The Renin-Angiotensin-Aldosterone System in the Paediatric Population: Reliable Highly Sensitive Bioanalytical Investigations via Immunoassay and Innovative Multiplex High-resolution Mass Spectrometry

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„There is always a bigger fish!”

Qui-Gon Jinn

I Erklärung zu Dissertation

Ich versichere an Eides statt, dass die vorliegende Dissertation von mir selbstständig 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.

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III Zusammenfassung

Kardiovaskuläre Erkrankungen zählen zu den Hauptursachen natürlicher Todesfälle in der westlichen Hemisphäre, jedoch ist die evidenzbasierte Therapie dieser Erkrankungen in der pädiatrischen Population limitiert. Dies ist unter anderem in dem mangelnden Wissen bezüglich humoraler Regelkreisläufe im heranwachsenden Organismus wie dem Renin-Angiotensin-Aldosteron-System begründet. Folglich umfasst die vorliegende Arbeit die umfangreiche Evaluierung des pädiatrischen Angiotensin-Aldosteron-Systems mittels der Entwicklung und Etablierung qualitätsgesicherter, bioanalytischer Methoden für die Erhebung pharmakodynamischer Daten in pädiatrisch-klinischen Studien.

Initial wurde die Bedeutung des Alters, des Geschlechts und therapeutischer Maßnahmen auf essenzielle Peptide des Renin-Angiotensin-Aldosteron-Systems in gesunden und kardiovaskulär erkrankten Kindern evaluiert, als auch altersbezogene Daten hinsichtlich der Plasma-Renin-Aktivität zusammengestellt. Beide systematischen Übersichtsarbeiten lassen eine altersabhängige Beeinflussung der untersuchten Parameter vermuten und zeigen relevante Datenlücken in der pädiatrischen Population auf.

Zweitens wurde ein den regulatorischen Anforderungen entsprechender Immunoassay für die Bestimmung der Plasma-Renin-Aktivität in Kindern im Rahmen der Guten Klinischen Laborpraxis etabliert. Das nötige Probenvolumen wurde auf ein in der Pädiatrie anwendbares Volumen von 100 µL reduziert und der Assay nach internationalen Richtlinien für den Einsatz in klinischen Studien validiert, um vorhandene Datenlücken zu schließen.

Drittens wurde ein Qualitätskontrollsystem für die Monitorierung bioanalytischer Methoden zur Erhebung pharmakodynamischer Daten in Kindern, im Kontext des „Labeling of Enalapril in Neonates up to Adolescents“ Projektes, entwickelt. Dieses ermöglichte die qualitätsgesicherte Messung der Plasma-Renin-Aktivität über den Studienzeitraum von 24 Monaten. Dabei garantierte die Überwachung der Langzeit- und Wiederholgenauigkeit sowie der Reproduzierbarkeit eine regulatorisch adäquate, hohe Datenqualität.

Viertens wurde eine bioanalytische Methode zur hochsensitiven Bestimmung von acht essenziellen Peptiden des Renin-Angiotensin-Aldosteron-Systems mittels Flüssigchromatografie gekoppelt mit hochauflösender Massenspektrometrie entwickelt, um zuverlässige Daten bei Kindern zu generieren. Die Methode ermöglichte die simultane Quantifizierung von Angiotensin I, Angiotensin II, Angiotensin-(1-7), Angiotensin III, Angiotensin IV, Angiotensin A, Alamandine und Angiotensin-(1-9) in 50 µL Plasma. Die durchgeführte Validierung gestattete den Einsatz der Methode im pädiatrischen Kollektiv des „Labeling of Enalapril in Neonates up to Adolescents“ Projektes und konnte wesentliche Unterschiede der untersuchten Parameter zwischen Kindern und Erwachsenen aufzeigen.

IV Summary

Cardiovascular diseases are the primary causes of natural deaths in the western hemisphere. However, the evidence-based pharmacotherapy in the paediatric population is limited. This constraint is affected by the lack of knowledge regarding humoral circuits of the maturing organism such as the renin-angiotensin-aldosterone system. In this regard, this thesis presents a comprehensive evaluation of the paediatric renin-angiotensin-aldosterone system as well as the development and implementation of quality-assured bioanalytical methods for the assessment of related pharmacodynamic datasets in paediatric, clinical studies.

First, the significance of age, gender and therapeutic measures on essential peptides of the renin-angiotensin-aldosterone system in healthy and cardiovascular diseased paediatric patients have been evaluated. Besides, age-related data on the plasma renin activity have been compiled. Both systematic reviews indicate an age-dependent influence on the peptides and plasma renin activity and also suggest a relevant lack of knowledge in the paediatric population.

Second, an immunoassay for the determination of the plasma renin activity in children was established under Good Clinical Laboratory Practice following regulatory requirements. The required sample volume was successfully downscaled to a volume of 100 µL applicable in paediatric research, and the assay has been validated in accordance with international bioanalytical guidelines to address the existing knowledge gap.

Third, a comprehensive quality control system for the monitoring of bioanalytical investigations of pharmacodynamic parameters in children was developed in the context of the “Labeling of Enalapril in Neonates up to Adolescents” project. The quality control system allowed the quality-assured evaluation of the plasma renin activity over the entire study period of 24 months. Moreover, the monitoring of long-term and inter-run accuracy, as well as reproducibility, ensured a high data quality in line with regulatory requirements.

Fourth, a bioanalytical method has been developed for the highly sensitive determination of eight essential peptides of the renin-angiotensin-aldosterone system by liquid chromatography coupled to high-resolution mass spectrometry, to obtain reliable data in children. This method enabled the simultaneous quantification of angiotensin I and angiotensin II, as well as angiotensin-(1-7), angiotensin III, angiotensin IV, angiotensin A, alamandine, and angiotensin-(1-9) in 50 µL plasma. The conducted validation empowered the method to be applied in the paediatric collective of the “Labeling of Enalapril in Neonates up to Adolescents” project and was able to demonstrate notable differences of the examined parameters in paediatrics as compared to adults.

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VI Abbreviations

ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor
AGA	Appropriate for gestational age
Ala	Alamandine
Ang1-7	Angiotensin-(1-7)
Ang1-9	Angiotensin-(1-9)
AngA	Angiotensin A
AngI	Angiotensin I
AngII	Angiotensin II
AngIII	Angiotensin III
AngIV	Angiotensin IV
ANP	Atrial natriuretic peptide
APA	Aspartyl aminopeptidase A
APN	Aminopeptidase N
AA	Amino acid
ASD	Atrial septal defect
AT_1-R	Angiotensin receptor subtype 1
AT_2-R	Angiotensin receptor subtype 2
AT_4-R	Angiotensin receptor subtype 4
BDG	Bidirectional Glenn
CCB	Calcium channel blocker
CE	Collision energy
CHD	Congenital heart disease
CID	Collision induced dissociation
CLSI	Clinical and Laboratory Standards Institute
Cps	Counts per second
CRF	Chronic renal failure
CS	Calibration standard
CV	Coefficient of variation
CVD	Cardiovascular disease
DCM	Dilated cardiomyopathy
DP	Declustering potential
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESI	Electrospray ionisation
ESRD	End-stage renal disease
FA	Formic acid
FDA	U.S. Food and Drug Administration
GA	Gestational age
GCLP	Good Clinical Laboratory Practice
GPCR	G-protein-coupled receptor
HETP	Height equivalent to a theoretical plate
HF	Heart failure
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HRMS	High-resolution mass spectrometry
HUT	Head-up tilt

IRAP	Cyclic insulin-regulating aminopeptidase
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IQR	Interquartile range
IS	Internal standard
IS-MF	Internal standard normalised matrix factor
ISR	Incurred sample reanalysis
LBA	Ligand-binding assay
LC	Liquid chromatography
LENA	Labeling of Enalapril from Neonates up to Adolescents
LLOQ	Lower Limit of Quantification
LoB	Limit of Blank
LoD	Limit of Detection
Mas-R	Mas receptor
MF	Matrix factor
MLBW	Moderate-low birthweight
MrgD	Mas-related g-protein-coupled receptor, member D
MS	Mass spectrometry
m/z	Mass-to-charge
NBW	Normal birthweight
OD	Optical density
PD	Pharmacodynamic
PK	Pharmacokinetic
PMSF	Phenylmethylsulfonyl fluoride
PRA	Plasma renin activity
QC	Quality control
QCS	Quality control system
Q-TOF	Quadrupole and time-of-flight mass spectrometer
RAAS	Renin-angiotensin-aldosterone system
RE	Relative error
RF	Renal failure
RfB	German Reference Institute for Bioanalytics
RIA	Radioimmunoassay
RSD	Relative standard deviation
RT	Room temperature
SD	Standard deviation
SE	Standard error
SGA	Small for gestational age
SPE	Solid-phase extraction
SRINS	Steroid-resistant idiopathic nephrotic syndrome
SST	System suitability test
TMB	Tetramethylbenzidine
TQMS	Triple quadrupole mass spectrometer
ULOQ	Upper Limit of Quantification
UV/VIS	Ultraviolet/visible spectroscopy
VLBW	Very-low birthweight
VSD	Ventricular septal defect

VII Figures

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1 Introduction

1.1 The renin-angiotensin-aldosterone system

Background

The renin-angiotensin-aldosterone system (RAAS) is a major regulator of blood pressure, perfusion, and fluid homeostasis. It subsequently plays a key role in the development and prevalence of high blood pressure and renal failure (RF). Besides, it is actively involved in the development and prognosis of leading causes of death in the western world, such as cardiovascular diseases (CVD), including heart failure (HF) and coronary arterial disease [1, 2]. Furthermore, an influence on cerebral perfusion and the progression of stress symptoms, depression, Alzheimer's disease, Parkinson's disease or diabetes was shown [3–8]. The RAAS is described by a complex enzymatic cascade wherein the prohormone angiotensinogen represents the primary precursor. Angiotensinogen is mainly generated in the liver but can also be expressed in other tissues like kidney, brain, heart, and adrenal gland [9]. After secretion into the blood, the circulating angiotensinogen is predominantly degraded by cleavage at the N-terminal end by the aspartyl protease renin [10]. Renin itself was first described by Robert Tigerstedt and his student Per Bergmann in 1898 as a substance located in the kidney of rabbits, which lowers blood pressure [3]. Renin is activated by eliminating an N-terminal propeptide from its precursor prorenin [11, 12]. The release of renin from the juxtaglomerular apparatus of the kidney is primarily triggered by the change of the sodium chloride concentration at the *macula densa*, renal baroreceptors responsive to altering arterial perfusion, the sympathetic stimulation via beta-1 receptors, and angiotensin II (AngII) triggered negative feedback on the juxtaglomerular apparatus [9, 13].

The degradation of angiotensinogen by renin presents the beginning of an enzymatic cascade involving several physiological important peptides, which are primarily investigated in animal and cell-culture studies, as well as increasingly in the adult population. To highlight the importance of these peptides in terms of physiological functions and the influence on pathophysiological processes, the following chapter provides an overview of important peptides of the RAAS and their corresponding target receptors based on *in-vitro,* animal *in-vivo,* as well as human adult *in-vivo* data (Figure 1-1).

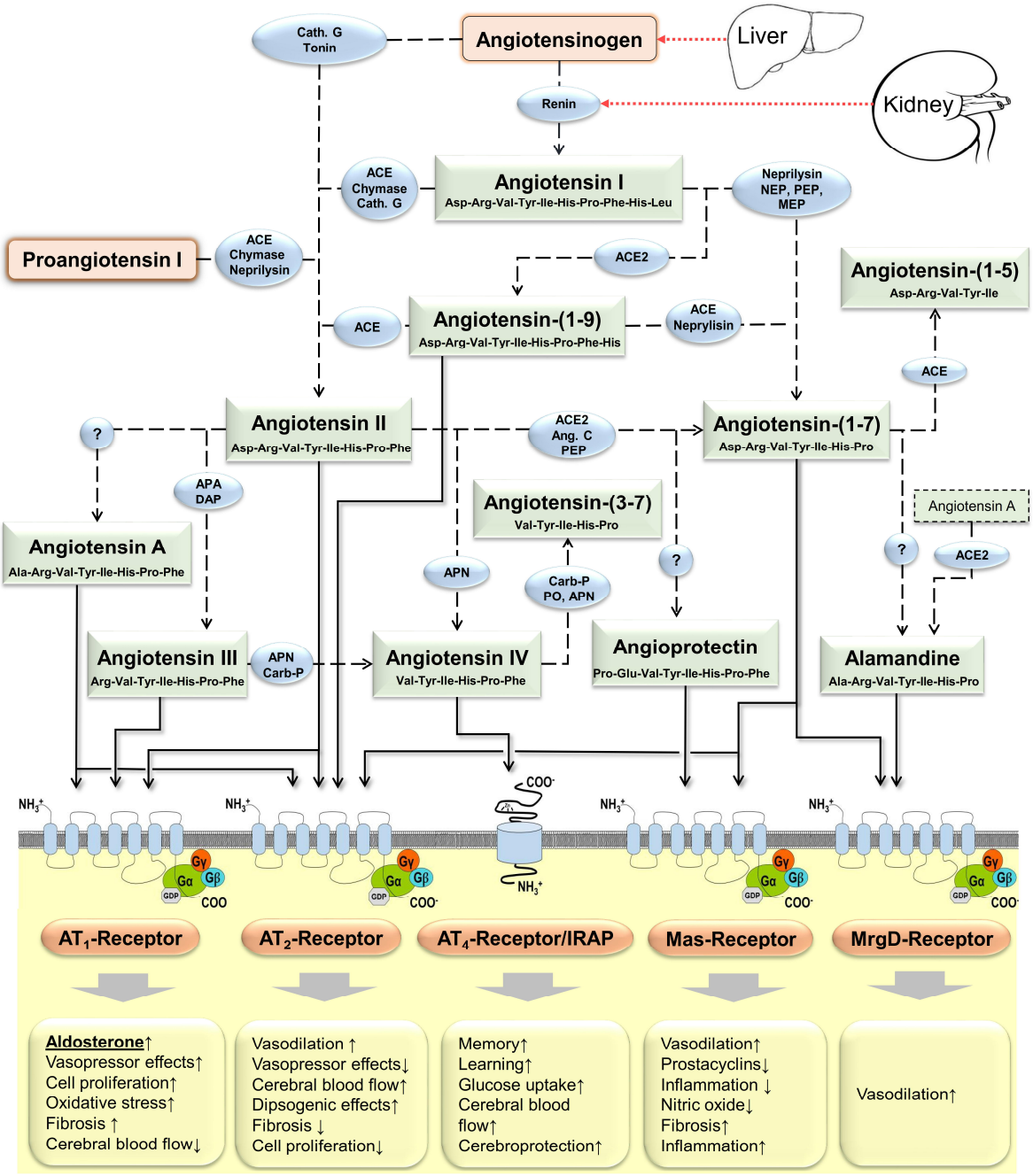


Figure 1-1: Schematic presentation of major components involved in the renin-angiotensin-aldosterone system. Blue ovals with dashed lines represent involved enzymes (unknown enzymes are expressed as question marks). Solid lines indicate peptide-receptor interaction. Big grey arrows represent effects triggered by activation of the receptor. Amino acids sequences are expressed using the three-letter code. Potential feedback mechanisms are not illustrated here. [6, 9, 10, 12, 14–22]. APN: aminopeptidase N; APA: aspartyl-aminopeptidase A; ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; NEP: neutral endopeptidase; PEP: prolyl endopeptidase; MEP: metalloen endopeptidase; Carb-P: carboxypeptidase C; PO: propyl oligopeptidase; DAP: aspartyl aminopeptidase; IRAP: cyclic insulin-regulating aminopeptidase; AT_1: angiotensin receptor subtype 1; AT_2: angiotensin receptor subtype 2; AT_4: angiotensin receptor subtype 4; MrgD: Mas-related G-protein-coupled Receptor, member D; Cath. G: Cathepsin G

Essential peptides of the renin-angiotensin-aldosterone system

The cleavage of angiotensinogen by the action of renin leads to the inactive interim peptide angiotensin I (AngI), which was first described by Page et al. in 1940 [23]. The conversion of AngI from angiotensinogen is the rate-limiting step of the whole RAAS metabolism and is applied for the assessment of the renin activity. The evaluation of the plasma renin activity (PRA) is a widely established approach for the determination of renin activity in plasma, which is technically less demanding than measurement of direct renin concentration. PRA is commonly utilised for evaluating metabolic dysfunctions (e.g. PRA-aldosterone ratio for the diagnosis of primary hyperaldosteronism, a cause of hypertension and a trigger for diverse CVDs, including HF) [24].

The degradation of AngI by the metalloprotease angiotensin-converting enzyme (ACE) results in the octapeptide AngII. Since Menendez and Fasciolo first isolated AngII from renal dog blood in 1939, the homeostatic regulation of blood pressure was mainly be ascribed to AngII [25], as it presents one of the most vasoconstrictive substances ever known and thus is the most examined peptide of the RAAS [26]. AngII plays an active role in inflammatory processes, coronary atherosclerosis and fibrosis, as well as in HF and hypertension [27]. Currently, two types of receptors regarding AngII interactions are clearly identified. The angiotensin receptor subtype 1 (AT_1-R) and subtype 2 (AT_2-R) belong to the family of G-protein-coupled receptors (GPCR). The AT_1-R is ubiquitous in the body (e.g. blood vessels, kidney, heart, liver, brain, and lung) and is responsible for the increase of blood pressure, vasoconstriction, the activity of the sympathetic nervous system and the release of the mineralocorticoid receptor agonist aldosterone from the *zona glomerulosa* (the outermost layer of the adrenal gland) [9, 28]. Aldosterone leads to elevated water and sodium retention and consequently, to increased blood volume [9, 29, 30]. In contrast to the AT_1-R, the AT_2-R constitutes the counterpart by causing primarily protective effects like vasodilation, modulation of cell proliferation, apoptosis and regenerative processes [31, 32].

Angiotensin III (AngIII), also known as des-aspartyl-angiotensin II, was first described by Campbell et al. in 1974 who demonstrated an AngIII triggered effect on aldosterone release, indicating a comparable effect like AngII [33]. AngIII is generated from AngII by the action of the aspartyl aminopeptidase A (APA) and possesses similar characteristics like its precursor peptide (e.g. addressing the AT_1-R and AT_2-R, inducing vasoconstriction and the release of ANP, proinflammatory effects, increase in glomerular filtration rate and influence on the release of vasopressin [8, 19–21, 33–36]. These observations even led to a theory that revaluates AngIII as actual physiologically relevant peptide of RAAS, reasoned by the potential conversion of AngII to AngIII before activation of the receptors [8, 34, 37–39]. This

thesis was also supported by Wright et al., who demonstrated an increase in blood pressure of spontaneously hypertensive rats by injection of APA. Conversely, the injection of aminopeptidase N (APN), which degrades AngIII, resulted in a decrease in blood pressure [40]. However, there is no consistent evidence for this thesis, as Clark et al. observed a constant activation of the mitogen-activated kinase cascade despite APA inhibition, indicating no exclusively stimulation by AngIII [41]. In addition, Wilson et al. were able to demonstrate an equivalent effect on the thirst and sodium appetite with aminopeptidase-resistant AngII and AngIII analogues [42], while administration of AngIII and AngII in baboons led to similar increases in salt and water intake [43]. However, new insights into the regulation of blood pressure as well as HF can be derived from these findings, in which AngIII is likely to play a decisive role.

The degradation of AngII or AngIII by the action of APN results in the hexapeptide Angiotensin IV (AngIV), which main target receptor significantly differs from the previously described components of the RAAS. The angiotensin receptor subtype 4 (AT_4-R) was initially described in 1992, and afterwards identified by Albiston et al. in 2001 as cyclic insulin-regulating aminopeptidase (IRAP), a type 2 transmembrane protein which belongs to the family of gluzincin aminopeptidases [4, 44, 45]. Moreover, a soluble IRAP in maternal serum, also known as oxytocinase, was described [45]. Besides neurons, brain, heart, kidney, and skeletal muscle, the membrane-bound IRAP is expressed in adipocytes in which the IRAP shows similarities to the glucose transporter type 4 [19, 45–47]. When stimulated with insulin, the receptor is transferred to the outside of the plasma membrane [46]. After this translocation, the IRAP is capable to enzymatically degrade potent vasoconstrictors such as vasopressin and AngIII, hormones like oxytocin, as well as neurokinin A and B, lys-bradykinin and somatostatin [45, 48, 49]. Thus, a decreased translocation of the IRAP by reduced insulin levels can lead to reduced degradation of vasopressin and further vasopressors [4, 46, 50, 51]. Consequently, AngIV could potentially play a role in the development of comorbidities like elevated blood pressure and HF in diabetes mellitus type 2 patients [52]. However, current research on AngIV focuses primarily on pathophysiological conditions of cognitive perception, Alzheimer's disease, epilepsy, and insulin release [9, 45, 53]. In this context, animal studies showed that AngIV plays a role in cognitive processes and sensorimotor functions [54]. Since high-affinity binding structures for AngIV are also present in humans and these are likewise localised in areas for cognitive and motor skills such as the hippocampus, neocortex, and cerebellum, the delayed progress and onset of Alzheimer's disease, observed under vascular dysregulation caused by administration of ARBs and ACE-inhibitors (ACEi) in several cohort studies, could

therefore potentially be awarded to increased conversion of AngII to AngIV [7, 55–57]. Thus, AngIV could become a potential target for Alzheimer's disease, Parkinson or dementia [49].

The discovery of a homologous enzyme to ACE, namely the angiotensin-converting enzyme 2 (ACE2), allowed further insights into an alternative pathway of the RAAS [58, 59]. In this, the leucine is cleaved from AngI by the action of ACE2 from the C-terminal end, which leads to the nonapeptide Angiotensin-(1-9) (Ang1-9). Ang1-9 could exhibit protective effects via direct agonising of the AT_2-R [60]. Besides, Ocaranza et al. demonstrated an anti-hypertrophic effect on cultured cardiac myocytes by the administration of Ang1-9 after myocardial infarction in rats [61]. Moreover, an increased plasma concentration of Ang1-9 was shown after experimental myocardial infarction and ACE/AT_1-R inhibition. The elevated Ang1-9 concentration thereby correlates with the attenuation of left heart hypertrophy [61]. Nevertheless, the mode of action and the cardiovascular effects of Ang1-9 are still not finally assessed.

The conversion of Ang1-9 by the ACE results in Angiotensin-(1-7) (Ang1-7) [59]. Alternatively, Ang1-7 could also be generated from AngII by the ACE2, angiotensinase C, and prolyl endopeptidase and could thus be synthesised via both pathways of the RAAS [62, 63]. Moreover, neprilysin is also capable to directly generate Ang1-7 out of AngI by bypassing the ACE and ACE2 pathways [64]. High-doses of Ang1-7 in animal models showed minor vasopressor effects, probably caused by weak AT_1-R stimulation [65]. Nevertheless, binding studies in human embryonic kidney-293 cells by Bosnyak et al. indicate higher effects on the AT_2-R (Table 1-1). [66] However, Ang1-7 mainly addresses the GPCR Mas (Mas-R) as well as the Mas-related GPCR, member D (MrgD) [35, 67, 68]. The Mas-R exhibits many similarities to the AT_2-R, including protective and AT_1-R antagonising effects like vasodilation, anti-inflammation as well as neurogenerative effects [18, 69–71]. Moreover, Ang1-7 possesses antiproliferative, anti-fibrotic and inhibitory effects on the growth of cardiomyocytes as well as vasodilative properties via the Mas-R. It thus is regarded as a functional antagonist of AngII [59, 67, 72]. As a consequence, the discovery of the ACE2/Ang1-7/Mas axis might provide new potentials for the treatment of hypertension and HF.

Table 1-1: Relative affinities of angiotensin peptides to the angiotensin receptor subtype 1 and 2 in human embryonic kidney-293 cells.

Ligand	Angiotensin receptor subtype 1		Angiotensin receptor subtype 2	
	IC_{50} value (M)	Percentage affinity of Angiotensin II	IC_{50} value (M)	Percentage affinity of Angiotensin II
Angiotensin II	7.92E-09	100%	5.22E-10	100%
Angiotensin III	2.11E-08	38%	6.48E-10	81%
Angiotensin IV	n/a	n/a	4.86E-08	1%
Angiotensin-(1-7)	n/a	n/a	2.46E-07	0.21%

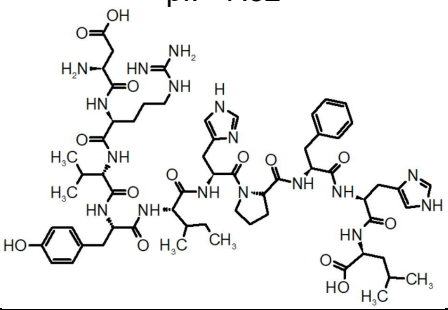
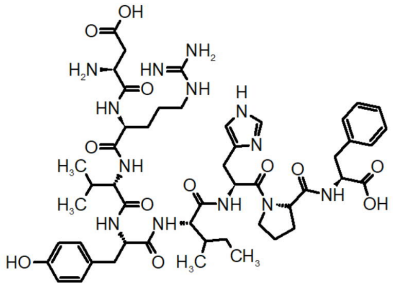
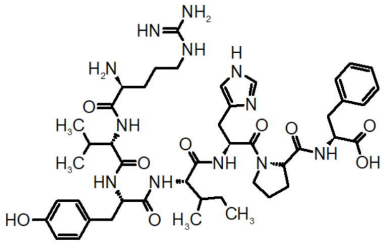
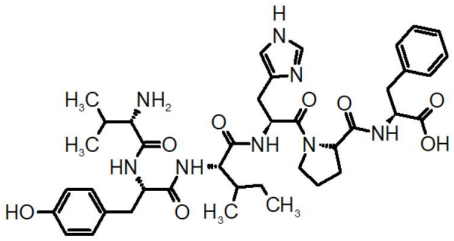
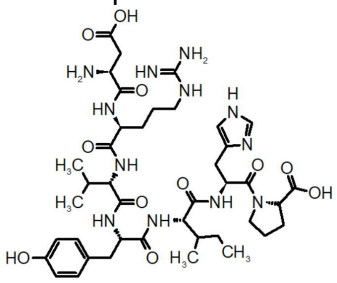
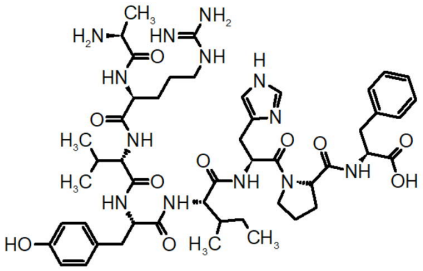
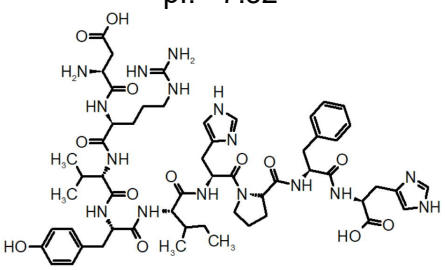
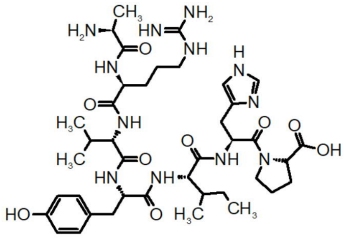
The table was modified according to [66]. IC_{50}: half maximal inhibitory concentration; n/a: not applicable; M: molar

In recent years, investigations indicated the existence of further peptides of the angiotensin-family that are derived from AngII. Jankowski et al. identified a novel peptide named angiotensin A (AngA), which is potentially generated from AngII by enzymatic decarboxylation and shows the same affinity on the AT_1-R, but higher affinity on AT_2-R like AngII [15]. Nevertheless, this assumption is still under discussion as other research demonstrated equal affinities of AngII and AngA to both receptor subtypes [73]. The obtained data suggest that AngA is rather dedicated to vasoconstriction than vasodilation and therefore shows similarities to AngII; yet with lower potency compared to AngII. However, experiments in the abdominal aorta of rabbits lead to the suggestion, that plasma concentrations of AngA could be significantly increased in pathophysiological conditions and hence compensate the lower potency compared to AngII [74]. All in all, there is conflicting evidence about the role of AngA in context of the RAAS and its impact on physiological functions and pathophysiological conditions like CVD.

In 2013, Lautner et al. published the discovery of the heptapeptide alamandine as well as the corresponding target receptor, which was identified as MrgD-R [17]. Alamandine could be directly formed from Ang1-7 and resembles its biological activity. As a result, alamandine is assigned protective properties similar to Ang1-7. However, the exact formation of this novel peptide and its role in CVDs is still not entirely understood. Nevertheless, the demonstrated reduction of long-term blood pressure in spontaneously hypertensive rats [17], the vasodilative effects in rabbit vessels [75], and the observed vasorelaxation in aortic rings from mice [76] suggests counteracting effects compared to AngII, AngIII and AngA.

In summary, all these described peptides might play a key role regarding the physiological function of the RAAS, which makes them interesting target structures for the investigation of CVDs (e.g. HF) and the related pharmacotherapy (Table 1-2 provides further information for all investigated physiological peptides of the RAAS within this thesis).

Table 1-2: Characteristics of the eight investigated angiotensin peptides.

Angiotensin I Sequence: DRVYIHPFHL Molecular mass: 1296.49 pI: ~7.52 	**Angiotensin II** Sequence: DRVYIHPF Molecular mass: 1046.19 pI: ~7.37 
Angiotensin III Sequence: RVYIHPF Molecular mass: 931.10 pI: ~9.95 	**Angiotensin IV** Sequence: VYIHPF Molecular mass: 774.91 pI: ~7.36 
Angiotensin-(1-7) Sequence: DRVYIHP Molecular mass: 899.02 pI: ~7.37 	**Angiotensin A** Sequence: ARVYIHPF Molecular mass: 1002.18 pI: ~9.95 
Angiotensin-(1-9) Sequence: DRVYIHPFH Molecular mass: 1183.34 pI: ~7.52 	**Alamandine** Sequence: ARVYIHP Molecular mass: 855.01 pI: ~9.95 

The amino acid sequence of the peptides is expressed as one-letter code. The theoretical isoelectric point (pI) and the molecular mass was estimated with the “Isoelectric Point Calculator” [77].

1.2 The renin-angiotensin-aldosterone system in paediatric heart failure

The various components of the RAAS are substantially involved in the development and prognosis of HF, hypertension, and cardiac remodelling. Nevertheless, evidence regarding the interaction of humoral parameters of the RAAS and their influence on HF is primarily available in adults, while only limited knowledge regarding the maturing RAAS in children suffering from HF exists [2]. However, the current data regarding circulating concentrations of angiotensin peptides in the paediatric population is inconsistent at all. While numerous studies are conducted in the context of, e.g. PRA and AngII in the adult RAAS, there is less evidence for children than for adults. Moreover, although paediatric studies for AngII and PRA exist, just sporadic evidence regarding AngI and Ang1-7 is available in the paediatric collective, and meaningful investigations of AngIII, AngIV, AngA, alamandine and Ang1-9 are lacking in all paediatric age groups [78]. Therefore, the identification and evaluation of significant developmental changes in the maturing RAAS, which are emphasised by the rapid maturational changes in the post-natal period, might be vital for a more in-depth understanding of paediatric HF [79].

Generally, HF with preserved ejection fraction is characterised by insufficient cardiac output and is thus associated with an undersupply of metabolising tissues with oxygen, leading to oedema, respiratory distress, fatigue and weakness as clinical symptoms (Figure 1-2) [80, 81]. The International Society for Heart and Lung transplantation defines paediatric HF as “a clinical and pathophysiologic syndrome that results from ventricular dysfunction, volume, or pressure overload, alone or in combination. In children, it leads to characteristic signs and symptoms, such as poor growth, feeding difficulties, respiratory distress, exercise intolerance, and fatigue, and is associated with circulatory, neurohormonal, and molecular abnormalities” [82].

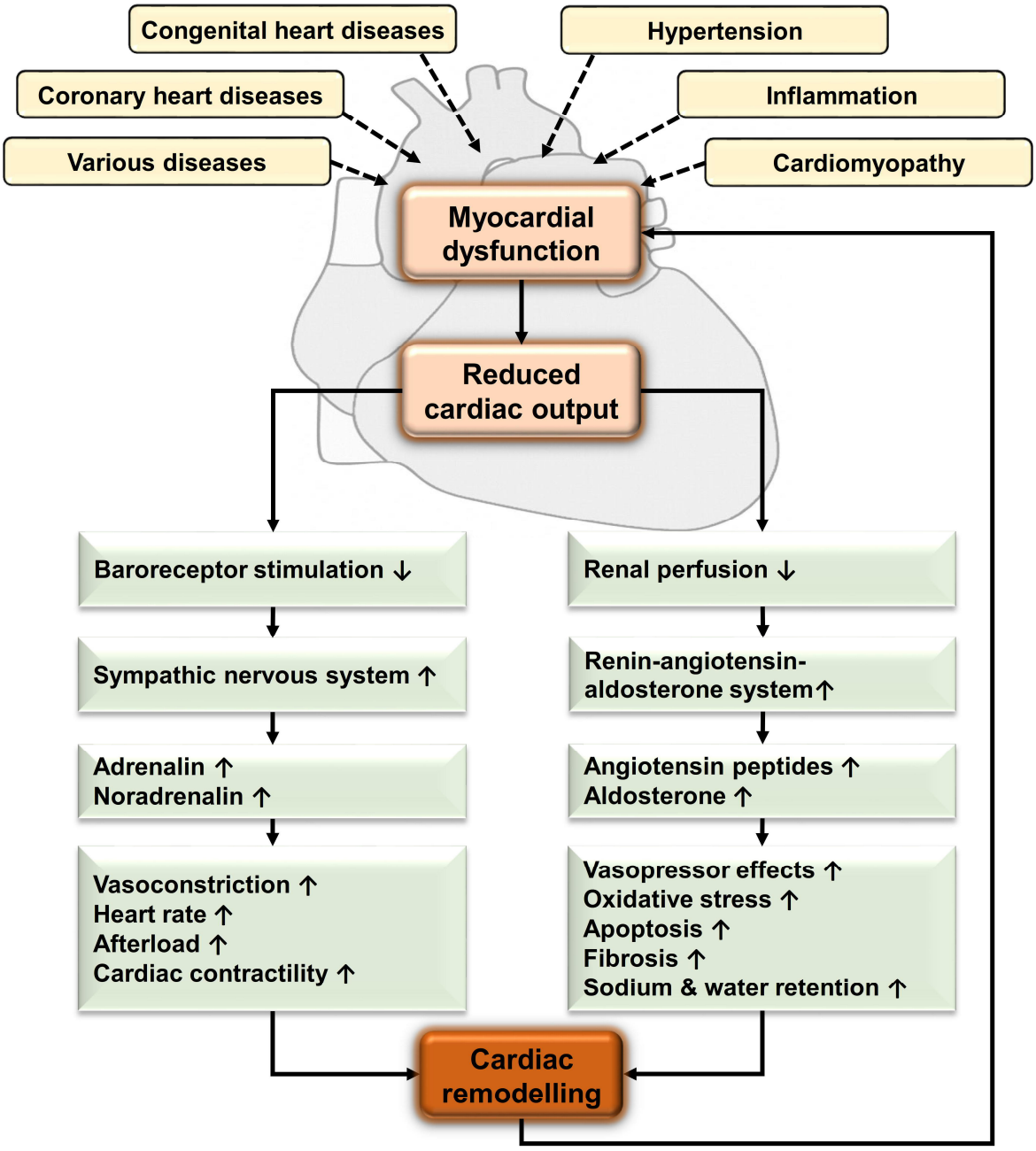


Figure 1-2: Pathophysiology of heart failure.

HF in paediatrics is the leading cause of mortality in children suffering from heart diseases [83] and, unlike the adult HF, often characterised by primary (e.g. congenital heart diseases (CHD)) rather than acquired aetiology [84, 85]. CHD could have several reasons (e.g. left-to-right-shunts, conotruncal lesions, single ventricle lesions, and valve diseases) [86] and is the primary cause for HF in children [87]. However, cardiomyopathies (e.g. dilated cardiomyopathy (DCM)) also contribute as a cause for HF in children with structurally normal hearts [86] and is characterised by systolic dysfunction and dilation mainly of the left ventricle [88]. In addition, there is evidence for shared genetic causes between CHD and cardiomyopathy, which could increase the likelihood of HF concerning CHD [86]. The

estimated annual incidence in the United States, Australia, United Kingdom, and Ireland amount to 1/100,000 children, with DCM as the most common (66%) cardiomyopathic diagnosis [89]. The clinical management in paediatric HF is generally conducted via heart transplantation or surgery, mechanical devices (e.g. pacemakers) and bridging drug therapy consisting of diuretics, beta-blockers and dominantly ACEis [90, 91]. However, studies showed that pharmacotherapy in adults could be only limited transferred to children, based on, e.g. distinctive age-dependent variability in metabolism, expression of target structures and body composition, as well as alterations in the ontogeny of maturing children [92, 93]. Yet, the pharmacological treatment strategies frequently overlap, due to the presumption of similar pathophysiological mechanisms [86, 89]. Nonetheless, evidence regarding effectivity and safety of the treatment in children is still lacking, emphasised by drugs for the treatment of paediatric HF commonly used in an off-label approach, putting the vulnerable population into jeopardy [92].

All in all, paediatric HF is a complex clinical syndrome, which is associated with congenital and acquired heart diseases, resulting in high mortality, morbidity and health-care costs [87, 94]. Although the pharmacological therapy of paediatric HF (as in adults) is predominantly based on RAAS antagonists, the paediatric RAAS has not been adequately investigated, and sophisticated evaluations of the various components are lacking.

1.3 Prerequisites and challenges for research and bioanalysis in children

Background

The off-label use of drugs and the exposure of non-evident doses are frequently the sole possibility for paediatricians to conduct treatment in children. This trend is apparent in the therapy of HF, in which several drugs are approved for HF in adults [95, 96], while only a few approved drugs are available to treat HF in all paediatric age groups. However, due to the rapid changes of factors influencing pharmacokinetics (PK) and pharmacodynamics (PD) within the maturing organism, particularly young children are prone to an increased risk of adverse effects [97]. Therefore, an increased amount of clinical investigations in paediatrics are mandatory. In Europe, this issue was addressed by the European Medicines Agency (EMA) in 2007 via the implementation of the “EU Paediatric Regulation”. The aim of this regulation states “that medicines for use in children are of high quality, ethically researched and authorised appropriately and improving the availability of information on the use of medicines for children” [98]. Moreover, the regulation substantially affects the pharmaceutical industry by the requirement for paediatric investigation plans, with the aim “that most medicines used by children are specifically authorised for such use with age-appropriate forms and formulations; and to increase the availability of high-quality information about medicines used by children” [99]. However, the number of sophisticated paediatric clinical studies remains limited. This still results in a lack of evidence-based pharmacotherapy in this population, attributed by multiple difficulties in the conduct of paediatric studies [92]. Besides the common hurdles within clinical research, paediatric studies are characterised by further difficulties in study conduct. Intricate study designs, issues of guardianship, researcher competencies, barriers to recruitment, and commercial sponsorship are challenges for the performance of paediatric studies [100]. This is emphasised by high rates of study discontinuation and non-publication, which results in a high amount of “lost” paediatric participants [101]. In this regard, a discontinuation rate of 40% was reported for randomised clinical controlled trials, which were conducted in the paediatric population [101]. However, besides the aforementioned hurdles, further obstacles are reasoned by the bioanalysis of paediatric samples.

Bioanalytical challenges

The bioanalytical investigation of paediatric study samples is confronted with challenging blood sampling conditions and restricted sample volumes [101, 102]. Ethical constraints required by the EMA permits withdrawable blood volume in children of 3% of the total blood volume during a period of four weeks or 1% at a single blood sampling [90]. For an estimated blood volume of 80-90 mL/kg, the withdrawable blood volume thus amounts to 2.4 mL/kg in four weeks and 0.8 ml/kg at a single time point. By the example calculation for one-month-old boy with a bodyweight of 4.5 kg (value according to the 50% percentile of the weight-for-age table provided by the World Health Organisation), the maximal allowed blood volume amounts to 10.8 mL in four weeks and 3.6 mL at a single time point. Since bioanalytical assays often rely on serum or plasma analysis, the available matrix is further reduced by approximately 45% to a maximal amount of plasma/serum of 5.9 mL (four weeks) and 2 mL (single sampling). This volume may be sufficient for the analysis of single parameters with conventional assays. However, the simultaneous investigations of PK, PD and safety parameters are common in paediatric clinical trials which require bioanalytical assays applicable for very low sample volumes. These restrictions limit the amount of available matrix for a comprehensive investigation of humoral parameters. Therefore, routinely applied bioanalytical assays are frequently inappropriate for application in paediatrics, as these assays usually require much larger volumes. Consequently, the bioanalytical assays applied within paediatric research had to be tailored for the paediatric purpose.

Due to the ethical constraints, paediatric clinical studies are typically only conducted once [90]. In this regard, from an ethical and analytical perspective, the most effective use of the rare sample material is essential to generate a reliable evidence base for future drug therapy. This is particularly important concerning the limited number of sophisticated paediatric clinical studies, which has led to a lack of evidence-based pharmacotherapy in this vulnerable population [92]. For this reason, the highest possible knowledge must be obtained from the limited sample material. In the context of bioanalysis, sophisticated considerations regarding the individual matrix (e.g. plasma for PRA) and characteristics of each parameter to be measured (e.g. rapid degradation of peptides in plasma) are indispensable. The latter is particularly crucial as the reported low half-life of peptides in plasma (e.g. 16±1 s for AngII and 14±1 s for AngIII) require a sufficient inhibition of plasma directly after the sampling and a fast sample processing [103]. Consequently, the precise planning of blood sampling and sample processing plays a vital role in the reliable conduction of subsequent bioanalysis [104].

Bioanalytical assays which are applied in paediatric clinical trials, have to address the ethical constraints given, e.g. by the EMA [79]. Besides, the used assays are obliged to maximum quality standards and have to be in line with Good Clinical Laboratory Practice (GCLP). In this regard, a maximum effort has to be made to guarantee the acquisition of the highest data quality from the unique paediatric samples, as this population may not be investigated a second time. Moreover, critical therapeutic decisions may be derived from the obtained findings, which makes the assurance of high data quality urgently necessary. In this regard, bioanalytical quality control systems (QCSs) can substantially contribute to reliable data quality and monitor process performance. However, their use in academia is not as widespread as in the pharmaceutical industry; therefore, this deficiency requires particular attention in academia-driven research projects.

1.4 The LENA project: Labeling of Enalapril from Neonates up to Adolescents

The LENA (Labeling of Enalapril from Neonates up to Adolescents) project (Seventh Framework Programme [FP7/2007-2013] under grant agreement n°602295 [LENA]) aimed to systematically investigate the PK and PD of a novel orodispersible dosage form (orodispersible minitablets) of the ACEi enalapril maleate in paediatrics suffering from HF. Especially as no long-lasting ACEi has been licensed for paediatric use below six years of age, the achievement of a safe and child-appropriate administration of enalapril was an important priority of the LENA project. All in all, a total of 102 children (>70% below one year of age) suffering from HF expressed as CHD or DCM participated within the international multi-centre project. Besides the evaluation of PK of enalapril and its active metabolite enalaprilat, the LENA project aimed for comprehensive investigations of the PD parameters AngI, PRA, renin, and aldosterone to bridge the knowledge gap of these humoral parameters in the maturing organism. These examinations played an essential role within the LENA project, as the change in PD parameters could serve as an indicator for the effect of enalapril.

Several quality tools (e.g. a feasibility study, simulation training, audit trails, standard operation procedures, form sheets, dual control principle, external audits) were implemented to meet the regulatory requirements and provide the most reliable data acquisition [102]. This demand for quality also had to be guaranteed in the bioanalytical investigation of the study samples. In this regard, the applied bioanalytical assays must additionally be able to deal with small sample volumes (50-100 µL) and simultaneously provide high sensitivity. Besides, all methods must be validated according to international regulatory guidelines to comply with the GCLP environment. Ultimately, the assay performance and quality had to be monitored over the entire study period to guarantee the continuous acquisition of reliable data from the unique paediatric collective (Figure 1-3).

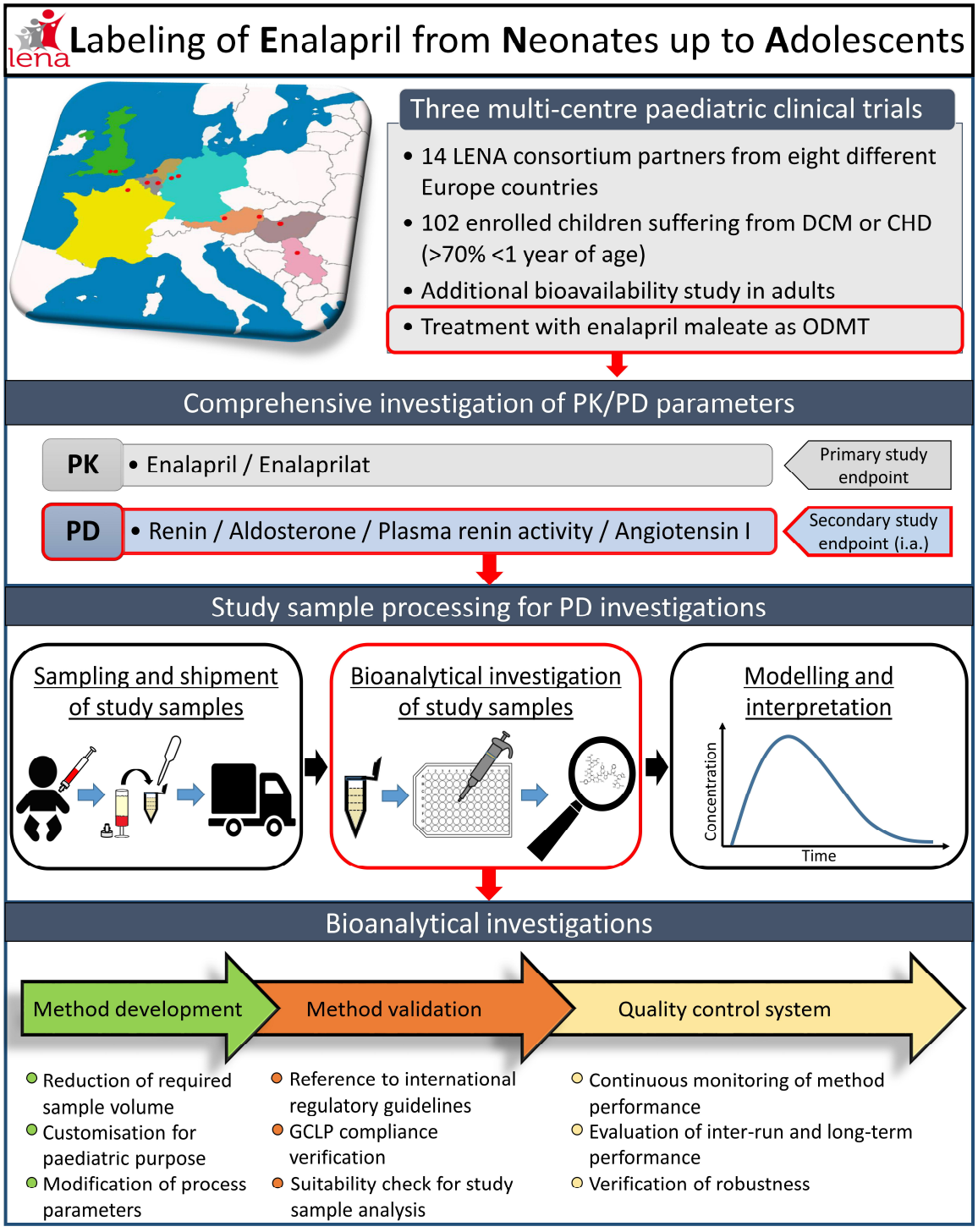


Figure 1-3: **Bioanalytical investigations within the "Labeling of Enalapril in Neonates up to Adolescents" project with a focus on the bioanalytical investigation of pharmacodynamic parameters.** DCM: dilative cardiomyopathy; CHD: congenital heart disease; ODMT: orodispersible minitablet; PK: pharmacokinetic; PD: pharmacodynamic; GCLP: Good Clinical Laboratory Practice

1.5 Highly-sensitive bioanalysis via immunoassay and liquid chromatography coupled to mass spectrometry methods

The lack of reliable PD data for children suffering from severe diseases like HF impedes the development of efficient treatment options and subsequently exposes the children to a heightened risk. Thus, the sophisticated bioanalytical investigation of humoral parameters in children with HF is a valuable approach to provide a remedy. To achieve this, the applied assays have to exhibit high sensitivity as the endogenous biomarkers commonly occur in sub nanogram per millilitre ranges. For this thesis, two different bioanalytical approaches were applied. First, an enzyme-linked immunosorbent assay (ELISA) was utilised for the analysis of the PRA. Second, a solid-phase extraction (SPE) with subsequent liquid chromatography (LC) coupled to high-resolution mass spectrometry (HRMS) was applied for the investigation of the angiotensin peptides AngI, AngII, Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9. Both techniques are explained in more detail in the following chapters.

Bioanalysis of plasma renin activity by enzyme-linked immunosorbent assays

Currently, radioimmunoassays (RIAs) are widely used and constitute a dominant role in PRA determination. On the one hand, RIAs generally represent simple and highly sensitive radiometric methods. On the other hand, several detriments, apart from the inherent risks and costs in handling radioactive material, are mostly based on the short stability of the radioactive tracers due to radiolysis as well as challenges in disposal management [105]. This analytical effort may contribute to PRA not being regularly assessed in clinical research. More easily implemented ligand binding assays (LBAs) such as ELISA have not yet been embraced in the routine determination of PRA values in children, although their simplicity in handling might facilitate the sophisticated monitoring of PRA. The availability of such a method could be instrumental in minimising the previous knowledge gap of PRA in very young children suffering from HF. As the bioanalytical technique of the ELISA constitutes the basis for the determination of PRA within the LENA project, the basic principle is subsequently explained.

Assays based on the principle of antibody-antigen interaction were first described in 1941 [106]. Since the first ELISA was developed in 1971 by modifying the already available RIA method, the ELISA approach was continuously improved and attained crucial importance and widespread application in bioanalytics [107]. The fundamental functionality of an ELISA is described by a selective interaction between an antigen and the corresponding antibody,

which is tagged with a colour-reaction catalysing enzyme (e.g. horseradish peroxidase (HRP)). After the antibody-antigen reaction, an enzyme-specific substrate is added, and the antigen could be quantified with an absorbance reader via the extent of the colour reaction. Nowadays, several subgroups of ELISA methods exist. Since the competitive ELISA was exclusively applied in the context of this thesis, this kind of type will be explained in more detail (Figure 1-4). During the first incubation step, the unlabelled target-antigen (analyte) competes with a labelled-antigen for the immobilised anti-antigen-antibody. After washing and removal of unbound residuals, the enzyme-labelled conjugate is added, which binds to the immobilised labelled-antigen-antibody complex. Subsequently, another washing step is conducted to remove surplus materials, and the colourimetric substrate is added. Finally, the reaction is stopped by acidification, and the optical density (OD) is measured with an absorbance reader. Due to the competitive mechanism, the quantity of the investigated antigen is higher, the lesser the OD.

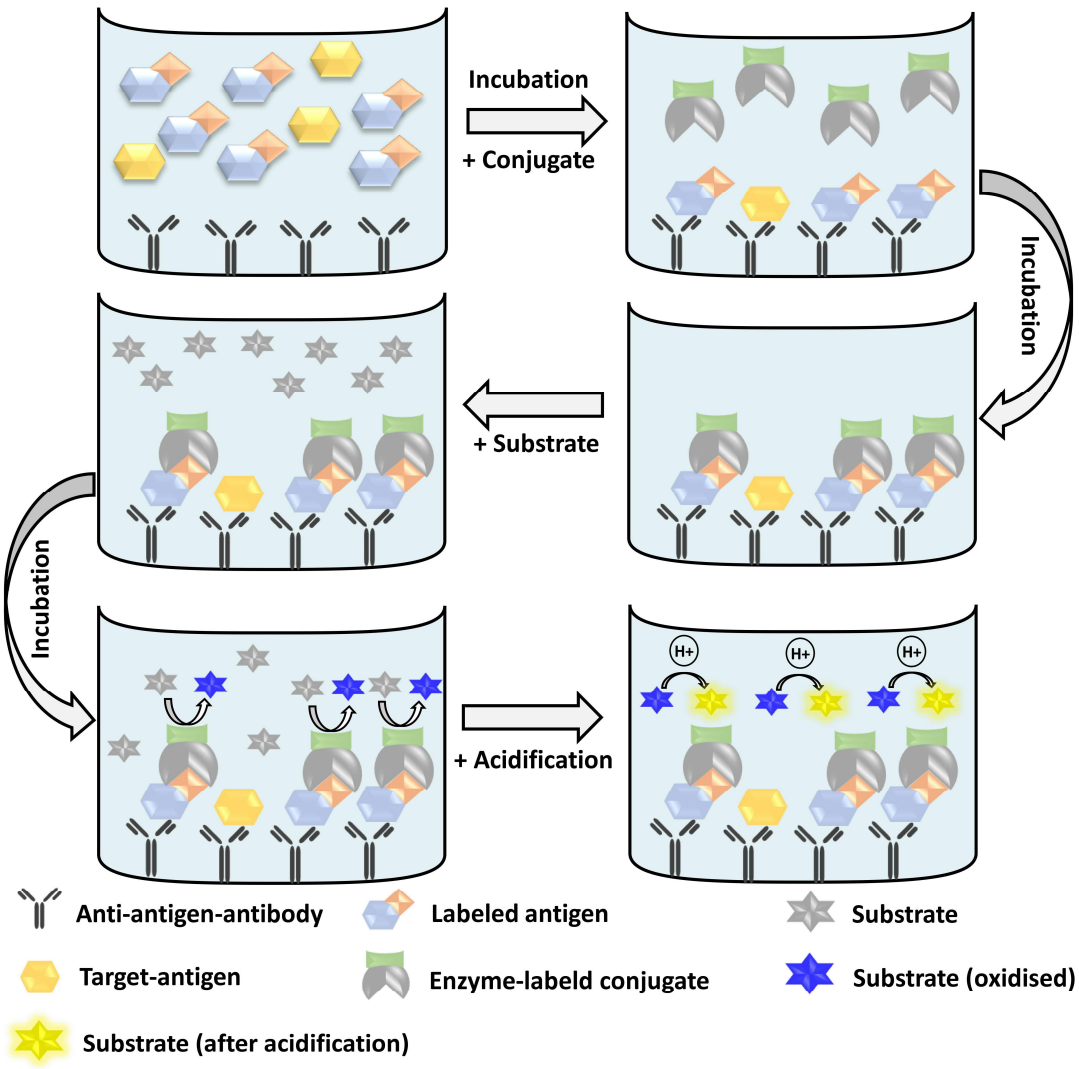


Figure 1-4: Schematic presentation of a competitive enzyme-linked immunosorbent assay procedure. H+: acid

Bioanalysis of peptides by solid-phase extraction and subsequent liquid chromatography coupled to high-resolution mass spectrometry

The LC-HRMS allows separation and subsequent accurate determination of analytes in complex matrices via their exact mass. In particular, the bioanalysis of complex analytes like physiological peptides has benefited from this technique. In contrast to the LBA, the combination of LC with mass spectrometry (MS) provides simultaneous quantification of various peptides with high selectivity over a broad analytical range [108]. However, the extraction and purification of the analytes from plasma with, e.g. SPE is a mandatory step prior to LC-MS analysis.

Solid-phase extraction

The determination of analytes in a complex matrix such as plasma is associated with numerous challenges. First, the components of the plasma can affect the LC by causing interfering peaks, the elevation of the baseline, and the clogging of capillaries. Secondly, matrix components can influence the ionisation of coeluting analytes in the ion source of the MS by causing amplification or suppression of the ion signal. Thirdly, they could contaminate the MS and thus affect the intensity of the analytes and the accuracy and precision of the conducted measurements. Therefore, the thorough purification of samples and the related removal of interfering matrix is a crucial element for reliable bioanalysis with LC-MS. Besides the conduction of a precipitation step to remove plasma components, liquid-liquid extractions and SPE are usually applied to purify the samples for subsequent analysis. Both techniques present widely applicable concepts for sample purification, which are universally used in several analytical fields. However, liquid-liquid separation exhibits various disadvantages. High quantities of required organic solvents, often inadequate separation of the phases, lower recovery levels and reduced sample throughput limit its application as opposed to SPE [109]. Moreover, the high sample throughput underlines the advantages of the SPE in a clinical setting like LENA. Therefore, SPE was applied as current “gold-standard” for the extraction of analytes in the context of this research.

Like liquid-liquid extraction, the SPE principle is also based on the interaction of the solved analyte with two phases. Yet, instead of two immiscible liquid phases, the SPE makes use of a liquid phase and a stationary phase. The selection of an appropriate stationary phase thereby strongly depends on the analytes of interest. According to the chemical properties of the analytes (e.g. hydrophobicity, polarity, and charge), different sorbent materials are applicable [110]. Depending on the sorbent material, various modes of interaction are achievable between the stationary phase and the analyte solution. The normal phase SPE

is characterised by a polar analyte which is dissolved in a non-polar solution as liquid phase (e.g. acetone) and a polar stationary phase. The interaction of the stationary phase and analyte occurs primarily by hydrogen bonding and π-π interactions of the polar groups. In contrast, the reversed-phase requires a polar liquid phase and a non-polar stationary phase. To obtain adequate retention, the analyte should be mid- to non-polar. In this case, the interaction of the analyte with the stationary phase is caused by Van der Waals forces. The elution of the analyte could be achieved by applying a solution with higher polarity (normal phase SPE) or lower polarity (reversed-phase SPE) than the analyte of interest. As a modification of the reversed-phase SPE, the ion-exchange SPE is characterised by additional interactions of ionic compounds with charged functional groups of the sorbent material (e.g. quaternary amines for anionic compounds or sulfonic acid groups for cationic compounds). The retention of the analyte is mainly caused by electrostatic interactions of the analyte with the functional groups of the stationary phase and could be subsequently eluted with a solution of higher ionic strength [111]. However, in all types of SPE, purification of the sample could be realised by washing steps which exhibit opposite properties to the elution solvent [110].

In the course of this thesis, an Oasis® Prime HLB (hydrophilic-lipophilic balance) SPE from Waters® was used for the extraction and purification of the analytes. The Prime HLB sorbents material represents a modified version of the reversed-phase stationary phase, which exhibits mixed properties for the retention of polar and non-polar compounds (Figure 1-5).

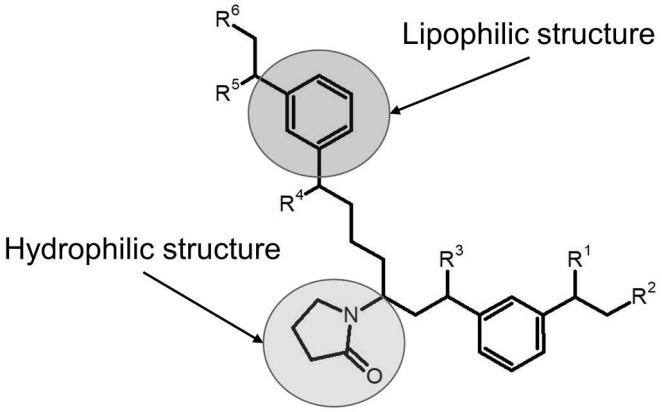


Figure 1-5: Chemical characteristics of the stationary phase for the solid-phase extraction. The applied Oasis® HLB Prime material was obtained from Waters®. R^1-R^6: unknown residuals.

This type of stationary phase provides advantages for both polar and non-polar analytes and is particularly suitable for substances with variable chemical properties like peptides.

High-performance liquid chromatography

The high-performance liquid chromatography (HPLC) is a powerful version of the LC and a reliable method for the qualitative and quantitative determination of mixtures of substances. In brief, the various analytes are solved in a liquid mobile phase which passed over a stationary phase (packed in high density within a column) under application of high pressure. The interactions of the analyte molecules with the two phases can be described with distribution coefficient k. It is defined as the quotient of the concentration in the stationary phase (C_{stat}) and the concentration in the mobile phase (C_{mob}) [112] (Equation 1-1).

$$k = \frac{C_{stat}}{C_{mob}}$$

Equation 1-1: Calculation of the distribution coefficient.

Thus, the efficiency of the separation process is dependent on distribution processes within the column but also impacted by further processes which could be described by the Van-Deemter equation (Equation 1-2).

$$HETP = A + \frac{B}{v} + C * v$$

Equation 1-2: The Van-Deemter equation. HETP: height equivalent to a theoretical plate; A: Eddy-diffusion; B: diffusion coefficient; C: mass transfer coefficient; v: linear velocity.

The height equivalent to a theoretical plate (HETP) represents a measure of the separation efficiency of the column and is influenced by the Eddy-diffusion, the diffusion coefficient, the mass transfer and the linear velocity. The Eddy-diffusion is almost independently of the linear velocity and is expressed as channelling of molecules triggered by non-homogenous packaging and inconsistent particle diameter of the column. The diffusion coefficient divided by the linear velocity of the mobile phase represents the longitudinal diffusion of the molecules and depends on the viscosity and temperature of the mobile phase. The mass transfer coefficient multiplied with the linear velocity describes the mass transfer between the mobile phase and the stationary phase. It is therefore linked to properties of the column and the mobile phase. Therefore, the lesser the HETP, the higher the separation efficacy of the analytes [112]. Consequently, various factors (e.g. hydrophilicity, isoelectric point, and size of the analyte) have to be recognised to achieve a sufficient separation and resolution of the analytes. This separation is indicated by the different retention time of each

analyte, which is substance-specific under constant conditions (e.g. temperature of the column, gradient, and the type of column). The different chemical characteristics of each analyte result in a separation of the mixture's components, which are subsequently transferred to the ion source of the MS.

Electrospray ionisation

The electrospray ionisation (ESI) represents a well-established method to transfer the analytes from the liquid phase to the gas phase and thus making them assessable to the MS. The ESI was developed in 1968 by Dole et al. and 1984 confirmed by Yamashita and Fenn [113, 114]. The mobile phase is transferred, ionised, and sprayed into an interface by means of a capillary on which a high voltage is applied (electrode). The applied voltage results in an electric field between the electrode and a counter-electrode. Depending on the polarity, positively charged ions (electrode as the anode) or negatively charged ions (electrode as the cathode) can be formed [115, 116]. When spraying the mobile phase, a Taylor cone occurs due to the charge repulsion of the resulting ions (Figure 1-6). Nebulisation is enhanced by an inert gas such as nitrogen. The resulting droplets now migrate to the counter-electrode [115, 116]. According to the Charge-Residue-Model by Dole et al., the solvent droplets are removed by an opposing, heated stream of gas (e.g. compressed air or nitrogen), which causes the analyte ions to cluster closer together[114]. These repel due to the same charge. The radius reduction of the droplet eventually reaches the Rayleigh limit. By exceeding this limit, the energy of the repulsive ions is greater than the surface tension, and it comes to a Coulomb explosion, whereby the droplets continue to disintegrate until the individual ions are present separately. The Ion-Evaporation-Model by Iribarne et al. [117] assumes an increase in the curvature of the droplet during evaporation. This is accompanied by an increase in the tension sufficient to desorb the ions from the surface. However, both mechanisms are not yet evidently proven.

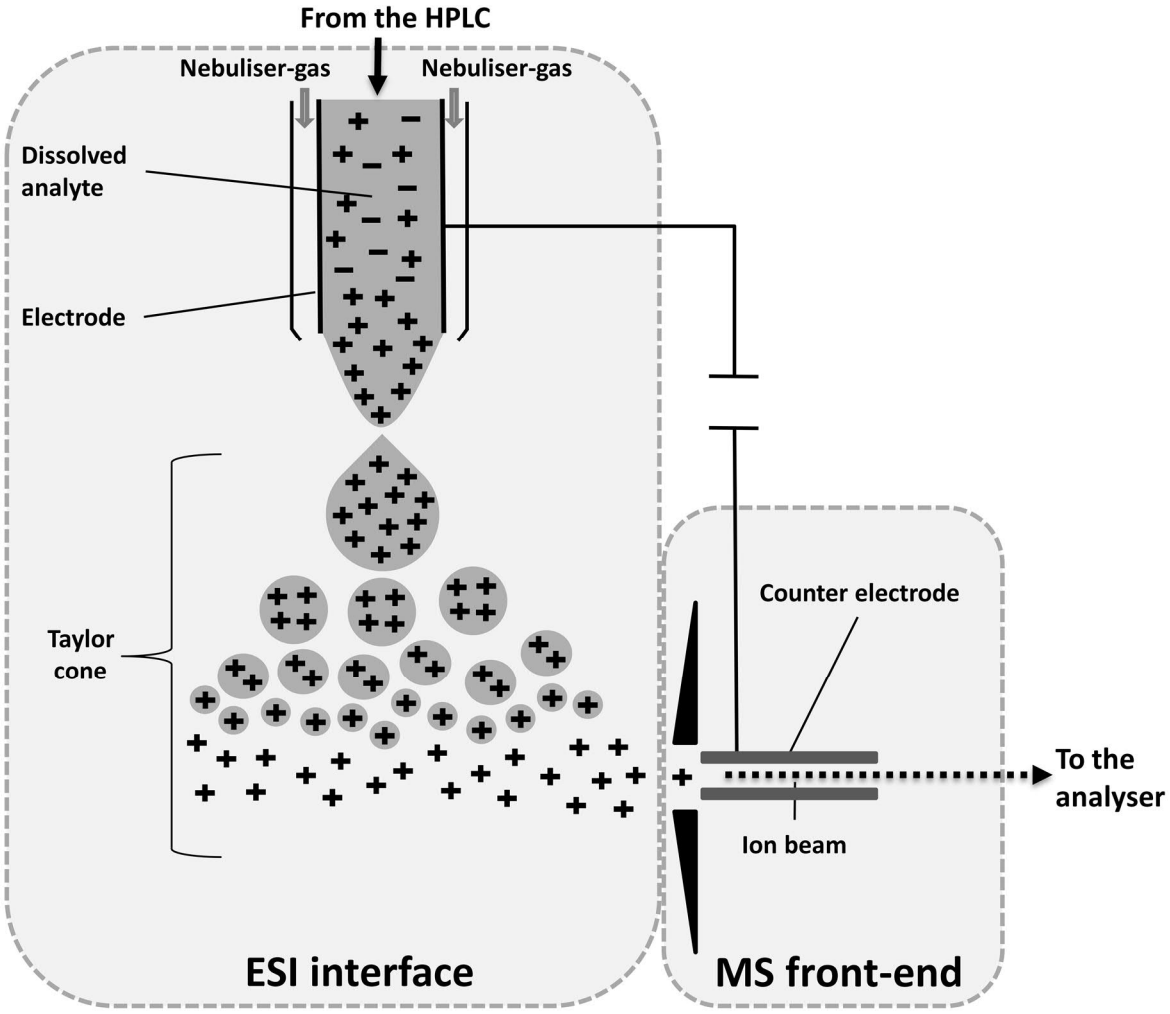


Figure 1-6: Schematic presentation of the vertical electrospray ionisation in the positive mode. HPLC: High-performance liquid chromatography; ESI: electrospray ionisation; MS: mass spectrometer.

High-resolution mass spectrometry

The principle of MS can be traced back to 1912 when Joseph John Thomson managed to separate the neon isotopes with mass 22 and 24 [118]. To this day, the original principle has often been modified and expanded. The triple quadrupole mass spectrometer (TQMS) is regularly used in targeted peptide quantification. However, a HRMS such as a hybrid of quadrupole and time-of-flight mass spectrometer (Q-TOF) provides additional advantages. While the TQMS represents an excellent tool to investigate predefined analytes, it is not suitable for additional untargeted screenings of the sample. Since the MS is a destructive process, potentially valuable information from the rare study material can be lost. In contrast, the technique of the high-resolution Q-TOF empowers the targeted screening and precise separation of very similar analytes and differentiate between metabolites and precursors, as well as the untargeted screening over a broad mass range. Therefore, this HRMS approach allows more profound insights into the maturing organism and can provide

more valuable data with less potential cross-reaction in comparison with, e.g. LBAs. Figure 1-7 describes the applied Q-TOF within this thesis.

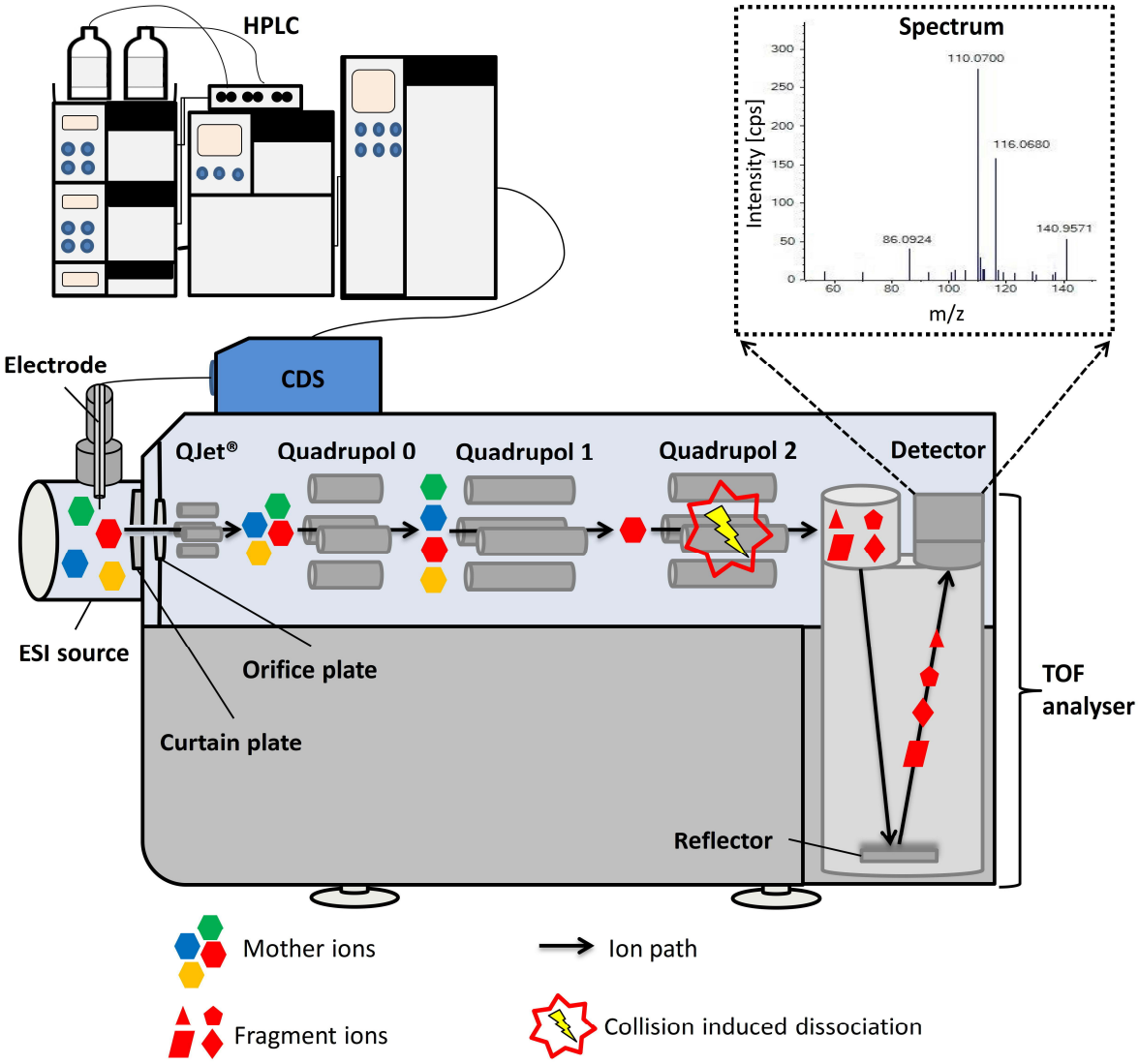


Figure 1-7: Descriptive scheme of the Sciex TipleTOF® 6600 and the related product Ion scan mode. HPLC: high-performance liquid chromatography; CDS: calibration delivery system; ESI: electrospray ionisation TOF: time-of-flight.

After the ESI of the analytes, the ions reach the front-end of the Q-TOF through the curtain plate and the orifice plate (counter-electrode). Subsequently, they pass through the QJet® into the analyser (mass filter) by a high vacuum. The vacuum is mandatory to prevent a collision with molecules of the air, which would result in an uncontrolled fragmentation and thus must be avoided [115, 116]. Due to the curtain gas (a stream of nitrogen behind the curtain plate) and the declustering potential (DP) (which is applied on the orifice plate), resulting clusters consisting of solvent molecules or other foreign ions will be resolved. For ion-focusing, the QJet® is arranged behind the orifice plate, followed by another quadrupole contributing to the further focusing and trajectory stabilisation of the ions. Thereafter, the ions reach two further series-connected quadrupoles, with the first quadrupole, serves as a

mass analyser. In this, an oscillation of the ions is achieved by a superimposed AC voltage. Consequently, ions that do not correspond to the selected mass-to-charge (m/z) ratio are neutralised at the rods and sucked from the vacuum [116, 119]. The last quadrupole represents the collision cell filled with an inert gas. The collision with the gas molecules causes the fragmentation of the ions. The degree of fragmentation is indicated by the collision energy (CE). The resulting fragments now enter the time-of-flight analyser. The ions are accelerated by pulses and hit the detector after a certain distance of the flight. A reflector additionally extends the trajectory of the individual ions. Since, depending on the m/z of the ions, the duration of the flight may be short (small m/z) or more extended (large m/z), it is possible to accurately differentiate between the charged fragments with a high resolution [112, 119].

Characteristics of peptide analysis by liquid chromatography coupled to mass spectrometry

Peptides are molecules which consist of two or more amino acids (AA), linked to a chain via peptide (amide) bonds. Depending on the amount of AA within this chain, peptides were classified as dipeptide (two AA), tripeptide (three AA) etc. Peptides with more than 10 AA are termed polypeptides [120]. All in all, twenty naturally occurring AA are directly encoded by triplet codons in the human genome.

In contrast to small molecules, the chromatographic separation of large molecules like peptides is characterised by further particularities. The size, frequently low solubility, non-specific binding to container walls, polypeptide folding due to AA side-chains and often suboptimal peak shape complicate the chromatographic separation [121]. Consequently, adequate knowledge about the analytes of interest is mandatory for the selection of SPE sorbents, mobile phases, injection solvents, chromatographic columns and the applied settings. Besides, the reported low half-life of peptides in biological fluids requires a fast sample processing [103].

In general, only molecules which carry at least one charge can be analysed with MS. This also applies to peptides, which are characterised (dependent on their AA sequence) by usually more than one charge state. In this regard, the isoelectric point of the peptide and the pH value of the surrounding milieu tributes to the kind of charge. The charge state of the molecule ion (precursor) is indicated by the superscript kind and the number of charges (e.g. $[M+3H]^{3+}$ for a precursor ion with three positive charges). The fragmentation of the peptide strongly depends on the energy of the collision-induced dissociation (CID) within the collision cell of the MS. The low-energy CID (≤100 eV) is a frequently applied technique for the fragmentation of peptides and occurs in mass spectrometers with quadrupole

collision cells [120]. The types of fragment ions which could be observed with low-energy CID depend on various factors like primary peptide sequence, the charge state of the peptide, and the amount of internal energy. Roepstorff and Fohlman first proposed an accepted nomenclature for this fragment ions (Figure 1-8) [122]. The most common resulting fragment ions at the low-energy CID are y and b ions. However, further potential fragment ions like x, z, a, and c are plausible. Moreover, internal fragment ions and neutral-loss fragment ions (loss of ammonia, water or carbon monoxide) could potentially be found [120].

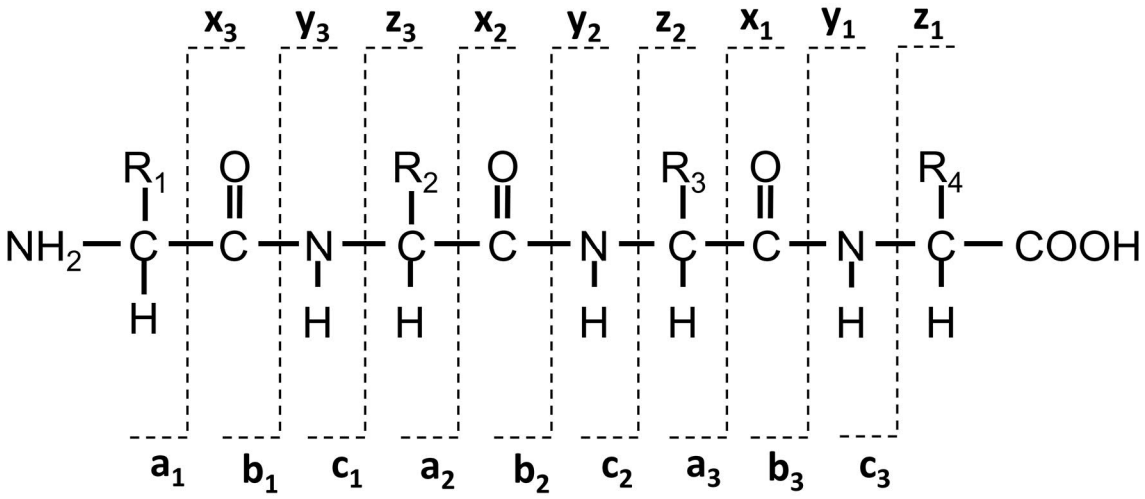


Figure 1-8: Schematic presentation of a peptide fragmentation with the corresponding nomenclature by Roepstorff and Fohlman [122]. Fragment ions comprising the N-terminal end are termed with x, y, or z, while fragment ions with the C-terminal end are termed a, b, or c. The numbering of the respective fragment ions base on the peptide bond and begins at the corresponding end (C-terminal for a, b, or c and N-terminal for x, y, or z).

2 Aims of this thesis

The lack of comprehensive data regarding humoral parameters in the paediatric population represents a barrier for the understanding of the progression and pathogenesis of HF in children. Moreover, the knowledge of pharmacodynamic effects of current pharmacotherapy on the maturing organism might contribute to a safer and more evidence-based pharmacotherapy in children. Therefore, this thesis aimed to enable deeper insights into the paediatric renin-angiotensin-aldosterone system by facilitating reliable bioanalysis of pharmacodynamic parameters in the context of the LENA project. Moreover, the assurance of a high-quality bioanalytical data acquisition throughout the entire study period, as well as a compilation of literature-based data to assist the categorisation of the obtained data within the LENA project were set as objectives.

Therefore, four principal aims were pursued during this thesis to meet these requirements. Each aim is again subdivided into sub-aims in the corresponding chapter.

1. **Systematic reviews of humoral parameters of the paediatric renin-angiotensin-aldosterone system.**

   The aim of the first part of this thesis encompassed the conduction of systematic reviews to provide an overview of angiotensin I, angiotensin II, angiotensin-(1-7), angiotensin III, and angiotensin IV values in healthy besides cardiovascular and renal diseased children to outline the current state of knowledge of the renin-angiotensin-aldosterone system in childhood. Moreover, this investigation aimed to compile literature-based plasma renin activity values in the healthy and cardiovascular diseased paediatric population to support the categorisation of obtained values within the LENA project.

2. **Customisation and validation of a plasma renin activity immunoassay applicable for paediatric research.**

   The second aim was the reliable analysis of plasma renin activity in neonates and children to obtain new perceptions of the renin-angiotensin-aldosterone system within the maturing organism. To achieve this, an enzyme-linked immunosorbent assay appropriate for the analysis of the plasma renin activity in Good Clinical Laboratory Practice settings must be developed. The assay had to be further customised to low-volume applicability to cope with the limited sample volumes being common in paediatric clinical trials like LENA. Moreover, the U.S. Food and Drug Administration

compliant validation of the assay had to guarantee the sophisticated analysis of valuable paediatric data obligatory to comply with international regulatory requirements.

3. **Implementation of a bioanalytical quality control system for the plasma renin activity immunoassay.**

   The third part of this thesis was dedicated to the substantiation of the high-quality acquisition of bioanalytical data within the unique paediatric population of the LENA project. Thus, the establishment of an easy-to-apply but comprehensive quality control system for the bioanalysis of the plasma renin activity by the validated enzyme-linked immunosorbent assay to support the reliability of the obtained data should be implemented. Besides, this paediatric-tailored approach should comprise well-established regulations as well as further scientific references and recommendations to handle the bioanalytical obstacles in academia-driven paediatric research in a Good Clinical Laboratory Practice environment.

4. **Development and validation of an innovative multiplex liquid chromatography high-resolution mass spectrometry method for the investigation of angiotensin peptides in plasma.**

   Lastly, the fourth aim of this thesis intended to enable unprecedented insights in the renin-angiotensin-aldosterone system of neonates and children. This aim should be reached by the development of a high-performance liquid chromatography coupled to high-resolution mass spectrometry method for the simultaneous quantification of angiotensin I, angiotensin II, angiotensin-(1-7), angiotensin III, angiotensin IV, angiotensin A, alamandine, and angiotensin-(1-9) in the paediatric collective. Thus, the applied highly sensitive method has to cope with the limited available plasma volumes in children and should be validated to comply with U.S. Food and Drug Administration guideline requirements.

3 Systematic reviews of humoral parameters of the paediatric renin-angiotensin-aldosterone system

Some aspects of this chapter are already published on the 18th of May 2019 as “Levels of angiotensin peptides in healthy and cardiovascular/renal diseased paediatric population – an investigative review” in the peer-reviewed journal Heart Failure Reviews (doi: 10.1007/s10741-019-09797-y).

3.1 Background and aim

Pharmacological strategies for the treatment of HF are predominantly based on the inhibition of the endogenous angiotensin peptides AngI and AngII and their target receptors. However, recent cardiovascular research focuses on novel peptides of the RAAS [16], which raises a controversial discussion whether AngI and AngII represent the most appropriate target structures or the less investigated peptides such as Ang1-7, AngIII, and AngIV could be more suitable [20]. Since the evidence-based pharmacotherapy of HF is limited in children, these peptides might represent interesting structures for a better understanding of paediatric HF. Yet, the current data regarding circulating concentrations of angiotensin peptides in children and neonates are inconsistent, which highlights the need for a meaningful evaluation of the maturing RAAS in order to facilitate future progress in paediatric care in all age groups. In addition, while numerous studies have been conducted in the context of PRA in the adult RAAS, a comprehensive picture of PRA values regarding the maturing RAAS is lacking, which makes a systematic compilation of available PRA values necessary. Ultimately, the compiled data sets might support the categorisation of the obtained data of the LENA collective as supportive reference. This is particularly noteworthy as the inclusion of healthy children as controls are limited in paediatric clinical trials like LENA.

Thus, the sub-aims of these systematic reviews were:

- To provide an overview of AngI, AngII, Ang1-7, AngIII, and AngIV values in healthy besides cardiovascular and renal diseased children to outline the current state of knowledge of the RAAS in childhood.

- To evaluate the impact of age, gender, and treatment on the plasma concentrations of the individual angiotensin peptides.

- To compile PRA values in the healthy and cardiovascular diseased paediatric population to support the categorisation of obtained values within the LENA project.

3.2 Methods

3.2.1 Search strategy for angiotensin peptides

A literature search was conducted in the MEDLINE database via PubMed between August 2018 and January 2019. For record identification, the search term “(Angiotensin I OR Angiotensin II OR Angiotensin-(1–7) OR Angiotensin III OR Angiotensin IV) AND (Child OR infant OR newborn OR toddler)” was defined. Filters were set as follows: humans, German, English, child: (birth-18 years). Healthy children were defined as children without any cardiovascular and renal diseases or other ailments with obvious evidence for influencing the RAAS. CVD was defined as congenital or acquired heart defects, including HF and DCM. The definition of subjects with RF in this literature search represents congenital or acquired nephrotic diseases, including chronic kidney diseases (stage G2-G5) as well as patients after surgical treatment of the kidney. Inclusion criteria were human in vivo serum/plasma values of AngI, AngII, Ang1-7, AngIII, and AngIV in paediatrics from birth to 18 years of age Exclusion criteria were values in adults (over 18 years, lack of obtained peptide values, *ex-vivo* and animal studies, imprecise date (e.g. undefined age and health status of population, undescribed analytical assay and matrix, no assignability of values and statistical operators), no possibility to access the publication, and diseases not fitting the inclusion criteria.

3.2.2 Search strategy for plasma renin activity

A further literature search was also conducted in the MEDLINE database via PubMed between January 2019 and February 2019. For record identification the search terms “Plasma renin activity AND (Heart failure OR dilated cardiomyopathy OR congenital heart defect OR congenital heart disease) AND (child OR neonate OR infant OR toddler OR paediatric)” besides “Plasma renin activity AND (Healthy) AND (child OR neonate OR infant OR toddler OR paediatric)” were defined. Detailed inclusion criteria and exclusion criteria are shown in Appendix 10.1-1.

In contrast to the systematic review of angiotensin peptides, the sole objective of this systematic review was the compilation of literature PRA values as a reference for the obtained data in the LENA collective. Therefore, the results of this search are expressed in a condensed format as figures and tables without detailed interpretation of the results.

3.2.3 Data processing

Of the included records, the age of the population, the number of participants, PRA and peptide values, sampling procedures, used analytical assays, matrices, characteristics of the collectives, statistical operators, and leading authors with publication date were transferred into a Microsoft® Excel® file (2016 MSO 16.0.4266.1011 64 bit). If required, graphical data was extracted via GetData Graph Digitizer® (Version 2.26.0.20), with a mean of three replicates. Peptide values were expressed as pg/mL and converted from other units, if necessary. PRA values were expressed as ng/ml/h and converted from ng/L/s, if necessary (Equation 3-1).

$$\text{ng/mL/h} = \left(\frac{\text{ng/L/s}}{1000} \right) * 60^{2}$$

Equation 3-1: Conversion of plasma renin activity values for standardisation of compiled literature data

Figures were designed with Origin® 2018b (Version 2018b 9.55). In this context, the age of the population was converted into months, followed by conversion into days in Microsoft® Excel®. For a graphical presentation of the age of study participant's, values given in mean±standard deviation (SD) and mean±standard error (SE) were unmodified transferred in Origin® 2018b. For records in which the range or interquartile range (IQR) was given, values were modified into a centre point of the range and a deviation (Equation 3-2, Equation 3-3).

$$\text{Center point of the range} = \left(\frac{\text{End point of the range} - \text{Start point of the range}}{2} \right) + \text{Start point of the range}$$

Equation 3-2: Calculation of the middle point of the range for graphical representation of age ranges in Origin® 2018b.

$$\text{Deviation} = \left(\frac{\text{End point of the range} - \text{Start point of the range}}{2} \right)$$

Equation 3-3: Calculation of the deviation of the centre point of the range for graphical representation of age ranges in Origin® 2018b.

3.3 Results

3.3.1 Angiotensin peptides in the paediatric population

Overall, 928 findings were screened for the angiotensin peptides literature search of which 78 were assessed for eligibility. Of these, 35 publications were classified as relevant for this review. Further details are provided in the PRISMA diagram [123] (Figure 3-1).

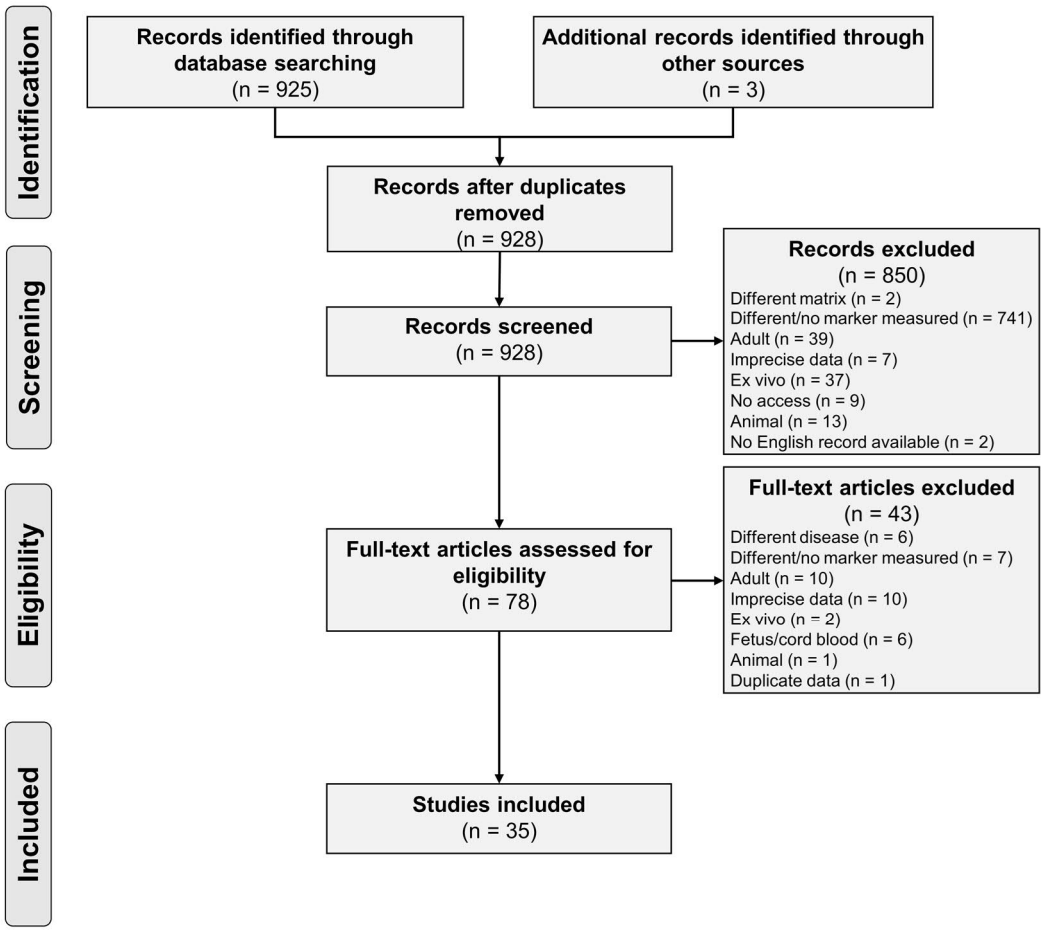


Figure 3-1: PRISMA flow diagram for the systematic review of angiotensin peptides in healthy and cardiovascular/renal diseased paediatric population.

Age-related change of angiotensin peptides during childhood

The ontogeny in children is related to various metabolic and enzymatic changes during childhood. Alteration in parameters occurs in different velocities, leading to continually changing conditions in the organism [92, 93, 124]. The compiled data anticipate that angiotensin peptides decrease rapidly in newborns during the first weeks of life, but remain higher in childhood compared to adult concentrations (Figure 3-2 and Appendix 10.1-2). If not otherwise indicated, statistical operators for the age and plasma concentrations in the following chapters were expressed as range and mean±SD, respectively.

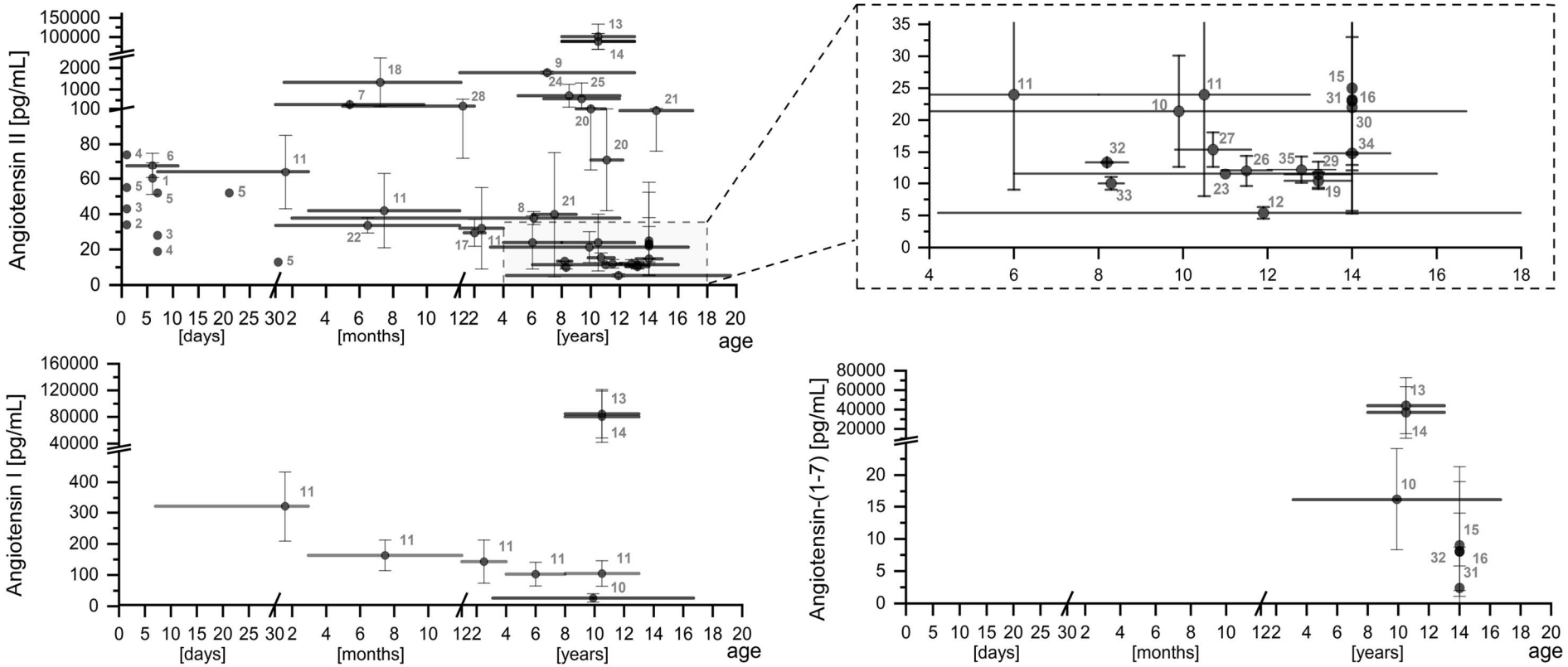


Figure 3-2: Age-related change of Angiotensin I, Angiotensin II, and Angiotensin-(1-7) levels in the healthy population during childhood. Concentrations (C) are expressed as mean±standard deviation (SD), unless other classified; Thick horizontal line: range of age of investigated population, unless other classified; SE: standard error; IQR: interquartile range; BW: Birthweight. 1) Pipkin, 1975 (n=25); 2) Miyawaki, 2006 (Very low BW, n=16); 3) Miyawaki, 2006 (Moderate low BW, n=16); 4) Miyawaki, 2006 (Normal BW, n=16); 5) Miyawaki, 2008 (Very low BW, n=9); 6) Pipkin, 1977 (n=46); 7) Tang, 2015 (n=30); 8) Pipkin, 1981 (n=63); 9) El-Deek, 2017 (n=10); 10) Simões e Silva, 2004 (n=32); 11) Fiselier, 1983 (n=9-16); 12) Gheissari, 2013 (age as mean±SD) (n=30); 13) Franco, 2008 (Appropriate for gestational age) (n=31); 14) Franco, 2008 (Small for gestational age) (n=35); 15) Washburn, 2015 (normotensive pregnancy, n=78); 16) Washburn, 2015 (preeclamptic pregnancy, n=49); 17) Zhang, 2018 (age as mean±SD) (n=30); 18): Zaher, 2015 (n=35); 19) Rittig, 2006 (C and age as mean±SE) (n=10); 20) Mahler, 2012 (age as mean±SD) (n=10); 21) Tiosano, 2001 (n=2-4); 22) Van Acker, 1983 (n=20); 23) Hjortdal, 2000 (C as median (IQR), n=33); 24) Al-Daghri, 2010 (n=150); 25) Al-Daghri, 2011 (C as median (IQR), age as mean±SD, n=131); 26) Kamperis, 2012 (age as mean±SD, n=11); 27) Kamperis, 2008 (age as mean±SD, n=10); 28) Cruces, 2012 (C and age as median (IQR), n=60); 29) Rittig, 2010 (age as mean±SD, n=10); 30) South, 2017 (C as median (IQR), n=50); 31) South, 2017 (antenatal corticosteroids, C as median (IQR), n=70); 32) Mahler, 2015 (prepuberty boys, C as mean±SE, n=9); 33) Mahler, 2015 (prepuberty girls, C as mean±SE, n=9); 34) Mahler, 2015 (puberty boys, C as mean±SE, n=10); 35) Mahler, 2015 (puberty girls, C as mean±SE, n=10).

Newborns

AngI values in newborns are reported by Fiselier et al. in the early 1980th only. The plasma concentrations of 9 newborns and infants (1 week-3 months) ranged from 191-468 pg/mL with a median of 302 pg/mL, which were higher than in older subjects (4 months-13 years) investigated in the same study [124]. Simultaneously measured AngII values revealed likewise higher concentration with a range of 30-117 pg/mL and a median of 58 pg/mL than in older subjects [124]. Pipkin et al. measured AngII concentrations in newborns with prematurity or jaundice. AngII levels significantly decreased from 178.4±26.2 pg/mL (venous cord blood) at birth to 60.3±9.2 pg/mL during the first six days but were higher than in adult controls (28.8±4.2 pg/mL) [125]. Later on, the same group investigated AngII values in 46 healthy newborns 1-11 days after birth. Newborns with less than four days of age showed non-significant higher AngII levels than newborns more than four days of age. Overall, the mean and SD was 67.7±7.1 pg/mL with a median of 50 and a wide range of 9.4-204 pg/mL, which is comparable with the further findings in this group [126]. An investigation of AngII levels in children with varying birthweights (1-7 days) indicated a sustained decrease in the strata of infants with normal birthweight (NBW) and moderate-low birthweight (MLBW) from birth (NBW: 74 pg/mL (range 3-443 pg/mL), MLBW: 43 pg/mL (range 5-438 pg/mL) (geometric mean (range))) to 7 days after birth (NBW: 19 pg/mL (range 1-127 pg/mL), MLBW: 8 pg/mL (range 5-254 pg/mL) (geometric mean (range))). In contrast, infants with a very-low birthweight (VLBW) showed a marked increase in in AngII concentrations of 34 pg/mL (range 5-201 pg/mL) to 76 pg/mL (range 7-1041 pg/mL) (geometric mean (range)) [127]. However, two years later, the same group noted a slight decline in AngII values from birth to day 35 in VLBW infants [128]. These contradict findings in the newborn underline the lack of comprehensive knowledge in this age group.

Infants and toddlers

The AngI plasma concentrations of 16 healthy infants (3-12 months) ranged from 94-244 pg/mL with a median of 157 pg/mL and thus tending to be lower than obtained concentrations from newborns beforehand [124]. The obtained AngII values were equivalent to a range of 12-82 pg/mL and a median of 37 pg/mL [124]. A further cohort of 20 infants (1-12months) exhibit comparable plasma concentrations of 33.6±3.4 pg/mL (mean±SE) [129]. In contrast, Cruces et al. found higher levels in healthy infants with a median and IQR of 10 (5-24) years of age, which amount to a median of 229 pg/mL with an IQR of 157-319 pg/mL. Moreover, 30 infants (30 days-10 months) showed AngII concentrations of 300±30 pg/mL [130] and even 35 infants (1.5-13 months) a concentration of 1330±1130 pg/mL [131]. These findings indicate the high variability which is not clarified yet.

Children and adolescents

The AngI values between a cohort of children with 1-4 years of age (n=10) and a cohort of children with 4-8 years of age (n=9) showed a decrease of plasma concentration from a median of 133 pg/mL (range 83-312 pg/mL) to a median of 99 pg/mL (range 63 183 pg/mL), respectively. The latter values did not notable differ to children with 8-13 years of age (n=10) [124] but were higher than in a cohort of 32 children and adolescents (3.1-16.7 years) with 26.4±13.4 pg/mL [132]. In opposition, remarkable higher concentrations were observed in 8-13 years old boys and girls with appropriate gestational birthweight (AGA) and small gestational birthweight (SGA). Obtained values regarding AGA boys and girls were 77.4±35.6 ng/mL and 85.3±43.7 ng/mL respectively, whereas values obtained from SGA boys and girls amount to 79.7±43.0 ng/mL and 89.1±31.4 ng/mL, correspondingly [133]. AngII values measured by the same group showed slightly higher concentration ranges. The mean plasma concentrations of boys and girls in the SGA and AGA group varied between 82.4 ng/mL and 117.8 ng/mL [133] compared to children (n=10) with the same age being born with NBW (median 22 pg/mL (range 12-67 pg/mL)) [124]. Several publications reported similar lower pg/mL levels of circulating AngII levels in children and adolescents, supported by relatively large numbers of participants, with a broad range of age for included paediatrics [124, 132, 134–143]. Apart from that, higher concentrations were found in 1-13 years old subjects (n=10) with 1780±70 pg/mL [144], as well as in 5-12 years (n=150) and 9.4±2.6 years (mean±SD)) old children (n=131) showing serum levels of 710±530 pg/mL and a median of 570 pg/mL (IQR 440-730 pg/mL), respectively [145, 146]. Ang1-7 plasma levels were reported in children and adolescents only. Concentrations of 16.2±7.9 pg/mL were found in 3.1-16.7 years old subjects (n=32) by Simões e Silva et al. in 2004. In adolescents (14 years) born preterm without (n=50) and with antenatal corticosteroids (n=70), low levels of Ang1-7 with a median of 2.4 pg/mL (IQR 1.3-6.3 pg/mL) versus 8.1 pg/mL (IQR 2.3-13.2 pg/mL) were observed [142]. Measured Ang1-7 levels of adolescents (14 years) born prematurely with VLBW under normotensive (n=78) or preeclamptic (n=49) pregnancy were 9±10 pg/mL and 8±6 pg/mL, respectively. Comparable to previous reported AngI and AngII values, remarkable high concentrations occurred in AGA and SGA children which amount to more than 31.7 ng/mL (mean) [133].

Sex-related differences

Sex hormones are believed to play a role in the regulation of the adult RAAS, which is reflected in altered blood pressure and pathogenesis of cardiovascular and renal diseases [147]. Whereas most of the reported studies in this review included a balanced number of male and female participants, sex-related deviations in the paediatric population regarding angiotensin peptides were reported infrequently. However, based on the growing interest in

personalised medicine approaches, gender could play an increasingly important role in prospective therapy of CVD and RF [148]. AngI and AngII values of 32 boys and 23 girls were obtained for children subdivided into groups of 1 week-3 months, 3 months-1 year, 1-4 years, 4-8 years, and 8-13 years. No significant differences ($p>0.05$) of plasma concentrations were found between males and females in the corresponding groups [124]. In contrast, findings of Pipkin et al., including 20 boys and 43 girls (2 months-12 years), showed evidence ($p<0.02$) of higher AngII plasma concentrations (34.7±4.5 pg/mL) in girls versus (24.9±5.5 pg/mL) in boys. Subdividing into roughly numerical-equal groups of younger than 8 and older than 8 years of age showed significant ($p<0.01$) lower AngII values in boys under 8 years of age. For boys older than 8 years, an equal (but not significant ($p>0.6$)) trend could be observed [149]. A further study was able to gather gender-specific data on AngI, AngII, and Ang1-7 in 66 children (8-13 years). However, no significant, sex-related variations (considering the high intergroup SD) could be ascertained for all RAAS peptides between AGA boys (AngI: 77.4±35.6 ng/mL, AngII: 92.3±18.9 ng/mL, Ang1-7: 31.7±24.3 ng/mL) and girls (AngI: 85.3±43.7 ng/mL, AngII: 82.4±23.1 ng/mL, Ang1-7: 45.8±30.1 ng/mL) as well as between SGA boys (AngI: 79.7±43.0 ng/mL, AngII: 117.8±36.8 ng/mL, Ang1-7: 41.6±31.2 ng/mL) and girls (AngI: 89.1±31.4 ng/mL, AngII: 86.6±29.6 ng/mL, Ang1-7: 46.2±27.0 ng/mL) [133]. In a similar subject, Washburn et al. investigated AngII and Ang1-7 values in VLBW infants of 14 years of age, which were born prematurity, either in normotensive or preeclampsia conditions. AngII and Ang1-7 values of normotensive male children were 19±10 pg/mL and 9±13 pg/mL, compared to normotensive female children of 29±13 pg/mL and 9±8 pg/mL, respectively. The preeclampsia born male children showed AngI and Ang1-7 values of 22±9 pg/mL and 10±9 pg/mL besides 24±10 pg/mL and 7±4 pg/mL, respectively for female preeclampsia born children [143]. Overall, no significant differences could be observed in circulating peptide concentrations between both genders. Further AngII values were obtained as part of a study including 73 boys (9.0±1.8 years (mean±SD)) and 77 girls (9.3±2 years (mean±SD)). Serum concentrations for boys were 650±300 pg/mL, while values in girls were slightly higher (730±600 pg/mL) but did not reach statistical significance ($p=0.45$) [145]. Supplementary evidence for similar, gender independent levels of AngII in the paediatric population was obtained by Mahler et al. No significant sex-related differences in baseline concentrations could be observed in prepuberty boys (13.4±0.1 pg/mL (mean±SE), 8.2±0.5 years, n=9) and girls (10.1±1 pg /mL (mean±SE), 8.3±0.3 years, n=9) besides puberty boys (14.8±0.2 pg/mL (mean±SE), 14.0±0.9 years, n=10) and girls (12.2±2.1 pg/mL (mean±SE), 12.8±0.8 years, n=10) ($p=0.879$) [150]. In contrast, the same group measured significant ($p=0.03$) different night time levels of AngII in boys

(71±29 pg/mL (mean±SE), 10.6±1.2 years, n=10) and girls (107±42 pg/mL (mean±SE), 10.6±1.2 years, n=10) [139].

Pharmacological intervention in cardiovascular/ renal diseased children

10.4±4.8 years (mean±SD) old children (n=32) with hypertensive chronic renal failure (CRF) receiving antihypertensive medications showed an elevation as compared to normotensive CRF children (12±4 years (mean±SD), n=28) without antihypertensive medication for plasma levels of AngI (171.8±85.4 pg/mL *vs.* 26.7±6.7 pg/mL), Ang1-7 (140.8±58 pg/mL *vs.* 18.6±6.8 pg/mL) and AngII (84.2±52.9 pg/mL *vs.* 22.4±9.1 pg/mL). In addition, a similar proportion was observed with respect to 11.2±4 years (mean±SD) old healthy controls (n=32) [151]. For patients in the same collective receiving antihypertensive medication either with (n=20) or without ACEi (n=14), a significant elevation for AngI (199.0±47.6 pg/mL *vs.* 135.3±44.6 pg/mL) and Ang1-7 (159.1±62.6 pg/mL *vs.* 114.7±39.7 pg/mL), as well as significantly lower levels for AngII (53.6±32.5 pg/mL *vs.* 127.9±45.6 pg/mL) could be observed. Additionally, antihypertensive medication led to a significant elevation of peptide levels in a hypertensive end-stage renal disease (ERSD) (n=18) group compared to ESRD patients without antihypertensive medication (n=3) for AngI (349±33.1 pg/mL *vs.* 230.4±47.6 pg/mL) and Ang1-7 (443.8±58.5 pg/mL *vs.* 271.4±32.9 pg/mL). Yet, AngII concentration did not raised significantly (117.2±25.6 pg/mL *vs.* 89.3±9.7 pg/mL) [151].
In contrast, antihypertensive treated ERSD children with additional ACEi (n=12) and without additional ACEi (n=9) showed comparable results for AngI, Ang1-7 and AngII [151]. Moreover, treatment of 11.6±4.8 years (mean±SD) old children suffering from essential hypertension with calcium channel blockers alone (n=12) or non-pharmacological treatment (n=3) showed no differences in RAAS profile before and after treatment for AngI (36.5±11.4 pg/mL *vs.* 35.7±11.2 pg/mL), Ang1-7 (78.5±20.4 pg/mL *vs.* 79.8±23.4 pg/mL) and AngII (21.8±10.6 pg/mL *vs.* 22.5±10.9 pg/mL) [132]. Moreover, administration of fosinopril in addition to prednisone in context of a randomized controlled trial in 8.7±3.5 years (mean±SD) old children (n=25) with steroid-resistant idiopathic nephrotic syndrome (SRINS) revealed no significant difference of AngII values as compared to 8.7±3.7 years (mean±SD) old SRINS children receiving prednisone alone (n=20) after 12 weeks of treatment (78.9±26.2 pg/mL *vs.* 79.0±35.8 pg/mL) [152] (Figure 3-3 and Appendix 10.1-3).

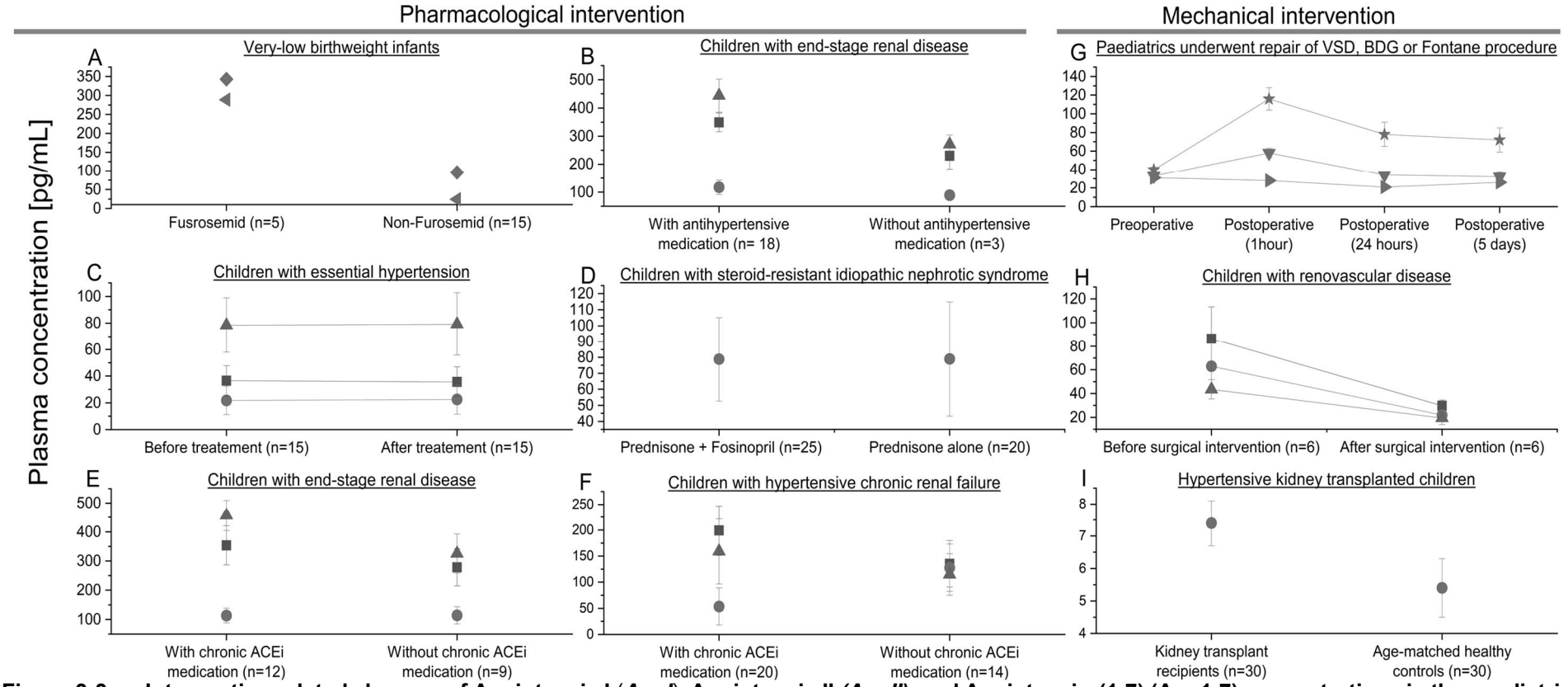


Figure 3-3: Intervention related changes of Angiotensin I (*AngI*), Angiotensin II *(AngII),* and Angiotensin-(1-7) (Ang1-7) concentrations in the paediatric population (expressed as mean±standard deviation (if not other classified)). Lines: identical patients before and after intervention; VSD: ventricular septal defect; BDG: bidirectional Glenn; CCB: calcium channel blocker ACEi: angiotensin-converting enzyme inhibitor; ESRD: end-stage renal disease; ■: AngI; ●: AngII; ▲: Ang1-7. A: AngII values in very-low birthweight children. ◆: AngII values 21 days after birth; ◀ AngII values 35 days after birth [128]. B: Children with ESRD treated with CCB + ACEi (n=12), CCB + loop-diuretic (n=2), CCB (n=4) ore untreated (n=3) [151]. C: Children with essential hypertension treated with CCB (n=12) or non-pharmacological (n=3)[132]. D: Normotensive children with steroid-resistant idiopathic nephrotic syndrome receiving prednisone + fosinopril (n=25) or prednisone alone (n=20) [152]. E: Children with ESRD receiving ACEi (n=12) or no ACEi (n=9) [151] F: Children with hypertensive chronic renal failure receiving ACEi (n=20) or no ACEi (n=14). [151]. G: AngII values (mean±standard error) of paediatrics underwent cardiac surgeries. ▶: AngII values in infants and toddlers (age 25±8 months, n=10) underwent repair of VSD; ▼: AngII values in infants and toddlers (age 13±5 months, n=15) underwent BDG procedure; ★: AngII values in children (age 36±10 months, n=8) underwent Fontane procedure [153] H: Children with renovascular disease before and after renal angioplasty (n=5) or nephrectomy (n=1) [132]. I: Hypertensive kidney transplanted children (n=30) and age-matched healthy controls (n=30)[154].

Mechanical intervention in cardiovascular/ renal diseased children

The impact on circulating AngII concentrations of newborns and infants before and after bidirectional Glenn (BDG) procedure (13±5 months (mean±SE), n=15), Fontane procedure (36±10 months (mean±SE), n=18) or elective ventricular septal defect (VSD) repair (25±8 months (mean±SE), n=10) during catheterisation was investigated by Mainwaring et al. AngII levels were slightly elevated in the BDG group at 1 hour after operation (58±5 pg/mL (mean±SE)), compared to preoperative measurements (33±4 pg/mL (mean±SE)), but returned to their preoperative values after 24 hours (34±4 pg/mL (mean±SE)) and stayed constant till day 5 after operation (32±5 pg/mL (mean±SE)). Preoperative AngII values in the Fontane group were 40±3 pg/mL (mean±SE) and increased significantly 1 hour after operation (116±12 pg/mL (mean±SE)), followed by a decrease in 24 hours (78±13 pg/mL (mean±SE)) and 5 days (72±13 pg/mL (mean±SE)) but remained at significant elevated levels [153]. In a different manner, renal surgical correction (renal angioplasty (n=5), nephrectomy (n=1)) in 6 children with renovascular hypertension led to a significant decrease in plasma concentrations of peptides levels from before (AngI: 86.5±26.9 pg/mL, Ang1-7: 43.5±8.2 pg/mL, AngII: 62.9±21.5 pg/mL) to 6 months after successful surgery (AngI: 29.7±4.8 pg/mL, Ang1-7: 19.5±5.5 pg/mL, AngII: 21.8±2.9 pg/mL) (Figure 3-3 and Appendix 10.1-3).

Figure 3-4 provides an overview of the overall impact of examined factors on plasma and serum concentrations of AngI, AngII, Ang1-7, AngIII, and Ang IV.

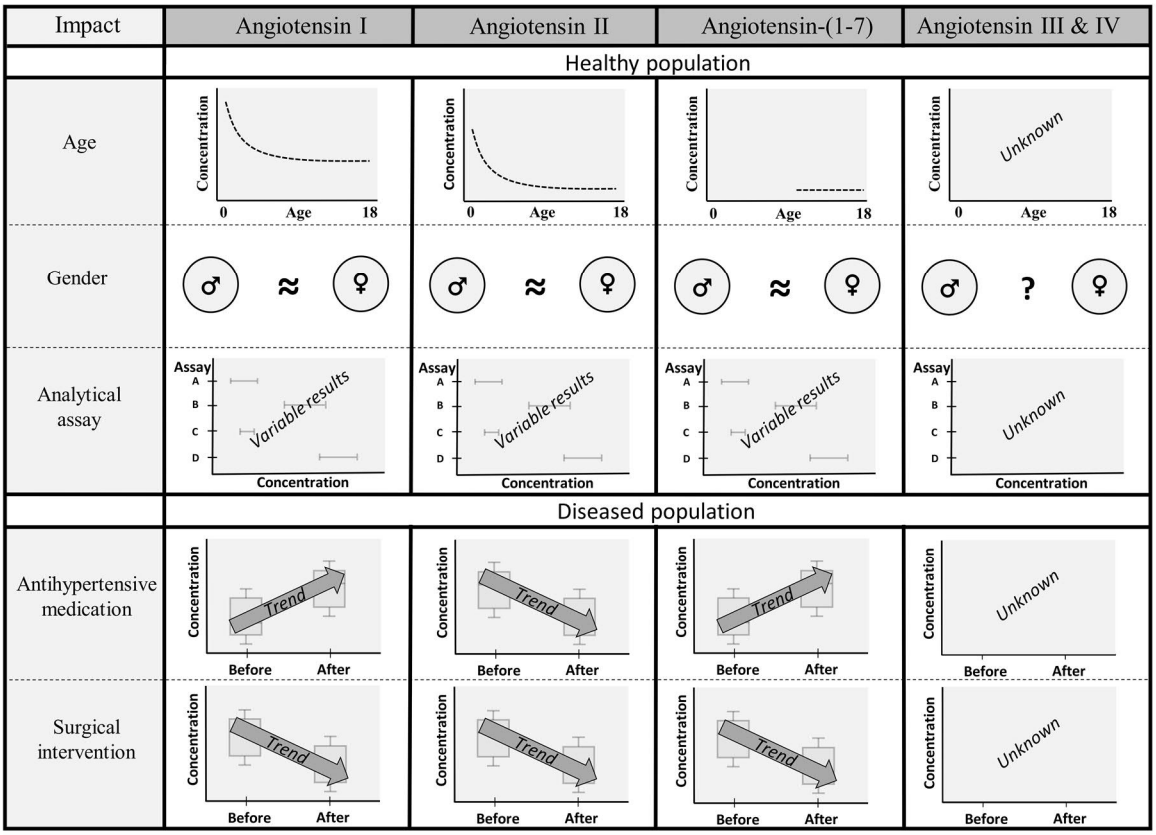


Figure 3-4: Impact of examined factors on plasma and serum concentrations of angiotensin peptides in the paediatric population.

3.3.2 Plasma renin activity in the paediatric population

In summary, 167 findings were screened for PRA, of which 104 records and 63 records were identified for healthy and diseased children, respectively. Furthermore, 5 records were added in the context of external full-text screening accomplished beforehand. After removal of 11 duplicates, title and abstract of 161 publications were screened, of which 62 full-text articles were assessed for eligibility. After the full-text screening, 34 publications were included for this literature search. Further details are provided in the PRISMA diagram [123] (Figure 3-5).

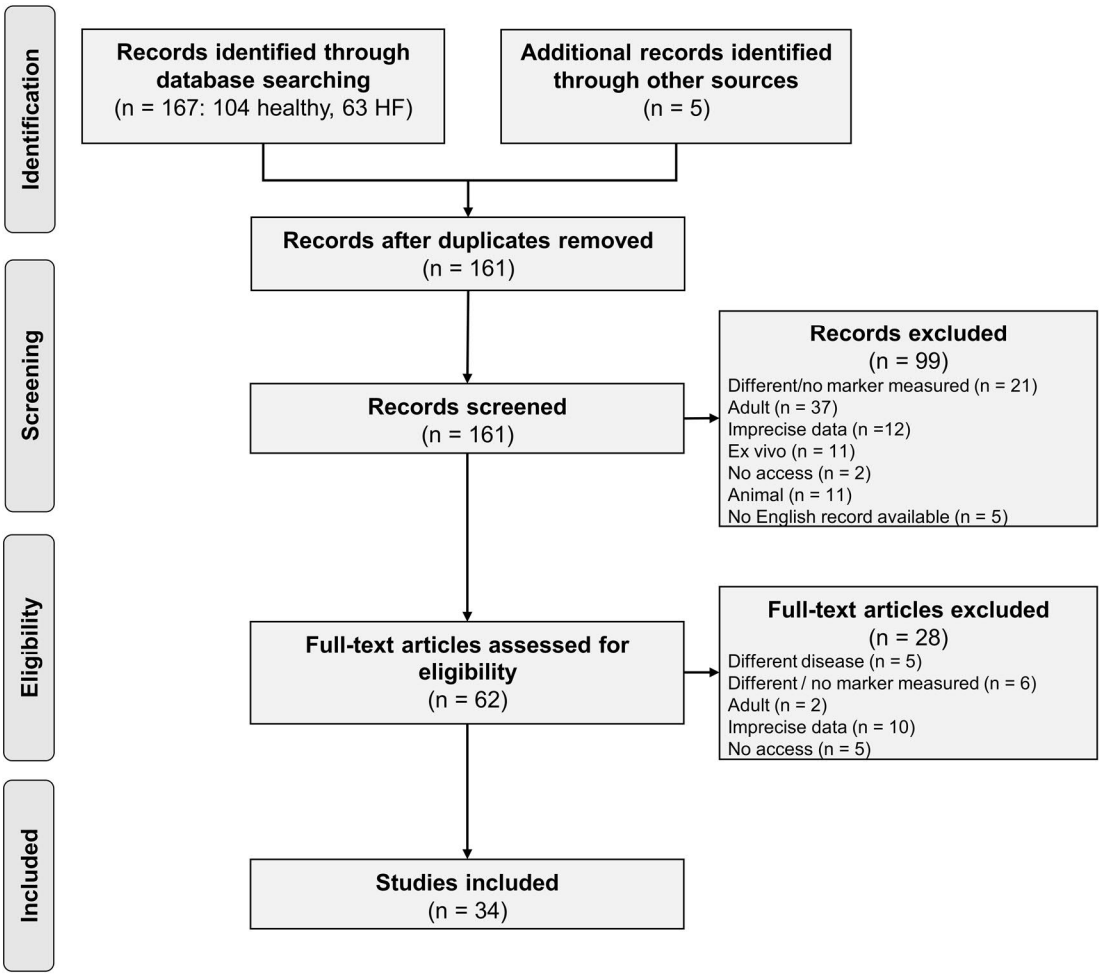


Figure 3-5: PRISMA flow diagram for the systematic review of plasma renin activity in the healthy and cardiovascular diseased paediatric population.

Plasma renin activity in healthy paediatrics

PRA concentrations of healthy paediatrics (n=1995) indicate an age-dependent decrease (Figure 3-6 and Appendix 10.1-4). Highest values were found in newborns and infants. The observed values for children and adolescents were lower but still higher than PRA in adult

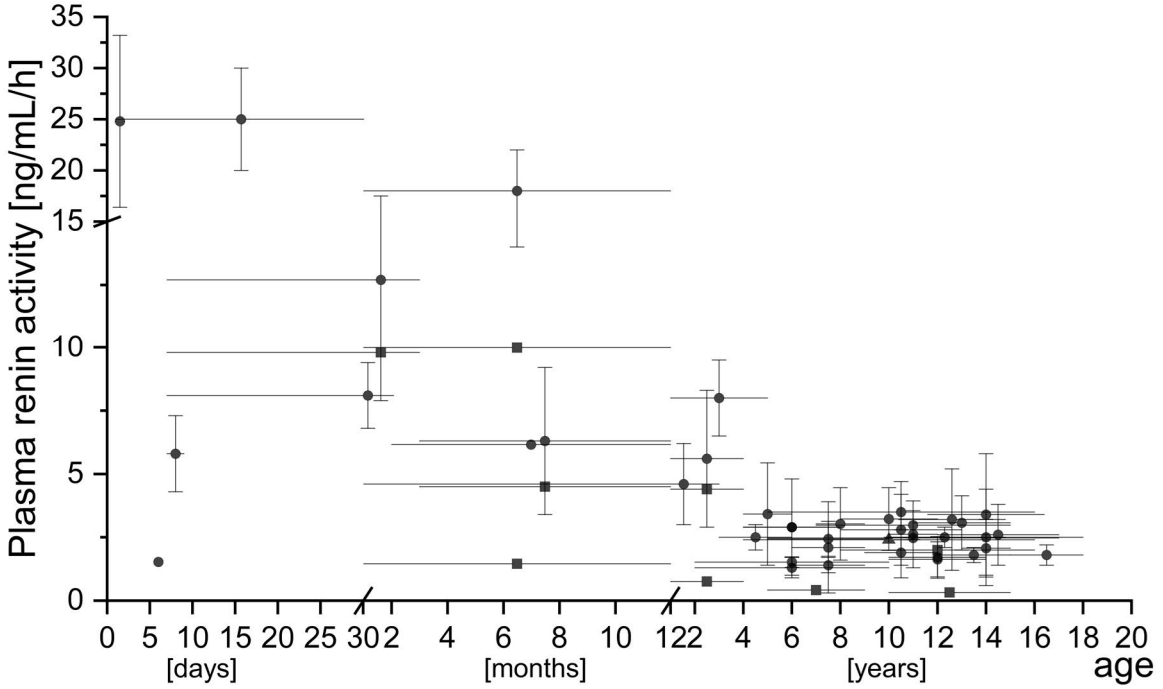


Figure 3-6: **Plasma renin activity in the healthy paediatric population.** For more detailed information, please refer to Appendix 10.1-4. Variation of age expressed as range or mean±SD. ■: PRA values as mean (range);▲: PRA values as median (IQR); ●: PRA values as mean±standard deviation (SD); The shown data was obtained from: Abd-Allah et al. 2004; Arvay et al. 1982; Blazy et al. 1989; Dechaux et al. 1982; Dillon et al. 1976, Dillon et al. 1975; Fiselier et al. 1984; Fiselier et al. 1983; Fukushige et al. 1993; Gjuric et al. 1982; Godard et al. 1979; Harshfield et al. 1992; Shibutani et al. 1988; Harshfield et al. 1991; Lall et al. 1995; Martinez-Aguayo et al. 2010; Stalker et al. 1976; Mardesic et al. 1979; Tiosano et al. 2011; Tu et al. 2017; Simsolo et al. 1988.

Plasma renin activity in cardiovascular diseased paediatrics

In general, the PRA values of paediatrics suffering from DCM (n=12) and CHD (n=272) showed higher levels than corresponding healthy controls. The results are shown in Figure 3-7 and Appendix 10.1-5.

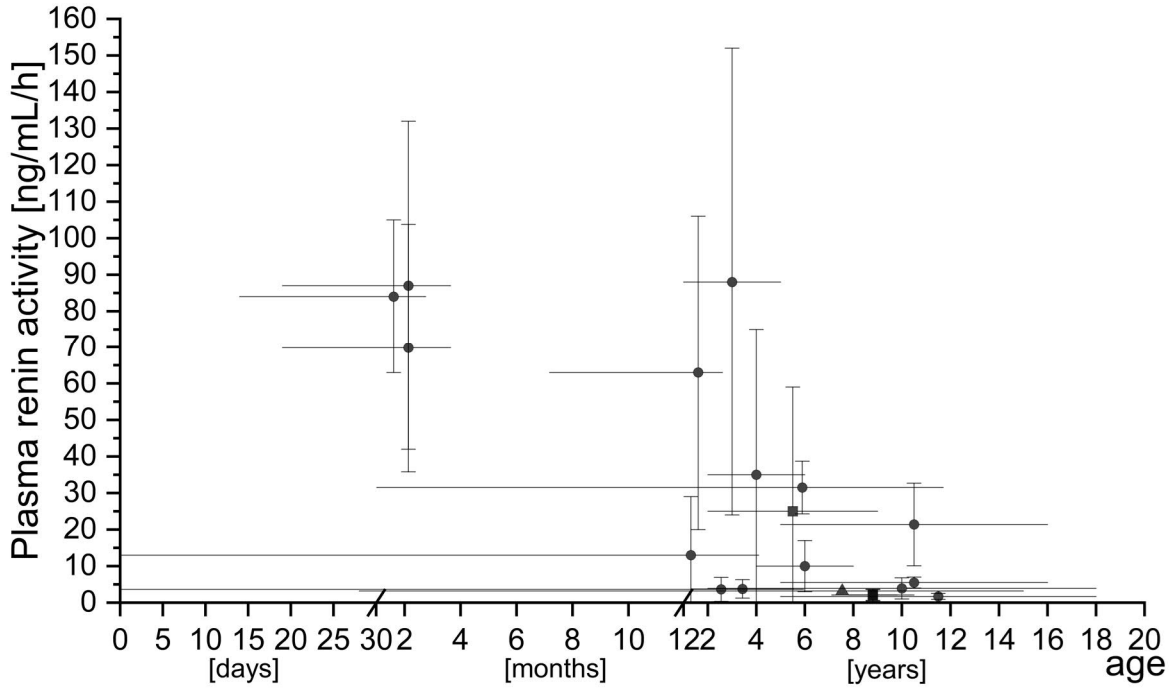


Figure 3-7: Plasma renin activity in cardiovascular (dilative cardiomyopathy, congenital heart disease) diseased paediatric population. For more detailed information, please refer to Appendix 10.1-5. Variation of age expressed as range, mean±standard deviation (SD) or mean±standard error (SE); ■: PRA values as mean±SE; ▲: PRA values as median (95% CI); ●: PRA values as mean±SD; The shown data was obtained from: Alvarez Kindelan et al. 1994; Buchhorn et al. 2001; Fallo et al. 1978; Parker et al. 1982; Scammel et al. 1987; Scammel et al. 1988; Scammel et al. 1989 Giardini et al. 2003; Stern et al. 1990; Saiki et al. 2015; Ationou et al. 1993; Gidding et al. 1985.

3.4 Limitations

These reviews include only MEDLINE database listed publications that were available in the English language (language bias). In addition, inclusion criteria not mentioned in the title or abstract could potentially be missed out. Incomplete reported data (e.g. unspecific age, concentration units, assays, health status of participants) was excluded, leading to a more particularised dataset. While blood sampling of entirely healthy children is ethically questionable, some of the reported values of healthy children were obtained during routine investigation or hospitalisation caused by various diseases. It was anticipated that these impacts are not to be RAAS-related. However, the influence of the plasma concentration cannot be completely ruled out. The lack of consistent and uniformly of reported raw data is another limitation of the accomplished literature search. A further limitation is the heterogeneity of reported statistical operators (e.g. mean±SD, mean±SE, and median (95% confidence interval)) of reported age and plasma concentrations, which represents an aggravating factor of data comparison.

3.5 Discussion

In the healthy paediatric population, AngI values were higher than AngII values. Yet, both plasma concentrations constantly decreased during early childhood and stayed constant afterwards, showing still higher values than in adults [16, 124]. In the case of Ang1-7, data in paediatrics is insufficiently available to generate a reliable statement, although the obtained levels appear to be low in the paediatric RAAS if compared to adults [16]. Whereas all values were obtained from healthy controls, the variation of determined values is high between each study. The phenomenon may be explained by different race, the impact of sampling, used inhibitor-cocktails, matrix, sample processing procedures and analytical principals of measurement (e.g. RIA, ELISA or ultraviolet/visible spectroscopy (UV/VIS)). Moreover, AngIII and AngIV, as well as recently investigated bioactive peptides of the RAAS like AngA, alamandine, and Ang1-9 could be subject to misinterpretation of angiotensin concentrations due to the missing selectivity of applied immunoassays [14, 15, 17]. Thus, the implications on the paediatric RAAS due to AngIII and AngIV besides further bioactive RAAS peptides is still insufficiently investigated. Consequently, an overestimation of AngII and Ang1-7 impact on the RAAS in paediatric subjects is conceivable.

In contrast to gender-specific variations of the RAAS in adults [147, 155], no clear evidence for differences between male and female infants and children regarding circulating AngI, AngII, and Ang1-7 concentrations in the paediatric population could be demonstrated. Hence, sex hormones which are believed to play a regulatory role in the adult RAAS [155], seems to be less distinctive for a discriminatory effect in children, suggesting that the gender-specific influence of the RAAS will be settled after childhood.

RAAS profiles could be subject to different states of hypertension and renal diseases [28, 150]. Because of conflicting evidence for the alteration of concentrations of RAAS components during and after treatment of cardiovascular and renal diseased children, evaluation of pharmacological and mechanical treatment effects in the very youngest are mandatory for a better prediction of therapeutic effects in the future. However, consistent evidence for changes of the angiotensin peptides related to antihypertensive drugs and surgical interventions in children is still discussed. A pharmacological intervention indicates an impact on circulating peptide concentrations due to the influence of antihypertensive medication which interferes with renal processes (especially ACEi), yet the evidence is still conflicting. However, normalisation of angiotensin peptides caused by surgical intervention is anticipated.

While PRA values for healthy paediatrics are necessary for a supplementary picture of the RAAS, appraisal of changes in PRA regarding diseased children is an indispensable approach for a better understanding of certain cardiovascular diseases. This review of reported PRA data facilitates an overview in an innately variable population. Moreover, the conditions of the maturing RAAS influenced by cardiovascular diseases were depicted. A coherent decrease of PRA values during the first two years of life regarding healthy paediatrics was observed. This results are in accordance with further findings and support the inverse correlation of PRA with age [124, 156–159]. Compared to children and adolescents, higher values occurred in newborns and infants, which show evidence for elevated activity of the RAAS in the very early period of childhood. Although PRA decreases during childhood, values were still higher in children and adolescents than in adults. The differences in obtained values regarding similar age groups may be assigned to different bioanalytical assays, sampling procedures, and positions during blood sampling. Although tendencies in higher PRA values for cardiovascular diseased paediatrics could be observed, variations between similar age groups exist. This unsteady values may be raised by various states of heart diseases and discrepancies in aetiology (e.g. ventricular septal defect vs. coarctation of the aorta) of the examined population [160–163]. In addition, the quantity and development of PRA values are probably influenced by pharmacological and mechanical interventions [164–167].

Since drugs in paediatric care are still prevalent used off-label [168], several initiatives like the European Commission aims to counteract the safety risks resulting from off-label use by increased funding of paediatric studies (e.g. the seventh framework program LENA trial). However, the direct comparison between control groups and severely diseased children is often limited due to ethical constraints regarding the inclusion of healthy children in clinical trials. Diligent compiled pharmacodynamic data from literature might support a meaningful assessment of obtained study results in an overall context and serve as a supportive reference for paediatric RAAS for this kind of trials.

3.6 Conclusion

A systematic review of angiotensin peptide concentrations during childhood as well as the identification of age, gender, and cardiac/ renal influences was successfully conducted. Moreover, literature PRA data were compiled as a supportive reference for the LENA project. The literature searches indicated a lack of reliable data for the various angiotensin peptides in all paediatric age groups as well as inconsistent data for PRA in healthy and cardiac diseased newborns and infants.

4 Customisation and validation of a plasma renin activity immunoassay applicable for paediatric research

Some aspects of this chapter are already published on the 23rd of October 2019 as "Customisation and validation of a low-volume plasma renin activity immunoassay: Enabling of regulatory compliant determination in paediatric trials" in the peer-reviewed journal Practical Laboratory medicine (doi: 10.1016/j.plabm.2019.e00144).

4.1 Background and aim

Among the humoral parameters involved in the RAAS, PRA is a valuable parameter for the evaluation of metabolic dysfunctions regarding CVD. Nevertheless, while corresponding reference values for the classification of disease severity are well established in adults, reliable data in children are inconsistent. Notably, the lack of data for children impedes the development of evidence-based medicine and subsequently puts children in jeopardy [92]. Moreover, high rates of off-label and unlicensed drug use in the paediatric population indicate a gap in current knowledge of PRA and further RAAS-related parameters in the very youngest [168]. A sophisticated investigation of PRA values in children suffering from HF are urgently required to overcome this hurdle. Consequently, small volume, regulatory guideline compliant bioanalytical assays tailored for paediatric application are necessary [169, 170]. In this context, the small available sample volume is an obstacle which should be addressed by the customisation of the assay and downscaling of the applicable sample volumes for paediatric research.

Therefore, the sub-aims of the present research were:

- To enable a downscaling of the required sample volume for the PRA ELISA by customisation of the assay to cope with the available sample volumes in paediatric research.

- To validate the customised microassay according to the FDA bioanalytical guideline and thus ensure the use for GCLP settings in the context of the LENA project.

- To show the applicability of the assay in a real-life approach by an example measurement of paediatric study samples.

4.2 Methods

The quantitative characterisation of PRA by ELISA is based on the determination of AngI values. The PRA can be calculated by comparing the AngI concentration in incubated human matrix with the AngI values of non-incubated matrix of the same sample. The measurement was accomplished by an in-house customised ELISA encompassing small volume applications. Units for PRA are expressed as ng/mL/h, while units for AngI are expressed as ng/mL. A comprehensive validation assured GCLP conformity of the developed assay using the FDA bioanalytical guideline, as well as further quality assurance tools (e.g. tracking software, audit trails, standard operation procedures, defined criteria for the validity of analytical runs, dual control principle, external audits, ring test participation).

4.2.1 Materials

Generation buffer, phenylmethylsulfonyl fluoride (PMSF), rabbit anti-AngI antibody-coated microwell plates, AngI-biotin conjugate, streptavidin-HRP conjugate concentrate, assay buffer, wash buffer concentrate, tetramethylbenzidine (TMB) and 1M sulphuric acid, as well as AngI calibration standards (CSs) and quality controls (QCs), were delivered by DRG Instruments GmbH (Springfield, USA). All CSs and QCs contained preservatives and emulated matrix (protein-based buffer) to mimic the effect of the human matrix. Water HPLC-grade was purchased from Fisher Scientific U.K. limited (Loughborough, United Kingdom). AngI stock solution, ethanol (>99.8% p.a.) and formic acid (FA) (≥98% p.a.) were obtained from Sigma-Aldrich (Steinheim, Germany). EIA buffer was purchased from Bertin Pharma (Montigny le Bretonneux, France). S-Monovetten® (Sarstedt AG & CO, Nümbrecht, Germany) were used to obtain the required plasma samples from healthy volunteers. All pipetting steps were performed using calibrated Eppendorf Research® pipettes in accordance with GCLP (Eppendorf, Hamburg, Germany).

4.2.2 Calibration standards

All seven non-zero CSs (0.2, 0.5, 1.5, 4, 10, 25, 60 ng/mL) were utilised as a ready-to-use solution (analyte in artificial matrix) without further processing and contained AngI solutions. The PRA represents a calculated ratio of AngI values at 0 °C and 37 °C (incubated over a fixed period of time).

4.2.3 In-house customised assay procedure

Pretreatment

Blood samples were thawed using a water bath (23±3 °C). After adding 1 µL PMSF to 100 µL sample volume, the samples were vortexed for 20 s. Next, 10 µL generation buffer was added followed by a further 20 s of vortexing. The final mixture was separated into two aliquots. One aliquot was placed in an ice bath (0 °C), while the other aliquot incubated at 37 °C was put into a water bath. After 90 min, the generation process was stopped by placing the incubated aliquot in the ice bath for 5 min.

Immunoassay

50 µL assay buffer was dispensed in every well of the microwell plate. Subsequently, 40 µL of CSs, blanks, QCs, as well as 0 °C and 37 °C aliquots were added, respectively. The microwell plate was incubated in the dark for 5 min at 500 rpm (24 °C) followed by a reduction of the shaking speed to 300 rpm for 15 min. Then, 80 µL AngI-biotin conjugate was added to each well and a second incubation for 5 min at 700 rpm in the dark (22 °C) was conducted. Afterwards, the shaking speed was reduced to 500 rpm, and the incubation continued for 55 min. During the first automatic washing step (Tecan HydroFlex™, Männedorf, Switzerland), the contents of the wells were rinsed with five times 500 µL wash buffer and the residual liquids were subsequently removed thoroughly. Then, 170 µL streptavidin-HRP conjugate concentrate was added to all wells and incubated for 30 min at 22 °C and 500 rpm. Then, a second washing procedure (5 times 500 µL) was conducted and 170 µL TMB solution was added to each well, followed by 10 min of incubation at 500 rpm (22 °C) in the dark. The enzyme reaction was stopped by adding 60 µL sulphuric acid and additional mixing for 3 min at 350 rpm (Figure 4-1). All incubation steps were performed by applying a ThermoMixer® by Eppendorf AG (Hamburg, Germany).

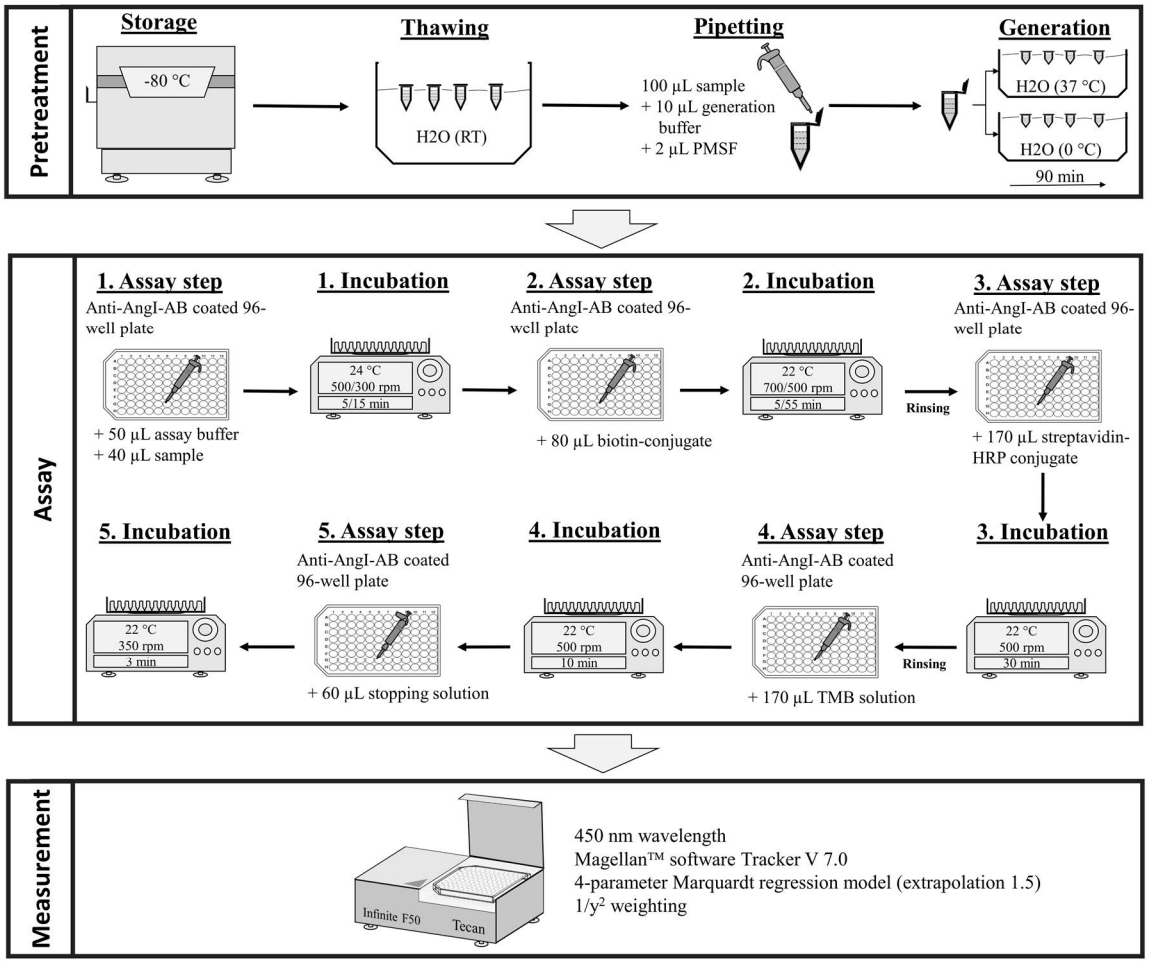


Figure 4-1: The modified plasma renin activity immunoassay procedure. PMSF: phenylmethylsulfonyl fluoride; AB: antibody; HRP: horseradish peroxidase; TMB: tetramethylbenzidine; Rinsing: automatic washing step with 5 x 500 µL wash buffer and subsequent residual removal.

4.2.4 Measurement of angiotensin I and determination of plasma renin activity

The OD measurement of AngI (450 nm) was performed using the absorbance reader infinite®F50 by Tecan (Männedorf, Switzerland). All calculations of concentration levels, as well as further statistical evaluations, were performed with the corresponding Magellan™ software Tracker V 7.0 based on the 4-parameter Marquardt regression model (extrapolation 1.5) with weighting $1/y^2$. PRA was calculated using Equation 4-1.

$$\mathrm{PRA}\left[\mathrm{ng/mL/h}\right] = \left(\frac{(\mathrm{AngI}\left[\mathrm{ng/mL}\right](37°\mathrm{C})) - (\mathrm{AngI}\left[\mathrm{ng/mL}\right](0°\mathrm{C}))}{1.5\ \mathrm{h}}\right) * 1.11$$

Equation 4-1: Calculation of the plasma renin activity

4.2.5 U.S. Food and Drug Administration based bioanalytical method validation

The method was validated based on the recommendations of the FDA bioanalytical guideline for LBAs [169]. Due to the nature of the assay, the accuracy, precision, total error, calibration curve/linearity, and matrix effect was assessed by the direct evaluation of AngI values. For the validation parameters stability, parallelism, and the between-run precision of the whole process, the calculation of PRA values (Equation 4-1) were conducted with the additional AngI generation step was included in the related validation runs.

Acceptance criteria of single analytical runs/ intra-run assessment

The assay was intended to be applied in the context of the LENA project, which aims to generate data for a new marketing authorization. Therefore, recommendations provided by regulatory guidance for the conduct of analytical runs were followed. As this intra-run evaluation includes comprehensive monitoring of QCs as well as CSs, this regulatory guidance appeared to be more suitable for the project aim than the sole application of Westgard rules commonly used in routine laboratories. In this regard, the acceptance criteria for a valid analytical run included CSs and QCs. The calibration curve in the PRA ELISA was established by no less than six ready-to-use CS. The CSs ranged from a concentration of 0.2 ng/mL to 60 ng/mL, containing CSs and a blank standard measured in duplicate. The back-calculated concentration of 75% of non-zero CSs including the LLOQ must not deviate by more than 25% and 20% for the calibrator at the LLOQ and the other calibrators, respectively. Exclusion of CSs was only acceptable if they failed the acceptance criteria or assignable causes. The validity of each run was assured by adding three QC levels (low, middle, high), at least measured in duplicate. The low and middle QC levels were directly delivered by DRG Instruments GmbH (Springfield, USA), while the high QC level was separately prepared in-house. A minimum of 50% of all samples per QC level must be within 20% of their nominal concentrations. Moreover, at least 67% of all QC samples must be within 20% of their respective nominal values.

Calibration curve/ linearity

The calibration curve consisted of seven non-zero CS levels (0.2, 0.5, 1.5, 4, 10, 25 and 60 ng/mL). The calibration curve was evaluated in 12 independent runs on 12 different days. Each calibration curve was formed of at least six CS levels marked with a maximal relative error (RE) of ±20% (±25% at Lower Limit of Quantification (LLOQ)) (Equation 4-2). Standards that did not comply with this criterion were excluded, and the calibration curve

was re-constituted. The linearity of each calibration curve was considered appropriate if the corresponding r-value was ≥0.995.

$$\text{Relative error}\,[\%] = \left(\frac{\text{measured concentration} - \text{nominal concentration}}{\text{nominal concentration}}\right) * 100$$

Equation 4-2: Calculation of the relative error [%].

Accuracy, precision, and total error

The accuracy, precision, and total error of the assay were investigated at five different concentration levels (0.2, 0.5, 1.5, 4, 10, 25 and 60 ng/mL) in quintuplicate. Within-run accuracy and precision were investigated in one run, while between-run accuracy and precision were obtained in six individual runs on four different days. For the evaluation of within-run and between-run accuracy, the RE (as a measure for accuracy) should not exceed ±20% (±25 for LLOQ). The coefficient of variation (CV) must be ≤20% (≤25% for LLOQ) for within-run and between-run precision (Equation 4-3). The total error was calculated as the sum of the absolute value of the RE and CV. The maximal limit of the total error for within-run and between-run investigation must be ≤30% (≤40% at LLOQ), following the recommendations of the FDA [169]. deviation mean

$$CV\,[\%] = \left(\frac{\text{standard deviation}}{\text{mean}}\right) * 100$$

Equation 4-3: Calculation of the coefficient of variation (CV) [%].

In addition, the between-run precision of PRA covering the whole process (sample pretreatment and determination) was assessed. Plasma samples of seven human sources were determined in triplicate at 15 different days concerning their PRA levels. The between-run precision (CV) per source must be ≤20%.

Matrix effect

Matrix effects are defined as direct or indirect influences on the detector response caused by interacting substances within the sample. However, clear recommendations for the assessment of the matrix effect of LBAs currently lack in regulatory guidelines such as FDA and EMA [169, 170]. Therefore, the matrix effect was obtained by the comparison of native (plasma with endogenous levels of AngI only) and AngI-spiked samples of the same human source. Five different human sources were investigated, and samples were measured in duplicate. Exact final angiotensin I concentrations (obtained by the spiking of native

samples with AngI working solution (1.05 µg/mL)) were dependent on the AngI concentration inherently present in native samples. To minimise matrix dilution, the spiked solution did not exceed 10% of the total volume. The determined AngI values of four out of five sources should not deviate more than ±20% compared to the nominal AngI values.

Parallelism

Plasma from one human source was spiked with AngI working solution (10.5 µg/mL) to reach high concentrations near the Upper Limit of Quantification (ULOQ). The following dilution steps were conducted: 1:2; 1:3; 1:4; and 1:5. All dilutions were performed using the highest concentration and the corresponding amount of EIA buffer to reach the intended dilution. In addition, a blank reduction was conducted by the investigation of native samples of the same source to determine the endogenous concentration of AngI still present in the native samples. The maximum deviation of the CV between the dilutions must not be more than 30%.

Stability

Several stability experiments were conducted in the context of the FDA guideline to exclude an influence on analyte concentration due to limited stability during the study period, including sample preparation, and assay procedure. Analyte stability was ensured by the evaluation of freeze and thaw stability, short-term stability and long-term stability. For all experiments, freshly drawn plasma was aliquoted, snap-frozen and stored at -80 °C to mimic the same conditions as for study samples.

Freeze and thaw stability

Freeze and thaw stability was examined by passing three freeze-thaw cycles of plasma from one human source. Snap-frozen human matrix was stored at -80 °C for a minimum of 24 h before being applied for the next freeze-thaw cycle. This procedure was repeated three times. After each freeze-thaw cycle, PRA was determined in triplicate and compared to the corresponding reference value (first value obtained). A maximum of ±20% RE of the mean value was allowed.

Short-term stability

Short-term stability was investigated by the determination of PRA in human plasma for up to 2 hours at room temperature (RT) and for up to 4 hours at 0 °C to guarantee appropriate stability during the entire assay procedure. For stability at RT, six aliquots were thawed and stored on ice. At time points 0, 0.25, 0.5, 1, 1.5 and 2 hours, one aliquot was placed on the

bench-top at RT. The sample for the reference value was continuously stored on ice. After the 2-hour time point, all samples were measured simultaneously. Using the same method, the 4-hour stability at 0 °C was assessed (time points of determination: 0, 2, 2.5, 3, 3.5 and 4 hours (reference value)). All samples were measured in triplicate. The mean values of PRA should not deviate by more than ±20% from the mean PRA of the reference value.

Long-term stability

The long-term stability was determined in seven human sources (three females, four males). Each human source was aliquoted and subsequently stored at -80 °C. Initial PRA reference values were obtained by calculating the mean of three independent analytical runs on two different days. Every 2 weeks, aliquots of each human source were thawed and measured in triplicate. The stability of AngI was proven if the deviation of the mean did not exceed ±20% of the reference value.

4.2.6 Interlaboratory quality assessment

Besides the in-house validation procedure, the reliability of the ELISA was investigated by participation in an interlaboratory ring test in January 2018. Two samples of unknown concentration were obtained from the German Reference Institute for Bioanalytics (RfB) (Bonn, Germany) and analysed in duplicate to verify the accuracy. The measured values were back-reported and compared against 13 RIA based PRA reference methods to assess the general accuracy of the assay as well as its comparability to established RIA assays.

4.2.7 Application of the validated method

Study samples

In order to demonstrate the applicability of the paediatric approach in the context of the EU-funded LENA project, example measurements were conducted using paediatric samples from three selected subjects suffering from HF (0.12, 0.37, and 0.69 years old). Blood samples were obtained before and 4 hours after application of 0.25 mg enalapril maleate. All paediatric samples were collected in line with the Declaration of Helsinki. Written informed consent from the parent(s)/legal representatives and assent from the patient according to national legislation and as far as achievable from the child were obtained. Blood sampling was conducted by trained study staff following a specific time-monitored sampling procedure adapted to paediatric needs (e.g. use of microneedles) and parameter characteristics (e.g. limited time window between sampling, sample preparation and freezing) [104]. Each time point of the sampling process, as well as the behaviour and

position of the neonate, was documented. In this context, please refer to the list of the LENA collaborators (Appendix 10.4-15) who performed the onward collection of paediatric study samples.

Incurred sample reanalysis

CSs and QCs were constructed in an emulated matrix. Since potential differences between this matrix and paediatric matrix might impact the PRA determination by altering factors such as protein binding, back-conversion metabolites and the effects of co-medication incurred sample reanalysis (ISR) was conducted to confirm the reproducibility of measured study samples and the robustness of the assay. In this regard, the here conducted ISR should demonstrate the robustness of the example measurement of the three paediatric patients. An overall evaluation of all performed ISR is shown in chapter 5. ISR was calculated as follows (Equation 4-4) [170, 171]:

$$\text{Difference } [\%] = \left(\frac{\text{repeat value} - \text{original value}}{\text{mean}} \right) * 100$$

Equation 4-4: Calculation of the percentage difference between the original sample value and measured repeat.

In accordance with the FDA international guideline**,** the percentage difference was allowed to vary no more than ±30% of the mean value in 67% of all repeats [169]. All ISR samples were obtained in the context of the LENA project.

4.3 Results

4.3.1 U.S. Food and Drug Administration based bioanalytical method validation

Calibration curve/ linearity

In all twelve runs, the RE of all evaluated CSs was within ±20% of the nominal values (including LLOQ) and thus showed compliance with the FDA guideline (Figure 4-2). All obtained r-values were ≥0.99884. Implementation of a 4-parameter Marquardt regression model (extrapolation 1.5) with weighting $1/y^2$ provided the best fit for a calibration range of 0.2 ng/mL to 60 ng/mL.

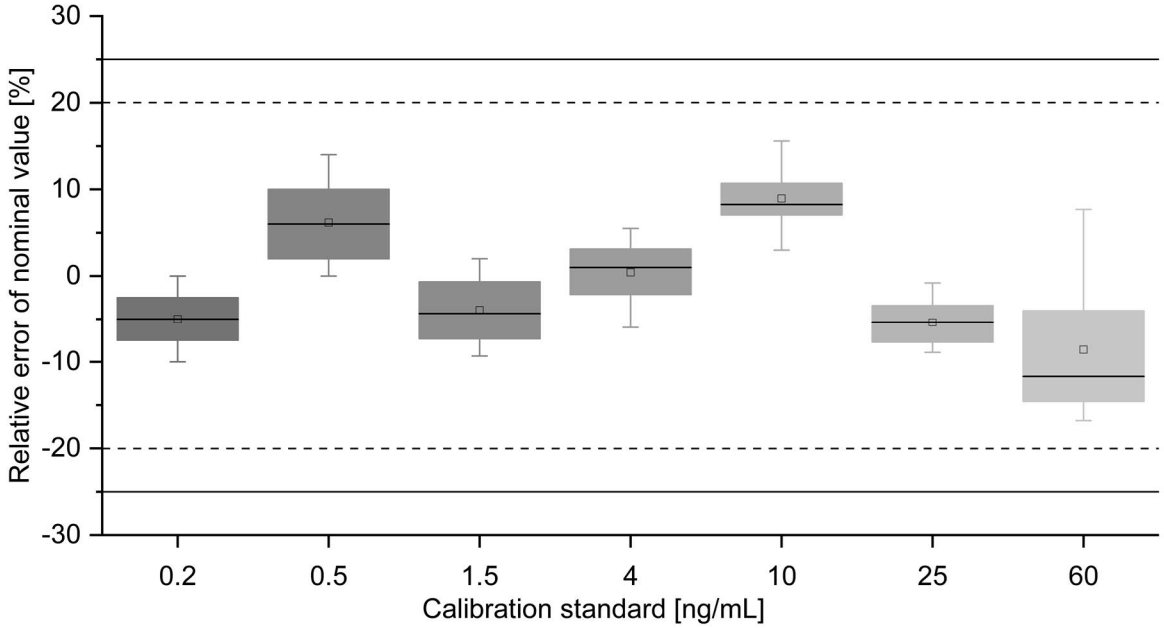


Figure 4-2: The relative error of all conducted calibration standards for the evaluation of linearity (12 runs) by the plasma renin activity immunoassay expressed as boxplots. Solid lines: acceptance limits regarding U.S. Food and Drug Administration (FDA) at LLOQ; Dashed line: acceptance limits regarding FDA for the other calibrators; Box: 25-75 %; ⌶: 1.5 interquartile range; —: median; ▫: mean.

Accuracy, precision, and total error

Within-run accuracy over all five concentration levels showed a mean RE between -8.6% and 5.5% of the nominal values (mean of five determinations). Within-run precision varied between 2.3% and 11.1% (CV), while the total error ranged between 1.0 and 18.6%. The RE for between-run accuracy was between 4.2% and 5.0%, while the between-run precision (CV) varied between 1.2% and 6.3%. Furthermore, the total error across all concentration levels was below 16.3%. For further details, please refer to Figure 4-3 and Appendix

10.2-1/Appendix 10.2-2). In summary, the customised assay showed an appropriate accuracy and precision in the context of the applied bioanalytical guideline. All results for accuracy and precision were within the required limit of ±20% (±25% at LLOQ). Moreover, the total error for within-run and between-run investigations did not exceed the required limits of ±30% (±40% at LLOQ).

The determined mean PRA levels for the seven investigated sources amount to 2.85, 4.84, 0.79, 2.87, 1.96, 0.9, and 3.74 ng/mL/h with a CV of 5.2%, 9.6%, 8.3%, 9.3%, 10.4%, 10.9%, and 9.0%, respectively. Therefore, the between-run precision of the whole process complied with the predefined limit of ≤20% (CV) (Appendix 10.2-3).

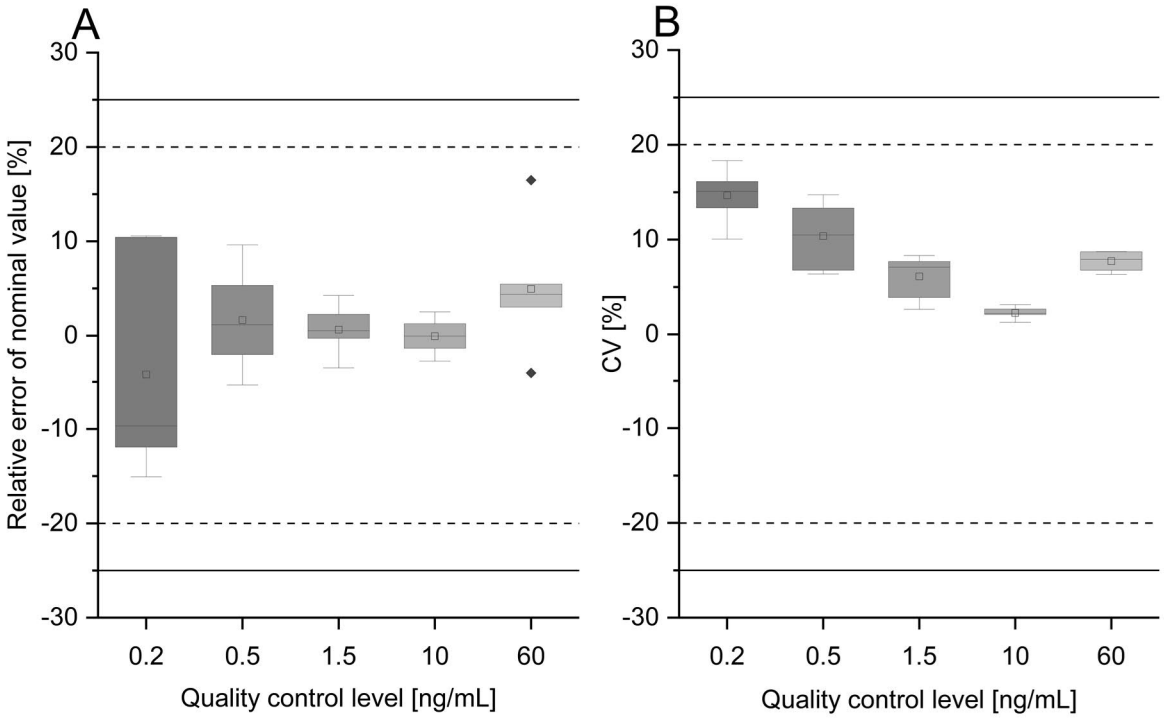


Figure 4-3: The relative error of between-run accuracy (A) and the coefficient of variation (CV) of between-run precision (B) for the plasma renin activity immunoassay. Five individual quality control samples (including the Lower Limit of Quantification (LLOQ) were determined over six runs in quintuplicate. The six determined mean angiotensin I concentration levels for each of the six runs are shown as boxplots for every individual quality control level. Solid lines: acceptance limits regarding U.S. Food and Drug Administration (FDA) at LLOQ; Dashed line: acceptance limits regarding FDA for the other calibrators. Box: 25-75 %; ⌶: 1.5 interquartile range; —: median; ▫: mean.

Matrix effect

The obtained Ang I concentrations in the five evaluated human sources ranged from 23.9 ng/mL to 29.0 ng/mL AngI (mean of two replicates each). The percentage deviations to the nominal concentration varied between -20.4% and -3.3%. Since 80% of the investigated sources were well within the specifications (±20%) and one source showed borderline

results (20.4%), no substantial matrix effect was claimed for the assay (Table 4-1 and Appendix 10.2-4).

Table 4-1: The results of the investigated matrix effect on the plasma renin activity determination. The mean values were obtained by the measurement of two replicates.

Source	Sex	Mean determined AngI concentration [ng/mL]	Mean deviation of AngI concentration to nominal value [%]
1	Female	26.3	12.5
2	Female	29.0	3.3
3	Female	26.9	10.4
4	Female	27.6	8.2
5	Female	23.9	20.4

Parallelism

All dilution steps were well within the calibration curve. The back-calculated mean AngI values were 35.1 ng/mL for the undiluted sample, 32.5 ng/mL for the 1:2 dilution, 29.5 ng/mL for the 1:3 dilution, 27.7 ng/mL for the 1:4 dilution, and 27.1 ng/mL for the 1:5 dilution step. The inter-run precision (CV) was 11.1% over the five assay runs and thus did not exceed the limit of 30%. For further details, please refer to Appendix 10.2-5.

Stability

Freeze-thaw stability

The RE of the stressed samples to the reference values showed a maximum of 11.3% after three freeze and thaw cycles. The within-run precision varied between 1.6 to 4.4% for PRA, while the between-run precision was 8.6% (CV). Since the deviations varied by no more than 20% from the reference values, accurate and precise sample analysis was not affected by the freeze-thaw cycles conducted (Appendix 10.2-7).

Short-term stability

All stability samples (2 hours bench-top and 4 hours at 0 °C) deviated by a maximum of +12.7% from the PRA reference value (Figure 4-4). Since PRA concentration level did not vary more than ±20%, the analyte can be considered stable at RT for at least 2 hours and at least 4 hours on ice (Appendix 10.2-8).

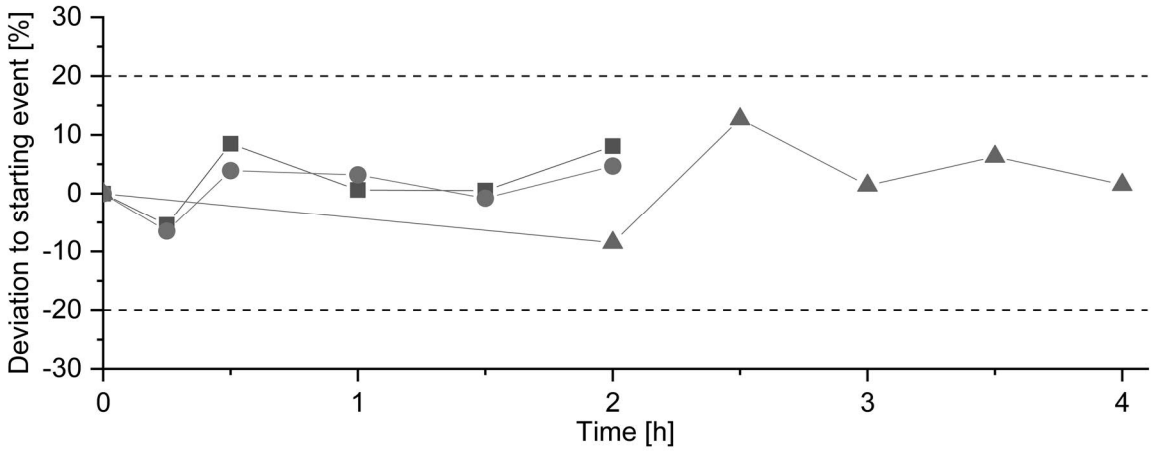


Figure 4-4: The percentage deviation of short-term stability for the plasma renin activity. Box: bench-top; Circle: 2 2 hours on ice; Triangle: 4 hours on ice. Grey dashed lines indicate the maximum accepted deviation regarding U.S. Food and Drug Administration guidelines.

Long-term stability

Long-term stability was proven for 20 runs over a time period of 260 days. However, the 18th run was excluded from the evaluation due to a processing error (deviation of water bath temperature). After the 20th run, one source exhibited a deviation of more than 20%, and the assessment of long-term stability was terminated. Overall, long-term stability for 260 days (ca. 37 weeks) was proven (Figure 4-5 and Appendix 10.2-6).

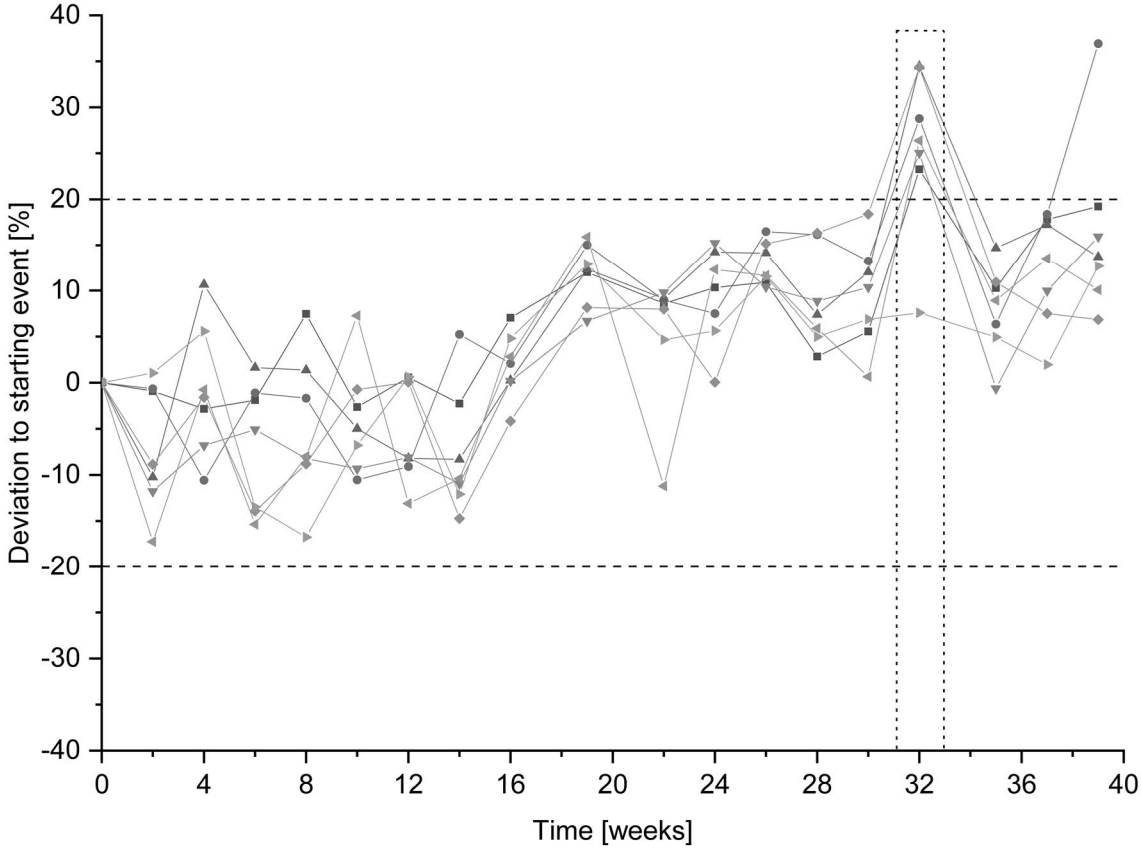


Figure 4-5: The percentage deviation of long-term stability in seven sources for the plasma renin activity. The different sources are indicated by the different symbols. Reference values of plasma renin activity were obtained by calculating the mean of three replicates of each source. Grey dashed lines indicate the maximum accepted deviation regarding U.S. Food and Drug Administration guidelines. The vertical dotted line marks the excluded run due to a processing error.

4.3.2 Interlaboratory quality assessment

In addition to the in-house validation, an external verification by participation in a ring test executed by the RfB was successfully conducted. The ring test allowed for a comparison of the developed ELISA with established RIAs used by contract laboratories (for further details, please refer to http://rfb.bio). As an ELISA reference value was lacking, the reported values were compared with RIA outcomes only. The latter allowed for a comparison of the easy-to-use ELISA to the more complex RIA setup. The interlaboratory test samples (sample A and sample B) were measured a total of three times. The back-reported mean concentration values of the two unknown samples were 2.2 ng/mL/h and 2.8 ng/mL/h PRA. The RfB calculated the maximal accepted deviation based on all submitted assay results of the 14 participants. All other participating methods were conducted on the basis of RIAs. A valid result within the specification limits for both reported samples was achieved. By passing the ring test, the external quality assessment verified the reliability of the validated small-volume microassay.

4.3.3 Application of the validated method in paediatric patients

Example paediatric study samples were successfully analysed to demonstrate the applicability of the assay using a real-life approach. Moreover, ISR was conducted to assess the comparability of the obtained data during the analytical runs within the study period, which is indispensable due to the unique data derived from the study population in the LENA project. All processes relevant for an accurate determination of humoral parameters (e.g. blood sampling, sample shipment, storage, and subsequent analysis) were conducted under GCLP conditions. The determined PRA values of the study samples and corresponding ISR are presented in Figure 4-6.

Study samples

The PRA values of three randomly selected neonates were determined before as well as 4 hours after the administration of enalapril maleate. All six values were obtained in independent analytical runs. All conducted analytical runs were declared as valid in regard to the FDA acceptance criteria of an analytical run. Thus, the essential reduction of the applied sample volume from 500 µL to 100 µL enabled the decisive determination of PRA, proving the applicability of the ELISA for paediatric trials.

Incurred sample reanalysis

Since the CS/QC matrix used in the validation and the paediatric study matrix is not compulsively comparable, ISR samples were included in the analytical runs. In brief, six ISR samples were measured collectively with the study samples (one for each analytical run, conducted on different days). The calculated difference for all six ISRs did not exceed ±30% and was thus well within the guideline limits, thereby substantiating the reproducibility of study samples under routine conditions. The paediatric-tailored microassay demonstrated the feasibility of obtaining high-quality data under GCLP conditions and complied with sophisticated guideline requirements. Therefore, the robustness of the microassay for the determination of PRA was proven, indicating the reliable determination of study samples in the context of the LENA project.

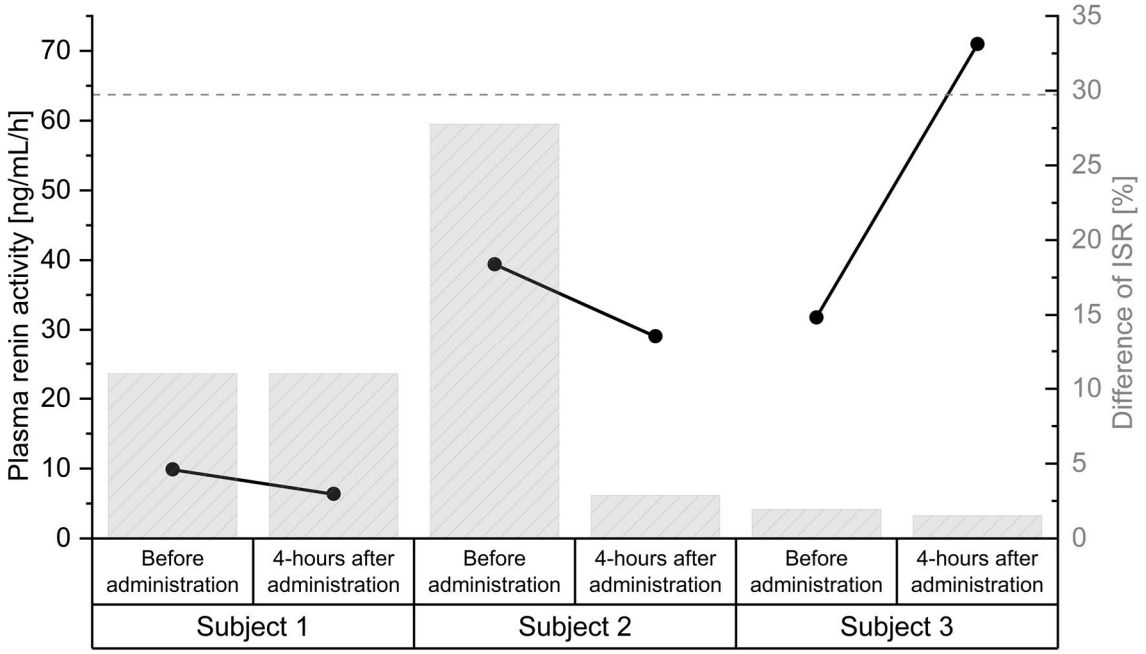


Figure 4-6: Example measurements of paediatric samples and the corresponding incurred sample reanalysis (ISR) by the plasma renin activity immunoassay. The plasma renin activity values (connected points/ left y-axis) of three infants with congenital heart diseases before and 4 hours after administration of enalapril maleate, as well as the relative deviation of each corresponding ISR (bars/ right y-axis). Subject 1: 0.69 years of age; Subject 2: 0.12 years of age; Subject 3: 0.37 years of age; Dashed line: maximum accepted deviation for ISR.

4.4 Discussion

The PRA ELISA presented here was successfully customised and validated regarding current international FDA bioanalytical guidelines. The assay serves the overall goal of application in vulnerable populations (e.g. elderly, severely diseased patients or children) by three properties. First, PRA determination was empowered by an easy-to-handle enzyme-linked immunosorbent assay instead of commonly applied radioimmunoassays. This could support the establishment of the assay in far more laboratories than the highly skilled laboratories with access to RIA. The latter facilitates wider data generation for PRA values of the RAAS in sensitive population and could subsequently contribute to safer and more rational drug therapy. Second, the assay properties—especially the required blood volume—have been adapted for use in the aforementioned populations to reduce the burden of additional blood sampling in the context of simultaneous research to routine care. Third, the assay was validated according to current FDA bioanalytical guideline to ensure reliable data generation and its application in a GCLP-compliant environment.

The recently published EMA concept paper on guidelines for the investigation of medicinal products in the term and preterm neonates demands a greater focus on organ and enzyme system maturation differences across several paediatric age groups [172]. However, these differences and their pharmacodynamic effects can only be sophisticatedly investigated if appropriate microassays are applied. The comprehensive understanding of adult RAAS has resulted in over 20 drugs being approved for HF in adults; however, only a few approved drugs are available to treat HF in all paediatric age groups. New potential target structures within the RAAS are continuously identified and provide further insight into the adult RAAS [16], while the intervention on these humoral parameters remains unknown in children. Therefore, urgently fostered research on PRA and the maturating RAAS can only be accomplished by the establishment of customised assays. Against this background, the ELISA PRA assay was customised and characterised by a five-fold reduction in required plasma volume. Based on ethical recommendations in clinical trials [79], the total blood loss for routine care and research in neonates must not exceed 3% over 4 weeks (1.2 mL to 1.4 mL plasma per kg body weight). Even LBAs, which were used for paediatric investigation of PRA (e.g. GammaCoat® Plasma Renin Activity 125I RIA Kit by DiaSorin Inc. [173]) require high sample volumes of 250 µL to 1000 µL for a single determination; therefore, this limits the amount of practicable analysis (e.g. determination of multiple PK/PD [174–176] parameters beside investigation of safety samples) [158, 177]. Although other small-volume RIA assays (50 µL up to 150 µL for PRA determination) appropriate for investigation in children exist, a validation regarding international guidelines has not been demonstrated

and subsequently does not allow for application in clinical trials. The substantial reduction in sample volume (500 µL to 100 µL) of the presented ELISA—without restricting the calibration range (0.2 ng/mL to 60 ng/mL)— also enables the examination in the diseased paediatric population (e.g. children suffering from HF) [173, 178–181].

The ELISA was successfully validated concerning the FDA bioanalytical guideline. This verification of quality is important in the context of applying the assay in a regulatory environment—a factor that most published assays lack [158, 174, 175, 177]. In particular, clinical studies in paediatrics are usually only performed once owing to ethical constraints. Therefore, it is of utmost importance that the generated bioanalytical data is of high quality and reliable as it will be used for decision making related to paediatric drug therapy. In addition to the successful in-house validation procedure of the presented ELISA, participation in external independent quality assurance (ring trial) confirmed the accurate performance of the customised ELISA. Furthermore, the comparability of the ELISA results with routinely applied RIA was assessed within this external quality assurance.

The outcome of this comparison indicated non-inferiority regarding the reported reference RIAs, thus highlighting the usefulness of the presented ELISA for routine application. Besides the applied analytical technique, the sample matrix used is also known to influence reliable determination. Protein binding, sample inhomogeneity, known or unknown metabolites and co-medication might affect accurate sample determination. For example, such effects can be prone to mature organisms and therefore require a corresponding investigation. Therefore, the applicability of the assay in paediatric research (e.g. the LENA project) was outlined by the random determination of six paediatric samples with corresponding ISR in different analytical runs. The conducted ISR confirmed the accurate performance of the assay in a real-application approach and the reproducibility of unknown sample results obtained in different runs. In conclusion, the in-house validation, external quality assurance and applicability demonstrated the assay’s reliability, fit-for-purpose and usefulness for accurate determination of PRA in vulnerable populations.

Notably, small-volume ELISAs are not only suitable for examination in children and can also unburden severely diseased adults. The total volume of blood withdrawal and the number of blood draws in critically ill patients is related to increased consumption of blood transfusions, higher incidence of anaemia and illness severity [182]. In addition, Ullman et al. reported that anaemia occurs in 95% of all patients admitted to intensive care units at day three, which increases the risk of severe events in critically ill patients [183]. The reduced required blood volume could also facilitate the ongoing monitoring of patients and

contribute to avoiding additional burdens due to increased blood loss. An application of GCLP-compliant small-volume bioanalytical assays can thus bring a decisive advantage, even in an adult population.

In brief, an FDA-compliant validation was successfully accomplished for the developed microassay, enabling a reliable investigation of PRA in pivotal trials. In addition, this downscaled ELISA is applicable for paediatric trials and successfully copes with limitations in blood volume, providing a valuable alternative to commonly used RIAs.

4.5 Conclusion

The development and validation of a fit-for-purpose PRA ELISA were accomplished for paediatric application, indicating non-inferiority to commonly used RIAs. The FDA-compliant PRA assay is able to accurately and precisely quantify PRA values in 100 µL plasma and is applicable for GCLP-compliant clinical studies, thus enabling sophisticated investigations in children within paediatric clinical studies.

5 Bioanalytical quality control system for the plasma renin activity immunoassay

Some aspects of this chapter are already published on the 28th of December 2019 as “A quality control system for ligand-binding assay of plasma renin activity: Proof-of-concept within a pharmacodynamic study” in the peer-reviewed Journal of Pharmaceutical and Biomedical Analysis (doi: 10.1016/j.jpba.2019.113090).

5.1 Background and aim

Paediatric studies are mostly characterised by intricate study designs, limited study populations, ethical constraints, challenging blood sampling conditions and restricted sample volumes [101, 102]. Accordingly, the number of successfully conducted paediatric clinical studies remains limited. However, since current publications indicate that clinical investigations in paediatric patients are not of high priority in the pharmaceutical industry, academic consortiums have been formed to address this insufficiency [184]. Thus, a GCLP-compliant environment had to be implemented for bioanalysis of PRA within the LENA project. In this regard, the PRA ELISA was validated in accordance with the regulatory requirements of the FDA to enable robust data acquisition (Chapter 4). However, validation usually investigates the performance of a method at one specific time point. Thus, additional quality measures that enable the investigation of assay comparability over the whole study period are necessary. Clear specifications for the evaluation of single analytical runs are stated in the bioanalytical guideline of the FDA [169]. Nevertheless, comprehensive recommendations for the evaluation of the inter-run performance of analytical runs within analytical life cycle management are not provided in this guideline. Therefore, recommendations of the EMA guideline on bioanalytical method validation were additionally considered to amend the requirements given by the FDA [170]. However, the recommendations of both guidelines still appear to be inadequate to guarantee sufficient inter-run monitoring of bioanalytical runs. Therefore, further scientific references and recommendations must be taken into account to ensure a qualitative data collection over the entire study period. However, this long-term monitoring is essential to investigate the assay’s performance and subsequently ensure the comparability of study results. Moreover, to bridge the requirements for sophisticated monitoring of bioanalytical runs within clinical studies and academic research, the necessary bioanalytical QCSs must be lean, easy-to-handle and cost-effective. This situation generates a demand for customised and extensive QCSs that are applicable to academia-driven paediatric studies in a GCLP environment.

In this regard, the sub-aims of this research were:

- To establish an easy-to-apply but comprehensive QCS for the bioanalysis of PRA within the LENA project to substantiate the high-quality data acquisition of this unique paediatric data set.

- To compensate the lack of homogenous regulatory requirements for inter- and intra-run, as well as long-term monitoring of analytical runs by tailoring the QCS to paediatric needs by including well-established guidelines, recommendations by reputable organisations and suggestions based on scientific discussions.

- To evaluate the inter-lot performance of the applied PRA ELISA kits throughout the entire study sample analysis period.

- To evaluate the implemented QCS as a proof-of-concept for applicability in the academic-paediatric environment.

5.2 Methods

5.2.1 Assay procedure

For the determination of PRA within the LENA project, the in-house customised small-volume ELISA was applied. This assay was validated according to FDA international bioanalytical guideline [169]. The initial validation involved assessing linearity, accuracy, precision, total error, parallelism, matrix effect as well as long- and short-term stability experiments. Due to shelf-life limitations, new ELISA kits from the vendor DRG Instruments GmbH (Marburg, Germany) were ordered as a replacement within the study period. New replacement lots were often equipped with new lots of chemicals, substances, and antibodies. Since the results provided in the vendor's quality control certificate appeared partly insufficient, partial validation was conducted for every new lot to ensure comparable assay performance between the initial and replacement lots. The validation procedure followed the recommendations issued in the FDA bioanalytical guidelines [169]. If necessary, these revalidations could involve minor changes in the method (e.g. slight variations in rotation speed during incubation steps or the implementation of an automatic strip washer). The RE for between-run accuracy and the CV for between-run precision for all five validations are presented in Table 5-1.

Table 5-1: The lot dependent results of between-run accuracy and precision for the plasma renin activity immunoassay.

Validations	Between-run accuracy (RE)	Between-run precision (CV)
Initial validation	-13.0-11.2%	2.8-13.9%
1st replacement lot	-7.8-7.5%	5.2-11.6%
2nd replacement lot	-4.2-4.9%	2.3-14.5%
3rd replacement lot	-14.1-6.4%	3.2-10.7%
4th replacement lot	4.2-5.0%	1.2-6.3%.

The individual between-run accuracy and precision comprised five different concentration levels (0.2, 0.5, 1.5, 10, and 60 ng/mL) analysed in quintuplicate obtained in six individual runs on four different days. RE: relative error; CV coefficient of variation

5.2.2 Quality control system

Since a single comprehensive QCS for short- and long-term evaluation has yet to be described for the bioanalysis of PD parameters, a customised QCS was established to guarantee reliable data analysis. The multi-step QCS included a daily system suitability test (SST), blank sample analysis, appraisal of calibration curve standards, assessment of inter- and intra-run accuracy and precision via QCs, ISR evaluation, and periodic interlaboratory testing (Figure 5-1). The QCS was applied within the bioanalysis of the three paediatric studies of the LENA project (registration numbers: Eudra CT2015-002335-17, Eudra

CT2015-002396-18, and Eudra CT2015-002397-21). The system presented here is specifically tailored for monitoring pharmacodynamic analysis of plasma renin activity by the competitive ELISA (secondary study endpoint). As described in chapter 4.2.7, written informed consent from the parent(s)/legal representatives and assent from the patient according to national legislation and as far as achievable from the child were obtained, and ethical guidelines were carefully followed.

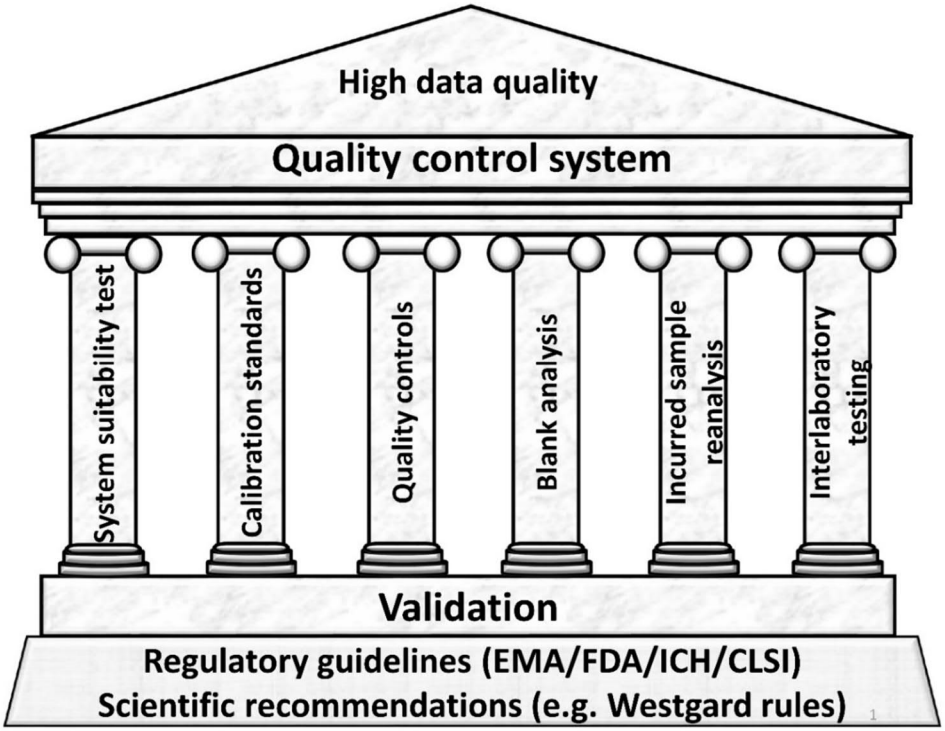


Figure 5-1: Schematic presentation of the established bioanalytical quality control system. EMA: European Medicine Agency; FDA: U.S. Food and Drug Administration; ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; CLSI: Clinical and Laboratory Standards Institute

System suitability test

Continuous daily equipment verification is crucial for reliable data generation. To evaluate potential daily variabilities in the accuracy or even malfunctions of the utilized absorbance reader (Tecan® Infinite F50), an SST was performed on each day of analysis. A fresh six-step dilution series of Alizarin Yellow GG was prepared and measured in quintuplicate. The dilutions covered the entire measuring range of the absorbance reader with the highest Alizarin solution close to the upper limit (maximal OD: 3). The OD was measured at 450 nm. On each measuring day, the obtained values of the fresh dilution series were compared to the measured reference values. Results within ±10% RE of the reference values were regarded as acceptable.

Overall evaluation of calibration standards

As mentioned before, the calibration curve of the utilised PRA immunoassay ranged from 0.2 to 60 ng/mL AngI, containing seven non-zero CS levels measured in duplicate. Every valid analytical run had to consist of at least six non-zero CS levels. Moreover, the RE of 75% of non-zero CSs should not deviate by more than 20% (25% at the LLOQ) [169]. The exclusion of CSs from the calibration curve was only acceptable if they failed the acceptance criteria or had assignable causes (e.g. pipetting errors). An overall assessment of CSs in valid analytical runs was performed to evaluate the overall performance of the calibration curve during the entire period of study sample analysis.

Overall evaluation of quality controls

For the QC assessment, liquid control material made and supplied by the manufacturer DRG was incorporated into all analytical runs. This material reflected emulated plasma (protein-based buffer with a non-mercury preservative). Patient specimens as QC material were omitted owing to the low volume of study samples, which would not allow for repeated measurement over several analytical runs. Three QC levels measured at least in duplicate were included in every run: one near the LLOQ (QC low), one in the mid-range (QC middle), and one near the ULOQ (QC high). As such, the number of QC levels fixed at three; however, the replicates per level could vary between 2–4 QC samples to comply with the guideline requirement of at least 5% of the number of study samples [169, 170]. The nominal concentration of QC low (0.97–1.21 ng/mL) and QC middle (4.35–6.51 ng/mL) altered slightly during the course of the study depending on the delivered lots, while the in-house prepared QC high concentration (42.5 ng/mL) remained constant. The three QC levels were carefully selected to allow for determination of acceptable method performance over the measuring interval of interest. Nevertheless, the lower level of QCs resulted in a minimum concentration of at least 0.97 ng/mL (~5x LLOQ) and did not fulfil recommendations in current regulatory guidelines (3x LLOQ). However, control material with lower levels was not supplied by the kit manufacturer, and laboratory-prepared material was not applicable.

Intra-run assessment

The intra-run assessment of QCs was scheduled in batch measurement mode by the conduct of the analytical run in a microplate format within one working day [185].
Although the Clinical and Laboratory Standards Institute (CLSI) guideline suggests a minimum of two levels for microplate approaches [185], the intra-run assessment met the stricter requirements of the FDA bioanalytical guideline [169]. Overall, 67% of all QC samples (at least 50% per concentration level) per analytical run should not deviate more

than ±20% from the nominal value to comply with the FDA bioanalytical guideline. Moreover, intra-run precision for each level must be within ±20% (CV) [169].

Inter-run assessment

In contrast to the between-run evaluation which is conducted during the validation of the assay and comprises a defined number of validation runs, the inter-run QCs assessment is dedicated to the examination of all analytical runs during the entire study period. The inter-run QCs investigation could therefore record possible variability over time and subsequently enable the assessment of the long-term performance of the assay. The latter is even more important in the context of long-lasting clinical studies to ensure comparability from the first to the last sample. Since the current EMA and FDA guidelines provide only recommendations for intra-run monitoring of analytical runs, further international recommendations were incorporated into this QCS. Considerations provided by current CLSI guidelines [185], the Westgard rules [186], and recommendations by van Bruijnsvoort et al. [187] were factored into the inter-run assessment. Subsequently, a multirule QC concept with the following rules was applied for the classification of long-term performance over the 24-month study period.

The multirule concept based on van Bruijnsvoort et al. [187]:

1) 1_{3S}: Not more than one measurement per QC level exceed the alert level (mean measured concentration of the QC level ±3 times the SD)

2) 3_{2S}: Not more than three consecutive measurements per QC level exceed the warning level (mean measured concentration of the QC level ±2 times the SD)

3) $10\bar{x}$: Not more than ten consecutive measurements of a QC level fall on the same side of the mean

Blank sample evaluation

All known standards (CSs and QCs) were based on a blank matrix that consisted of emulated plasma and conservatives. Thus, the blank standard potentially included some residual amounts of AngI that might impact the LLOQ level of the calibration curve. To evaluate the blank levels, two blank samples were included in each analytical run and were individually compared to the corresponding LLOQ sample to determine the blank-to-LLOQ ratio (Equation 5-1).

$$\text{Blank} - \text{to} - \text{LLOQ ratio}\,[\%] = \left(\frac{\text{Measured blank concentration}\left[\frac{\text{ng}}{\text{mL}}\right]}{\text{Measured LLOQ concentration}\left[\frac{\text{ng}}{\text{mL}}\right]} \right) * 100$$

Equation 5-1: Calculation of the blank-to-Lower Limit of Quantification ratio (LLOQ).

The blank sample assessment was performed considering the two following guidelines, as the blank evaluation for ligand-binding assays is not provided in the EMA or FDA guidelines. The ICH M10 draft guideline for Bioanalytical Method Validation requires that the concentration of the blank should not exceed the LLOQ concentration [171]. Additionally, calculation of the Limit of Blanks ($LoB = mean_{blanks} + 1.645 \times SD_{blanks}$) and the Limit of Detection ($LoD = LoB + 1.645 \times SD_{LLOQ\ samples}$) was performed in compliance with guideline EP17 (Protocols for Determination of Limits of Detection and Limits of Quantitation) published by the CLSI [188].

Incurred sample reanalysis

Due to potential differences between the matrix of CS/QC samples and the paediatric study samples (e.g. in protein binding, back-conversion metabolites, and sample inhomogeneity), study samples were reanalysed to confirm the reproducibility of the assay [170]. ISR was advised to ensure that the same study samples measured on different days were comparable and that the acquired data was of consistent quality. The EMA and FDA guidelines recommend reanalysing 10% of study samples if the number of study samples is less than 1000 and a further 5% if the number of study samples exceeds 1000 [169, 170]. Moreover, it is recommended to enclose study samples for ISR with concentration levels at c_{max} and within the elimination phase. This requirement indicates that this quality measure was established primarily for PK investigations. Thus, specifications of ISR applied to humoral parameters require certain adaptations. The bioanalytical analysis was conducted blinded and randomly because the merging of the acquired bioanalytical data with patient characteristics was scheduled in the study protocol to be conducted after the bioanalytical data acquisition and clearance period. This format did not permit the selection of samples at c_{max} and within the elimination phase for ISR, as demanded by the FDA [169]. A lag time at the beginning of the study was established to observe the residual blood volume applicable to the ISR during the initial months of the study. This lag time was meant to verify that the ISR can be used in paediatric studies enrolling younger children. Randomly selected, already analysed study samples were repeatedly measured, and the nominal concentration of the first valid result of a study sample (original) and the results of repeated measurement of the same corresponding study sample (repeat) were compared for the

PRA value to ensure comparability and reproducibility through continuous bioanalysis. For the ISR, at least 67% of the incurred samples should be within ±30% of the nominal concentration of the mean of the original and reanalysed value, which was calculated using the following equations (Equation 4-4 and Equation 5-2) [170].

$$ISR\ [\%] = \left(\frac{\text{number of ISR with difference} [\%] \leq 30\%}{\text{total number of ISR pairs}} \right) * 100\%$$

Equation 5-2: Calculation of incurred sample reanalysis (ISR) pairs [%] meeting the guideline criteria.

Moreover, the paired-sample Wilcoxon signed-rank test was conducted to evaluate whether the original and repeat differed significantly. The test was conducted in Origin® 2018b at a level of significance of 0.05.

Interlaboratory testing

Apart from the internal QC procedure, the accuracy of the assay was investigated by periodic (every 6 months) participation in interlaboratory tests during the LENA project. This evaluation was enclosed into the QCS within the LENA project because interlaboratory testing is a standard requirement for German laboratories involved in diagnosis and clinical analysis (e.g. hospitals and clinical research organizations). Moreover, the CLSI highly encourages the implementation of interlaboratory testing into a meaningful QCS [185]. Two samples with unknown concentrations were obtained from the RfB for each individual interlaboratory testing and analysed in duplicate to provide verification of reliable data acquisition. The measured values were back-reported and then compared to established assays used by other laboratories. Due to the lack of participating ELISA PRA methods, the reported values were compared to RIA-based methods (for further details, please refer to http://rfb.bio).

5.3 Results

A total of 168 analytical runs were conducted throughout the 24-month study period. Two analytical runs were rejected due to failing the predefined CSs specifications. Of the remaining 166 runs, 142 were characterised by also fulfilling the QC requirements and were classified as valid (Figure 5-2).

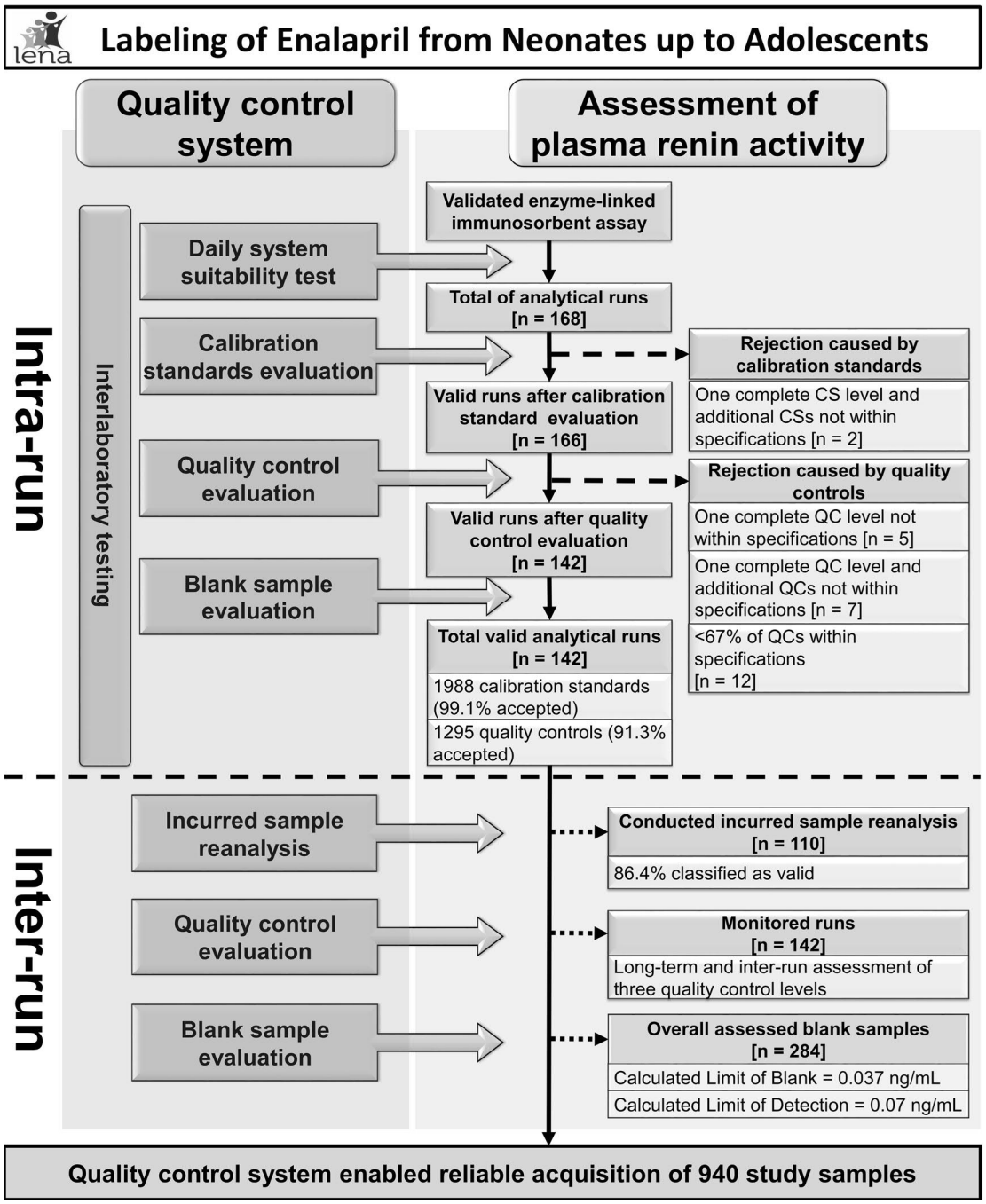


Figure 5-2: Flow diagram of the customised bioanalytical quality control system. CS: calibration standard; QC: quality control

5.3.1 System suitability test

The SST was successfully implemented and performed daily prior to sample analysis. All 163 conducted SST runs showed a RE of less than 10% and thus confirmed the suitability of the equipment (Figure 5-3 and Appendix 10.3-2/Appendix 10.3-3).

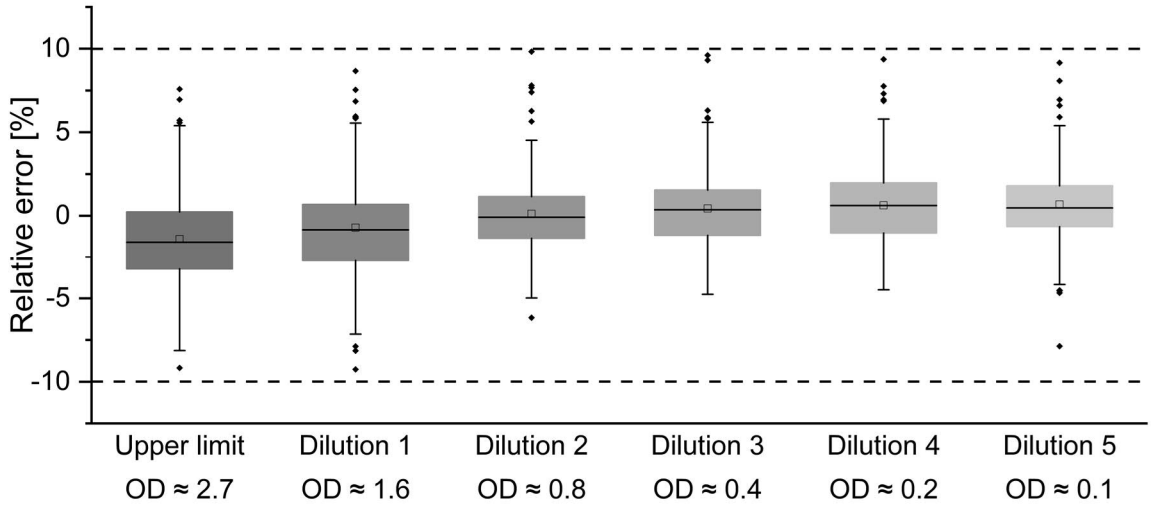


Figure 5-3: Boxplots of the relative error for the conducted system suitability tests for all analytical runs of the plasma renin activity immunoassay from August 2016 to August 2018 (n = 163). Dashed lines indicate the maximally allowed deviation of ±10%. Optical density (OD) values provided as reference. Exact OD values varied upon corresponding stock solution. ▪: interquartile range; ⌶: 1.5 interquartile range; — : Median; □: Mean; ♦: Outlier.

5.3.2 Overall evaluation of calibration standards

All 166 valid calibration curves consisted of seven CS levels. No entire level had to be excluded due to accuracy failure or any other cause. However, two calibration curves had to be rejected due to failing the required specifications. One complete concentration level plus additional CSs failed to reach accuracy limits, thus resulting in <75% CSs availability in both calibration curves. Overall, 1970 valid CSs (including all LLOQ CSs) did not exceed the limit of ±20% RE and were utilised for the quantification of unknown study samples. The RE for each CS level (mean of two replicates per run) for the valid analytical runs is presented in Figure 5-4. For further information, please refer to Appendix 10.3-1.

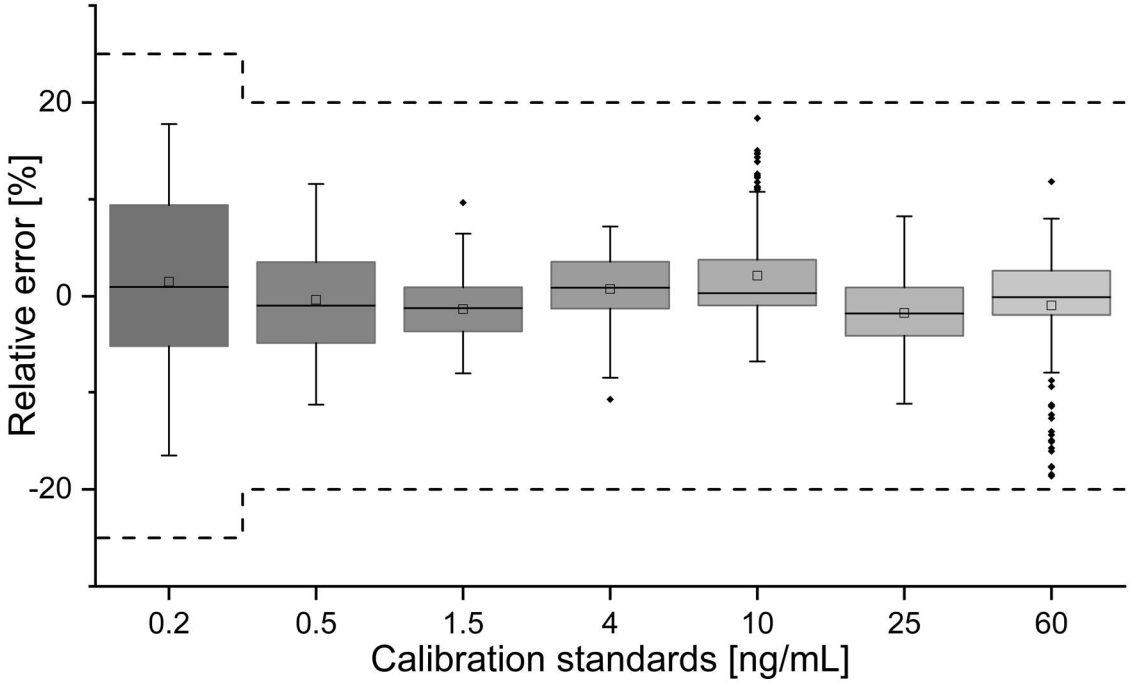


Figure 5-4: Boxplots of the relative error for all calibration standards (0.2, 0.5, 1.5, 4, 10, 25, 60 ng/mL AngI) in valid bioanalytical runs by the plasma renin activity immunoassay (n = 142). Dashed lines indicate the maximally allowed deviation in regulatory bioanalytical guidelines by the European Medicine Agency and the U.S. Food and Drug Administration (±20%; ±25% for the Lower Limit of Quantification). ▪: interquartile range; ⌶: 1.5 interquartile range; —: Median; □: Mean; ♦: Outlier.

5.3.3 Overall evaluation of quality controls

Intra-run assessment

Following the evaluation of calibration curve compliance to the FDA bioanalytical guideline specifications [169], 166 analytical runs were included for QC intra-run analysis. In five runs, one complete QC level was not within the required specification. Seven runs contained one complete QC level plus additional QC samples that exceeded the rejection limits. A further 12 runs were characterised by <67% valid QCs. Overall, 142 valid runs comprising 1308 QCs were applied for the subsequent evaluation of 940 PRA study samples. The relatively high amount of QCs in relation to the PRA study samples resulted from the high effort needed to determine the enzyme activity. This was caused by the duplicate measurement of an incubated and non-incubated AngI sample to obtain the corresponding PRA value. The number of QCs was therefore adjusted to AngI samples and the corresponding guideline specifications. In total, 91.3% of the 1308 QCs distributed over three concentration levels were in accordance with the predefined FDA requirements of intra-run accuracy of ±20%. Thus, 114 QCs (8.7%) showed a higher RE than 20%, without affecting the validity of the run (<20% RE for at least 50% per QC level and 67% of all QC levels).

Inter-run assessment

Evaluation of the inter-run assessment was scheduled and conducted as a post-hoc evaluation. The advanced investigation of inter-run accuracy by applying a combined assessment system derived from the CLSI guideline [185], the Westgard rules [186], and recommendations by van Bruijnsvoort et al. [187] showed compliance with the first two rules for all QC levels. In accordance with the first rule, in no concentration level, two subsequent QCs values exceeded the alert limit (mean measured concentration ±3 times the SD). The second rule was also met, implying that no QC level four consecutive values surpassed the warning level (mean measured concentration ±2 times the SD). However, transient shifts characterised by more than 10 consecutive QC values on one side of the mean were detected. The QC middle and QC high levels exceeded the objective threshold by 1 and 2 times, respectively. No assignable cause for the violation was identified by following the procedure described in CLSI guideline C24 on trend troubleshooting [185]. To more closely investigate whether this finding identified a substantial shift in assay performance, the effected consecutive runs were assessed using the Westgard rule 4_{1s} (4 consecutive control measurements exceed the same mean ±1 times the SD), and it was noted that this rule was not violated [186]. Further details are presented in the Levey Jennings plots in Figure 5-5.

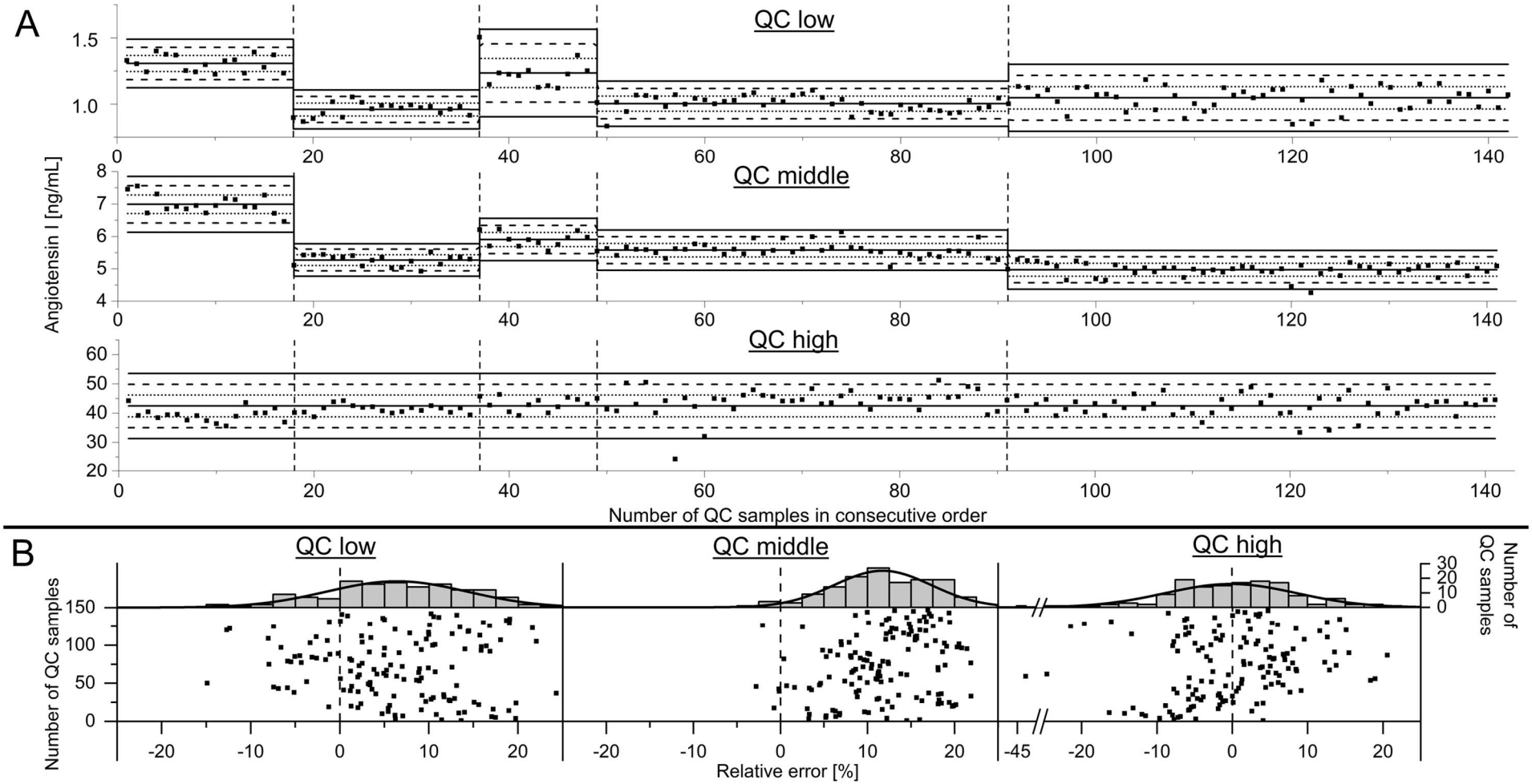


Figure 5-5: The long-term and inter-run evaluation of quality control (QC) samples of the plasma renin activity immunoassay. A: Levey-Jennings plot with the results (means of at least duplicate) of three levels of QCs in consecutive order over 24 months. The middle horizontal solid line indicates the overall mean of QCs of the same level. Horizontal dotted lines indicate control limits (mean ±1 standard deviation (SD)). Horizontal dashed lines indicate the warning limit (mean ±2 SD). The outer horizontal solid lines indicate the alert limit (mean ±3 SD). Vertical dashed lines represent the implementation of replacement lots. B: The relative error of the back-calculated QC concentrations of all QC samples obtained in valid runs. The results were depicted separately for the three QC concentration levels with corresponding marginal histograms. Each point reflects the mean QC standard per run. Dashed vertical lines indicate a relative error of zero %.

By comparing the three QC levels, it became apparent that the QC low and QC middle levels mainly exhibited values above the nominal concentration. Additional plotting of the lot-dependent RE for all measured QC values indicated a trend toward a positive deviation of the measured QCs derived by the vendor in relation to the specified concentration. Only 21.3% of the measured QC low values were below the nominal concentration, and even less were below the nominal concentration for the QC middle value (5.6% below the nominal concentration). In contrast, the in-house QC high level exhibited a homogenous distribution (47.3% below the nominal concentration) (Figure 5-5). The reason for this finding could not be clarified since further independent measures (e.g. patient specimens) could not be established as quality controls due to limited sample volumes. For further information, please refer to Appendix 10.3-4.

5.3.4 Blank sample evaluation

Of all 284 evaluated blank samples, 197 samples exhibited concentrations below the detection limit of the absorbance reader. Of the remaining 87 blanks, 67 exhibited a blank-to-LLOQ ratio below 20%, while 20 blanks ranged between 20% and 47%. Consequently, all blanks showed a blank-to-LLOQ ratio below 100%, thus fulfilling the ICH guideline requirements of blank concentrations below the LLOQ [171]. The LoB was 0.037 ng/mL, while the estimated LoD was 0.064 ng/mL. The LLOQ of 0.2 ng/mL was predefined by the vendor and verified by validation prior to sample analysis. The theoretical value was also confirmed by the mean of all 142 measured LLOQ samples (0.203 ng/mL) (Figure 5-6 and Appendix 10.3-5).

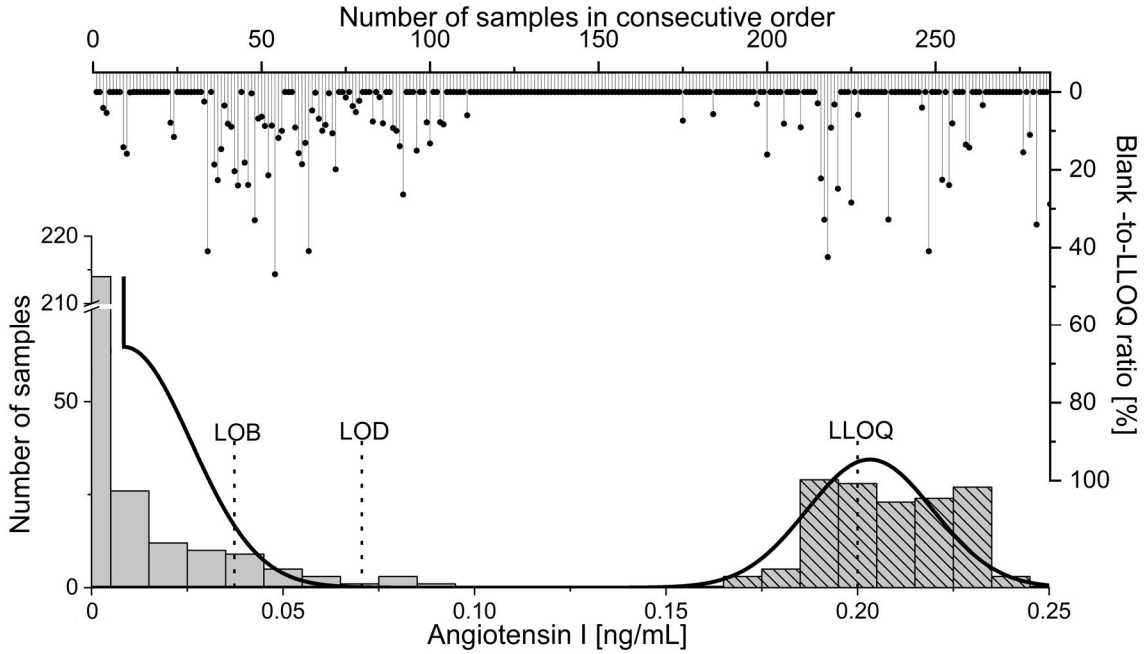


Figure 5-6: The overall evaluation of blank samples in all analytical runs by the plasma renin activity immunoassay. Upper and right axis: Each data point represents the ratio of a blank sample (two replicates of blank samples per run) and the corresponding Lower Limit of Quantification (LLOQ) sample (blank-to-LLOQ ratio) for all 142 valid analytical runs. Lower and left axis: The histogram of blank samples (filled bars) and LLOQ samples (striped bars) with a distribution curve (black solid curve) modified according to [14]. The Limit of Blank (LoB), Limit of Detection (LoD) and LLOQ are depicted as dotted lines. Values "<minimum" were defined as zero, and the distribution curve was artificially increased.

Since no measured blank sample violated the predefined rejection limits, no substantial effect on the reliable determination of CSs and QCs was claimed for the emulated matrix.

5.3.5 Incurred sample reanalysis

After a lag time of 5 months for evaluation of residual blood volume, ISR for plasma renin activity was implemented from this time point onwards the ISR samples (repeats) were measured. Samples obtained within the 5 months evaluation time were not re-assessed after ISR was implemented due to uncertainties in the stability. Out of all 940 study samples, 110 ISR pairs (11.7%) were analysed within the 19-month ISR period, including four replacement lots with corresponding revalidations. A total of 95 ISR pairs (86.4%) met the specifications of a deviation of <±30% between the original value and the repeat. The mean PRA of all measured originals was 24.6 ng/mL/h (±16.4 ng/mL/h SD) with a median of 21.8 ng/mL/h and an interquartile range of 13.7–33.0 ng/mL/h. The mean of the repeats was 24.0 ng/mL/h (±15.0 ng/mL/h SD) with a median of 19.1 ng/mL/h and an interquartile range of 11.9–33.0 ng/mL/h. The utilized ISR samples covered a range of 5.9–70.5 ng/mL/h. The PRA values acquired with the presented assay were based on the determination of AngI concentration values. With regard to the ISR, the evaluated samples covered a range of

0.4–8.3 ng/mL (assay range: 0.2–60 ng/mL). As a result, the concentration range of the ISR is not congruent with the range of the assay range. However, the obtained concentrations of ISR pairs were in the lower and most critical range of the assay. Since different lots of ELISA kits with partially slightly modified methods were used over the 24-month study period, the comparability of the ISR pairs between the lots was further investigated. A total of nine ISR experiments were conducted in a lot-overlapping approach, characterised by the measurement of original and repeat in different lots and after different revalidations. Lot-overlapping ISR was accomplished between the second and third (n = 1), third and fourth (n = 2), fourth and fifth (n = 5), and second and fifth (n = 1) lot. Every calculated difference between the nine ISR experiments was below the rejection limit of ±30%, thus indicating consistent performance over time. The ISR pairs, which were evaluated within the second (n = 7), third (n = 11), and fifth (n = 50) replacement lot, showed no significant differences after applying the paired-sample Wilcoxon signed-rank test (level of significance $\alpha = 0.05$). Yet, a significant distinction of the ISR pairs within the fourth (n = 42) replacement lot could be detected. However, within the fourth replacement lot, 83% of the ISR pairs were within the limit of <±30% and thus in accordance with the guideline specifications. By comparing the mean differences between ISR pairs regarding the different lots, PRA values within the second, third, fourth, and fifth replacement lots showed mean differences between original and repeat of −0.2%, −6.1%, −11.3%, and −2.9%, respectively. For further details, please refer to Figure 5-7 and Appendix 10.3-6.

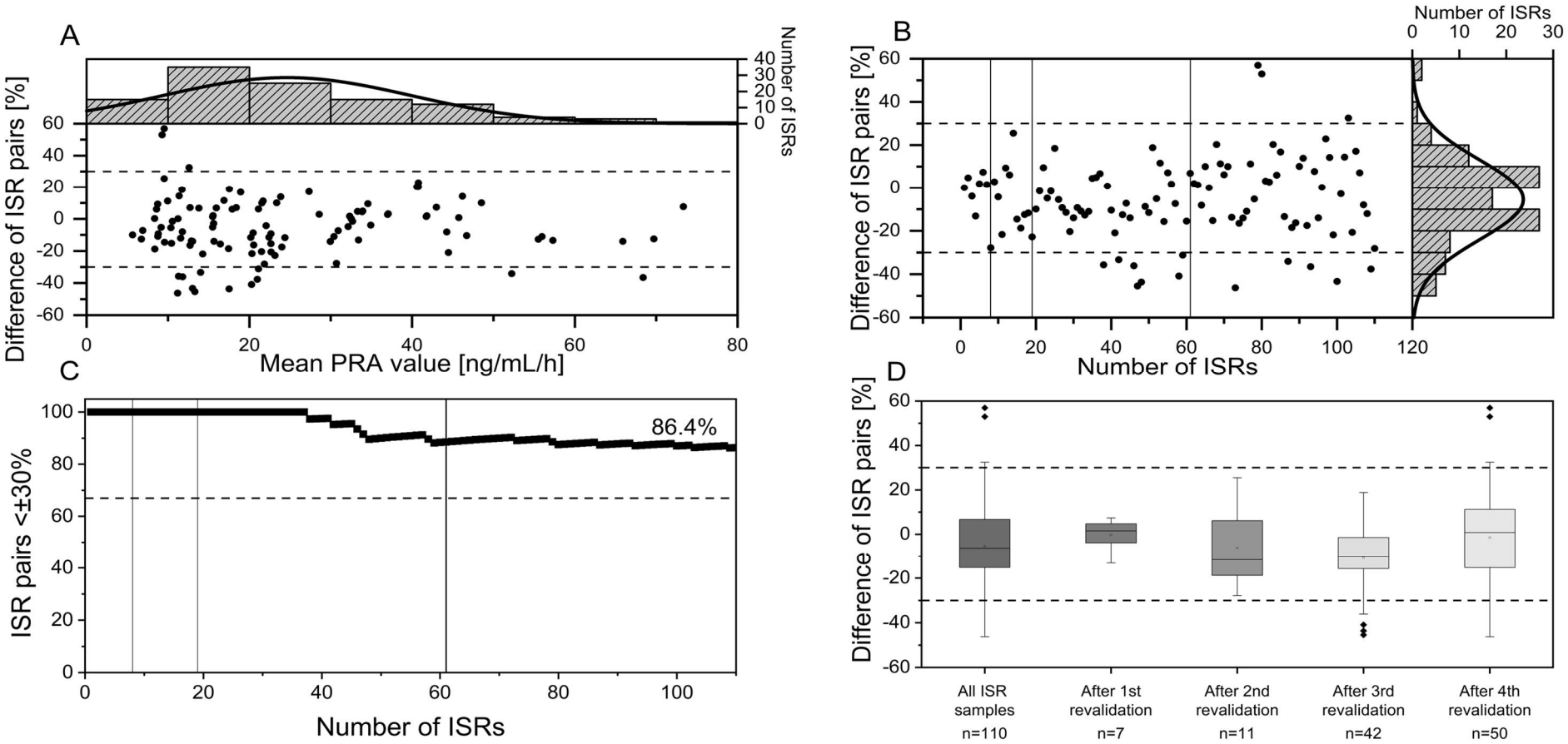


Figure 5-7: **Assessment of incurred sample reanalysis (ISR) by the plasma renin activity immunoassay.** A: Percentage difference plot of ISR pairs distributed over the concentration range with a marginal histogram of all plasma renin activity (PRA) values. Dotted lines indicate the ±30% acceptance thresholds given by the European Medicine Agency (EMA) and the U.S. Food and Drug Administration (FDA). B: Percentage difference plot of ISR pairs in consecutive order with a marginal histogram of the difference between the ISR pairs. Dotted lines indicate the acceptance thresholds given by the EMA and FDA of ±30% deviation, which is required to be fulfilled in at least 67% of all ISR pairs. Vertical solid lines indicate the implementation of replacement lots. C: Cumulative ISR samples indicating the overall ISR performance against the 67% regulatory limit (dotted black line). Vertical solid lines indicate the implementation of replacement lots. The cumulative ISR plot was presented, as recommended by Rudzki et al. [189]. D: Boxplots of the difference in ISR between the different assay lots. Dashed lines indicate the EMA and FDA guideline limits for the ISR difference of ±30%, which is required to be fulfilled in at least 67% of all ISR pairs. ▪: interquartile range; ⌶: 1.5 interquartile range; —: Median; □: Mean; ♦: Outlier.

5.3.6 Interlaboratory testing

The external verification by interlaboratory testing was conducted three times, which allowed for a comparison of the developed assay with the established assays used by other laboratories. Interlaboratory testing was conducted in January 2017, July 2017, and January 2018. Measured concentrations of 2.3 ng/mL/h and 8.1 ng/mL/h PRA were observed for sample A and sample B in January 2017, with 2.9 ng/mL/h and 2.2 ng/mL/h observed in July 2017, and 2.2 ng/mL/h and 2.8 ng/mL/h observed in January 2018. Participation in January 2017 and 2018 resulted in passing the interlaboratory tests, thus verifying the reliability of the validated assay. The evaluation of the conducted interlaboratory test in July 2017 could not be accomplished by the RfB due to a lack of applicable consensus values (<4), which are mandatory for the appraisal of reported values. These findings confirmed the accuracy of the established PRA ELISA and demonstrated the comparability between the developed easy-to-use ELISA assay and the more complex RIAs commonly applied in routine clinical analysis.

5.4 Discussion

The long-term monitoring of bioanalysis of the humoral parameter PRA within the LENA project was successfully conducted by the implementation of a multi-step QCS. Since a comprehensive QCS for the special demands of bioanalytics within long-lasting paediatric studies was lacking, a customised QCS was developed based on well-established guidelines (EMA and FDA), the recommendations given of reputable organisations (e.g. CLSI), and suggestions based on current scientific discussions. Moreover, an advantageous characteristic of the applied easy-to-handle QCS represents its applicability in academia-driven projects, enabling the acquisition of unique, high-quality data with moderate effort.

A fit-for-purpose SST regarding analytical equipment is strongly recommended prior to sample analysis [190]. However, clear specifications are not currently mentioned in regulatory guidelines (e.g. EMA, FDA) [169, 170]. For chromatographic assays, Broadhurst et al. recommended an acceptable peak area of ±10% by the use of analytes in matrix-free solution [190]. These specifications were also considered suitable to ensure the appropriate performance qualification of the used absorbance reader because chromatographic specifications are commonly more rigorous than those for ligand-binding assays [170]. While investigations of the intra-run monitoring of bioanalytical runs are well defined, recommendations for the assessment of the inter-run performance of the assay are lacking in EMA and FDA guidelines [169, 170]. Therefore, the recommendations of the CLSI guideline [185], Westgard rules [186], and suggestions from van Bruijnsvoort et al. [187] were combined in the QCS to monitor deviations caused by process influencing factors (e.g. analyst, temperature, or humidity). Although the collected PRA data was not used to decide on the treatment of paediatric patients directly, the aim was to ensure the high quality of first-time PRA data in very young children with HF at the highest level. Therefore, the modified Westgard rules by van Bruijnsvoort et al. seemed appropriate, as they represented a rational consideration of the required QC standard specifications over the duration of the study and its rigour with regard to the risk of patient harm in the case of misjudgements of the analytical run [187]. Although CLSI recommended establishing two concentration levels based on clinical decision values [185], the selection of QC concentration and levels per analytical run was made on the stricter requirements issued by regulatory agencies since clinical decision values of PRA in children suffering from HF were lacking. All QC levels showed compliance with the first two rules (1_{3S} and 3_{2S}) in the inter-run QC assessment. However, the third rule ($10\bar{x}$) was not fully met, and no assignable cause for the violation could be identified. As proposed by Westgard et al., violation of this rule contributes the

least to error detection compared to the other rules [186]. Therefore, it was classified as a transient out-of-control condition with less impact. Further reassessment by applying the Westgard rule "4_{1s}" (not more than four consecutive results exceed the mean ±1 SD in the same direction) to the effected consecutive runs [186] as well as the application of the "10_{1s}" rule (10 consecutive results exceed the mean ±1 SD in the same direction) on all QC samples recommended by the CLSI [185] confirmed this argumentation. The post-hoc analysis presented here showed that the implementation of such a QCS in academic research is feasible. Thus, it is recommended to establish continuous monitoring of inter-run QC in line with current recommendations by CLSI for future approaches whenever appropriate. Retrospectively, continuous monitoring through performing a recalculation of statistical boundaries after every 40 samples, as proposed by *van* Bruijnsvoort et al. [187], would have been impractical due to the low number of analytical runs per lot and the recommendations of the CLSI guideline appearing more suitable [185]. Moreover, since deviations between the nominal versus the observed concentration of the QC low and QC middle levels were identified, the information of the certificate of analysis provided by the vendor appeared questionable. To avoid this mismatch, an in-house verification of the provided reference values is recommended and should be established by utilizing at least 20 corresponding QC values [185]. The determination of blank-to-LLOQ ratios, as well as the estimation of LoB and LoD, was demonstrated to be of high importance for reliable data acquisition due to potential interfering substances within the emulated matrices of the CSs and QCs [188, 191]. The results of the blank sample evaluation complied with recently published recommendations by Azadeh et al., who suggests a blank-to-LLOQ ration below 90 % for competitive ELISA [192], and were in line with the requirements of the ICH M10 draft guideline [171]. Continuous monitoring was established in the context of this setting, which resulted in an LoB/LoD assessment using a total of 284 blank samples and comprised five different lots over a 24-month study period. This approach was selected because different lots of chemicals could contain differing concentrations of interfering substances, making a temporally delimitated determination less sensitive to trends or fluctuations in long-term bioanalysis.

While the EMA and FDA guidelines on bioanalytical method validation and the ICH M10 draft guideline demand a fixed ratio of ISR (10% for <1000 study samples and additional 5% for >1000 study samples) [169–171], Rudzki et al. suggested that the number of incurred samples could be reduced to a fixed amount (e.g. 30 samples) without undermining the meaningfulness of the analysis [193, 194]. An ISR rate of 11.7% representing 110 ISR pairs was achieved in accordance with both of the aforementioned approaches. More than 86% of the blinded and randomly selected ISR pairs met the guideline criteria (≥67%).

Despite these good results, the applicability of further guideline recommendations remains limited. Any PK or PD analyses were not scheduled before the finalization of the bioanalysis and data clearance. The subsequent lack of feedback from PD evaluation prevented the assignment of analysed sample values to those near the c_{max} or concentrations close to the elimination phase. Furthermore, AngI and the resulting PRA values in this unique population have not been previously investigated, resulting in a lack of reference values for the classification of maximal and minimal values, as recommended by the EMA and FDA [169, 170]. Finally, the maturation of paediatric organisms [78, 158], possible influence of circadian rhythm [195, 196], and blood sampling conditions [197] complicate compliance with the aforementioned regulatory requirement. However, the extensive number of analysed incurred samples provided an appropriate distribution within the sampling profiles covering various sampling time points. The lack of recommendations regarding the reliable long-term and between-run monitoring of QCs and CSs as well as blank sample requirements indicate a topic for improvement in current EMA/FDA guidelines and also the ICH draft M10 guideline [169–171]. Since these missing items are presently only addressed by scientific references (e.g. Westgard), clear suggestions should be included in future regulatory guidance. In addition to the fact that between-run specifications should be covered by regulatory guidelines, the required number of QCs per run should be re-considered. Within the LENA project, 1308 QC were necessary to fulfil all requirements given in the guidelines. A reduced number of QCs or a fixed number as recommended for ISR by Rudzki et al. [193, 194], could contribute to an increased throughput of study samples without undermining the meaningfulness of analysis.

In summary, the combination of well-established regulatory guidelines with alternative recommendations derived from current scientific discussions into one comprehensive customised QCS applicable to academic research has been demonstrated. This lean and cost-effective QCS complies with GCLP requirements and should encourage other academic researchers to establish comparable systems when investigating similar research topics.

5.5 Conclusion

The customised bioanalytical QCS comprised regulatory guidelines, international recommendations, and current scientific discussions. The multi-step QCS successfully monitored the short- and long-term performance of the bioanalysis of the humoral parameter PRA via ligand-binding assay and substantially contributed to the generation of reliable data. The applicability of this combined system in an academic environment was confirmed for the PRA within the FP7- funded LENA project.

6 Development and validation of an innovative multiplex liquid chromatography high-resolution mass spectrometry method for the investigation of angiotensin peptides in plasma

6.1 Background and aim

Of 165 newly labelled drugs in the United States between 2003 and 2012, just 28% provided paediatric study data at the time of approval, including only five medicines with data for neonates [184]. In Europe, this constraint is increasingly addressed by the "EU Paediatric Regulation" [98]. Although this regulation affects the pharmaceutical industry in Europe by the requirement of paediatric investigation plans, the amount of evidence-based medicines for the paediatric population is limited, and the number of meaningful clinical trials for children is still underrepresented compared to adults [92]. Nevertheless, paediatric research is increasingly addressed by academic consortiums like LENA. The comprehensive evaluation angiotensin peptides in the population of the LENA project is a rational approach to gain valuable new insight into the maturing RAAS and the paediatric HF. In the past decades, it became apparent that the angiotensin peptides AngI and AngII could not be the only physiological important peptides of the RAAS. Indeed, novel angiotensin peptides such as AngIII and AngA, who are characterised by primarily AngII-like characteristics, as well as the potential functional counter-peptides Ang1-7, alamandine, and Ang1-9 are increasingly addressed by cardiovascular research. However, while there is little evidence regarding in-vivo data for these novel peptides in adults, sophisticated investigations in the healthy and cardiovascular diseased paediatric population are almost entirely lacking [78]. Thus, the development and GCLP compliant validation of bioanalytical methods applicable to paediatric research in an academic environment is a crucial step to support the evidence-based medicine for children in the future. Yet, the simultaneous quantification of complex analytes like peptides in biological fluids is one of the significant challenges in bioanalytics and is associated with various obstacles. Deficient endogenous concentrations, similar properties of the peptides to be analysed, short half-life, a challenging matrix with potential interfering compounds, a small available sample volume, as well as an adequate accuracy and precision of the method present hurdles to be overcome.

Therefore, the sub-aims of this project were:

- To develop a multiplex LC-HRMS method for the simultaneous determination of the essential angiotensin peptides AngI, AngII, Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9 in human plasma.

- To provide an acceptable sensitivity and calibration range of the method by applying minimal sample volumes of 50 µL plasma and thus enable a meaningful investigation of these humoral parameters within the paediatric population of the LENA project.

- To achieve adequate sample purification and extraction steps by taking the short half-life of the analytes and the complex matrix into account.

- To perform a fit-for-purpose validation according to the FDA bioanalytical guideline to ensure the reliable determination of all peptides in a GCLP environment.

- To show the applicability of the method by example measurements of adult and paediatric human sources.

6.2 Methods

6.2.1 Materials

Consumables

Reaction tubes 1.5 mL Protein LB, Biosphere® filter tips 20 µL, Biosphere® filter tips 100 µL, pipette tips 1250 µL, pipette tips 10 mL, falcon tubes 50 ml, S-Monovetten® 9 mL K3E, and S-Monovetten® 1.2 mL K3E were purchased from Sarstedt AG & Co. KG (Nümbrecht, Germany). Oasis® Prime HLB µElution plates and AQUITY UPLC® 700 µL Round 96-Well plates were obtained from Waters® GmbH (Eschborn, Germany). Eppendorf tubes 5.0 mL, Combitips advanced® 10 mL, Combitips advanced® 5 mL, and Combitips advanced® 2.5 mL were purchased from Eppendorf AG (Hamburg, Germany).

Chemicals and reagents

Methanol MS-grade and water MS-grade were provided by Honeywell (Seelze, Germany). Methanol HPLC-grade and water HPLC-grade were obtained by Fisher Scientific U.K. Limited (Loughborough, United Kingdom). FA (>98%) p.a. and dichloroacetic acid (DCA) were purchased from Sigma-Aldrich (Steinheim, Germany) and dimethyl sulfoxide (DMSO) (99.9%) was supplied by AppliChem GmbH (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) p.a. (≥99%) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as well as 4-(Hydroxymercuri) benzoic acid sodium salt, pepstatin A (>90%), and 1,10 phenanthroline monohydrate (Sigma-Aldrich, Steinheim, Germany) were used. Aliskiren hemifumarate was delivered from MSN Laboratories Limited (Rudraram, India). Angiotensin I human acetate salt hydrate (≥90%), angiotensin II human acetate (≥93%), angiotensin III trifluoroacetate salt hydrate (≥98%), angiotensin IV trifluoroacetate salt hydrate (≥95%), angiotensin-(1-7) acetate salt hydrate (≥90%), angiotensin A trifluoroacetate salt hydrate (≥98%), alamandine trifluoroacetate salt hydrate (≥98%), and synthetic [Val^5]-angiotensin I (99%) were purchased from Sigma-Aldrich (Steinheim, Germany) while angiotensin-(1-9) trifluoroacetate salt hydrate (≥98%) was delivered by Adooq Bioscience (Irvine, United States) and ([ring-D_5]Phe^8)-angiotensin II acetate salt (≥98%) was obtained from Bachem AG (Bubendorf, Switzerland).

Preparation of standard solutions

0.1% FA was used as a reconstitution solution to obtain the first stock solution (1 mg/mL) for every analyte and IS each. The first stock solution was diluted with 0.1% FA to obtain the second stock solution (10 µg/mL). Subsequently, the analyte working solution

(100 ng/mL) was prepared by spiking 0.1% FA with the 10 µg/mL stock solution of every analyte. By analogy, the internal standard (IS) working solution (100 ng/mL) was prepared by spiking 0.1% FA with the 10 µg/mL stock solution of [Val^5]-angiotensin I and ([ring-D_5]Phe^8)-angiotensin II.

Instruments

For the preparation of stock solutions and processing of validation and study samples, Research® plus pipettes 0.5-10 µL, 10-100 µL, 0.1-10 mL, and 1-10 mL, as well as a Multipette® E3 (Eppendorf AG, Hamburg, Germany) were used. Furthermore, a TX4 Digital IR Vortex Mixer (VELP Scientifica, Usmate, Italy), a ThermoMixer® (Eppendorf AG, Hamburg, Germany), and a Centrifuge 5415R (Eppendorf AG, Hamburg, Germany) were applied. The SPE was performed using a Positive Pressure-96 Manifold from Waters® GmbH (Eschborn, Germany). The HPLC analysis was conducted with a Nexera XR HPLC system (Shimadzu, Duisburg, Deutschland) including a CBM-20A controller bus module, two LC20AD XR pumps, a DGU 20A5R degasser, a SIL-30AC autosampler, a FCV-11AL valve unit, and a CTO-20AC column oven. The HPLC was coupled to a TripleTOF® 6600 mass spectrometer from AB Sciex (Concord, Canada), which was equipped with a calibration delivering system, a roughing pump, and a Turbo V ESI source.

Software

Acquisition of data was performed using the Analyst® software version 1.7.1 (AB Sciex, Darmstadt, Germany). Processing of the generated data was accomplished with the MultiQuant® software version 3.0.2 (AB Sciex, Darmstadt, Germany). The appraisal of the raw data was further conducted with Microsoft Office® 2016 (Microsoft Corp., Redmond, WA, United States) and Origin® pro 2018b (OriginLab Corporation, Northampton, United States).

6.2.2 Optimisation steps during method development

During the method development, several optimisation steps were necessary to overcome the bioanalytical hurdles which are reasoned in the complex matrix, the restricted sample volume, as well as the complex characteristics of endogenous peptides.

These optimisation steps included:

1. The generation of an appropriate blank plasma to prevent the influence of endogenous peptides on method performance.

2. The removal of interfering matrix to avoid ion suppression or enhancement and to reduce the impact on accuracy and precision of the method.

3. The enhancement of the sensitivity of the method to enable an adequate LLOQ for the quantification of the peptides in a sub nanogram per millilitre range.

Optimisation of the blank plasma generation

In contrast to the bioanalytical analysis of exogenous substances (e.g. pharmaceutical drugs, herbal components or environmental toxins), the analysis of endogenous peptides, which are physiologically present in the matrix, is accompanied with further difficulties. The pre-existing endogenous concentrations in the matrix could significantly impact the accurate determination of CSs and QCs. Moreover, the endogenous plasma concentrations could increase the LLOQ. In addition, the generation of AngI by the action of renin could occur during sample preparation, which would additionally distort the quantification of AngI and all of the subordinate angiotensin peptides. Therefore, the simultaneous measurement of a blank sample with the calibration curve and the subsequent blank value reduction seemed not appropriate to guarantee a precise determination. Moreover, in LC-MS analysis, this technique is related to several inaccuracies (e.g. differing measurement time points of the CSs and the blank, as well as the higher impact on the lower part of the calibration curve and the corresponding influence on the regression). Thus, the highest possible removal of physiologically present peptides with additional inhibition of post-sampling generation of peptides prior to CS and QC preparation was conducted to bypass this issue. In this regard, the renin inhibitor aliskiren was spiked directly in S-Monovetten® to inhibit the generation of angiotensin peptides instantly. After centrifugation, the plasma was incubated at 37 °C for 90 min to degrade the remaining angiotensin peptides in the sample. This processed plasma was used for the preparation of CSs and QCs.

Optimisation of peptide purification and removal of interfering matrix

The removal of interfering substances is vital for the accurate and precise quantification of peptides in biological samples. An insufficient purification of the samples results in coeluting matrix components which could lead to an increase or suppression of analyte signals. Moreover, the accuracy and precision, as well as the individual matrix effect, could be significantly impacted by various amounts and compositions of matrix components. During the method development, several modifications had to be applied to minimise the effect of the matrix and enable a satisfactory method performance and recovery of the analytes. It became apparent that the standard SPE protocol provided by the vendor of the utilised SPE plates was not sufficient to provide adequate sample purification. This was characterised

by frequent clogging of the guard-column and the capillaries of the HPLC system, as well as by insufficient accuracy and precision which could be assigned to a high effect of matrix components on the analytes. Therefore, the SPE protocol was modified to provide better sample purification without severe loss of analyte. However, although the customised SPE protocol yielded beneficial effects on the guard column lifetime and clogging frequency without a downturn in recovery, the measurement of accuracy and precision still lacked for satisfactory results. Thus, a precipitation step before SPE was implemented to achieve additional removal of matrix components, and the SPE protocol was finally adopted.

Optimisation of sensitivity

Reliable reference values of angiotensin peptides in children and neonates suffering from HF are inconsistent or entirely lacking [78]. The calibration range is thus just limited predictable for this unique population using existing data. Consequently, a sufficient method sensitivity in combination with a broad dynamic calibration range is a crucial element for the quantification of these peptides in paediatric study samples. Thus, the increase in signal intensity of the peptides represented a further hurdle to be overcome.

First, most of the peptides are subject to unspecific adsorption on container walls (e.g. glass and polypropylene), which could result in a significant loss of analyte and reduced signals [198]. Silanisation and bovine serum albumin treatment of the glassware were evaluated but considered as inadequate to reduce peptide adsorption on the container walls effectively. Therefore, no glassware was used during sample preparation and subsequent measurement of the peptides. Moreover, the plasma samples for CSs and QCs, as well as the study samples, were exclusively prepared in 1.5 mL protein low bind reaction tubes and AQUITY UPLC® 700 µL Round 96-Well plates.

Second, depending on the AS sequence, peptides could have multiple charge states which could decrease the MS signal of the selected precursor ion. The state of charge is strongly dependent on the surrounding milieu and the isoelectric point of the respective peptide. In order to achieve the highest possible sensitivity, the peptide should be preferable present in only one charge state (desirable in the state in which the peptide is most frequently present under the given conditions), since only one kind of charge state of the precursor ion could be considered for the targeted screening and quantification of the peptide. Besides acidification of the injection solvent and mobile phases (0.1% FA), DMSO was added as supercharging reagent. While the specific mechanism of DMSO as supercharging reagent is still not clearly identified, an increase in the droplet surface tension of the ESI which is directly proportional to the charge availability of the droplet is proposed [199].

Third, peptides are related to an increased fragmentation compared to small molecules, which further reduces the signal intensity and yield higher background (Figure 6-1).

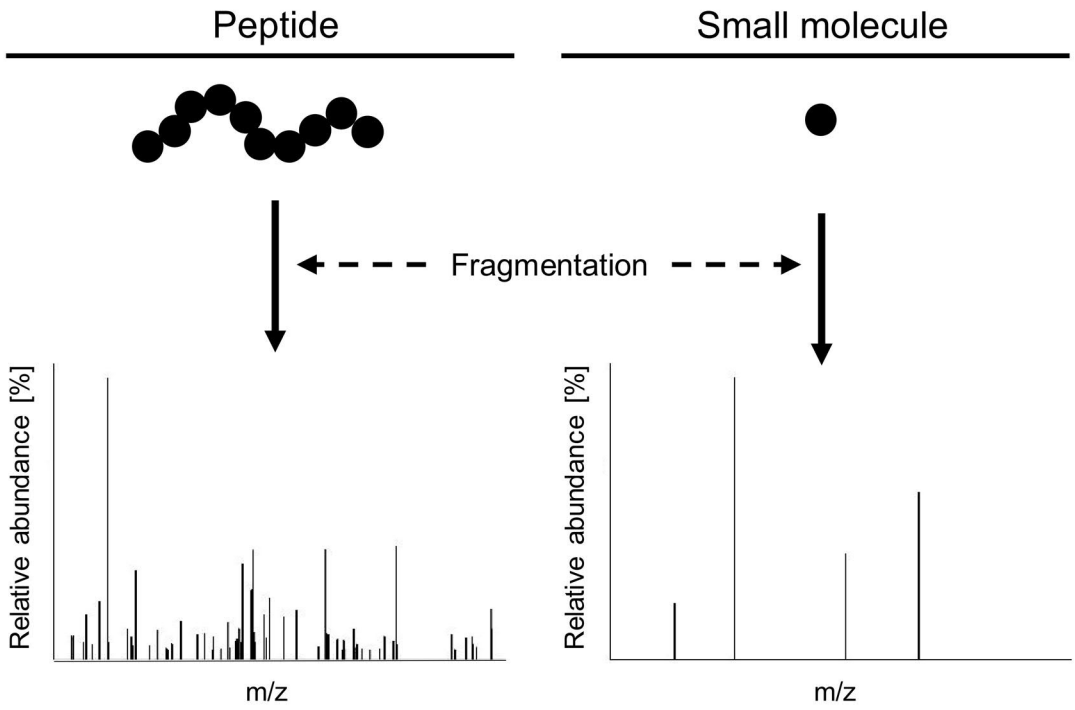
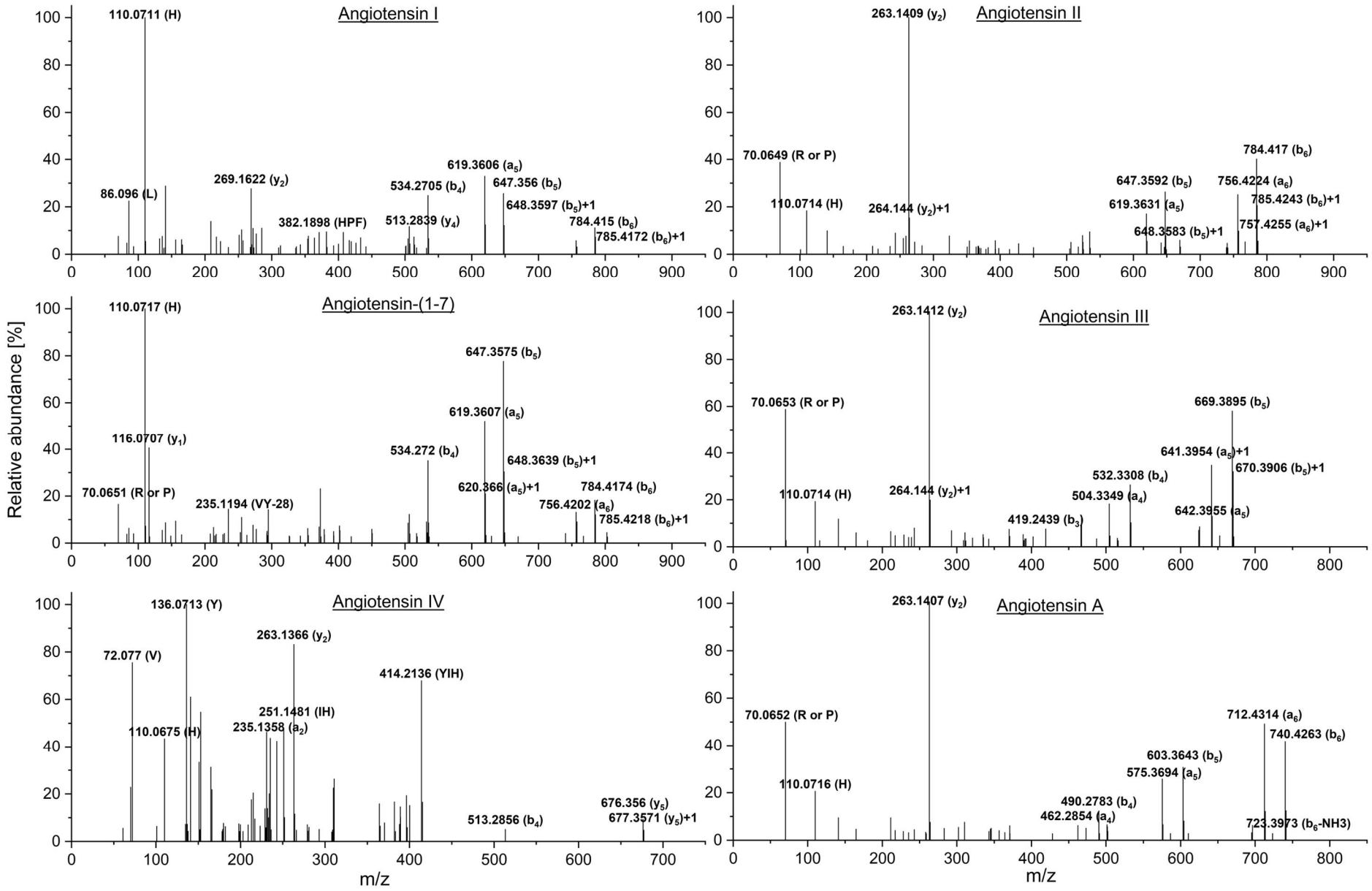


Figure 6-1: Schematic presentation of the difference of a product ion scan fragmentation between a peptide and small molecule. m/z: mass-to-charge ratio.

The applied Q-TOF technique enables the measurement and summation of several fragment ions, which results in increased signal intensity. Moreover, this approach allows the concurrent conduction of untargeted screening comprising broad mass ranges and thus might provide advanced insights into the unique population of the LENA project. As a consequence, the Q-TOF was the method of choice.

The following figure (divided in part A (on the next page) and part B (continued on the following page)) shows the mass spectrum of each analyte and IS with corresponding m/z of the fragment ions (Figure 6-2).

Part A


Angiotensin I
110.0711 (H)
86.096 (L)
269.1622 (y2)
382.1898 (HPF)
513.2839 (y4)
534.2705 (b4)
619.3606 (a5)
647.356 (b5)
648.3597 (b5)+1
784.415 (b6)
785.4172 (b6)+1
Angiotensin II
263.1409 (y2)
70.0649 (R or P)
110.0714 (H)
264.144 (y2)+1
619.3631 (a5)
647.3592 (b5)
648.3583 (b5)+1
756.4224 (a6)
757.4255 (a6)+1
784.417 (b6)
785.4243 (b6)+1
Angiotensin-(1-7)
110.0717 (H)
116.0707 (y1)
70.0651 (R or P)
235.1194 (VY-28)
534.272 (b4)
619.3607 (a5)
620.366 (a5)+1
647.3575 (b5)
648.3639 (b5)+1
756.4202 (a6)
784.4174 (b6)
785.4218 (b6)+1
Angiotensin III
263.1412 (y2)
70.0653 (R or P)
110.0714 (H)
264.144 (y2)+1
419.2439 (b3)
504.3349 (a4)
532.3308 (b4)
641.3954 (a5)+1
642.3955 (a5)
669.3895 (b5)
670.3906 (b5)+1
Angiotensin IV
136.0713 (Y)
72.077 (V)
110.0675 (H)
235.1358 (a2)
251.1481 (IH)
263.1366 (y2)
414.2136 (YIH)
513.2856 (b4)
676.356 (y5)
677.3571 (y5)+1
Angiotensin A
263.1407 (y2)
70.0652 (R or P)
110.0716 (H)
462.2854 (a4)
490.2783 (b4)
575.3694 (a5)
603.3643 (b5)
712.4314 (a6)
740.4263 (b6)
723.3973 (b6-NH3)
Relative abundance [%]
m/z

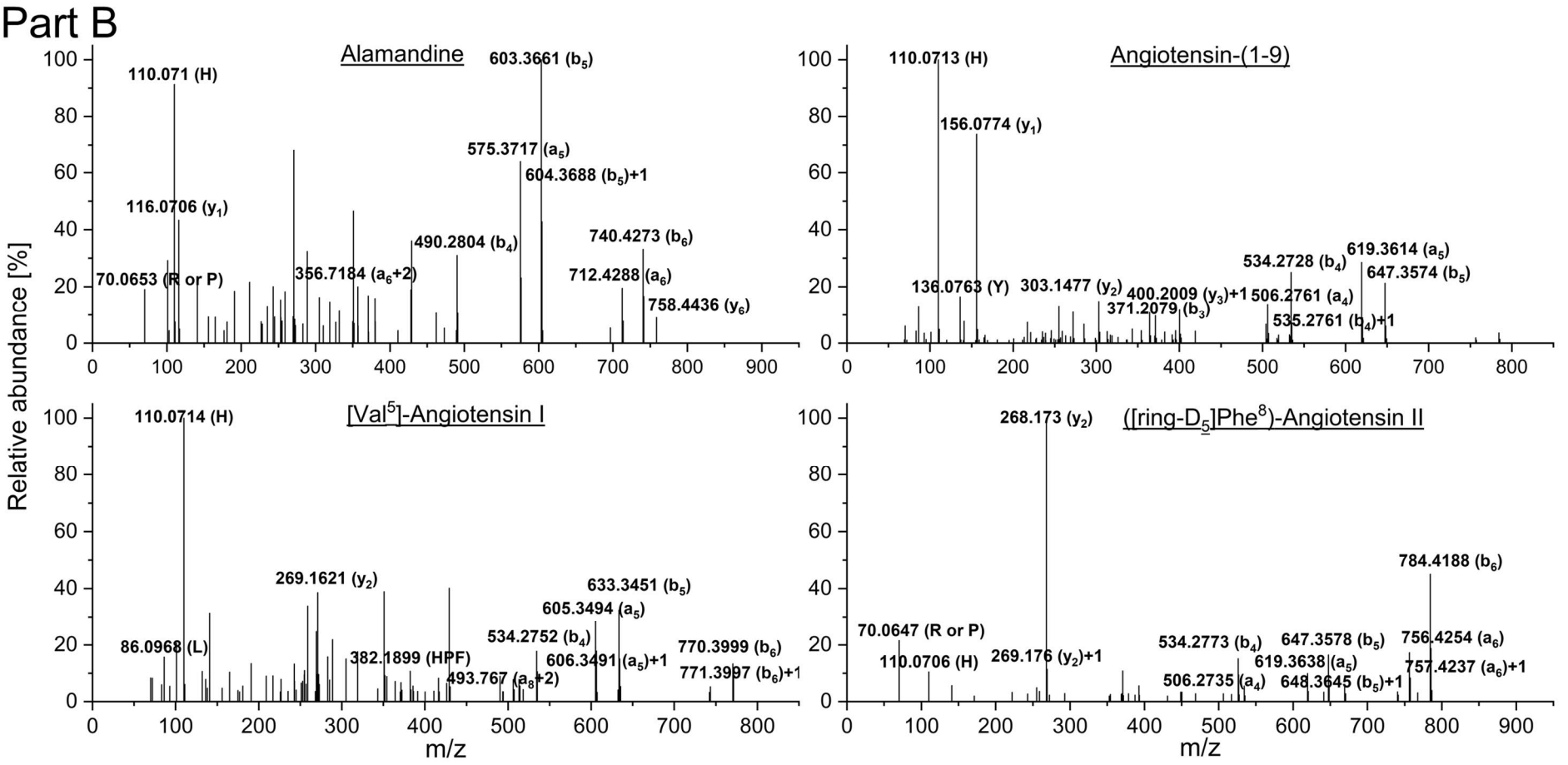


Figure 6-2: Example mass spectra of product ion scans for every individual analyte and internal standard included in the liquid chromatography coupled to high-resolution mass spectrometry method. Each fragment ion is indicated by its mass-to-charge ratio (m/z) and the type of fragment in the parentheses. The “y” and “z” fragments comprise the N-terminal end, while the “a” and “b” fragments comprise the C-terminal end. The numbering of the respective fragment ions based on the peptide bond and begins at the corresponding end (according to the nomenclature by Roepstorff and Fohlman[122]). A “+1” behind the parenthesis represents the isotope signal of the corresponding fragment. A “+2” behind the type of fragment within the parenthesis indicates a double charged fragment. A single capital letter in the parentheses indicates the corresponding amino acid as one-letter code, while multiple capital letters indicate internal ions which are caused by double backbone cleavage. The “-28” behind the internal ion indicates the loss of carbon monoxide.

6.2.3 Final assay procedure

Blood sampling and preparation of plasma for calibration standards and quality controls

Adult human plasma was used as matrix for the preparation of all CSs and QCs samples, as paediatric plasma samples could not be applied due to ethical constraints. The blood sampling from adults in context of this study was permitted by the Ethical Commission of the Heinrich-Heine-University Düsseldorf (Study number 6116) and written informed consent from the volunteers was obtained. The blood sampling was always conducted by trained staff.

The blood was explicitly collected and prepared to free the plasma from pre-existing analytes to generate a blank matrix applicable for the preparation of CSs and QCs. 500 µL solution (500 ng/mL in water HPLC-grade) of the renin-inhibitor aliskiren was added to 9 mL K3E S-Monovetten® to prevent the post-sampling generation of AngI. The blood was sampled from a healthy adult volunteer at RT. Subsequently, the S-Monovetten® were centrifuged at 2000 g for 10 min at RT. Afterwards, the plasma supernatant was incubated for 90 min at 37 °C to degrade the angiotensin peptides still present in the plasma. Finally, the incubated plasma was again centrifuged at 16100 g for 20 min at RT, and the supernatant was frozen at -20 °C.

CSs and QCs had to be prepared for all validation runs and analytical runs. As the plasma for CSs and QCs should be as comparable as possible with the study samples, the plasma was spiked with a freshly prepared inhibitor cocktail (79.95 parts 0.03 M 4-(Hydroxymercuri) benzoic acid in EDTA, 10.66 parts 0.01 M 1,10-phenanthroline in DMSO, and 9.38 parts 0.03 M pepstatin A in DMSO) to mimic the study sample plasma. For the preparation of CS and QC dilution series, a plasma sample was spiked with an initial concentration of the analyte working solution (100 ng/mL). This step was conducted to keep the dilution of the plasma within the CS and QC series as low as possible. Subsequently, two independent dilution series were prepared for the CSs and the QCs from the initially spiked plasma sample, respectively (Figure 6-3).

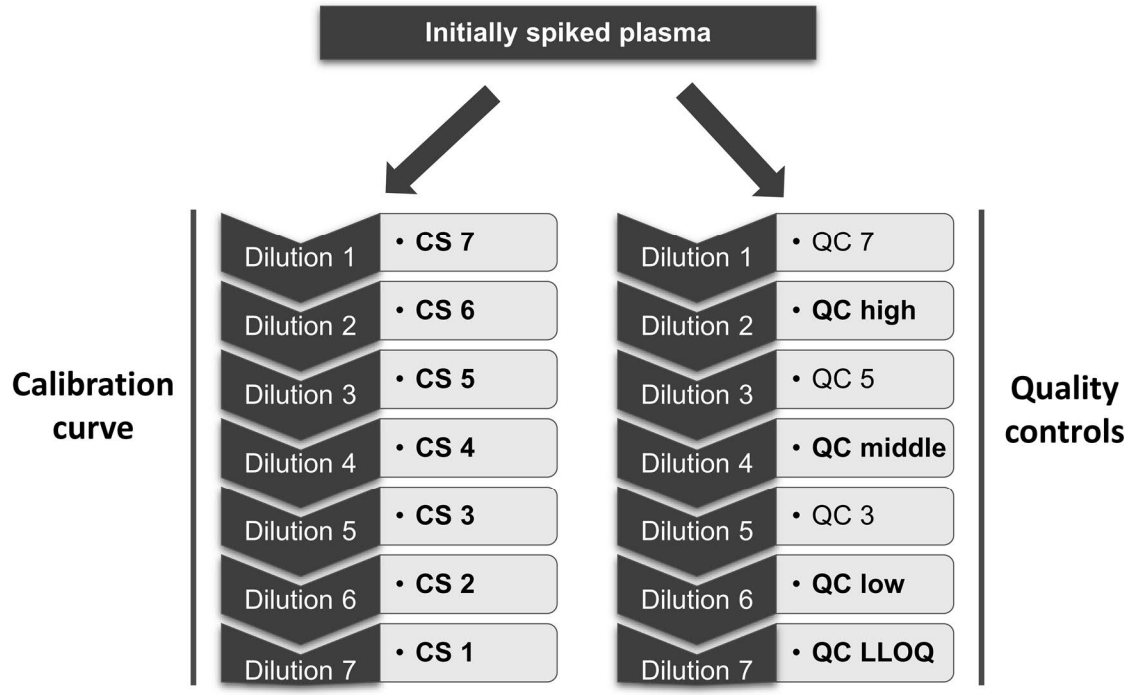


Figure 6-3: Preparation of calibration standards (CS) and quality controls (QC) for the liquid chromatography coupled to high-resolution mass spectrometry method. LLOQ: Lower Limit of Quantification.

Precipitation step and internal standards

A precipitation step was conducted to remove interfering matrix compounds, such as large proteins (Figure 6-4). 50 µL plasma of each study sample, CS, and QC were pipetted into protein low-bind reaction tubes. The precipitation solution (55 parts water MS-grade, 40 parts methanol MS-grade, and 5 parts DCA) was spiked with IS solution (100 ng/mL) to obtain a precipitation-IS solution with a concentration of 800 pg/mL. The precipitation-IS solution was stored at -20 °C. Subsequently, 200 µL cold precipitation-IS solution was added quickly to each tube. The tubes were then vortexed for 3 minutes and centrifuged at 16100 g for 10 minutes at RT. After centrifugation, 200 µL supernatant was removed and directly loaded on the SPE plate.

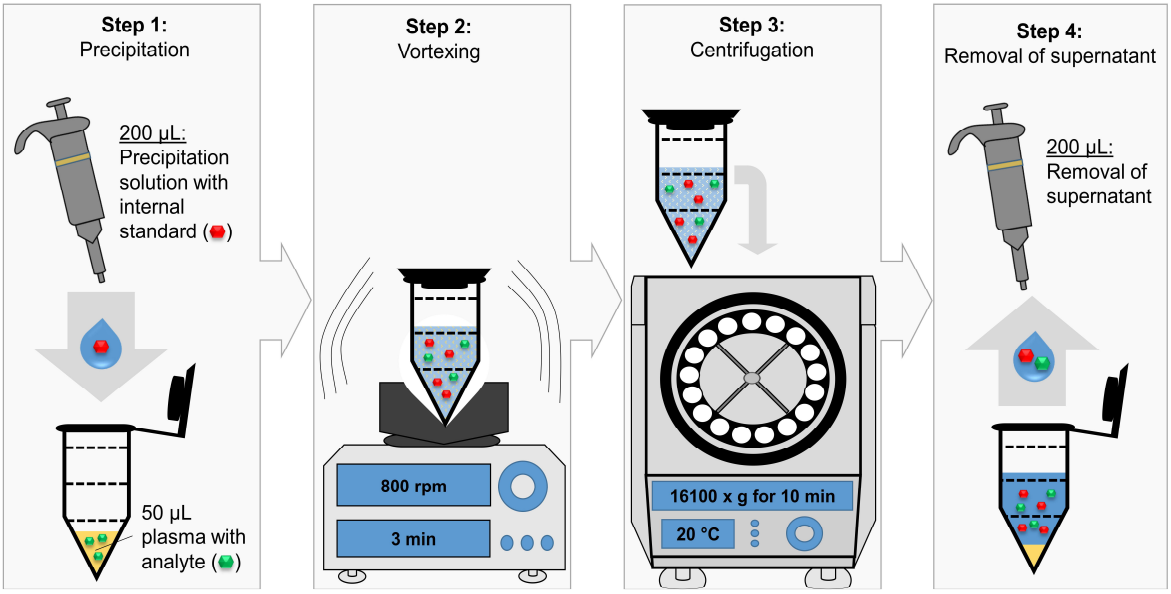


Figure 6-4: The conducted precipitation procedure using 50 µL plasma prior to solid-phase extraction and liquid chromatography coupled to high-resolution mass spectrometry.

Solid-phase extraction

The SPE was conducted to further purify the sample prior to the injection to the HPLC and to concentrate the analytes after precipitation. For the conduction of the SPE, an Oasis® Prime HLB µElution plate in combination with a Positive Pressure-96 Manifold were used. The pressure was applied on the µElution plate until a slow and uniform drop speed was reached for every cartridge. The subsequent step was only performed when the complete liquid had run through the sorbent material due to the pressure applied. Before loading the samples on the SPE cartridges, the sorbent material in each cartridge was preconditioned at the first step with 200 µL preconditioning solution (60 parts methanol HPLC-grade, 39.9 parts water HPLC-grade, and 0.1 parts FA) to remove impurities as well as manufacturing remnants. In the second step, the sorbent material was equilibrated with 200 µL water. In the third step, 400 µL water was applied on the cartridge and 200 µL of the supernatant of the precipitation step was directly spiked. The fourth step comprised the addition of 200 µL washing solution (95 parts water HPLC-grade and 5 parts FA) to remove any matrix components remaining on the sorbent material. The passed liquids in step 1-4 were discarded. The elution of the analytes from the sorbent material was achieved in the last step by applying 2 times 50 µL elution solution (60 parts methanol MS-grade, 39.9 parts water MS-grade, and 0.1 parts FA) with the application of pressure after each addition of 50 µL (Figure 6-5). The elution step was collected in an AQUITY UPLC® 700 µL Round 96-Well plate and evaporated using a ThermoMixer® at 50 °C and 350 rpm for 45 minutes under N_2-gas. Finally, the residue was reconstituted in 50 µL injection solvent (39.9 parts

water MS-grade, 30 parts methanol MS-grade, 30 parts DMSO, and 0.1 parts FA) and vortexed for 5 minutes and 500 rpm at RT.

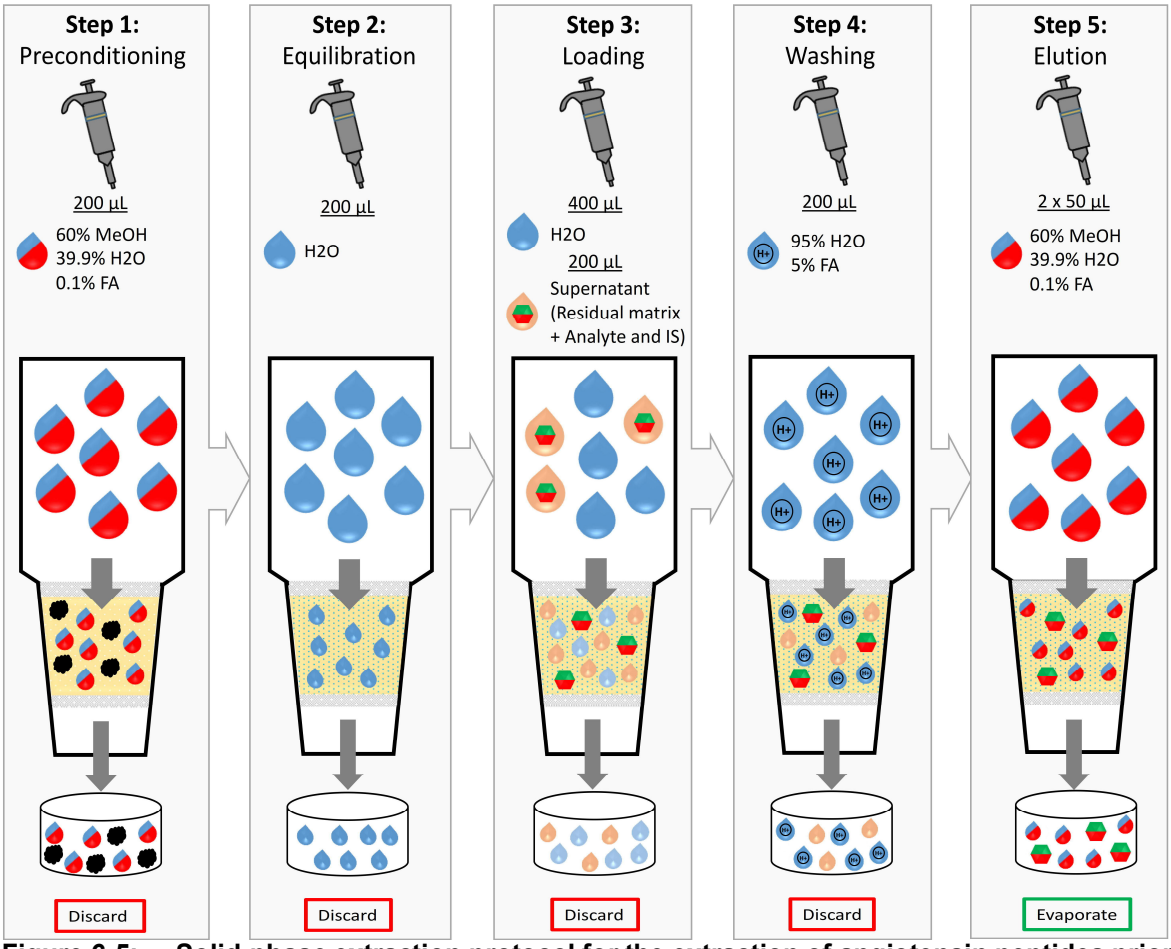


Figure 6-5: Solid-phase extraction protocol for the extraction of angiotensin peptides prior to liquid chromatography coupled to high-resolution mass spectrometry. A µElution 96-well plate with an HLB (hydrophilic-lipophilic balance) prime sorbents material (expressed as yellow area) by Waters® was used. IS: internal standard; MeOH: methanol: H2O: water; FA: formic acid; Matrix: supernatant of precipitated plasma; Black particles: potential impurities.

HPLC settings

The autosampler of the HPLC was cooled to 15 °C. The separation of the analytes was achieved with reversed-phase separation conducted with an XBridge C18 4.6 x 150 mm 2.5 µm column (Waters GmbH, Eschborn, Germany). Moreover, an XBridge BEH C18 XP VanGuard Cartridge 130 Å, 2.5 µm, 2.1 mm x 5 mm (Waters GmbH, Eschborn, Germany) was applied to protect the analytical column from larger impurities. The column was tempered to 60 °C, and the flow rate was set to 0.4 mL/min. The total run time of the method was 11 minutes, including 2.5 min equilibration time prior to injection of 20 µL of injection solvent. The mobile phase A consists of 98.9 parts water MS-grade, 1 part DMSO, and 0.1 parts FA, while the mobile phase B was 98.9 parts methanol MS-grade, 1 part DMSO,

and 0.1 parts FA. After injection, the mobile phase B concentration rose in several steps from 10% up to 85% in 3.7 minutes. Then, the high mobile phase B concentration stayed constant to provide consistent conditions for the ionisation of the analytes (Table 6-1).

Table 6-1: The applied gradient for the liquid chromatography

Time (minutes)	0	0.2	0.7	2.7	3.2	3.7	8.5
Mobile phase B concentration	10%	10%	30%	50%	70%	85%	85%

ESI and HRMS settings

The ESI was run in positive ionisation mode with an ion source temperature of 550 °C and the ion source gas 1 and 2 both at 50 psi. The curtain gas was set at 30 psi, and the ion spray voltage floating was 5500 V. All analytes were measured via the product ion scan in the high sensitivity mode with nitrogen as the collision gas. The DP (95 V), the ion release delay (48 ms) and the ion release width (17 ms) were equal for all analytes while the CE was individually set for each analyte (Table 6-2).

Table 6-2: The precursor ion and collision energy for all analytes and internal standards regarding the high-resolution mass spectrometry.

Substance	Molecule ion (m/z)	Collision energy (eV)
Angiotensin I	433.1	27
Angiotensin II	523.9	30
Angiotensin-(1-7)	450.5	27
Angiotensin III	466.5	27
Angiotensin IV	388.3	25
Angiotensin A	501.8	30
Alamandine	428.3	25
(Val^5)-Angiotensin I	428.6	25
([ring-D_5]Phe^8)-Angiotensin II	526.3	29

m/z: mass-to-charge ratio; eV:electron volt.

Statistical evaluation

The results of the conducted measurements were evaluated with the MultiQuant® Software. The MQ4 algorithm was used for the integration of the peaks with a Gaussian smooth width of 2 points, a RT half time window of 30 seconds, a minimal peak width of 3 points, a minimal peak height of 50 counts per second (cps), a baseline subtraction window of 0.2 min and a peak splitting of 3 points. The quantification of each analyte was achieved by summation of various fragments of each individual ion (Appendix 10.4-1). For the calibration curve, a quadratic regression with a weighting of $1/x^2$ was applied.

6.2.4 U.S. Food and Drug Administration based bioanalytical method validation

The method validation was performed according to the FDA bioanalytical guideline recommendations for chromatographic assays [169]. However, these recommendations notably differ from the recommendations for LBAs (see chapter 4.2.5). Therefore, the applied validation parameters for the developed LC-HRMS assay are exclusively described in this chapter. In this context, the linearity of the calibration curve, within-run and between-run accuracy and precision, the matrix effect and recovery, the sensitivity and the LLOQ, as well as the autosampler stability were evaluated. Moreover, a LC-HRMS SST was established.

System suitability test

An SST was conducted every working day prior to analysis. Injection solvent was spiked with the analyte and IS working solution (100 ng/mL) to obtain a final concentration of 500 pg/mL. This SST sample was measured in duplicate. The RT should not deviate by more than 0.2 minutes for each analyte. Moreover, the measured peak area should be within ±30% of the peak area obtained with the primary SST sample of the same batch.

Acceptance criteria of single analytical runs/ intra-run assessment

The acceptance criteria for a valid analytical run included CSs and QCs. The applied calibration curve for every angiotensin peptide consisted of seven non-zero CS levels measured in duplicate (Table 6-3). The back-calculated concentration of 75% of non-zero CSs including the LLOQ must not deviate by more than 20% and 15% for the calibrator at the LLOQ and the other calibrators, respectively. Exclusion of CSs was only acceptable if they failed the acceptance criteria or due to assignable causes.

Table 6-3: Nominal concentrations of the applied calibration standards for the liquid chromatography coupled to high-resolution mass spectrometry method.

	The nominal concentration of calibration standards (pg/mL)						
Analyte	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Angiotensin I	25.4	50.7	101.5	203.0	405.9	811.8	1623.6
Angiotensin II	22.3	44.5	89.1	178.1	356.3	712.5	1425.0
Angiotensin-(1-7)	24.3	48.5	97.0	194.0	388.0	776.0	1552.0
Angiotensin III	31.0	62.0	124.0	248.0	496.0	992.0	1984.0
Angiotensin IV	22.9	45.8	91.6	183.3	366.6	733.1	1466.2
Angiotensin A	21.8	43.7	87.4	174.8	349.5	699.0	1398.0
Alamandine	19.0	38.1	76.1	152.2	304.4	608.8	1217.6
Angiotensin-(1-9)	31.0	62.0	124.0	248.1	496.1	992.2	1984.4

Values are rounded to one decimal place.

Three QC levels (a QC within three times the LLOQ (QC low), a QC in the mid-range (QC middle), and a QC at the upper range of the calibration curve (QC high)) were measured in duplicate in every run to guarantee the validity. The QCs were always prepared in a calibration curve independent dilution series (Table 6-4). The total amount of QCs per run must be at least 5% of the unknown samples. Moreover, ≥67% of the QCs and at least 50% of all samples per QC level should not deviate by more than 15% of their nominal concentration.

Table 6-4: Nominal concentrations of the implemented quality controls for the liquid chromatography coupled to high-resolution mass spectrometry method.

	Nominal concentrations of the quality controls (pg/mL)		
Analyte	**Low**	**Middle**	**High**
Angiotensin I	50.7	203.0	811.8
Angiotensin II	44.5	178.1	712.1
Angiotensin-(1-7)	48.5	194.0	776.0
Angiotensin III	62.0	248.0	992.0
Angiotensin IV	45.8	183.3	733.1
Angiotensin A	43.7	174.8	699.0
Alamandine	38.1	152.2	608.8
Angiotensin-(1-9)	62.0	248.1	992.2

Values are rounded to one decimal place.

Calibration curve/ linearity

The calibration curve was evaluated in 6 independent runs on 6 different days. Each calibration curve must contain at least six CS levels marked with a maximal RE of ±15% (±20% at LLOQ). 75% of non-zero CSs, including the LLOQ, have to fulfil the criteria mentioned above. CSs that did not comply with this criterion were excluded, and the

calibration curve was re-constituted. Besides, a blank sample (matrix without standards and IS) and a zero sample (matrix with IS) were measured in duplicate.

Accuracy and precision

The accuracy and precision of the assay were investigated at four different concentration levels, including the QC low, QC middle, and QC high, as well as an additional QC at the LLOQ in quintuplicate. All QC samples were prepared independently from the CSs. Within-run accuracy and precision were investigated in one run, while between-run accuracy and precision were obtained in three individual runs on three different days. For the evaluation of within-run and between-run accuracy, the RE should not deviate by more than ±15% (±20 at LLOQ). The CV must be ≤15% (≤20% at LLOQ) for within-run and between-run precision.

Sensitivity

Five LLOQ samples were measured in quintuplicate in three different runs. The RE should not exceed ±20% of the nominal concentration, while the CV should be ≤20%. In addition, the analyte response at the LLOQ should be at least five times the analyte response of the zero calibrator of the same source.

Matrix effect and recovery

The FDA bioanalytical guideline currently lacks clear recommendations regarding the matrix effect for chromatographic assays. However, general requirements for the evaluation of the matrix effect for these kinds of assays are provided by the EMA bioanalytical guideline. Since the EMA has a comparable claim for the bioanalytical method validation, the recommendations from the EMA bioanalytical guideline seemed appropriate and were applied for the investigation of the matrix effect [170].

Matrix effects are defined as direct or indirect influences on the detector response caused by interacting substances within the sample. The matrix effect was evaluated in six individual human sources using a low and a high concentration level (Table 6-5). In this regard, the matrix factor (MF) was calculated as the ratio between the peak area of each analyte in the presence of the matrix (post-spiked-samples) to the peak area of each analyte in the absence of the matrix (solvent samples). In addition, the IS normalised MF (IS-MF) was calculated (MF of the analyte divided by the MF of the IS). The CV of the IS-MF from six sources should not exceed 15% to fulfil the EMA requirements. The spiked solution should not exceed 10% of the total volume to minimise matrix dilution.

Table 6-5: Applied concentration levels for the investigation of the matrix effect for the liquid chromatography coupled to high-resolution mass spectrometry method.

	Concentration level (pg/mL)	
Analytes	**Low**	**High**
Angiotensin I	50.7	1217.7
Angiotensin II	44.5	1068.8
Angiotensin-(1-7)	48.5	1164.0
Angiotensin III	62.0	1488.0
Angiotensin IV	45.8	1099.7
Angiotensin A	43.7	1048.5
Alamandine	38.1	913.2
Angiotensin-(1-9)	62.0	1217.7

Values are rounded to one decimal place.

The recovery of an analyte is enunciated as the complete transfer from the matrix in the solution, which will be measured and is defined as the ratio between the peak area in the presence of the matrix. The complete transfer stands for 100% recovery (post-spiked samples) to the peak area to samples which are spiked before extraction (pre-spiked samples). The recovery was calculated for each analyte and IS in one adult human source using three different concentration levels (QC low, QC middle, QC high). Since the evaluation of the recovery is not subject to any rejection limits of the FDA, it was only performed to evaluate the effectiveness of the extraction procedure.

Autosampler stability

Measurement of a sample batch via HPLC-HRMS can take several hours depending on the number of samples and injections. Moreover, it may be necessary to restart a lot due to equipment malfunction, which may also result in a longer autosampler storage time. The autosampler stability was determined to ensure that no degradation took place during this time. QC low, QC middle, and QC high samples in adult human matrix at four time points within 24 hours were determined in duplicate to verify the autosampler stability. The 24 hours present a regular study sample analysis. The mean RE at each level should not exceed ±15% of the nominal value to comply with FDA guideline recommendations.

6.2.5 Application of the validated method

Cardiac diseased patients

In order to demonstrate the applicability of the validated method in the context of the EU-funded LENA project, example measurements were conducted using three study samples from paediatric subjects suffering from HF. As mentioned in chapter 4.3.3, written informed

consent from the parent(s)/legal representatives and assent from the patient according to national legislation and as far as achievable from the child were obtained. Ethical guidelines such as the Declaration of Helsinki and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines for Good Clinical Practice and Good Manufacturing Practice were carefully followed. The blood sampling was conducted by trained study staff following a specific time-monitored sampling procedure adapted to paediatric needs (e.g. use of microneedles) and a parameter-adopted sampling procedure (e.g. limited time window between sampling, sample preparation and freezing, as well as the addition of the peptide cocktail to the sampling device prior to blood sampling) [104]. Cooling conditions were consistently applied during blood sampling and subsequent sample preparation (centrifugation at 2000 g for 10 min at 0-4 °C, subsequent snap freezing of supernatant, and storage at -80 °C until analysis) to guarantee analyte stability. In this context, please refer to the list of the LENA collaborators (Appendix 10.4-15) who performed the onward collection of paediatric study samples.

Healthy adults

In addition to the paediatric study samples, the plasma of six healthy adult volunteers was analysed (Table 6-6). To mimic the blood sampling and sample preparation procedure applied for the paediatric study sample in the LENA project, the blood sampling was conducted with 9 mL K3E S-Monovetten®, which are previously spiked with inhibitor cocktail. Moreover, the blood sampling and sample preparation were conducted according to the procedure applied for the paediatric study samples (e.g. in regard to the cooling, centrifugation, and storage conditions). The plasma of each source was measured in duplicate.

Table 6-6: Characteristics of the six investigated healthy adult volunteers for the determination of the matrix effect.

Source	Sex	Age (years)
1	Male	28
2	Male	29
3	Female	26
4	Male	26
5	Female	29
6	Male	27

6.3 Results

6.3.1 Optimisation steps

Optimisation of the blank plasma generation

The conducted sample processing ensured a reduction of baseline levels for AngI, AngII, and Ang1-9 in comparison to the native unprocessed matrix of the same source, which resulted in an improvement of the LLOQ for these peptides (Figure 6-6). Since the baselines of Ang1-7, AngIII, AngIV, AngA, and alamandine were already sufficiently low in native plasma, this sample processing had no additional effect on these peptides.

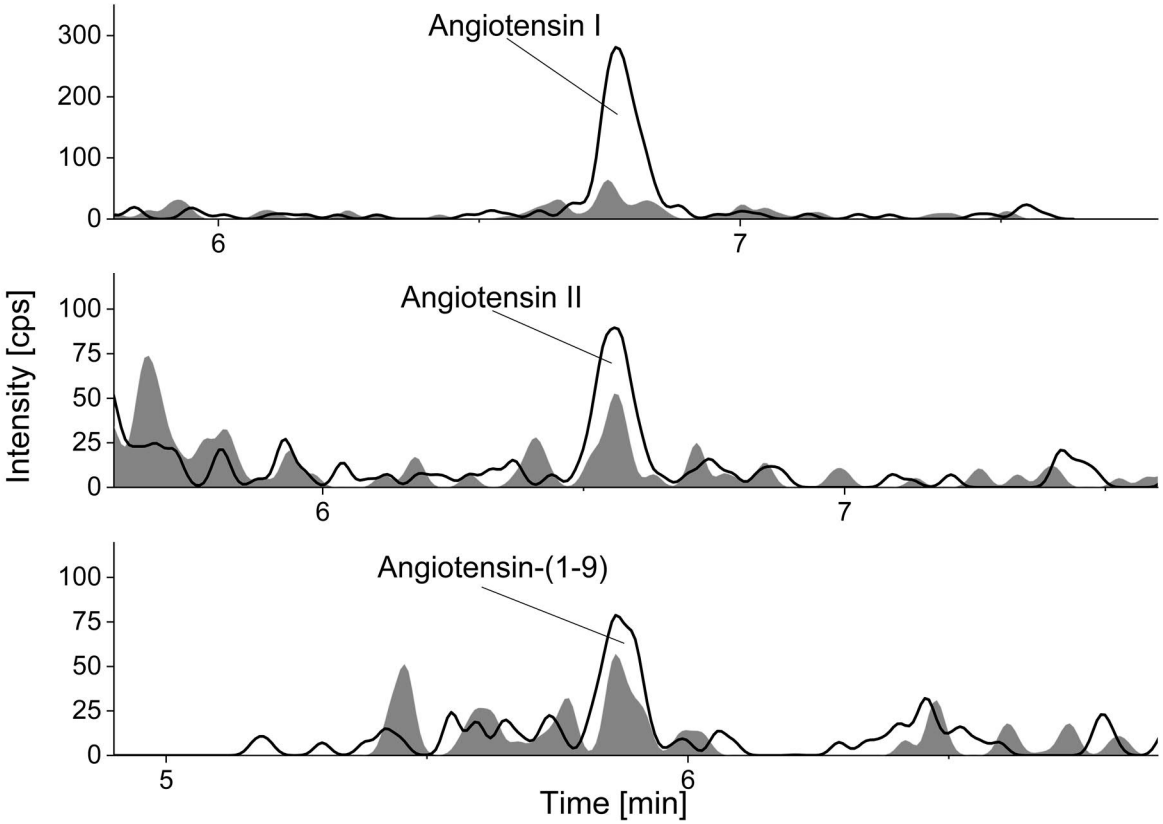


Figure 6-6: Example product ion scans of Angiotensin I, Angiotensin II, and Angiotensin-(1-9) of native and processed matrix of the same source. The black area represents the processed matrix (with spiked aliskiren and incubation step). The solid black line represents the native sample without spiked aliskiren and incubation step.

Optimisation of peptide purification and removal of interfering matrix

The SPE protocol was successfully modified to improve the removal of interfering matrix (Figure 6-7). In addition, the 96-well plate approach for the SPE enabled a high-throughput of study samples which resulted in a potential measurement of up to 74 study samples per plate. Due to the application of a Positive Pressure-96 Manifold for the SPE, the entire

process time (from the beginning of sample preparation until the submitting of the samples to the HPLC-HRMS) amounts to <4 hours and could be efficiently conducted during one regular working day. Besides, the utilisation of a µElution SPE notably reduced the required amount of chemical reagents and organic solvents.

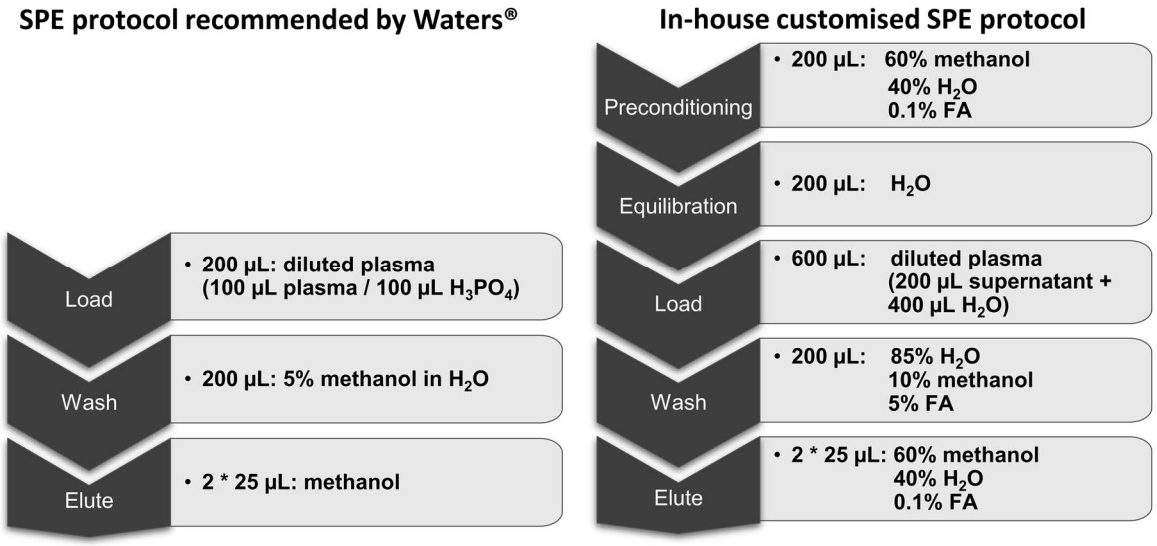


Figure 6-7: The in-house customised solid-phase extraction protocol compared to the standard protocol. The standard protocol was recommended by Waters® [200]. SPE: solid-phase extraction; FA: formic acid.

The applied precipitation step was performed with a precipitation solution tailored to the chemical properties of the analytes. The mix of 60 parts water, 40 parts methanol and 5 parts DCA enables acceptable extraction conditions for the analytes, as well as sufficient precipitation. Moreover, the supernatant could be directly loaded on the SPE while preventing breakthrough of the analytes due to the high amounts of water. By referring to the time-of-flight MS scans of a plasma sample with only SPE and a plasma sample of the same source with combined precipitation and SPE, it gets obvious, that this combination of extraction techniques effectively reduced matrix components (Figure 6-8).

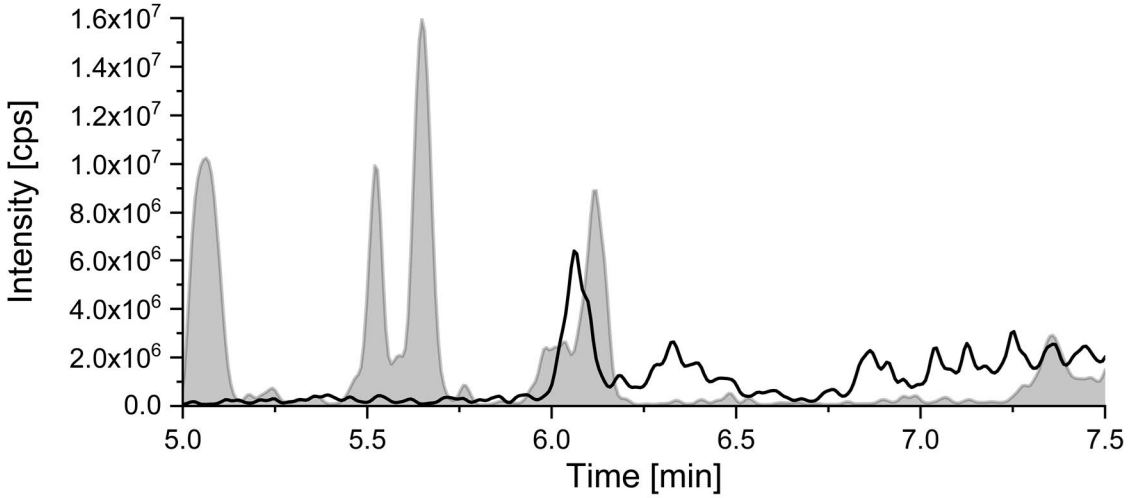


Figure 6-8: Example time-of-flight mass spectra with and without precipitation step prior to solid-phase extraction. The same human plasma matrix was purified with solid-phase extraction (SPE) only (grey filled area) and with an additional prior conducted precipitation step (solid black line).

Optimisation of sensitivity

The required sensitivity of the method was obtained by the avoidance of glassware, which would result in loss of analytes due to adsorption of the peptides. Moreover, the addition of 1% DMSO to the mobile phase significantly impacted the charge states of the peptides by shifting them to a defined number of charges per molecule. The modification of the mobile phase with DMSO enabled an up to threefold increase in signal intensity of the analytes (Figure 6-9). All in all, a LLOQ of 25.4 pg/mL for AngI, 22.3 pg/mL for Ang II, 24.3 pg/mL for Ang1-7, 31.0 pg/mL for AngIII, 22.9 pg/mL for AngIV, 21.8 pg/mL AngA, 19.0 pg/mL for alamandine, and 31.0 pg/mL for Ang1-9 could be successfully established.

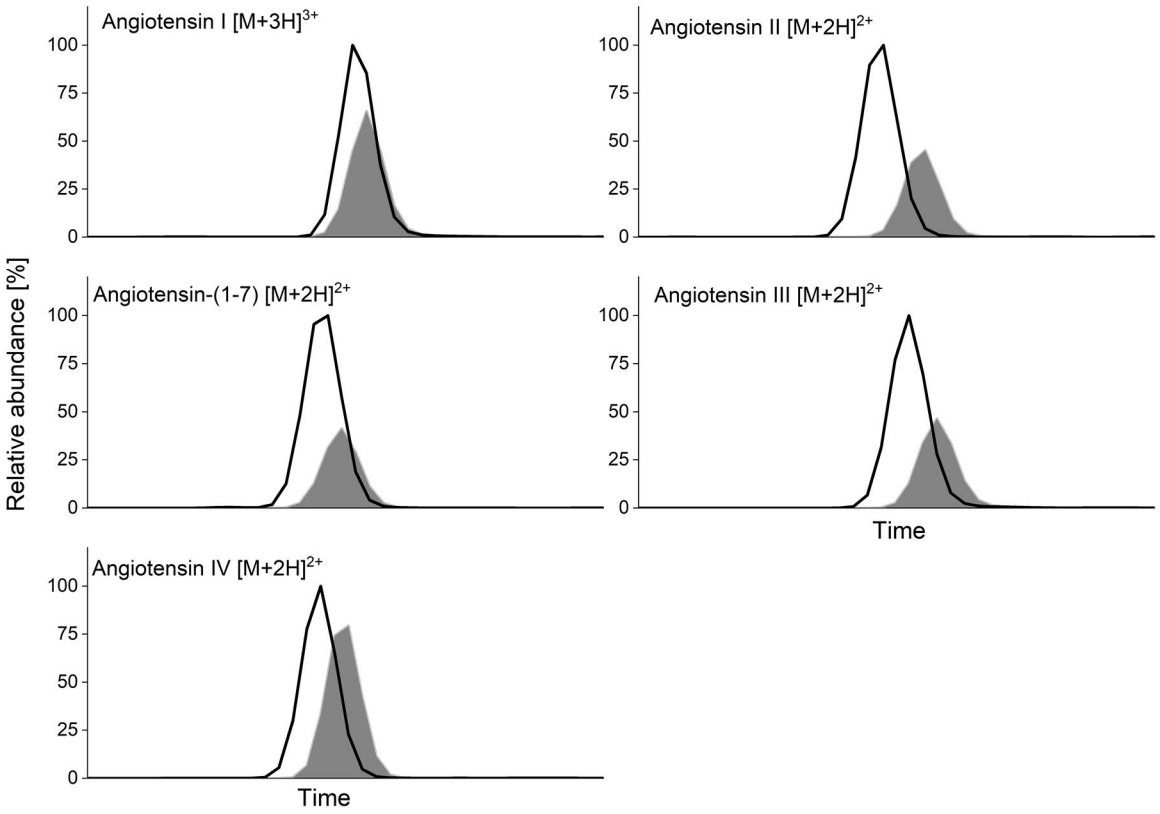


Figure 6-9: Schematic presentation of the influence on the sensitivity of angiotensin peptides by the addition of 1% dimethyl sulfoxide (DMSO) to the mobile phase. Equal analyte concentrations (1 ng/mL) were measured with the addition of 0.1% formic acid only (dark grey area) and with the addition of 0.1% formic acid +1% DMSO to the mobile phase (black solid line). Angiotensin A, alamandine, and angiotensin-(1-9) were included posterior to this experiment and are thus not shown.

6.3.2 U.S. Food and Drug Administration based bioanalytical method validation

System suitability test

The SST was successfully established and performed daily prior to every analytical run. The analytical run was not submitted until the SST showed acceptable results according to the predefined criteria. If an SST failed these criteria, appropriate steps were performed to recover the performance of the device (e.g. cleaning of the MS). An example chromatogram of the SST sample comprising the eight investigated analytes and the two ISs, as well as the applied HPLC gradient, is shown in Figure 6-10.

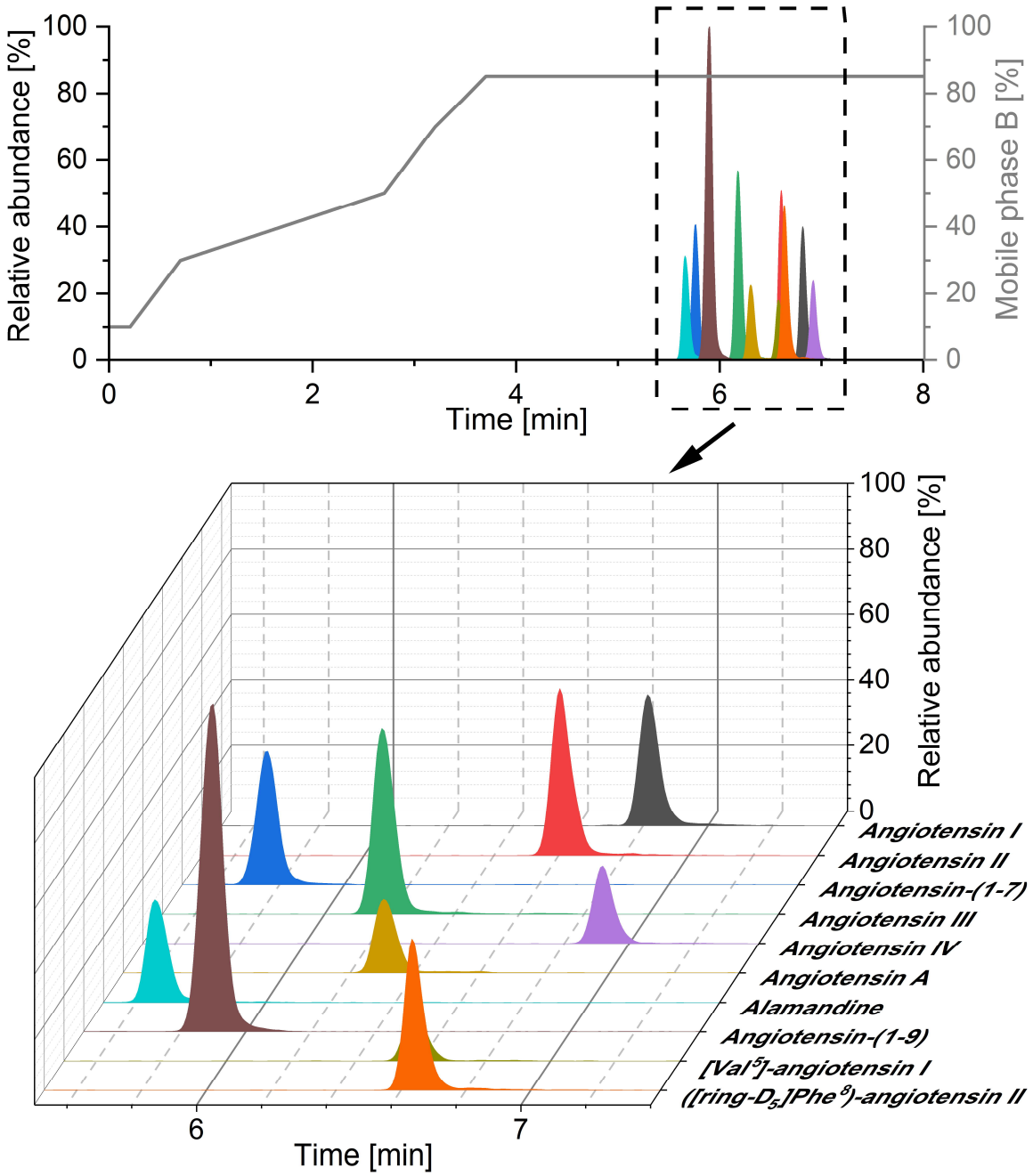


Figure 6-10: Example chromatogram of the system suitability test sample. The concentration of each analyte and internal standard of this measurement amounts to 500 pg/mL. The different peptides are indicated by the different colours of the peaks. The applied gradient is indicated by the grey line and the left y-axis of the upper chromatogram.

Calibration curve/ linearity

All six conducted evaluation runs fulfilled the criteria required by the FDA and thus could be included in the evaluation of linearity. In all six runs, the RE of the evaluated CSs was within the guideline limits of ±15% (±20%at LLOQ) of the nominal values (Figure 6-11). All obtained r-values were ≥0.99152 (Appendix 10.4-2). Implementation of a quadratic regression model with a weighting of $1/x^2$ provided the best fit for the calibration range of all

analytes. Summarising, the obtained results showed an adequate calibration curve for all 8 investigated analytes and confirmed the linearity of the developed method.

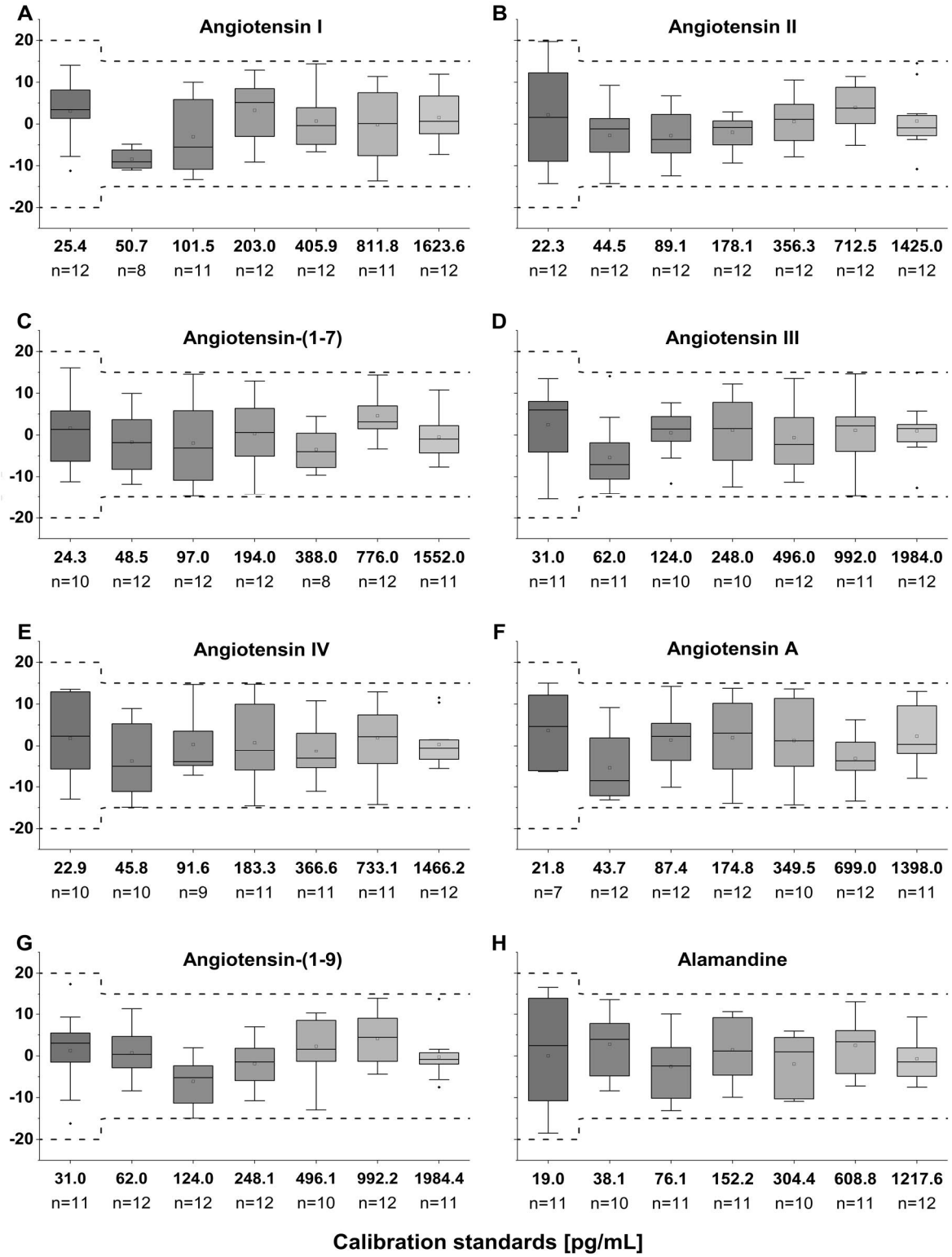


Figure 6-11: The relative error of all conducted calibration standards (CSs) for each analyte (A-H) expressed as boxplots. A duplicate measurement was accomplished for each of the 6 valid runs. The variable number of CSs was caused by the exclusion of single CSs in compliance with the requirements given by the U.S. Food and Drug Administration (FDA). Nominal values were rounded to one decimal place. The dashed lines indicate the acceptance limits according to the FDA. Box: 25-75 %; ⌶: 1.5 interquartile range; —: median; ▫: mean.

Accuracy and precision

In total, 3 runs were conducted for the assessment of between-run accuracy and precision. The within-run accuracy and precision were evaluated in one run.

Within-run accuracy over all four concentration levels showed a mean RE between -2.8% and +12.7% for AngI, -6.8% and 3.5% for AngII, -4.9% and 6.6% for Ang1-7, -1.1% and 7.7% for AngIII, -6.4% and 1.4% for AngIV, -3.8% and 7.3% for AngA, -3.6% and 3.3% for alamandine, and -2.5% and 5.2% for Ang1-9 of the nominal values (mean of five determinations). The RE for between-run accuracy was between -7.6% and 4.8% for AngI, -6.6% and 7.2% for AngII, -7.5% and 2.6% for Ang1-7, -12.8% and 3.1% for AngIII, -2.1% and 5.9% for AngIV, -3.2% and 3.1% for AngA, -3.1% and 1.1% for alamandine, and -4.5% and 0.9% for Ang1-9 (Figure 6-12).

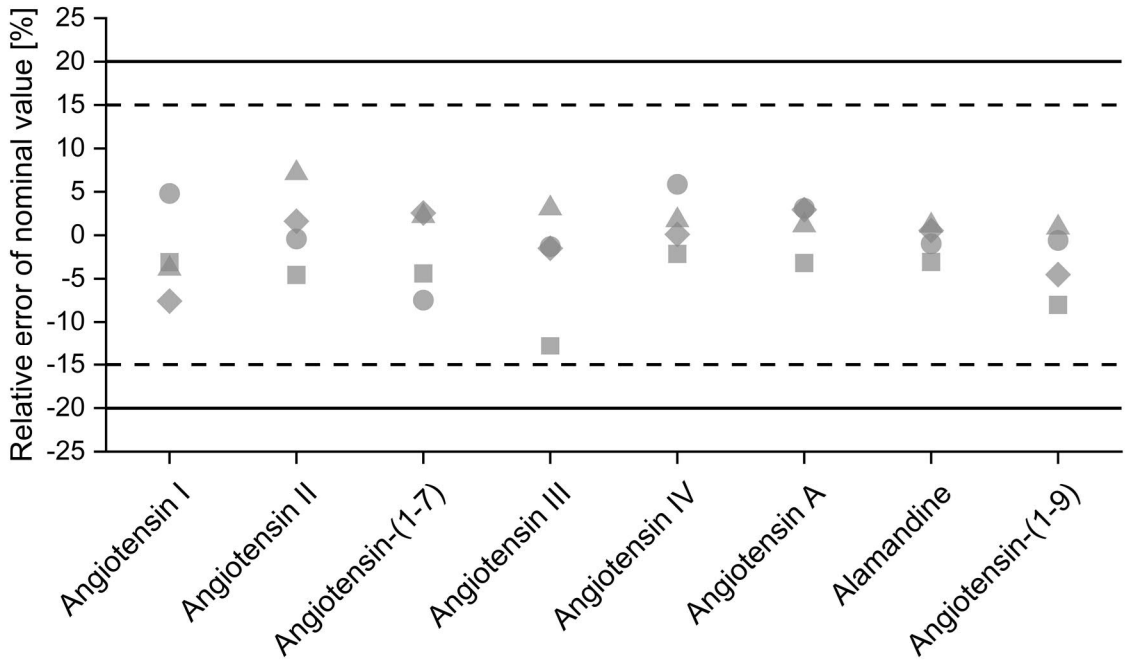


Figure 6-12: Results of between-run accuracy for the three conducted runs. The solid lines indicate the acceptance limits according to the U.S. Food and Drug Administration (FDA) at Lower Limit of Quantification (LLOQ). The dashed lines indicate the acceptance limits according to the FDA for the other quality controls (QCs). Box: QC at the LLOQ level; Circle: QC at the low concentration level; Rhombus: QC at the middle concentration level; Triangle: QC at the high concentration level.

The within-run precision over all four concentration levels showed a CV between 6.9% and 13.6% for AngI, 3.5% and 9.7% for AngII, 7.8% and 9.7% for Ang1-7, 4.3% and 7.4% for AngIII, 5.5% and 15.2% for AngIV, 5.8% and 10.7% for AngA, 5.6% and 11.0% for alamandine, and 2.7% and 6.0% for Ang1-9. The between-run precision (CV) varied between 5.9% and 9.5% for AngI, 4.5% and 8.5% for AngII, 8.7% and 10.8% for Ang1-7,

7.1% and 12.8% for AngIII, 6.8% and 12.9% for AngIV, 10.4% and 12.4% for AngA, 9.0% and 10.3% for alamandine, and 5.2% and 5.9% for Ang1-9 (Figure 6-13).

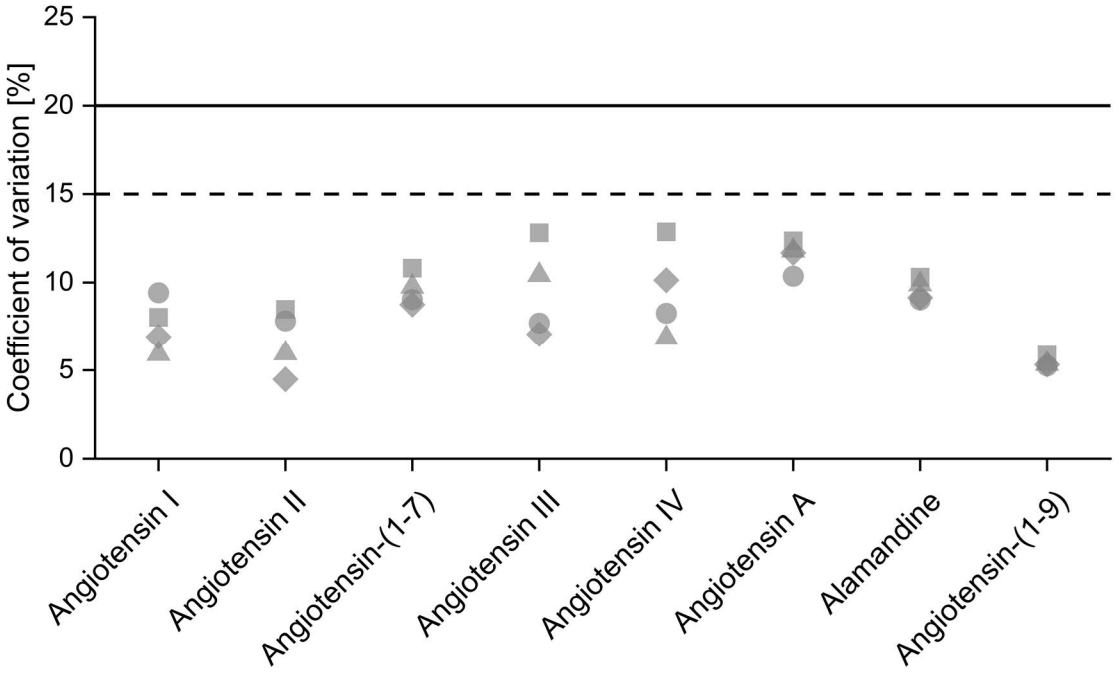


Figure 6-13: Results of between-run precision for the three conducted runs. The solid lines indicate the acceptance limits according to the U.S. Food and Drug Administration (FDA) at Lower Limit of Quantification (LLOQ). The dashed lines indicate the acceptance limits according to the FDA for the other quality controls (QCs). Box: QC at the LLOQ level; Circle: QC at the low concentration level; Rhombus: QC at the middle concentration level; Triangle: QC at the high concentration level.

In summary, the developed and optimised HPLC-HRMS assay showed an appropriate accuracy and precision in the context of the applied FDA bioanalytical guideline.

Sensitivity

The sensitivity of the method was investigated in three different runs by a fivefold determination of the LLOQ. The maximal RE of the LLOQ over all 3 runs was 4.1% for AngI, 11.3% for AngII, 5.7% for Ang1-7, 18.5% for AngIII, 7.8% for AngIV, 9.3% for AngA, 5.5% for alamandine, and 12.0% for Ang1-9. The maximal CV of the LLOQ over all 3 runs was 9.1% for AngI, 9.7% for AngII, 15.3% for Ang1-7, 19.0% for AngIII, 15.2% for AngIV, 15.1% for AngA, 11.8% for alamandine, and 6.1% for Ang1-9. The RE of the LLOQ samples for all analytes was below ±20%, while the CV was ≤20% and thus fulfilled guideline criteria. Moreover, the analyte response at the LLOQ was ≥5 times the response at the zero calibrator. The only exception was AngI with an analyte response of ≥5 times the response in the second run and responses of ~3 times in run one and three. However, despite the low injection volume of 20 µL and the overall applied sample volume of 50 µL per sample,

the determination at the LLOQ showed sufficient accuracy and precision. Representative chromatograms for the LLOQ and the zero sample are shown in Figure 6-14.

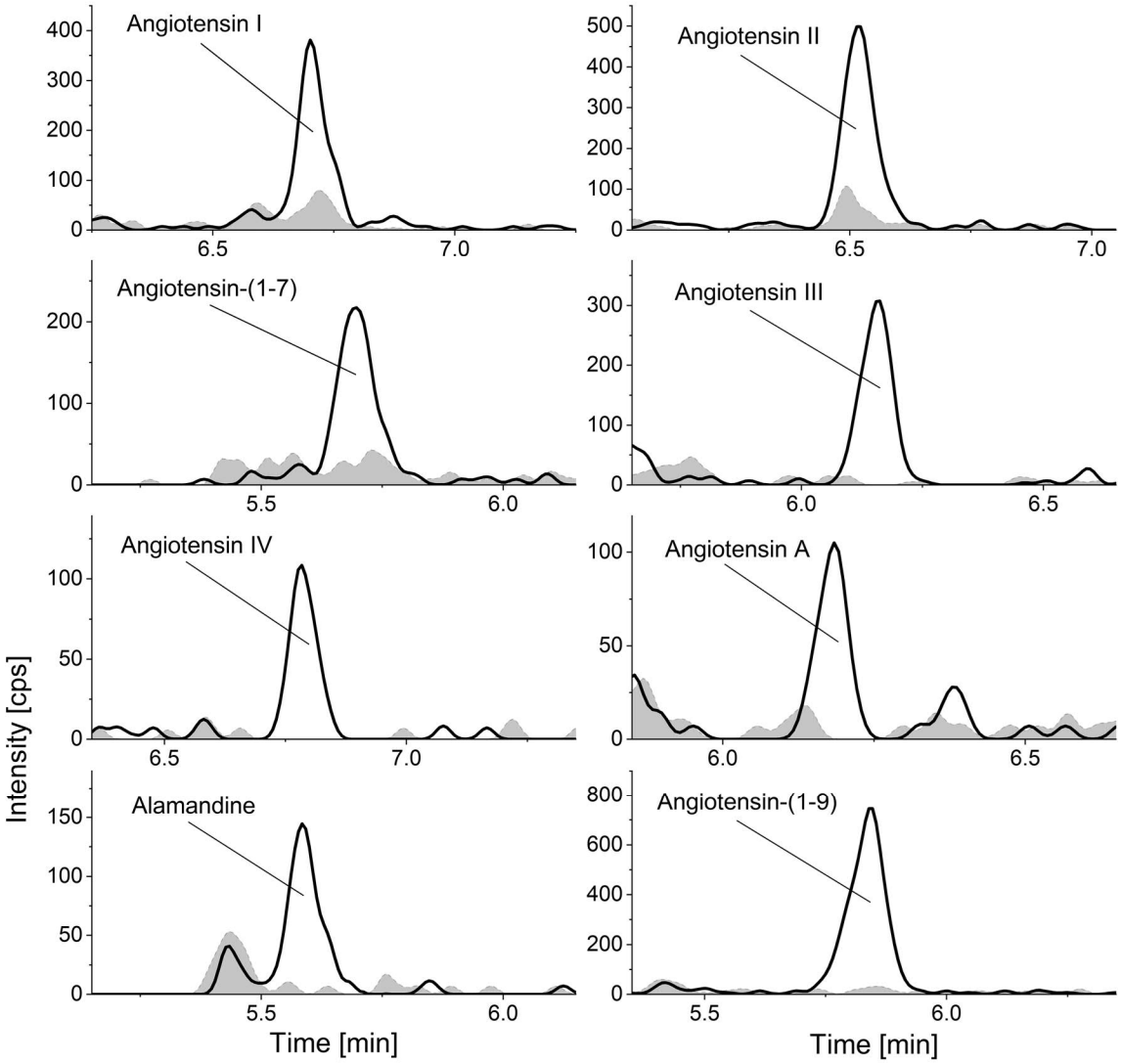


Figure 6-14: Example presentation of analyte signals at the Lower Level of Quantification (LLOQ) and the zero sample in human plasma. Light grey area: LLOQ sample; Dark grey area: zero sample.

Matrix effect and recovery

The matrix effect was successfully investigated in six human sources. The unprocessed native plasma of every source was used for the investigation of the matrix effect. Therefore, a blank reduction was applied for every analyte. The calculated MF over both concentrations and all six sources ranged between 0.76 and 0.96 for AngI, 0.74 and 0.87 for AngII, 0.8 and 0.97 for Ang1-7, 0.56 and 0.75 for AngIII, 0.7 and 0.84 for AngIV, 0.37 and 0.46 for AngA, 0.74 and 0.87 for alamandine, and 0.87 and 1.05 for Ang1-9. For the ISs, the MF varied between 0.8 and 0.96 for [Val_5]-Angiotensin I and 0.65 and 0.86 for ([ring-D_5]Phe^8)-Angiotensin II.

After the normalisation of the MF of the analyte with the MF of the corresponding IS, the IS-MF ranged between 0.9 and 1.12 for AngI, 0.9 and 1.16 for AngII, 1.10 and 1.24 for Ang1-7, 0.83 and 1.06 for AngIII, 0.92 and 1.09 for AngIV, 0.53 and 0.7 for AngA, -1.06 and 1.23 for alamandine, and 1.26 and 1.31 for Ang1-9 (Figure 6-15).

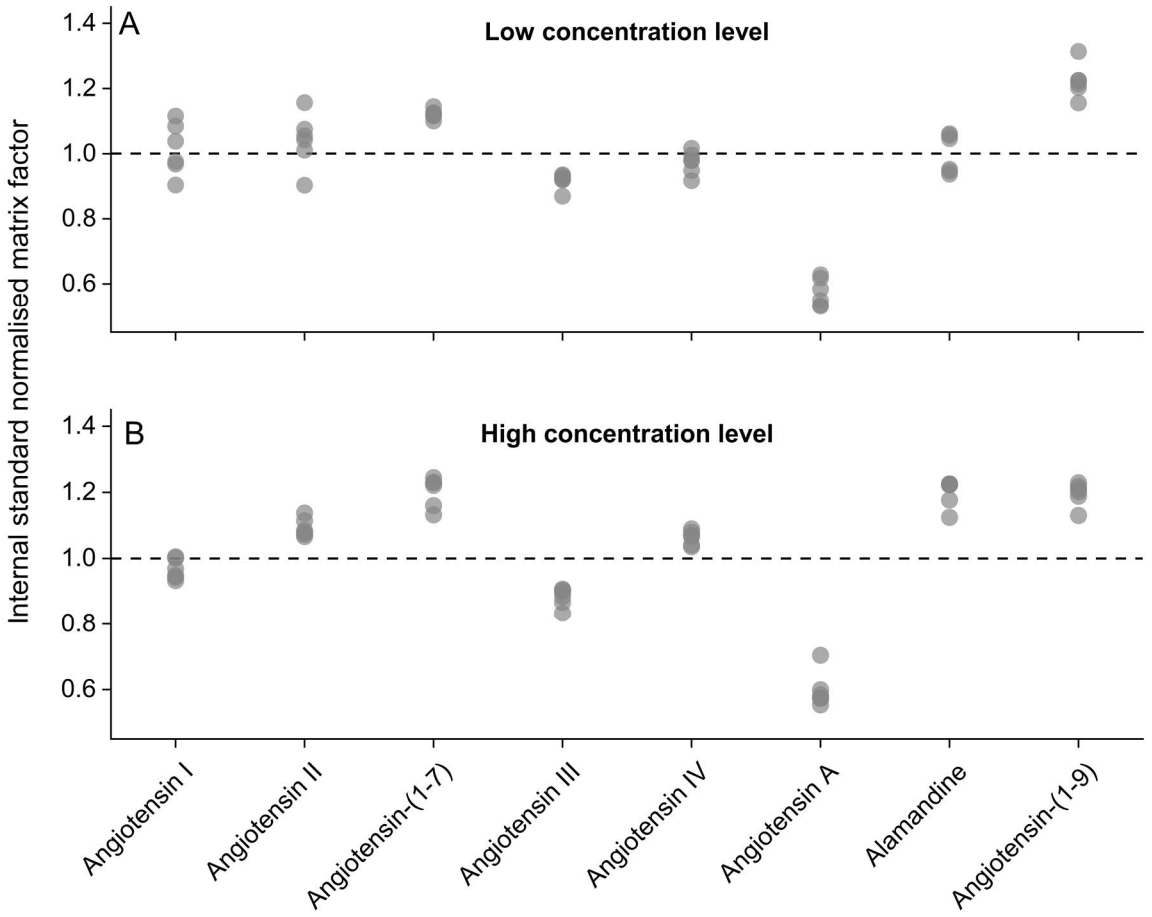


Figure 6-15: Results of the evaluation for the internal standard normalised matrix factor (IS-MF) of six different human sources. The dashed line demonstrates a calculated IS-MF of 1, which is characterised by a complete compensation of the matrix effects from the IS and analyte signal. The individual sources are indicated by the circles (n=6). A: The calculated IS-MF of the low concentration sample. B: The calculated IS-MF of the high concentration sample.

The inter-source variability (CV) of the low concentration and the high concentration between all six investigated sources was 7.8% and 3.2% for AngI, 8.0% and 2.5% for AngII, 1.3% 3.8% for Ang1-7, 2.6% and 3.2% for AngIII, 3.7% and 2.0% for AngIV, 7.5% and 9.0% for AngA, 6.0% and 3.5% for alamandine, and 4.2% and 3.0% for Ang1-9, respectively. Since all investigated sources were well within the specifications of an inter-source variability of ≤15%, no substantial influence of the different matrices on the assay was ascertained.

The recovery of each analyte was investigated by comparing the peak areas of post-spiked samples with the peak areas of pre-spiked samples of three concentration levels measured in triplicate (Figure 6-16). Except for AngI and [Val^5]-Angiotensin I, all samples showed a uniform recovery over the three investigated concentration levels. Yet, the recovery of AngI showed a nearly doubled value at QC low while the corresponding [Val^5]-Angiotensin I IS value almost halved. Nevertheless, the other QCs behaved normally. Moreover, all three investigated samples of the QC low showed comparable values. Thus, the atypical behaviour of AngI and [Val^5]-Angiotensin I was awarded to a processing error during the preparation of the QC low. Due to the sufficient signal and the accurate and precise determination at LLOQ, the recovery was regarded as sufficient for all analytes.

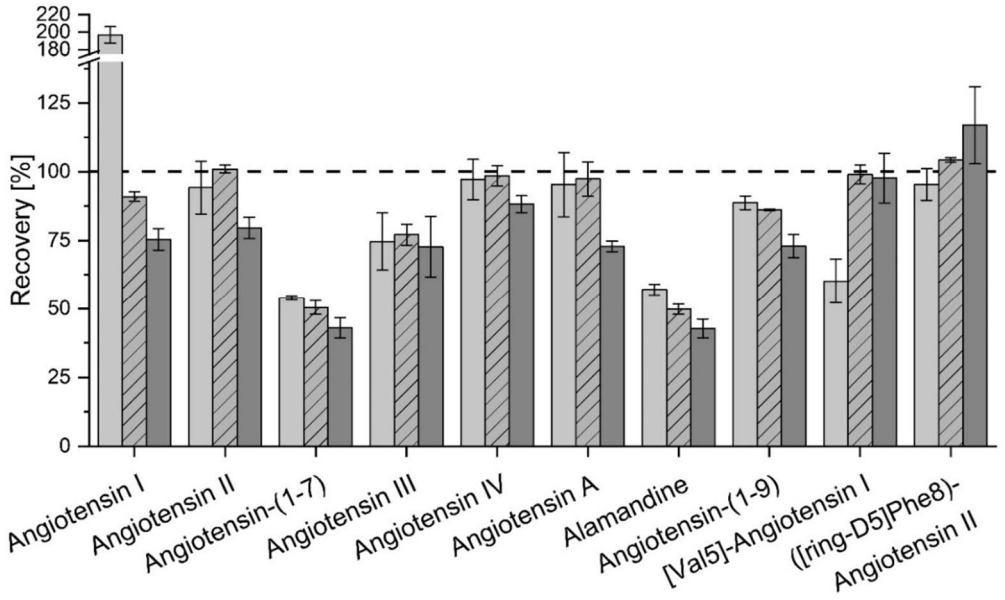


Figure 6-16: The percentage recovery of all analytes and internal standards. Three quality controls (QCs) were spiked with the analytes and internal standards before (pre-spiked) and after (post-spiked) extraction. The percentage of recovery was determined by comparing pre-spiked samples and post-spiked samples (n=3). Error bars: standard error of the mean; QC low: light grey filled bars; QC middle: striped bars; QC high: dark grey bars.

Autosampler stability

The stability of the samples for all three concentration levels in the autosampler was proven for 24.2 h run time (Figure 6-17). Each concentration level was measured on four time points (~3.2, ~10.2, ~17.2, and ~24.2 h) in duplicate after placing the samples in the autosampler. The mean RE of every investigated concentration level was below the rejection limit of ±15% for all 8 analytes.

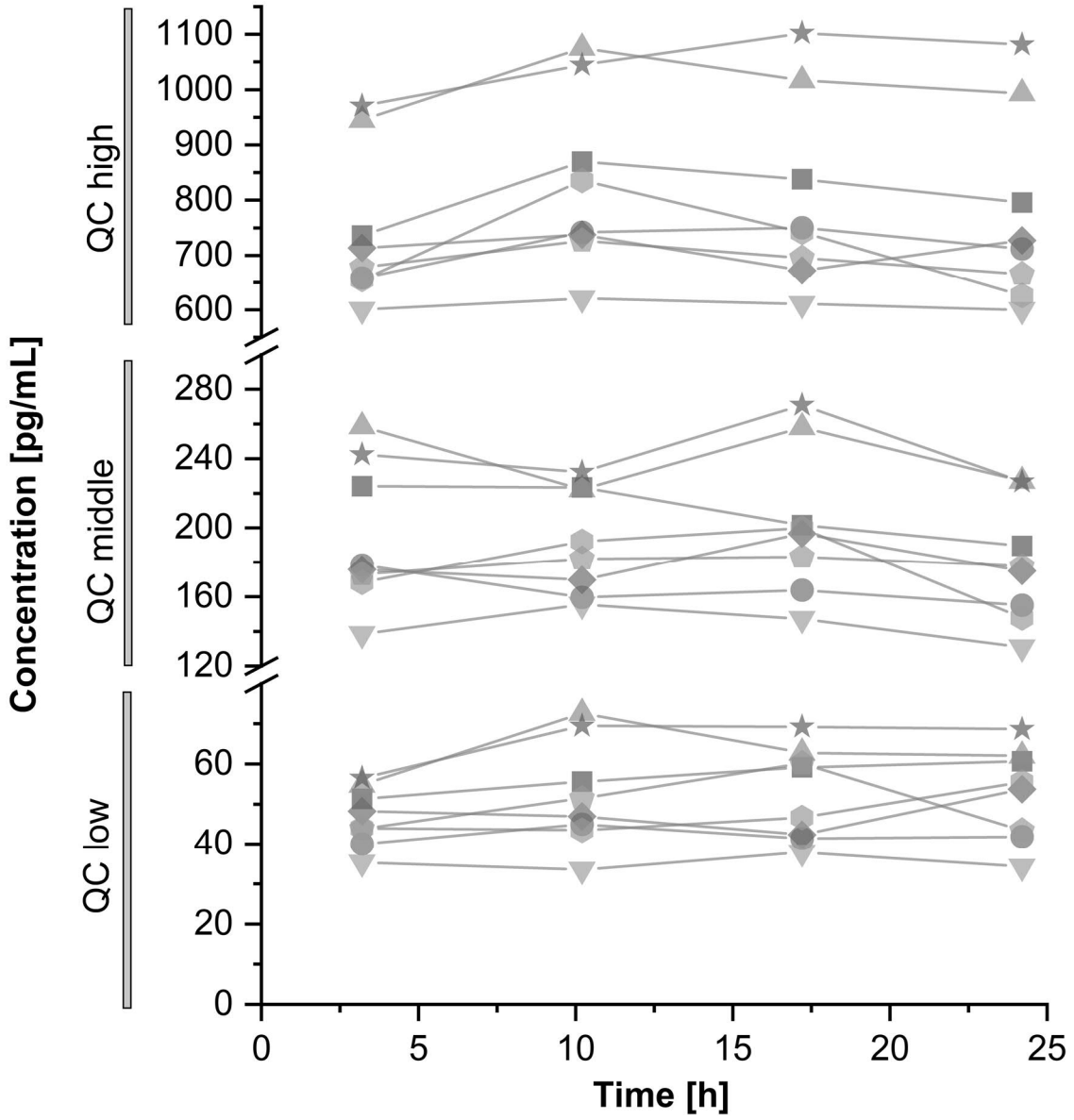


Figure 6-17: Results of the autosampler stability expressed as mean measured values over 24.2 hours. For each time point, the concentrations of the analytes were measured in duplicate. Box: angiotensin I; Circle: angiotensin II; Rhombus: angiotensin-(1-7); Triangle up: angiotensin III; Hexagon: angiotensin IV; Pentagon: angiotensin A; Triangle down: alamandine; Star: angiotensin-(1-9).

6.3.3 Application of the method

Cardiac diseased paediatrics

To demonstrate the suitability of the developed method in the context of a paediatric application, three randomly selected study samples from the LENA collective were analysed. All processes relevant for an accurate determination of the humoral parameters (e.g. blood sampling, sample shipment, storage, and subsequent analysis) were conducted under GCLP conditions. All conducted runs were declared as valid with regard to the FDA acceptance criteria of an analytical run (6.2.4). The measured analyte concentrations are shown in Table 6-7. Example chromatograms for the three different paediatric study samples are provided in Figure 6-18.

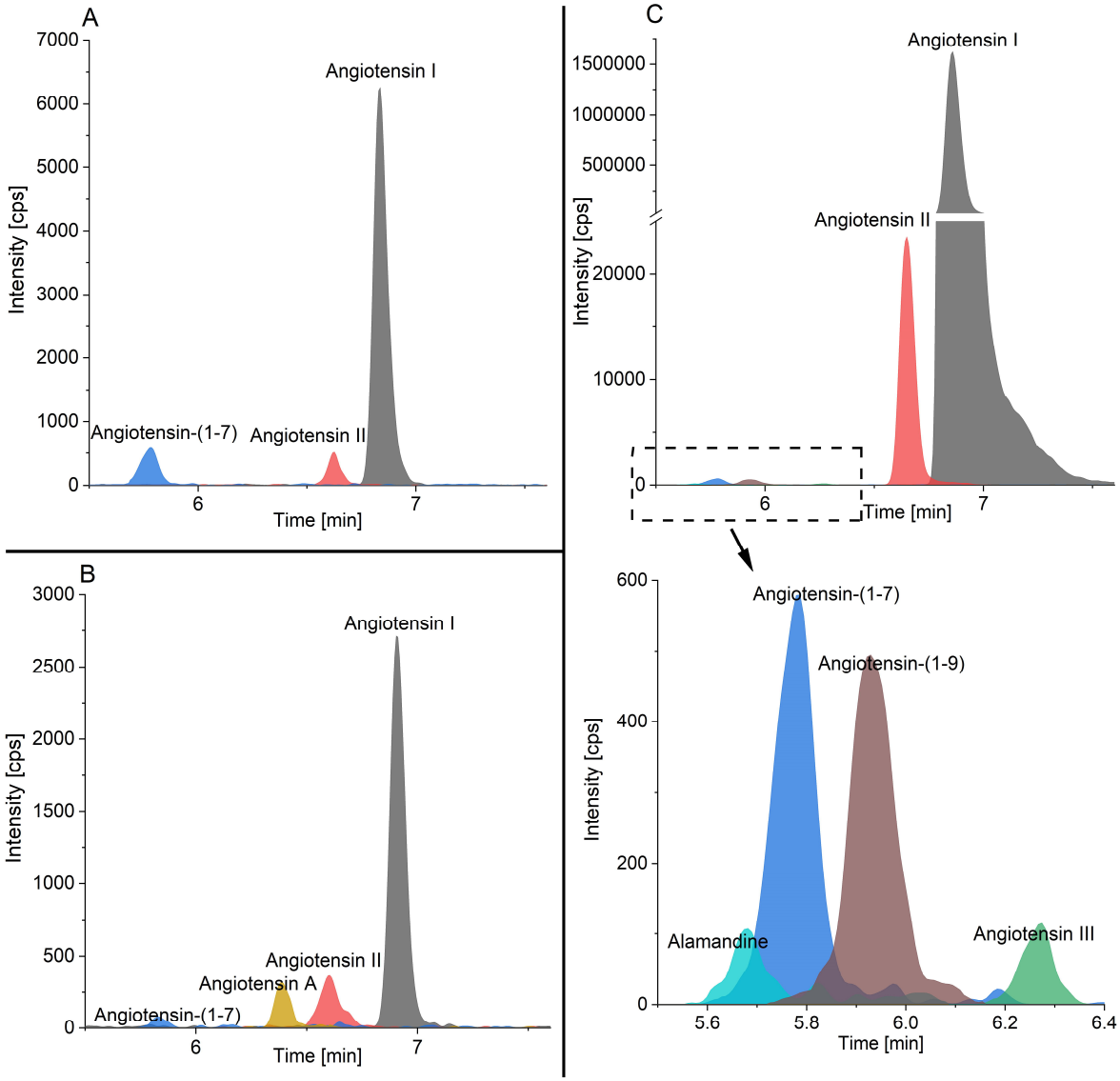


Figure 6-18: Example chromatograms of three children (A, B, and C) suffering from heart failure. The different detectable signals of the analytes are indicated by the different colours and the tagged names of the analytes. The chromatograms of analytes with no detectable signal are not shown.

Healthy adults

The plasma samples of two female and four male healthy adult volunteers were successfully analysed to demonstrate the applicability of the assay using a real-life approach. All sources demonstrated a quantifiable or detectable amount of AngI. AngII was detectable in three sources, while Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9 concentrations were not detectable. The measured analyte concentrations are shown in Table 6-7. Example chromatograms for the six investigated adult sources are provided in Figure 6-19.

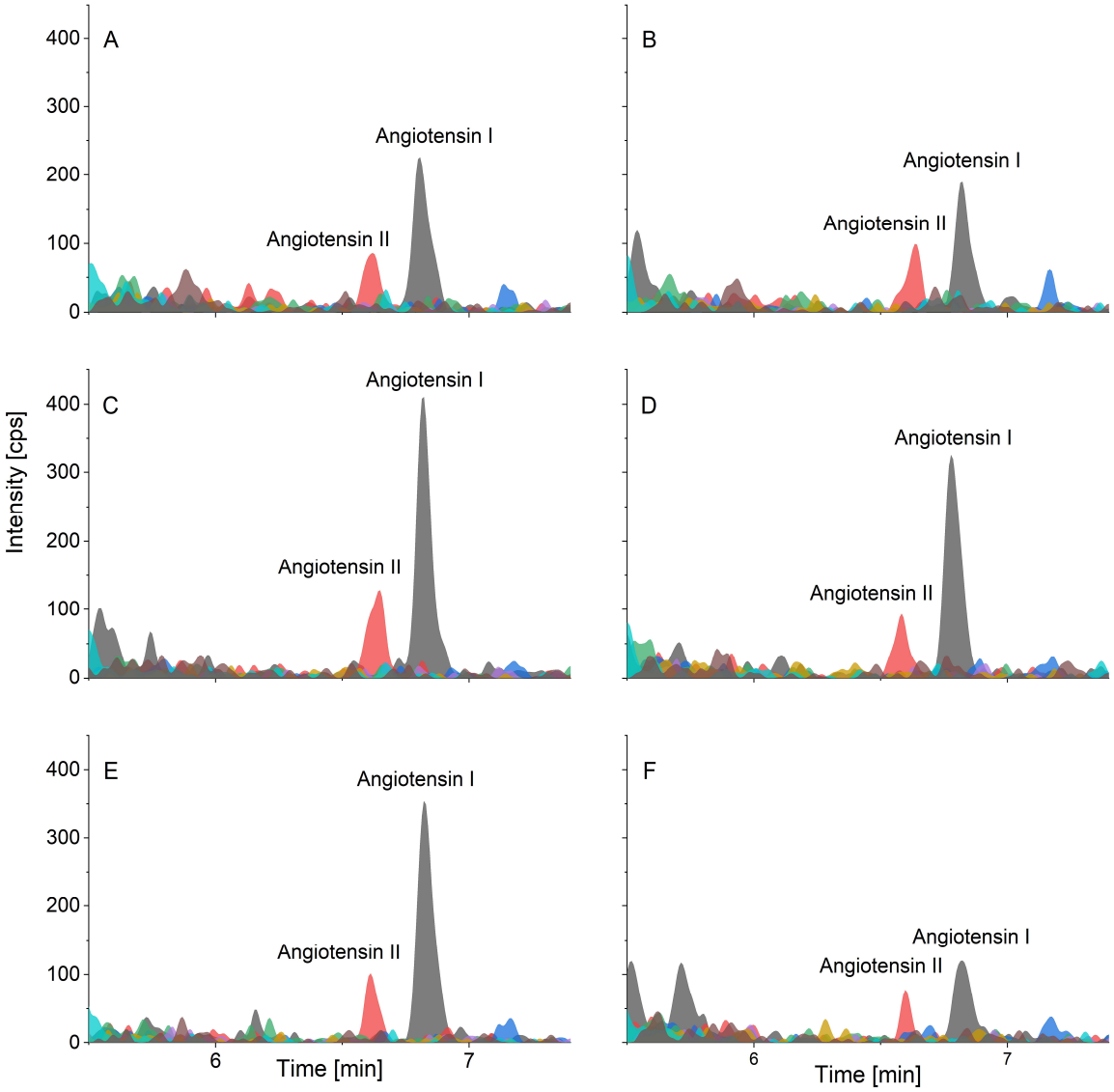


Figure 6-19: Example chromatograms of six healthy adult volunteers. No detectable concentrations were found for angiotensin-(1-7), angiotensin III, angiotensin IV, angiotensin A, alamandine, and angiotensin-(1-9). A: 28 years old male; B: 29 years old male; C: 26 years old female; D: 26 years old male; E: 29 years old female; F: 27 years old male.

Comparison of healthy adults and cardiac diseased paediatrics

Notably, different plasma concentrations were obtained for the healthy adult population in comparison with the cardiac diseased paediatric population (Table 6-7). To demonstrate that important information will be delineated by analysing the samples of the paediatric population, a few chromatograms of paediatric plasma samples are shown from the LENA project. Due to the preliminary status of the data analysis, information about age, gender, medication, disease are not given.

Table 6-7: Measured plasma concentrations of eight angiotensin peptides in healthy adults and paediatrics suffering from heart failure.

Source	AngI	AngII	Ang1-7	AngIII	AngIV	AngA	Ala	Ang1-9
Plasma concentrations (pg/mL) in healthy adults								
1	27.64	nd	nd	nd	nd	nd	nd	nd
2	22.05*	nd	nd	nd	nd	nd	nd	nd
3	40.82	2.71*	nd	nd	nd	nd	nd	nd
4	36.91	0.71*	nd	nd	nd	nd	nd	nd
5	42.62	0.50*	nd	nd	nd	nd	nd	nd
6	12.04*	nd	nd	nd	nd	nd	nd	nd
Plasma concentrations (pg/mL) in cardiac diseased paediatrics								
7	287338.25*	1998.87*	109.81	18.28*	nd	nd	25.	40.21
8	1230.57	62.26	37.14	nd	nd	nd	nd	nd
9	858.50	89.87	41.74	nd	nd	611.42	nd	nd

*The expressed value represents the mean of a duplicate measurement. Source 1: 28 years old male; Source 2: 29 years old male; Source 3: 26 years old female; Source 4: 26 years old male; Source 5: 29 years old female; Source 6: 27 years old male. Source 7-9: cardiac diseased paediatrics; *: extrapolated values; nd: not detectable.*

In summary, the developed low volume HPLC-HRMS method demonstrated the feasibility of measuring several angiotensin peptides within a cardiac diseased paediatric collective. Moreover, the FDA compliant validation enabled the assay for the assessment of high-quality data under GCLP conditions. Therefore, the sophisticated multiplex evaluation of eight important peptides of the RAAS within the LENA collective by application of this bioanalytical method was proven.

6.4 Discussion

The developed LC-HRMS method enabled the reliable determination of AngI, AngII, Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9 in a child-appropriate volume of 50 µL plasma. Moreover, the FDA compliant validation facilitated the applicability within clinical trials like the LENA project. The method thus contributes to a reliable quantification of these humoral parameters within the RAAS of children and neonates. The thereby achieved knowledge about the broad spectrum of angiotensin peptides could provide further valuable insights into the maturing organism.

In paediatric research, withdrawable blood volumes were limited to 3% of the total blood volume during a period of four weeks or 1% at a single blood sampling [90]. For an estimated blood volume of 80-90 mL/kg, the total withdrawable blood volume thus amounts to 0.8 mL/kg at a single time point. Since bioanalytical assays often rely on serum or plasma analysis, the available matrix is further reduced. This volume may be sufficient for the analysis of single parameters with conventional assays. However, the simultaneous investigations of PK, PD and safety parameters in clinical routine (as conducted in the LENA project) are common in paediatric clinical trials. This requires bioanalytical assays which can cope with low sample volumes. Schulz et al. reported a method for the quantification of AngII with SPE and LC-MS, which utilised 500 µL plasma [201]. The same amount of plasma was applied for the quantification of AngI by Fredline et al. using LC-MS [202]. Even methods with downscaled sample volumes for the determination of AngI required at least 150 µL sample matrix [203]. These volumes are not feasible for the bioanalysis in LENA, as an evaluation of several PK/PD and safety parameters in children was necessary. In contrast, the here presented method requires a total amount of 50 µL plasma volume for the reliable determination of all analytes without infringing the ethical considerations and therefore demonstrates applicability in paediatric clinical trials.

The simultaneous measurement of various angiotensin peptides represents a vital technique to evaluate the status and activation of the RAAS in an overall context. An LC-MS method comprising the determination of AngI, AngII, Ang1-7, AngII, AngIV, and Ang1-9 in plasma from adults with HF was previously demonstrated [16]. While the LLOQ of the method for the different peptides ranged from 1.5 pg/mL to 4 pg/mL, the amount of utilised plasma to achieve these limits was not reported. Another LC-MS method for the determination of AngI, AngII, AngIII, AngIV, and Ang1-7 in 50 µL mice plasma with an LLOQ of 5 pg/mL was shown by Olkowicz et al. [204]. However, to the author's knowledge, no method exists for the additional investigation of the novel peptides AngA and alamandine

in human plasma. Besides, the establishment of an extremely low LLOQ seemed not appropriate for the investigations within the LENA collective. Instead, a wide calibration range was established to cover a broad bandwidth of possible peptide concentrations and thus enable comprehensive insights in the maturing organism. This is particularly of high importance since it permits the correlation between all these peptides and might generate relevant knowledge regarding the cardiac diseased LENA collective. Moreover, this method comprised an FDA compliant validation which empowered the method for the usage in a GCLP environment. Furthermore, the performed measurements of adult and paediatric samples substantiated the applicability of the low-volume method for the matrix of adults and children.

The observed matrix effect on the investigated peptides has proven to be a crucial factor for the quantification of the analytes within this research. The ion enhancement or ion suppression may be caused by co-eluting matrix components which could influence the ionisation efficiency of the analytes and significantly impact the accuracy and precision of the method [205]. Dams et al. reported suppression of signal intensity of up to -50% on small molecules due to the matrix effects of plasma by the usage of LC-TQMS with ESI and beforehand conducted purification step with SPE [206]. In addition, the same group detected a reduction in signal intensity of up to -75% after protein precipitation under the same conditions. However, the impact of matrix components on endogenous peptides is still poorly understood. In this regard, Ahn et al. proposed a 70%-rule for the quantification of endogenous peptides in a complex matrix [207]. This rule divides the suppression of the analyte signal by the matrix in normal (<70% ion suppression) and abnormal (>70% ion suppression). The abnormal ion suppression prevents a reliable quantification of the peptides, while the normal ion suppression still provides adequate performance of the method. The in this thesis developed LC-HRMS method combined a precipitation step with a subsequent performed SPE to provide sufficient analyte extraction and sample purification. This technique resulted in a detected MF between 0.37 and 1.05 for the eight peptides, which is equivalent to a matrix effect of -63% to +5%. Thus, these results comply with the 70%-rule. In fact, the validation demonstrated a low inter-source variability of ≤9% for the IS-MF as well as an accurate and precise determination of all peptides in plasma.

The conducted measurements of healthy adult volunteers (26-29 years) showed mean AngI levels of 30.35 pg/mL (n=6), mean AngII levels of 1.3 pg/mL (n=3, values were extrapolated), and no detectable concentrations of Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9. These results comply with the findings of Basu et al. in 36 healthy adults (46-58 years) with comparable mean AngI and AngII levels of 6 pg/mL and

5.1 pg/mL, respectively, as well as Ang1-7, AngIII, AngIV, and Ang1-9 levels <3 pg/mL [16]. In contrast, the measured peptide concentrations of the three paediatric patients indicated considerable divergent conditions of the RAAS in children suffering from HF. As a consequence, these investigations are of high priority to enable meaningful insights into feedback mechanisms and functionalities of the paediatric RAAS as well as to achieve further evidence regarding the prevalence and development of paediatric HF. Moreover, potential new target structures for the pharmacotherapy of HF might be derived from these findings.

6.5 Conclusion

The developed low-volume HPLC-HRMS method was able to accurately and precisely quantify AngI, AngII, Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9 in 50 µL human plasma. The conducted FDA compliant validation enabled the highly sensitive method to be applied under GCLP in clinical studies and thus substantially contributes to the sophisticated investigations of the RAAS in the paediatric population of the LENA project.

7 Overall discussion and perspective

This thesis facilitated the decisive evaluation of several humoral parameters of the maturing RAAS within a unique paediatric population. In this regard, the thorough compilation and interpretation of reference values in literature, the development and validation of a customised low-volume PRA immunoassay, as well as the subsequent establishment of a fit-for-purpose QCS for the reliable analysis of PRA in paediatric clinical trials were conducted. Moreover, an innovative highly sensitive low-volume HPLC-HRMS method for the simultaneous quantification of the well-established angiotensin peptides AngI and AngII, as well as the novel peptides Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9 was developed and validated according to the FDA bioanalytical guideline, allowing valuable insights in the RAAS of children and neonates.

The conducted literature review of circulating angiotensin peptides as well as PRA provided a systematic characterisation of plasma levels within healthy and diseased paediatrics. Age was identified as a major factor on circulating AngI, AngII, and Ang1-7 levels, as well as PRA, which is reflected in an age-dependent decrease during childhood. In contrast to the data obtained in adults, no gender-related differences in angiotensin levels were identified. The observed increase in peptide concentrations regarding cardiac and renal diseased children are influenced by surgical repair, while evidence for a pharmacological impact is conflicting. However, the performed review also revealed a lack of consistent data for AngI, AngII, Ang1-7 and PRA, particularly for children below one year of age. Moreover, evidence about potential promising targets for future pharmacotherapy of CVD like AngIII, AngIV, AngA, and alamandine are still lacking. Therefore, the limited evidence-based medicine in this vulnerable age group could potentially be dedicated to the sparse data available. Besides, a comprehensive investigation of these parameters might contribute to a deeper understanding of the maturing organism and pathophysiological processes of paediatric HF. In this regard, innovative treatment strategies for children suffering from CVD might be fostered by an in-depth analysis of the paediatric RAAS. All in all, these findings emphasised the need for reliable bioanalytical investigations applicable in paediatric clinical trials.

To investigate the PRA in the cardiac diseased paediatric LENA collective, an ELISA was successfully customised for paediatric application. The microassay enabled the sophisticated determination of PRA in 100 µL plasma and thus showed applicability in trials with frequent blood samplings. Moreover, the low volume required facilitates the simultaneous determination of several PK/PD and safety parameters mandatory in pivotal clinical trials and could reduce the burden of additional blood samplings. As the ELISA

technique is characterised by a secure handling and a simple implementation in bioanalytical routine, this kind of low-volume assay could advance the quantity of conducted paediatric PRA data. Besides, the FDA compliant validation allowed the microassay to be applied in regulatory, clinical trials and assured the high-quality of obtained data. This might contribute to more homogenous obtained data sets due to a more accurate and precise assay performance. Ultimately, this research might contribute to a reliable basis of PRA data in LENA and further clinical trials to achieve an improved evidence-based pharmacotherapy of HF in the very youngest.

The customised QCS was successfully established for the analysis of PRA within the academia-driven LENA project and verified the high-quality of obtained data. The assurance of continuous quality during long-lasting study sample analysis represents a significant hurdle in sophisticated bioanalytical investigations. Yet, it might contribute to an increased assessment of continuously high data. The lack of homogenous regulatory recommendations regarding the inter-run and long-term evaluation was successfully compensated by the application of Westgard rules, CLSI guideline requirements and suggestions of scientific literature. Moreover, the QCS facilitated a careful monitoring of inter-lot dependent deviations and no severe impact on method performance could be detected. However, while systems for quality assurance are already established in the pharmaceutical industry, feasible approaches for comprehensive QCS in academic research are still underrepresented. This, in particular, is worrying as this kind of research is increasingly being addressed by academic consortiums. This proof-of-concept study might initiate other researchers to implement similar systems within their bioanalytics to substantiate the obtained data quality.

The developed and validated low-volume multiplex HPLC-HRMS method reliable quantified eight angiotensin peptides of the RAAS in 50 µL plasma. The low required sample volume and the regulatory compliant validation provided the opportunity for the investigation of these essential humoral parameters in paediatric clinical trials. This is of particular importance, as comprehensive datasets of angiotensin peptides in children and neonates are lacking. In this regard, the sophisticated evaluation of AngI and AngII, as well as the novel effector peptides Ang1-7, AngIII, AngIV, AngA, alamandine, and Ang1-9 in the unique population of the LENA project might facilitate a more in-depth knowledge of the paediatric RAAS. Ultimately, the establishment of a reliable database encompassing these crucial parameters of the RAAS could support the evidence-based therapy of paediatric HF in the future.

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9 References

1. Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abera SF, Abyu G, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. J Am Coll Cardiol. 2017;70:1–25. doi:10.1016/j.jacc.2017.04.052.
2. Sayer G, Bhat G. The renin-angiotensin-aldosterone system and heart failure. Cardiol Clin. 2014;32:21-32, vii. doi:10.1016/j.ccl.2013.09.002.
3. Wright JW, Kawas LH, Harding JW. The development of small molecule angiotensin IV analogs to treat Alzheimer's and Parkinson's diseases. Prog Neurobiol. 2015;125:26–46. doi:10.1016/j.pneurobio.2014.11.004.
4. Bundel D de, Smolders I, Vanderheyden P, Michotte Y. Ang II and Ang IV: Unraveling the mechanism of action on synaptic plasticity, memory, and epilepsy. CNS Neurosci Ther. 2008;14:315–39. doi:10.1111/j.1755-5949.2008.00057.x.
5. Pelegrini-da-Silva A, Rosa E, Guethe LM, Juliano MA, Prado WA, Martins AR. Angiotensin III modulates the nociceptive control mediated by the periaqueductal gray matter. Neuroscience. 2009;164:1263–73. doi:10.1016/j.neuroscience.2009.09.004.
6. Stragier B, Bundel D de, Sarre S, Smolders I, Vauquelin G, Dupont A, et al. Involvement of insulin-regulated aminopeptidase in the effects of the renin-angiotensin fragment angiotensin IV: A review. Heart Fail Rev. 2008;13:321–37. doi:10.1007/s10741-007-9062-x.
7. Royea J, Zhang L, Tong X-K, Hamel E. Angiotensin IV Receptors Mediate the Cognitive and Cerebrovascular Benefits of Losartan in a Mouse Model of Alzheimer's Disease. J Neurosci. 2017;37:5562–73. doi:10.1523/JNEUROSCI.0329-17.2017.
8. Reaux A, Fournie-Zaluski MC, Llorens-Cortes C. Angiotensin III: A central regulator of vasopressin release and blood pressure. Trends in Endocrinology & Metabolism. 2001;12:157–62. doi:10.1016/S1043-2760(01)00381-2.
9. Atlas SA. The renin-angiotensin aldosterone system: Pathophysiological role and pharmacologic inhibition. J Manag Care Pharm. 2007;13:9–20. doi:10.18553/jmcp.2007.13.s8-b.9.
10. Streatfeild-James RM, Williamson D, Pike RN, Tewksbury D, Carrell RW, Coughlin PB. Angiotensinogen cleavage by renin: Importance of a structurally constrained N-terminus. FEBS Lett. 1998;436:267–70.
11. Jan Danser AH, Batenburg WW, van Esch JHM. Prorenin and the (pro)renin receptor--an update. Nephrol Dial Transplant. 2007;22:1288–92. doi:10.1093/ndt/gfl846.
12. Li W, Peng H, Seth DM, Feng Y. The Prorenin and (Pro)renin Receptor: New Players in the Brain Renin-Angiotensin System? Int J Hypertens. 2012;2012:290635. doi:10.1155/2012/290635.
13. Brown MJ. Direct renin inhibition a new way of targeting the renin system. J Renin Angiotensin Aldosterone Syst. 2006;7:S8. doi:10.3317/jraas.2006.035.
14. Jankowski V, Tölle M, Santos RAS, Günthner T, Krause E, Beyermann M, et al. Angioprotectin: An angiotensin II-like peptide causing vasodilatory effects. FASEB J. 2011;25:2987–95. doi:10.1096/fj.11-185470.
15. Jankowski V, Vanholder R, van der Giet M, Tölle M, Karadogan S, Gobom J, et al. Mass-spectrometric identification of a novel angiotensin peptide in human plasma. Arterioscler Thromb Vasc Biol. 2007;27:297–302. doi:10.1161/01.ATV.0000253889.09765.5f.
16. Basu R, Poglitsch M, Yogasundaram H, Thomas J, Rowe BH, Oudit GY. Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure. J Am Coll Cardiol. 2017;69:805–19. doi:10.1016/j.jacc.2016.11.064.
17. Lautner RQ, Villela DC, Fraga-Silva RA, Silva N, Verano-Braga T, Costa-Fraga F, et al. Discovery and characterization of alamandine: A novel component of the renin-angiotensin system. Circ Res. 2013;112:1104–11. doi:10.1161/CIRCRESAHA.113.301077.
18. Iwai M, Horiuchi M. Devil and angel in the renin-angiotensin system: ACE-angiotensin II-AT1 receptor axis vs. ACE2-angiotensin-(1-7)-Mas receptor axis. Hypertens Res. 2009;32:533–6. doi:10.1038/hr.2009.74.
19. Jackson L, Eldahshan W, Fagan SC, Ergul A. Within the Brain: The Renin Angiotensin System. Int J Mol Sci 2018. doi:10.3390/ijms19030876.
20. Yugandhar VG, Clark MA. Angiotensin III: A physiological relevant peptide of the renin angiotensin system. Peptides. 2013;46:26–32. doi:10.1016/j.peptides.2013.04.014.
21. Wysocki J, Ye M, Batlle D. Plasma and Kidney Angiotensin Peptides: Importance of the Aminopeptidase A/Angiotensin III Axis. Am J Hypertens. 2015;28:1418–26. doi:10.1093/ajh/hpv054.

22. Bujak-Gizycka B, Madej J, Wołkow PP, Olszanecki R, Drabik L, Rutowski J, Korbut R. Measurement of angiotensin metabolites in organ bath and cell culture experiments by liquid chromatography - electrospray ionization - mass spectrometry (LC-ESI-MS). J Physiol Pharmacol. 2007;58:529–40.
23. Page IH. A crstalline pressor substance (angiotonin) resulting from the reaction between renin and renin-activator. Journal of Experimental Medicine. 1940;71:29–42. doi:10.1084/jem.71.1.29.
24. Olson N, DeJongh B, Hough A, Parra D. Plasma renin activity-guided strategy for the management of hypertension. Pharmacotherapy. 2012;32:446–55. doi:10.1002/j.1875-9114.2012.01031.x.
25. Muñoz JM, Braun-Menéndez E, Fasciolo JC, Leloir LF. Hypertensin: The Substance Causing Renal Hypertension. Nature. 1939;144:980. doi:10.1038/144980a0.
26. Schulz A, Jankowski J, Zidek W, Jankowski V. Absolute quantification of endogenous angiotensin II levels in human plasma using ESI-LC-MS/MS. Clin Proteomics. 2014;11:37. doi:10.1186/1559-0275-11-37.
27. Li W, Li J, Hao P, Chen W, Meng X, Li H, et al. Imbalance between angiotensin II and angiotensin-(1-7) in human coronary atherosclerosis. J Renin Angiotensin Aldosterone Syst 2016. doi:10.1177/1470320316659618.
28. Ferrario CM. Role of angiotensin II in cardiovascular disease therapeutic implications of more than a century of research. J Renin Angiotensin Aldosterone Syst. 2006;7:3–14. doi:10.3317/jraas.2006.003.
29. Mulrow PJ. Angiotensin II and aldosterone regulation. Regul Pept. 1999;80:27–32.
30. Coppage WS, Island DP, Cooner AE, Liddle GW. The metabolism of aldosterone in normal subjects and in patients with hepatic cirrhosis. J Clin Invest. 1962;41:1672–80. doi:10.1172/JCI104624.
31. Keidar S, Kaplan M, Gamliel-Lazarovich A. ACE2 of the heart: From angiotensin I to angiotensin (1-7). Cardiovasc Res. 2007;73:463–9. doi:10.1016/j.cardiores.2006.09.006.
32. Rodriguez-Pallares J, Quiroz CR, Parga JA, Guerra MJ, Labandeira-Garcia JL. Angiotensin II increases differentiation of dopaminergic neurons from mesencephalic precursors via angiotensin type 2 receptors. Eur J Neurosci. 2004;20:1489–98. doi:10.1111/j.1460-9568.2004.03621.x.
33. Campbell WB, Brooks SN, Pettinger WA. Angiotensin II- and angiotensin 3-induced aldosterone release vivo in the rat. Science. 1974;184:994–6.
34. Fink GD, Bruner CA. Hypertension during chronic peripheral and central infusion of angiotensin III. Am J Physiol. 1985;249:E201-8. doi:10.1152/ajpendo.1985.249.2.E201.
35. Batt CM, Klein EW, Harding JW, Wright JW. Pressor responses to amastatin, bestatin and Plummer's inhibitors are suppressed by pretreatment with the angiotensin receptor antagonist sarthran. Brain Res Bull. 1988;21:731–5.
36. Park BM, Oh Y-B, Gao S, Cha SA, Kang KP, Kim SH. Angiotensin III stimulates high stretch-induced ANP secretion via angiotensin type 2 receptor. Peptides. 2013;42:131–7. doi:10.1016/j.peptides.2013.01.018.
37. Harding JW, Felix D. The effects of the aminopeptidase inhibitors amastatin and bestatin on angiotensin-evoked neuronal activity in rat brain. Brain Research. 1987;424:299–304. doi:10.1016/0006-8993(87)91474-0.
38. Harding JW, Felix D. Angiotensin-sensitive neurons in the rat paraventricular nucleus: Relative potencies of angiotensin II and angiotensin III. Brain Research. 1987;410:130–4. doi:10.1016/S0006-8993(87)80033-1.
39. Harding JW, Yoshida MS, Dilts RP, Woods TM, Wright JW. Cerebroventricular and Intravascular Metabolism of [125 I]Angiotensins in Rat. J Neurochem. 1986;46:1292–7. doi:10.1111/j.1471-4159.1986.tb00652.x.
40. Wright JW, Mizutani S, Murray CE, Amir HZ, Harding JW. Aminopeptidase-induced elevations and reductions in blood pressure in the spontaneously hypertensive rat. J Hypertens. 1990;8:969–74.
41. Clark MA, Nguyen C, Tran H. Distinct Molecular Effects of Angiotensin II and Angiotensin III in Rat Astrocytes. Int J Hypertens. 2013;2013:782861. doi:10.1155/2013/782861.
42. Wilson WL, Roques BP, Llorens-Cortes C, Speth RC, Harding JW, Wright JW. Roles of brain angiotensins II and III in thirst and sodium appetite. Brain Research. 2005;1060:108–17. doi:10.1016/j.brainres.2005.08.032.
43. Blair-West JR, Carey KD, Denton DA, Madden LJ, Weisinger RS, Shade RE. Possible contribution of brain angiotensin III to ingestive behaviors in baboons. Am J Physiol Regul Integr Comp Physiol. 2001;281:R1633-6. doi:10.1152/ajpregu.2001.281.5.R1633.

44. Albiston AL, McDowall SG, Matsacos D, Sim P, Clune E, Mustafa T, et al. Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. J Biol Chem. 2001;276:48623–6. doi:10.1074/jbc.C100512200.
45. Chai SY, Fernando R, Peck G, Ye S-Y, Mendelsohn FAO, Jenkins TA, Albiston AL. The angiotensin IV/AT4 receptor. Cell Mol Life Sci. 2004;61:2728–37. doi:10.1007/s00018-004-4246-1.
46. Keller SR. Role of the insulin-regulated aminopeptidase IRAP in insulin action and diabetes. Biol Pharm Bull. 2004;27:761–4.
47. Wright JW, Harding JW. The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning, and memory. Prog Neurobiol. 2004;72:263–93. doi:10.1016/j.pneurobio.2004.03.003.
48. Matsumoto H, Nagasaka T, Hattori A, Rogi T, Tsuruoka N, Mizutani S, Tsujimoto M. Expression of placental leucine aminopeptidase/oxytocinase in neuronal cells and its action on neuronal peptides. Eur J Biochem. 2001;268:3259–66.
49. Albiston AL, Fernando R, Ye S, Peck GR, Chai SY. Alzheimer's, angiotensin IV and an aminopeptidase. Biol Pharm Bull. 2004;27:765–7.
50. Herbst JJ, Ross SA, Scott HM, Bobin SA, Morris NJ, Lienhard GE, Keller SR. Insulin stimulates cell surface aminopeptidase activity toward vasopressin in adipocytes. Am J Physiol. 1997;272:E600-6. doi:10.1152/ajpendo.1997.272.4.E600.
51. Burbach JP, Liu B. Measurement of vasopressin-converting aminopeptidase activity and vasopressin metabolites. Meth Enzymol. 1989;168:385–97.
52. Garvey WT, Maianu L, Zhu JH, Brechtel-Hook G, Wallace P, Baron AD. Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. J Clin Invest. 1998;101:2377–86. doi:10.1172/JCI1557.
53. Beyer CE, Dwyer JM, Platt BJ, Neal S, Luo B, Ling H-P, et al. Angiotensin IV elevates oxytocin levels in the rat amygdala and produces anxiolytic-like activity through subsequent oxytocin receptor activation. Psychopharmacology (Berl). 2010;209:303–11. doi:10.1007/s00213-010-1791-1.
54. Wright JW, Stubley L, Pederson ES, Kramár EA, Hanesworth JM, Harding JW. Contributions of the brain angiotensin IV-AT4 receptor subtype system to spatial learning. J Neurosci. 1999;19:3952–61.
55. Zhuang S, Li J, Wang X, Wang H-f, Zhang W-j, Wang H-y, Xing C-m. Renin-angiotensin system-targeting antihypertensive drugs and risk of vascular cognitive impairment: A meta-analysis. Neurosci Lett. 2016;615:1–8. doi:10.1016/j.neulet.2016.01.011.
56. Mogi M, Horiuchi M. Effects of angiotensin II receptor blockers on dementia. Hypertens Res. 2009;32:738–40. doi:10.1038/hr.2009.110.
57. Shibasaki Y, Mori Y, Tsutumi Y, Masaki H, Sakamoto K, Murasawa S, et al. Differential kinetics of circulating angiotensin IV and II after treatment with angiotensin II type 1 receptor antagonist and their plasma levels in patients with chronic renal failure. Clin Nephrol. 1999;51:83–91.
58. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res. 2000;87:E1-9.
59. Iwata M, Cowling RT, Yeo SJ, Greenberg B. Targeting the ACE2-Ang-(1-7) pathway in cardiac fibroblasts to treat cardiac remodeling and heart failure. J Mol Cell Cardiol. 2011;51:542–7. doi:10.1016/j.yjmcc.2010.12.003.
60. Kittana N. Angiotensin-converting enzyme 2-Angiotensin 1-7/1-9 system: Novel promising targets for heart failure treatment. Fundam Clin Pharmacol. 2018;32:14–25. doi:10.1111/fcp.12318.
61. Ocaranza MP, Lavandero S, Jalil JE, Moya J, Pinto M, Novoa U, et al. Angiotensin-(1-9) regulates cardiac hypertrophy in vivo and in vitro. J Hypertens. 2010;28:1054–64. doi:10.1097/hjh.0b013e328335d291.
62. Maier C, Schadock I, Haber PK, Wysocki J, Ye M, Kanwar Y, et al. Prolylcarboxypeptidase deficiency is associated with increased blood pressure, glomerular lesions, and cardiac dysfunction independent of altered circulating and cardiac angiotensin II. J Mol Med. 2017;95:473–86. doi:10.1007/s00109-017-1513-9.
63. Kehoe K, Gielis JF, Vliegen G, van Elzen R, Verkerk R, Driessens E, et al. Dysregulation of the renin-angiotensin system during lung ischemia-reperfusion injury. Exp Lung Res. 2016;42:277–85. doi:10.1080/01902148.2016.1207725.
64. Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem J. 2004;383:45–51. doi:10.1042/BJ20040634.

65. van Twist DJL, Kroon AA, Leeuw PW de. Angiotensin-(1-7) as a strategy in the treatment of hypertension? Curr Opin Nephrol Hypertens. 2014;23:480–6. doi:10.1097/MNH.0000000000000050.
66. Bosnyak S, Jones ES, Christopoulos A, Aguilar M-I, Thomas WG, Widdop RE. Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors. Clin Sci. 2011;121:297–303. doi:10.1042/CS20110036.
67. Gava E, Castro CH de, Ferreira AJ, Colleta H, Melo MB, Alenina N, et al. Angiotensin-(1-7) receptor Mas is an essential modulator of extracellular matrix protein expression in the heart. Regul Pept. 2012;175:30–42. doi:10.1016/j.regpep.2012.01.001.
68. Santos RAS, Simoes E Silva AC, Maric C, Silva DMR, Machado RP, Buhr I de, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A. 2003;100:8258–63. doi:10.1073/pnas.1432869100.
69. Ho JK, Nation DA. Cognitive benefits of Angiotensin IV and Angiotensin-(1-7): A systematic review of experimental studies. Neurosci Biobehav Rev 2018. doi:10.1016/j.neubiorev.2018.05.005.
70. Jiang F, Yang J, Zhang Y, Dong M, Wang S, Zhang Q, et al. Angiotensin-converting enzyme 2 and angiotensin 1-7: novel therapeutic targets. Nat Rev Cardiol. 2014;11:413–26. doi:10.1038/nrcardio.2014.59.
71. Villela D, Leonhardt J, Patel N, Joseph J, Kirsch S, Hallberg A, et al. Angiotensin type 2 receptor (AT2R) and receptor Mas: A complex liaison. Clin Sci. 2015;128:227–34. doi:10.1042/CS20130515.
72. Iwata M, Cowling RT, Gurantz D, Moore C, Zhang S, Yuan JX-J, Greenberg BH. Angiotensin-(1-7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. Am J Physiol Heart Circ Physiol. 2005;289:H2356-63. doi:10.1152/ajpheart.00317.2005.
73. Yang R, Smolders I, Vanderheyden P, Demaegdt H, van Eeckhaut A, Vauquelin G, et al. Pressor and renal hemodynamic effects of the novel angiotensin A peptide are angiotensin II type 1A receptor dependent. Hypertension. 2011;57:956–64. doi:10.1161/HYPERTENSIONAHA.110.161836.
74. Hrenak J, Paulis L, Simko F. Angiotensin A/Alamandine/MrgD Axis: Another Clue to Understanding Cardiovascular Pathophysiology. Int J Mol Sci 2016. doi:10.3390/ijms17071098.
75. Habiyakare B, Alsaadon H, Mathai ML, Hayes A, Zulli A. Reduction of angiotensin A and alamandine vasoactivity in the rabbit model of atherogenesis: Differential effects of alamandine and Ang(1-7). Int J Exp Pathol. 2014;95:290–5. doi:10.1111/iep.12087.
76. Le Tran Y, Forster C. Angiotensin-(1-7) and the rat aorta: Modulation by the endothelium. J Cardiovasc Pharmacol. 1997;30:676–82. doi:10.1097/00005344-199711000-00019.
77. Kozlowski LP. IPC – Isoelectric Point Calculator. Biol Direct. 2016;11:1–16. doi:10.1186/s13062-016-0159-9.
78. Suessenbach FK, Burckhardt BB. Levels of angiotensin peptides in healthy and cardiovascular/renal-diseased paediatric population-an investigative review. Heart Fail Rev. 2019;24:709–23. doi:10.1007/s10741-019-09797-y.
79. European Medicines Agency, Committee for Medicinal Products for Human Use and Paediatric Committee. Guideline on the Investigation of Medicinal Products in the Term and Preterm neonate. 2010. https://www.ema.europa.eu/en/investigation-medicinal-products-term-preterm-neonate. Accessed 15 Apr 2019.
80. Gazewood JD, Turner PL. Heart Failure with Preserved Ejection Fraction: Diagnosis and Management. Am Fam Physician. 2017;96:582–8.
81. Masarone D, Valente F, Rubino M, Vastarella R, Gravino R, Rea A, et al. Pediatric Heart Failure: A Practical Guide to Diagnosis and Management. Pediatrics & Neonatology. 2017;58:303–12. doi:10.1016/j.pedneo.2017.01.001.
82. Kirk R, Dipchand AI, Rosenthal DN, Addonizio L, Burch M, Chrisant M, et al. The International Society for Heart and Lung Transplantation Guidelines for the management of pediatric heart failure: Executive summary. Corrected. J Heart Lung Transplant. 2014;33:888–909. doi:10.1016/j.healun.2014.06.002.
83. Wittlieb-Weber CA, Lin KY, Zaoutis TE, O'Connor MJ, Gerald K, Paridon SM, et al. Pediatric versus adult cardiomyopathy and heart failure-related hospitalizations: A value-based analysis. J Card Fail. 2015;21:76–82. doi:10.1016/j.cardfail.2014.10.011.
84. Fridman MD, Mital S. Perspective on precision medicine in paediatric heart failure. Clin Sci. 2017;131:439–48. doi:10.1042/CS20160414.
85. Masarone D, Valente F, Rubino M, Vastarella R, Gravino R, Rea A, et al. Pediatric Heart Failure: A Practical Guide to Diagnosis and Management. Pediatr Neonatol. 2017;58:303–12. doi:10.1016/j.pedneo.2017.01.001.

86. Hinton RB, Ware SM. Heart Failure in Pediatric Patients With Congenital Heart Disease. Circ Res. 2017;120:978–94. doi:10.1161/CIRCRESAHA.116.308996.
87. Rossano JW. Clinical management of patients with acute heart failure. Cardiol Young. 2015;25 Suppl 2:67–73. doi:10.1017/S1047951115000852.
88. Daubeney PEF, Nugent AW, Chondros P, Carlin JB, Colan SD, Cheung M, et al. Clinical features and outcomes of childhood dilated cardiomyopathy: Results from a national population-based study. Circulation. 2006;114:2671–8. doi:10.1161/CIRCULATIONAHA.106.635128.
89. Das BB, Scholl F, Vandale B, Chrisant M. Sacubitril/Valsartan: Potential treatment for paediatric heart failure. Cardiol Young. 2018;28:1077–81. doi:10.1017/S1047951118001014.
90. Committee for Medicinal Products for Human Use. European Union. Ethical considerations for clinical trials on medicinal products conducted with the paediatric population. Eur J Health Law. 2008;15:223–50.
91. Patel MD, Mohan J, Schneider C, Bajpai G, Purevjav E, Canter CE, et al. Pediatric and adult dilated cardiomyopathy represent distinct pathological entities. JCI Insight 2017. doi:10.1172/jci.insight.94382.
92. Momper JD, Mulugeta Y, Burckart GJ. Failed Pediatric Drug Development Trials. Clin Pharmacol Ther. 2015;98:245–51. doi:10.1002/cpt.142.
93. De A, Shah P, Szmuszkovicz J, Bhombal S, Azen S, Kato RM. A retrospective review of infants receiving sildenafil. J Pediatr Pharmacol Ther. 2018;23:100–5. doi:10.5863/1551-6776-23.2.100.
94. Knudson JD, Cabrera AG. The Pathophysiology of Heart Failure in Children: The Basics. Curr Cardiol Rev. 2016;12:99–103.
95. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J. 2016;37:2129–200. doi:10.1093/eurheartj/ehw128.
96. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Colvin MM, et al. 2017 ACC/AHA/HFSA Focused Update of the 2013 ACCF/AHA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Heart Failure Society of America. Circulation. 2017;136:e137-e161. doi:10.1161/CIR.0000000000000509.
97. Kern SE. Challenges in conducting clinical trials in children: Approaches for improving performance. Expert Rev Clin Pharmacol. 2009;2:609–17. doi:10.1586/ecp.09.40.
98. European Commission. Regulation (EC) No 1901/2006 of the European Parliament and of the Council of 12 December 2006 on medicinal medicines for pediatric use and amending Regulation (EEC) No 1768/92, Directive 2001/20/EC, Directive 2001/83/EC and Regulation (EC) No 726/2004. https://ec.europa.eu/health//sites/health/files/files/eudralex/vol-1/reg_2006_1901/reg_2006_1901_en.pdf. Accessed 22 Nov 2019.
99. European Commission. State of Paediatric Medicines in the EU 10 years of the EU Paediatric Regulation. https://ec.europa.eu/health/sites/health/files/files/paediatrics/docs/2017_childrensmedicines_report_en.pdf. Accessed 22 Nov 2019.
100. Podany AT. Ethical considerations in pediatric research 2017: PedSAP.
101. Pica N, Bourgeois F. Discontinuation and Nonpublication of Randomized Clinical Trials Conducted in Children. Pediatrics 2016. doi:10.1542/peds.2016-0223.
102. Bajcetic M, Wildt SN de, Dalinghaus M, Breitkreutz J, Klingmann I, Lagler FB, et al. Orodispersible minitablets of enalapril for use in children with heart failure (LENA): Rationale and protocol for a multicentre pharmacokinetic bridging study and follow-up safety study. Contemp Clin Trials Commun. 2019;15:100393. doi:10.1016/j.conctc.2019.100393.
103. Al-Merani SA, Brooks DP, Chapman BJ, Munday KA. The half-lives of angiotensin II, angiotensin II-amide, angiotensin III, Sar1-Ala8-angiotensin II and renin in the circulatory system of the rat. The Journal of Physiology. 1978;278:471–90.
104. Ciplea AM, Laeer S, Bengt Burckhardt B. A feasibility study prior to an international multicentre paediatric study to assess pharmacokinetic/pharmacodynamic sampling and sample preparation procedures, logistics and bioanalysis. Contemp Clin Trials Commun. 2018;12:32–9. doi:10.1016/j.conctc.2018.08.008.
105. Sink TD, Lochmann RT, Fecteau KA. Validation, use, and disadvantages of enzyme-linked immunosorbent assay kits for detection of cortisol in channel catfish, largemouth bass, red pacu, and golden shiners. Fish Physiology and Biochemistry. 2008;34:95–101. doi:10.1007/s10695-007-9150-9.

106. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol Med. 1941;47:200–2.
107. Aydin S. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides. 2015;72:4–15. doi:10.1016/j.peptides.2015.04.012.
108. Ewles M, Goodwin L. Bioanalytical approaches to analyzing peptides and proteins by LC--MS/MS. Bioanalysis. 2011;3:1379–97. doi:10.4155/bio.11.112.
109. Ötles S, Kartal C. Solid-Phase Extraction (SPE): Principles and Applications in Food Samples. Acta Sci Pol Technol Aliment. 2016;15:5–15. doi:10.17306/J.AFS.2016.1.1.
110. Żwir-Ferenc A, Biziuk M. Solid Phase Extraction Technique--Trends, Opportunities and Applications. Polish Journal of Environmental Studies. 2006;15.
111. Hennion M-C. Solid-phase extraction: Method development, sorbents, and coupling with liquid chromatography. Journal of Chromatography A. 1999;856:3–54. doi:10.1016/S0021-9673(99)00832-8.
112. Steinhilber, D. and A. Dominik. Instrumentelle Analytik. 2nd ed. Stuttgard: Deutscher Apotheken Verlag Stuttgard; 2002.
113. Yamashita, M. and J.B. Fenn. Electrospray ion source. Another variation on the free-jet theme. . The Journal of Physical Chemistry. 1984:4451–9.
114. Clegg GA, Dole M. Molecular beams of macroions. 3. Zein and polyvinylpyrrolidone. Biopolymers. 1971;10:821–6. doi:10.1002/bip.360100506.
115. Gey MH, editor. Instrumentelle Analytik und Bioanalytik. 2nd ed. Berlin Heidelberg.: Springer-Verlag; 2008.
116. Gross JH. Massenspektrometrie. Springer Spektrum. 2012.
117. Iribarne, J. and B. Thomson. On the evaporation of small ions from charged droplets. The Journal of Chemical Physics. 1976:2287–94.
118. Lohninger, H., et al, editor. Teach/Me Instrumentelle Analytik: Springer-Verlag GmbH & Co. KG.; 2003.
119. Becker JS. Inorganic mass spectrometry: Principles and applications: Wiley Online Library; 2007.
120. Hughes AB. Amino acids, peptides and proteins in organic chemistry, analysis and function of amino acids and peptides: John Wiley & Sons; 2013.
121. Mant CT, Chen Y, Yan Z, Popa TV, Kovacs JM, Mills JB, et al. HPLC Analysis and Purification of Peptides. In: Fields GB, editor. Peptide characterization and application protocols. Totowa, NJ: Humana Press; 2007. p. 3–55. doi:10.1007/978-1-59745-430-8_1.
122. Roepstorff P, Fohlman J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomedical mass spectrometry. 1984.
123. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Syst Rev. 2015;4:1. doi:10.1186/2046-4053-4-1.
124. Fiselier TJ, Lijnen P, Monnens L, van Munster P, Jansen M, Peer P. Levels of renin, angiotensin I and II, angiotensin-converting enzyme and aldosterone in infancy and childhood. Eur J Pediatr. 1983;141:3–7.
125. Broughton Pipkin F, Smales OR. Proceedings: Blood pressure and angiotensin II in the newborn. Arch Dis Child. 1975;50:330.
126. Broughton Pipkin F, Smales OR. A study of factors affecting blood pressure and angiotensin II in newborn infants. J Pediatr. 1977;91:113–9.
127. Miyawaki M, Okutani T, Higuchi R, Yoshikawa N. Plasma angiotensin II concentrations in the early neonatal period. Arch Dis Child Fetal Neonatal Ed. 2006;91:F359-62. doi:10.1136/adc.2005.089573.
128. Miyawaki M, Okutani T, Higuchi R, Yoshikawa N. The plasma angiotensin II level increases in very low-birth weight infants with neonatal chronic lung disease. Early Hum Dev. 2008;84:375–9. doi:10.1016/j.earlhumdev.2007.10.005.
129. van Acker KJ, Scharpé SL, Lynen PJ, Amery AK. Comparative study of active and inactive plasma renin in healthy infants and adults. J Clin Chem Clin Biochem. 1983;21:775–8.
130. Tang Y-J, Zhang Z-Z, Chen S-Q, Chen S-M, Li C-J, Chen J-W, et al. Effect of topical propranolol gel on plasma renin, angiotensin II and vascular endothelial growth factor in superficial infantile hemangiomas. J Huazhong Univ Sci Technol Med Sci. 2015;35:759–62. doi:10.1007/s11596-015-1503-5.
131. Zaher H, Rasheed H, El-Komy MM, Hegazy RA, Gawdat HI, Abdel Halim DM, et al. Propranolol versus captopril in the treatment of infantile hemangioma (IH): A randomized controlled trial. J Am Acad Dermatol. 2016;74:499–505. doi:10.1016/j.jaad.2015.09.061.

132. Simões e Silva AC, Diniz JSS, Regueira Filho A, Santos RAS. The renin angiotensin system in childhood hypertension: Selective increase of angiotensin-(1-7) in essential hypertension. J Pediatr. 2004;145:93–8. doi:10.1016/j.jpeds.2004.03.055.
133. Franco MCP, Casarini DE, Carneiro-Ramos MS, Sawaya AL, Barreto-Chaves MLM, Sesso R. Circulating renin-angiotensin system and catecholamines in childhood: Is there a role for birthweight? Clin Sci. 2008;114:375–80. doi:10.1042/CS20070284.
134. Zhang C, Chen S, Zhou G, Jin Y, Zhang R, Yang H, et al. Involvement of the renin-angiotensin system in the progression of severe hand-foot-and-mouth disease. PLoS ONE. 2018;13:e0197861. doi:10.1371/journal.pone.0197861.
135. Tiosano D, Schwartz Y, Braver Y, Hadash A, Gepstein V, Weisman Y, Lorber A. The renin-angiotensin system, blood pressure, and heart structure in patients with hereditary vitamin D-resistance rickets (HVDRR). J Bone Miner Res. 2011;26:2252–60. doi:10.1002/jbmr.431.
136. Hjortdal VE, Stenbøg EV, Ravn HB, Emmertsen K, Jensen KT, Pedersen EB, et al. Neurohormonal activation late after cavopulmonary connection. Heart. 2000;83:439–43.
137. Kamperis K, Rittig S, Bower WF, Djurhuus JC. Effect of indomethacin on desmopressin resistant nocturnal polyuria and nocturnal enuresis. J Urol. 2012;188:1915–22. doi:10.1016/j.juro.2012.07.019.
138. Kamperis K, Rittig S, Radvanska E, Jorgensen KA, Djurhuus JC. The effect of desmopressin on renal water and solute handling in desmopressin resistant monosymptomatic nocturnal enuresis. J Urol. 2008;180:707-13; discussion 713-4. doi:10.1016/j.juro.2008.04.047.
139. Mahler B, Kamperis K, Schroeder M, Frøkiær J, Djurhuus JC, Rittig S. Sleep deprivation induces excess diuresis and natriuresis in healthy children. Am J Physiol Renal Physiol. 2012;302:F236-43. doi:10.1152/ajprenal.00283.2011.
140. Gheissari A, Sabri M, Pirpiran M, Merrikhi A. Possible correlation among echocardiographic measures, serum brain natriuretic peptide, and angiotensin II levels in hypertensive kidney transplanted children. Exp Clin Transplant. 2013;11:128–33. doi:10.6002/ect.2012.0140.
141. Rittig S, Matthiesen TB, Pedersen EB, Djurhuus JC. Circadian variation of angiotensin II and aldosterone in nocturnal enuresis: Relationship to arterial blood pressure and urine output. J Urol. 2006;176:774–80. doi:10.1016/S0022-5347(06)00594-5.
142. South AM, Nixon PA, Chappell MC, Diz DI, Russell GB, Snively BM, et al. Antenatal corticosteroids and the renin-angiotensin-aldosterone system in adolescents born preterm. Pediatr Res. 2017;81:88–93. doi:10.1038/pr.2016.179.
143. Washburn LK, Brosnihan KB, Chappell MC, Diz DI, Gwathmey TM, Nixon PA, et al. The renin-angiotensin-aldosterone system in adolescent offspring born prematurely to mothers with preeclampsia. J Renin Angiotensin Aldosterone Syst. 2015;16:529–38. doi:10.1177/1470320314526940.
144. El-DeeK SEM, Sayed AA, Nassar AY, Mohey-Eldeen ZM, Eldeeb HM, Meki A-RMA. Role of some vasoactive mediators in scorpion envenomed children: Possible relation to envenoming outcome. Toxicon. 2017;127:77–84. doi:10.1016/j.toxicon.2017.01.006.
145. Al-Daghri NM, Al-Attas OS, Alokail MS, Alkharfy KM, Draz HM. Relationship between resistin and aPAI-1 levels with insulin resistance in Saudi children. Pediatr Int. 2010;52:551–6. doi:10.1111/j.1442-200X.2010.03091.x.
146. Al-Daghri NM, Al-Attas OS, Alokail MS, Alkharfy KM, Yakout SM, Sabico SB, et al. Parent-offspring transmission of adipocytokine levels and their associations with metabolic traits. PLoS ONE. 2011;6:e18182. doi:10.1371/journal.pone.0018182.
147. Miller JA, Anacta LA, Cattran DC. Impact of gender on the renal response to angiotensin II. Kidney Int. 1999;55:278–85. doi:10.1046/j.1523-1755.1999.00260.x.
148. Legato MJ, Johnson PA, Manson JE. Consideration of Sex Differences in Medicine to Improve Health Care and Patient Outcomes. JAMA. 2016;316:1865–6. doi:10.1001/jama.2016.13995.
149. Broughton Pipkin F, Smales OR, O'Callaghan M. Renin and angiotensin levels in children. Arch Dis Child. 1981;56:298–302.
150. Mahler B, Kamperis K, Ankarberg-Lindgren C, Djurhuus JC, Rittig S. The effect of puberty on diurnal sodium regulation. Am J Physiol Renal Physiol. 2015;309:F873-9. doi:10.1152/ajprenal.00319.2014.
151. Simões e Silva AC, Diniz JSS, Pereira RM, Pinheiro SVB, Santos RAS. Circulating renin angiotensin system in childhood chronic renal failure: Marked increase of Angiotensin-(1-7) in end-stage renal disease. Pediatr Res. 2006;60:734–9. doi:10.1203/01.pdr.0000246100.14061.bc.
152. Yi Z, Li Z, Wu X-C, He Q-N, Dang X-Q, He X-J. Effect of fosinopril in children with steroid-resistant idiopathic nephrotic syndrome. Pediatr Nephrol. 2006;21:967–72. doi:10.1007/s00467-006-0096-4.

153. Mainwaring RD, Lamberti JJ, Moore JW, Billman GF, Nelson JC. Comparison of the hormonal response after bidirectional Glenn and Fontan procedures. Ann Thorac Surg. 1994;57:59-63; discussion 64.
154. Seguias L, Zilleruelo G, Strauss J, Abitbol C, Montane B. Angiotensin-II and endothelin-1 levels in children with renoprival hypertension. Pediatr Nephrol. 2001;16:493–6.
155. Komukai K, Mochizuki S, Yoshimura M. Gender and the renin-angiotensin-aldosterone system. Fundam Clin Pharmacol. 2010;24:687–98. doi:10.1111/j.1472-8206.2010.00854.x.
156. Dillon MJ, Gillin ME, Ryness JM, Swiet M de. Plasma renin activity and aldosterone concentration in the human newborn. Arch Dis Child. 1976;51:537–40.
157. Fiselier T, Monnens L, van Munster P, Jansen M, Peer P, Lijnen P. The renin-angiotensin-aldosterone system in infancy and childhood in basal conditions and after stimulation. Eur J Pediatr. 1984;143:18–24.
158. Dillon MJ, Ryness JM. Plasma renin activity and aldosterone concentration in children. BMJ. 1975;4:316–9. doi:10.1136/bmj.4.5992.316.
159. Sassard J, Sann L, Vincent M, Francois R, Cier JF. Plasma renin activity in normal subjects from infancy to puberty. J Clin Endocrinol Metab. 1975;40:524–5. doi:10.1210/jcem-40-3-524.
160. Ationu A, Singer DR, Smith A, Elliott M, Burch M, Carter ND. Studies of cardiopulmonary bypass in children: Implications for the regulation of brain natriuretic peptide. Cardiovasc Res. 1993;27:1538–41.
161. Alvarez Kindelan A, Pérez Navero JL, La Ibarra de Rosa I, Concha Ruiz M, Montilla López P, Romanos Lezcano A. Relationship between hemodynamic changes and blood hormone concentrations after cardiac surgery in children with congenital heart disease. Crit Care Med. 1994;22:1754–61.
162. Buchhorn R, Hammersen A, Bartmus D, Bürsch J. The pathogenesis of heart failure in infants with congenital heart disease. Cardiol Young. 2001;11:498–504.
163. Parker FB, Streeten DH, Farrell B, Blackman MS, Sondheimer HM, Anderson GH. Preoperative and postoperative renin levels in coarctation of the aorta. Circulation. 1982;66:513–4.
164. Scammell AM, Diver MJ. Plasma renin activity in infants with congenital heart disease. Arch Dis Child. 1987;62:1136–8.
165. Stern H, Weil J, Genz T, Vogt W, Bühlmeyer K. Captopril in children with dilated cardiomyopathy: Acute and long-term effects in a prospective study of hemodynamic and hormonal effects. Pediatr Cardiol. 1990;11:22–8. doi:10.1007/BF02239543.
166. Fallo F, Armanini D, Maragno I, Mantero F. Plasma renin activity in coarctation of the aorta before and after surgical correction. Br Heart J. 1978;40:1415–8.
167. Gidding SS, Rocchini AP, Beekman R, Szpunar CA, Moorehead C, Behrendt D, Rosenthal A. Therapeutic effect of propranolol on paradoxical hypertension after repair of coarctation of the aorta. N Engl J Med. 1985;312:1224–8. doi:10.1056/NEJM198505093121904.
168. Gore R, Chugh PK, Tripathi CD, Lhamo Y, Gautam S. Pediatric Off-Label and Unlicensed Drug Use and Its Implications. Curr Clin Pharmacol. 2017;12:18–25. doi:10.2174/1574884712666170317161935.
169. U.S. Food and Drug Administration, U.S. Department ofof Health and Human Services. Guidance for Industry. Bioanalytical Method Validation. 2018. https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064964.htm. Accessed 10 Apr 2019.
170. European Medicines Agency, Committee fo Medicinal Products for Human Use and Paediatric Committee. Guideline on Bioanalytical Method Validation. 2011. https://www.ema.europa.eu/en/bioanalytical-method-validation. Accessed 10 Apr 2019.
171. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Bioanalytical Method Validation M10. 2019. Accessed 12 Sep 2019.
172. European Medicines Agency, Committee for Medicinal Products for Human Use and Paediatric Committee. Concept paper on the need for revision of the guideline on the investigation of medicinal products in the term and preterm neonate. 2019. https://www.ema.europa.eu/en/documents/scientific-guideline/adopted-concept-paper-need-revision-guideline-investigation-medicinal-products-term-preterm-neonate_en.pdf. Accessed 18 Jul 2019.
173. DiaSorin Inc. GammaCoat® Plasma Renin Activity 125I RIA Kit. http://catalog.diasorin.com/en/ricerca-hypertension/U,4,,,,. Accessed 6 Jun 2019.
174. Blazy I, Guillot F, Laborde K, Dechaux M. Comparison of plasma renin and prorenin in healthy infants and children as determined with an enzymatic method and a new direct immunoradiometric assay. Scand J Clin Lab Invest. 2011;49:413–8. doi:10.1080/00365518909089115.

175. Poulsen K, Jorgensen J. An easy radioimmunological microassay of renin activity, concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody. J Clin Endocrinol Metab. 1974;39:816–25. doi:10.1210/jcem-39-5-816.
176. DRG International I. Plasma Renin Activity (PRA) ELISA. https://store.drg-international.com/pc_product_detail.asp?key=85626BE0FA20493AB28205FFCA88B5D4. Accessed 5 Jun 2019.
177. Abd-Allah NM, Hassan FH, Esmat AY, Hammad SA. Age dependence of the levels of plasma norepinephrine, aldosterone, renin activity and urinary vanillylmandelic acid in normal and essential hypertensives. Biol Res. 2004;37:95–106.
178. Eagle Bioscience. Plasma Renin Activity ELISA Assay Kit. https://eaglebio.com/product/renin-elisa-assay-kit/. Accessed 5 Jun 2019.
179. DEMEDITEC Diagnostics GmbH. Plasma Renin Activity (PRA) ELISA. https://www.demeditec.com/en/products/plasma-renin-activity-pra-elisa-demse5600. Accessed 5 Jun 2019.
180. Lonati C, Bassani N, Gritti A, Biganzoli E, Morganti A. Measurement of plasma renin concentration instead of plasma renin activity decreases the positive aldosterone-to-renin ratio tests in treated patients with essential hypertension. J Hypertens. 2014;32:627–34. doi:10.1097/HJH.0000000000000076.
181. MyBioSource I. Plasma Renin Activity (PRA), ELISA Kit. https://www.mybiosource.com/pra-human-elisa-kits/plasma-renin-activity/495044. Accessed 5 Jun 2019.
182. Shaffer C. Diagnostic blood loss in mechanically ventilated patients. Heart & Lung. 2007;36:217–22. doi:10.1016/j.hrtlng.2006.09.001.
183. Ullman AJ, Keogh S, Coyer F, Long DA, New K, Rickard CM. 'True Blood' The Critical Care Story: An audit of blood sampling practice across three adult, paediatric and neonatal intensive care settings. Australian Critical Care. 2016;29:90–5. doi:10.1016/j.aucc.2015.06.002.
184. Samiee-Zafarghandy S, Mazer-Amirshahi M, van den Anker JN. Trends in paediatric clinical pharmacology data in US pharmaceutical labelling. Arch Dis Child. 2014;99:862–5. doi:10.1136/archdischild-2013-305605.
185. Curtis A. Parvin, Nils B. Person, Nikola Baumann, Lili Duan, A. Paul DurhamValerio M. Genta, Jeremie Gras, Greg Miller, Megan E. Sawchuk, editor. C24 - Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions. 4th ed. Wayne: Clinical and Laboratory Standards Institut; 2016.
186. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. Clin Chem. 1981;27:493–501.
187. van Bruijnsvoort M, Meijer J, van den Beld C. The application of control charts in regulated bioanalysis for monitoring long-term reproducibility. Bioanalysis. 2017;9:1955–65. doi:10.4155/bio-2017-0163.
188. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. The clinical biochemist reviews. 2008;29:S49.
189. Rudzki PJ, Biecek P, Kaza M. Comprehensive graphical presentation of data from incurred sample reanalysis. Bioanalysis. 2017;9:947–56. doi:10.4155/bio-2017-0038.
190. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, Dunn WB. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics. 2018;14:72. doi:10.1007/s11306-018-1367-3.
191. Sanagi MM, Ling SL, Nasir Z, Hermawan D, Ibrahim WAW, Abu Naim A. Comparison of signal-to-noise, blank determination, and linear regression methods for the estimation of detection and quantification limits for volatile organic compounds by gas chromatography. J AOAC Int. 2009;92:1833–8.
192. Azadeh M, Sondag P, Wang Y, Raines M, Sailstad J. Quality Controls in Ligand Binding Assays: Recommendations and Best Practices for Preparation, Qualification, Maintenance of Lot to Lot Consistency, and Prevention of Assay Drift. AAPS J. 2019;21:89. doi:10.1208/s12248-019-0354-6.
193. Rudzki PJ, Buś-Kwaśnik K, Kaza M. Incurred sample reanalysis: Adjusted procedure for sample size calculation. Bioanalysis. 2017;9:1719–26. doi:10.4155/bio-2017-0142.
194. Rudzki PJ, Biecek P, Kaza M. Incurred Sample Reanalysis: Time to Change the Sample Size Calculation? AAPS J. 2019;21:28. doi:10.1208/s12248-019-0293-2.
195. Armbruster H, Vetter W, Uhlschmid G, Zaruba K, Beckerhoff B, Nussberger J, et al. Circadian rhythm of plasma renin activity and plasma aldosterone in normal man and in renal allograft recipients. Proc Eur Dial Transplant Assoc. 1975;11:268–76.

196. Ohashi N, Isobe S, Ishigaki S, Yasuda H. Circadian rhythm of blood pressure and the renin-angiotensin system in the kidney. Hypertens Res. 2017;40:413–22. doi:10.1038/hr.2016.166.
197. Emdin M, Fatini C, Mirizzi G, Poletti R, Borrelli C, Prontera C, et al. Biomarkers of activation of renin-angiotensin-aldosterone system in heart failure: How useful, how feasible? Clinica Chimica Acta. 2015;443:85–93.
198. Duncan MR, Lee JM, Warchol MP. Influence of surfactants upon protein/peptide adsorption to glass and polypropylene. Int J Pharm. 1995;120:179–88. doi:10.1016/0378-5173(94)00402-Q.
199. Miladinović SM, Fornelli L, Lu Y, Piech KM, Girault HH, Tsybin YO. In-spray supercharging of peptides and proteins in electrospray ionization mass spectrometry. Anal Chem. 2012;84:4647–51. doi:10.1021/ac300845n.
200. Zhang X, Iraneta PC, Marszalkowski [JR] FJ, Fountain KJ. A Simplified Solid Phase Extraction (SPE) Protocol for Bioanalysis Using Oasis HLB. 2014. https://www.waters.com/webassets/cms/library/docs/720005140en.pdf. Accessed 11 Feb 2020.
201. Schulz A, Jankowski J, Zidek W, Jankowski V. Absolute quantification of endogenous angiotensin II levels in human plasma using ESI-LC-MS/MS. Clin Proteom. 2014;11:1–9. doi:10.1186/1559-0275-11-37.
202. Fredline VF, Kovacs EM, Taylor PJ, Johnson AG. Measurement of Plasma Renin Activity with Use of HPLC-Electrospray-Tandem Mass Spectrometry. Clin Chem. 1999;45:659–64. doi:10.1093/clinchem/45.5.659.
203. Bystrom CE, Salameh W, Reitz R, Clarke NJ. Plasma renin activity by LC-MS/MS: Development of a prototypical clinical assay reveals a subpopulation of human plasma samples with substantial peptidase activity. Clin Chem. 2010;56:1561–9. doi:10.1373/clinchem.2010.146449.
204. Olkowicz M, Radulska A, Suraj J, Kij A, Walczak M, Chlopicki S, Smolenski RT. Development of a sensitive, accurate and robust liquid chromatography/mass spectrometric method for profiling of angiotensin peptides in plasma and its application for atherosclerotic mice. J Chromatogr A. 2015;1393:37–46. doi:10.1016/j.chroma.2015.03.012.
205. Zhou W, Yang S, Wang PG. Matrix effects and application of matrix effect factor. Bioanalysis. 2017;9:1839–44. doi:10.4155/bio-2017-0214.
206. Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid. J Am Soc Mass Spectrom. 2003;14:1290–4. doi:10.1016/S1044-0305(03)00574-9.
207. Ahn SH, Bae YJ, Moon JH, Kim MS. Matrix suppression as a guideline for reliable quantification of peptides by matrix-assisted laser desorption ionization. Anal Chem. 2013;85:8796–801. doi:10.1021/ac401967n.
208. Cruces P, Diaz F, Puga A, Erranz B, Donoso A, Carvajal C, et al. Angiotensin-converting enzyme insertion/deletion polymorphism is associated with severe hypoxemia in pediatric ARDS. Intensive Care Med. 2012;38:113–9. doi:10.1007/s00134-011-2381-3.
209. Nakagawa Y, Toya K, Natsume H, Nasuda K, Takeuchi H, Kubota A, et al. Long-term follow-up of a girl with the neonatal form of Bartter's syndrome. Endocr J. 1997;44:275–81.
210. Saiki H, Kuwata S, Kurishima C, Masutani S, Senzaki H. Vulnerability of Coronary Circulation After Norwood Operation. Ann Thorac Surg. 2016;101:1544–51. doi:10.1016/j.athoracsur.2015.10.077.
211. Tokiwa K, Iwai N, Nakamura K, Shiraishi I, Hayashi S, Onouchi Z. Pulmonary hypertension as a fatal complication of extrahepatic portal hypertension. Eur J Pediatr Surg. 1993;3:373–5. doi:10.1055/s-2008-1066049.
212. Friedman A, Chesney RW, Ball D, Goodfriend T. Effective use of captopril (angiotensin I-converting enzyme inhibitor) in severe childhood hypertension. J Pediatr. 1980;97:664–7.
213. Wagoner AL, Shaltout HA, Fortunato JE, Diz DI. Distinct neurohumoral biomarker profiles in children with hemodynamically defined orthostatic intolerance may predict treatment options. Am J Physiol Heart Circ Physiol. 2016;310:H416-25. doi:10.1152/ajpheart.00583.2015.
214. Blazy I, Guillot F, Laborde K, Dechaux M. Comparison of plasma renin and prorenin in healthy infants and children as determined with an enzymatic method and a new direct immunoradiometric assay. Scand J Clin Lab Invest. 1989;49:413–8.
215. Godard C, Geering JM, Geering K, Vallotton MB. Plasma renin activity related to sodium balance, renal function and urinary vasopressin in the newborn infant. Pediatr Res. 1979;13:742–5.
216. García del Río C, Acuña D, Bustamante M, Soler A, García-Torres L, Osorio C. Increased activity of the renin-angiotensin-aldosterone system during the perinatal period. Rev Esp Fisiol. 1982;38:171–6.
217. Gjurić G, Mardesić D, Plavsić V, Dumić M. Plasma renin activity and plasma aldosterone concentration in infants with various potassium intakes. Eur J Pediatr. 1982;139:139–41.

218. Stalker HP, Holland NH, Kotchen JM, Kotchen TA. Plasma renin activity in healthy children. J Pediatr. 1976;89:256–8.
219. Mardesić D, Gjurić G, Dumić M, Vlatković G, Plavsić V, Vlatković M. Plasma renin activity and plasma aldosterone concentration in infants with various sodium intakes. Eur J Pediatr. 1979;132:49–54.
220. Lall SB, Peshin SS, Karmarkar MG. Plasma renin activity in normal healthy volunteers: Effect of physiological variables. Indian J Physiol Pharmacol. 1995;39:51–4.
221. Fukushige J, Shimomura K, Ueda K. Influence of upright activity on plasma renin activity and aldosterone concentration in children. Eur J Pediatr. 1994;153:284–6.
222. Martinez-Aguayo A, Aglony M, Campino C, Garcia H, Bancalari R, Bolte L, et al. Aldosterone, plasma Renin activity, and aldosterone/renin ratio in a normotensive healthy pediatric population. Hypertension. 2010;56:391–6. doi:10.1161/HYPERTENSIONAHA.110.155135.
223. Tu W, Eckert GJ, Decker BS, Howard Pratt J. Varying Influences of Aldosterone on the Plasma Potassium Concentration in Blacks and Whites. Am J Hypertens. 2017;30:490–4. doi:10.1093/ajh/hpx006.
224. Dechaux M, Broyer M, Lenoir G, Limal JM, Sachs C. Nyctohemeral rhythm of plasma renin activity and plasma aldosterone in children. Pediatr Res. 1982;16:354–6.
225. Arvay G, Szathmáry G, Reuter M. Changes in angiotensin-converting enzyme activity and angiotensin I level in asthmatic and healthy children after submaximal physical work. J Allergy Clin Immunol. 1982;69:178–80.
226. Shibutani Y, Sakamoto K, Katsuno S, Yoshimoto S, Matsuura T. An epidemiological study of plasma renin activity in schoolchildren in Japan: Distribution and its relation with family history of hypertension. J Hypertens. 1988;6:489–93.
227. Harshfield GA, Alpert BS, Pulliam DA. Renin-angiotensin-aldosterone system in healthy subjects aged ten to eighteen years. J Pediatr. 1993;122:563–7.
228. Simsolo R, Grunfeld B, Gimenez M, Lopez M, Berri G, Becú L, Barontini M. Long-term systemic hypertension in children after successful repair of coarctation of the aorta. Am Heart J. 1988;115:1268–73.
229. Harshfield GA, Alpert BS, Pulliam DA, Willey ES, Somes GW, Stapelton FB. Sodium excretion and racial differences in ambulatory blood pressure patterns. Hypertension. 1991;18:813–8.
230. Giardini A, Formigari R, Bronzetti G, Prandstraller D, Donti A, Bonvicini M, Picchio FM. Modulation of neurohormonal activity after treatment of children in heart failure with carvedilol. Cardiol Young. 2003;13:333–6.
231. Scammell AM, Diver MJ. Plasma aldosterone and renin activity. Arch Dis Child. 1988;64:139–41.
232. Scammell AM, Arnold R. The effect of the first dose of captopril on blood pressure in infants in heart failure. Int J Cardiol. 1989;22:377–9.

10 Appendix

10.1 Systematic reviews of humoral parameters of the paediatric renin-angiotensin-aldosterone system

Appendix 10.1-1: Inclusion and exclusion criteria for the plasma renin activity systematic literature search.

Inclusion criteria	Exclusion criteria
Plasma renin activity measured in the blood (Serum, Plasma, and whole blood)	No plasma renin activity data are given
0-18 years	Animal studies or ex vivo data
	Fetal or umbilical cord blood
	Disease not (only) heart failure
	Language not English or German
	Preterm infants (<2,5kg birthweight, <37 gestational weeks)
	End-stage heart failure requiring intensive care unit support
	Uncorrected primary obstructive valvular disease, significant systemic ventricular outflow obstruction or dilated restrictive or hypertrophic cardiomyopathy
	Uncorrected severe peripheral stenosis of large arteries
	Severe renal impairment <30mL/1.73m2

Appendix 10.1-2: Angiotensin values, specifications and analytics of investigated healthy population.

Age	Statistical operator	N	AngI [pg/mL]	AngII [pg/mL]	Ang1-7 [pg/mL]	Statistical operator	Sampling procedure	Analytic	Collective	Reference
1-7 days	Range	16		Day 0: 34 (5-201) Day 7: 76 (7-1041)		Mean (range)	Day 0: at birth Day 7: supine	Plasma / ELISA	VLBW newborns 27.9±2.9 weeks GA (mean±SD)	Miyawaki et al. 2006 [127]
1–7 days	Range	16		Day 0: 43 (5-438) Day 7: 28 (5-254)		Mean (range)	Day 0: at birth Day 7: supine	Plasma / ELISA	MLBW newborns 33.8±1.5 weeks GA (ean±SD)	Miyawaki et al. 2006 [127]
1–7 days	Range	14		Day 0: 74 (3-443) Day 7: 19 (1-127)		Mean (range)	Day 0: at birth Day 7: supine	Plasma / ELISA	NBW newborns 38.9±1.6 weeks GA (mean±SD)	Miyawaki et al. 2006 [127]
1-11 days	Range	46		67.7±7.1 (9.4 – 204)§		Mean±SD (range)	Supine in the morning	Plasma + Inhibitor / RIA	Newborn and infants with prematurity or jaundice	Pipkin et al. 1977 [126]
1–35 days	Range	9		Day 0: 55 / Day 7: 52 Day 21:52 / Day 35: 13		Mean	Supine in the morning	Plasma / ELISA	VLBW newborns and infants	Miyawaki et al. 2008 [128]
6 days	mean	25		60.3±9.2		Mean±SD	n/a	Plasma / RIA	Newborns with prematurity or jaundice	Pipkin et al. 1975 [125]
1 week–3 months	Range	9	321±112#	64±31#		Mean±SD	Sober/supine in the morning	Plasma + Inhibitor / RIA	Newborns, infants, and toddlers	Fiselier et al. 1983 [124]
30 days–10 months	Range	30		300±30		Mean±SD	n/a	Plasma / ELISA	Infants and toddlers	Tang et al. 2015 [130]
1.5–13 months	Range	35		1330±1130		Mean±SD	n/a	Plasma / ELISA	Infants and toddlers	Zaher et al. 2016 [131]
1–12 months	Range	20		33.6±4.3		Mean±SE	Recumbent in the afternoon	Plasma / RIA	Infants and toddlers	Van Acker et al. 1983 [129]
2 months –12 years	Range	63		37.8±3.7 (5-103)		Mean±SD (range)	Sober/supine in the morning	Plasma + Inhibitor / RIA	Infants, toddlers, and children	Pipkin et al. 1981 [149]
3-12 months	Range	16	163±49#	42±21#		Mean±SD	Sober/supine in the morning	Plasma + Inhibitor / RIA	Infants and toddlers	Fiselier et al. 1983 [124]
10 (5–24) months	Median (IQR)	60		229 (157–319)		Median (IQR)	n/a	Plasma / ELISA	Infants and toddlers with pneumonia	Cruces et al. 2012 [208]

Age	Statistical operator	N	AngI [pg/mL]	AngII [pg/mL]	Ang1-7 [pg/mL]	Statistical operator	Sampling procedure	Analytic	Collective	Reference
1–4 years	Range	10	143±69#	32±23# (n=11)		Mean±SD	Sober/supine in the morning	Plasma + Inhibitor / RIA	Infants, toddlers, and children	Fiselier et al. 1983 [124]
1-13 years	Range	10		1780±70		Mean±SD	n/a	Plasma / ELISA	Infants, toddlers, and children	El-Deek et al. 2017 [144]
24.1±8.6 months	Mean±SD	30		29.5±7.6§		Mean±SD	n/a	Serum / ELISA	Infants and toddlers and children	Zhang et al. 2018 [134]
3.1–16.7 years	Range	32	26.4±13.4	21.4±8.7	16.2±7.9	Mean±SD	Sober/supine in the morning	Plasma + Inhibitor / RIA	Children and adolescents	Simões e Silva et al. 2004 [132]
4–8 years	Range	9	103±38#	24±15#		Mean±SD	Sober/supine in the morning	Plasma + Inhibitor / RIA	Children	Fiselier et al. 1983 [124]
5–12 years	Range	150		710±530		Mean±SD	Fasting	Serum / RIA	Children	Al-Daghri et al. 2010 [145]
6 and 9 years		2		39.9±35.5#		Mean±SD	Supine	Plasma / RIA	Male children	Tiosano et al. 2011 [135]
6–16 years	Range	33		11.5 (8.4 – 15.7)		Median (IQR)	Fasting	Plasma / RIA	Children and adolescents	Hjortdal et al. 2000 [136]
8-13 years	Range	31	♂: 77380±35570 ♀: 85280±43670	♂: 92340±18860 ♀: 82380±23140	♂: 31680±24260 ♀: 45750±30120	Mean±SD	n/a	Plasma +Inhibitor HPLC-UV/VIS	AGA birthweight children and adolescents	Franco et al. 2008 [133]
8-13 years	Range	35	♂: 79740±42980 ♀: 89110±31360	♂: 117810±36790 ♀: 86630±29600	♂: 41590±31150 ♀: 46160±27040	Mean±SD	n/a	Plasma + Inhibitor / HPLC-UV/VIS	SGA birthweight children and adolescents	Franco et al. 2008 [133]
8–13 years	Range	10	105±41#	24±16#		Mean±SD	Sober/supine in the morning	Plasma + Inhibitor/ RIA	Children and adolescents	Fiselier et al. 1983 [124]
8.2±0.5 years	Mean±SD	9		13±0.1#		Mean±SE	Supine at 12.00 h	Plasma / RIA	Prepuberty boys	Mahler et al. 2015 [150]
8.3±0.3 years	Mean±SD	9		10.1±1#		Mean±SE	Supine at 12.00 h	Plasma / RIA	Prepuberty girls	Mahler et al. 2015 [150]
9.4±2.6 years	Mean±SD	131		570 (440 – 730)		median (IQR)	Fasting	Serum / RIA	Children and adolescents	Al-Daghri et al. 2011 [146]

Age	Statistical operator	N	AngI [pg/mL]	AngII [pg/mL]	Ang1-7 [pg/mL]	Statistical operator	Sampling procedure	Analytic	Collective	Reference
10.7±0.9	Mean±SD	10		15.4±2.7#		Mean±SD	Sitting in the morning	Plasma / RIA	Children and adolescents	Kamperis et al. 2008 [138]
10.0±1 year	Mean±SD	10		Girls: 107±42		Mean±SD	Supine in the night	Plasma / RIA	Female children	Mahler et al. 2012 [139]
11.1±1.1 years	Mean±SD	10		Boys: 71±29		Mean±SD	Supine in the night	Plasma / RIA	Male children and adolescents	Mahler et al. 2012 [139]
11.5±0.6	Mean±SD	11		12±2.4#		Mean±SD	Sitting in the morning	Plasma / RIA	Children and adolescents	Kamperis et al. 2012 [137]
11.9±7.7 years	Mean±SD	30		5.4±0.9		Mean±SD	n/a	Serum / ELISA	Children and adolescents	Gheissari et al. 2013 [140]
12–17 years	Range	4		99.0±22.9#		Mean±SD	Supine	Plasma / RIA	Female adolescents	Tiosano et al. 2011 [135]
12.8±0.8 years	Mean±SD	10		12.2±2.1#		Mean±SE	Supine at 12.00 h	Plasma / RIA	Puberty girls	Mahler et al. 2015 [150]
13.2±0.8 years	Mean±SD	10		Day: 10.4±1.3 Night: 22.3±3.1		Mean±SE	Sitting at day Supine at night	Plasma / RIA	Children and adolescents	Rittig et al. 2006 [141]
14±0.9 years	Mean±SD	10		14.8±0.2#		Mean±SE	Supine at 12.00 h	Plasma / RIA	Puberty boys	Mahler et al. 2015 [150]
14 years	Mean	50		22 (16.3–30.4)§	2.4 (1.3–6.3)§	Median (IQR)	Sitting	Plasma	Adolescents born preterm	South et al. 2017 [142]
14 years	Mean	70		23.3 (17.9–34.8)§	8.1 (2.3–13.2)§	Median (IQR)	Sitting	Plasma	Adolescents born preterm (ANCS)	South et al. 2017 [142]
14 years	Mean	78		25±13#	9±10§	Mean±SD	Sitting	Plasma + Inhibitor / RIA	Adolescents / prematurely with VLBW / normotensive pregnancy	Washburn et al. 2015 [143]
14 years	Mean	49		23±10#	8±6§	;ean±SD	Sitting	Plasma + Inhibitor / RIA	Adolescents / prematurely with VLBW / preeclamptic pregnancy	Washburn et al. 2015 [143]

n/a: data not available; SD: standard deviation; IQR: interquartile range; VLBW: very low birthweight; MLBW: moderate low birthweight; NBW: normal birthweight; GA: gestational age; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay; HPLC: high-performance liquid chromatography; UV/VIS: ultraviolet/visible spectroscopy; AGA: appropriate for gestational age; SGA: small for gestational age; ANCS: antenatal corticosteroids; §: values calculated from pmol/L to pg/mL; #: values generated via GetData Graph Digitizer 2.26.0.20, mean of three times conduct.

Appendix 10.1-3: Angiotensin values, specifications and analytics of investigated cardiovascular and renal diseased population.

Age	Statistical operator	N	AngI [pg/mL]	AngII [pg/mL]	Ang1-7 [pg/mL]	Statistical operator	Sampling procedure	Analytic	Collective	Reference
4 months	Single value	1		622		Single value	n/a	Plasma / RIA	A female infant with Bartter's syndrome	Nakagawa et al. 1997 [209]
1 year up to 16 years	Single value	1	No treatment: 3875 2.4 years of treatment: 541 15 years of treatment: 536	No treatment: 860 2.4 years of treatment: 85 15 years of treatment: 151		Single value	n/a	Plasma / RIA	A female child with Bartter's syndrome treated with Indomethacin	Nakagawa et al. 1997 [209]
13±5 months	Mean±SD	15		33±4		Mean±SD	n/a	Plasma / RIA	Infants and toddlers (before) undergoing BDG procedure	Mainwaring et al. 1994 [153]
1.6±1 years	Mean±SD	29		199±223		Mean±SD	During cardiac catheterization	n/a	Hypoplastic left heart syndrome infants, toddlers, and children undergoing Norwood procedure	Saiki et al. 2016 [210]
1.3±2.8 years	Mean±SD	27		38±55		Mean±SD	During cardiac catheterization	n/a	Infants, toddlers, and children suffering from pulmonary atresia with AP shunt	Saiki et al. 2016 [210]
25±8 months	Mean±SD	10		31±2		Mean± SE	n/a	Plasma / RIA	Infants, toddlers, and children with VSD	Mainwaring et al. 1994 [153]
36±10 months	Mean±SD	18		40±3		Mean± SE	n/a	Plasma / RIA	Children (before) undergoing Fontane procedure	Mainwaring et al. 1994 [153]
2.2-17.9 years	Range	23	26.7±6.7	22.4±9.1	18.6±6.8	Mean±SD	Sober/supine in the morning	Plasma + Inhibitor RIA	Normotensive children and adolescents with CRF	Simões e Silva et al. 2006 [151]

Age	Statistical operator	N	AngI [pg/mL]	AngII [pg/mL]	Ang1-7 [pg/mL]	Statistical operator	Sampling procedure	Analytic	Collective	Reference
2.2-17.2 Years	Range	34	171.8±85.4*	84.2±52.9*	140.8±58.0*	Mean±SD	Sober/supine in the morning. Before medication	Plasma + Inhibitor / RIA	Hypertensive children and adolescents with CRF	Simões e Silva et al. 2006 [151]
2.2–15.5 years	Range	12	81.4±24.8*	59.3±17.0*	41.0±10.5*	Mean±SD	Supine in the morning. untreated	Plasma + Inhibitor / RIA	Children and adolescents with renovascular hypertension	Simões e Silva et al. 2004 [132]
2.7–17.7 years	Range	12	Untreated: 36.5±11.4 Treated: 35.7±11.2	Untreated: 21.8±10.6 Treated: 22.5±10.9	Untreated: 78.5±20.4* Treated: 79.8±23.4*	Mean±SD	Supine in the morning	Plasma + Inhibitor / RIA	Children and adolescents with essential hypertension	Simões e Silva et al. 2004 [132]
5.8±3.6 years	Mean±SD	30		13±7		Mean±SD	During cardiac catheterization	n/a	Children with VSD or repaired VSD/ASD	Saiki et al. 2016 [210]
5.9-16.8 Years	Range	21	332.1±54.5*	113.2±25.8*	419.2±82.2*	Mean±SD	Supine in the morning.	Plasma + Inhibitor / RIA	Children and adolescents with ESRD	Simões e Silva et al. 2006 [151]
6–18 years	Range	8		42 (15–102)		Median (IQR)	During cardiac catheterization	Plasma / RIA	Children and adolescents underwent BDG with pulmonary atresia, pulmonary stenosis, tricuspid atresia or transposition of the great arteries	Hjortdal et al. 2000 [136]
8.7±3.5 years	Mean±SD	25		78.9±26.2		Mean±SD	In the morning. After 12 weeks of treatment	Serum / RIA	Normotensive children and adolescents with SRINS receiving prednisone+fosinopril	YI et al. 2006 [152]
8.7±3.7 years	Mean±SD	20		79.0±35.8		Mean±SD	In the morning. After 12 weeks of treatment	Serum / RIA	Normotensive children and adolescents with SRINS receiving prednisone	YI et al. 2006 [152]

Age	Statistical operator	N	AngI [pg/mL]	AngII [pg/mL]	Ang1-7 [pg/mL]	Statistical operator	Sampling procedure	Analytic	Collective	Reference
10 years	Single value	1	Inferior vena cava: 990 Right ventricle: 1200	Inferior vena cava: 16 Right ventricle:10		Single value	n/a	n/a	A female child with pulmonary hypertension and extrahepatic portal hypertension	Tokiwa et al. 1992 [211]
10.4±4.6	Mean±SD	6	Before medication: 80-2100 With medication: 390-5370	Before medication 53-3530 With medication: 0-700		Range	Supine in the morning before/after captopril administration	n/a	Children and adolescents with severe hypertension and diverse renal diseases	Friedman et al. 1980 [212]
12.4±5.3 years	Mean±SD	6		30.16±15.81		Mean±SD	Before haemodialysis	Serum + EDTA / RIA	Anephric hypertensive adolescents receiving haemodialysis	Seguias et al. 2001 [154]
13.8±3.7 years	Mean±SD	6		35.8±10.5		Mean±SD	Before haemodialysis	Serum + EDTA / RIA	Anephric normotensive adolescents receiving haemodialysis	Seguias et al. 2001 [154]
14.7±3.4 years	Mean±SD	30		7.4±0.7*		Mean±SD	n/a	Serum / ELISA	Adolescent kidney transplant recipients	Gheissari et al. 2013 [140]
15±0.4 years	Mean±SD	18		Supine position: 27.7±3# After 15 min of HUT test: 38.1±4.2#	Supine position: 12.9±1.8# After 15 min of HUT test: 15.8±1.7#	Mean±SD	Supine During HUT test	RIA	Adolescents with symptoms for orthostatic intolerance with normal HUT test	Wagoner et al. 2016 [213]
15±0.4 years	Mean±SD	30		Supine position: 30±2.2# After 15 min of HUT test: 48.6±3.6#	Supine position: 14.9±1.1# After 15 min of HUT test: 16.5±1.6#	Mean±SD	Supine During HUT test	RIA	Adolescents with symptoms for orthostatic intolerance with abnormal HUT test	Wagoner et al. 2016 [213]

*n/a: data not available; SD: standard deviation; IQR: interquartile Range; SE: standard error; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay; BDG: bidirectional Glenn, ASD: atrial septal defect; VSD: ventricular septal defect; CRF: chronic renal failure; ESRD: end-stage renal disease; HUT: head-up tilt; *: significant higher values compared to healthy control group; #: values generated via GetData Graph Digitizer 2.26.0.20, mean of three times conduct.*

Appendix 10.1-4: Plasma renin activity, specifications and analytics of investigated healthy population.

Age (Range)	Statistical operator	N	PRA (ng/mL/h)	Statistical operator	Sampling procedure	Analytic	Matrix	Collective	Reference
16 h-1 month	Range	17	25±5 (1.5-70)	Mean±SD (range)	Supine after 2h rest	RIA	Plasma EDTA	Newborns	Blazy et al. 1989 [214]
1-2 days	Range	10	24.8±8.4 (3.7-96)	Mean±SD (range)	Supine in the morning	RIA	Plasma EDTA	Newborns	Godard et al. 1979 [215]
2-6 days	Range	15	24.7±2.84	Mean±SEM	Recumbent position in the morning	RIA	Plasma	Newborns	Garcia del Rio et al. 1982 [216]
6 days		21	1.53	Mean	Supine in the morning	RIA	Plasma EDTA	Newborns	Dillon et al. 1976 [156]
7-9 days	Range	9	5.8±1.5 (1.1-13.8)	Mean±SD (range)	Supine in the morning	RIA	Plasma EDTA	Newborns	Godard et al. 1979 [215]
12-22 days	Range	10	8.73±0.95	Mean±SEM	Recumbent position in the morning	RIA	Plasma	Newborns	Garcia del Rio et al. 1981 [216]
1 week-3 months	Range	15	9.8 (4.0-23.8)	Mean (range)	supine/fasting in the morning	RIA	Plasma EDTA	Newborns and infants	Fiselier et al. 1984 [157]
1 week-3 months	Range	9	12.7±4.8#	Mean±SD	supine/fasting in the morning	RIA	Plasma EDTA	Newborns and infants	Fiselier et al. 1983 [124]
4-9 weeks	Range	9	8.1±1.3 (3.5-12.4)	Mean±SD (Range)	Supine in the morning	RIA	Plasma EDTA	Infants	Godard et al. 1979 [215]
1 month-1 year	Range	18	1.459 (0.472-3.130)	Mean (range)	Supine in the morning/midday	RIA	Plasma	Newborns and infants	Dillon et al. 1975 [158]
1 month-1 year	Range	11	18±4 (0.6-40)	Mean±SD (range)	Supine after 2h rest	RIA	Plasma EDTA	Newborns and infants	Blazy et al. 1989 [214]
1-12 months	Range	20	9.99 (3.9-32.0)	Mean (range)	Supine in the morning	RIA	Plasma	Infants	Gjuric et al. 1982 [217]

Age (Range)	Statistical operator	N	PRA (ng/mL/h)	Statistical operator	Sampling procedure	Analytic	Matrix	Collective	Reference
1 month-3 years	Range	14	4.6±1.6	Mean±SD	Sitting/supine	RIA	Plasma	Newborns and infants	Stalker et al. 1976 [218]
2-12 months	Range	20	6.16	Mean	Supine in the morning	RIA	Plasma	Infants	Mardesic et al. 1979 [219]
3-12 months	Range	32	4.5 (1.5-10.2)	Mean (Range)	Supine/fasting in the morning	RIA	Plasma EDTA	Infants	Fiselier et al. 1984 [157]
3-12 months	Range	16	6.3±2.9#	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Infants	Fiselier et al. 1983 [124]
1-4 years	Range	11	5.6±2.7#	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Infants and children	Fiselier et al. 1983 [124]
1-4 years	Range	20	4.4 (1.7-11.8)	Mean (range)	Supine/fasting in the morning	RIA	Plasma EDTA	Infants and children	Fiselier et al. 1984 [157]
1-4 years	Range	18	0.757 (0.110-2.610)	Mean (range)	Supine in the morning/midday	RIA	Plasma	Infants and children	Dillon et al. 1975 [158]
1-5 years	Range	10	8±1.5 (0.8-16.4)	Mean±SD (range)	Supine after 2h rest	RIA	Plasma EDTA	Infants and children	Blazy et al. 1989 [214]
2-10 years	Range	4	1.52±0.21	Mean±SD	Upright position	RIA	Plasma EDTA	Boys	Lall et al. 1995 [220]
2-10 years	Range	2	1.30±0.4	Mean±SD	Upright position	RIA	Plasma EDTA	Girls	Lall et al. 1995 [220]
3-6 years	Range	17	2.5±0.5	Mean±SD	Sitting/supine	RIA	Plasma	Children	Stalker et al. 1976 [218]
4-6 years	Range	36	3.42±2.02	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Children	Fukushige et al. 1993 [221]
4-8 years	Range	17	2.9 (0.9-9.2)	Mean (range)	Supine/fasting in the morning	RIA	Plasma EDTA	Children	Fiselier et al. 1984 [157]
4-8 years	Range	9	2.9±1.9#	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Children	Fiselier et al. 1983 [124]

Age (Range)	Statistical operator	N	PRA (ng/mL/h)	Statistical operator	Sampling procedure	Analytic	Matrix	Collective	Reference
4-16 years	Range	211	2.4 (1.6-3.2)	Median (Q1-Q3)	Sitting	RIA	Plasma	Children and adolescents	Martinez-Aguayo et al. 2010 [222]
5-9 years	Range	24	0.417 (0.131-0.834)	Mean (range)	Supine in the morning/midday	RIA	Plasma	Children	Dillon et al. 1975 [158]
5-10 years	Range	25	2.44±0.69 (1.2-3.9)	Mean±SD (range)	12 h fasting / after 30 min in sitting position	RIA	Plasma EDTA	Children	Abd-Allah et al. 2004 [177]
5-16 years	Range	19	3.5±0.7 (0.6-11)	Mean±SD (range)	supine after 2h rest	RIA	Plasma EDTA	Children and adolescents	Blazy et al. 1989 [214]
5-17 years	Range	302	2.47 (0.09)	geometric mean	Sitting in the morning	RIA	Plasma	Children and adolescents	Tu et al. 2017 [223]
6 and 9 years	Range	2	2.1±1.8#	Mean±SD	Supine	RIA	Plasma	Prepubertal boys	Tiosano et al. 2011[135]
6-9 years	Range	24	1.4±0.3	Mean±SD	Sitting/supine	RIA	Plasma	Children	Stalker et al. 1976 [218]
7-9 years	Range	38	3.03±1.43	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Children	Fukushige et al. 1993 [221]
7-15 years	Range	8	2.98±0.57	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Children and adolescents	Dechaux et al. 1982 [224]
8-13 years	Range	9	2.8±1.9#	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Children and adolescents	Fiselier et al. 1983 [124]
8-16 years	Range	19	2 (0.9-7.6)	mean (range)	Supine/fasting in the morning	RIA	Plasma EDTA	Children and adolescents	Fiselier et al. 1984 [157]
8-12 years	Range	16	Before exercise: 3.22±1.24 After exercise: 5.72±1.45	Mean±SD	Sitting	RIA	Plasma	Children and adolescents	Arvay et al. 1982 [225]
9-12 years	Range	16	1.9±0.5	Mean±SD	Sitting/supine	RIA	Plasma	Children and adolescents	Stalker et al. 1976 [218]

Age (Range)	Statistical operator	N	PRA (ng/mL/h)	Statistical operator	Sampling procedure	Analytic	Matrix	Collective	Reference
10-12 years	Range	41	2.62±1.32	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Children and adolescents	Fukushige et al. 1993 [221]
10-14 years	Range	190	1.71±0.82	Mean±SD	Supine	RIA	Plasma EDTA	Boys	Shibutani et al. 1988 [226]
10-14 years	Range	188	1.63±0.69	Mean±SD	Supine	RIA	Plasma EDTA	Girls	Shibutani et al. 1988 [226]
10-15 years	Range	19	0.321 (0.55-0.899)	mean (Range)	Supine in the morning/midday	RIA	Plasma	Children and adolescents	Dillon et al. 1975 [158]
10-18 years	Range	195	2.5±1.9 (0.1-13.5)	Mean±SD (range)	Sitting	RIA	Plasma EDTA	Children and adolescents	Harshfield et al. 1993 [227]
11-15 years	Range	24	3.07±1.07 (1.6-5.4)	Mean±SD (range)	12 h fasting / after 30 min sitting position	RIA	Plasma EDTA	Adolescents	Abd-Allah et al. 2004 [177]
12-15 years	Range	16	1.8±0.3	Mean±SD	Sitting/supine	RIA	Plasma	Adolescents	Stalker et al. 1976 [218]
12.3±2.5 years	Mean±SD	24	2.5±0.4	Mean±SD	Supine/fasting in the morning	RIA	Plasma	Adolescents	Simsolo et al. 1988 [228]
12.6±2.2 years	Mean±SD	74	3.2±2	Mean±SD	Sitting	RIA	Plasma EDTA	Adolescents	Harshfield et al. 1991 [229]
12-17 years	Range	4	2.6±1.2#	Mean±SD	Supine	RIA	Plasma	Pubertal girls	Tiosano et al. 2011 [135]
13-15 years	Range	41	2.07±1.14	Mean±SD	Supine/fasting in the morning	RIA	Plasma EDTA	Adolescents	Fukushige et al. 1993 [221]
14±2.4 years	Mean±SD	66	3.4±2.4	Mean±SD	Sitting	RIA	Plasma EDTA	Adolescents	Harshfield et al. 1991 [229]
15-18 years	Range	10	1.8±0.4	Mean±SD	Sitting/supine	RIA	Plasma	Adolescents	Stalker et al. 1976 [218]

SD: standard deviation; SEM: standard error of the mean; § values calculated from pmol/L to pg/mL; #: values generated via GetData Graph Digitizer 2.26.0.20, mean of three times conduct.

Appendix 10.1-5: Plasma renin activity, specifications and analytics of investigated diseased population.

Age	Statistical operator	N	PRA (ng/mL/h)	Statistical operator	Sampling procedure	Analytic	Matrix	Collective	Reference
1 month-11.7 years	Range	9	Baseline:31.5±7.2 Carvedilol: 21.5±5.7	Mean±SD	n/a	RIA	Plasma	Children and infants with severe cardiac failure receiving carvedilol	Giardini et al. 2003 [230]
14-84 days	Range	11	84±21 (57-126)	Mean±SD (Range)	Supine in the morning	RIA	Plasma EDTA	Infants with CHD with left to right shunts	Scammell et al. 1987 [164]
19-111 days	Range	8	87±45	Mean±SD	Supine in the morning	RIA	Plasma EDTA	Children with CHD with left to right shunts receiving diuretics and captopril	Scammell et al. 1988 [231]
19-111 days	Range	6	69.8± 34	Mean±SD	In the morning	RIA	Plasma	CHD children with left to right shunts and PH or high pulmonary blood flow receiving captopril	Scammel et al. 1989 [232]
4 weeks-15 years	Range	12	Before: 3.2 (1.5-36.2) After 2 days: 29.4 (13-52) After 3 months: 12.4 (8.7-28.2)	Median (95% confidence interval)	Supine	RIA	Plasma EDTA	Children with DCM receiving digitalis and/or furosemide	Stern et al. 1990 [165]
1.3±2.8	Mean±SD	27	16±16	Mean±SD	During catheterization	n/a	Plasma	1-ventricular circulation other than HLHS and pulmonary atresia with AP shunt	Saiki et al. 2016 [210]
1.6±1	Mean±SD	29	63±43	Mean±SD	During catheterization	n/a	Plasma	HLHS patients after the Norwood procedure	Saiki et al. 2016 [210]
2-9 years	Range	9	25 (34)	mean (SEM)	Before elective cardiac surgery	RIA	Plasma EDTA	Children with Fallot's tetralogy, TCPC, and ASD undergoing cardiopulmonary bypass	Ationu et al. 1993 [160]
2-18 years	Range	22	3.9±2.9*	Mean±SD	Upright position	RIA	Plasma	Children with coarctation of the aorta	Fallo et al. 1978 [166]
30.6±40.7 months	Mean±SD	31	13.1±11.8	Mean±SD	Supine in the morning	RIA	Plasma EDTA	Diverse congenital diseases	Alvarez Kindelan et al. 1994 [161]
3±2 years	Mean±SD	10	88±64	Mean±SD	n/a	RIA	Plasma	Children with CHD and left-to-right shunt (RR ≤ 50 min-1)	Buchhorn et al. 2001 [162]

Age	Statistical operator	N	PRA (ng/mL/h)	Statistical operator	Sampling procedure	Analytic	Matrix	Collective	Reference
41.1±25.4 months	Mean±SD	21	13.57±9.1$	Mean±SD	Supine in the morning	RIA	Plasma EDTA	Children with CHD	Alvarez Kindelan et al. 1994 [161]
4±2 years	Mean±SD	7	35±40	Mean±SD	n/a	RIA	Plasma	Children with CHD and left-to-right shunt (RR ≤ 50 min-1)	Buchhorn et al. 2001 [162]
5-16 years	Range	8	21.4±11.3$	Mean±SD	Upright	RIA	Plasma	Children with coarctation of the thoracic aorta receiving furosemide	Parker et al. 1982 [163]
5-16 years	Range	8	5.5±1.5	Mean±SD	Upright	RIA	Plasma	Children with corrected coarctation of the thoracic aorta receiving furosemide	Parker et al. 1982 [163]
5-18 years	Range	12	1.7±0.8*	Mean±SD	Upright position	RIA	Plasma	Children with coarctation of aorta after surgery	Fallo et al. 1978 [166]
5.8±3.6 years	Mean±SD	30	6±5	Mean±SD	during catheterization	n/a	n/a	Children with repaired VSD	Saiki et al. 2015 [210]
6±2 years	Mean±SD	10	10±7	Mean±SD	n/a	RIA	Plasma	Children with CHD and left-to-right shunt (RR ≤ 50 min-1)	Buchhorn et al. 2001 [162]
8.8±1 years	Mean±SD	7	2.1±1.6#	mean±SEM	supine in the morning before surgery	RIA	Plasma	Children before repair of coarctation of the aorta	Gidding et al. 1985 [167]
8.8±1.7 years	Mean±SD	7	2.1±1.5#	mean±SEM	supine in the morning before surgery	RIA	Plasma	Children before repair of coarctation of aorta receiving propranolol	Gidding et al. 1985 [167]

SD: standard deviation; SEM: standard error of the mean; § values calculated from pmol/L to pg/mL; #: values generated via GetData Graph Digitizer 2.26.0.20, mean of three times conduct, HLHS: hypoplastic left heart syndrome, ASD atrial septal defect, CHD: congenital heart disease, TCPC: total cavopulmonary connection, PH: pulmonary hypertension, RR: respiratory rate $: recalculated from ng/l/s.

10.2 Customisation and validation of a plasma renin activity immunoassay

Appendix 10.2-1: Inter-run accuracy and precision results for the plasma renin activity assay validation.

	Validity of the analytical run							Accuracy and Precision									
	NV (ng/mL)	Rep 1 (ng/mL)	Rep 2 (ng/mL)	Mean	AE (%)	Stdev	CV (%)	Sample	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)
Run 1	Calibration curve								QC 0.2		QC 0.5		QC 1.5		QC 10		QC 60	
	0.2	0.190		0.190	5.000			1	0.210	5.1	0.402	-19.7	1.409	-6.0	9.941	-0.6	7.000	-88.3
	0.5	0.503		0.503	0.698			2	0.185	-7.3	0.518	3.5	1.361	-9.3	9.635	-3.7	59.387	-1.0
	1.5	1.501	1.513	1.507	0.447	0.009	0.573	3	0.183	-8.4	0.532	6.3	1.631	8.8	9.751	-2.5	72.679	21.1
	4	3.964	3.992	3.978	0.551	0.020	0.507	4	0.176	-12.0	0.461	-7.7	1.588	5.9	10.219	2.2	60.418	0.7
	10	9.998	10.039	10.018	0.184	0.029	0.291	5	0.159	-20.3	0.455	-9.1	1.526	1.7	9.840	-1.6	60.628	1.0
	25	25.557	24.601	25.079	0.316	0.676	2.695	Mean	0.183	-8.6	0.473	-5.3	1.503	0.2	9.877	-1.2	52.022	-13.3
	60	61.487	58.199	59.843	0.262	2.325	3.885	Std	0.018		0.052		0.115		0.222		25.751	
								CV (%)	10.057		11.075		7.678		2.247		49.500	
	Quality controls							TE (%)	18.613		16.413		7.470		1.020		36.204	
	0.98	0.940	0.978	0.959	2.173	0.027	2.803											
	5.04	4.765	4.682	4.724	6.278	0.058	1.233											
	42.5	43.016	40.077	41.547	2.244	2.078	5.002											
Run 2	Calibration curve								QC 0.2		QC 0.5		QC 1.5		QC 10		QC 60	
	0.2	0.168	0.159	0.164	18.163	0.007	4.091	1	0.128	-36.2	0.518	3.6	1.456	-2.9	9.762	-2.4	60.069	0.1
	0.5	0.545	0.565	0.555	11.014	0.015	2.645	2	0.170	-15.0	0.442	-11.6	1.519	1.2	10.012	0.1	61.878	3.1
	1.5	1.418	1.524	1.471	1.953	0.075	5.077	3	0.206	3.1	0.475	-5.0	1.397	-6.9	9.954	-0.5	69.550	15.9
	4	3.811	4.158	3.985	0.379	0.245	6.156	4	0.205	2.6	0.492	-1.6	1.384	-7.7	9.241	-7.6	64.059	6.8
	10	9.823	10.298	10.061	0.607	0.336	3.336	5	0.171	-14.3	0.523	4.6	1.479	-1.4	9.681	-3.2	59.851	-0.2
	25	25.186	25.048	25.117	0.468	0.098	0.389	Mean	0.176	-11.9	0.490	-2.0	1.447	-3.5	9.730	-2.7	63.081	5.1
	60	58.994	62.155	60.575	0.958	2.235	3.690	Std	0.032		0.033		0.056		0.305		3.992	
								CV (%)	18.317		6.771		3.891		3.134		6.329	
	Quality controls							TE (%)	30.247		8.784		7.427		5.833		11.465	
	0.98	0.983	1.038	1.010	3.078	0.039	3.870											
	5.04	5.144	5.088	5.116	1.505	0.039	0.764											
	42.5	41.392	41.097	41.245	2.954	0.209	0.506											

	Validity of the analytical run							Accuracy and Precision									
	NV (ng/mL)	Rep 1 (ng/mL)	Rep 2 (ng/mL)	Mean	AE (%)	Stdev	CV (%)	Sample	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)
Run 3	Calibration curve								QC 0.2		QC 0.5		QC 1.5		QC 10		QC 60	
	0.2	0.207	0.182	0.195	2.633	0.018	9.103	1	0.184	-8.2	0.427	-14.7	1.423	-5.2	10.363	3.6	69.969	16.6
	0.5	0.500	0.515	0.508	1.539	0.011	2.079	2	0.185	-7.3	0.548	9.5	1.339	-10.8	10.072	0.7	71.679	19.5
	1.5	1.453	1.554	1.503	0.230	0.072	4.793	3	0.196	-2.1	0.605	21.1	1.590	6.0	10.240	2.4	78.658	31.1
	4	3.808	4.121	3.964	0.896	0.221	5.585	4	0.141	-29.3	0.556	11.1	1.608	7.2	10.388	3.9	67.101	11.8
	10	9.926	10.376	10.151	1.510	0.318	3.135	5	0.143	-28.4	0.605	21.1	1.519	1.3	10.191	1.9	62.058	3.4
	25	24.607	24.643	24.625	1.500	0.025	0.103	Mean	0.170	-15.1	0.548	9.6	1.496	-0.3	10.251	2.5	69.893	16.5
	60	60.308	60.797	60.553	0.921	0.346	0.571	Std	0.026		0.073		0.114		0.129		6.107	
								CV (%)	15.090		13.332		7.624		1.263		8.738	
	Quality controls							TE (%)	30.166		22.958		7.914		3.771		25.227	
	0.98	1.081	1.094	1.088	10.974	0.009	0.826											
	5.04	5.087	4.910	4.999	0.819	0.125	2.498											
	42.5	45.847	39.804	42.826	0.766	4.273	9.978											
Run 4	Calibration curve								QC 0.2		QC 0.5		QC 1.5		QC 10		QC 60	
	0.2	0.191	0.204	0.197	1.393	0.009	4.600	1	0.181	-9.5	0.504	0.9	1.578	5.2	9.660	-3.4	58.846	-1.9
	0.5	0.511	0.493	0.502	0.478	0.013	2.508	2	0.188	-6.1	0.462	-7.5	1.493	-0.5	9.843	-1.6	54.603	-9.0
	1.5	1.460	1.554	1.507	0.457	0.067	4.416	3	0.228	14.1	0.517	3.4	1.479	-1.4	10.216	2.2	68.941	14.9
	4	3.880	4.094	3.987	0.334	0.151	3.797	4	0.247	23.6	0.532	6.4	1.521	1.4	9.794	-2.1	63.341	5.6
	10	9.751	10.246	9.998	0.017	0.350	3.504	5	0.261	30.7	0.548	9.7	1.490	-0.7	9.821	-1.8	63.341	5.6
	25	24.538	25.817	25.178	0.710	0.904	3.592	Mean	0.221	10.6	0.513	2.6	1.512	0.8	9.867	-1.3	61.814	3.0
	60	56.140	63.598	59.869	0.218	5.274	8.809	Std	0.036		0.033		0.040		0.208		5.391	
								CV (%)	16.125		6.367		2.638		2.106		8.722	
	Quality controls							TE (%)	26.692		8.937		3.455		3.440		11.746	
	0.98	1.071	1.038	1.055	7.607	0.024	2.233											
	5.04	4.949	5.124	5.036	0.073	0.123	2.449											
	42.5	45.847	39.804	42.826	0.766	4.273	9.978											

	Validity of the analytical run							Accuracy and Precision									
	NV (ng/mL)	Rep 1 (ng/mL)	Rep 2 (ng/mL)	Mean	AE (%)	Stdev	CV (%)	Sample	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)	Value (ng/mL)	Dev (%)
Run 5	Calibration curve								QC 0.2		QC 0.5		QC 1.5		QC 10		QC 60	
	0.2	0.186	0.206	0.196	2.105	0.014	7.216	1	0.216	8.0	0.435	-13.0	1.431	-4.6	9.915	-0.9	65.657	9.4
	0.5	0.476	0.535	0.506	1.137	0.042	8.254	2	0.229	14.3	0.485	-2.9	1.368	-8.8	9.915	-0.9	66.573	11.0
	1.5	1.463	1.538	1.500	0.030	0.053	3.539	3	0.262	31.1	0.644	28.8	1.625	8.3	10.127	1.3	56.236	-6.3
	4	3.934	4.070	4.002	0.047	0.096	2.389	4	0.180	-10.0	0.527	5.3	1.590	6.0	10.423	4.2	62.247	3.7
	10	9.790	10.115	9.952	0.476	0.230	2.311	5	0.218	8.9	0.542	8.5	1.658	10.5	10.254	2.5	60.178	0.3
	25	25.405	25.131	25.268	1.072	0.194	0.767	Mean	0.221	10.4	0.527	5.3	1.534	2.3	10.127	1.3	62.178	3.6
	60	57.790	61.194	59.492	0.847	2.407	4.046	Std	0.029		0.078		0.128		0.220		4.205	
								CV (%)	13.339		14.723		8.313		2.173		6.763	
	Quality controls							TE (%)	23.774		20.056		10.582		3.441		10.393	
	0.98	1.054	1.119	1.087	10.872	0.046	4.250											
	5.04	4.803	4.917	4.860	3.570	0.081	1.657											
	42.5	45.440	35.738	40.589	4.496	6.860	16.902											
Run 6	Calibration curve								QC 0.2		QC 0.5		QC 1.5		QC 10		QC 60	
	0.2	0.193	0.189	0.191	4.363	0.003	1.549	1	0.215	7.7	0.480	-4.0	1.481	-1.3	10.130	1.3	61.572	2.6
	0.5	0.512	0.507	0.509	1.897	0.004	0.817	2	0.155	-22.3	0.502	0.5	1.436	-4.2	9.710	-2.9	60.828	1.4
	1.5	1.512	1.506	1.509	0.601	0.005	0.320	3	0.187	-6.7	0.583	16.5	1.660	10.7	10.055	0.6	60.308	0.5
	4	3.923	3.964	3.943	1.416	0.029	0.726	4	0.149	-25.5	0.467	-6.7	1.659	10.6	10.452	4.5	68.047	13.4
	10	10.196	10.070	10.133	1.330	0.089	0.879	5	0.186	-7.2	0.462	-7.7	1.584	5.6	10.227	2.3	56.648	-5.6
	25	25.308	24.282	24.795	0.820	0.725	2.926	Mean	0.178	-10.8	0.499	-0.3	1.564	4.3	10.115	1.1	57.564	-4.1
	60	57.940	62.670	60.305	0.508	3.345	5.546	Std	0.027		0.049		0.102		0.271		4.132	
								CV (%)	15.061		9.911		6.545		2.681		7.177	
	Quality controls							TE (%)	25.866		10.172		10.809		3.828		11.237	
	0.98	1.141	1.107	1.124	14.720	0.024	2.157											
	5.04	4.805	4.823	4.814	4.491	0.013	0.263											
	42.5	39.032	33.627	36.330	14.519	3.822	10.520											
Inter-run	Inter-run accuracy								4.23%		-1.65%		-0.62%		0.06%		-1.82%	
	Inter-run precision								12.11%		5.28%		2.60%		1.99%		9.76%	
	Inter-run total error								7.89%		6.93%		3.23%		2.05%		11.58%	

Six runs were conducted over 6 different days. QC: quality control; NV: nominal value; Rep: replicate; AE: absolute error; Std: standard deviation; CV: coefficient of deviation; Dev: deviation from nominal value; TE: total error.

Appendix 10.2-2: Results of the conducted one-way ANOVA for inter-run accuracy and precision for the plasma renin activity assay validation

0.2 ng/mL

Description

Group	Count	Sum	Mean	Variance	Value 1	Value 2	Value 3	Value 4	Value 5
Run 1	5	0.9144	0.1829	0.0003	0.2103	0.1855	0.1833	0.1759	0.1595
Run 2	5	0.8807	0.1761	0.0010	0.1276	0.1701	0.2062	0.2053	0.1715
Run 3	5	0.8492	0.1698	0.0007	0.1835	0.1853	0.1959	0.1414	0.1432
Run 4	5	1.1057	0.2211	0.0013	0.1810	0.1878	0.2282	0.2473	0.2615
Run 5	5	1.1044	0.2209	0.0009	0.2159	0.2285	0.2622	0.1800	0.2177
Run 6	5	0.8920	0.1784	0.0007	0.2155	0.1553	0.1865	0.1490	0.1857

ANOVA

Source of variation	Square sum	Degree of freedom	Mean square sum	F-value	P-value	Critical F-value
Difference between groups	0.01345715	5	0.002691431	3.29729728	0.02087196	2.620654148
Within groups	0.01959009	24	0.000816254			

Calculation of repeatability (%)	Calculation of time-different intermediate precision (%)	Calculation of variation between days
14.92	**17.54**	**0.0003**

0.5 ng/mL

Description

Group	Count	Sum	Mean	Variance	Value 1	Value 2	Value 3	Value 4	Value 5
Run 1	5	2.3665	0.4733	0.0027	0.4016	0.5175	0.5316	0.4613	0.4545
Run 2	5	2.4497	0.4899	0.0011	0.5179	0.4421	0.4749	0.4918	0.5231
Run 3	5	2.7407	0.5481	0.0053	0.4266	0.5477	0.6053	0.5557	0.6053
Run 4	5	2.5643	0.5129	0.0011	0.5045	0.4624	0.5169	0.5322	0.5483
Run 5	5	2.6333	0.5267	0.0060	0.4352	0.4854	0.6438	0.5266	0.5424
Run 6	5	2.4935	0.4987	0.0024	0.4802	0.5023	0.5825	0.4667	0.4617

ANOVA

Source of variation	Square sum	Degree of freedom	Mean square sum	F-value	P-value	Critical F-value
Difference between groups	0.01799256	5	0.003598512	1.1539867	0.36007178	2.620654148
Within groups	0.07483994	24	0.003118331			

Calculation of repeatability (%)	Calculation of time-different intermediate precision (%)	Calculation of variation between days
10.99	**11.13**	**0.00008**

1.5 ng/mL

Description

Group	Count	Sum	Mean	Variance	Value 1	Value 2	Value 3	Value 4	Value 5
Run 1	5	7.5156	1.5031	0.0133	1.4094	1.3607	1.6314	1.5882	1.5259
Run 2	5	7.2348	1.4470	0.0032	1.4564	1.5186	1.3970	1.3841	1.4787
Run 3	5	7.4783	1.4957	0.0130	1.4227	1.3386	1.5901	1.6077	1.5192
Run 4	5	7.5613	1.5123	0.0016	1.5781	1.4926	1.4794	1.5210	1.4902
Run 5	5	7.6702	1.5340	0.0163	1.4306	1.3675	1.6247	1.5897	1.6577
Run 6	5	7.8198	1.5640	0.0105	1.4805	1.4363	1.6602	1.6587	1.5841

ANOVA

Source of variation	Square sum	Degree of freedom	Mean square sum	F-value	P-value	Critical F-value
Difference between groups	0.03859528	5	0.007719056	0.80098054	0.55996887	2.620654148
Within groups	0.23128819	24	0.009637008			

Calculation of repeatability (%)	Calculation of time-different intermediate precision (%)	Calculation of variation between days
6.50	**6.40**	**-0.0003**

10 ng/mL

Description

Group	Count	Sum	Mean	Variance	Value 1	Value 2	Value 3	Value 4	Value 5
Run 1	5	49.3866	9.8773	0.0493	9.9414	9.6346	9.7512	10.2190	9.8404
Run 2	5	48.6503	9.7301	0.0930	9.7620	10.0120	9.9538	9.2412	9.6813
Run 3	5	51.2540	10.2508	0.0168	10.3630	10.0720	10.2400	10.3880	10.1910
Run 4	5	49.3330	9.8666	0.0432	9.6602	9.8425	10.2160	9.7936	9.8207
Run 5	5	50.6338	10.1268	0.0484	9.9149	9.9149	10.1270	10.4230	10.2540
Run 6	5	50.5739	10.1148	0.0735	10.1300	9.7099	10.0550	10.4520	10.2270

ANOVA

Source of variation	Square sum	Degree of freedom	Mean square sum	F-value	P-value	Critical F-value
Difference between groups	0.98833837	5	0.197667675	3.6591025	0.01331519	2.620654148
Within groups	1.2964994	24	0.054020808			

Calculation of repeatability (%)	Calculation of time-different intermediate precision (%)	Calculation of variation between days
2.33	**2.79**	**0.0239**

60 ng/mL

Description

Group	Count	Sum	Mean	Variance	Value 1	Value 2	Value 3	Value 4	Value 5
Run 1	5	316.3970	63.2794	29.6802	63.2850	59.3870	72.6790	60.4180	60.6280
Run 2	5	315.4070	63.0814	15.9392	60.0690	61.8780	69.5500	64.0590	59.8510
Run 3	5	349.4650	69.8930	37.3008	69.9690	71.6790	78.6580	67.1010	62.0580
Run 4	5	309.0720	61.8144	29.0663	58.8460	54.6030	68.9410	63.3410	63.3410
Run 5	5	310.8910	62.1782	17.6829	65.6570	66.5730	56.2360	62.2470	60.1780
Run 6	5	307.4030	61.4806	17.0702	61.5720	60.8280	60.3080	68.0470	56.6480

ANOVA

Source of variation	Square sum	Degree of freedom	Mean square sum	F-value	P-value	Critical F-value
Difference between groups	248.363151	5	49.67263011	2.03105219	0.11025247	2.620654148
Within groups	586.95839	24	24.45659957			

Calculation of repeatability (%)	Calculation of time-different intermediate precision (%)	Calculation of variation between days
7.77	**8.41**	**4.2027**

The repeatability, different intermediate precision, and variation between days were calculated for all five quality control levels (0.2, 0.5, 1.5, 10, and 60ng/mL).

Appendix 10.2-3: Results of precision over the whole process for the plasma renin activity assay validation.

Run	Mean value source 1	Mean value source 2	Mean value source 3	Mean value source 4	Mean value source 5	Mean value source 6	Mean value source 7	Date of run
Run 1	2.76	4.32	0.76	2.89	1.91	0.89	3.57	08.05.2017
Run 2	2.73	4.68	0.68	2.53	1.78	0.75	3.77	23.05.2017
Run 3	2.68	4.21	0.84	2.67	1.92	0.90	3.94	06.06.2017
Run 4	2.70	4.66	0.77	2.72	1.68	0.77	3.23	20.06.2017
Run 5	2.96	4.63	0.77	2.63	1.78	0.84	3.11	04.07.2017
Run 6	2.68	4.21	0.72	2.60	1.93	0.98	3.48	18.07.2017
Run 7	2.77	4.28	0.70	2.64	1.95	0.79	3.75	01.08.2017
Run 8	2.69	4.96	0.70	2.55	1.66	0.82	3.28	15.08.2017
Run 9	2.95	4.81	0.76	2.87	1.87	0.94	3.91	29.08.2017
Run 10	3.08	5.42	0.86	3.06	2.11	1.05	4.21	19.09.2017
Run 11	2.99	5.13	0.83	3.15	2.10	0.81	3.90	10.10.2017
Run 12	3.04	5.06	0.87	3.30	1.95	1.02	3.94	24.10.2017
Run 13	3.05	5.48	0.87	3.17	2.24	1.02	4.16	07.11.2017
Run 14	2.83	5.47	0.82	3.12	2.27	0.96	3.92	21.11.2017
Run 15	2.91	5.33	0.85	3.17	2.31	0.92	3.99	05.12.2017
Mean	**2.85**	**4.84**	**0.79**	**2.87**	**1.96**	**0.90**	**3.74**	
CV	**5.2%**	**9.6%**	**8.3%**	**9.3%**	**10.4%**	**10.9%**	**9.0%**	

Seven human sources were investigated in 15 different runs on 15 different days. The shown plasma renin activity values were obtained by a triplicate measurement of an incubated and non-incubated sample for each source. CV: coefficient of variation.

Appendix 10.2-4: Results of matrix effect experiments for the plasma renin activity assay validation.

Source	Value	Native AngI values (ng/mL)	Spiked AngI values (ng/mL)	Corrected AngI values (ng/mL)	Deviation from nominal* concentration (%)
1	1	0.3147	23.7690	26.0343	-12.5134
	2	0.4862	24.3220	26.4577	
	Mean	0.4005	24.0455	26.2460	
	Std	0.0857	0.2765	0.2117	
	CV [%]	21.4123	1.1499	0.8067	
2	1	0.8734	27.3680	29.4090	-3.2637
	2	0.9957	26.7910	28.6328	
	Mean	0.9346	27.0795	29.0209	
	Std	0.0611	0.2885	0.3881	
	CV [%]	6.5405	1.0654	1.3373	
3	1	1.1586	25.2070	26.6937	-10.3634
	2	1.0982	25.5020	27.0882	
	Mean	1.1284	25.3545	26.8910	
	Std	0.0302	0.1475	0.1972	
	CV [%]	2.6764	0.5818	0.7335	
4	1	0.7996	26.6180	28.6584	-8.2229
	2	0.7152	24.5060	26.4078	
	Mean	0.7574	25.5620	27.5331	
	Std	0.0422	1.0560	1.1253	
	CV [%]	5.5725	4.1311	4.0871	
5	1	0.7967	22.5830	24.1828	-20.4106
	2	0.6960	21.9310	23.5709	
	Mean	0.7464	22.2570	23.8768	
	Std	0.0504	0.3260	0.3059	
	CV [%]	6.7488	1.4647	1.2814	

Native angiotensin I (AngI) values were obtained by the measurement of native samples. Spiked AngI values were obtained by the spiking of native samples with AngI working solution (1.05 µg/mL) to reach a nominal AngI concentration of 30° ng/mL. Corrected AngI values were obtained by subtraction of native AngI values from spiked AngI values. Std: standard deviation; CV: coefficient of variation.

Appendix 10.2-5: Results of the evaluation of parallelism for the plasma renin activity assay validation.

Resulted concentration of the human source	Value	Measured Ang I (ng/mL)	Blank corrected Ang I (ng/mL)	Back calculated mean AngI value (ng/mL)	Deviation from starting value (%)
Blank value (0 ng/mL)	1	0.5351			
	2	0.6320			
	3	0.5270			
	Mean	0.5647			
	Std	0.0477			
	CV [%]	8.4470			
Starting value (60 ng/ml)	1	37.7120	37.1770	37.1770	0
	2	34.2100	33.5780		
	3	35.0350	34.5080		
	Mean	35.6523	35.0877		
	Std	1.4948	1.5254		
	CV [%]	4.1928	4.3473		
Dilution 1 (30 ng/ml)	1	17.2370	16.7020	33.4039	-10.15
	2	16.5940	15.9620		
	3	16.6410	16.1140		
	Mean	16.8240	16.2593		
	Std	0.2927	0.3191		
	CV [%]	1.7396	1.9624		
Dilution 2 (20 ng/ml)	1	10.3390	9.8040	29.5060	-20.63
	2	10.6290	9.9970		
	3	10.2320	9.7050		
	Mean	10.4000	9.8353		
	Std	0.1677	0.1213		
	CV [%]	1.6127	1.2330		
Dilution 3 (15 ng/ml)	1	7.5491	7.0141	27.7341	-25.40
	2	7.3831	6.7511		
	3	7.5624	7.0354		
	Mean	7.4982	6.9335		
	Std	0.0816	0.1293		
	CV [%]	1.0878	1.8644		
Dilution 4 (12.5 ng/ml)	1	6.0130	5.4780	27.3898	-26.33
	2	6.1087	5.4767		
	3	5.8206	5.2936		
	Mean	5.9808	5.4161		
	Std	0.1198	0.0866		
	CV [%]	2.0032	1.5994		
Mean of all back calculated AngI values (ng/mL)				31.0421	
Std of all back calculated AngI values (ng/mL)				4.1790	
Precision between samples of dilution series				13.46%	

The blank corrected angiotensin I (AngI) value was obtained by subtracting the measured blank value (measurement of the human source without spiked AngI) from the measured AngI value. The back calculated mean AngI value (n=3) was obtained by multiplying the blank corrected AngI value with the dilution factor. Std: standard deviation; CV: coefficient of variation.

Appendix 10.2-6: Long term stability of plasma renin activity in 7 human sources for the plasma renin activity assay validation.

Run	Week	Value	Source 1			Source 2			Source 3			Source 4			Source 5			Source 6			Source 7		
			Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)
1.1	1	1	0.5380	4.1713	2.6886	1.0649	8.2719	5.3332	0.5199	1.4797	0.7102	0.5860	4.4510	2.8601	1.5677	4.3414	2.0525	0.4579	1.7174	0.9321	1.9462	7.4794	4.0946
		2	0.5590	4.4622	2.8883	1.1389	8.7485	5.6311	0.4582	1.5995	0.8446	0.5499	4.3860	2.8387	1.5792	4.2017	1.9407	0.4976	1.6943	0.8856	1.9658	7.7144	4.2540
		3	0.5269	4.3189	2.8061	1.1515	7.8855	4.9832	0.4498	1.5207	0.7925	0.6013	4.6316	2.9824	1.5398	4.4436	2.1488	0.4508	1.8296	1.0203	2.0468	6.8149	3.5284
		Mean	0.5413	4.3175	2.7943	1.1184	8.3020	5.3158	0.4759	1.5333	0.7824	0.5791	4.4895	2.8937	1.5622	4.3289	2.0473	0.4688	1.7471	0.9460	1.9863	7.3362	3.9590
		Std	0.0133	0.1188	0.0820	0.0382	0.3530	0.2648	0.0313	0.0497	0.0553	0.0216	0.1039	0.0633	0.0165	0.0992	0.0851	0.0206	0.0591	0.0559	0.0435	0.3809	0.3113
		CV (%)	3.0158	3.3695	2.9330	4.1833	5.2073	4.9815	8.0481	3.2422	7.0686	4.5589	2.8348	2.1878	1.2961	2.8045	4.1547	5.3785	4.1436	5.9065	2.6860	6.3591	7.8642
1.2	1	1	0.5144	4.2468	2.7620	1.1515	7.4891	4.6898	0.4428	1.4344	0.7338	0.4408	4.3725	2.9095	1.5335	4.0883	1.8906	0.4057	1.5733	0.8641	1.8570	7.3102	4.0354
		2	0.6321	4.3557	2.7555	1.1212	7.6666	4.8436	0.4159	1.5823	0.8632	0.4381	4.4505	2.9692	1.4831	4.0251	1.8811	0.4099	1.6678	0.9309	1.9016	6.6336	3.5017
		3	0.6187	4.4580	2.8411	1.2168	7.0774	4.3368	0.4747	1.3965	0.6822	0.5733	4.3653	2.8061	1.4484	4.2387	2.0648	0.3768	1.6968	0.9768	1.9047	6.9991	3.7699
		Mean	0.5884	4.3535	2.7862	1.1632	7.4110	4.6234	0.4444	1.4711	0.7597	0.4841	4.3961	2.8949	1.4883	4.1174	1.9455	0.3974	1.6460	0.9239	1.8878	6.9810	3.7690
		Std	0.0526	0.0862	0.0389	0.0399	0.2468	0.2121	0.0240	0.0802	0.0761	0.0631	0.0386	0.0674	0.0349	0.0896	0.0845	0.0147	0.0527	0.0463	0.0218	0.2765	0.2179
		CV (%)	10.9530	2.4260	1.3973	4.2027	4.0783	4.5884	6.6224	6.6728	10.0215	15.9680	1.0749	2.3273	2.8759	2.6653	4.3420	4.5364	3.8558	5.0118	1.4127	6.3591	5.7808
1.3	1	1	0.6168	4.2468	2.6862	1.1684	6.8940	4.2369	0.4469	1.4799	0.7644	0.6665	4.4252	2.7814	1.4388	3.9402	1.8510	0.4414	1.5006	0.7838	1.9431	6.7377	3.5480
		2	0.6406	4.3557	2.7492	1.2588	7.2708	4.4489	0.4310	1.5533	0.8305	0.6578	4.5146	2.8540	1.4554	4.0211	1.8986	0.3625	1.6283	0.9367	1.8654	6.7568	3.6196
		3	0.6285	4.4580	2.8339	1.2005	6.9639	4.2649	0.5159	1.4559	0.6956	0.5549	4.6757	3.0494	1.4331	4.0992	1.9729	0.4453	1.7098	0.9357	1.9351	6.7043	3.5292
		Mean	0.6289	4.3535	2.7562	1.2092	7.0429	4.3169	0.4646	1.4964	0.7635	0.6264	4.5385	2.8949	1.4424	4.0202	1.9075	0.4164	1.6129	0.8854	1.9145	6.7329	3.5656
		Std	0.0097	0.0862	0.0605	0.0374	0.1637	0.0940	0.0368	0.0414	0.0551	0.0507	0.1037	0.1132	0.0095	0.0649	0.0502	0.0381	0.0861	0.0719	0.0349	0.0217	0.0390
		CV (%)	1.8962	2.6285	2.1946	3.7877	2.8481	2.1779	9.7111	3.3918	7.2121	0.8005	2.7973	3.9091	0.8005	1.9770	2.6292	11.2160	6.5353	8.1151	2.2310	0.3947	1.0927
Reference PRA value (Mean of first 3 runs)			0.59	4.34	2.75	1.16	7.59	4.71	0.46	1.50	0.76	0.56	4.47	2.87	1.50	4.16	1.95	0.43	1.67	0.91	1.93	7.02	3.73
2	2	1	0.5228	4.0988	2.6462	1.0588	7.2337	4.5694	0.4740	1.3362	0.6380	0.5269	4.0229	2.5871	1.2922	3.5228	1.6506	0.4048	1.5264	0.8300	1.5300	6.4791	3.6623
		2	0.5026	4.2713	2.7888	1.0978	7.3008	4.5902	0.4212	1.3687	0.7011	0.5572	3.8788	2.4580	1.3856	3.7725	1.7663	0.3763	1.2907	0.6767	1.7093	6.2013	3.3241
		3	0.5462	4.2637	2.7510	1.0716	7.6597	4.8752	0.4811	1.4431	0.7119	0.4773	3.9193	2.5471	1.2213	3.8060	1.9127	0.3824	1.4004	0.7533	1.6757	6.4666	3.5453
		Mean	0.5239	4.2113	2.7287	1.0761	7.3981	4.6783	0.4588	1.3827	0.6837	0.5205	3.9403	2.5307	1.2997	3.7004	1.7765	0.3878	1.4058	0.7533	1.6383	6.7329	3.7700
		Std	0.0178	0.0796	0.0603	0.0162	0.1870	0.1395	0.0267	0.0447	0.0326	0.0329	0.0607	0.0539	0.0673	0.1263	0.1072	0.0123	0.0963	0.0626	0.0778	0.1281	0.1403
		CV (%)	4.1562	2.3144	2.2102	1.8501	3.0963	2.9818	7.1319	3.3918	4.7655	0.8005	2.7973	2.1314	6.3418	4.1821	6.0353	3.8727	8.3899	8.3073	5.8180	2.4582	3.7203
		Dev (%)		99.0820			99.3427			89.7629			88.2255			91.1486			82.7687			101.0554	
3	4	1	0.5128	3.9671	2.5562	1.0084	6.7470	4.2466	0.5264	1.6245	0.8126	0.5767	4.0471	2.5681	1.7850	4.2804	1.8466	0.5268	1.7247	0.8864	1.9766	7.1011	3.7921
		2	0.5724	4.1201	2.6253	1.0830	6.8544	4.2708	0.4404	1.5877	0.8490	0.5418	4.0768	2.6159	1.8004	4.0360	1.6543	0.4526	1.6327	0.8733	2.0447	7.6064	4.1157
		3	0.5507	4.3977	2.8468	1.1621	6.7260	4.1173	0.4580	1.6289	0.8664	0.4860	4.3190	2.8364	1.5824	4.6272	2.2532	0.4259	1.7096	0.9499	2.2379	7.5188	3.9079
		Mean	0.5453	4.1616	2.6761	1.0845	6.7758	4.2116	0.4749	1.6137	0.8427	0.5348	4.1476	2.6734	1.7226	4.3145	1.9180	0.4684	1.6890	0.9032	2.0864	7.4088	3.9386
		Std	0.0246	0.1782	0.1239	0.0628	0.0562	0.0674	0.0371	0.0185	0.0224	0.0373	0.1218	0.1168	0.0993	0.2426	0.2496	0.0427	0.0403	0.0335	0.1107	0.2205	0.1339

Run	Week	Value	Source 1			Source 2			Source 3			Source 4			Source 5			Source 6			Source 7		
			Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)	Angl 0° C (ng/mL)	Angl 37° C (ng/mL)	PRA (ng/mL/h)
		CV (%)	5.5342	4.1616	4.6316	7.0892	1.0165	1.6003	9.5675	1.4005	2.6619	8.5510	3.5957	4.3708	7.0629	6.8856	13.0147	11.1620	2.9204	3.7040	6.4974	3.6447	3.3985
		Dev (%)	97.1735			89.4320			110.6432			93.2013			98.4079			99.2369			105.5741		
4	6	1	0.4515	3.9498	2.5888	0.9075	7.2239	4.6741	0.4458	1.4007	0.7066	0.4623	3.8916	2.5377	1.3967	3.7889	1.7702	0.3794	1.4443	0.7881	1.5073	6.0584	3.3678
		2	0.5141	4.3112	2.8099	0.9742	7.4268	4.7749	0.3977	1.5247	0.8340	0.4866	4.2403	2.7778	1.3674	3.3911	1.4975	0.4015	1.3854	0.7281	1.5459	5.7159	3.0858
		3	0.5419	4.1998	2.7069	1.0736	7.1826	4.5207	0.4246	1.4809	0.7817	0.4553	4.2285	2.7922	1.2578	3.6452	1.7667	0.3625	1.4369	0.7950	1.6472	6.0137	3.2312
		Mean	0.5025	4.1536	2.7018	0.9851	7.2778	4.6566	0.4227	1.4688	0.7741	0.4681	4.1476	2.7229	1.3406	3.6084	1.6781	0.3811	1.4222	0.7704	1.5668	5.9293	3.2283
		Std	0.0378	0.1511	0.0903	0.0682	0.1067	0.1045	0.0197	0.0513	0.0523	0.0134	0.1617	0.1167	0.0598	0.1645	0.1277	0.0160	0.0262	0.0300	0.0590	0.1520	0.1152
		CV (%)	9.2166	4.5490	3.3434	8.4848	1.7953	2.2448	5.7086	4.2832	6.7535	3.5022	4.8066	4.2868	5.4590	5.5820	7.6107	5.1281	2.2541	3.9004	4.6128	3.6447	3.5669
		Dev (%)	98.1070			98.8815			101.6365			94.9243			86.1003			84.6433			86.5349		
5	8	1	0.5615	4.2670	2.7421	1.1410	7.1698	4.4613	0.4876	1.4809	0.7350	0.7481	4.1643	2.5280	1.5719	4.0654	1.8452	0.4796	1.6717	0.8822	1.7842	7.0261	3.8790
		2	0.6610	4.8485	3.0988	1.1313	7.7449	4.8941	0.5352	1.6234	0.8053	0.7328	4.1653	2.5401	1.5769	3.5570	1.4653	0.4926	1.5492	0.7819	1.8363	5.3406	2.5932
		3	0.6712	4.7752	3.0370	1.2504	7.3790	4.5352	0.4827	1.5311	0.7758	0.6483	4.4727	2.8300	1.3720	4.1036	2.0214	0.4712	1.6174	0.8482	1.9251	5.7708	2.8458
		Mean	0.6312	4.6302	2.9593	1.1742	7.4312	4.6302	0.5018	1.5451	0.7720	0.7097	4.2674	2.6327	1.5069	3.9087	1.7773	0.4811	1.6128	0.8374	1.8485	6.0458	3.1060
		Std	0.0495	0.2586	0.1556	0.0540	0.2377	0.1890	0.0237	0.0590	0.0288	0.0439	0.1451	0.1396	0.0954	0.2492	0.2321	0.0088	0.0501	0.0417	0.0582	0.7151	0.5562
		CV (%)	9.6012	6.8396	5.2592	5.6358	3.9172	4.0822	5.7804	4.6765	3.7296	7.5704	4.1650	5.3037	7.7556	7.7968	13.0566	2.2467	3.8070	4.9755	3.8538	14.4850	17.9088
		Dev (%)	107.4550			98.3213			101.3677			91.7801			91.1866			92.0073			83.2567		
6	10	1	0.5641	4.0321	2.5663	1.1964	6.9697	4.2722	0.5363	1.4957	0.7099	0.6180	4.0300	2.5249	1.5840	4.1348	1.8876	0.4328	1.6697	0.9153	1.9590	6.3390	3.2412
		2	0.5841	4.2771	2.7328	1.1986	7.0324	4.3170	0.4691	1.5505	0.8003	0.6053	4.0648	2.5600	1.7536	4.2531	1.8496	0.4978	1.7288	0.9110	1.9548	7.1200	3.8222
		3	0.5682	4.2783	2.7455	1.1926	6.6678	4.0516	0.5633	1.4564	0.6609	0.6970	4.3696	2.7177	1.5891	4.3827	2.0673	0.4694	1.9590	1.1023	1.9412	6.4941	3.3691
		Mean	0.5722	4.1958	2.6815	1.1959	6.8900	4.2136	0.5229	1.5009	0.7237	0.6401	4.1548	2.6009	1.6422	4.2569	1.9349	0.4667	1.7858	0.9762	1.9517	6.6510	3.4775
		Std	0.0086	0.1158	0.0816	0.0025	0.1592	0.1160	0.0396	0.0386	0.0577	0.0406	0.1525	0.0838	0.0788	0.1012	0.0949	0.0266	0.1248	0.0892	0.0076	0.3376	0.2493
		CV (%)	1.8339	3.3796	3.0447	0.2527	2.8293	2.7527	9.2828	3.1506	7.9771	7.7633	4.4968	3.2238	5.8770	2.9131	4.9058	6.9782	2.3876	9.1364	0.4773	6.2185	7.1685
		Dev (%)	97.3690			89.4760			95.0189			90.6713			99.2710			107.2554			93.2163		
7	12	1	0.5416	4.0706	2.6114	1.0850	7.0316	4.4005	0.4459	1.2555	0.5991	0.5579	4.1738	2.6758	1.4677	4.1069	1.9530	0.4636	1.4755	0.7488	1.5819	6.7974	3.8595
		2	0.5411	4.3814	2.8418	1.0960	6.8961	4.2921	0.4024	1.4542	0.7783	0.6079	4.0555	2.5512	1.4735	3.9904	1.8625	0.3866	1.4802	0.8093	1.6722	6.6460	3.6806
		3	0.5421	4.4024	2.8566	1.0442	6.6538	4.1511	0.4144	1.3878	0.7203	0.5274	4.1485	2.6796	1.3965	4.1473	2.0356	0.3615	1.4628	0.8150	1.7236	6.5028	3.5366
		Mean	0.5416	4.2848	2.7700	1.0751	6.8605	4.2812	0.4209	1.3658	0.6992	0.5644	4.1259	2.6355	1.4459	4.0815	1.9504	0.4039	1.4728	0.7910	1.6592	6.7329	3.7545
		Std	0.0004	0.1517	0.1122	0.0223	0.1563	0.1021	0.0184	0.0826	0.0747	0.0332	0.0509	0.0597	0.0350	0.0665	0.0707	0.0435	0.0073	0.0299	0.0586	0.1203	0.1321
		CV (%)	0.0882	2.3144	4.0523	2.5400	2.7899	2.3848	5.3418	7.4064	10.6802	7.2040	1.5105	2.2634	2.9651	1.9968	3.6243	13.1780	0.6119	3.7861	4.3244	2.2160	3.5175
		Dev (%)	100.5810			90.9111			91.8094			91.8795			100.0671			86.9087			100.6409		
8	14	1	0.5345	4.0305	2.5870	1.1081	7.6637	4.8511	0.4404	1.3565	0.6779	0.4843	4.0020	2.6031	1.3973	3.5690	1.6071	0.3550	1.3182	0.7128	1.7057	6.3645	3.4475
		2	0.5644	4.3619	2.8102	1.1180	7.8177	4.9578	0.3758	1.4146	0.7687	0.4721	3.8451	2.4960	1.4915	3.6345	1.5858	0.4137	1.5017	0.8051	1.6228	6.1495	3.3498
		3	0.5648	4.1830	2.6775	1.1409	7.9771	5.0588	0.4789	1.3548	0.6482	0.5348	4.0010	2.5650	1.3096	3.7344	1.7944	0.3551	1.6105	0.9290	1.7142	5.8248	3.0418

Run	Week	Value	Source 1 Angl 0° C (ng/mL)	Source 1 Angl 37° C (ng/mL)	Source 1 PRA (ng/mL/h)	Source 2 Angl 0° C (ng/mL)	Source 2 Angl 37° C (ng/mL)	Source 2 PRA (ng/mL/h)	Source 3 Angl 0° C (ng/mL)	Source 3 Angl 37° C (ng/mL)	Source 3 PRA (ng/mL/h)	Source 4 Angl 0° C (ng/mL)	Source 4 Angl 37° C (ng/mL)	Source 4 PRA (ng/mL/h)	Source 5 Angl 0° C (ng/mL)	Source 5 Angl 37° C (ng/mL)	Source 5 PRA (ng/mL/h)	Source 6 Angl 0° C (ng/mL)	Source 6 Angl 37° C (ng/mL)	Source 6 PRA (ng/mL/h)	Source 7 Angl 0° C (ng/mL)	Source 7 Angl 37° C (ng/mL)	Source 7 PRA (ng/mL/h)
		Mean	0.5546	4.1918	2.6916	1.1223	7.8195	4.9559	0.4317	1.3753	0.6983	0.4971	3.9494	2.5547	1.3995	3.6460	1.6624	0.3746	1.4768	0.8156	1.6809	6.1129	3.2797
		Std	0.0142	0.1354	0.0916	0.0137	0.1280	0.0848	0.0425	0.0278	0.0513	0.0272	0.0737	0.0443	0.0743	0.0680	0.0937	0.0276	0.1206	0.0886	0.0412	0.2218	0.1729
		CV (%)	3.1308	3.9576	3.4049	1.4987	2.0040	1.7107	12.0670	2.4753	7.3423	6.6933	2.2866	1.7345	6.5010	2.2842	5.6363	9.0385	10.0020	10.8616	3.0030	3.6447	5.2707
		Dev (%)	97.7342			105.2379			91.6812			89.0617			85.2928			89.6135			87.9135		
9	16	1	0.5346	4.3880	2.8515	1.0855	7.8662	5.0177	0.5315	1.5245	0.7348	0.6160	4.5199	2.8889	1.5550	4.4604	2.1500	0.5575	1.6322	0.7952	1.8538	7.3215	4.0461
		2	0.5716	4.9540	3.2430	1.1925	7.5882	4.7328	0.4679	1.5780	0.8214	0.5958	4.3610	2.7863	1.6450	3.8677	1.6448	0.5079	1.8016	0.9573	1.7602	7.5287	4.2687
		3	0.6099	4.3255	2.7496	1.2178	7.5287	4.6701	0.5166	1.5094	0.7347	0.5166	4.4969	2.9454	1.5741	4.0186	1.8089	0.4768	1.9020	1.0546	1.9375	6.5506	3.4137
		Mean	0.5720	4.5558	2.9480	1.1653	7.6610	4.8069	0.5053	1.5373	0.7636	0.5761	4.4593	2.8735	1.5914	4.1156	1.8679	0.5141	1.7786	0.9357	1.8505	7.1336	3.9095
		Std	0.0307	0.2827	0.2127	0.0573	0.1471	0.1513	0.0271	0.0294	0.0409	0.0429	0.0701	0.0659	0.0387	0.2515	0.2104	0.0332	0.1113	0.1070	0.0724	0.4208	0.3622
		CV (%)	6.5762	7.5955	7.2144	6.0257	2.3513	3.1471	6.5765	2.3445	5.3520	4.4708	1.9210	2.2921	2.9791	7.5182	11.2650	7.9192	7.6690	11.4336	4.7937	7.2256	9.2638
		Dev (%)	107.0467			102.0732			100.2646			100.1770			95.8363			102.8100			104.7952		
10	19	1	0.5600	4.1722	2.6730	1.0720	8.2325	5.2988	0.5250	1.5652	0.7698	0.5683	4.4942	2.9052	1.5951	4.2270	1.9476	0.4390	1.8458	1.0410	1.7669	7.6647	4.3644
		2	0.5522	4.9944	3.2872	1.1245	8.6204	5.5470	0.4391	1.6875	0.9238	0.5932	4.9176	3.2001	1.4781	4.4384	2.1906	0.4349	1.8636	1.0572	1.7365	7.4191	4.2051
		3	0.3585	4.8073	3.2921	1.1115	8.4125	5.4027	0.4998	1.6797	0.8731	0.5020	4.6571	3.0748	1.5965	4.5498	2.1854	0.3627	1.8030	1.0658	1.7767	7.2687	4.0641
		Mean	0.4903	4.6580	3.0841	1.1027	8.4218	5.4162	0.4880	1.6441	0.8556	0.5545	4.6896	3.0600	1.5566	4.4051	2.1079	0.4122	1.8375	1.0547	1.7600	7.4508	4.2112
		Std	0.0932	0.3519	0.2907	0.0223	0.1585	0.1018	0.0360	0.0559	0.0641	0.0385	0.1744	0.1208	0.0555	0.1339	0.1134	0.0350	0.0254	0.0103	0.0171	0.1632	0.1227
		CV (%)	23.2870	7.5955	9.4254	2.4777	2.3051	1.8790	9.0441	1.0200	7.4919	8.5001	4.5544	3.9491	4.3651	3.7226	5.3778	10.4130	1.6927	0.9746	2.6829	2.6829	2.9129
		Dev (%)	111.9885			115.0114			112.3341			106.6776			108.1490			115.8789			112.8823		
11	22	1	0.4753	4.4287	2.9255	1.0073	7.8939	5.0961	0.4308	1.5302	0.8136	0.4813	4.7424	3.1532	1.4597	4.3137	2.1120	0.4023	1.6337	0.9112	1.6473	7.1851	4.0980
		2	0.5308	4.6193	3.0255	1.0014	8.1532	5.2923	0.3711	1.6042	0.9125	0.4832	4.6499	3.0834	1.4967	4.2113	2.0088	0.4415	1.6843	0.9197	1.6367	6.9555	3.9359
		3	0.5373	4.6180	3.0197	1.1216	7.8873	5.0066	0.4285	1.4653	0.7672	0.5084	4.8464	3.2101	1.3057	4.2690	2.1928	0.3757	1.1779	0.5936	1.6947	6.6668	3.6794
		Mean	0.5144	4.5553	2.9903	1.0434	7.9781	5.1317	0.4101	1.5332	0.8311	0.4910	4.7462	3.1489	1.4207	4.2647	2.1045	0.4065	1.4986	0.8082	1.6596	6.9358	3.9044
		Std	0.0278	0.0895	0.0458	0.0553	0.1238	0.1193	0.0276	0.0567	0.0606	0.0124	0.0803	0.0518	0.0827	0.0419	0.0753	0.0270	0.2277	0.1517	0.0252	0.2121	0.1723
		CV (%)	6.6213	2.4076	1.5328	6.4908	1.9010	2.3253	8.2489	4.5343	7.2914	8.5001	2.0707	1.6458	7.1298	1.2034	3.5788	8.1400	4.3324	18.7767	1.8600	3.7444	4.4141
		Dev (%)	108.5806			108.9705			109.1201			109.7771			107.9769			88.7942			104.6590		
12	24	1	0.5318	4.5089	2.9430	1.1321	8.1559	5.1976	0.4602	1.6921	0.9116	0.4398	5.1372	3.4761	1.4133	3.8450	1.7995	0.4195	1.6282	0.8944	1.7476	7.4378	4.2107
		2	0.5355	4.7668	3.1312	1.1222	7.8344	4.9670	0.4765	1.5515	0.7955	0.5186	4.9808	3.3020	1.3748	3.9957	1.9395	0.3953	1.8002	1.0396	1.7033	7.0157	3.9312
		3	0.5284	-	-	-	7.9127	5.8554	0.4343	1.6210	0.8781	0.4782	4.7159	3.1359	1.2442	4.0986	2.1123	0.3502	1.8804	1.1323	1.7899	6.7644	3.6811
		Mean	0.5319	4.6379	3.0384	1.1272	7.9677	5.0620	0.4570	1.6215	0.8617	0.4789	4.9446	3.3047	1.3441	3.9798	1.9504	0.3883	1.7696	1.0221	1.7469	7.0726	3.9410
		Std	0.0029	0.1290	0.0941	0.0050	0.1369	0.3764	0.0174	0.0574	0.0488	0.0322	0.1739	0.1389	0.0724	0.1041	0.1279	0.0287	0.1052	0.0979	0.0354	0.2778	0.2163
		CV (%)	1.0168	3.9310	3.0964	0.6198	2.3051	7.4357	3.8932	1.0200	5.6612	11.2730	4.3066	4.2025	6.5937	3.2052	6.5593	9.0584	7.2823	9.5797	2.4788	4.8110	5.4891
		Dev (%)	110.3289			107.4905			114.2214			115.2067			100.0683			112.3031			105.6402		
13	26	1	0.5315	4.7612	3.1300	1.0812	8.3900	5.4085	0.5304	1.6563	0.8332	0.5788	4.9907	3.2648	1.5350	4.6112	2.2764	0.4863	1.8126	0.9815	1.7404	7.5667	4.3115

Run	Week	Value	Source 1 Angl 0° C (ng/mL)	Source 1 Angl 37° C (ng/mL)	Source 1 PRA (ng/mL/h)	Source 2 Angl 0° C (ng/mL)	Source 2 Angl 37° C (ng/mL)	Source 2 PRA (ng/mL/h)	Source 3 Angl 0° C (ng/mL)	Source 3 Angl 37° C (ng/mL)	Source 3 PRA (ng/mL/h)	Source 4 Angl 0° C (ng/mL)	Source 4 Angl 37° C (ng/mL)	Source 4 PRA (ng/mL/h)	Source 5 Angl 0° C (ng/mL)	Source 5 Angl 37° C (ng/mL)	Source 5 PRA (ng/mL/h)	Source 6 Angl 0° C (ng/mL)	Source 6 Angl 37° C (ng/mL)	Source 6 PRA (ng/mL/h)	Source 7 Angl 0° C (ng/mL)	Source 7 Angl 37° C (ng/mL)	Source 7 PRA (ng/mL/h)
		2	0.5399	4.8247	3.1707	1.1248	8.8134	5.6896	0.4684	1.7506	0.9489	0.6015	4.8758	3.1630	1.4568	4.4459	2.2119	0.4760	1.7994	0.9793	1.7535	7.5432	4.2844
		3	0.6308	4.4954	2.8598	1.1828	8.4218	5.3569	0.4948	1.6110	0.8260	0.5433	4.6950	3.0722	1.5320	4.5658	2.2450	0.4319	1.9019	1.0878	1.8822	7.1371	3.8886
		Mean	0.5674	4.6938	3.0535	1.1296	8.5417	5.4850	0.4979	1.6726	0.8693	0.5745	4.8538	3.1667	1.5079	4.5410	2.2444	0.4647	1.8380	1.0162	1.7920	7.4157	4.1615
		Std	0.0449	0.1426	0.1380	0.0416	0.1925	0.1462	0.0254	0.0581	0.0563	0.0239	0.1217	0.0787	0.0362	0.0697	0.0263	0.0236	0.0455	0.0507	0.0640	0.1972	0.1933
		CV (%)	9.7024	3.7219	4.5182	4.5145	2.3051	2.6653	6.2503	4.2579	6.4768	5.1013	3.0707	2.4845	2.9374	1.8812	1.1725	6.2231	3.0343	4.9850	4.3726	3.2567	4.6440
		Dev (%)	110.8778			116.4728			114.1410			110.3967			115.1551			111.6508			111.5500		
14	28	1	0.5004	4.6335	3.0585	1.0256	7.8971	5.0849	0.5382	1.6676	0.8358	0.5004	4.7482	3.1433	1.4587	4.2355	2.0548	0.4125	1.4867	0.7949	1.6894	6.8636	3.8289
		2	0.5630	4.3398	2.7949	1.0614	8.2232	5.2997	0.5303	1.6592	0.8354	0.4867	4.6537	3.0836	1.2401	4.2907	2.2574	0.4164	1.7725	1.0035	1.6294	6.9154	3.9116
		3	0.5903	4.1610	2.6423	1.0971	9.2375	6.0239	0.5261	1.5835	0.7825	0.5638	4.8073	3.1402	1.3510	4.7150	2.4894	0.3919	1.8686	1.0927	1.6976	7.1156	4.0093
		Mean	0.5512	4.3781	2.8319	1.0614	8.4526	5.4695	0.5315	1.6368	0.8179	0.5170	4.7364	3.1224	1.3499	4.4137	2.2672	0.4069	1.7093	0.9637	1.6721	6.9649	3.9166
		Std	0.0376	0.1948	0.1719	0.0292	0.5708	0.4017	0.0050	0.0378	0.0250	0.0336	0.0633	0.0274	0.0892	0.2142	0.1775	0.0107	0.1622	0.1248	0.0304	0.1087	0.0737
		CV (%)	8.3581	5.4486	6.0702	3.3685	8.2698	7.3443	1.1580	0.8419	3.0584	7.9595	1.6366	0.8789	8.0958	5.9438	7.8303	3.2326	4.3324	11.6210	2.2237	1.9103	1.8827
		Dev (%)	102.8296			116.1444			107.3864			108.8522			116.3233			105.8849			104.9863		
15	30	1	0.5761	4.5750	2.9592	1.0563	8.0487	5.1744	0.4367	1.5570	0.8291	0.5144	4.8445	3.2043	1.5372	4.5845	2.2550	0.4466	1.5904	0.8464	1.7273	7.3658	4.1725
		2	0.5986	4.7340	3.0602	1.0571	8.5508	5.5453	0.4109	1.6629	0.9265	0.5815	4.8047	3.1251	1.4108	4.6589	2.4036	0.4065	1.6612	0.9285	1.7176	7.0234	3.9263
		3	0.5837	4.2341	2.7013	1.0743	8.2092	5.2798	0.4674	1.5536	0.8038	0.5308	4.8098	3.1664	1.4399	4.4990	2.2638	0.3614	1.6767	0.9733	1.7406	6.9615	3.8635
		Mean	0.5861	4.5144	2.9069	1.0626	8.2696	5.3332	0.4383	1.5912	0.8531	0.5423	4.8197	3.1653	1.4626	4.5808	2.3075	0.4048	1.6428	0.9161	1.7285	7.1169	3.9874
		Std	0.0093	0.2085	0.1511	0.0083	0.2094	0.1561	0.0231	0.0507	0.0529	0.0286	0.0177	0.0323	0.0540	0.0653	0.0681	0.0348	0.0376	0.0526	0.0094	0.1778	0.1334
		CV (%)	4.7005	5.6573	5.1985	0.9553	3.1010	2.9265	6.4519	3.9050	6.2000	6.4552	0.4500	1.0212	4.5235	4.5541	2.9503	10.5310	2.8015	5.7367	0.6678	3.7444	3.3444
		Dev (%)	105.5542			113.2494			112.0122			110.3480			118.3879			100.6508			106.8839		
16 (excluded)	32	1	0.5679	5.3220	3.5180	1.1288	9.5632	6.2415	0.4246	1.7824	1.0048	0.4848	5.2374	3.5169	1.7627	4.9126	2.3309	0.3721	1.7530	1.0219	2.0840	7.6313	4.1050
		2	0.6258	5.2749	3.4404	1.2481	9.7002	6.2546	0.4001	1.9741	1.1647	0.4590	5.4690	3.7074	1.5701	5.1724	2.6657		1.9422	1.4372	1.9718	7.3753	3.9986
		3	0.5956	4.9547	3.2257	1.2294	8.9265	5.6959	0.6238	1.8438	0.9028	0.5833	5.3589	3.5339	1.4947	5.3251	2.8345		2.0844	1.5425	1.9945	7.3130	3.9357
		Mean	0.5964	5.1839	3.3947	1.2021	9.3966	6.0640	0.4829	1.8668	1.0241	0.5090	5.3551	3.5861	1.5655	5.1367	2.6427	0.3721	1.9265	1.1503	2.0168	7.4399	4.0131
		Std	0.0236	0.1632	0.1236	0.0524	0.3371	0.2603	0.1002	0.0799	0.1078	0.0536	0.0946	0.0861	0.1128	0.1703	0.2093	0.0000	0.1357	0.2248	0.0484	0.1377	0.0699
		CV (%)	4.8526	5.6573	3.6417	0.9553	4.3937	4.2933	25.4110	3.9050	10.5278	12.8940	2.1636	2.4004	4.5235	4.5541	7.9189		8.6283	19.5391	2.9411	2.2676	1.7413
		Dev (%)	123.2665			128.7673			134.4607			125.0184			134.3699			126.3815			107.5722		
17	35	1	0.5331	4.5644	2.9832	1.0866	7.8721	5.0213	0.3487	1.5170	0.8645	0.5131	4.1428	2.6860	1.4477	4.1428	1.9944	0.3808	1.5719	0.8814	1.7687	6.9631	3.8439
		2	0.5707	4.8200	3.1445	1.1170	7.6975	4.8696	0.3501	1.5395	0.8802	0.4313	4.0552	2.6817	1.4578	4.0552	1.9221	0.3802	1.6548	0.9432	1.7006	6.9466	3.8820
		3	0.5523	4.5835	2.9831	1.1085	8.0445	5.1326	0.3769	1.5601	0.8756	0.5606	4.8617	3.1828	1.3895	4.8617	2.5694	0.2869	1.8402	1.1494	1.7376	7.1667	4.0175
		Mean	0.5520	4.6560	3.0369	1.1040	7.8714	5.0078	0.3586	1.5389	0.8734	0.5017	4.3532	2.8502	1.4317	4.3532	2.1620	0.3493	1.6890	0.9914	1.7356	7.0255	3.9145
		Std	0.0154	0.1163	0.0761	0.0128	0.1417	0.1078	0.0130	0.0176	0.0066	0.0534	0.3613	0.2352	0.0301	0.3613	0.2896	0.0441	0.1122	0.1146	0.0278	0.1001	0.0745
		CV (%)	3.4077	3.0580	2.5048	1.4218	2.2044	2.1530	4.4337	1.4009	0.7524	13.0360	10.1650	8.2532	2.5747	4.5541	13.3967	15.4690	8.1340	11.5593	1.9650	1.7450	1.9037

			Source 1			Source 2			Source 3			Source 4			Source 5			Source 6			Source 7		
Run	Week	Value	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)
		Dev (%)	110.2744			106.3405			114.6789			99.3623			110.9231			108.9208			104.9287		
18	37	1	0.5739	5.0203	3.2903	1.2076	8.3159	5.2601	0.5614	1.6844	0.8310	0.5621	4.7946	3.1320	1.5501	4.2578	2.0037	0.3664	1.7720	1.0401	1.7748	6.7074	3.6501
		2	0.5782	5.0539	3.3120	1.1501	8.8308	5.6837	0.5203	1.6915	0.8667	0.5574	4.7763	3.1220	1.5600	4.1693	1.9309	0.3857	1.8335	1.0714	1.7582	6.6631	3.6296
		3	0.6826	4.9127	3.1303	1.2915	9.0996	5.7780	0.3841	1.7094	0.9807	0.4649	4.8017	3.2092	1.4443	4.6206	2.3505	0.3756	1.7111	0.9882	1.7572	7.3421	4.1328
		Mean	0.6116	4.9956	3.2442	1.2164	8.7488	5.5740	0.4886	1.6951	0.8928	0.5281	4.7909	3.1544	1.5181	4.3492	2.0950	0.3759	1.7722	1.0333	1.7634	6.9042	3.8042
		Std	0.0503	0.0602	0.0811	0.0581	0.3252	0.2252	0.0758	0.0105	0.0638	0.0447	0.0107	0.0390	0.0524	0.1953	0.1831	0.0079	0.0500	0.0343	0.0081	0.3102	0.2325
		CV (%)	10.0660	1.4766	2.4984	5.8426	4.5517	4.0404	18.9910	0.7607	7.1505	10.3780	0.2732	1.2354	4.2226	5.4983	8.7378	1.1459	3.4524	3.3188	0.5620	5.5020	6.1125
		Dev (%)	117.8021			118.3621			117.2210			109.9692			107.4883			113.5248			101.9725		
19	39	1	0.5601	5.2417	3.4644	1.1556	9.5998	6.2487	0.5041	1.6221	0.8273	0.5502	4.9840	3.2810	1.7091	4.4664	2.0404	0.4266	1.7436	0.9746	1.6627	7.4613	4.2910
		2	0.6334	5.2246	3.3975	1.2428	9.8835	6.3941	0.4791	1.6274	0.8498	0.5045	5.1086	3.4070	1.7564	4.4676	2.0063	0.3927	1.7367	0.9945	1.6540	7.3732	4.2322
		3	0.6496	4.6878	2.9883	1.3214	10.3780	6.7019	0.4007	1.6454	0.9211	0.4963	4.9397	3.2882	1.6630	4.6372	2.2009	0.3612	1.7622	1.0367	1.6427	7.1667	4.0878
		Mean	0.6143	5.0514	3.2834	1.2399	9.9538	6.4483	0.4613	1.6316	0.8661	0.5170	5.0108	3.3254	1.7095	4.5237	2.0825	0.3935	1.7475	1.0019	1.6531	7.3337	4.2036
		Std	0.0389	0.2572	0.2105	0.0677	0.3216	0.1889	0.0440	0.0100	0.0400	0.0237	0.0715	0.0578	0.0381	0.0802	0.0849	0.0267	0.0108	0.0259	0.0082	0.1235	0.0854
		CV (%)	3.4077	3.0580	6.4096	1.4218	2.2044	2.9299	4.4337	1.4009	4.6143	13.0360	10.1650	1.7376	2.5747	4.5541	4.0746	15.4690	8.1340	2.5859	1.9650	1.7450	2.0311
		Dev (%)	119.2250			136.9280			113.7112			115.9301			106.8480			110.0835			112.6799		

In every run, incubated (37° C) and non-incubated (0° C) samples were measured in triplicate and corresponding plasma renin activity (PRA) values were calculated. The mean PRA value of the first three runs (1.1, 1.2, 1.3) was implemented as a reference value for the subsequently conducted stability runs. The stability for every subsequently run was assessed as a percentage deviation from the reference value for each individual source. The deviation should not be more than ±20% to comply with U.S. Food and Drug Administration guideline recommendations. Run number 16 was exluded due to processing error. The investigation of long-term stability was stopped at run 19 (PRA value of Source 2 exceeded ±20%). AngI: angiotensin I; Std: standard deviation; CV: coefficient of variation; Dev: deviation from the reference value.

Appendix 10.2-7: Results of freeze and thaw stability for the plasma renin activity assay validation.

Freeze and thaw cycles	Value	AngI 0° C (ng/mL)	AngI 37° C (ng/mL)	PRA (ng/mL/h)
Reference value	**1**	0.8480	9.0720	6.0858
	2	0.9198	9.1089	6.0599
	3	0.8828	8.6880	5.7758
	4	0.7729	9.3234	6.3274
	5	0.8503	9.0856	6.0941
	Mean	0.8548	9.0072	6.0328
	Std	0.0543	0.2296	0.1960
	CV [%]	6.3514	2.5490	3.2495
	Dev (%)		**100.0000**	
1 Cycle	**1**	0.7852	9.7022	6.5392
	2	0.8569	9.7164	6.4969
	3	0.8980	9.6201	6.3962
	4	0.8489	9.5642	6.3912
	5	0.7801	9.2085	6.1808
	Mean	0.8338	9.5623	6.4009
	Std	0.0503	0.2073	0.1386
	CV [%]	6.0353	2.1675	2.1659
	Dev (%)		**106.1014**	
2 Cycle	**1**	1.0197	8.2726	5.3188
	2	0.7616	8.0946	5.3775
	3	0.8142	7.9187	5.2100
	4	0.8176	n/a	n/a
	5	0.5757	n/a	n/a
	Mean	0.7978	8.0953	5.3515
	Std	0.1585	0.1770	0.0850
	CV [%]	19.8710	2.1858	1.5885
	Dev (%)		**88.7070**	
3 Cycle	**1**	0.8341	7.7451	5.0681
	2	0.8702	8.2923	5.4429
	3	0.8004	8.4459	5.6067
	4	0.7265	8.0694	5.3848
	5	0.7007	8.4595	5.6898
	Mean	0.7863	8.2024	5.4385
	Std	0.0715	0.3001	0.2406
	CV [%]	9.0883	3.6589	4.4239
	Dev (%)		**90.1489**	
Inter-run precision			**8.59%**	

The mean plasma renin activity (PRA) value of the first measurement after thawing the samples was implemented as a reference value for the subsequently conducted stability runs. The stability for every subsequently run was assessed as a percentage deviation from the reference value. The inter-run precision should not deviate by more than ±20%. AngI: angiotensin I; Std: standard deviation; CV: coefficient of variation; Dev: deviation from the reference value; n/a: not available

Appendix 10.2-8: Results of short term stability for the plasma renin activity assay validation.

Stability at room temperature (ca. 20° C)						Stability on ice (0° C)					
Time (h)	Value	Measured Angl 0° C (ng/mL)	Measured Angl 37° C (ng/mL)	PRA (ng/mL/h)	Deviation from 0 hours value (%)	Time (h)	Value	Measured Angl 0° C (ng/mL)	Measured Angl 37° C (ng/mL)	PRA (ng/mL/h)	Deviation from 0 hours value (%)
0	1	0.7227	9.2477	6.3085	0	0	1	0.6678	7.6422	5.1610	0
	2	0.7176	9.1421	6.2342			2	0.7221	7.7855	5.2269	
	3	0.7223	8.5324	5.7795			3	0.6314	7.7920	5.2988	
	Mean	0.7209	8.9741	6.1074			Mean	0.6738	7.7399	5.2289	
	Std	0.0028	0.3861	0.2864			Std	0.0456	0.0847	0.0689	
	CV [%]	0.3949	4.3026	4.6890			CV [%]	6.7687	1.0940	1.3180	
0.25	1	0.7529	8.5509	5.7185	5.73	2	1	0.6743	7.1511	4.7497	10.80
	2	0.7730	8.5695	5.7175			2	0.6880	7.5338	5.0203	
	3	0.6974	8.7325	5.8924			3	0.6065	6.8473	4.5766	
	Mean	0.7411	8.6177	5.7762			Mean	0.6563	7.1774	4.7822	
	Std	0.0391	0.0999	0.1007			Std	0.0436	0.3440	0.2236	
	CV [%]	5.2817	1.1594	1.7433			CV [%]	6.6441	4.7929	4.6764	
0.5	1	0.8732	10.1440	6.7986	-2.56	2.5	1	0.7595	8.1778	5.4401	-1.69
	2	0.8738	10.1440	6.7981			2	0.8186	8.0800	5.3250	
	3	0.8907	9.5240	6.3311			3	0.7454	8.1147	5.4042	
	Mean	0.8764	9.4239	6.2681			Mean	0.7745	8.1242	5.3898	
	Std	0.0099	0.3580	0.2698			Std	0.0389	0.0496	0.0589	
	CV [%]	1.1307	3.7984	4.3038			CV [%]	5.0168	0.6103	1.0925	
1	1	0.9852	9.4436	6.2028	-3.19	3	1	0.7161	8.0765	5.3976	-3.01
	2	1.0131	9.4702	6.2019			2	0.7260	8.3249	5.5725	
	3	0.9762	9.8680	6.5207			3	0.6749	8.0662	5.4203	
	Mean	0.9915	9.5939	6.3084			Mean	0.7057	8.1559	5.4635	
	Std	0.0193	0.2377	0.1838			Std	0.0271	0.1465	0.0951	
	CV [%]	1.9424	2.4778	2.9133			CV [%]	3.8424	1.7960	1.7407	
1.5	1	0.9100	6.8642	4.3664	-3.71	3.5	1	0.7318	8.3909	5.6167	-8.78
	2	0.9341	8.8063	5.7729			2	0.7685	9.1968	6.1808	
	3	0.9247	9.4926	6.2831			3	0.8385	8.5145	5.6291	
	Mean	0.9230	9.5717	6.3424			Mean	0.7796	8.7007	5.8088	
	Std	0.0122	1.3633	0.9927			Std	0.0542	0.4340	0.3221	
	CV [%]	1.3172	14.2429	15.6513			CV [%]	6.9501	4.9884	5.5458	
2	1	0.9735	9.6886	6.3911	-10.94	4	1	0.7891	8.8100	5.8820	-10.11
	2	1.0436	11.1920	7.4422			2	0.6856	9.5384	6.4921	
	3	1.0605	10.2510	6.7397			3	0.6920	7.9270	5.3057	
	Mean	1.0258	10.3770	6.8575			Mean	0.7222	8.7603	5.8946	
	Std	0.0462	0.7596	0.5354			Std	0.0580	0.8069	0.5933	
	CV [%]	4.4999	7.3201	7.8069			CV [%]	8.0314	9.2113	10.0648	

Stability for plasma renin activity (PRA) was measured at room temperature for 2 hours and on ice for 4 hours. Angl: angiotensin I; Std: standard deviation; CV: coefficient of variation.

10.3 Bioanalytical quality control system for the plasma renin activity immunoassay

Appendix 10.3-1: Results of calibration standards evaluation for every conducted valid run in the context of the bioanalytical quality control system.

			Calibration standards (ng/mL)						
Run	**Date**	**Lot**	**0.2**	**0.5**	**1.5**	**4**	**10**	**25**	**60**
1	26.08.2016	1	0.185	0.53639	1.40625	4.08905	10.897	23.949	52.6085
2	26.08.2016	1	0.18633	0.54438	1.4018	3.99525	11.1195	24.7645	51.364
3	29.08.2016	1	0.185	0.53134	1.4289	4.03445	10.864	23.6905	54.7245
4	29.08.2016	1	0.185	0.53663	1.42255	4.08785	10.7185	23.7175	53.219
5	30.08.2016	1	0.19084	0.53128	1.419	3.9591	11.4745	24.2505	50.3535
6	31.08.2016	1	0.18995	0.5387	1.40885	4.0372	11.262	23.6565	51.023
7	02.09.2016	1	0.18	0.54798	1.3892	4.07045	11.2265	24.096	49.3585
8	29.09.2016	1	0.18976	0.53603	1.417	4.08705	10.774	23.8165	53.127
9	20.10.2016	1	0.18396	0.54933	1.4085	4.0635	10.9605	23.739	51.566
10	04.11.2016	1	0.18584	0.55276	1.39335	3.99695	11.504	24.1265	48.921
11	07.11.2019	1	0.187	0.54399	1.41605	3.9718	11.4335	23.9065	49.4115
12	09.11.2016	1	0.18934	0.54493	1.3989	3.94805	11.8375	23.313	50.931
13	10.11.2016	1	0.18932	0.54007	1.406	4.0714	11.101	22.8125	55.4855
14	25.11.2016	1	0.18644	0.55304	1.3792	4.1163	10.976	24.7355	48.8255
15	28.11.2016	1	0.19029	0.54702	1.38645	4.08295	10.897	25.018	50.555
16	29.11.2016	1	0.18796	0.53931	1.4196	4.06185	10.809	24.005	52.3955
17	30.11.2016	1	0.19736	0.51047	1.4734	4.01465	10.32	24.5975	58.0445
18	01.12.2016	2	0.19894	0.50365	1.4994	3.9015	10.934	22.841	59.6155
19	14.12.2016	2	0.19948	0.50158	1.51055	3.83105	11.1765	23.6495	55.2095
20	19.12.2016	2	0.19761	0.50912	1.48295	3.90745	11.2435	22.709	56.254
21	21.12.2016	2	0.20151	0.49302	1.5366	3.82005	10.694	24.029	59.874
22	11.01.2017	2	0.20412	0.4852	1.5587	3.80615	10.4405	24.427	63.2135
23	13.01.2017	2	0.20003	0.49973	1.50875	3.90825	10.532	24.29	59.084
24	16.01.2017	2	0.20199	0.49263	1.52885	3.8892	10.3535	23.9855	63.3
25	20.01.2017	2	0.20197	0.49182	1.53375	3.8632	10.478	23.829	62.5765
26	30.01.2017	2	0.20031	0.49792	1.5296	3.75605	11.388	23.8	54.354
27	31.01.2017	2	0.20345	0.48467	1.57085	3.72255	10.789	24.825	57.9795
28	01.02.2017	2	0.20047	0.49744	1.52065	3.8634	10.6165	24.561	57.174
29	02.02.2017	2	0.20287	0.48995	1.53785	3.8626	10.3745	24.389	61.6565
30	03.04.2017	2	0.20161	0.49218	1.5441	3.77935	10.9695	23.3595	60.724
31	06.04.2017	2	0.20829	0.46587	1.64495	3.5707	10.8405	24.045	67.1
32	07.04.2017	2	0.20261	0.4869	1.57185	3.6808	11.077	24.5165	57.018
33	19.04.2017	2	0.20194	0.49125	1.54905	3.7259	11.4695	22.2015	62.163
34	21.04.2017	2	0.20259	0.48812	1.5621	3.7132	11.1305	24.0185	57.4255
35	24.04.2017	2	0.20185	0.49148	1.5492	3.7468	10.9885	24.456	56.6275
36	03.05.2017	2	0.20528	0.47849	1.5969	3.6599	10.8345	24.598	61.6525
37	05.05.2017	3	0.21561	0.46365	1.52085	4.1072	9.5564	25.8345	59.273
38	10.05.2017	3	0.21935	0.46956	1.46705	4.1683	9.8411	24.6875	60.7155
39	12.05.2017	3	0.21793	0.46543	1.49265	4.13485	9.7859	24.976	60.336

			Calibration standards (ng/mL)						
Run	**Date**	**Lot**	**0.2**	**0.5**	**1.5**	**4**	**10**	**25**	**60**
40	15.05.2017	3	0.21598	0.45569	1.54365	4.1132	9.31645	26.3935	58.669
41	16.05.2017	3	0.22307	0.45424	1.50535	4.15055	9.5577	25.6165	59.5685
42	17.05.2017	3	0.22107	0.44579	1.53605	4.13585	9.45365	25.851	59.3775
43	18.05.2017	3	0.21471	0.45908	1.53785	4.1123	9.37425	26.2445	58.8455
44	19.05.2017	3	0.21606	0.47325	1.4792	4.1616	9.66335	25.287	59.963
45	24.05.2017	3	0.21374	0.47314	1.50095	4.0615	9.9944	24.6485	60.694
46	29.05.2017	3	0.22111	0.4436	1.53605	4.1744	9.38595	25.8105	59.529
47	30.05.2017	3	0.21842	0.45484	1.5355	4.11605	9.4725	26.0125	59.114
48	31.05.2017	3	0.20911	0.47755	1.5186	4.0379	9.8522	25.225	59.8465
49	01.06.2017	4	0.22088	0.47697	1.4406	4.2076	9.8438	24.137	62.5465
50	08.06.2017	4	0.22764	0.45636	1.4711	4.1542	9.98845	24.081	61.6625
51	14.06.2017	4	0.2249	0.47307	1.4472	4.14205	10.088	23.727	62.503
52	21.06.2017	4	0.22813	0.46368	1.41725	4.2542	10.087	23.542	63.063
53	05.07.2017	4	0.2215	0.47412	1.47935	4.0236	10.3655	23.194	63.254
54	07.07.2017	4	0.22264	0.47536	1.45225	4.15885	9.9435	24.013	62.8105
55	10.07.2017	4	0.21665	0.49013	1.4213	4.194	9.96975	23.979	62.0845
56	12.07.2017	4	0.22561	0.47031	1.4174	4.26225	9.90905	23.7445	62.4475
57	14.07.2017	4	0.23555	0.44949	1.4595	4.20005	9.8554	24.0805	62.949
58	19.07.2017	4	0.2193	0.47796	1.4506	4.1558	9.9775	24.2445	61.351
59	27.07.2017	4	0.22591	0.46666	1.4589	4.16395	9.8756	24.4725	61.25
60	28.07.2017	4	0.21473	0.48009	1.4783	4.08715	9.9737	24.593	60.8185
61	22.08.2017	4	0.22004	0.48307	1.4315	4.21335	9.7477	24.719	61.0555
62	23.08.2017	4	0.22923	0.46987	1.41925	4.2338	9.9436	23.715	62.7205
63	24.08.2017	4	0.2285	0.46171	1.45235	4.1647	10.03745	23.9635	61.769
64	28.08.2017	4	0.21878	0.47877	1.45805	4.1426	9.88245	24.67	60.796
65	30.08.2017	4	0.22172	0.47789	1.4346	4.16625	10.0675	23.9085	61.78
66	01.09.2017	4	0.23188	0.46647	1.40785	4.2029	10.327	22.6535	64.05
67	17.10.2017	4	0.22738	0.4539	1.4861	4.14025	9.90505	24.3415	61.493
68	18.10.2017	4	0.21521	0.48192	1.4517	4.2516	9.3904	26.1415	58.9805
69	25.10.2017	4	0.22628	0.47146	1.4236	4.2499	9.775	24.4185	61.5315
70	26.10.2017	4	0.22416	0.47122	1.44555	4.17485	9.95485	24.23	61.574
71	02.11.2017	4	0.2247	0.4725	1.4377	4.1844	9.9724	24.1	61.6945
72	10.11.2017	4	0.21879	0.47584	1.4716	4.11635	9.9211	24.569	61.0325
73	13.11.2017	4	0.2224	0.46903	1.4654	4.15305	9.8831	24.576	60.9955
74	14.11.2017	4	0.21707	0.49578	1.40005	4.21385	9.9859	23.9645	61.9345
75	15.11.2017	4	0.22465	0.47058	1.44405	4.17385	10.0145	23.826	62.3245
76	22.11.2017	4	0.22098	0.47642	1.44145	4.19615	9.8232	24.6195	60.925
77	23.11.2017	4	0.21334	0.48765	1.44735	4.13925	10.031	24.1245	61.5195
78	27.11.2017	4	0.22165	0.47157	1.46605	4.1588	9.8299	24.9155	60.609
79	28.11.2017	4	0.22157	0.47687	1.43845	4.21595	9.74665	24.722	61.0895
80	29.11.2017	4	0.21534	0.49113	1.43135	4.1584	10.0311	24.0675	61.6705
81	01.12.2017	4	0.21674	0.4831	1.45505	4.13365	9.9408	24.5305	60.917
82	04.12.2017	4	0.22312	0.4749	1.45195	4.1247	10.08045	24.0645	61.515
83	12.12.2017	4	0.21979	0.48655	1.4194	4.17175	10.1555	23.3905	63.042
84	13.12.2017	4	0.22784	0.46676	1.43615	4.18605	10.05635	23.675	62.3655

			Calibration standards (ng/mL)						
Run	**Date**	**Lot**	**0.2**	**0.5**	**1.5**	**4**	**10**	**25**	**60**
85	04.01.2018	4	0.21241	0.4886	1.4485	4.17195	9.8316	24.663	60.898
86	05.01.2018	4	0.22265	0.47483	1.42425	4.16635	10.319	23.0755	62.893
87	10.01.2018	4	0.21957	0.47369	1.47665	4.101	9.95965	24.518	61.573
88	11.01.2018	4	0.22598	0.47434	1.38375	4.2875	10.2153	23.263	63.5815
89	15.01.2018	4	0.21976	0.47491	1.4743	4.0795	10.15635	23.879	62.161
90	16.01.2018	4	0.21085	0.49556	1.44995	4.12855	9.9273	24.5475	61.096
91	17.01.2018	5	0.19963	0.48958	1.5469	3.87655	10.1955	24.921	59.915
92	18.01.2018	5	0.19655	0.50697	1.49665	3.9912	10.0505	24.8615	60.22
93	19.01.2018	5	0.18357	0.52185	1.5031	3.978	9.9112	25.721	58.5745
94	22.01.2018	5	0.20205	0.49605	1.51005	3.94425	10.242	24.2555	61.205
95	29.01.2018	5	0.19407	0.51028	1.50635	3.9279	10.264	24.456	60.823
96	30.01.2018	5	0.19937	0.49584	1.52015	3.9791	9.90055	25.524	59.143
97	01.02.2018	5	0.18617	0.52116	1.5064	3.9172	10.166	24.9365	59.794
98	02.02.2018	5	0.1958	0.51241	1.4764	4.04785	9.94565	25.033	60.174
99	13.02.2018	5	0.18602	0.51382	1.51995	3.97935	9.826	26.1845	58.9265
100	19.02.2018	5	0.19167	0.51757	1.47995	4.02685	9.97805	25.0225	60.4435
101	21.02.2018	5	0.19225	0.50487	1.5211	3.95665	9.96125	25.4265	59.362
102	22.02.2018	5	0.18457	0.51617	1.51735	3.953	9.93265	25.7075	58.5165
103	26.02.2018	5	0.19857	0.50909	1.47595	4.0119	10.1575	24.352	61.01
104	28.02.2018	5	0.18535	0.52499	1.4925	3.9425	10.208	24.631	60.7235
105	01.03.2018	5	0.19185	0.50882	1.50475	3.9999	9.89495	25.467	59.277
106	05.03.2018	5	0.19286	0.51472	1.48535	4.0287	9.922	25.3065	59.503
107	06.03.2018	5	0.18252	0.53095	1.48865	3.98885	9.93965	25.477	59.292
108	12.03.2018	5	0.20627	0.49478	1.4833	4.0371	10.19045	24.069	61.6475
109	13.03.2018	5	0.20974	0.49232	1.4602	4.0367	10.52	22.4355	64.8015
110	15.03.2018	5	0.20933	0.49446	1.46895	4.0143	10.362	23.4625	62.7605
111	16.03.2018	5	0.20917	0.49659	1.45915	4.04095	10.377	22.9785	63.9775
112	19.03.2018	5	0.19325	0.51672	1.4761	4.0512	9.91185	25.166	59.9095
113	21.03.2018	5	0.17895	0.52895	1.5034	3.93295	10.1685	24.7825	60.1425
114	22.03.2018	5	0.18429	0.51748	1.5136	3.9569	9.96915	25.4525	59.119
115	27.03.2018	5	0.18209	0.52408	1.50255	3.95815	10.0309	25.22	59.591
116	28.03.2018	5	0.19566	0.50457	1.502	4.02875	9.7977	25.6345	59.3435
117	13.04.2018	5	0.16786	0.53601	1.51135	3.949	9.9098	25.9945	57.632
118	16.04.2018	5	0.17805	0.52991	1.4968	3.9694	10.02285	25.2295	59.606
119	17.04.2018	5	0.19295	0.50211	1.5281	3.9294	10.0715	25.186	59.3975
120	19.04.2018	5	0.16812	0.55787	1.44855	4.00615	10.0638	25.072	59.691
121	14.05.2018	5	0.17267	0.53061	1.51695	3.9075	10.096	25.333	58.852
122	15.05.2018	5	0.18117	0.52665	1.49515	3.9487	10.1595	24.7365	60.209
123	16.05.2018	5	0.19733	0.50267	1.5043	3.9907	9.9934	25.1505	59.8425
124	18.05.2018	5	0.17892	0.53261	1.4899	3.9724	10.00645	25.32	59.3155
125	07.06.2018	5	0.18951	0.50723	1.5353	3.8462	10.4045	24.154	61.3825
126	11.06.2018	5	0.18312	0.52209	1.50845	3.98465	9.8007	26.0405	58.452
127	12.06.2018	5	0.18454	0.51975	1.5025	4.0004	9.8088	25.9115	58.532
128	14.06.2018	5	0.18948	0.51735	1.4996	3.9368	10.238	24.437	61.0285
129	19.06.2018	5	0.20632	0.48272	1.52865	3.9791	9.9404	25.359	59.328

			Calibration standards (ng/mL)						
Run	**Date**	**Lot**	**0.2**	**0.5**	**1.5**	**4**	**10**	**25**	**60**
130	06.07.2018	5	0.18978	0.51455	1.49475	4.0362	9.78085	25.7825	58.83
131	11.07.2018	5	0.19386	0.51263	1.4845	4.0504	9.81665	25.561	59.6195
132	12.07.2018	5	0.20031	0.49803	1.5035	4.0127	9.90375	25.328	59.5485
133	16.07.2018	5	0.18223	0.51114	1.5299	3.99435	9.58695	27.0605	56.4205
134	18.07.2018	5	0.19148	0.52136	1.46825	4.04505	9.9691	24.973	60.141
135	20.07.2018	5	0.19071	0.50821	1.51345	3.98515	9.8989	25.6145	58.8725
136	24.07.2018	5	0.18615	0.53061	1.45915	4.0076	10.23535	23.9555	62.1535
137	25.07.2018	5	0.17086	0.53829	1.5141	3.94255	9.95995	25.9795	58.0105
138	30.07.2018	5	0.20282	0.47908	1.5588	3.92685	9.8555	26.007	58.1185
139	31.07.2018	5	0.18218	0.53031	1.4793	4.01595	9.9063	25.5655	59.3155
140	01.08.2018	5	0.1942	0.50174	1.5271	3.9439	10.0051	25.3935	59.136
141	02.08.2018	5	0.16697	0.54341	1.50115	3.90505	10.1894	25.177	59.468
142	06.08.2018	5	0.19376	0.5096	1.50235	3.9564	10.143	24.7065	60.423

The calibrations standard values were expressed as the mean of two replicate (where applicable).

Appendix 10.3-2: Results of system suitability tests before valid analytical runs in the context of the bioanalytical quality control system.

	Relative error (%)					
Date	**Upper limit**	**Dilution 1**	**Dilution 2**	**Dilution 3**	**Dilution 4**	**Dilution 5**
26.08.2016	1.8502	1.7297	3.0188	2.6905	2.8118	2.1861
29.08.2016	1.2398	1.2422	2.0387	0.3617	0.6077	2.5141
30.08.2016	2.7009	1.9367	2.9009	3.6518	2.0952	3.6696
31.08.2016	1.4954	-0.2671	0.1677	1.3577	1.8050	1.7021
02.09.2016	1.3335	1.6281	2.8189	0.6050	4.2145	-1.0541
29.09.2016	-0.1128	-0.1985	0.9959	-0.2212	0.9838	1.1001
20.10.2016	-2.3190	-0.8400	0.2634	1.0105	-0.0087	4.3006
04.11.2016	-1.0405	-1.4312	-1.0807	-1.2191	-0.6132	-2.2966
07.11.2016	1.0292	-1.3916	-1.1605	-1.2482	-0.9153	4.6236
09.11.2016	-4.0784	-3.2509	-1.6054	-2.2413	-0.5110	0.4747
10.11.2016	-3.0007	-3.0453	-1.5498	-2.4024	-0.8106	0.4135
25.11.2016	-2.6590	-0.6517	-1.7329	-1.5803	-1.4141	0.3663
28.11.2016	-0.5194	1.0738	1.4755	1.8078	-1.5571	-0.7709
29.11.2016	0.2263	3.2014	1.8239	3.0654	-1.0699	0.1209
30.11.2016	-0.4415	0.4308	0.9222	2.1517	-1.9398	1.6022
01.12.2016	-1.6287	-0.5236	-0.7531	1.4787	-1.2961	1.2999
14.12.2016	1.4900	-0.3015	0.6925	2.1201	3.5108	1.8620
19.12.2016	-2.2670	-1.8556	-0.5928	-0.8747	0.3901	-0.5477
21.12.2016	-1.8408	1.3867	2.4290	1.7445	2.3224	3.3485
11.01.2017	1.0469	3.4595	-0.4485	-1.7940	-1.6288	-7.8721
13.01.2017	1.2189	3.4130	0.4157	-1.2923	-0.6722	-4.6754
16.01.2017	-1.3835	-2.7283	-2.1535	-3.7511	-2.3576	-0.3228
20.01.2017	-2.4954	-3.7087	-2.0625	-2.5768	-1.6071	-1.3833
30.01.2017	-2.1338	0.9629	-0.0928	-0.0836	0.1089	0.0156
31.01.2017	-1.4089	0.7769	1.1525	1.6272	2.8494	1.6260
01.02.2017	-0.0855	2.5898	2.5395	2.4236	1.8240	1.4071
02.02.2017	-4.4943	-1.4941	-1.5521	-1.5190	-0.6261	-0.2345
03.04.2017	-1.8138	-4.5383	0.3316	1.3359	0.4535	-0.0764
06.04.2017	-0.0646	-7.1438	-3.7319	2.8230	5.1791	-0.1833
07.04.2017	7.5882	5.0912	2.1566	1.9937	0.5443	0.5866
19.04.2017	5.7108	6.8524	2.6376	0.7148	-2.1686	-4.1667
21.04.2017	-2.5002	-1.6128	0.1971	1.4155	2.0952	1.4014
24.04.2017	-3.2317	-3.2891	-0.6595	-1.2099	0.1159	0.2491
03.05.2017	-1.7978	-2.0573	-0.2501	-0.6791	0.7935	1.1367
05.05.2017	0.4183	-2.9297	-2.2656	-0.8926	-0.7632	0.5194
10.05.2017	-2.1138	-2.7890	-2.7167	-2.0410	-0.3681	1.9358
12.05.2017	4.6385	4.5225	5.6501	5.1436	5.0009	3.8244
15.05.2017	0.2412	-2.1311	-1.4913	-1.1763	-1.1954	-1.5980
16.05.2017	-1.0276	-2.2752	-0.8811	-1.4726	-1.3553	-1.1201
17.05.2017	0.0419	-2.7855	1.3452	1.2931	2.0120	1.4486
18.05.2017	0.6187	-0.6964	0.4241	-0.1571	0.8250	-0.6272
19.05.2017	0.0175	-2.8275	-1.5290	-2.6041	-1.9362	-0.9707
24.05.2017	-1.3369	-2.4667	-1.3426	-1.3130	-0.8023	-2.0664
29.05.2017	-0.4018	-1.8395	-0.2159	0.4559	0.9901	0.5279
30.05.2017	-0.4814	-0.8383	0.0807	-0.0638	0.3414	0.8899
31.05.2017	-9.1712	-4.0106	-1.5015	-0.0274	0.9645	1.4178
01.06.2017	-0.0727	-1.9661	-1.6747	-0.6519	-0.5804	-1.8854
08.06.2017	-5.6313	-2.8082	-1.6806	-1.1935	-1.0470	-0.7420
14.06.2017	-4.2953	-4.1792	-2.5138	-1.4076	-1.7985	-0.6208
21.06.2017	-4.0285	-1.3063	0.7773	0.4180	1.4323	1.9554
05.07.2017	-2.7800	-3.9812	-0.7080	-1.0204	-0.8062	-0.4533

	Relative error (%)					
Date	**Upper limit**	**Dilution 1**	**Dilution 2**	**Dilution 3**	**Dilution 4**	**Dilution 5**
07.07.2017	-2.4241	-1.9846	1.0219	2.3808	3.3775	5.2282
10.07.2017	-1.9053	-0.9238	-0.9204	-0.3945	0.9165	-0.2115
12.07.2017	-2.7868	-1.2277	-2.1689	-0.4036	-0.0764	0.3022
14.07.2017	-3.9870	-3.6474	-3.5401	-4.7572	-3.9630	-3.1278
19.07.2017	-2.0549	0.1055	-2.5292	-3.1568	-3.2226	-4.0394
27.07.2017	-0.8797	2.1772	-0.3603	-2.0455	-2.7217	-2.8320
28.07.2017	-1.6907	1.1537	0.1002	-0.9162	-1.6781	-2.1017
22.08.2017	-2.3056	-2.5149	-0.6307	0.4238	0.0620	-0.2047
23.08.2017	-1.6865	0.3018	0.2340	0.2952	1.3205	1.7947
24.08.2017	-1.3165	-2.6910	-1.5299	-1.0952	-0.1507	-0.3149
28.08.2017	-2.7291	-3.7913	-1.9512	0.3238	-1.0280	0.4251
30.08.2017	-1.9925	-0.0440	-0.9953	0.3571	0.5760	1.4326
01.09.2017	0.5428	0.6659	1.5221	1.5428	1.8991	0.2429
17.10.2017	-1.4986	-1.3908	-0.5552	1.2566	1.8339	2.6630
18.10.2017	-2.6423	-1.7370	-1.1009	-0.1381	0.7541	1.7906
25.10.2017	1.0939	0.3782	3.2417	1.2511	1.0112	1.0721
26.10.2017	1.0725	-1.6430	2.0492	0.4791	0.6137	-0.1379
02.11.2017	-0.5964	2.3039	2.3906	1.9830	2.6282	3.2706
10.11.2017	0.3035	-0.2718	0.9422	1.0799	1.4312	1.6199
13.11.2017	0.9785	0.2409	-0.4917	0.9682	1.5960	1.5119
14.11.2017	0.8642	0.1730	1.6422	1.9737	1.9776	1.5736
15.11.2017	-3.0460	-4.5559	-1.3934	-1.0822	-1.7407	-1.6598
22.11.2017	-3.5923	-3.3745	-2.3979	-1.5933	-2.1483	-1.8426
23.11.2017	-1.9811	-2.8111	-0.3818	0.1105	-1.1208	0.0152
27.11.2017	-3.2750	-1.8513	-1.7986	-1.2156	-2.1109	-1.9205
28.11.2017	-2.8339	-4.7926	-1.0941	-1.4366	-3.2130	-2.4044
29.11.2017	-3.4556	-2.9353	-1.7193	-1.9385	-2.0939	-1.9205
01.12.2017	-4.3203	-4.2080	-1.5173	-1.8004	-3.0265	-2.2834
04.12.2017	-4.4558	-1.9426	-0.6685	-0.4743	-2.0685	-0.6351
12.12.2017	-3.7713	-2.9782	-0.7792	-1.4462	-0.9252	-0.1536
13.12.2017	-4.3914	-3.2489	-0.8342	-1.5881	-1.3621	-0.5069
04.01.2018	-3.4992	-9.2547	-6.1639	-4.6895	-3.2590	1.4387
05.01.2018	-0.0665	-1.6376	2.2147	3.1453	0.2180	-1.5453
10.01.2018	4.4537	4.2164	2.8101	4.3428	2.1709	0.1683
11.01.2018	5.5699	4.3904	3.5699	5.5932	2.1622	0.2907
15.01.2018	4.9527	4.6612	3.4204	4.3284	1.6827	0.0459
16.01.2018	4.5683	5.5445	4.5191	4.4823	1.2903	-0.2754
17.01.2018	2.7166	4.4356	3.0368	3.9725	1.0811	1.2852
18.01.2018	-2.9996	-0.0186	-0.3920	-1.1122	-0.6550	-1.4741
19.01.2018	-1.5915	-0.1299	-0.0263	-0.2325	0.1106	-0.6468
22.01.2018	-1.6226	0.6370	0.5760	0.3054	0.9528	0.2557
29.01.2018	-0.5120	0.4700	-0.6406	-1.3629	-0.5359	-1.9555
30.01.2018	-1.3493	0.3649	-0.0669	-0.9390	-0.2127	-0.8724
01.02.2018	1.3966	0.7780	1.1274	0.4813	0.6756	-0.0155
02.02.2018	-0.0073	-0.6023	-0.0098	-0.4065	-1.0307	1.3132
13.02.2018	-0.4426	-1.3363	-0.1744	-0.4532	-0.3291	-0.2317
19.02.2018	-2.1932	-0.4927	0.1824	0.7216	1.4282	0.9519
21.02.2018	-1.6440	0.6735	1.5824	2.6015	3.3177	3.6173
22.02.2018	-1.7250	-0.4428	0.9440	1.4432	2.0225	2.9192
26.02.2018	-3.3866	-3.1369	-0.8306	-0.2469	0.6653	1.7452
28.02.2018	-1.2961	3.9164	6.2747	9.3126	7.3149	5.3924
01.03.2018	-3.9225	0.4379	0.9887	2.0684	3.5440	6.9464
05.03.2018	-7.9951	-2.3560	-3.3472	-3.5519	-3.7535	-4.5221
06.03.2018	-7.1799	-3.7375	-4.9822	-4.7452	-4.4867	-4.5532
12.03.2018	-6.9991	-4.8107	-4.5746	-3.5004	-3.3170	-2.6107

	Relative error (%)					
Date	**Upper limit**	**Dilution 1**	**Dilution 2**	**Dilution 3**	**Dilution 4**	**Dilution 5**
13.03.2018	-5.6211	-3.8794	-3.9259	-2.4100	-1.6673	-1.2898
15.03.2018	-8.1281	-2.6274	-2.2017	-0.1264	1.3268	1.3364
16.03.2018	-7.7256	-5.3534	-4.1237	-2.1948	-1.4054	-1.1500
19.03.2018	-1.4414	-2.5673	2.3536	0.6189	2.0696	1.8821
21.03.2018	4.7154	8.6713	9.8342	9.6106	9.3650	9.1606
22.03.2018	2.3538	5.8924	7.6759	5.8821	7.7585	8.0780
27.03.2018	4.2745	5.8378	7.8042	5.8217	6.8702	6.5956
28.03.2018	5.3977	7.5447	7.3950	6.3098	6.9552	5.9127
13.04.2018	-1.4856	-0.5588	-0.5273	0.7128	0.6255	0.4779
16.04.2018	-1.8659	-2.1726	-0.9545	0.4736	0.1911	-0.8787
17.04.2018	-2.6976	-0.8226	-0.9422	0.6472	-0.1390	-0.4471
19.04.2018	-0.0746	1.6263	-0.3125	1.2005	1.3552	1.0945
14.05.2018	-1.7168	0.4189	-0.2876	-0.2996	1.2932	1.2879
15.05.2018	-0.9841	1.7137	0.0999	0.9923	2.2697	3.5967
16.05.2018	0.6974	2.4056	0.7799	1.0345	2.4809	3.2983
18.05.2018	-0.9912	0.9077	0.3924	0.1498	0.6862	0.9581
07.06.2018	-1.2043	-1.4847	-0.3078	-0.6663	-0.3800	-0.1383
11.06.2018	-1.5659	0.1248	-0.4314	-0.8097	1.5288	1.6439
12.06.2018	-5.8844	-3.2190	-3.7762	-3.8081	-1.7879	-2.2891
14.06.2018	-3.3460	2.2271	2.4043	2.4200	3.4289	3.4721
19.06.2018	0.4705	0.7798	0.4169	0.7588	1.0192	0.7682
06.07.2018	-4.1696	-1.7526	-0.5388	1.2951	1.8949	2.7500
11.07.2018	-4.5569	-0.6450	0.8022	2.0338	3.4143	3.2656
12.07.2018	-2.3441	0.1217	1.6382	2.8473	3.0388	4.1563
16.07.2018	-4.8769	0.9067	2.2035	3.1418	4.1303	3.7031
18.07.2018	-2.2946	-3.7021	1.1513	1.1601	1.2416	0.8931
20.07.2018	-5.1186	-0.0242	1.2833	0.6101	1.6124	1.5861
24.07.2018	-5.4411	-7.8814	0.1919	1.4790	2.5522	2.5562
25.07.2018	-5.0270	-1.6488	0.9403	1.1185	2.0090	1.5245
30.07.2018	-3.6998	-2.5667	-1.1513	0.4113	1.8624	0.7391
31.07.2018	-3.5267	-0.5315	1.2065	1.5529	1.7762	1.7709
01.08.2018	-0.1284	1.1619	1.7914	3.0477	2.4930	2.6531
02.08.2018	2.1072	0.6622	1.1166	0.9417	1.8083	1.0832
06.08.2018	0.9306	-1.2556	0.4491	1.3724	1.9751	2.6217

Appendix 10.3-3: Results of system suitability tests before invalid analytical runs in the context of the bioanalytical quality control system.

	Relative error (%)					
Date	**Upper limit**	**Dilution 1**	**Dilution 2**	**Dilution 3**	**Dilution 4**	**Dilution 5**
25.08.2016	3.8186	3.6664	3.8207	3.7410	3.2744	2.7327
13.10.2016	0.2290	-3.5177	-0.6697	-0.3592	-0.5476	2.0202
27.10.2016	-2.0885	-2.2490	-2.0893	-1.6368	-1.3063	-1.5057
16.12.2016	-2.1538	-0.4153	0.1312	-0.9093	-0.7439	-0.1878
04.04.2017	2.7911	-3.6894	-1.1683	2.0575	3.9819	3.1312
05.04.2017	-1.0305	-2.7361	-1.2856	0.9215	3.5737	1.6954
22.05.2017	-0.5942	-2.0531	-1.5549	-1.7420	-0.9849	-1.7174
07.06.2017	-3.6493	-3.1449	-1.5979	-1.3347	-4.0530	-3.4222
08.01.2018	6.9633	5.9506	3.5201	3.8763	1.8309	0.3519
25.01.2018	-1.9340	0.0247	-0.8031	-0.8022	2.0332	-0.6468
31.01.2018	-0.1524	0.3890	0.0933	-0.5280	-0.6236	-0.2781
05.02.2018	-2.1838	-3.8334	-2.1713	-2.8502	-1.6283	-0.8188
06.02.2018	0.2829	-0.2008	0.1990	-0.5467	-1.5070	-0.2317
07.02.2018	-1.5707	-1.9512	0.2579	0.9859	-0.7448	-0.5407
02.03.2018	-6.5182	-3.4908	-3.7137	-0.9032	-1.1610	1.5229
08.03.2018	-7.9405	-2.2696	-1.7387	0.2808	1.1697	3.0769
09.03.2018	-6.7604	-8.1411	-4.3600	-2.8640	-1.6061	-0.8858
12.04.2018	1.1654	2.9633	4.4638	4.6594	5.7929	4.6636
23.04.2018	-1.4643	-0.0753	-0.0317	0.7316	1.2249	0.3546
18.06.2018	-0.3265	0.0437	-0.3127	-0.1897	0.1123	0.3841
09.07.2018	-5.4697	-2.5193	0.4518	1.8935	2.7943	3.7656
17.07.2018	-5.7593	0.0304	2.3147	3.4176	3.6500	3.7813
23.07.2018	-3.3638	-2.2165	0.9786	1.0445	1.0691	1.7247

Appendix 10.3-4: Results of quality control evaluation for all three quality control levels in the context of the bioanalytical quality control system.

		Quality control low			Quality control middle			Quality control high		
Run	Date	NV	MV (ng/mL)	RE (%)	NV	MV (ng/mL)	RE (%)	NV	MV (ng/mL)	RE (%)
1	26.08.2016	1.17	1.3298	13.6539	6.51	7.4548	14.5127	42.5	44.2350	4.0824
2	26.08.2016	1.17	1.3038	11.4388	6.51	7.5512	15.9931	42.5	39.2120	-7.7365
3	29.08.2016	1.17	1.2430	6.2393	6.51	6.7219	3.2546	42.5	40.4380	-4.8518
4	29.08.2016	1.17	1.4011	19.7500	6.51	7.3048	12.2093	42.5	38.4000	-9.6471
5	30.08.2016	1.17	1.3747	17.4936	6.51	6.8484	5.1978	42.5	39.4250	-7.2353
6	31.08.2016	1.17	1.3697	17.0684	6.51	6.9228	6.3406	42.5	39.5800	-6.8706
7	02.09.2016	1.17	1.2528	7.0769	6.51	6.8511	5.2400	42.5	37.6135	-11.4977
8	29.09.2016	1.17	1.2427	6.2115	6.51	6.9512	6.7773	42.5	39.1245	-7.9424
9	20.10.2016	1.17	1.2970	10.8547	6.51	6.7258	3.3141	42.5	37.4030	-11.9929
10	04.11.2016	1.17	1.2256	4.7479	6.51	6.9542	6.8230	42.5	36.4220	-14.3012
11	07.11.2019	1.17	1.3265	13.3718	6.51	7.1685	10.1156	42.5	35.5585	-16.3329
12	09.11.2016	1.17	1.3340	14.0192	6.51	7.1386	9.6563	42.5	38.9575	-8.3353
13	10.11.2016	1.17	1.2336	5.4338	6.51	6.9206	6.3072	42.5	43.6060	2.6024
14	25.11.2016	1.17	1.3921	18.9808	6.51	6.9007	6.0019	42.5	40.0380	-5.7929
15	28.11.2016	1.17	1.2772	9.1603	6.51	7.2708	11.6859	42.5	40.1010	-5.6447
16	29.11.2016	1.17	1.3715	17.2201	6.51	6.7102	3.0758	42.5	41.7140	-1.8494
17	30.11.2016	1.17	1.2338	5.4487	6.51	6.4624	-0.7316	42.5	36.9015	-13.1729
18	01.12.2016	0.88	0.8980	2.0477	4.53	5.1113	12.8322	42.5	40.1865	-5.4435
19	14.12.2016	0.88	0.8691	-1.2426	4.53	5.4239	19.7334	42.5	40.3225	-5.1235
20	19.12.2016	0.88	0.8912	1.2733	4.53	5.4318	19.9073	42.5	38.7350	-8.8588
21	21.12.2016	0.88	0.9294	5.6091	4.53	5.4429	20.1523	42.5	41.7925	-1.6647
22	11.01.2017	0.88	1.0174	15.6188	4.53	5.3509	18.1220	42.5	43.8815	3.2506
23	13.01.2017	0.88	0.9010	2.3804	4.53	5.3674	18.4846	42.5	44.2373	4.0877
24	16.01.2017	0.88	1.0542	19.7966	4.53	5.4077	19.3758	42.5	42.5538	0.1265
25	20.01.2017	0.88	1.0128	15.0918	4.53	5.0885	12.3284	42.5	41.9588	-1.2735
26	30.01.2017	0.88	0.9650	9.6625	4.53	5.2659	16.2450	42.5	42.1228	-0.8877
27	31.01.2017	0.88	0.9892	12.4068	4.53	5.3552	18.2152	42.5	40.7383	-4.1453
28	01.02.2017	0.88	0.9880	12.2742	4.53	5.0328	11.0988	42.5	40.0305	-5.8106
29	02.02.2017	0.88	0.9704	10.2736	4.53	5.0422	11.3057	42.5	40.5340	-4.6259
30	03.04.2017	0.88	0.9935	12.8986	4.53	5.2384	15.6374	42.5	41.8595	-1.5071
31	06.04.2017	0.88	0.9725	10.5068	4.53	4.9295	8.8179	42.5	40.8610	-3.8565
32	07.04.2017	0.88	0.9832	11.7273	4.53	5.5214	21.8841	42.5	42.5120	0.0282
33	19.04.2017	0.88	0.9335	6.0787	4.53	5.1448	13.5723	42.5	41.7115	-1.8553
34	21.04.2017	0.88	0.9613	9.2421	4.53	5.3512	18.1280	42.5	40.2720	-5.2424
35	24.04.2017	0.88	0.9827	11.6685	4.53	5.3663	18.4614	42.5	41.7710	-1.7153
36	03.05.2017	0.88	0.9156	4.0415	4.53	5.3010	17.0204	42.5	39.3860	-7.3271
37	05.05.2017	1.21	1.5043	24.3182	5.71	6.2063	8.6918	42.5	45.7550	7.6588
38	10.05.2017	1.21	1.1479	-5.1322	5.71	5.7027	-0.1283	42.5	42.7310	0.5435
39	12.05.2017	1.21	1.2350	2.0620	5.71	6.2246	9.0127	42.5	46.3850	9.1412
40	15.05.2017	1.21	1.2244	1.1901	5.71	5.9141	3.5740	42.5	40.4485	-4.8271
41	16.05.2017	1.21	1.2152	0.4256	5.71	5.6994	-0.1861	42.5	39.2045	-7.7541
42	17.05.2017	1.21	1.2537	3.6074	5.71	5.8999	3.3257	42.5	42.8580	0.8424
43	18.05.2017	1.21	1.1266	-6.8967	5.71	5.8021	1.6130	42.5	44.3840	4.4329
44	19.05.2017	1.21	1.1389	-5.8802	5.71	5.5504	-2.7955	42.5	40.0465	-5.7729
45	24.05.2017	1.21	1.1201	-7.4339	5.71	5.7503	0.7062	42.5	42.1300	-0.8706
46	29.05.2017	1.21	1.2261	1.3265	5.71	5.9657	4.4772	42.5	45.3610	6.7318
47	30.05.2017	1.21	1.3689	13.1281	5.71	6.1778	8.1918	42.5	44.7065	5.1918
48	31.05.2017	1.21	1.2510	3.3843	5.71	5.9732	4.6090	42.5	43.0155	1.2129
49	01.06.2017	0.98	1.0114	3.2041	5.04	5.5423	9.9668	42.5	45.0870	6.0871
50	08.06.2017	0.98	0.8342	-14.8806	5.04	5.6231	11.5699	42.5	41.3285	-2.7565
51	14.06.2017	0.98	1.0134	3.4082	5.04	5.4123	7.3864	42.5	40.7735	-4.0624
52	21.06.2017	0.98	0.9436	-3.7163	5.04	5.6790	12.6786	42.5	50.2900	18.3294
53	05.07.2017	0.98	1.0676	8.9388	5.04	5.6005	11.1200	42.5	43.0535	1.3024
54	07.07.2017	0.98	1.0645	8.6225	5.04	5.5930	10.9712	42.5	50.5595	18.9635
55	10.07.2017	0.98	1.0507	7.2092	5.04	5.4994	9.1141	42.5	40.0795	-5.6953
56	12.07.2017	0.98	0.9807	0.0730	5.04	5.3228	5.6116	42.5	44.2625	4.1471
57	14.07.2017	0.98	1.0699	9.1684	5.04	5.6168	11.4449	42.5	24.2918	-42.8428
58	19.07.2017	0.98	0.9998	2.0189	5.04	5.6033	11.1766	42.5	45.1535	6.2435
59	27.07.2017	0.98	1.0427	6.3929	5.04	5.7709	14.5010	42.5	42.2855	-0.5047
60	28.07.2017	0.98	1.0311	5.2143	5.04	5.7375	13.8398	42.5	32.0430	-24.6047
61	22.08.2017	0.98	1.0016	2.2046	5.04	5.6059	11.2272	42.5	44.9700	5.8118

		Quality control low			Quality control middle			Quality control high		
Run	**Date**	**NV**	**MV (ng/mL)**	**RE (%)**	**NV**	**MV (ng/mL)**	**RE (%)**	**NV**	**MV (ng/mL)**	**RE (%)**
62	23.08.2017	0.98	1.0208	4.1658	5.04	5.4524	8.1816	42.5	44.5200	4.7529
63	24.08.2017	0.98	1.0284	4.9388	5.04	5.6100	11.3095	42.5	41.4100	-2.5647
64	28.08.2017	0.98	1.0670	8.8725	5.04	5.4629	8.3914	42.5	46.1290	8.5388
65	30.08.2017	0.98	1.0851	10.7245	5.04	5.9533	18.1215	42.5	47.9800	12.8941
66	01.09.2017	0.98	0.9919	1.2189	5.04	5.4758	8.6473	42.5	46.0915	8.4506
67	17.10.2017	0.98	1.0315	5.2551	5.04	5.5663	10.4415	42.5	45.6595	7.4341
68	18.10.2017	0.98	1.0176	3.8393	5.04	5.9515	18.0858	42.5	44.0825	3.7235
69	25.10.2017	0.98	1.0679	8.9643	5.04	5.5177	9.4787	42.5	44.1980	3.9953
70	26.10.2017	0.98	1.0776	9.9592	5.04	5.6159	11.4266	42.5	44.6450	5.0471
71	02.11.2017	0.98	1.1037	12.6174	5.04	5.9939	18.9256	42.5	48.3635	13.7965
72	10.11.2017	0.98	1.0488	7.0235	5.04	5.5695	10.5055	42.5	43.1665	1.5682
73	13.11.2017	0.98	1.0013	2.1755	5.04	5.6654	12.4092	42.5	43.5125	2.3824
74	14.11.2017	0.98	1.0360	5.7092	5.04	6.1418	21.8601	42.5	45.9045	8.0106
75	15.11.2017	0.98	0.9022	-7.9408	5.04	5.6618	12.3363	42.5	47.7115	12.2624
76	22.11.2017	0.98	1.0047	2.5163	5.04	5.6320	11.7455	42.5	43.1315	1.4859
77	23.11.2017	0.98	0.9377	-4.3199	5.04	5.5416	9.9529	42.5	41.2620	-2.9129
78	27.11.2017	0.98	0.9263	-5.4837	5.04	5.5232	9.5873	42.5	45.4650	6.9765
79	28.11.2017	0.98	0.9226	-5.8617	5.04	5.0598	0.3934	42.5	44.7990	5.4094
80	29.11.2017	0.98	0.9932	1.3439	5.04	5.5022	9.1706	42.5	44.8635	5.5612
81	01.12.2017	0.98	0.9644	-1.5918	5.04	5.4497	8.1295	42.5	44.5930	4.9247
82	04.12.2017	0.98	0.9911	1.1301	5.04	5.3051	5.2604	42.5	41.0565	-3.3965
83	12.12.2017	0.98	0.9546	-2.5934	5.04	5.4450	8.0357	42.5	45.4360	6.9082
84	13.12.2017	0.98	0.9494	-3.1245	5.04	5.3714	6.5759	42.5	51.2385	20.5612
85	04.01.2018	0.98	0.9305	-5.0485	5.04	5.5561	10.2391	42.5	45.3755	6.7659
86	05.01.2018	0.98	0.9361	-4.4801	5.04	5.5446	10.0124	42.5	45.6055	7.3071
87	10.01.2018	0.98	1.0286	4.9541	5.04	5.4728	8.5868	42.5	49.0810	15.4847
88	11.01.2018	0.98	0.9691	-1.1168	5.04	5.9779	18.6096	42.5	48.2690	13.5741
89	15.01.2018	0.98	0.9783	-0.1725	5.04	5.3282	5.7178	42.5	39.4160	-7.2565
90	16.01.2018	0.98	1.0453	6.6582	5.04	5.2836	4.8338	42.5	40.5990	-4.4729
91	17.01.2018	0.97	1.0023	3.3299	435.00	4.9885	14.6776	42.5	44.3970	4.4635
92	18.01.2018	0.97	1.1337	16.8763	4.35	5.2821	21.4270	42.5	45.9340	8.0800
93	19.01.2018	0.97	1.1240	15.8763	4.35	5.2558	20.8230	42.5	40.8185	-3.9565
94	22.01.2018	0.97	1.0590	9.1701	4.35	5.2332	20.3035	42.5	42.9650	1.0941
95	29.01.2018	0.97	1.1064	14.0619	4.35	5.1802	19.0845	42.5	44.6950	5.1647
96	30.01.2018	0.97	1.0187	5.0155	4.35	5.0798	16.7776	42.5	39.1350	-7.9177
97	01.02.2018	0.97	0.9060	-6.5943	4.35	4.6544	6.9983	42.5	41.2945	-2.8365
98	02.02.2018	0.97	1.1243	15.9072	4.35	5.2425	20.5161	42.5	43.8410	3.1553
99	13.02.2018	0.97	1.1297	16.4639	4.35	5.1691	18.8299	42.5	40.3980	-4.9459
100	19.02.2018	0.97	1.0711	10.4253	4.35	4.6932	7.8891	42.5	43.2965	1.8741
101	21.02.2018	0.97	1.0739	10.7088	4.35	4.6531	6.9678	42.5	39.2000	-7.7647
102	22.02.2018	0.97	1.0534	8.5938	4.35	5.1238	17.7874	42.5	43.9495	3.4106
103	26.02.2018	0.97	0.9378	-3.3209	4.35	5.0225	15.4598	42.5	41.8440	-1.5435
104	28.02.2018	0.97	0.9945	2.5247	4.35	4.8759	12.0885	42.5	46.5610	9.5553
105	01.03.2018	0.97	1.1842	22.0799	4.35	5.0308	15.6494	42.5	41.5930	-2.1341
106	05.03.2018	0.97	0.9564	-1.4067	4.35	4.9120	12.9184	42.5	43.1630	1.5600
107	06.03.2018	0.97	1.1451	18.0541	4.35	5.0238	15.4897	42.5	47.8605	12.6129
108	12.03.2018	0.97	1.0643	9.7242	4.35	5.0313	15.6615	42.5	39.4185	-7.2506
109	13.03.2018	0.97	0.8921	-8.0299	4.35	4.7307	8.7517	42.5	41.9445	-1.3071
110	15.03.2018	0.97	1.0038	3.4887	4.35	4.9926	14.7713	42.5	39.8670	-6.1953
111	16.03.2018	0.97	0.9445	-2.6327	4.35	4.8792	12.1655	42.5	36.8160	-13.3741
112	19.03.2018	0.97	0.9938	2.4487	4.35	4.9657	14.1540	42.5	40.0590	-5.7435
113	21.03.2018	0.97	1.1226	15.7320	4.35	4.8989	12.6172	42.5	44.6615	5.0859
114	22.03.2018	0.97	1.0670	10.0000	4.35	5.0039	15.0310	42.5	41.4925	-2.3706
115	27.03.2018	0.97	1.0925	12.6237	4.35	5.0758	16.6845	42.5	47.4375	11.6177
116	28.03.2018	0.97	1.0462	7.8544	4.35	5.0470	16.0230	42.5	48.9215	15.1094
117	13.04.2018	0.97	1.0668	9.9742	4.35	4.9279	13.2839	42.5	43.5230	2.4071
118	16.04.2018	0.97	1.1099	14.4201	4.35	4.9060	12.7810	42.5	46.0895	8.4459
119	17.04.2018	0.97	1.1107	14.5026	4.35	4.9993	14.9270	42.5	39.8785	-6.1682
120	19.04.2018	0.97	0.8468	-12.6969	4.35	4.4601	2.5299	42.5	40.2185	-5.3682
121	14.05.2018	0.97	1.0302	6.2005	4.35	5.1128	17.5362	42.5	33.3825	-21.4529
122	15.05.2018	0.97	0.8501	-12.3634	4.35	4.2609	-2.0483	42.5	41.7720	-1.7129
123	16.05.2018	0.97	1.1799	21.6340	4.35	4.8578	11.6724	42.5	45.0560	6.0141
124	18.05.2018	0.97	1.1009	13.4897	4.35	4.9928	14.7759	42.5	34.1570	-19.6306
125	07.06.2018	0.97	0.8980	-7.4258	4.35	4.7904	10.1247	42.5	44.7510	5.2965

		Quality control low			Quality control middle			Quality control high		
Run	**Date**	**NV**	**MV (ng/mL)**	**RE (%)**	**NV**	**MV (ng/mL)**	**RE (%)**	**NV**	**MV (ng/mL)**	**RE (%)**
126	11.06.2018	0.97	1.1334	16.8428	4.35	5.1962	19.4517	42.5	47.8525	12.5941
127	12.06.2018	0.97	1.0679	10.0928	4.35	5.0790	16.7586	42.5	35.6720	-16.0659
128	14.06.2018	0.97	1.0028	3.3843	4.35	5.0455	15.9891	42.5	43.4075	2.1353
129	19.06.2018	0.97	1.1547	19.0438	4.35	4.8787	12.1529	42.5	39.8195	-6.3071
130	06.07.2018	0.97	1.1264	16.1211	4.35	5.1498	18.3856	42.5	48.5550	14.2471
131	11.07.2018	0.97	0.9585	-1.1809	4.35	4.8922	12.4649	42.5	39.9385	-6.0271
132	12.07.2018	0.97	0.9708	0.0771	4.35	4.9808	14.5000	42.5	41.5130	-2.3224
133	16.07.2018	0.97	1.1516	18.7165	4.35	5.0684	16.5138	excluded from evaluation		
134	18.07.2018	0.97	1.0188	5.0343	4.35	5.1038	17.3287	42.5	43.9840	3.4918
135	20.07.2018	0.97	1.1551	19.0851	4.35	4.7251	8.6218	42.5	42.5180	0.0424
136	24.07.2018	0.97	1.0169	4.8361	excluded from evaluation			42.5	43.8430	3.1600
137	25.07.2018	0.97	1.0781	11.1479	4.35	5.0934	17.0897	42.5	44.0655	3.6835
138	30.07.2018	0.97	1.0724	10.5593	4.35	5.1927	19.3718	42.5	38.8840	-8.5082
139	31.07.2018	0.97	0.9786	0.8840	4.35	4.7839	9.9753	42.5	43.2150	1.6824
140	01.08.2018	0.97	1.0976	13.1546	4.35	5.0139	15.2615	42.5	42.8085	0.7259
141	02.08.2018	0.97	0.9723	0.2381	4.35	4.9206	13.1178	42.5	44.5220	4.7577
142	06.08.2018	0.97	1.0697	10.2729	4.35	5.0860	16.9195	42.5	44.5220	4.7577

NV: nominal value (lot dependent); MV: measured value (mean of 2-4 replicate); RE: relative error. Excluded quality controls were removed from the evaluation due to processing errors and the concomitant variation in nominal concentration.

Appendix 10.3-5: Results of the blank sample evaluation in the context of the bioanalytical quality control system.

Run	Date	LLOQ (ng/mL	Blank 1 (ng/mL)	Blank 1-to-LLOQ ratio	Blank 2 (ng/mL)	Blank 2-to-LLOQ ratio
1	26.08.2016	0.191	<min	0	<min	0
2	26.08.2016	0.18946	0.00779	4.11%	0.01017	5.37%
3	29.08.2016	0.19147	<min	0	<min	0
4	29.08.2016	0.18876	<min	0	<min	0
5	30.08.2016	0.19325	0.02739	14.17%	0.03062	15.84%
6	31.08.2016	0.18945	0.00018467	0.10%	<min	0
7	02.09.2016	0.18744	<min	0	<min	0
8	29.09.2016	0.18976	<min	0	<min	0
9	20.10.2016	0.18396	<min	0	<min	0
10	04.11.2016	0.18584	<min	0	<min	0
11	07.11.2019	0.187	<min	0	<min	0
12	09.11.2016	0.18934	0.01496	7.90%	0.02188	11.56%
13	10.11.2016	0.18932	<min	0	<min	0
14	25.11.2016	0.18644	<min	0	<min	0
15	28.11.2016	0.19029	<min	0	<min	0
16	29.11.2016	0.18796	<min	0	<min	0
17	30.11.2016	0.19736	0.00491	2.49%	0.08072	40.90%
18	01.12.2016	0.19894	<min	0	0.03711	18.65%
19	14.12.2016	0.19948	0.04514	22.63%	0.02928	14.68%
20	19.12.2016	0.19761	0.00695	3.52%	0.01616	8.18%
21	21.12.2016	0.20151	0.01805	8.96%	0.04099	20.34%
22	11.01.2017	0.19039	0.04572	24.01%	<min	0
23	13.01.2017	0.20003	0.03628	18.14%	0.04777	23.88%
24	16.01.2017	0.20199	0.00078449	0.39%	0.06654	32.94%
25	20.01.2017	0.20197	0.0138	6.83%	0.01284	6.36%
26	30.01.2017	0.20031	0.01748	8.73%	0.04289	21.41%
27	31.01.2017	0.20345	0.01756	8.63%	0.09525	46.82%
28	01.02.2017	0.20047	0.02371	11.83%	0.02005	10.00%
29	02.02.2017	0.20287	<min	0	<min	0
30	03.04.2017	0.20161	<min	0	0.01844	9.15%
31	06.04.2017	0.20829	0.03278	15.74%	0.0386	18.53%
32	07.04.2017	0.20261	0.02654	13.10%	0.08285	40.89%
33	19.04.2017	0.20194	0.00961	4.76%	0.00041132	0.20%
34	21.04.2017	0.20259	0.01391	6.87%	0.0202	9.97%
35	24.04.2017	0.20185	0.01708	8.46%	0.00069585	0.34%
36	03.05.2017	0.20528	0.02178	10.61%	0.0408	19.88%
37	05.05.2017	0.21561	<min	0	<min	0
38	10.05.2017	0.21935	0.00312	1.42%	<min	0
39	12.05.2017	0.21793	0.00793	3.64%	0.01127	5.17%
40	15.05.2017	0.21598	0.00491	2.27%	<min	0
41	16.05.2017	0.22307	<min	0	<min	0
42	17.05.2017	0.22107	0.01673	7.57%	<min	0
43	18.05.2017	0.21471	0.00292	1.36%	0.01736	8.09%
44	19.05.2017	0.21606	<min	0	<min	0

Run	Date	LLOQ (ng/mL	Blank 1 (ng/mL)	Blank 1-to-LLOQ ratio	Blank 2 (ng/mL)	Blank 2-to-LLOQ ratio
45	24.05.2017	0.21374	0.01984	9.28%	0.02128	9.96%
46	29.05.2017	0.22111	0.03079	13.93%	0.05822	26.33%
47	30.05.2017	0.21842	<min	0	<min	0
48	31.05.2017	0.21782	<min	0	0.03283	15.07%
49	01.06.2017	0.22088	<min	0	<min	0
50	08.06.2017	0.23689	0.01849	7.81%	0.03128	13.20%
51	14.06.2017	0.2249	<min	0	<min	0
52	21.06.2017	0.22813	0.01771	7.76%	0.01906	8.35%
53	05.07.2017	0.2215	<min	0	<min	0
54	07.07.2017	0.22264	<min	0	<min	0
55	10.07.2017	0.21665	<min	0	<min	0
56	12.07.2017	0.22561	0.01358	6.02%	<min	0
57	14.07.2017	0.23555	<min	0	<min	0
58	19.07.2017	0.2193	<min	0	<min	0
59	27.07.2017	0.22591	<min	0	<min	0
60	28.07.2017	0.21473	<min	0	<min	0
61	22.08.2017	0.22004	<min	0	<min	0
62	23.08.2017	0.22923	<min	0	<min	0
63	24.08.2017	0.2285	<min	0	<min	0
64	28.08.2017	0.21878	<min	0	<min	0
65	30.08.2017	0.22172	<min	0	<min	0
66	01.09.2017	0.23188	<min	0	<min	0
67	17.10.2017	0.22738	<min	0	<min	0
68	18.10.2017	0.21521	<min	0	<min	0
69	25.10.2017	0.22628	<min	0	<min	0
70	26.10.2017	0.22416	<min	0	<min	0
71	02.11.2017	0.2247	<min	0	<min	0
72	10.11.2017	0.21879	<min	0	<min	0
73	13.11.2017	0.2224	<min	0	<min	0
74	14.11.2017	0.21707	<min	0	<min	0
75	15.11.2017	0.22465	<min	0	<min	0
76	22.11.2017	0.22098	<min	0	<min	0
77	23.11.2017	0.21334	<min	0	<min	0
78	27.11.2017	0.22165	<min	0	<min	0
79	28.11.2017	0.22157	<min	0	<min	0
80	29.11.2017	0.21534	<min	0	<min	0
81	01.12.2017	0.21674	<min	0	<min	0
82	04.12.2017	0.22312	<min	0	<min	0
83	12.12.2017	0.21979	<min	0	<min	0
84	13.12.2017	0.22784	<min	0	<min	0
85	04.01.2018	0.21241	<min	0	<min	0
86	05.01.2018	0.22265	<min	0	<min	0
87	10.01.2018	0.21957	<min	0	<min	0
88	11.01.2018	0.22598	0.01664	7.36%	<min	0
89	15.01.2018	0.21976	<min	0	<min	0
90	16.01.2018	0.21085	<min	0	<min	0
91	17.01.2018	0.19963	<min	0	<min	0

Run	Date	LLOQ (ng/mL	Blank 1 (ng/mL)	Blank 1-to-LLOQ ratio	Blank 2 (ng/mL)	Blank 2-to-LLOQ ratio
92	18.01.2018	0.19655	<min	0	0.01124	5.72%
93	19.01.2018	0.18357	<min	0	<min	0
94	22.01.2018	0.20205	<min	0	<min	0
95	29.01.2018	0.19407	<min	0	<min	0
96	30.01.2018	0.19937	<min	0	<min	0
97	01.02.2018	0.18617	<min	0	<min	0
98	02.02.2018	0.1958	<min	0	<min	0
99	13.02.2018	0.18602	0.00576	3.10%	<min	0
100	19.02.2018	0.19167	<min	0	0.03082	16.08%
101	21.02.2018	0.19225	<min	0	<min	0
102	22.02.2018	0.18457	<min	0	<min	0
103	26.02.2018	0.19857	0.01625	8.18%	<min	0
104	28.02.2018	0.18535	<min	0	<min	0
105	01.03.2018	0.19185	<min	0	0.01744	9.09%
106	05.03.2018	0.19286	<min	0	<min	0
107	06.03.2018	0.18252	<min	0	<min	0
108	12.03.2018	0.20627	0.006	2.91%	0.04584	22.22%
109	13.03.2018	0.20974	0.06882	32.81%	0.08896	42.41%
110	15.03.2018	0.20933	0.01921	9.18%	0.00674	3.22%
111	16.03.2018	0.20917	0.05201	24.86%	<min	0
112	19.03.2018	0.19325	<min	0	<min	0
113	21.03.2018	0.17895	0.05081	28.39%	<min	0
114	22.03.2018	0.18429	0.01079	5.85%	<min	0
115	27.03.2018	0.18209	<min	0	<min	0
116	28.03.2018	0.19566	<min	0	<min	0
117	13.04.2018	0.16786	<min	0	<min	0
118	16.04.2018	0.17805	<min	0	0.05833	32.76%
119	17.04.2018	0.19295	<min	0	<min	0
120	19.04.2018	0.16812	<min	0	<min	0
121	14.05.2018	0.17267	<min	0	<min	0
122	15.05.2018	0.18117	<min	0	<min	0
123	16.05.2018	0.19733	<min	0	0.00796	4.03%
124	18.05.2018	0.17892	<min	0	0.07319	40.91%
125	07.06.2018	0.18951	<min	0	<min	0
126	11.06.2018	0.18312	<min	0	0.0413	22.55%
127	12.06.2018	0.18454	<min	0	0.04416	23.93%
128	14.06.2018	0.18948	0.01532	8.09%	<min	0
129	19.06.2018	0.20632	<min	0	<min	0
130	06.07.2018	0.18978	0.02568	13.53%	0.02706	14.26%
131	11.07.2018	0.19386	<min	0	<min	0
132	12.07.2018	0.20031	<min	0	0.00684	3.41%
133	16.07.2018	0.18223	<min	0	<min	0
134	18.07.2018	0.19148	<min	0	<min	0
135	20.07.2018	0.19071	<min	0	<min	0
136	24.07.2018	0.18615	<min	0	<min	0
137	25.07.2018	0.17086	<min	0	<min	0
138	30.07.2018	0.20282	<min	0	0.03143	15.50%

Run	Date	LLOQ (ng/mL	Blank 1 (ng/mL)	Blank 1-to-LLOQ ratio	Blank 2 (ng/mL)	Blank 2-to-LLOQ ratio
139	31.07.2018	0.18218	<min	0	0.02009	11.03%
140	01.08.2018	0.1942	<min	0	0.06613	34.05%
141	02.08.2018	0.16697	<min	0	<min	0
142	06.08.2018	0.19376	<min	0	0.05581	28.80%

The LLOQ values are expressed as the mean of two replicates.

Appendix 10.3-6: Results of the incurred sample reanalysis evaluation in the context of the bioanalytical quality control system.

		Original		Repeat		
Run	**Cryo ID**	**Date**	**PRA (ng/mL/h)**	**Date**	**PRA (ng/mL/h)**	**Difference (%)**
1	ISA 00019	30.01.2017	32.5096	30.01.2017	32.5311	0.07
2	ISA 00019	31.01.2017	32.5096	31.01.2017	34.0857	4.73
3	ISA 00266	01.02.2017	35.6055	01.02.2017	34.2444	-3.90
4	ISA 00266	02.02.2017	35.6055	02.02.2017	31.2091	-13.16
5	ISA 01514	19.04.2017	41.4116	19.04.2017	42.2327	1.94
6	ISA 01514	21.04.2017	41.4116	21.04.2017	44.5801	7.11
7	ISA 01514	24.04.2017	41.4116	24.04.2017	42.0524	1.52
8	ISA 01048	10.05.2017	34.9678	10.05.2017	26.4423	-27.77
9	ISA 01307	12.05.2017	28.1598	12.05.2017	28.9854	2.89
10	ISA 01090	15.05.2017	22.5216	15.05.2017	21.5914	-4.22
11	ISA 01090	16.05.2017	22.5216	16.05.2017	18.1184	-21.67
12	ISA 01144	17.05.2017	8.3258	17.05.2017	9.1395	9.32
13	ISA 01144	18.05.2017	8.3258	18.05.2017	8.8495	6.10
14	ISA 01144	19.05.2017	8.3258	19.05.2017	10.7588	25.50
15	ISA 01384	24.05.2017	10.2935	24.05.2017	8.8936	-14.59
16	ISA 01508	29.05.2017	9.1739	29.05.2017	7.6010	-18.75
17	ISA 01078	30.05.2017	74.0614	30.05.2017	65.3507	-12.50
18	ISA 01505	31.05.2017	25.7787	31.05.2017	22.9351	-11.67
19	ISA 01505	01.06.2017	25.7787	01.06.2017	20.5063	-22.78
20	ISA 00824	08.06.2017	5.9314	08.06.2017	5.3714	-9.91
21	ISA 01347	14.06.2017	32.9162	14.06.2017	32.4423	-1.45
22	ISA 01347	21.06.2017	32.9162	21.06.2017	36.2043	9.51
23	ISA 01347	05.07.2017	32.9162	05.07.2017	31.3664	-4.82
24	ISA 00813	07.07.2017	10.6350	07.07.2017	10.4720	-1.54
25	ISA 00813	10.07.2017	10.6350	10.07.2017	12.7973	18.46
26	ISA 00813	12.07.2017	10.6350	12.07.2017	10.0683	-5.47
27	ISA 01382	14.07.2017	23.6891	14.07.2017	21.5904	-9.27
28	ISA 01382	19.07.2017	23.6891	19.07.2017	21.1194	-11.47
29	ISA 01382	27.07.2017	23.6891	27.07.2017	19.3114	-20.36
30	ISA 01452	28.07.2017	16.7604	28.07.2017	14.5668	-14.00
31	ISA 00887	22.08.2017	9.2220	22.08.2017	8.4085	-9.23
32	ISA 00887	23.08.2017	9.2220	23.08.2017	8.2708	-10.88
33	ISA 01042	24.08.2017	59.0385	24.08.2017	51.9795	-12.72
34	ISA 01042	28.08.2017	59.0385	28.08.2017	52.8781	-11.01
35	ISA 01122	30.08.2017	33.1048	30.08.2017	34.6136	4.46
36	ISA 01122	01.09.2017	33.1048	01.09.2017	34.7594	4.88
37	ISA 01535	17.10.2017	13.3304	17.10.2017	14.2595	6.73
38	ISA 01535	18.10.2017	13.3304	18.10.2017	9.2931	-35.69
39	ISA 00944	25.10.2017	45.5585	25.10.2017	45.9456	0.85
40	ISA 00907	26.10.2017	49.1481	26.10.2017	44.2951	-10.39
41	ISA 00907	02.11.2017	49.1481	02.11.2017	39.8181	-20.97
42	ISA 01208	10.11.2017	16.3354	10.11.2017	11.6685	-33.33
43	ISA 00895	13.11.2017	7.1571	13.11.2017	6.3104	-12.57

		Original		Repeat		
Run	**Cryo ID**	**Date**	**PRA (ng/mL/h)**	**Date**	**PRA (ng/mL/h)**	**Difference (%)**
44	ISA 00895	14.11.2017	7.1571	14.11.2017	6.6598	-7.20
45	ISA 01183	15.11.2017	13.9212	15.11.2017	12.1049	-13.96
46	ISA 01183	22.11.2017	13.9212	22.11.2017	9.6651	-36.09
47	ISA 01208	23.11.2017	16.3354	23.11.2017	10.2919	-45.39
48	ISA 01169	27.11.2017	21.3466	27.11.2017	13.7038	-43.61
49	ISA 01169	28.11.2017	21.3466	28.11.2017	19.5582	-8.74
50	ISA 01169	29.11.2017	21.3466	29.11.2017	19.0225	-11.51
51	ISA 00969	01.12.2017	15.8942	01.12.2017	19.1901	18.79
52	ISA 00969	04.12.2017	15.8942	04.12.2017	15.1002	-5.12
53	ISA 00969	12.12.2017	15.8942	12.12.2017	17.8489	11.59
54	ISA 01792	13.12.2017	17.7241	13.12.2017	15.1420	-15.71
55	ISA 01792	04.01.2018	17.7241	04.01.2018	19.0357	7.14
56	ISA 12717	05.01.2018	32.0390	05.01.2018	32.6083	1.76
57	ISA 12717	10.01.2018	32.0390	10.01.2018	29.7664	-7.35
58	ISA 00842	11.01.2018	24.4111	11.01.2018	16.1242	-51.39
59	ISA 00842	15.01.2018	24.4111	15.01.2018	17.8229	-36.96
60	ISA 00842	16.01.2018	24.4111	16.01.2018	20.9061	-16.77
61	ISA 01216	17.01.2018	15.3899	17.01.2018	16.4821	6.85
62	ISA 01216	18.01.2018	15.3899	18.01.2018	15.7025	2.01
63	ISA 01216	19.01.2018	15.3899	19.01.2018	15.6047	1.39
64	ISA 01342	22.01.2018	46.0793	22.01.2018	42.4592	-8.18
65	ISA 01342	29.01.2018	46.0793	29.01.2018	50.9507	10.04
66	ISA 01644	30.01.2018	11.2208	30.01.2018	11.2275	0.06
67	ISA 01644	01.02.2018	11.2208	01.02.2018	9.6334	-15.22
68	ISA 01692	02.02.2018	36.6599	02.02.2018	44.9110	20.23
69	ISA 01336	19.02.2018	20.4666	19.02.2018	22.9089	10.66
70	ISA 01336	21.02.2018	20.4666	21.02.2018	21.7684	5.98
71	ISA 01336	22.02.2018	20.4666	22.02.2018	22.6204	9.52
72	ISA 01790	26.02.2018	13.7691	26.02.2018	12.0034	-13.70
73	ISA 01790	28.02.2018	13.7691	28.02.2018	8.5947	-46.27
74	ISA 01790	01.03.2018	13.7691	01.03.2018	11.6702	-16.50
75	ISA 01673	05.03.2018	32.0567	05.03.2018	27.8517	-15.10
76	ISA 01673	06.03.2018	32.0567	06.03.2018	28.7315	-11.57
77	ISA 01242	12.03.2018	9.4004	12.03.2018	10.5181	11.22
78	ISA 01242	13.03.2018	9.4004	13.03.2018	8.9220	-5.22
79	ISA 12701	15.03.2018	6.8239	15.03.2018	12.2536	44.31
80	ISA 12701	16.03.2018	6.8239	16.03.2018	11.7428	41.89
81	ISA 01223	19.03.2018	36.4737	19.03.2018	37.6543	3.19
82	ISA 01223	21.03.2018	36.4737	21.03.2018	37.4974	2.77
83	ISA 01223	22.03.2018	36.4737	22.03.2018	44.6993	20.27
84	ISA 01414	13.04.2018	17.3435	13.04.2018	18.4239	5.86
85	ISA 01414	16.04.2018	17.3435	16.04.2018	20.5255	15.50
86	ISA 13207	17.04.2018	61.1424	17.04.2018	53.4710	-13.39
87	ISA 13207	19.04.2018	61.1424	19.04.2018	43.3271	-34.11
88	ISA 01013	14.05.2018	19.0540	14.05.2018	15.8261	-20.40
89	ISA 00942	15.05.2018	22.1902	15.05.2018	18.8529	-16.26

		Original		Repeat		
Run	**Cryo ID**	**Date**	**PRA (ng/mL/h)**	**Date**	**PRA (ng/mL/h)**	**Difference (%)**
90	ISA 00942	16.05.2018	22.1902	16.05.2018	24.5411	10.06
91	ISA 00942	18.05.2018	22.1902	18.05.2018	25.5057	13.90
92	ISA 01711	07.06.2018	26.1062	07.06.2018	21.8921	-19.25
93	ISA 01542	11.06.2018	80.8745	11.06.2018	55.9026	-36.51
94	ISA 01622	12.06.2018	70.5247	12.06.2018	76.1573	7.40
95	ISA 01622	14.06.2018	70.5247	14.06.2018	61.2830	-15.08
96	ISA 00149	19.06.2018	8.3514	19.06.2018	8.3721	0.25
97	ISA 01665	19.06.2018	36.1158	19.06.2018	45.4487	20.54
98	ISA 01785	06.07.2018	42.8970	06.07.2018	49.4774	14.25
99	ISA 12708	11.07.2018	15.8376	11.07.2018	12.7141	-24.57
100	ISA 12708	12.07.2018	15.8376	12.07.2018	10.2027	-55.23
101	ISA 12708	16.07.2018	15.8376	16.07.2018	15.3968	-2.86
102	ISA 13328	18.07.2018	10.5506	18.07.2018	12.1936	14.45
103	ISA 13328	20.07.2018	10.5506	20.07.2018	14.6470	32.51
104	ISA 01186	24.07.2018	24.9992	24.07.2018	20.3224	-23.01
105	ISA 01186	25.07.2018	24.9992	25.07.2018	29.6870	15.79
106	ISA 01065	30.07.2018	12.2498	30.07.2018	13.1569	7.14
107	ISA 01065	31.07.2018	12.2498	31.07.2018	11.3087	-7.99
108	ISA 01065	01.08.2018	12.2498	01.08.2018	10.8561	-12.06
109	ISA 01022	02.08.2018	24.9282	02.08.2018	17.0302	-46.38
110	ISA 01022	06.08.2018	24.9282	06.08.2018	18.7736	-32.78

ISR: incurred sample reanalysis; PRA: plasma renin activity.

10.4 Development and validation of an innovative multiplex liquid chromatography high-resolution mass spectrometry method for the investigation of angiotensin peptides in plasma

Appendix 10.4-1: The applied MultiQuant® parameters for the individual quantification of all analytes and internal standards by the liquid chromatography high-resolution mass spectrometry method.

Peptide	Angiotensin I	Angiotensin II	Angiotensin-(1-7)	Angiotensin III
Precursor	433.1 $[M+3H]^{3+}$	523.9 $[M+2H]^{2+}$	450.5 $[M+2H]^{2}$	466.5 $[M+2H]^{2}$
Fragment 1	86.0923-86.1023	70.0595-70.0695	70.0595-70.0695	70.0595-70.0695
Fragment 2	110.0655-110.0755	110.0655-110.0755	110.0655-110.0755	110.0655-110.0755
Fragment 3	269.1540-269.1640	263.1331-263.1431	116.0635-116.0735	263.1350-263.1450
Fragment 4	382.1816-382.1916	264.1386-264.1486	235.1123-235.1223	264.1375-264.1475
Fragment 5	513.2783-513.2883	619.3472-619.3572	534.2605-534.2705	419.2373-419.2473
Fragment 6	534.2648-534.2748	647.3498-647.3598	619.3529-619.3629	504.3225-504.3325
Fragment 7	619.3529-619.3629	648.3480-648.3580	620.3631-620.3731	532.3202-532.3302
Fragment 8	647.3426-647.3526	756.4172-756.4272	647.3426-647.3526	642.3820-642.3920
Fragment 9	648.3478-648.3578	757.4130-757.4230	648.3478-648.3578	641.3889-641.3989
Fragment 10	784.4106-784.4206	784.4106-784.4206	756.4137-756.4237	669.3768-669.3868
Fragment 11	785.4264-785.4364	785.4264-785.4364	784.4106-784.4206	670.3785-670.3885
Fragment 12			785.4147- 85.4247	

Peptide	Angiotensin IV	Angiotensin A	Alamandine	Angiotensin-(1-9)
Precursor	388.3 $[M+2H]^{2}$	501.8 $[M+2H]^{2}$	428.3 $[M+2H]^{2}$	395.2 $[M+2H]^{2}$
Fragment 1	72.0756-72.0856	70.0595-70.0695	70.0595-70.0695	86.0923-86.1023
Fragment 2	110.0655-110.0755	110.0655-110.0755	110.0655-110.0755	110.0655-110.0755
Fragment 3	136.0704-136.0804	263.1336-263.1436	116.0637-116.0737	136.0688-136.0788
Fragment 4	235.1369-235.1469	462.2777-462.2877	356.7204-356.7304	156.0708-156.0808
Fragment 5	251.1463-251.1563	490.2745-490.2845	490.2720-490.2820	303.1391-303.1491
Fragment 6	263.1333-263.1433	575.3628-575.3728	575.3605-575.3705	371.1983-371.2083
Fragment 7	414.2075-414.2175	603.3571-603.3671	603.3586-603.3686	400.1949-400.2049
Fragment 8	513.2783-513.2883	712.4226-712.4326	604.3638-604.3738	506.2681-506.2781
Fragment 9	676.3409-676.3509	723.3962-723.4062	712.4254-712.4354	534.2657-534.2757
Fragment 10	677.3473-677.3573	740.4168-740.4268	740.4236-740.4336	535.2648-535.2748
Fragment 11			758.4381-758.4481	619.3529-619.3629
Fragment 12				647.3426-647.3526

Peptide	[Val^{5}]- Angiotensin I	([ring-D_5]Phe^{8})-Angiotensin II
Precursor	428.6 $[M+3H]^{3+}$	526.2 $[M+2H]^{2}$
Fragment 1	86.0923-86.1023	70.0595-70.0695
Fragment 2	110.0655-110.0755	110.0655-110.0755
Fragment 3	269.1540-269.1640	268.1666-268.1766
Fragment 4	382.1816-382.1916	269.1540-269.1640
Fragment 5	493.7589-493.7689	506.2661-506.2761
Fragment 6	534.2648-534.2748	534.2606-534.2706
Fragment 7	605.3381-605.3481	619.3529-619.3629
Fragment 8	606.3407-606.3507	647.3426-647.3526
Fragment 9	633.3321-633.3421	648.3478-648.3578
Fragment 10	770.3914-770.4014	756.4135-756.4235
Fragment 11	771.3910-771.4010	757.4142-757.4242
Fragment 12		784.4106-784.4206

Appendix 10.4-2: Evaluation of linearity for all 8 analytes by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin I

	Run 1		Run 2		Run 3		Run 4		Run 5		Run 6	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
25.4	26.257	**3.5%**	26.557	**4.7%**	23.389	**-7.8%**	28.922	**14.0%**	27.107	**6.8%**	27.837	**9.7%**
	25.75	**1.5%**	26.211	**3.3%**	27.741	**9.3%**	22.526	**-11.2%**	25.744	**1.5%**	25.678	**1.2%**
50.7	47.745	**-5.9%**	45.132	**-11.1%**	37.211	**-26.7%**	46.482	**-8.4%**	45.254	**-10.8%**	45.508	**-10.3%**
	45.758	**-9.8%**	42.474	**-16.3%**	29.384	**-42.1%**	48.282	**-4.8%**	47.402	**-6.6%**	23.304	**-54.1%**
101.5	107.383	**5.8%**	89.854	**-11.5%**	93.155	**-8.2%**	107.883	**6.3%**	75.425	**-25.7%**	87.939	**-13.3%**
	111.583	**10.0%**	91.33	**-10.0%**	95.857	**-5.5%**	104.217	**2.7%**	102.167	**0.7%**	90.477	**-10.8%**
203.0	198.422	**-2.2%**	225.431	**11.1%**	214.782	**5.8%**	218.715	**7.8%**	204.884	**1.0%**	211.985	**4.5%**
	184.416	**-9.1%**	221.463	**9.1%**	229.062	**12.9%**	215.234	**6.1%**	195.38	**-3.7%**	194.224	**-4.3%**
405.9	422.508	**4.1%**	420.668	**3.6%**	398.856	**-1.7%**	378.815	**-6.7%**	438.358	**8.0%**	382.476	**-5.8%**
	405.361	**-0.1%**	420.105	**3.5%**	379.567	**-6.5%**	464.159	**14.4%**	389.358	**-4.1%**	403.184	**-0.7%**
811.8	894.697	**10.2%**	785.975	**-3.2%**	831.303	**2.4%**	701.067	**-13.6%**	872.317	**7.5%**	812.386	**0.1%**
	750.011	**-7.6%**	1000.029	**23.2%**	807.523	**-0.5%**	701.986	**-13.5%**	851.166	**4.8%**	903.911	**11.3%**
1623.6	1652.644	**1.8%**	1641.667	**1.1%**	1733.917	**6.8%**	1626.235	**0.2%**	1608.267	**-0.9%**	1816.592	**11.9%**
	1574.64	**-3.0%**	1596.658	**-1.7%**	1504.884	**-7.3%**	1744.171	**7.4%**	1548.409	**-4.6%**	1729.756	**6.5%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.99653		**0.9965**		**0.99609**		**0.99195**		**0.99738**		**0.99454**	

Angiotensin II

	Run 1		Run 2		Run 3		Run 4		Run 5		Run 6	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
22.3	21.728	**-2.4%**	20.009	**-10.2%**	20.541	**-7.8%**	19.769	**-11.2%**	19.087	**-14.3%**	21.545	**-3.3%**
	23.5	**5.5%**	25.354	**13.8%**	24.526	**10.1%**	25.69	**15.4%**	24.616	**10.5%**	26.653	**19.7%**
44.5	44.218	**-0.7%**	41.563	**-6.7%**	43.78	**-1.7%**	48.634	**9.2%**	47.057	**5.7%**	38.209	**-14.2%**
	41.854	**-6.0%**	44.854	**0.7%**	44.497	**-0.1%**	41.479	**-6.9%**	45.328	**1.8%**	38.17	**-14.3%**
89.1	95.055	**6.7%**	86.406	**-3.0%**	85.043	**-4.5%**	77.989	**-12.4%**	89.565	**0.6%**	81.498	**-8.5%**
	84.266	**-5.4%**	93.322	**4.8%**	84.682	**-4.9%**	78.562	**-11.8%**	90.013	**1.1%**	92.086	**3.4%**
178.1	174.926	**-1.8%**	161.399	**-9.4%**	183.201	**2.8%**	172.954	**-2.9%**	179.809	**0.9%**	170.249	**-4.4%**
	178.226	**0.1%**	168.199	**-5.6%**	181.12	**1.7%**	179.042	**0.5%**	178.32	**0.1%**	166.83	**-6.3%**
356.3	346.861	**-2.6%**	373.669	**4.9%**	335.72	**-5.8%**	368.226	**3.4%**	346.523	**-2.7%**	337.629	**-5.2%**
	365.697	**2.7%**	354.603	**-0.5%**	393.642	**10.5%**	375.554	**5.4%**	328.195	**-7.9%**	372.268	**4.5%**
712.5	765.295	**7.4%**	755.858	**6.1%**	722.147	**1.4%**	771.956	**8.3%**	778.785	**9.3%**	718.139	**0.8%**
	697.714	**-2.1%**	777.934	**9.2%**	707.782	**-0.7%**	793.191	**11.3%**	675.976	**-5.1%**	722.939	**1.5%**

1425	1413.759	**-0.8%**	1377.908	**-3.3%**	1459.926	**2.5%**	1447.787	**1.6%**	1403.639	**-1.5%**	1594.59	**11.9%**
	1408.896	**-1.1%**	1391.54	**-2.3%**	1371.69	**-3.7%**	1270.704	**-10.8%**	1445.66	**1.4%**	1631.442	**14.5%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.99867		**0.99626**		**0.99769**		**0.99291**		**0.99683**		**0.99177**	

Angiotensin-(1-7)

	Run 1		**Run 2**		**Run 3**		**Run 4**		**Run 5**		**Run 6**	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
24.3	21.526	**-11.2%**	22.193	**-8.5%**	40.575	**67.3%**	23.472	**-3.2%**	25.381	**4.7%**	24.637	**1.6%**
	28.142	**16.0%**	28.113	**15.9%**	24.514	**1.1%**	40.561	**67.3%**	22.727	**-6.3%**	25.642	**5.7%**
48.5	43.908	**-9.5%**	42.763	**-11.8%**	53.318	**9.9%**	51.715	**6.6%**	51.431	**6.0%**	47.37	**-2.3%**
	48.164	**-0.7%**	44.082	**-9.1%**	47.852	**-1.3%**	49.117	**1.3%**	47.271	**-2.5%**	44.894	**-7.4%**
97	105.258	**8.5%**	111.096	**14.5%**	82.693	**-14.7%**	108.018	**11.4%**	93.319	**-3.8%**	94.579	**-2.5%**
	85.408	**-12.0%**	99.762	**2.8%**	82.774	**-14.7%**	90.719	**-6.5%**	99.955	**3.0%**	87.551	**-9.7%**
194	203.986	**5.1%**	176.864	**-8.8%**	196.762	**1.4%**	185.098	**-4.6%**	183.079	**-5.6%**	185.406	**-4.4%**
	205.606	**6.0%**	193.435	**-0.3%**	219.005	**12.9%**	166.288	**-14.3%**	207.037	**6.7%**	212.501	**9.5%**
388	368.144	**-5.1%**	358.337	**-7.6%**	529.418	**36.4%**	382.155	**-1.5%**	225.503	**-41.9%**	350.535	**-9.7%**
	376.746	**-2.9%**	357.247	**-7.9%**	504.105	**29.9%**	396.987	**2.3%**	226.665	**-41.6%**	405.269	**4.5%**
776	788.099	**1.6%**	883.351	**13.8%**	806.933	**4.0%**	831.808	**7.2%**	773.064	**-0.4%**	793.656	**2.3%**
	819.812	**5.6%**	887.525	**14.4%**	786.878	**1.4%**	827.987	**6.7%**	749.838	**-3.4%**	789.345	**1.7%**
1552	1529.1	**-1.5%**	1432.434	**-7.7%**	1485.236	**-4.3%**	1542.001	**-0.6%**	1521.55	**-2.0%**	1719.146	**10.8%**
	1542.384	**-0.6%**	1536.759	**-1.0%**	1586.086	**2.2%**	1478.532	**-4.7%**	1605.976	**3.5%**	1869.612	**20.5%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.99594		**0.99116**		**0.99584**		**0.99625**		**0.99829**		**0.9962**	

Angiotensin III

	Run 1		**Run 2**		**Run 3**		**Run 4**		**Run 5**		**Run 6**	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
31	32.853	**6.0%**	33.483	**8.0%**	34.064	**9.9%**	22.879	**-26.2%**	31.123	**0.4%**	28.763	**-7.2%**
	31.727	**2.3%**	26.205	**-15.5%**	29.73	**-4.1%**	33.22	**7.2%**	32.984	**6.4%**	35.177	**13.5%**
62	55.431	**-10.6%**	70.714	**14.1%**	60.821	**-1.9%**	55.476	**-10.5%**	54.732	**-11.7%**	85.101	**37.3%**
	59.176	**-4.6%**	64.619	**4.2%**	53.236	**-14.1%**	57.684	**-7.0%**	57.61	**-7.1%**	55.697	**-10.2%**
124	125.665	**1.3%**	122.106	**-1.5%**	128.076	**3.3%**	98.603	**-20.5%**	132.501	**6.9%**	122.233	**-1.4%**
	109.531	**-11.7%**	117.133	**-5.5%**	100.496	**-19.0%**	133.546	**7.7%**	129.45	**4.4%**	125.855	**1.5%**
248	232.986	**-6.1%**	197.66	**-20.3%**	274.14	**10.5%**	267.268	**7.8%**	250.072	**0.8%**	199.212	**-19.7%**
	278.252	**12.2%**	253.393	**2.2%**	257.697	**3.9%**	217.045	**-12.5%**	247.495	**-0.2%**	229.623	**-7.4%**
496	544.804	**9.8%**	513.797	**3.6%**	461.821	**-6.9%**	519.29	**4.7%**	493.064	**-0.6%**	460.965	**-7.1%**
	563.044	**13.5%**	476.14	**-4.0%**	505.187	**1.9%**	457.552	**-7.8%**	439.89	**-11.3%**	475.557	**-4.1%**
992	845.33	**-14.8%**	1013.648	**2.2%**	737.521	**-25.7%**	1016.256	**2.4%**	1133.839	**14.3%**	1034.662	**4.3%**

	991.439	**-0.1%**	889.525	**-10.3%**	953.075	**-3.9%**	1136.958	**14.6%**	1015.599	**2.4%**	1001.861	**1.0%**
1984	1969.83	**-0.7%**	2008.814	**1.3%**	1963.476	**-1.0%**	2097.202	**5.7%**	1926.774	**-2.9%**	2028.508	**2.2%**
	2033.875	**2.5%**	2018.832	**1.8%**	2033.102	**2.5%**	1732.457	**-12.7%**	1938.946	**-2.3%**	2279.515	**14.9%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.99391		**0.99604**		**0.99649**		**0.99319**		**0.99592**		**0.9952**	

Angiotensin IV

	Run 1		**Run 2**		**Run 3**		**Run 4**		**Run 5**		**Run 6**	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
22.9	21.753	**-5.1%**	19.944	**-12.9%**	22.173	**-3.2%**	24.71	**7.9%**	35.082	**53.1%**	21.591	**-5.8%**
	29.626	**29.3%**	25.865	**12.9%**	25.916	**13.1%**	19.94	**-13.0%**	26.009	**13.5%**	25.268	**10.3%**
45.8	49.891	**8.9%**	48.648	**6.2%**	40.707	**-11.2%**	33.681	**-26.5%**	39.732	**-13.3%**	44.342	**-3.2%**
	44.833	**-2.2%**	42.547	**-7.1%**	42.631	**-7.0%**	48.244	**5.3%**	38.988	**-14.9%**	37.63	**-17.9%**
91.6	76.294	**-16.7%**	68.444	**-25.3%**	88.015	**-4.0%**	103.031	**12.4%**	86.548	**-5.6%**	85.027	**-7.2%**
	105.085	**14.7%**	65.937	**-28.0%**	87.994	**-4.0%**	87.142	**-4.9%**	94.86	**3.5%**	89.489	**-2.3%**
183.3	160.526	**-12.4%**	206.5	**12.7%**	232.136	**26.7%**	210.284	**14.7%**	172.35	**-6.0%**	201.508	**9.9%**
	177.895	**-2.9%**	177.544	**-3.1%**	189.848	**3.6%**	181.187	**-1.1%**	196.161	**7.0%**	156.556	**-14.6%**
366.6	377.53	**3.0%**	346.642	**-5.4%**	355.076	**-3.1%**	325.95	**-11.1%**	405.968	**10.8%**	348.711	**-4.9%**
	349.77	**-4.6%**	340.445	**-7.1%**	398.246	**8.6%**	458.869	**25.2%**	364.103	**-0.7%**	364.251	**-0.6%**
733.1	787.02	**7.4%**	763.245	**4.1%**	827.761	**12.9%**	628.756	**-14.2%**	826.962	**12.8%**	730.229	**-0.4%**
	676.25	**-7.8%**	899.653	**22.7%**	746.494	**1.8%**	700.418	**-4.5%**	748.892	**2.2%**	785.013	**7.1%**
1466.2	1487.752	**1.5%**	1474.604	**0.6%**	1385.024	**-5.5%**	1420.89	**-3.1%**	1384.023	**-5.6%**	1485.851	**1.3%**
	1458.842	**-0.5%**	1456.683	**-0.6%**	1432.293	**-2.3%**	1634.834	**11.5%**	1410.773	**-3.8%**	1618.013	**10.4%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.9961		**0.99459**		**0.99539**		**0.99186**		**0.99332**		**0.99565**	

Angiotensin A

	Run 1		**Run 2**		**Run 3**		**Run 4**		**Run 5**		**Run 6**	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
21.8	22.849	**4.6%**	29.753	**36.2%**	33.59	**53.8%**	23.525	**7.7%**	20.489	**-6.2%**	24.491	**12.1%**
	11.243	**-48.5%**	20.458	**-6.3%**	21.56	**-1.3%**	11.606	**-46.9%**	25.113	**15.0%**	66.749	**205.6%**
43.7	43.213	**-1.1%**	45.798	**4.8%**	40.113	**-8.2%**	38.105	**-12.8%**	38.667	**-11.5%**	37.942	**-13.2%**
	38.101	**-12.8%**	47.673	**9.1%**	47.443	**8.6%**	38.71	**-11.4%**	40.14	**-8.1%**	39.79	**-8.9%**
87.4	91.05	**4.2%**	89.88	**2.9%**	78.948	**-9.6%**	88.85	**1.7%**	78.551	**-10.1%**	87.735	**0.4%**
	91.321	**4.5%**	88.771	**1.6%**	92.845	**6.3%**	94.409	**8.0%**	99.815	**14.2%**	80.562	**-7.8%**
174.8	160.405	**-8.2%**	150.303	**-14.0%**	195.175	**11.7%**	189.823	**8.6%**	162.8	**-6.8%**	189.622	**8.5%**
	186.746	**6.9%**	166.676	**-4.6%**	196.892	**12.7%**	198.732	**13.7%**	173.456	**-0.7%**	166.592	**-4.7%**
349.5	331.768	**-5.1%**	352.431	**0.8%**	357.381	**2.3%**	332.5	**-4.9%**	468.38	**34.0%**	355.021	**1.6%**
	395.385	**13.1%**	389.031	**11.3%**	299.287	**-14.4%**	328.769	**-5.9%**	397.071	**13.6%**	462.422	**32.3%**

699	655.893	**-6.2%**	724.227	**3.6%**	605.224	**-13.4%**	677.055	**-3.1%**	742.457	**6.2%**	732.908	**4.9%**
	685.897	**-1.9%**	619.121	**-11.4%**	684.555	**-2.1%**	663.029	**-5.1%**	668.209	**-4.4%**	657.437	**-5.9%**
1398	1403.172	**0.4%**	1438.616	**2.9%**	1531.492	**9.5%**	1554.846	**11.2%**	1394.111	**-0.3%**	1808.457	**29.4%**
	1417.026	**1.4%**	1386.619	**-0.8%**	1367.738	**-2.2%**	1286.533	**-8.0%**	1369.416	**-2.0%**	1579.921	**13.0%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.99658		**0.99584**		**0.9931**		**0.99343**		**0.994**		**0.99348**	

Alamandine

	Run 1		Run 2		Run 3		Run 4		Run 5		Run 6	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
19.0	18.04	**-5.2%**	22.182	**16.6%**	15.501	**-18.5%**	17.627	**-7.4%**	19.645	**3.2%**	21.686	**14.0%**
	19.755	**3.8%**	16.983	**-10.8%**	21.786	**14.5%**	30.747	**61.6%**	19.491	**2.4%**	16.601	**-12.8%**
38.1	36.21	**-4.8%**	38.992	**2.5%**	37.983	**-0.2%**	40.802	**7.2%**	52.203	**37.2%**	40.108	**5.4%**
	41.871	**10.0%**	31.844	**-16.3%**	43.247	**13.7%**	40.997	**7.7%**	34.863	**-8.4%**	35.942	**-5.5%**
76.1	72.368	**-4.9%**	72.756	**-4.4%**	41.7	**-45.2%**	74.229	**-2.5%**	68.382	**-10.1%**	77.589	**2.0%**
	76.368	**0.4%**	83.738	**10.0%**	66.113	**-13.1%**	82.276	**8.1%**	74.575	**-2.0%**	67.047	**-11.9%**
152.2	168.299	**10.6%**	117.34	**-22.9%**	161.672	**6.2%**	153.96	**1.2%**	155.416	**2.1%**	151.789	**-0.3%**
	138.148	**-9.2%**	153.062	**0.6%**	145.19	**-4.6%**	137.065	**-9.9%**	166.112	**9.1%**	167.129	**9.8%**
304.4	308.986	**1.5%**	283.735	**-6.8%**	319.297	**4.9%**	271.73	**-10.7%**	209.51	**-31.2%**	322.433	**5.9%**
	273.096	**-10.3%**	271.217	**-10.9%**	317.795	**4.4%**	310.121	**1.9%**	190.334	**-37.5%**	305.756	**0.4%**
608.8	645.329	**6.0%**	688.67	**13.1%**	578.154	**-5.0%**	629.459	**3.4%**	642.96	**5.6%**	595.961	**-2.1%**
	636.219	**4.5%**	685.257	**12.6%**	582.776	**-4.3%**	714.289	**17.3%**	614.593	**1.0%**	564.691	**-7.2%**
1217.6	1158.687	**-4.8%**	1152.827	**-5.3%**	1126.409	**-7.5%**	1209.458	**-0.7%**	1156.723	**-5.0%**	1179.867	**-3.1%**
	1242.26	**2.0%**	1190.527	**-2.2%**	1331.259	**9.3%**	1238.155	**1.7%**	1238.776	**1.7%**	1282.395	**5.3%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.99653		**0.99152**		**0.99262**		**0.99649**		**0.99743**		**0.99547**	

Angiotensin-(1-9)

	Run 1		Run 2		Run 3		Run 4		Run 5		Run 6	
NV (pg/mL)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)	MV (pg/mL)	RE (%)
31.0	30.536	**-1.5%**	31.426	**1.4%**	30.686	**-1.0%**	27.71	**-10.6%**	25.98	**-16.2%**	32.056	**3.4%**
	32.703	**5.5%**	31.946	**3.0%**	32	**3.2%**	36.399	**17.4%**	33.881	**9.3%**	42.519	**37.1%**
62.0	62.658	**1.0%**	64.514	**4.0%**	61.829	**-0.3%**	65.254	**5.2%**	69.002	**11.3%**	66.144	**6.7%**
	61.146	**-1.4%**	56.817	**-8.4%**	59.974	**-3.3%**	57.379	**-7.5%**	64.446	**3.9%**	60.484	**-2.5%**
124.0	118.293	**-4.6%**	119.735	**-3.5%**	116.758	**-5.9%**	107.443	**-13.4%**	120.142	**-3.1%**	112.209	**-9.5%**
	105.456	**-15.0%**	122.007	**-1.6%**	125.653	**1.3%**	107.724	**-13.1%**	126.426	**1.9%**	115.723	**-6.7%**
248.1	244.331	**-1.5%**	221.356	**-10.8%**	247.665	**-0.2%**	247.578	**-0.2%**	237.702	**-4.2%**	230.687	**-7.0%**
	259.702	**4.7%**	244.456	**-1.4%**	257.38	**3.8%**	235.955	**-4.9%**	265.247	**6.9%**	227.907	**-8.1%**
496.1	503.746	**1.5%**	503.391	**1.5%**	534.481	**7.7%**	543.841	**9.6%**	431.842	**-13.0%**	477.801	**-3.7%**

	538.211	**8.5%**	489.444	**-1.3%**	605.939	**22.1%**	546.889	**10.2%**	391.69	**-21.0%**	503.991	**1.6%**
992.2	988.133	**-0.4%**	1131.055	**14.0%**	948.639	**-4.4%**	1042.949	**5.1%**	1035.042	**4.3%**	1014.179	**2.2%**
	1037.046	**4.5%**	1073.35	**8.2%**	969.847	**-2.3%**	1099.822	**10.8%**	968.631	**-2.4%**	1089.855	**9.8%**
1984.4	1966.68	**-0.9%**	1871.11	**-5.7%**	1997.923	**0.7%**	1945.219	**-2.0%**	1976.743	**-0.4%**	2257.783	**13.8%**
	1947.269	**-1.9%**	1946.944	**-1.9%**	1993.943	**0.5%**	1834.706	**-7.5%**	2015.2	**1.6%**	2532.518	**27.6%**
	Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient		Correlation coefficient	
	0.9979		**0.99589**		**0.99896**		**0.99243**		**0.99532**		**0.99544**	

Relative error: RE; MV: measured value; NV: nominal value.

Appendix 10.4-3: The accuracy and precision of angiotensin I by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin I																			
		Run 1					Run 2					Run 3					Bwtween-run RE	Between-run CV	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	24.274	23.8375	6.9%	-0.5%	11.7%	29.474	27.222	9.1%	4.7%	11.3%	22.564	23.298	8.4%	4.1%	9.1%	**-3.1%**	**8.0%**	**10.6%**
		23.401					24.97					24.032							
	2	29.998	25.064				28.656	28.4505				29.606	28.227						
		20.13					28.245					26.848							
	3	28.621	28.277				21.116	22.3415				26.659	26.405						
		27.933					23.567					26.151							
	4	25.015	24.604				29.75	27.037				26.442	26.2015						
		24.193					24.324					25.961							
	5	25.97	24.489				26.06	27.757				27.125	29.106						
		23.008					29.454					31.087							
Low	1	44.932	43.449	13.6%	-2.8%	14.9%	45.994	47.5115	5.9%	-6.5%	8.9%	52.446	50.1175	8.7%	-5.2%	10.7%	**4.8%**	**9.4%**	**11.6%**
		41.966					49.029					47.789							
	2	50.078	44.9165				45.302	44.6795				49.201	52.007						
		39.755					44.057					54.813							
	3	54.565	59.7175				41.174	46.7965				57.217	51.2315						
		64.87					52.419					45.246							
	4	52.078	52.2235				44.84	46.1025				43.154	43.546						
		52.369					47.365					43.938							
	5	43.25	46.319				55.724	52.1025				43.834	43.6395						
		49.388					48.481					43.445							
Middle	1	213.013	206.5395	3.7%	5.6%	6.6%	192.369	207.1145	7.6%	4.5%	9.6%	191.393	192.9465	6.4%	1.4%	6.6%	**-3.9%**	**5.9%**	**7.7%**
		200.066					221.86					194.5							
	2	190.008	213.223				225.913	223.409				191.969	195.4415						
		236.438					220.905					198.914							
	3	227.189	227.189				240.296	223.1035				232.345	224.016						
							205.911					215.687							
	4	214.554	215.4095				203.46	221.101				208.59	202.484						
		216.265					238.742					196.378							
	5	216.535	209.605				183.759	185.8025				219.695	214.404						
		202.675					187.846					209.113							
High	1	993.923	963.4535	7.8%	12.7%	8.2%	783.694	789.737	6.6%	2.6%	10.4%	888.814	875.33	2.2%	7.3%	3.2%	**-7.6%**	**6.9%**	**8.5%**
		932.984					795.78					861.846							
	2	840.042	843.24				1001.605	870.3575				856.4	845.975						
		846.438										835.55							
	3	930.482	924.8555				739.11	758.987				858.502	880.918						
		919.229					743.213					903.334							
	4	851.705	842.1835				774.761	862.662				895.592	859.915						
		832.662					826.154					824.238							
	5	941.393	1002.505				899.17	884.019				893.056	894.797						
		1063.617					884.019					896.538							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-4: The accuracy and precision of angiotensin II by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin II																		
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	22.352	22.59	9.7%	3.5%	10.1%	23.898	22.531	7.0%	-1.0%	9.5%	24.961	25.2805	4.9%	11.3%	6.8%	**-4.6%**	**8.5%**	**9.8%**
		22.828					21.164					25.6							
	2	20.588	21.4685				21.906	21.443				25.015	23.489						
		22.349					20.98					21.963							
	3	25.454	25.9725				17.371	19.7255				25.298	25.9195						
		26.491					22.08					26.541							
	4	19.213	20.5455				24.2	23.8415				25.442	23.467						
		21.878					23.483					21.492							
	5	25.821	24.6425				21.14	22.6985				26.305	25.7265						
		23.464					24.257					25.148							
Low	1	43.106	42.8595	8.9%	-1.4%	10.3%	44.261	44.038	7.3%	2.3%	9.9%	49.117	49.795	8.5%	0.3%	9.8%	**-0.4%**	**7.8%**	**9.8%**
		42.613					43.815					50.473							
	2	44.71	49.396				46.032	46.1095				43.149	39.9275						
		54.082					46.187					36.706							
	3	45.694	45.898				38.933	42.701				42.416	42.85						
		46.102					46.469					43.284							
	4	44.88	42.1945				49.524	43.8655				44.885	43.8265						
		39.509					38.207					42.768							
	5	38.03	39.102				52.279	51.146				51.08	47.0145						
		40.174					50.013					42.949							
Middle	1	158.032	165.5475	3.5%	-6.8%	4.4%	152.763	163.1725	5.1%	-3.3%	7.7%	149.708	149.943	6.4%	-11.3%	8.3%	**7.2%**	**6.0%**	**7.7%**
		173.063					173.582					150.178							
	2	170.715	170.701				153.078	166.227				163.346	166.8835						
		170.687					179.376					170.421							
	3	165.868	165.868				173.733	184.0715				146.02	163.213						
							194.41					180.406							
	4	159.269	156.61				174.293	168.9035				137.022	144.4705						
		153.951					163.514					151.919							
	5	175.058	170.995				186.085	178.649				163.941	165.6						
		166.932					171.213					167.259							
High	1	759.909	767.52	6.3%	-0.6%	6.5%	726.563	733.043	3.3%	0.0%	6.1%	745.204	694.3175	2.1%	-4.3%	6.2%	**1.6%**	**4.5%**	**6.4%**
		775.131					739.523					643.431							
	2	717.515	717.3615				750.642	710.553				625.485	676.685						
		717.208										727.885							
	3	717.621	709.9635				670.464	673.616				655.502	699.4915						
		702.306					662.796					743.481							
	4	665.817	642.1675				684.436	716.3905				667.643	667.475						
		618.518					776.89					667.307							
	5	737.475	705.4705				655.891	729.85				680.142	670.448						
		673.466					729.85					660.754							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-5: The accuracy and precision of angiotensin-(1-7) by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin-(1-7)																		
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	22.892	23.621	9.1%	4.7%	10.6%	25.773	27.259	9.6%	5.7%	14.9%	22.203	24.7565	15.3%	2.8%	16.5%	-4.4%	10.8%	13.7%
		24.35					28.745					27.31							
	2	23.839	22.409				25.141	23.478				18.805	19.8575						
		20.979					21.815					20.91							
	3	25.738	26.0955				31.081	26.8255				25.889	29.0255						
		26.453					22.57					32.162							
	4	30.916	28.036				32.315	28.086				28.947	28.215						
		25.156					23.857					27.483							
	5	27.402	26.767				23.196	22.5705				22.501	22.7695						
		26.132					21.945					23.038							
Low	1	57.438	56.2225	7.8%	6.6%	10.3%	47.784	45.796	6.3%	2.9%	11.1%	50.112	52.0135	11.0%	13.0%	11.0%	-7.5%	9.0%	11.1%
		55.007					43.808					53.915							
	2	51.299	53.66				47.393	50.8185				52.223	49.7715						
		56.021					54.244					47.32							
	3	53.995	48.757				40.474	48.9175				54.215	54.519						
		43.519					57.361					54.823							
	4	52.021	53.512				52.659	49.5315				54.928	52.505						
		55.003					46.404					50.082							
	5	51.303	46.2965				52.916	54.47				66.722	65.105						
		41.29					56.024					63.488							
Middle	1	192.431	205.0605	9.7%	0.9%	10.1%	158.588	172.743	7.9%	-7.9%	9.3%	188.106	185.981	10.4%	0.4%	11.3%	2.2%	9.8%	10.7%
		217.69					186.898					183.856							
	2	203.239	193.137				147.1	159.566				187.853	204.573						
		183.035					172.032					221.293							
	3	218.222	218.222				190.828	197.2935				177.064	186.77						
							203.759					196.476							
	4	198.249	195.1895				176.171	178.263				165.281	172.244						
		192.13					180.355					179.207							
	5	156.637	166.681				191.629	185.404				234.848	224.782						
		176.725					179.179					214.716							
High	1	791.44	807.278	9.1%	-4.9%	9.1%	790.893	818.929	6.4%	0.4%	8.0%	831.671	814.7735	11.3%	-3.2%	11.2%	2.6%	8.7%	9.4%
		823.116					846.965					797.876							
	2	816.817	775.206				760.574	742.5995				773.685	814.1255						
		733.595										854.566							
	3	671.185	677.2295				724.625	719.353				637.976	669.032						
		683.274					698.647					700.088							
	4	662.776	654.9265				740.059	776.03				814.304	809.937						
		647.077					850.691					805.57							
	5	767.019	775.5165				701.369	837.237				652.246	648.183						
		784.014					837.237					644.12							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-6: The accuracy and precision of angiotensin III by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin III																			
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	33.762	32.41	6.4%	6.1%	8.2%	34.152	35.3805	19.0%	18.5%	19.1%	36.08	36.0965	7.8%	13.8%	11.1%	-12.8%	12.8%	14.3%
		31.058					36.609					36.113							
	2	33.232	34.4525				28.646	29.0105				28.151	31.038						
		35.673					29.375					33.925							
	3	36.327	35.346				51.837	48.147				36.063	38.623						
		34.365					44.457					41.183							
	4	30.115	29.9935				33.022	36.2455				34.583	35.585						
		29.872					39.469					36.587							
	5	28.894	32.1875				36.362	34.967				30.184	34.971						
		35.481					33.572					39.758							
Low	1	59.131	58.738	4.3%	-1.1%	7.8%	60.458	64.628	6.6%	2.5%	7.9%	73.876	73.5315	11.4%	2.5%	12.2%	-1.3%	7.7%	9.4%
		58.345					68.798					73.187							
	2	63.523	62.0345				60.612	59.494				69.868	67.83						
		60.546					58.376					65.792							
	3	66.261	60.125				63.025	65.518				69.202	62.9735						
		53.989					68.011					56.745							
	4	63.628	60.152				68.246	69.008				53.46	57.3825						
		56.676					69.77					61.305							
	5	60.943	65.585				63.413	59.115				54.674	56.113						
		70.227					54.817					57.552							
Middle	1	218.431	247.5765	7.4%	1.9%	9.5%	261.03	229.768	8.3%	-1.1%	10.9%	215.809	216.111	12.9%	-10.1%	14.5%	3.1%	10.4%	12.3%
		276.722					198.506					216.413							
	2	256.032	238.3445				241.794	258.3525				257.826	244.1205						
		220.657					274.911					230.415							
	3	285.113	285.113				254.397	263.798				241.402	255.3775						
							273.199					269.353							
	4	249.489	242.69				223.297	217.53				196.757	218.402						
		235.891					211.763					240.047							
	5	235.304	249.4505				270.626	257.044				203.47	180.9525						
		263.597					243.462					158.435							
High	1	1125.27	1145.255	4.8%	7.7%	5.3%	1040.344	1070.621	5.7%	-1.1%	8.4%	1113.128	1062.824	7.0%	-2.1%	8.8%	-1.5%	7.1%	8.5%
		1165.24					1100.898					1012.52							
	2	1099.002	1053.3615				869.678	928.604				956.007	997.155						
		1007.721										1038.303							
	3	1036.834	1031.475				987.53	963.2335				878.528	977.9295						
		1026.116					945.398					1077.331							
	4	1055.333	1019.6175				981.069	944.873				872.762	880.215						
		983.902					1042.691					887.668							
	5	1100.12	1094.2945				847.055	998.298				952.508	936.045						
		1088.469					998.298					919.582							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-7: The accuracy and precision of angiotensin IV by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin IV																		
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	22.262	25.251	15.2%	-5.6%	20.0%	26.433	24.7335	11.4%	7.8%	13.9%	21.471	20.71	10.7%	4.3%	14.1%	-2.1%	12.9%	16.4%
		28.24					23.034					19.949							
	2	27.675	23.63				23.521	21.5415				21.598	26.124						
		19.585					19.562					30.65							
	3	19.783	22.7095				24.554	22.2255				23.263	21.879						
		25.636					19.897					20.495							
	4	19.618	17.1925				26.139	27.8025				24.28	24.237						
		14.767					29.466					24.194							
	5	20.367	19.3015				24.576	27.1335				27.796	26.555						
		18.236					29.691					25.314							
Low	1	39.668	36.329	14.0%	-6.4%	15.4%	39.348	43.528	3.9%	-6.7%	9.0%	49.917	44.434	5.1%	-4.5%	12.2%	5.9%	8.3%	12.1%
		32.99					47.708					38.951							
	2	39.716	42.4545				40.39	44.744				47.665	43.3065						
		45.193					49.098					38.948							
	3	49.693	52.53				36.06	40.34				47.653	45.237						
		55.367					44.62					42.821							
	4	39.21	42.909				42.34	42.02				37.773	40.124						
		46.608					41.7					42.475							
	5	36.549	40.184				42.62	43.067				38.799	45.743						
		43.819					43.514					52.687							
Middle	1	197.239	177.469	5.5%	-3.5%	9.8%	162.413	178.8175	4.9%	-6.7%	8.1%	216.517	186.9065	4.0%	5.0%	9.5%	1.7%	6.8%	10.3%
		157.699					195.222					157.296							
	2	144.327	165.8695				159.043	165.5775				208.594	204.818						
		187.412					172.112					201.042							
	3	192.094	192.094				189.327	175.537				183.449	192.3395						
							161.747					201.23							
	4	165.315	171.713				168.085	176.245				168.828	185.233						
		178.111					184.405					201.638							
	5	183.122	177.0945				156.37	158.9915				189.018	193.0825						
		171.067					161.613					197.147							
High	1	817.907	824.265	11.1%	1.4%	10.6%	618.984	640.5155	9.5%	-7.0%	13.6%	837.06	782.478	7.1%	5.3%	7.8%	0.1%	10.1%	11.3%
		830.623					662.047					727.896							
	2	707.842	716.6315				882.171	755.048				738.468	762.897						
		725.421										787.326							
	3	744.182	725.7365				627.925	615.069				748.152	769.413						
		707.291					638.807					790.674							
	4	610.061	627.483				591.331	747.2705				685.507	695.474						
		644.905					714.104					705.441							
	5	820.348	821.8025				780.437	651.702				834.41	849.3835						
		823.257					651.702					864.357							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-8: The accuracy and precision of angiotensin A by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin A																			
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	16.49	21.5195	10.7%	-3.8%	18.1%	26.089	23.1485	15.1%	4.1%	18.0%	29.658	25.131	9.5%	9.3%	15.5%	**-3.2%**	**12.4%**	**17.4%**
		26.549					20.208					20.604							
	2	15.959	18.4675				23.075	24.663				23.474	23.782						
		20.976					26.251					24.09							
	3	21.04	18.8935				25.789	24.044				27.611	23.978						
		16.747					22.299					20.345							
	4	20.6	23.524				21.84	25.1175				21.329	20.202						
		26.448					28.395					19.075							
	5	23.128	22.6535				18.582	16.7375				28.592	26.228						
		22.179					14.893					23.864							
Low	1	37.515	42.2235	6.8%	0.5%	11.9%	48.087	46.3695	12.3%	-4.5%	13.3%	40.622	39.432	12.7%	-5.3%	13.2%	**3.1%**	**10.4%**	**12.6%**
		46.932					44.652					38.242							
	2	46.103	44.6275				48.394	45.64				47.871	45.807						
		43.152					42.886					43.743							
	3	54.19	47.37				45.837	42.332				39.554	36.533						
		40.55					38.827					33.512							
	4	41.503	45.507				43.253	40.6175				37.45	37.096						
		49.511					37.982					36.742							
	5	40.775	39.724				31.617	33.555				51.123	48.056						
		38.673					35.493					44.989							
Middle	1	180.921	178.101	7.3%	6.3%	6.9%	191.12	182.7475	12.2%	-8.0%	13.0%	146.929	142.8045	12.9%	-1.7%	12.6%	**1.1%**	**11.8%**	**12.1%**
		175.281					174.375					138.68							
	2	178.967	174.743				184.407	179.599				198.835	198.007						
		170.519					174.791					197.179							
	3	201.575	201.575				150.935	150.433				191.263	189.057						
							149.931					186.851							
	4	179.053	175.162				119.095	137.3935				170.419	160.488						
		171.271					155.692					150.557							
	5	197.091	199.4655				153.3	153.2775				172.986	168.725						
		201.84					153.255					164.464							
High	1	797.212	787.027	5.8%	7.3%	7.0%	746.271	769.8955	14.9%	-12.1%	15.4%	742.253	683.221	1.9%	-3.9%	6.7%	**2.9%**	**11.7%**	**12.5%**
		776.842					793.52					624.189							
	2	789.567	745.7535				607.588	586.966				653.809	671.7055						
		701.94										689.602							
	3	805.425	756.321				566.344	534.5005				664.335	677.049						
		707.217					531.901					689.763							
	4	691.989	677.7525				537.1	569.659				654.448	650.425						
		663.516					605.6					646.402							
	5	778.264	781.5735				533.718	609.544				743.433	675.4585						
		784.883					609.544					607.484							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-9: The accuracy and precision of alamandine by the liquid chromatography high-resolution mass spectrometry method.

Alamandine																			
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	21.368	20.84	11.0%	3.3%	11.0%	17.254	17.7725	9.7%	0.4%	13.4%	23.535	21.4965	11.8%	5.5%	16.1%	-3.1%	10.3%	13.4%
		20.312					18.291					19.458							
	2	21.315	22.313				21.017	18.6645				21.528	19.825						
		23.311					16.312					18.122							
	3	15.773	16.4885				18.098	17.053				17.497	17.1065						
		17.204					16.008					16.716							
	4	18.96	19.611				18.08	20.734				17.948	18.782						
		20.262					23.388					19.616							
	5	18.979	19.0535				19.978	21.3085				19.076	23.19						
		19.128					22.639					27.304							
Low	1	38.846	35.8115	5.6%	2.3%	11.4%	40.743	39.5305	9.5%	6.3%	9.5%	35.82	38.3295	8.3%	-5.7%	9.2%	-1.0%	9.0%	11.0%
		32.777					38.318					40.839							
	2	39.744	37.923				33.311	34.47				33.598	30.982						
		36.102					35.629					28.366							
	3	43.72	39.1385				44.937	44.685				35.615	35.288						
		34.557					44.433					34.961							
	4	43.26	40.405				42.864	41.005				36.395	36.7975						
		37.55					39.146					37.2							
	5	36.11	41.3475				43.566	42.56				38.442	38.0105						
		46.585					41.554					37.579							
Middle	1	151.384	154.2255	9.8%	0.3%	11.4%	139.256	149.442	7.3%	2.1%	9.3%	131.875	133.696	12.5%	-5.8%	13.2%	1.1%	9.9%	11.4%
		157.067					159.628					135.517							
	2	129.12	151.0045				125.64	138.101				129.923	137.5105						
		172.889					150.562					145.098							
	3	173.369	173.369				151.763	161.0605				132.637	132.6995						
							170.358					132.762							
	4	159.061	153.796				156.929	164.492				128.65	137.8165						
		148.531					172.055					146.983							
	5	131.767	131.225				168.466	163.798				161.862	175.3545						
		130.683					159.13					188.847							
High	1	582.79	604.9455	8.6%	-3.6%	8.6%	691.304	681.2365	6.2%	4.7%	7.6%	681.931	631.6595	11.6%	-2.7%	11.8%	0.5%	9.1%	9.9%
		627.101					671.169					581.388							
	2	653.718	636.108				680.117	634.6365				684.503	666.7275						
		618.498										648.952							
	3	564.606	573.496				589.156	574.813				520.175	526.3985						
		582.386					560.66					532.622							
	4	490.571	506.143				588.966	657.754				637.658	623.37						
		521.715					683.48					609.082							
	5	627.937	614.58				632.028	637.781				506.411	512.2515						
		601.223					637.781					518.092							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-10: The accuracy and precision of angiotensin-(1-9) by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin-(1-9)																		
		Run 1					Run 2					Run 3					Inter-run accuracy	Inter-run precision	Between-run CV-I
QC	Nr	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)	Value (pg/mL)	Mean (pg/mL)	CV-M (%)	RE (%)	CV-I (%)			
LLOQ	1	30.589	31.0365	6.0%	5.2%	7.1%	32.978	32.3075	6.1%	6.9%	6.2%	36.252	33.59	4.7%	12.0%	7.5%	**-8.0%**	**5.9%**	**7.3%**
		31.484					31.637					30.928							
	2	28.661	30.971				30.34	30.1275				33.118	34.1115						
		33.281					29.915					35.105							
	3	35.537	35.445				35.036	34.7545				31.68	34.9875						
		35.353					34.473					38.295							
	4	30.837	31.8025				33.573	35.085				33.172	33.4795						
		32.768					36.597					33.787							
	5	35.387	33.881				33.189	33.3965				38.425	37.422						
		32.375					33.604					36.419							
Low	1	63.237	62.437	2.7%	-2.5%	6.2%	57.827	62.4	7.3%	0.3%	8.8%	66.505	63.982	3.1%	4.0%	4.7%	**-0.6%**	**5.2%**	**7.1%**
		61.637					66.973					61.459							
	2	56.703	60.861				53.056	56.5955				67.249	63.549						
		65.019					60.135					59.849							
	3	58.721	59.878				55.675	59.257				69.205	67.7725						
		61.035					62.839					66.34							
	4	56.235	61.18				70.642	68.392				63.446	62.406						
		66.125					66.142					61.366							
	5	61.118	58.026				63.087	64.236				65.887	64.6665						
		54.934					65.385					63.446							
Middle	1	244.491	250.1565	3.3%	-2.0%	7.1%	239.237	235.122	7.0%	0.5%	7.0%	211.741	222.8565	6.0%	-1.2%	7.0%	**0.9%**	**5.4%**	**6.8%**
		255.822					231.007					233.972							
	2	249.944	243.178				228.027	231.348				255.799	262.0045						
		236.412					234.669					268.21							
	3	260.353	230.3295				246.123	245.735				235.257	251.4895						
		200.306					245.347					267.722							
	4	243.032	249.4515				257.93	262.849				239.754	239.9005						
		255.871					267.768					240.047							
	5	251.748	242.8				280.868	271.474				244.915	249.027						
		233.852					262.08					253.139							
High	1	1058.607	1065.2375	5.6%	5.0%	6.4%	1060.797	1092.5355	7.2%	3.8%	8.7%	1015.187	1013.902	3.9%	4.8%	5.1%	**-4.5%**	**5.3%**	**6.6%**
		1071.868					1124.274					1012.617							
	2	1126.392	1064.5185				985.999	972.849				1013.447	1084.21						
		1002.645										1154.973							
	3	1047.404	1051.172				959.699	937.219				963.088	997.519						
		1054.94					898.936					1031.95							
	4	933.328	938.9815				975.502	1039.1845				1090.049	1082.5975						
		944.635					1140.88					1075.146							
	5	1135.679	1087.6255				937.489	1107.194				1026.592	1021.0965						
		1039.572					1107.194					1015.601							

Five samples were investigated for each of the four quality control (QC) levels.The mean of each sample was obtained by two injections of the same sample; CV-M: Coefficient of variation between samples; CV-I Coefficient of variation between injections; RE: relative error.

Appendix 10.4-11: The evaluation of the sensitivity in three different runs by the liquid chromatography high-resolution mass spectrometry method.

Angiotensin I

		Run 1				Run 2				Run 3			
QC	**Nr**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**
	1	1430.5	23.8375			1445.5	27.222			1536.5	23.298		
	2	1508	25.064			1545	28.4505			1872	28.227		
LLOQ	**3**	1480.5	28.277	**6.9%**	**-0.4%**	1185	22.3415	**9.1%**	**3.8%**	1933.5	26.405	**8.4%**	**4.1%**
	4	1356.5	24.604			1450.5	27.037			1756	26.2015		
	5	1388	24.489			1306	27.757			1844.5	29.106		
LLOQ and Zero		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**	
LLOQ		1432.7		**3:1**		1386.4		**5:1**		1788.5		**3:1**	
Zero		499.95				288.7				524.25			

Angiotensin II

		Run 1				Run 2				Run 3			
QC	**Nr**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**
	1	2100	22.59			1738	22.531			1711	25.2805		
	2	1995.5	21.4685			1747	21.443			1584	23.489		
LLOQ	**3**	2384.5	25.9725	**9.7%**	**3.5%**	1366	19.7255	**7.0%**	**-1.0%**	1856	25.9195	**4.9%**	**11.3%**
	4	1796.5	20.5455			1591.5	23.8415			1628	23.467		
	5	2237	24.6425			1543	22.6985			1744	25.7265		
LLOQ and Zero		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**	
LLOQ		2102.7		**6:1**		1597.1		**10:1**		1704.6		**11:1**	
Zero		352.2				156.65				155.15			

Angiotensin-(1-7)

		Run 1				Run 2				Run 3			
QC	**Nr**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**
	1	1140.5	23.621			1168.5	27.259			1711	24.7565		
	2	1087.45	22.409			1063.7	23.478			1584	19.8575		
LLOQ	**3**	1245	26.0955	**9.1%**	**4.7%**	1051.25	26.8255	**9.6%**	**5.7%**	1856	29.0255	**15.3%**	**2.8%**
	4	1285	28.036			1052.55	28.086			1628	28.215		
	5	1264	26.767			856.65	22.5705			1744	22.7695		
LLOQ and Zero		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**	

LLOQ	1204.39		**6:1**		1038.53		**7:1**		1704.6		**12:1**		
Zero	188.75				140.35				144.05				

Angiotensin III

		Run 1				**Run 2**				**Run 3**			
QC	**Nr**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**
	1	1352.5	32.41			1320	35.3805			1224.5	36.0965		
	2	1455.5	34.4525			1083.5	29.0105			1012.8	31.038		
LLOQ	**3**	1479.5	35.346	**6.4%**	**6.1%**	1762	48.147	**19.0%**	**18.5%**	1393.5	38.623	**7.8%**	**13.8%**
	4	1156	29.9935			1176.5	36.2455			1245.5	35.585		
	5	1293	32.1875			1164	34.967			1157	34.971		
LLOQ and Zero		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**	
LLOQ		1347.3		**22:1**		1301.2		**37:1**		1206.66		**29:1**	
Zero		60.1				34.795				42.07			

Angiotensin IV

		Run 1				**Run 2**				**Run 3**			
QC	**Nr**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**
	1	481.95	25.251			433.8	24.7335			488.9	20.71		
	2	455.25	23.63			378.85	21.5415			628.2	26.124		
LLOQ	**3**	420	22.7095	**15.2%**	**-5.6%**	340.1	22.2255	**11.4%**	**7.8%**	551.45	21.879	**10.7%**	**4.3%**
	4	295.25	17.1925			434.5	27.8025			601.15	24.237		
	5	348.75	19.3015			429.3	27.1335			637.6	26.555		
LLOQ and Zero		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**		**Mean peak area**		**Response ratio**	
LLOQ		400.24		**25:1**		403.31		**37:1**		581.46		**42:1**	
Zero		15.98				10.89				13.855			

Angiotensin A

		Run 1				**Run 2**				**Run 3**			
QC	**Nr**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**	**Peak area**	**MV (pg/mL)**	**CV(%)**	**RE (%)**
	1	419.6	21.5195			284.1	23.1485			234.4	25.131		
	2	363.3	18.4675			326.6	24.663			220.4	23.782		
LLOQ	**3**	369.45	18.8935	**10.7%**	**-3.8%**	270.05	24.044	**15.1%**	**4.1%**	237.85	23.978	**9.5%**	**9.3%**
	4	436.25	23.524			270.6	25.1175			180.65	20.202		
	5	433.1	22.6535			156.4	16.7375			249.9	26.228		

LLOQ and Zero	Mean peak area	Response ratio	Mean peak area	Response ratio	Mean peak area	Response ratio
LLOQ	404.34	9:1	261.55	20:1	224.64	8:1
Zero	42.79		13.305		29.01	

Alamandine

		Run 1				Run 2				Run 3			
QC	Nr	Peak area	MV (pg/mL)	CV(%)	RE (%)	Peak area	MV (pg/mL)	CV(%)	RE (%)	Peak area	MV (pg/mL)	CV(%)	RE (%)
LLOQ	1	633.7	20.84	11.0%	3.3%	512.6	17.7725	9.7%	0.4%	435.6	21.4965	11.8%	5.5%
	2	691.35	22.313			575.65	18.6645			401.9	19.825		
	3	452.4	16.4885			446.15	17.053			347.8	17.1065		
	4	551.05	19.611			525.75	20.734			382.45	18.782		
	5	546.2	19.0535			556.35	21.3085			468.8	23.19		

LLOQ and Zero	Mean peak area	Response ratio	Mean peak area	Response ratio	Mean peak area	Response ratio
LLOQ	574.94	7:1	523.3	26:1	407.31	16:1
Zero	85.19		20.33		25.3	

Angiotensin-(1-9)

		Run 1				Run 2				Run 3			
QC	Nr	Peak area	MV (pg/mL)	CV(%)	RE (%)	Peak area	MV (pg/mL)	CV(%)	RE (%)	Peak area	MV (pg/mL)	CV(%)	RE (%)
LLOQ	1	3695	31.0365	6.0%	5.2%	3236	32.3075	6.1%	6.9%	3266	33.59	4.7%	12.0%
	2	3685.5	30.971			3150	30.1275			3363.5	34.1115		
	3	4267	35.445			3212	34.7545			3582.5	34.9875		
	4	3591.5	31.8025			3086.5	35.085			3379	33.4795		
	5	3999.5	33.881			2990.5	33.3965			3720.5	37.422		

LLOQ and Zero	Mean peak area	Response ratio	Mean peak area	Response ratio	Mean peak area	Response ratio
LLOQ	3847.7	33:1	3135	11:1	3462.3	14:1
Zero	116.45		297.25		241.75	

QC: quality control; MV: measured value; CV: coefficient of variation; RE: relative error.

Appendix 10.4-12: The evaluation of the matrix effect of six different human sources on analytes and internal standards by the liquid chromatography high-resolution mass spectrometry method.

		Source 1			Source 2			Source 3			Source 4			Source 5			Source 6			Control		CV of IS-MF	
		Blank	QC	QC high	Blank	QC	QC high	Blank	QC	QC high	Blank	QC	QC high	Blank	QC	QC high	Blank	QC	QC high	low	high	QC low	QC
AngI	Area	560	2004	103299	1579	1863	93388	1443	1633	87334	1642	1738	90360	529	1821	89304	866	1943	94220	2081	115248		
	MF		-4%	-10%		-10%	-19%		-22%	-24%		-17%	-22%		-12%	-23%		-7%	-18%			7.8%	3.2%
	IS-MF		8%	-7%		-2%	0%		-10%	-6%		-3%	-3%		4%	-5%		12%	1%				
AngII	Area	299	3326	123151	583	2746	108367	416	3346	109534	359	3202	111157	208	3130	109234	419	3140	109481	3829	147217		
	MF		-13%	-16%		-28%	-26%		-13%	-26%		-16%	-24%		-18%	-26%		-18%	-26%			8.0%	2.5%
	IS-MF		1%	8%		-10%	8%		16%	14%		7%	7%		5%	7%		4%	11%				
Ang1-7	Area	0	4358	113067	0	3994	106683	0	3801	103588	0	4015	105700	0	3845	109517	0	3994	107003	4509	128767		
	MF		-3%	-12%		-11%	-17%		-16%	-20%		-11%	-18%		-15%	-15%		-11%	-17%			1.3%	3.8%
	IS-MF		12%	13%		12%	22%		12%	23%		14%	16%		10%	23%		13%	24%				
AngIII	Area	0	4613	124567	0	4508	113582	0	4361	107242	0	4416	118083	0	4425	116183	0	4527	103023	6171	185233		
	MF		-25%	-33%		-27%	-39%		-29%	-42%		-28%	-36%		-28%	-37%		-27%	-44%			2.6%	3.2%
	IS-MF		-13%	-13%		-8%	-10%		-6%	-12%		-8%	-10%		-7%	-9%		-7%	-17%				
AngIV	Area	0	1656	45785	0	1527	41508	0	1509	39597	0	1523	41583	0	1444	41895	0	1418	41237	1965	56700		
	MF		-16%	-19%		-22%	-27%		-23%	-30%		-23%	-27%		-27%	-26%		-28%	-27%			3.7%	2.0%
	IS-MF		-2%	4%		-2%	8%		2%	7%		0%	4%		-5%	7%		-8%	9%				
AngA	Area	0	1768	36088		1386	26175		1528	24852		1400	26798		1482	27403		1366	24433	3275	66105		
	MF		-46%	-45%		-58%	-60%		-53%	-62%		-57%	-59%		-55%	-59%		-58%	-63%			7.5%	9.0%
	IS-MF		-37%	-30%		-47%	-42%		-38%	-43%		-45%	-43%		-42%	-40%		-47%	-45%				
Ala	Area	97	2621	77939	36	2743	74306	72	2598	71499	195	2396	74465	144	2639	75584	175	2439	73115	3256	89378		
	MF		-19%	-13%		-16%	-17%		-20%	-20%		-26%	-17%		-19%	-15%		-25%	-18%			6.0%	3.5%
	IS-MF		-6%	12%		6%	23%		6%	22%		-5%	18%		5%	22%		-5%	23%				
Ang1-9	Area	124	9418	303443	88	9328	259695	182	8130	247618	46	8455	254638	81	8494	266502	113	8136	258854	8948	318383		
	MF		5%	-5%		4%	-18%		-9%	-22%		-6%	-20%		-5%	-16%		-9%	-19%			4.2%	3.0%
	IS-MF		22%	23%		31%	20%		20%	19%		21%	13%		22%	21%		16%	22%				
IS 1	Area	0	36492	29653	0	37707	24985	0	35687	24813	0	35457	24998	0	34645	25232	0	34413	25118	41105	30882		
	MF		-11%	-4%		-8%	-19%		-13%	-20%		-14%	-19%		-16%	-18%		-16%	-19%				
IS 2	Area		43828	43967	0	40472	38468	0	38522	37095	0	39677	40132	0	39522	39192	0	40128	37852	50995	56700		
	MF		-14%	-22%		-21%	-32%		-24%	-35%		-22%	-29%		-22%	-31%		-21%	-33%				

The quality controls (QC) constitute of plasma samples spiked with analyte after extraction.The control samples represent a pure solution of the analyte. Before calculating the matrix factor (MF), the peak area of the blank was subtracted from the measured area (mean of three replicate) of the QCs. The MF was obtained by calculating the ratio of the QC area to the area of the control. The internal standard normalised MF (IS-MF) was calculated by dividing the MF of the analyte by the MF of the IS. AngI: angiotensin I; AngII: angiotensin II; Ang1-7: angiotensin-(1-7); AngIII: angiotensin III: AngIV: angiotensin IV; AngA: angiotensin A; Ala: alamandine; Ang1-9: angiotensin-(1-9): CV: coefficient of variation.

Appendix 10.4-13: Results of the recovery of the analytes and internal standards by the liquid chromatography high-resolution mass spectrometry method.

	Peak area (counts) of the Blank	Peak area (counts) of spiked samples before extraction			Peak area (counts) of spiked samples after extraction			Recovery (%)		
		QC low	QC middle	QC high	QC low	QC middle	QC high	QC low	QC middle	QC high
AngI	1002	3307	29706	65936	1679	32663	87436	**197%**	**91%**	**75%**
AngII	363	2442	38155	84242	2593	37785	105837	**94%**	**101%**	**80%**
Ang1-7	0	1961	18030	43188	3633	35737	100534	**54%**	**50%**	**43%**
AngIII	73	2305	23862	57220	3087	30950	78675	**75%**	**77%**	**73%**
AngIV	0	1309	13407	33158	1348	13608	37580	**97%**	**99%**	**88%**
AngA	0	883	7922	14885	926	8134	20424	**95%**	**97%**	**73%**
Ala	0	1363	12785	30915	2387	25682	72266	**57%**	**50%**	**43%**
Ang1-9	268	6855	77260	170865	7723	89670	233992	**89%**	**86%**	**73%**
IS 1	0	19352	22445	22140	32135	22670	22660	**60%**	**99%**	**98%**
IS 2	0	33997	38993	44085	35637	37397	37706	**95%**	**104%**	**117%**

AngI: angiotensin I; AngII: angiotensin II; Ang1-7: angiotensin-(1-7); AngIII: angiotensin III: AngIV: angiotensin IV; AngA: angiotensin A; Ala: alamandine; Ang1-9: angiotensin-(1-9): QC: quality control

Appendix 10.4-14: Evaluation of the autosampler stability for all analytes by the liquid chromatography high-resolution mass spectrometry method.

	Quality control low					Quality control high					Quality control high				
	Calculated concentration (pg/mL) at:				RE (%)	Calculated concentration (pg/mL) at:				RE (%)	Calculated concentration (pg/mL) at:				RE (%)
	3.2 h	10.2 h	17.2 h	24.2 h		3.2 h	10.2 h	17.2 h	24.2 h		3.2 h	10.2 h	17.2 h	24.2 h	
AngI	51.33	55.64	59.15	60.70	**11.8%**	224.07	223.45	201.71	189.55	**3.3%**	736.22	869.90	837.97	795.88	**-0.2%**
AngII	40.04	44.97	41.42	41.89	**-5.5%**	178.50	159.65	163.79	155.14	**-7.8%**	658.53	742.31	750.09	711.81	**0.4%**
Ang1-7	48.23	46.98	42.36	53.71	**-1.4%**	175.76	169.72	196.74	174.92	**-7.6%**	713.31	737.87	672.42	727.52	**-8.1%**
AngIII	54.78	72.70	62.78	62.04	**1.7%**	258.71	222.28	258.16	227.04	**-2.6%**	945.18	1075.29	1016.61	993.21	**1.6%**
AngIV	44.00	43.53	46.68	55.59	**3.6%**	168.39	192.25	199.84	147.69	**-3.4%**	655.51	835.59	742.20	626.14	**-2.5%**
AngA	43.88	51.51	60.15	43.40	**13.8%**	173.07	182.09	183.15	177.79	**2.4%**	677.93	726.80	695.19	665.65	**-1.1%**
Ala	35.36	33.53	37.94	34.29	**-7.3%**	138.52	155.38	147.16	130.87	**-6.1%**	600.60	620.90	611.22	600.02	**-0.1%**
Ang1-9	56.55	69.50	69.30	68.66	**6.4%**	242.47	232.25	270.96	226.78	**-2.0%**	970.97	1044.84	1102.06	1081.50	**5.8%**

The autosampler stability was assessed over 24.2 hours for three different concentration levels (low, middle, high). For each timepoint, every concentration level was measured in duplicate. The first 3.2 hours were required for the determination of the calibration curve. Consequently, the first measurement of quality controls was 3.2 hours after placing the samples in the autosampler. AngI: angiotensin I; AngII: angiotensin II; Ang1-7: angiotensin-(1-7); AngIII: angiotensin III: AngIV: angiotensin IV; AngA: angiotensin A; Ala: alamandine; Ang1-9: angiotensin-(1-9):

Appendix 10.4-15: LENA collaborator list

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11 List of publications

Some parts of this dissertation have already been published in international peer-reviewed journals or were presented at conferences beforehand:

Publications in journals

Fabian Konstantin Suessenbach, Nina Makowski, Martin Feickert, Tanja Gangnus, Jutta Tins, Bjoern Bengt Burckhardt, on behalf of the LENA consortium. **Implementation of a customised quality control system for ligand-binding assays in pharmacodynamic determination: Proof-of-concept within an academic environment.** J Pharm Biomed Anal (2020) 181:113090. doi:10.1016/j.jpba.2019.113090

F.K. Suessenbach, J. Tins, B.B. Burckhardt, on behalf of the LENA consortium. **Customisation and validation of a low-volume plasma renin activity immunoassay: Enabling of regulatory compliant determination in paediatric trials.** Pract Lab Med (2019) 17:e00144. doi:10.1016/j.plabm.2019.e00144

F.K. Suessenbach, B.B. Burckhardt. **Levels of angiotensin peptides in healthy and cardiovascular/renal-diseased paediatric population—an investigative review.** Heart Fail Rev (2019) 24(5):709–723. doi:10.1007/s10741-019-09797-y

Conference contributions

F.K. Suessenbach, M. Feickert, J. Tins, B.B. Burckhardt. **Reliable acquisition of plasma renin activity in the maturating renin-angiotensin-aldosterone-system by a validated small-volume assay in context of the LENA project.** ESDPPP (2019), Basel, Switzerland - Poster

F.K. Suessenbach, T. Gangnus, I. Burdman, N. Makowski, S. Läer, B.B. Burckhardt. **Compilation of available plasma renin activity levels in the healthy and cardiac diseased paediatric population.** ESDPPP (2019), Basel, Switzerland - Poster

T. Gangnus, F.K. Suessenbach, N. Makowski, I. Burdman, S. Läer, B.B. Burckhardt. **Reference ranges of blood NT-proBNP in paediatric heart failure and healthy controls: compilation of literature data.** ESDPPP (2019), Basel, Switzerland - Poster

I. Farahani, A. Laven, S. Farahani, M.A. Deters, M. Feickert, F.K. Suessenbach, H. Schwender, S. Laeer. **Effectiveness of OSCEs in Training German Pharmacy Students in Consultation on Self-Medication – A Randomised Controlled Investigation.** ESDPPP (2019), Basel, Switzerland - Poster

F.K. Suessenbach, N. Makowski, B.B. Burckhardt. **Evaluation of renin-angiotensin-aldosterone-system peptides in complex biological matrices in paediatric patients: Development and validation of a low-volume LC-HRMS Method.** DPhG annual meeting (2018), Hamburg, Germany - Poster

Makowski N, Süßenbach FK, Burckhardt BB. **A novel GCLP-compliant bioanalytical LC-HRMS method for the reliable determination of aldosterone, precursor and metabolite facilitating further insight into paediatric maturation of the renin-angiotensin-aldosterone-system.** DPhG annual meeting (2018), Hamburg, Germany – Poster