer S.* Tailored bioanalytical methods for determination of aliskiren, enalapril and enalaprilat in serum and urine for systematic pharmacokinetic investigations in paediatric patients. 14th biannual congress of the European Society for Developmental Perinatal and Paediatric Pharmacology, 2013, Salzburg (A)

***Burckhardt BB**, Ramusovic S, Jaffan L, Läer S.* Identification of children at risk for high blood pressure out of routine blood pressure monitoring data. Annual conference of the Austrian and German pharmaceutical association, 2011, Innsbruck (A)