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Development and validation of low-volume assays as a tool for the reliable determination of parameters associated with the renin-angiotensin-aldosterone system in clinical investigations of children and adults

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Julia Christina Anna Schäfer
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Referent:	Prof. Dr. Stephanie Läer
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I. Erklärung zur Dissertation

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation mit dem Titel

‘Development and validation of low-volume assays as a tool for the reliable determination of parameters associated with the renin-angiotensin-aldosterone system in clinical investigations of children and adults’

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.

Düsseldorf, den Julia Christina Anna Schäfer

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III. Zusammenfassung

In der vorliegenden Arbeit werden zwei bioanalytische Assays zur Konzentrationsbestimmung von Parametern des Renin-Angiotensin-Aldosteron-Systems aus kleinen Probenvolumina vorgestellt. Dieser kleinvolumige Ansatz ist für Untersuchungen an pädiatrischen Patienten von besonderer Bedeutung, da das verfügbare Probenvolumen in dieser Population eingeschränkt ist. Das Renin-Angiotensin-Aldosteron-System ist an der Pathophysiologie von Herz-Kreislauf-Erkrankungen beteiligt und Angriffspunkt verschiedener kardiovaskulärer Wirkstoffe. Daher wird durch diesen Informationsgewinn einerseits ein tieferes Verständnis der zu Grunde liegenden Erkrankung und andererseits eine Optimierung der zugehörigen Pharmakotherapie ermöglicht. Dies ist insbesondere für die Therapie pädiatrischer Patienten von zentraler Bedeutung, da für diese junge Population wenige Daten verfügbar sind und die Pharmakotherapie häufig von Erwachsenen extrapoliert werden muss.

Beide Assays wurden auf der Basis von zwei kommerziell erhältlichen Immunoassays entwickelt. Das benötigte Probenvolumen, der Assayaufbau sowie die Assaydurchführung wurden angepasst, um eine Anwendung im Bereich der Pädiatrie und eine Validierung entsprechend internationaler bioanalytischer Leitlinien zu ermöglichen.

Die Zuverlässigkeit der entwickelten Assays wurde im Anschluss durch eine interne Validierung gemäß aktueller bioanalytischer Leitlinien der Europäischen Arzneimittel-Agentur und der US-amerikanischen Behörde für Lebensmittel- und Arzneimittelsicherheit bestätigt. Dieses Ergebnis konnte im Weiteren durch die erfolgreiche Teilnahme an einem externen Ringversuch untermauert werden. Da die Assays aktuellen Anforderungen der Europäischen Arzneimittel-Agentur und der US-amerikanischen Behörde für Lebensmittel- und Arzneimittelsicherheit entsprechen, sind sie für eine Anwendung unter den Bedingungen von ‚Good Clinical Laboratory Practice' geeignet.

Beide Assays werden derzeit im Rahmen der pädiatrischen Studien des EU-geförderten Projektes ‚Labeling of Enalapril from Neonates up to Adolescents' (LENA) an Proben herzinsuffizienter Kinder eingesetzt (FP7-Programm; EU grant agreement n°602295). Damit liefern die entwickelten Assays einen bedeutsamen Beitrag zur Erhebung dringend benötigter pharmakodynamischer Daten in der pädiatrischen Population.

IV. Summary

The present work introduces two bioanalytical assays for the measurement of parameters related to the renin-angiotensin-aldosterone system in small sample volumes. This low-volume approach is especially valuable for investigations in paediatric patients, as the sample volume is limited in this vulnerable population. The renin-angiotensin-aldosterone system is involved in the pathophysiology of cardiovascular diseases and is the target of several cardiovascular drugs. Therefore, the gathered data will facilitate a deeper understanding of the underlying diseases and an optimisation of the corresponding pharmacotherapies. This is of great significance for the therapy of paediatric patients, as pharmacotherapy often has to be extrapolated from adults due to the lack of data available in the young population.

The development of both assays was based on two commercially available immunoassays. The required sample volume, the assay setup, and the assay procedure were adjusted to allow for a paediatric application and a validation according to international bioanalytical guidelines.

The reliability of the developed assays was confirmed by an in-house validation according to current bioanalytical guidelines of the European Medicines Agency and the U.S. Food and Drug Administration. These findings were further substantiated, as both assays successfully passed an external ring test. Due to their compliance with current requirements of the European Medicines Agency and the U.S. Food and Drug Administration, the assays are suitable for application under conditions of ‘Good Clinical Laboratory Practice’.

Currently, both assays are applied to samples of paediatric patients suffering from heart failure in the clinical studies of the EU-funded project ‘Labeling of Enalapril from Neonates up to Adolescents’ (LENA) (FP7 Programme; EU grant

agreement n°602295). Consequently, the developed assays support the collection of urgently required reliable data on the paediatric population.

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VIII. Abbreviations

ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
ARB	Angiotensin receptor blocker
ARR	Aldosterone/renin ratio
CLIA	Chemiluminescence immunoassay
Conc.	Concentration
CV	Coefficient of variation
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
FP7	European Union's Research and Innovation funding programme
GCLP	Good Clinical Laboratory Practice
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
h	Hour
IU	International Unit
L	Litre
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
LENA	Labeling of Enalapril from Neonates up to Adolescents
LLOQ	Lower limit of quantification
M	Molar concentration
ME	Matrix effect
mL	Millilitre
MS_{bg}	Mean squared error between group

MS_{wg}	Mean squared error within group
ng	nanogram
nm	Nanometre
OD	Optical density
PDCO	Paediatric Committee
pg	Picogram
PIP	Paediatric investigation plan
PK	Pharmacokinetics
pmol	Picomole
PQ	Performance qualification
PRA	Plasma renin activity
PUMA	Paediatric-use marketing authorisation
QC	Quality Control
R	Correlation coefficient
RAAS	Renin-angiotensin-aldosterone system
RIA	Radioimmunoassay
Rpm	Revolutions per minute
s^2	Repetition variance
SD	Standard deviation
TMB	Tetramethylbenzidine
ULOQ	Upper limit of quantification
$\bar{x}$	Mean
µ	Micro
°C	Degree Celsius

1 Introduction

1.1 Drug use in children and developments of paediatric research

A significant number of paediatric patients are treated with unlicensed drugs or drugs prescribed outside the terms of their license regarding indication, age group, dosage or route of administration, which is referred to as ‘off-label use’. This can be attributed to the fact that medicines tailored to paediatric patients are often missing, and therapies that are exclusively based on clinical experience or extrapolated from adults are inevitable. While both unlicensed and off-label use are widely practiced in paediatrics in general, there is an especially large proportion in neonatal intensive care units: between 80 % and 93 % (4). Although off-label or unlicensed use may be advantageous to paediatric patients, as it offers a therapeutic option that experts assume to be beneficial to the individual patient, it comes along with an exposure to unknown risks: Drug formulations might be inadequate for children, and appropriate dosage information is sparse in this young population, which differs significantly from adults regarding pharmacokinetics (PK) and pharmacodynamics (PD). In a report on the off-label use of medicines in children from 2004, the European Medicines Agency (EMA) demonstrated the association of off-label use with more adverse reactions in children than adults and pointed out that adverse reactions in children may additionally be more severe or different from those in adults (5).

This demonstrates the unmet need for child-appropriate medicines and consequently the requirement for research on paediatric patients. Therefore, both the EU and U.S. legislation have forced the research in the field of paediatrics for years, which was followed by several improvements. According to Spielberg (2010), the ‘United States legislation including the Best Pharmaceuticals for Children Act and the Pediatric Research Equity Act have changed the drug development process, leading to new knowledge about the safe, effective use of

medicines in children'. (6) In 2007, the Paediatric Regulation (EC) No 1901/2006 (7) came into effect to improve the health of children in the European Union (EU). This objective was to be accomplished by supporting the development and availability of high-quality medicines and better access to information on drug use in children. The release of the Paediatric Regulation came along with the establishment of the Paediatric Committee (PDCO), which is the corresponding scientific committee of the EMA. The main task of the PDCO is the assessment of the paediatric investigation plans (PIPs) comprising all studies that have to be performed on children during the development of child-appropriate therapeutics. Apart from incentives for the development of new drugs, the Paediatric Regulation introduced the so-called paediatric-use marketing authorisation (PUMA), which was intended to promote research on already established drugs that are authorised and no longer under the protection of a patent or supplementary protection certificate. In the case of a granted PUMA, the product gains 10 years of market protection.

To assure that the funds are directed to the most urgently required medicines within the paediatric population, a priority list of off-patent drugs was created providing a basis for the EU's funding programmes. The Seventh Framework Programme (FP7) was the European Union's Research and Innovation funding programme from 2007 to 2013 (8). The EU-funded project 'Labeling of Enalapril from Neonates up to Adolescents' (LENA), which investigates a new drug formulation of the angiotensin-converting enzyme (ACE) inhibitor enalapril in children, represents one example of projects funded by the FP7 (9). The FP7 funding programme is followed by Horizon 2020 providing nearly €80 billion of funding between 2014 and 2020 (10).

As a result of the introduction of the Paediatric Regulation, more high-quality research was recorded in the realm of paediatrics between 2006 and 2015 as stated in the 10-year Report to the European Commission (EC) by the EMA and

the PDCO (11). While the proportion of clinical trials including children was 9.3 % in comparison to all clinical trials per year in 2006, it had a share of 11.5 % in 2015. In parallel, more neonates, a neglected subgroup regarding the development of medicines, have been included in clinical trials in recent years. While 470 neonates were planned to be included in clinical trials from 2007 to 2009, the number increased to more than 13,000 from 2013 to 2015, according to the EudraCT database. This progress is further reflected in the authorisation of 238 new medicines for use in children and 39 new child-appropriate pharmaceutical forms in the EU between 2007 and 2015.

Despite these positive trends, there are still challenges to be overcome. For instance, only three PUMAs have been authorised up to September 2016 (12). Furthermore, about 100 PIPs were completed while more than 700 were ongoing at the end of 2015, which may be attributed to the long duration of clinical development programmes, the rarity of the disease and challenges during recruitment (11).

In summary, the Paediatric Regulation has promoted research and medicines development in the field of paediatrics and led to a new awareness of the need for investigations in this young population. However, processes that are highly dependent on external conditions will demand time and must continue to be encouraged.

1.2 Challenges of clinical investigations in paediatric patients

Clinical investigations of paediatric patients are faced with several obstacles that are often more challenging than in clinical studies of adults. Turner et al. (2014) stated that 'Children. . .can be more vulnerable to the effects of studies than people in older age groups' (13). This leads to '. . .additional safety considerations, such as growth and development, in pediatric trials' (14). Furthermore, paediatric diseases are often rare and the patient population heterogeneous (11), which results in the challenge of properly recruiting patients. This is a major problem, as the validity of obtained data depends on the inclusion of a sufficient number of appropriate patients. In addition to the rarity of childhood diseases and the heterogeneity of the population, there are other factors, such as the availability of other medicines, albeit off-label, that impedes the recruitment into clinical trials (11). According to Dr. Nelson, Associate Professor of Anaesthesiology and Critical Care of the Children's Hospital of Philadelphia, the willingness of clinicians to use medicines with lacking sufficient clinical data results in further delays of required research (14).

A further challenge in pediatric investigations is that children are not able to provide consent for themselves. In this context, Dr. Nelson pointed out that there are three internationally accepted ethical principles, that should serve as basis for research on paediatric patients: First, children should only be involved in research if this is necessary in order to answer an important scientific question about the health and welfare of children. Second, risks and benefits have to be comparable to available alternatives, and third, research without direct benefit to children must be restricted to the minimal risk (14).

Moreover, investigations in paediatric drug development are faced with formulation challenges (15), as the pharmaceutical formulation has to meet the special needs and characteristics of this young population.

Apart from the aforementioned challenges regarding the conduct of paediatric trials, there are technical barriers (e.g., proper endpoints) or economic barriers, such as high risk and a relatively small market size, too. This makes studies on paediatric patients unattractive for many companies (14).

From a bioanalytical point of view, most assays cannot handle current ethical and analytical burdens (16). Because the sample volume is limited in paediatric patients, the availability of reliable bioanalytical assays running with a low sample volume is a necessary prerequisite for systematic investigations in this population. The guideline on the investigation of medicinal products in the term and preterm neonate by the EMA (17) recommends that '. . .the trial-related blood loss (including any losses in the manoeuvre) should not exceed 3 % of the total blood volume during a period of four weeks and should not exceed 1 % at any single time'. With a blood volume estimated at 80 - 90 mL/kg body weight (17), this corresponds to approximately 1.2 - 1.4 mL and 0.4 - 0.45 mL serum/plasma respectively. The sample volume available for scientific investigations according to these ethical recommendations is additionally reduced by the volume required for the determination of safety parameters. As the available data on the paediatric population is limited, systematic investigations are required. However, this comes along with the requirement of several samplings over an extended period of time to support the validity of obtained data, which emphasises the need for low-volume assays. Despite many endeavours to support the development of child-appropriate assays, including new analytical procedures (e.g., dried blood spots) and alternative ways of sampling (e.g., capillary or urinary), child-appropriate analytical assays that have been validated to assure a reliable data collection are still sparse.

1.3 Heart failure in children

Heart failure is a cardiovascular disease known in children and adults. The disease is characterised by structural or functional abnormalities of the heart that result in an insufficient blood circulation and an undersupply of body tissues with oxygen. The etiology of the disease differs significantly between paediatric and adult patients. While heart failure is due to coronary artery disease, hypertension, or heart attack in adults, it is usually caused by congenital heart disease or cardiomyopathy in the young leading to clinical symptoms like respiratory distress, exercise intolerance, and growth failure. Congenital heart disease shows a proportion of more than 60 % in children suffering from heart failure and includes structural abnormalities, such as left-to-right shunt lesions (e.g., ventricular septal defects and patent ductus arteriosus), pulmonary valve stenosis, and aortic stenosis. Catheter procedures or surgery are the usual therapeutic approaches in congenital heart disease, which might be supported by pharmacotherapy. In paediatric heart failure patients without structural abnormalities of the heart, cardiomyopathy is the most relevant cause. In these cases, the established myocardial dysfunction requires long-term pharmacotherapy. (18)

Despite the considerable differences between children and adults suffering from heart failure, pharmacotherapy in children is extrapolated from adults and based on clinical experience, as no sufficient pharmacokinetic and pharmacodynamic data is available in children. This approach raises questions regarding the efficacy and safety of current pharmacotherapies in children and calls for clinical investigations in all age groups of this seriously ill population. The need for research on paediatric heart failure is emphasised by the scarcity of child-appropriate drug formulations leading to dosing errors and following further uncertainties regarding safety and efficacy of the therapy.

1.4 The renin-angiotensin-aldosterone system as a target of pharmacotherapy in heart failure

The renin-angiotensin-aldosterone system (RAAS) is a cascade of several endogenous parameters, which play an important role in the body's salt and fluid homeostasis. The endopeptidase renin is released by the juxtaglomerular apparatus of the kidneys. Low sodium concentrations or a renal artery hypotension trigger the secretion of the enzyme, which is the rate-limiting step of the RAAS. Renin converts angiotensinogen into angiotensin I (Ang I). The angiotensin-converting enzyme (ACE) acts on Ang I to form angiotensin II (Ang II), which leads to vasoconstriction and an increased blood pressure. Additionally, Ang II is followed by the release of aldosterone, which is produced by the zona glomerulosa of the adrenal cortex. The determination of aldosterone levels and the comparison to renin concentrations by the so-called aldosterone/renin ratio (ARR) is used as a screening test for primary aldosteronism, a differential diagnosis of hypertension. Primary aldosteronism is characterized by an excessive production of aldosterone in the adrenal glands and by low renin levels, and it is associated with an elevated blood pressure.

Aldosterone levels are also altered in cardiac diseases. In heart failure patients, the activation of the RAAS is increased to compensate for the impaired function of the heart. However, long-term effects of a RAAS activation promote the pathogenesis of the disease. Ang II is a growth factor for myocytes and fibroblasts (19, 20), leads to phenotypic alterations comparable to those seen during remodeling (21), and activates other neurohormonal systems (22). Elevated aldosterone levels support the progression of left ventricular dysfunction by cardiac hypertrophy, fibrosis, and inflammation (23), subsequently contributing to remodeling processes and promoting the pathogenesis of the disease. Several classes of drugs that block different steps of the system (**Fig. 1.1**) are available and are commonly used as therapeutics in heart failure or hypertension. Although

increased aldosterone levels can be lowered by angiotensin receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEi), a recurrence of elevated plasma levels has been observed in up to 40 % of symptomatic heart failure patients (24). This is an important observation which might be used for monitoring the progression of the disease and that further emphasises the need for accurate and precise bioanalytical assays.

Little is known about the alterations of parameters associated with the RAAS in paediatric heart failure patients. The investigation plans of clinical studies within this population should address the corresponding parameters. This would facilitate a deeper understanding of the underlying pathomechanisms in this young population and provide the necessary prerequisite for an optimised pharmacotherapy in children.

The main incentive of the present work was to support the challenging but urgently needed collection of reliable data on this vulnerable population from a bioanalytical perspective. The availability of child-appropriate bioanalytical assays is a major prerequisite for meaningful study outcomes. Therefore, two bioanalytical assays were developed that are able to provide reliable and meaningful data despite a limited sample volume and can be used to promote research on paediatric patients suffering from cardiovascular diseases.

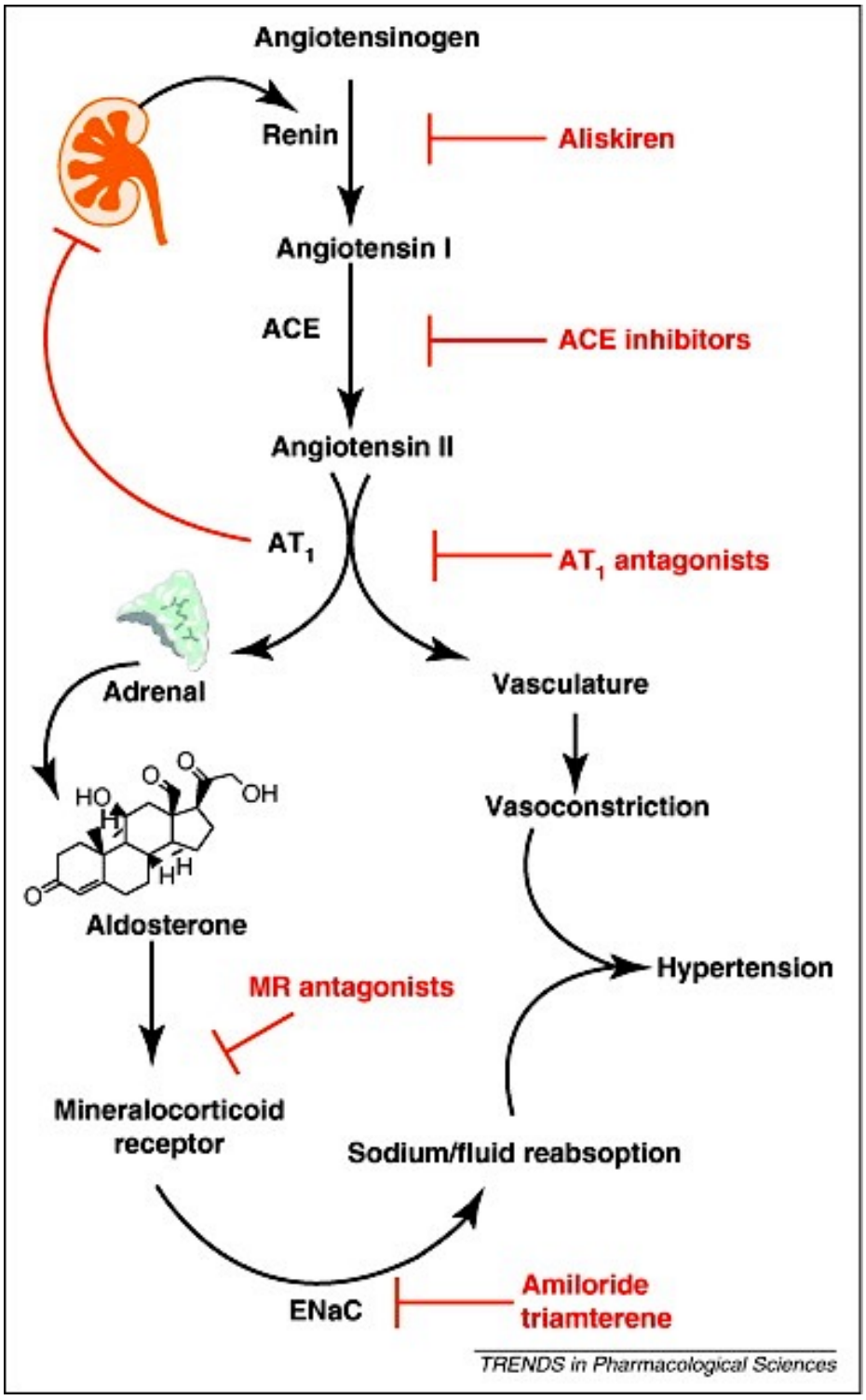

Fig. 1.1 The renin-angiotensin-aldosterone system (RAAS) (reprinted from Luther et al. (25)). Due to certain stimuli, renin is secreted by the kidneys. The enzyme renin converts angiotensinogen to angiotensin I, which is the substrate of the angiotensin-converting enzyme. The product angiotensin II leads to vasoconstriction in the systemic vasculature, followed by an increased blood pressure. Angiotensin II additionally triggers the release of aldosterone in the adrenal cortex leading to sodium and fluid reabsorption as well as potassium loss in the kidneys, which results in an elevated blood pressure. Several classes of active ingredients are available that are used as RAAS blockers (marked in red).

2 Aim of the thesis

2.1 Literature search

The aim of the first step of this work was to gather available data on physiological parameters associated with cardiac diseases. Therefore, a literature search was conducted on the parameters aldosterone, ang I, plasma renin activity (PRA), and renin in healthy and diseased subjects of all age groups. After the evaluation of the collected data, the parameters aldosterone and renin were selected for the subsequent assay developments.

2.2 Development, validation, and external verification of a low-volume aldosterone and renin immunoassay

The major part of this project was dedicated to developing two bioanalytical assays for the reliable determination of aldosterone and renin concentrations that are appropriate for the application to paediatric studies. The assays were developed on the basis of two commercially available enzyme-linked immunosorbent assays (ELISA), as this analytical method combines a low sample volume and a practicability, that is beneficial to routine analysis. The development process was associated with two major challenges: The assays were supposed to run with the lowest sample volume possible and the assay setup and procedure had to be changed to allow for a subsequent validation according to the current bioanalytical guidelines of EMA (26) and the U.S. Food and Drug Administration (FDA) (27), that was not possible using the unmodified commercially available ELISAs. While the low-volume approach was necessary because the assays were intended to be used with paediatric samples, the validation in compliance with current bioanalytical guidelines can be attributed to the fact that both assays were supposed to be used in clinical studies under conditions of Good Clinical Laboratory Practice (GCLP). An implementation under the terms of GCLP assures that the requirements of Good Clinical Practice (GCP) that are applicable to the

analysis of clinical trial samples are met at a bioanalytical laboratory working under Good Laboratory Practice (GLP).

Apart from this in-house validation, an additional external verification by participation in a ring test was intended. This is the common quality assurance required by the German Medical Association for laboratory medicine (Rili-BÄK (28)), which allows for a comparison of the developed assays with established assays used by contract laboratories.

2.3 Proof of concept

As most of the in-house validation tests were based on a real human matrix, both assays had already been tested for applicability to human samples during the validation process. In the last step, it was intended to verify these findings. Therefore, both assays were used to obtain physiological aldosterone and renin profiles of a healthy adult over a period of twelve hours.

Another intention of this step was the evaluation of the assays' applicability to a clinical study setting compliant with the requirements of GCLP, which was realised by implementing both assays in the clinical studies of the EU-funded project 'Labeling of Enalapril from Neonates up to Adolescents' (LENA).

2.4 Development of a performance qualification test for a microplate reader

As the immunoassays were intended to be established under conditions of GCLP, a performance qualification (PQ) test for the corresponding microplate reader used for the measurements was required. This test was hoped to be practicable and simple, as it would be needed to verify the adequate reader performance by regular test runs prior to the analytics.

3 Methods

3.1 Literature search

A literature search was performed to gain an overview of available data on physiological parameters associated with the cardiovascular system in adults and children of all age groups. Subjects with and without underlying cardiac diseases were included. The available literature data was intended to estimate both the range of concentration levels expected in subjects suffering from a cardiac disease and the possible impact of the use of ACE inhibitors on physiological parameters in these patients.

The following physiological parameters were covered by the literature search:

- Aldosterone
- Ang I
- PRA
- Renin

The literature search was primarily conducted using the MEDLINE database via PubMed. Search details are given in **Table 3.1**. Furthermore, results obtained by a free text search were added.

Table 3.1 Search details used during the literature search in the MEDLINE database via PubMed.

Parameter	Search details
Aldosterone	((((("adult"[MeSH Terms] OR "adolescent"[MeSH Terms]) OR "child"[MeSH Terms]) OR "infant"[MeSH Terms]) AND "heart failure"[MeSH Terms]) AND "aldosterone"[MeSH Terms]
Angiotensin I	((((("adult"[MeSH Terms] OR "adolescent"[MeSH Terms]) OR "child"[MeSH Terms]) OR "infant"[MeSH Terms]) AND "heart failure"[MeSH Terms]) AND "angiotensin i"[MeSH Terms]
Plasma renin activity	((((("adult"[MeSH Terms] OR "adolescent"[MeSH Terms]) OR "child"[MeSH Terms]) OR "infant"[MeSH Terms]) AND "heart failure"[MeSH Terms]) AND plasma renin activity[Text Word]
Renin	((((("adult"[MeSH Terms] OR "adolescent"[MeSH Terms]) OR "child"[MeSH Terms]) OR "infant"[MeSH Terms]) AND "heart failure"[MeSH Terms]) AND "renin"[MeSH Terms]

For each of the four physiological parameters included, data was extracted from the obtained literature according to the following categories:

- Age group
    - Newborn (0 - 27 days)
    - Infant and Toddler (28 days - 23 months)
    - Preschool child (2 - 5 years)
    - Child (6 - 11 years)
    - Adolescent (12 - 17 years)
    - Adult (≥18 years)
- Number of subjects
- Healthy or no cardiac disease vs. cardiac disease
- Pharmacotherapy with ace inhibitor vs. pharmacotherapy without ACE inhibitor or no pharmacotherapy
- Mean or median concentration found
- Minimum and maximum concentration found

For reasons of comparison, the following parameters aldosterone, ang I, and renin were converted into pg/mL if necessary. Corresponding conversion factors are given in **Table 3.2**.

Table 3.2 Conversion factors used during the data extraction process.

Parameter	pg/mL	Equivalent to
Aldosterone	1	2.774 pmol/L (adapted from (29))
Angiotensin I	1	0.772 pmol/L angiotensin I (according to (29))
Renin	1	1.67 μIU/mL (according to (30))

The determined concentration levels were visualised in data charts that reflect data density and illustrate the differences between populations with or without cardiac diseases as well as discrepancies between the different age groups and a possible effect of the use of ACEi. Underlying values were rounded to the nearest integer (except for PRA values).

3.2 Development, validation, and external verification of a low-volume aldosterone and renin immunoassay

In the following sections, the development and validation processes for both assays are described. The development process starts with the selection of appropriate commercial immunoassays and shows the stepwise adjustment of these assays to allow for a validation according to the current bioanalytical guidelines of the EMA and the FDA.

3.2.1 Immunoassays

Immunoassays are widely used in analytics and are an established analytical technique in routine work by clinical services. By use of immunoassays, the detection and quantification of biological compounds, usually proteins, become feasible in a rapid and easy way. As this is a decisive advantage over methods of higher complexity, immunoassays are an important technique for clinical screening, diagnosis, or monitoring processes. Despite the availability of a variety of immunoassay methods, the test principle is always based on an immunoreaction between antigen and antibody. As this antigen-antibody reaction represents the core of the assay principle, the characteristics of this interaction regarding specificity and strength of binding are of particular importance and can be influenced by the antibody used (e.g., monoclonal versus polyclonal antibody).

The analyte quantification is usually realised by use of a tracer (e.g., enzyme- or radioactive-labelled component) leading to a measurable signal related to the corresponding analyte concentration. The various kinds of immunoassays available differ regarding the kind of detection used for quantification (e.g., chemiluminescence immunoassay (CLIA), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA)) and also with respect to their design (e.g., sandwich immunoassay, competitive immunoassay).

The enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA), which were able to replace radioactive labelling material with enzymes for signal generation, go back to the research group of Peter Perlmann and Eva Engvall at Stockholm University in Sweden and to the research group of Anton Schuurs and Bauke van Weemen in the Netherlands (31). The ELISA technique belongs to the heterogeneous immunoassays, which include - in contrast to the homogeneous assays - washing steps to remove anything other than the antigen-antibody complex (32).

The present work focuses on two ELISAs with different assay principles. While the aldosterone assay is a competitive assay using polyclonal antibodies, the renin assay is based on the sandwich principle and applies monoclonal antibodies (1, 33). In a competitive ELISA, the analyte molecule competes with a tracer (analyte molecule labelled with enzyme) for binding to a limited number of antibody sites. This leads to a signal, which is inversely proportional to the analyte concentration. In a sandwich ELISA, such as the renin assay presented here, the analyte of interest binds to a first antibody (capture antibody) before a further antibody (detection antibody) is added. Because the analyte is surrounded by antibodies, this assay principle is referred to as a ‘sandwich immunoassay’. The use of enzyme-labelled antibodies facilitates the signal generation, which in this case is proportional to the analyte concentration. In the case of both ELISAs, the enzyme horseradish peroxidase oxidises a substrate to a product that is detected spectrophotometrically (1, 33).

The present work was based on the competitive aldosterone immunoassay by DRG Instruments GmbH (aldosterone ELISA kit EIA-5298 (1)) and the sandwich immunoassay for the determination of renin concentrations by the same vendor (renin ELISA kit EIA-5125 (33)). The corresponding detailed assay principles are given in **Fig. 3.1** and **Fig. 3.2**.

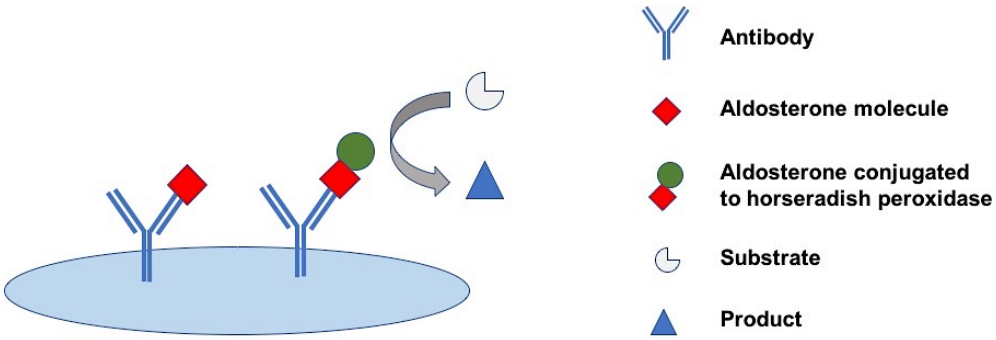

Fig. 3.1 Test principle of the aldosterone immunoassay (competitive immunoassay). The microtiter wells are coated with a polyclonal anti-aldosterone antibody. The aldosterone molecule of the patient sample competes with the enzyme conjugate (aldosterone conjugated with horseradish peroxidase) for binding to the antibody. During the following substrate incubation, the peroxidase catalyses the conversion of substrate (colourless) to product (blue). This step only takes place when the enzyme conjugate is bound to the antibody. Subsequently, the developed intensity of colour is inversely proportional to the aldosterone concentration in the patient specimen (1).

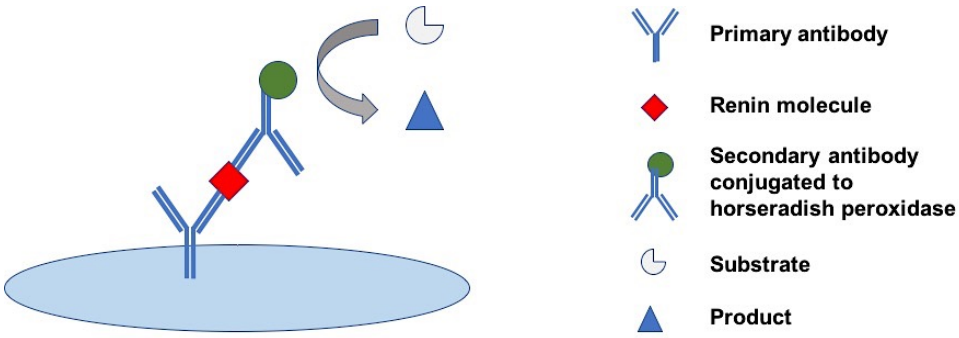

Fig. 3.2 Test principle of the renin immunoassay (sandwich immunoassay). The microtiter wells are coated with a monoclonal anti-renin antibody. The endogenous renin of the patient specimen binds to this primary antibody. In the following step, the enzyme conjugate (anti-renin antibody conjugated with horseradish peroxidase) binds to the renin molecule. During substrate incubation, the peroxidase catalyses the conversion of substrate (colourless) to product (blue), so that the intensity of the developed colour is proportional to the renin concentration in the patient specimen (33).

The final substrate reaction of both assays is based on the oxidation of 3,3',5,5'-tetramethylbenzidine by horseradish peroxidase. Both components are frequently used in colorimetric enzyme immunoassays. As described by Bally et al. ((34), **Fig. 3.3**), the reaction shows a transfer of two electrons. The first electron transfer results in a cation radical (B) that is in equilibrium with a charge-transfer

complex of blue colour (C). The cation radical is oxidized in a second electron transfer step to the yellow diimine derivative (D).

Finally, the enzyme reaction is terminated by acidification (0.5 M H_2SO_4) and photometric detection is performed at 450 nm, which is the absorption maximum of the diimine (34).

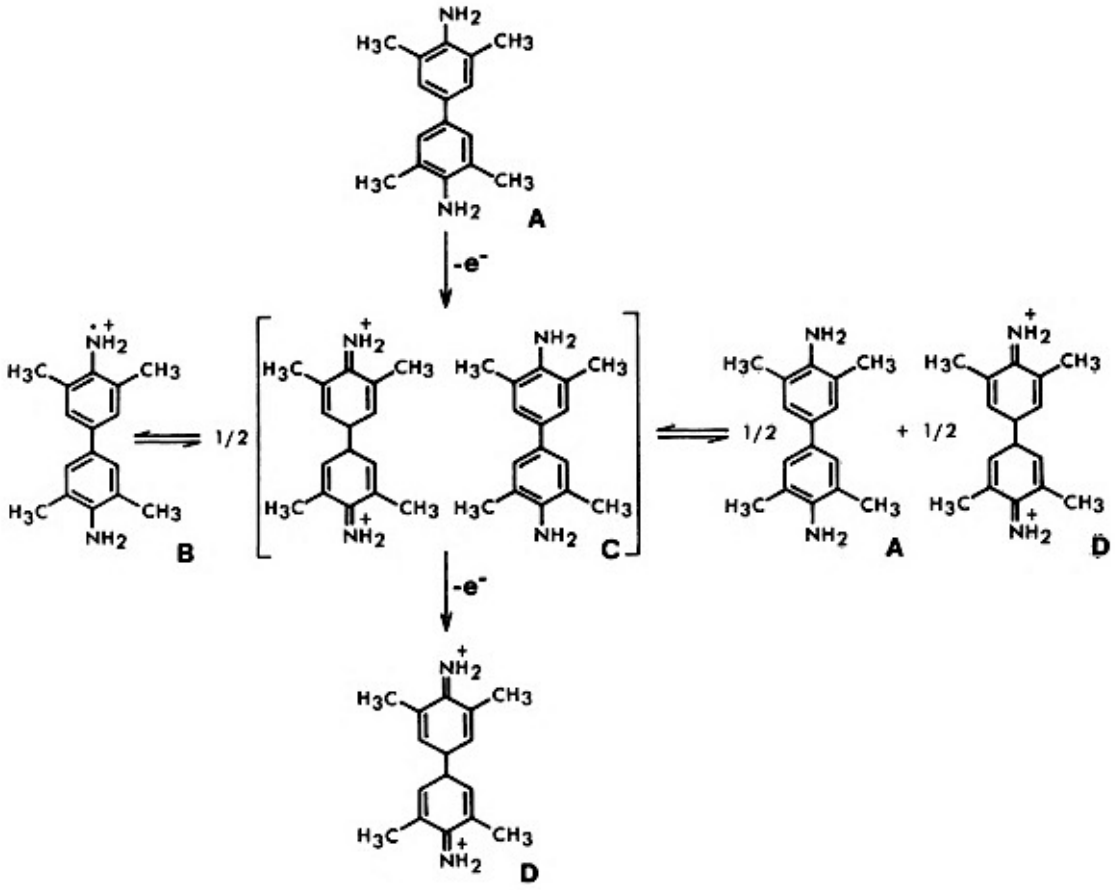

Fig. 3.3 Oxidation of 3,3',5,5'-tetramethylbenzidine by peroxidase. The oxidation can be subdivided into two steps. In the first oxidation step of 3,3',5,5'-tetramethylbenzidine (A), the transfer of one electron leads to a cation radical (B) that is in equilibrium with a charge-transfer complex coloured in blue (C). The second electron transfer results in the diimine of yellow colour (D) (reprinted from Bally et al. (34)).

3.2.2 Selection of commercial immunoassays as a basis for the following development of guideline-compliant low-volume assays

To gain an overview of commercially available immunoassays that might serve as a basis for the development of guideline-compliant low-volume assays, specifications of several immunoassays were collected. Three aldosterone immunoassays requiring a preferably low sample volume were selected (1–3). As both assays were intended to be used under routine conditions in clinical studies, assays with very complex or time-consuming assay procedures (e.g., overnight incubation steps) were excluded. The selected assays especially differed with regard to the antibodies used (monoclonal/polyclonal) and the assay range covered (**Table 3.3**).

Table 3.3 Three commercially available immunoassays for the determination of aldosterone concentrations. The assays differ regarding assay principle (competitive/sandwich immunoassay), antibodies (monoclonal, polyclonal), and assay range.

Assay	Sample volume required [µL]	Assay principle	Monoclonal/ polyclonal antibodies	Calibration standards	
				lowest [pg/mL]	highest [pg/mL]
Aldosterone ELISA (Ref. EIA-5298) by DRG Instruments GmbH (1)	50	competitive	polyclonal	0	1000
Aldosterone Assay (Cat. No. KGE016) by R&D Systems, Inc. (2)	100*	competitive	monoclonal	0	6000
Human Aldosterone (ALD) Elisa kit (Cat. No. E01A0774) by BlueGene Biotech CO., LTD (3)	50	sandwich immunoassay	monoclonal/ polyclonal	0	1000

*Pre-dilution step (serum/plasma), e.g., 100 µL sample + 200 µL of Calibrator Diluent (suggested by the vendor (2))

To facilitate a comparison of the selected immunoassays, serum and plasma samples of five different donors were measured over a period of up to eight weeks. During this time, the samples were stored at -20 °C. The reliability of the assay performances was compared by assessing the reproducibility of the obtained values.

The subsequent sections contain parts of the manuscripts ‘Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes’ by Schaefer et al. (35) and ‘Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations’ by Schaefer et al. (36).

3.2.3 Material

The following material was used during assay development and the subsequent validation process.

Aldosterone assay

Microtiter wells, lyophilised aldosterone standard ‘1000 pg/mL’, enzyme conjugate (aldosterone conjugated to horseradish peroxidase), substrate (tetramethylbenzidine, TMB) solution, stop solution (0.5 M sulfuric acid), and wash solution were components of the commercially available aldosterone ELISA kit EIA-5298 by DRG Instruments GmbH (Marburg, Germany) (1). Additional aldosterone standard 1000 pg/mL (lyophilised), artificial blank matrix, assay buffer, and aldosterone stock solution 100 ng/mL were also obtained from this vendor. All standards used are intended to mimic the matrix of study samples, and they contain treated human samples with preservatives. Double-distilled water was purchased from Carl Roth GmbH and Co. KG (Karlsruhe, Germany).

Renin assay

Microtiter wells, assay buffer, lyophilised renin standards, artificial blank matrix, renin stock solution, anti-human renin antibody conjugated to horseradish peroxidase, wash solution, TMB solution, and 0.5 M sulfuric acid were required. The artificial blank matrix as well as all standards contain treated human samples to mimic the matrix of study samples. These components were either obtained from the commercially available renin ELISA kit EIA-5125 by DRG Instruments GmbH (Marburg, Germany) (33) or were separately supplied by this vendor. For double-distilled water, see section ‘Aldosterone assay’.

Serum and plasma samples

Serum and EDTA plasma samples were donated by healthy volunteers who provided written, informed consent (study no. 5392 at local Ethics Committee at Heinrich-Heine-University Duesseldorf). Samples were collected using S-Monovettes® by Sarstedt AG & Co. (Nümbrecht, Germany).

3.2.4 Assay development

In the following sections, the development process of the two ELISAs for the determination of aldosterone and renin concentrations in small sample volumes is described. The development process of both assays was based on the corresponding immunoassays by DRG Instruments GmbH (Marburg, Germany), which had been selected before (see sections 3.2.2 and 4.2.1).

3.2.4.1 Aldosterone assay

The development of the aldosterone immunoassay was based on the commercial aldosterone immunoassay by DRG Instruments GmbH (1).

Adjustment of calibration standards and QC samples

In the first step of the development process, an adjustment of the calibration standards became necessary. According to regulatory recommendations of the EMA (26) and the FDA (27), calibration curves of ligand-binding assays require a minimum of six duplicate non-zero calibrator concentrations. The commercially available aldosterone ELISA kit by DRG Instruments GmbH (1) includes only five non-zero calibrator concentrations plus one zero standard. Instead of using these separate lyophilisates, which have to be individually reconstituted prior to use, a serial dilution of the highest standard ‘1000 pg/mL’ with the artificial blank matrix was performed. By this, a sixth non-zero calibrator concentration was introduced and an impact on the assay performance due to possible variations of single standards could be excluded.

Detailed preparation of calibration standards and QC samples

The lyophilised aldosterone standard ‘1000 pg/mL’ was reconstituted with 1.0 mL double-distilled water. The prepared solution corresponds to the highest calibration standard, the upper limit of quantification (ULOQ). The remaining calibration

standards were obtained by serial dilution of the ULOQ (1000 pg/mL) with the artificial blank matrix (see **Table 3.4**).

Table 3.4 Preparation of calibration standards (aldosterone assay).

Calibration Standard	Volume 1	Volume 2
1 (1000 pg/mL)	(Reconstitution of lyophilised standard '1000 pg/mL' with 1.0 mL double-distilled water)	
2 (500 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 1
3 (250 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 2
4 (125 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 3
5 (62.5 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 4
6 (31.3 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 5

For the preparation of Quality Control (QC) samples, an independent lyophilised aldosterone standard '1000 pg/mL' was reconstituted with double-distilled water and diluted with the artificial blank matrix to obtain the following concentration levels: 500 pg/mL, 250 pg/mL, 125 pg/mL, and 83.3 pg/mL (**Table 3.5**).

Table 3.5 Preparation of Quality Control samples (aldosterone assay).

QC sample	Volume 1	Volume 2
1 (500 pg/mL)	500 µL	500 µL
	artificial blank matrix	standard '1000 pg/mL'
2 (250 pg/mL)	500 µL	500 µL
	artificial blank matrix	QC sample 1
3 (125 pg/mL)	500 µL	500 µL
	artificial blank matrix	QC sample 2
4 (83.3 pg/mL)	250 µL	500 µL
	artificial blank matrix	QC sample 3

QC: Quality Control

Calibration standards and QC samples were frozen at -80 °C to apply the same treatment to calibration standards and QC samples as to unknown samples. Prior to analysis, standards and QC samples were thawed together with unknown samples using a water bath at 23 ± 3 °C. This procedure was applied in the case of both assays to standardise sample preparation and minimise the effect of surrounding conditions, especially temperature variations.

Reduction of sample volume and adjustment of assay procedure

As the required sample volume is crucial to bioanalytical assays intended for use in paediatric investigations, the development process was followed by a reduction of the required sample volume to 40 µL. In parallel with this volume reduction step, several adjustments of the assay procedure recommended by the vendor (**Appendix 9.1)** became necessary to optimise the assay performance and allow for the following validation process:

- As the first step of the assay procedure, an equilibration step was introduced
  (40 µL assay buffer, incubation for 5 minutes at room temperature)
- An additional incubation step after the pipetting of calibration standards, QC samples, and unknown samples was added (incubation for 30 minutes at 300 rpm in a water bath tempered at 23 °C)*
- The incubation step after adding the enzyme conjugate was changed. The duration of this step was reduced by applying an additional shaking (7 minutes at 300 rpm, 1 minute at 700 rpm, and 29 minutes at 300 rpm instead of 60 minutes at room temperature).
- The washing procedure was increased (8 times with 300 µL instead of 5 times with 300 µL)
- Constant temperature conditions were applied during all incubation steps by use of a tempered water bath (23 °C)

*In the meantime, an additional incubation step (30 minutes at room temperature) has also been introduced by the vendor, according to the revised manual (02/2017, **Appendix 9.2**).

The complete assay procedure is given below.

Final assay procedure and calculation of results

All standards, QC samples, and unknown samples had to reach room temperature (water bath at 23 ± 3 °C). After dispensing 40 µL assay buffer into each well, the microtiter plate was incubated for 5 minutes at room temperature. 40 µL of each standard, QC sample, unknown sample, and blank (artificial blank matrix) were pipetted into the respective wells and mixed for 1 minute at 500 rpm in the dark, followed by an incubation at 300 rpm for 30 minutes in the dark. After adding 150 µL enzyme conjugate (aldosterone conjugated to horseradish peroxidase), the microplate was incubated for 7 minutes at 300 rpm, for 1 minute at 700 rpm, and 29 minutes at 300 rpm in the dark. Afterwards, the wells were emptied and rinsed 8 times with 300 µL wash buffer. Between all washing steps, the liquid was removed thoroughly. Next, 200 µL TMB solution were added to each well, followed by an incubation for 30 minutes in the dark (300 rpm). Then, the microtiter plate was incubated for an additional 5 minutes in daylight, without shaking. The enzyme reaction was stopped by pipetting 100 µL 0.5 M sulfuric acid into each well. After mixing for 1 minute at 300 rpm, the optical density (OD) was determined at 450 nm. To guarantee constant conditions, all incubation steps were performed using a ThermoMixer® by Eppendorf AG (Hamburg, Germany) tempered at 23 °C.

The measurement of the OD was performed using the microplate reader infinite®F50 by Tecan (Männedorf, Switzerland). The calculations of concentrations were performed by the Magellan™ software Tracker V 7.0. The concentration values obtained are given in pg/mL, and 1 pg/mL aldosterone is equivalent to 2.774 pmol/L (adapted from (29)).

3.2.4.2 Renin assay

The development of the renin immunoassay was based on the commercial ELISA kit EIA-5125 by DRG Instruments GmbH (33).

Adjustment of calibration standards and QC samples

As described above, at least six non-zero standards are required for ligand-binding assays, according to regulatory recommendations of the EMA (26) and the FDA (27). As the preselected renin immunoassay (ELISA kit EIA-5125 by DRG Instruments GmbH (33)) is based on only five non-zero standards plus one zero standard, the calibration standards had to be adjusted. Comparable to the aldosterone assay, this was realised by a serial dilution of the highest standard '128 pg/mL' with the artificial matrix.

Detailed preparation of calibration standards and QC samples

The lyophilised renin standard '128 pg/mL' was reconstituted with 1.0 mL double-distilled water to obtain the highest calibration standard (128 pg/mL). Starting from this high concentration, a serial dilution with the artificial blank matrix was performed to obtain the remaining calibration standards (**Table 3.6**).

Table 3.6 Preparation of calibration standards (renin assay).

Calibration Standard	Volume 1	Volume 2
1 (128 pg/mL)	(Reconstitution of lyophilised standard '128 pg/mL' with 1.0 mL double-distilled water)	
2 (64 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 1
3 (32 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 2
4 (16 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 3
5 (8 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 4
6 (4 pg/mL)	500 µL artificial blank matrix	500 µL Calibration Standard 5

Independent QC samples were obtained by reconstitution of further lyophilised renin standards 128 pg/mL, 64 pg/mL, and 32 pg/mL with double-distilled water. Standard 8 pg/mL was prepared by diluting standard 16 pg/mL with the artificial blank matrix prior to analysis. Standard 8 pg/mL was used as the low-concentration QC sample within three times the lower limit of quantification (LLOQ).

The same freeze and thaw cycle prior to the sample analysis as described for the aldosterone assay in section 3.2.4.1 was applied to the calibration standards and QC samples of the renin assay. This procedure was additionally intended to avoid cryoactivation, which results in misleading renin concentrations.

Reduction of sample volume and adjustment of assay procedure

The required sample volume was reduced to the minimum volume (40 µL) that still allowed for the best assay performance. In parallel, an optimisation of the assay performance was realised by the following adjustments of the assay procedure, that is recommended by the vendor (**Appendix 9.5**):

- The duration of the substrate incubation step was reduced by an additional shaking (11 minutes at 150 rpm instead of 15 minutes)
- Constant temperature conditions were applied during all incubation steps by use of a tempered water bath (20 ± 3 °C)

The final assay procedure is given below.

Final assay procedure and calculation of results

All standards, QC samples, and unknown samples were brought to room temperature using a water bath at 23 ± 3 °C. After equilibration with 150 µL assay buffer, 40 µL of standards, QC samples, unknown samples, and the blank (artificial blank matrix) were added to the corresponding wells. The microplate was incubated for 90 minutes at 300 rpm in the dark. Subsequently, the wells were emptied and rinsed 4 times with 300 µL wash buffer. Between all washing steps, the wash buffer was thoroughly removed. 100 µL anti-human renin antibody conjugated to horseradish peroxidase were added to each well and incubated for 90 minutes while shaking (300 rpm) in the dark. Contents of the wells were emptied and rinsed 4 times with 300 µL wash buffer. Then, 100 µL TMB solution were added to each well, and the microplate was incubated for 11 minutes in the dark (150 rpm). The enzyme reaction was stopped by adding 100 µL 0.5 M sulfuric acid to each well. Contents were mixed for 1 minute at 500 rpm, and the OD was determined at 450 nm.

As described for the aldosterone assay above, the OD was measured using the microplate reader infinite®F50 by Tecan (Männedorf, Switzerland). The calculations of concentrations were carried out with the corresponding Magellan™ software Tracker V 7.0. An additional step for blank correction preceded all calculations of renin concentrations. All concentration values are given in pg/mL, whereas 1 pg/mL renin is equal to 1.67 μIU/mL (30).

3.2.5 Bioanalytical method validation

Both immunoassays have been validated in accordance with current EMA and FDA regulatory guidelines. The procedure and the acceptance criteria of this validation process are described in the following sections.

3.2.5.1 Preparation of calibration standards and Quality Control samples

All calibration standards and QC samples used during the validation of the assays were prepared as described in the previous sections 3.2.4.1 and 3.2.4.2 respectively.

3.2.5.2 Assay procedure and calculation of results

The assay procedures and the corresponding calculation of results applied during all validation processes have been described in the previous sections 3.2.4.1 and 3.2.4.2 (sections 'Final assay procedure and calculation of results') respectively.

3.2.5.3 Bioanalytical method validation according to bioanalytical guidelines of EMA and FDA

Both assays have been partially validated according to the current bioanalytical guidelines of the EMA (26) and the FDA (27). A partial validation is justified, as modifications were made to adapt to limited sample volumes for paediatric investigations. In the case of a discrepancy in the guideline criteria, the stricter criteria were applied.

The validation covered the following parameters: Accuracy, precision, parallelism, dilutional linearity (aldosterone assay), matrix effect (ME), short-term stability, freeze-thaw stability, long-term stability, processed sample stability, and impact of haemolysed blood samples. Because selectivity and specificity had already been investigated by the vendor (1, 33) and no modifications to the antibodies were

made, both validation parameters were regarded as sufficiently investigated and were excluded from the validation.

Calibration curve and QC levels

The calibration curve of the aldosterone assay consisted of six duplicate non-zero calibration standards covering the concentration range from 31.3 pg/mL to 1000 pg/mL aldosterone in serum. Each assay run included four QC levels, one within three times the LLOQ (83.3 pg/mL), two in the medium range of the assay (125 pg/mL and 250 pg/mL) and one with a high concentration (500 pg/mL). The calibration standards of the renin assay covered a concentration range from 4 pg/mL to 128 pg/mL. The following QC levels were included in each assay run: one within three times the LLOQ (8 pg/mL), two in the medium range of the assay (32 pg/mL and 64 pg/mL) and one with a high concentration (128 pg/mL). In all assay runs, calibration standards and QC levels were determined in duplicate.

To evaluate the regression model of the calibration curve and the suitability of QC levels, six independent assay runs over several days were performed. Within-run and between-run accuracy of calibration standards and QC levels were calculated. For the calibration standards, values had to be within the limits of ±20 % of the nominal values (±25 % at the LLOQ) for all six calibration standards. With regard to the QC levels, mean values of replicates must be within the limit of ±20 % of the nominal values for at least three out of four QC levels. Additionally, at least one value of each duplicate determination per concentration level had to be within the limits of ±20 % of the nominal value.

Accuracy and precision

In order to evaluate accuracy and precision of the aldosterone assay, the following five concentration levels were determined in six independent assay runs over three different days. Each concentration level was determined in fivefold replicate analysis per assay run.

Concentration level 1	1000	pg/mL
Concentration level 2	500	pg/mL
Concentration level 3	125	pg/mL
Concentration level 4	62.5	pg/mL
Concentration level 5	31.3	pg/mL

For evaluation of the renin assay, the following concentration levels were determined in six assay runs on four different days.

Concentration level 1	128	pg/mL
Concentration level 2	64	pg/mL
Concentration level 3	16	pg/mL
Concentration level 4	8	pg/mL
Concentration level 5	4	pg/mL

Mean values of within-run and between-run accuracy had to be within ±20 % (±25 % at LLOQ) of the nominal values. Within-run and between-run precision, reported as a coefficient of variation (CV), were regarded as acceptable if a limit of 20 % (25 % at LLOQ) was not exceeded. Within-run precision was calculated according to **Formula 3.1**. Time-different intermediate precision (between-run) was evaluated by one-way ANOVA (see **Formula 3.2**). Following recommendations of EMA (26), the total error of the method was calculated as the sum of the absolute value of the relative error [%] and the CV [%] and had to be less than the limit of

30 %. These calculations were based on the corresponding values obtained by between-run accuracy and precision.

$$\mathrm{CV}\,[\%] = \frac{\mathrm{SD}}{\bar{x}} * 100$$

Formula 3.1
Within-run precision

CV	Coefficient of variation
SD	Standard deviation of five replicates per concentration level and assay run
$\bar{x}$	Mean concentration of five replicates per concentration level and assay run

$$\mathrm{CV}\,[\%] = \frac{\sqrt{\frac{\mathrm{MS_{bg}} - \mathrm{MS_{wg}}}{\mathrm{n}} + \mathrm{s}^2}}{\bar{\mathrm{x}}} * 100$$

Formula 3.2
Time-different intermediate precision/ between-run precision

CV	Coefficient of variation
MS_{bg}	Mean squared error between group
MS_{wg}	Mean squared error within group
n	Amount of repetitions per day
s^2	Repetition variance
$\bar{x}$	Mean

Parallelism

The samples of three different sources were spiked at high aldosterone and renin concentrations respectively. After a serial dilution of the spiked samples with the artificial blank matrix, native and spiked samples as well as dilutions of spiked samples were measured, with three replicates each. The different sources were analysed in separate assay runs over two days. The values obtained for diluted samples were back-calculated and were compared to the values determined for spiked samples that had not been diluted prior to analysis. The maximum deviation

accepted was ±20 %. Precision between serially diluted samples was reported as CV and had to be ≤30 % according to the EMA (26).

Dilutional linearity (Aldosterone assay)

In contrast to parallelism, samples of three different sources were spiked at concentrations exceeding the ULOQ. A serial dilution of the spiked samples with the artificial blank matrix was performed and dilutions as well as native samples were measured in three assay runs over two days. Native samples and dilutions of spiked samples were measured with three replicates each. The values obtained were back-calculated and compared to theoretically calculated values of spiked samples. The percentage deviations had to be less than ±20 % of the calculated values and precision between diluted samples was not allowed to exceed 20 % (CV).

Matrix effect

The effect of the matrix on accurate determinations was investigated. As aldosterone and renin are inherently present in native samples, the possible effect of the matrix was assessed by a comparison of spiked biological samples with native samples.

Therefore, native samples of ten human sources (including one haemolysed source) were spiked using one solution with a lower concentration level and one with a higher concentration level. The volume of the spiking solution added to serum or plasma was ≤10 % of the total sample volume to minimise the effect of matrix dilution. The concentrations of native and spiked samples were determined (three replicates each) in parallel with the actual concentrations of the spiking solutions in all assay runs. After the inherent amount of aldosterone and renin in native samples had been combined with the amount added by spiking, the theoretical values of the samples were calculated and compared to the actually

determined values. The percentage deviations of actually determined values from calculated values (nominal values) were determined and had to be within ±20 % for at least 80 % of the matrices evaluated.

Stability

The stability investigations performed during the validation covered short-term stability, freeze-thaw stability, long-term stability, and processed sample stability. Native samples were used to assess lower concentration levels, and spiked samples were used for the evaluation of higher concentration levels. In all assay runs for the assessment of short-term, freeze-thaw, and long-term stability, samples of all concentration levels were determined with six replicates each, and mean values were calculated. Mean values were not allowed to exceed ±20 % of the reference value that had been obtained by fresh samples on the day of blood collection (day 0). This acceptance criterion was chosen because no loss of sample stability can be claimed as long as values do not exceed the accuracy limit of the assay (±20 %).

For the evaluation of short-term stability, aliquots of two samples with different concentration levels (one native sample, one spiked at a higher concentration) were placed on the laboratory bench (room temperature) on day 0. The following day, samples were measured with six replicates each and the percentage deviations of the mean values from the reference values were calculated.

To investigate the freeze-thaw stability of aldosterone, aliquots of two samples with different concentration levels were stored at -80 °C for 44 days after blood collection, and they underwent three freeze and thaw cycles (all of the freezing steps overnight) over the last three days of this period. A storage period of at least one month prior to analysis was chosen to mimic the usual treatment of study samples. For renin, four freeze and thaw cycles were performed on the days directly after blood collection. All freezing steps were performed as snap freezing

to avoid cryoactivation and/or to standardise sample treatment. After the last freeze and thaw cycle, values were determined with six replicates per concentration level. Mean values were calculated and compared to the reference values.

For the assessment of long-term stability, aliquots of three samples with different concentration levels were stored at -80 °C (snap frozen). The three concentration levels comprised one native sample, one sample spiked at a medium concentration level, and a third sample spiked at a higher concentration level. All concentration levels were determined at regular intervals over an extended period of time with six replicates per concentration level. For eight weeks, determinations were performed weekly and afterwards every two weeks or monthly. Samples were regarded as stable as long as values were within ±20 % of the reference values.

To assess processed sample stability, replicate readings of a typical validation run were performed at regular intervals over two hours at the end of an analytical run. During the first hour, readings were performed every 5 to 10 minutes and after that at intervals of 30 minutes. Mean values for both calibration standards and QC levels had to be within ±20 % of the nominal values. Values for unknown samples had to be within ±20 % of values obtained by the immediate measurement after stopping the enzyme reaction.

Impact of haemolysed blood samples on accurate sample measurement

Blood was collected from three different human sources. For each source, two aliquots were prepared, of which one was haemolysed in an ultrasonic bath. Both haemolysed and non-haemolysed samples of each source were measured with three replicates each. The percentage deviations of the values obtained through haemolysed samples from the values obtained through non-haemolysed samples were calculated to assess the impact of haemolysis on accurate sample measurement. In the case of deviations ≤20 %, the impact of haemolysis on an accurate sample measurement was regarded as negligible.

3.2.6 External verification

The reliable assay performance was additionally investigated by participation in an external ring test, which is the common quality assurance demanded by the German Medical Association for laboratory medicine (Rili-BÄK (28)). Two samples with unknown concentration levels of the respective parameter were received from the Reference Institute for Bioanalytics (Bonn, Germany). All samples were analysed in duplicate using the bioanalytical assays presented in this work, and mean values were reported to the Reference Institute for Bioanalytics. Values that had been obtained by the aldosterone assay were compared to the results of a liquid chromatography tandem-mass spectrometry (LC-MS/MS) method, while the reported renin concentrations were compared to the median of values obtained from participants in the same subgroup (same method and company combination).

3.3 Proof of concept

To further confirm the applicability of both low-volume assays to human samples, time-concentration profiles of a healthy adult were obtained for aldosterone and renin over twelve hours.

Furthermore, the developed assays are currently used in the paediatric studies of the EU-funded project ‘Labeling of Enalapril from Neonates up to Adolescents’ (LENA), which allows for an assessment of the assays’ applicability to a clinical setting under conditions of GCLP and to paediatric samples.

The subsequent sections contain parts of the manuscripts ‘Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes’ by Schaefer et al. (35) and ‘Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations’ by Schaefer et al. (36).

3.3.1 Twelve-hour profiles

The aldosterone and renin concentrations of a 30-year-old healthy volunteer (female) were determined over 12 hours. The first sample was collected after night’s rest at 6 a.m., the last one at 6 p.m. to monitor the parameter concentrations according to the circadian rhythm of the volunteer. In total, nine sample collections were performed. During the daytime, the subject was supine for at least 30 minutes prior to all other sampling points. Serum and plasma samples were taken every hour or every two hours. A snack was served between 11:00 a.m. and 11:30 a.m. Samples of all blood collections were analysed on the basis of the methods described in the previous section 3.2.4.1 and 3.2.4.2 All concentrations were determined in duplicate and mean concentrations were calculated for each time a sample was taken. The aldosterone and renin profiles over 12 hours were compiled by plotting the obtained mean concentrations against the corresponding time.

3.3.2 Application to study population

Both assays are used in the clinical studies of the LENA project, which collects pharmacokinetic and pharmacodynamic data on children suffering from heart failure. In this context, the assays are applied to paediatric samples and are exposed to a clinical setting under conditions of GCLP.

3.4 Development of a performance qualification test for a microplate reader

The implementation of a GCLP system involves several requirements regarding routine processes within the laboratory setting. These requirements include period tests to demonstrate that the technical equipment is in good working order. A practicable and rapid PQ test was developed in order to support the implementation of the developed immunoassays in the GCLP environment of the LENA studies.

To this end, Alizarin Yellow (dye content 50 %) by Sigma-Aldrich® was selected. It shows an absorption at 450 nm (37), the maximum absorption of the yellow diimine measured at the end of the enzyme reaction of both assays (34).

An aqueous solution of the selected dye was prepared, and the OD was measured at 450 nm. The concentration of the dye was adjusted until the OD was in the range of the highest value of the calibration curves of both developed assays. In the following, this solution will be referred to as ‘stock solution’. A serial dilution was prepared from this stock solution to cover the entire range of the calibration curves in both assays. For all dilutions, double-distilled water, purchased from Carl Roth GmbH and Co. KG (Karlsruhe, Germany), was used.

The PQ test was intended to be performed on each working day prior to sample analysis. The stock solution was stored in the dark at 2 - 8 °C. The stability of the aqueous solution under these storage conditions was assessed by regular determinations of the OD of the stock solution and the corresponding dilutions over an extended period of time. Results within ±10 % of the values obtained by the fresh stock solution and its dilutions were regarded as acceptable.

4 Results

4.1 Literature search

Twenty-nine papers were selected, and data was extracted as described in section 3.1. The found mean and median concentrations of each parameter are shown in **Fig. 4.1** - **Fig. 4.7**. Values are categorised according to the different age groups and corresponding subgroups (healthy or no cardiac disease, cardiac disease - medication with ACEi, and cardiac disease - medication without ACEi or with no medication).

Aldosterone

The highest data density was obtained for aldosterone. This applies especially to the adult age group (≥18 years), for which numerous mean values in all subgroups were found (**Fig. 4.1**). This age group showed generally higher values among patients with cardiac diseases than among the healthy population or those without cardiac disease. The highest value found in this age group was a mean value of 1170 pg/mL (subgroup with cardiac disease - medication without ACEi or with no medication).

Significantly less data was found for all groups of younger subjects. In the age group of very young children (0 days - 23 months), the values also showed a high variation ranging between 3 pg/mL and 2531 pg/mL. Median values found during the literature search are shown in **Fig. 4.2**.

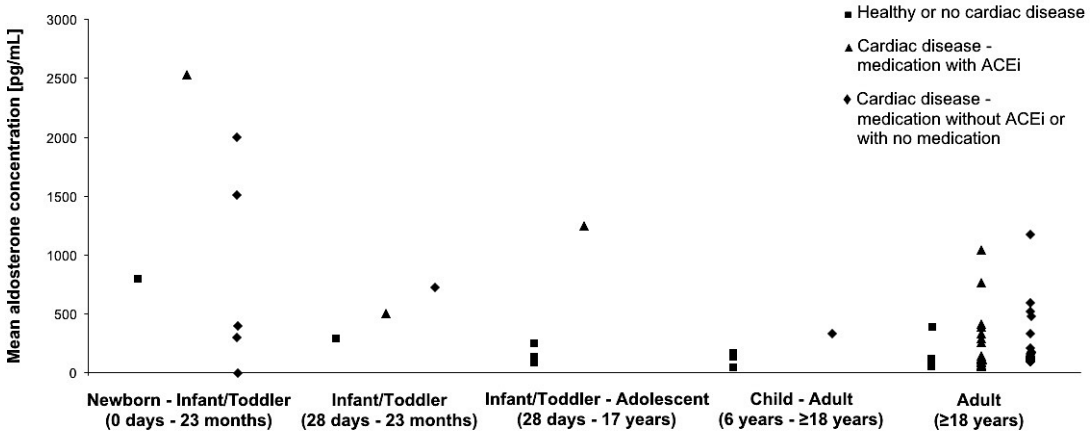

Fig. 4.1 Mean aldosterone concentrations found by literature search. Data was extracted from the sources (38–58).

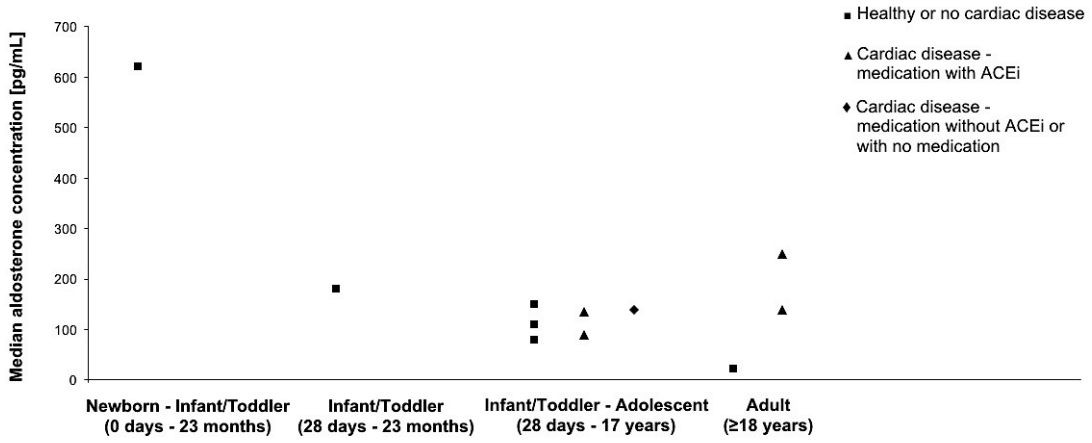

Fig. 4.2 Median aldosterone concentrations found by literature search. Data was extracted from the sources (59–62).

Angiotensin I

For ang I, few median values were found that could be clearly assigned to the corresponding categories. The little data found clearly differs between the adult and the younger age group but is not sufficient to indicate a trend (**Fig. 4.3**).

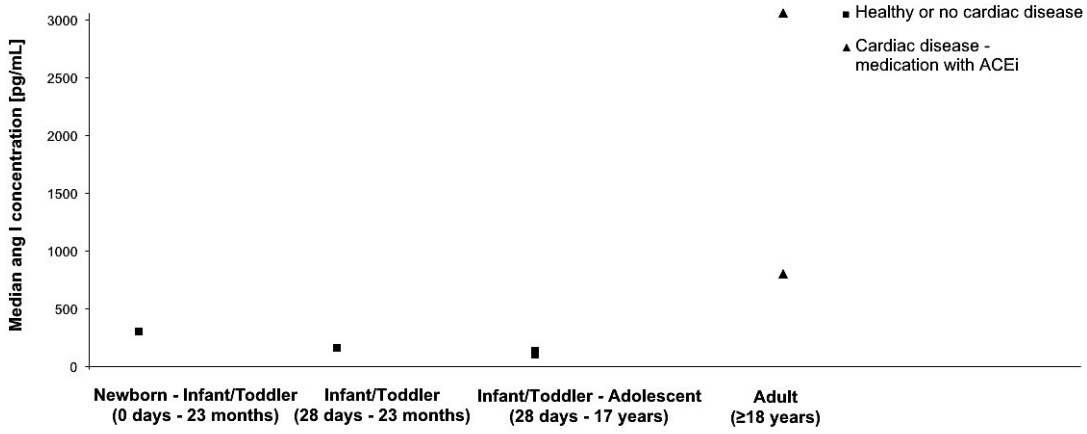

Fig. 4.3 Median angiotensin I concentrations found by literature search. Data was extracted from the sources (59, 61). Ang I: angiotensin I

Plasma renin activity

The number of values found for PRA was approximately as high as for aldosterone (**Fig. 4.4** - **Fig. 4.5**). However, similar to the finding with aldosterone, significantly less data was found for the younger age groups than for adults. The mean values found for the youngest age group (from 0 days to 23 months) show the highest variation (subgroup with cardiac disease without ACEi or with no medication).

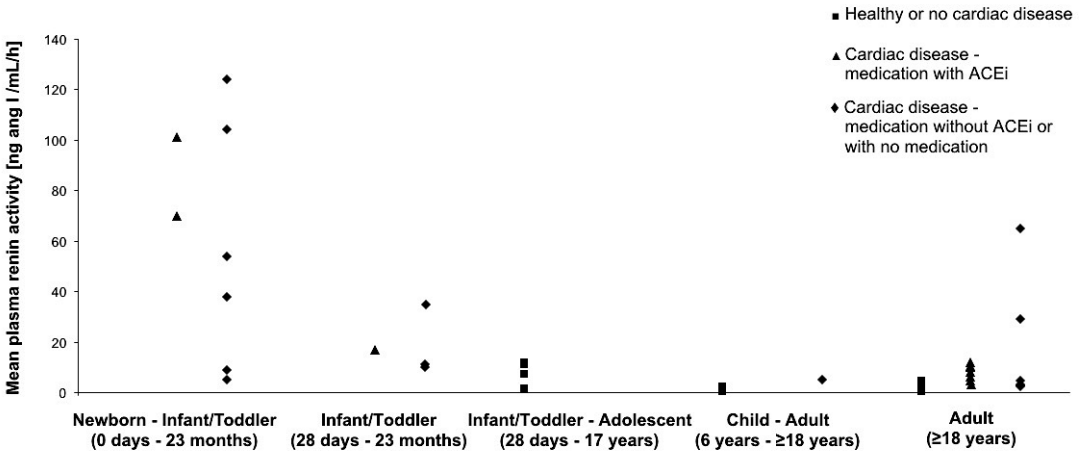

Fig. 4.4 Mean plasma renin activity found by literature search. Data was extracted from the sources (39–41, 45, 47–49, 51, 52, 54, 55, 57, 58, 63–65). Ang I: angiotensin I

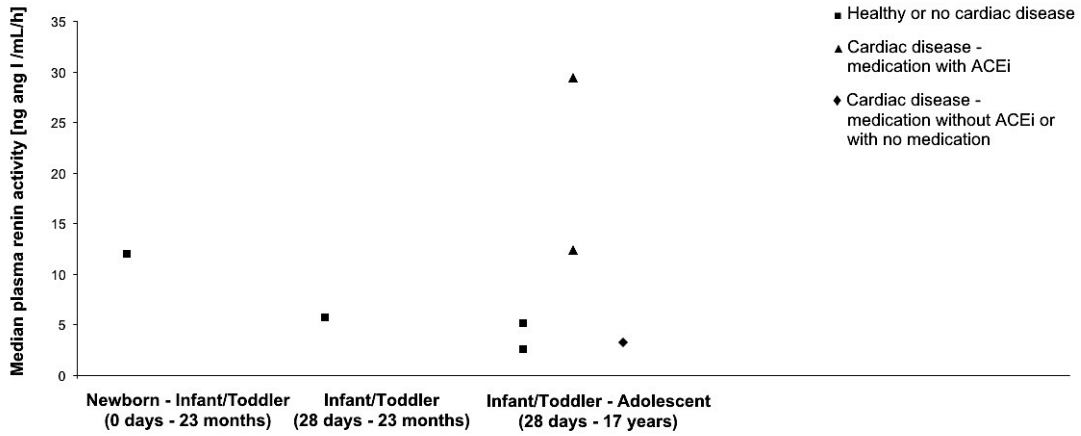

Fig. 4.5 Median plasma renin activity found by literature search. Data was extracted from the sources (59, 60). Ang I: angiotensin I

Renin

Concentration values for the parameter renin were found for the age group between 28 days and 23 months (Infant/Toddler) and adults (≥18 years) (**Fig. 4.6** - **Fig. 4.7**). Especially in the age group of adults, the data revealed a high variation between a median value of 8 pg/mL in the subgroup of healthy subjects or those without cardiac disease and a mean value of 9300 pg/mL in the subgroup suffering from cardiac disease (on ACEi).

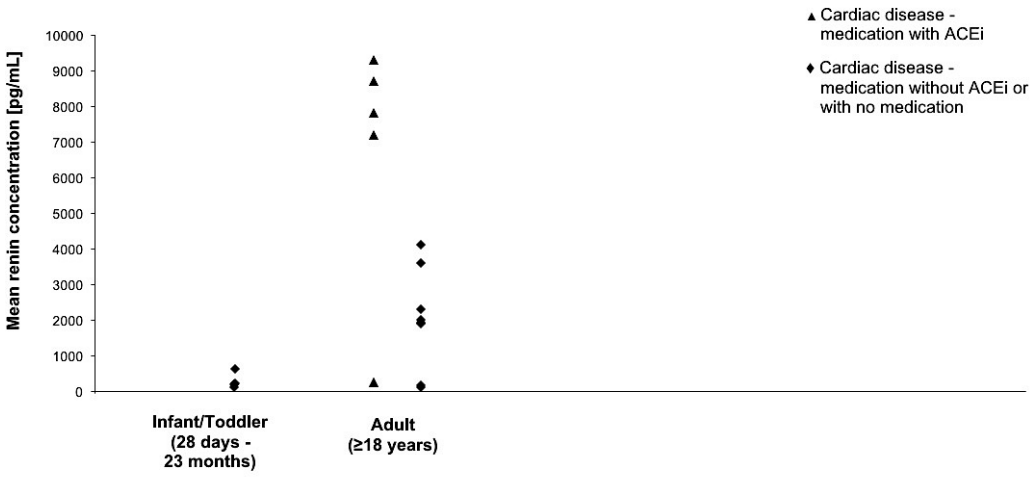

Fig. 4.6 Mean renin concentrations found by literature search. Data was extracted from the sources (42, 43, 64, 66).

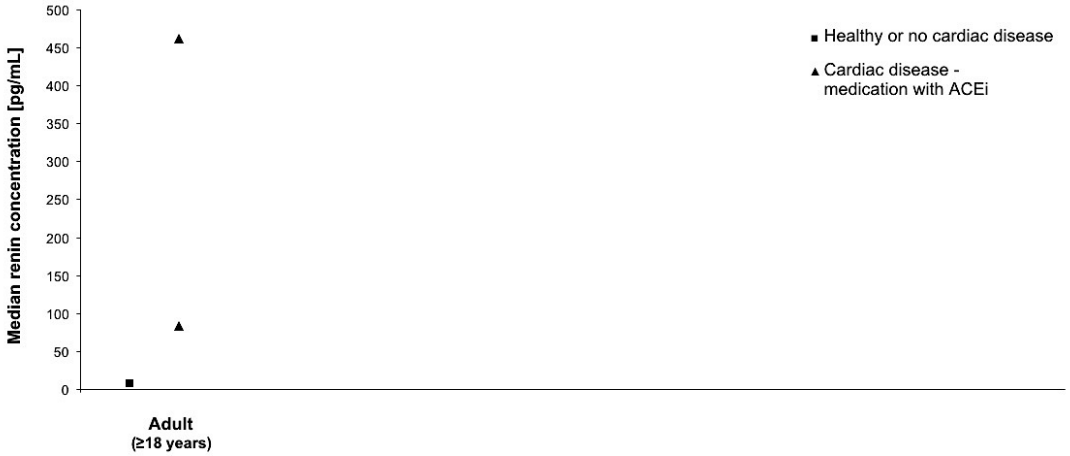

Fig. 4.7 Median renin concentrations found by literature search. Data was extracted from the sources (61, 62).

4.2 Development, validation, and external verification of a low-volume aldosterone and renin immunoassay

Two immunoassays for the determination of aldosterone and renin concentrations in small sample volumes (40 µL serum/plasma) have been successfully developed. The reliability of the assays was confirmed by a bioanalytical method validation in accordance with the current international guidelines of EMA and FDA. Additionally, both assays passed a ring test by the Reference Institute for Bioanalytics (Bonn, Germany), which served as external quality assurance.

Both assays have been implemented in the GCLP-compliant setting of the LENA project and are currently applied to paediatric samples.

4.2.1 Selection of commercial immunoassays as a basis for the following development of guideline-compliant low-volume assays

Aldosterone concentrations that were determined by use of the three selected immunoassays are given in **Table 4.1**. In the case of the Aldosterone Assay (Cat. No. KGE016) by R&D Systems, Inc. (2), the calibration standards did not show the expected values in the assay run at day seven and at week eight. Therefore, no further evaluation of the corresponding aldosterone concentrations was possible. Regarding the Human Aldosterone (ALD) Elisa kit (Cat. No. E01A0774) by BlueGene Biotech CO., LTD (3), a defective batch falsified the measurement of fresh samples and those frozen for seven days. The best performance was achieved using the Aldosterone ELISA (Ref. EIA-5298) by DRG Instruments GmbH (1). In each assay run performed, an evaluable calibration curve was obtained. All values obtained for donors 1, 2 , 4, and 5 were within ±20 % of their mean, and precision did not exceed 20 % (CV). Some of the values obtained for donor 3 were not within ±20 % of their mean, and precision exceeded 20 %. A possible explanation for this variation is that the obtained data was at the lower end of the calibration curve.

Consequently, the latter aldosterone assay provided by DRG Instruments GmbH was selected as basis for the development of a low-volume assay suitable for validation according to current bioanalytical guidelines.

For the development of a child-appropriate low-volume renin immunoassay, the ELISA kit EIA-5125 based on a sandwich immunoassay and the use of monoclonal antibodies of the same vendor (DRG Instruments GmbH) was chosen.

Table 4.1 Comparison of aldosterone concentrations obtained by three different immunoassays. Three commercially available immunoassays for the determination of aldosterone concentrations (1–3) were compared in several assay runs over an extended period of time (storage at -20 °C in the meantime). Each assay run included serum and plasma samples of five different donors.

	DRG Instruments GmbH Aldosterone concentration [pg/mL]				R&D Systems, Inc. Aldosterone concentration [pg/mL]				BlueGene Biotech CO., LTD Aldosterone concentration [pg/mL]			
Sample treatment	**Fresh**	**Frozen for 7 days at -20 °C**	**Frozen for 4 weeks at -20 °C**	**Frozen for 8 weeks at -20 °C**	**Fresh**	**Frozen for 7 days at -20°C**	**Frozen for 4 weeks at -20°C**	**Frozen for 8 weeks at -20°C**	**Fresh**	**Frozen for 7 days at -20°C**	**Frozen for 4 weeks at -20°C**	**Frozen for 8 weeks at -20°C**
Donor 1/Serum	232.0 *1	242.0 *1	246.8 *1	185.3 *1	387.1 *2	Calibration standards did not show expected values → no evaluation possible	No determination performed	Calibration standards did not show expected values → no evaluation possible	No determination possible → Batch defective according to vendor		93.3 *3	83.5 *3
Donor 1/Plasma	230.6 *1	248.5 *1	243.4 *1	197.3 *1	351.6 *2						81.0 *3	64.7 *3
Donor 2/Serum	214.0 *1	221.6 *1	252.6 *1	200.9 *1	334.9 *2						88.7 *3	77.4 *3
Donor 2/Plasma	200.4 *1	205.9 *1	220.7 *1	179.0 *1	386.1 *2						79.4 *3	64.7 *3
Donor 3/Serum	17.0 *1	13.8 *1	19.3 *1	8.5 *1	20.9 *2						22.3 *3	18.6 *3
Donor 3/Plasma	16.2 *1	12.9 *1	22.4 *1	4.4 *1	20.9 *2						19.4 *3	15.3 *3
Donor 4/Serum	146.3 *1	155.0 *1	152.2 *1	115.4 *1	480.4 *2						27.5 *3	22.1 *3
Donor 4/Plasma	141.0 *1	156.1 *1	158.6 *1	125.8 *1	517.2 *2						18.1 *3	18.0 *3
Donor 5/Serum	87.8 *1	86.8 *1	99.2 *1	75.1 *1	279.4 *2						21.4 *3	20.7 *3
Donor 5/Plasma	93.2 *1	85.7 *1	96.8 *1	80.3 *1	391.3 *2						18.4 *3	17.2 *3

*1 Calculation based on the 4-parameter Marquardt regression model
*2 Determination without correction for wavelength and without subtraction of non-specific binding
*3 Standard 0 pg/mL removed; linear regression with double-logarithmic plot

4.2.2 Bioanalytical method validation

Both assays have been validated according to EMA and FDA recommendations. The detailed results of the in-house validation are presented in this chapter.

The subsequent sections contain parts of the manuscripts ‘Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes’ by Schaefer et al. (35) and ‘Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations’ by Schaefer et al. (36).

4.2.2.1 Aldosterone assay

Calibration curve and Quality Control levels

The regression model of the calibration curve and the suitability of QC levels were assessed in six assay runs. The best curve fitting was obtained by applying the 4-parameter Marquardt regression model (extrapolation 1.5, **Formula 4.1**). Details on the corresponding regression parameters are given in **Table 4.2**. Regarding between-run accuracy, the relative error of the calibration standards ranged from -2.1 % to +1.5 %, while the relative error of the QC levels was between -2.3 % and +1.1 %. All determined values of calibration standards and QC levels were within the required limits of ±20 % (±25 % at LLOQ). A typical standard curve is shown in **Fig. 4.8**.

$$y = D + \frac{A - D}{1 + (\frac{X}{C})^B}$$

Formula 4.1
Equation of the 4-parameter logistic regression model (taken from 67).

A, D Asymptomatic ends

B Controls transition between the two asymptotes

C Transition point midway between the two asymptotes where the curve changes direction

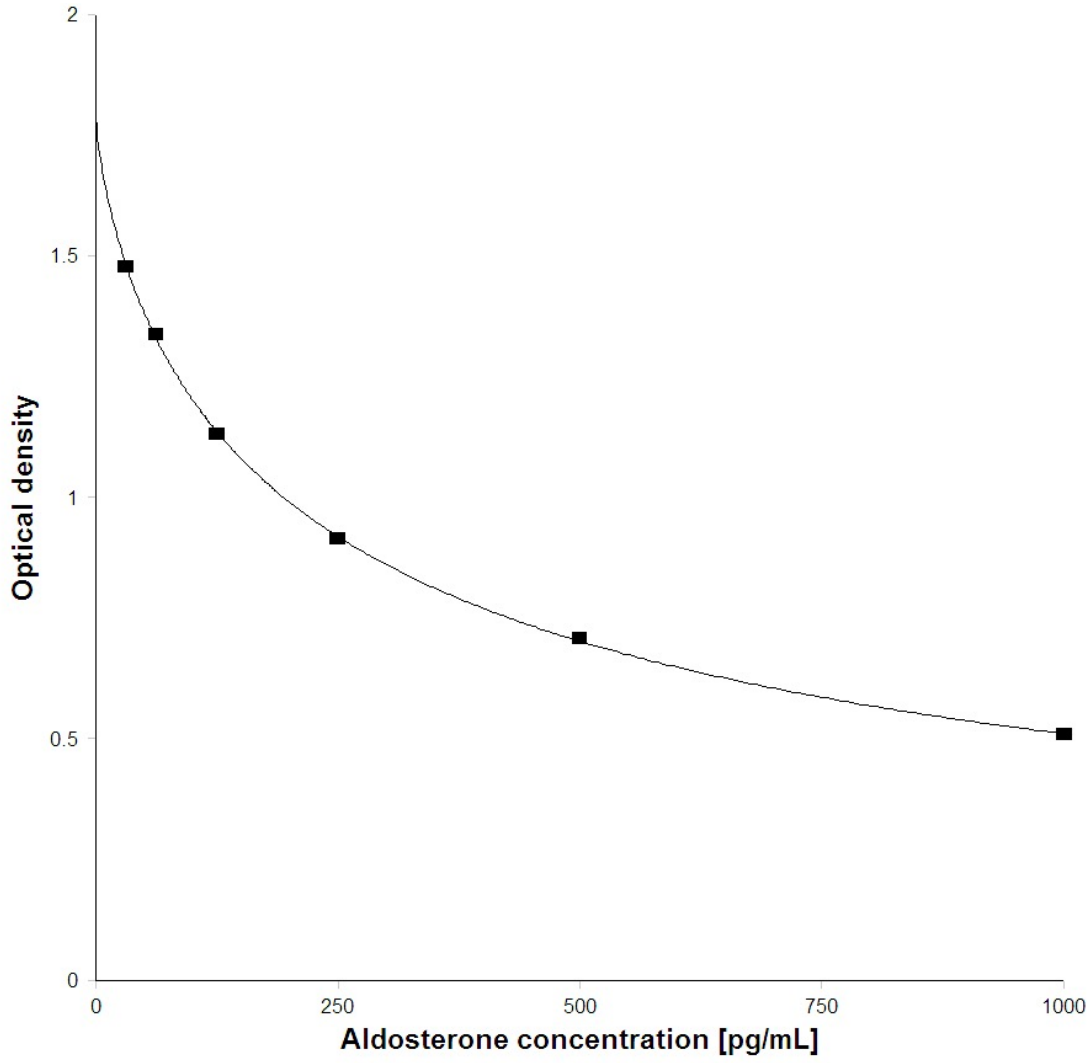

Fig. 4.8 Aldosterone standard curve. The calibration curve consists of six non-zero calibration standards ranging from 31.3 pg/mL to 1000 pg/mL.

Table 4.2 Regression parameters of six assay runs (aldosterone assay). For each run, all regression parameters as well as the mean squared error and the correlation coefficient are given.

	Regression model 4-parameter Marquardt (extrapolation 1.0)					
Assay run	**A**	**B**	**C**	**D**	**d**	**r**
1st	1.8	0.9	249.9	0.2	0.0045	1.0
2nd	1.8	0.9	214.4	0.2	0.0036	1.0
3rd	1.8	0.7	246.1	0.053	0.0059	1.0
4th	1.6	0.9	226.5	0.2	0.0067	1.0
5th	1.7	0.8	254.3	0.071	0.003	1.0
6th	1.7	0.8	259.2	0.1	0.0026	1.0

d: mean squared error; r: correlation coefficient

Accuracy and precision

In six independent assay runs, the within-run accuracy over all five different concentration levels showed a relative error between -11.1 % and +9.0 %. For between-run accuracy, the relative error ranged from -3.8 % to -0.8 %. All results were within ±20 % of the nominal values (±25 % at LLOQ) and thus complied with guideline requirements. The within-run precision over all five concentration levels was between 1.2 % and 11.8 % (CV), while between-run precision (one-way ANOVA) ranged from 4.9 % to 8.9 % (CV) across the five different concentration levels tested. None of the results exceeded the guideline limits of 20 % (25 % at LLOQ). The accuracy results for all five replicates per concentration level in six assay runs are shown in **Fig. 4.9**. Details on within-run and between-run accuracy (relative error) and precision (CV) are given in **Table 4.3** and **Table 4.4**.

At the LLOQ (31.3 pg/mL), the relative error of the within-run accuracy ranged from -9.6 % to +3.5 %, while between-run accuracy showed a relative error of -3.8 %. Within-run precision was between 3.7 % and 11.8 % (CV) and between-run precision (one-way ANOVA), reported as CV, was 8.8 %. At the ULOQ (1000 pg/mL), the relative error of the within-run accuracy ranged from -7.5 % to +1.2 %, while the relative error of the between-run accuracy was -3.3 %. Within-run precision varied from 2.0 % to 4.3 % and between-run precision (one-way ANOVA) was 4.9 %. All values were within the required guideline limits.

The total error of the method was between 8.0 % (concentration level 1000 pg/mL) and 12.5 % (concentration level 31.3 pg/mL) and thus was also in compliance with guideline requirements.

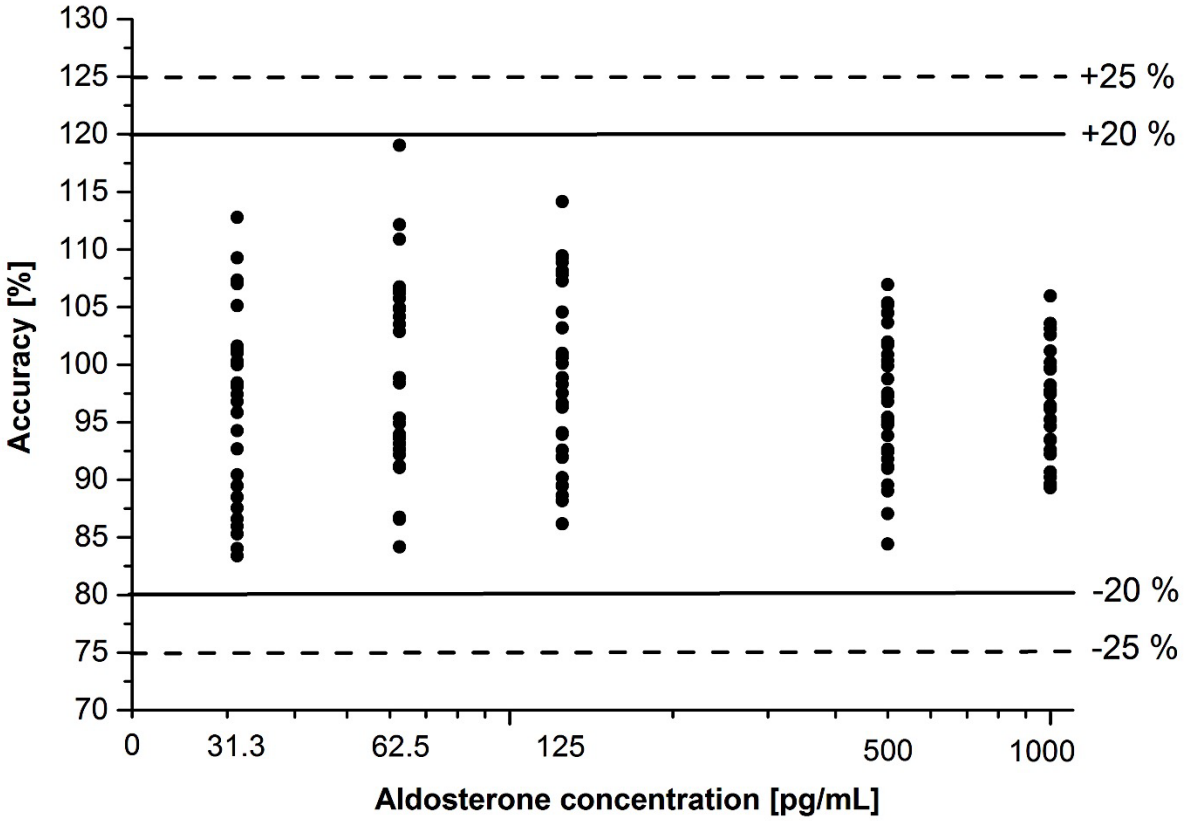

Fig. 4.9 Accuracy of the aldosterone assay. Five concentration levels (31.3 pg/mL, 62.5 pg/mL, 125 pg/mL, 500 pg/mL, and 1000 pg/mL) were determined in six independent assay runs. For each concentration level, values obtained for five replicates in six assay runs are shown (30 determinations per concentration level). Dashed black lines indicate the ±25 % limit for the LLOQ, while the continuous black lines highlight the accuracy limits for all other concentration levels.

Table 4.3 Within-run accuracy (relative error) and within-run precision (CV) of the aldosterone assay.

Nominal concentration level [pg/mL]	Assay run	Mean concentration found ± SD [pg/mL]	Relative error (within-run) [%]	CV (within-run) [%]*
31.3 (LLOQ)	1st	31.3 ± 1.6	±0.0	5.2
	2nd	30.9 ± 3.6	-1.3	11.8
	3rd	29.2 ± 1.1	-6.7	3.7
	4th	28.3 ± 2.3	-9.6	8.1
	5th	32.4 ± 1.8	+3.5	5.7
	6th	28.6 ± 2.7	-8.6	9.4
62.5	1st	68.1 ± 4.1	+9.0	6.0
	2nd	61.2 ± 3.6	-2.1	5.9
	3rd	56.5 ± 3.0	-9.6	5.3
	4th	57.5 ± 2.1	-8.0	3.6
	5th	66.7 ±1.6	+6.7	2.4
	6th	62.0 ± 3.8	-0.8	6.1
125	1st	135.7 ± 4.4	+8.6	3.2
	2nd	115.1 ± 4.9	-7.9	4.3
	3rd	116.0 ± 6.9	-7.2	5.9
	4th	118.9 ± 4.1	-4.9	3.5
	5th	132.7 ± 4.8	+6.2	3.6
	6th	118.7 ± 5.6	-5.0	4.7
500	1st	524.9 ± 6.2	+5.0	1.2
	2nd	444.6 ± 16.2	-11.1	3.7
	3rd	489.2 ± 11.2	-2.2	2.3
	4th	462.0 ± 14.2	-7.6	3.1
	5th	509.0 ± 10.3	+1.8	2.0
	6th	471.2 ± 7.5	-5.8	1.6
1000 (ULOQ)	1st	1005.4 ± 43.0	+0.5	4.3
	2nd	926.3 ± 27.4	-7.4	3.0
	3rd	1011.5 ± 20.1	+1.2	2.0
	4th	942.0 ± 21.7	-5.8	2.3
	5th	990.4 ± 24.0	-1.0	2.4
	6th	924.7 ± 25.2	-7.5	2.7

CV: coefficient of variation; SD: standard deviation; *calculated with **Formula 3.1** (section 3.2.5.3)

Table 4.4 Between-run accuracy (relative error) and between-run precision (CV) of the aldosterone assay.

Nominal concentration level [pg/mL]	Mean concentration found ± SD [pg/mL]	Relative error (between-run) [%]	CV (between-run) [%]*
31.3 (LLOQ)	30.1 ± 2.6	-3.8	8.8
62.5	62.0 ± 5.2	-0.8	8.9
125	122.9 ± 9.6	-1.7	8.2
500	483.5 ± 29.8	-3.3	6.6
1000 (ULOQ)	966.7 ± 45.1	-3.3	4.9

CV: coefficient of variation; SD: standard deviation; *calculated by one-way ANOVA (**Formula 3.2**, section 3.2.5.3)

Parallelism

After spiking, the aldosterone concentrations of the three different sources ranged from 600.0 pg/mL to 634.1 pg/mL. All back-calculated concentrations of diluted samples were within ±20 % of concentrations determined for the corresponding spiked sample. Precision between serially diluted samples ranged from 3.6 % to 5.6 % (CV) over all three assay runs and thus complied with guideline requirements. Results of parallelism are shown in **Table 4.5**.

Table 4.5 Parallelism of the aldosterone assay. The values of the spiked samples and the back-calculated values of the samples in dilution series are given. For the back-calculated values, the percentage deviations from the spiked samples were calculated.

	1st source	2nd source	3rd source
(Spiked sample)	634.1 pg/mL	611.7 pg/mL	600.0 pg/mL
Dilution factor	**Back-calculated concentration [pg/mL] (deviation from spiked sample [%])**		
2	635.2 (+0.2)	646.9 (+5.8)	571.0 (-4.8)
4	616.9 (-2.7)	633.8 (+3.6)	556.4 (-7.3)
8	555.8 (-12.3)	609.4 (-0.4)	515.3 (-14.1)
16	605.4 (-4.5)	663.2 (+8.4)	n/a
CV [%]	5.6	3.6	5.3

n/a: not applicable (value not reported because the obtained optical density was outside the assay range); CV: coefficient of variation

Dilutional linearity

For three different sources, the relative error ranged from -7.4 % to +10.5 % and was within the acceptance limits of ±20 %. Details are shown in **Table 4.6**. Over all three assay runs, precision ranged from 2.9 % to 7.4 % (CV).

Table 4.6 Dilutional linearity of the aldosterone assay. The values of the spiked samples were theoretically calculated, while the values of the serially diluted samples were back-calculated. The percentage deviations of back-calculated from theoretically calculated values are given in brackets.

	1st source	2nd source	3rd source
(Spiked sample)	2663.4 pg/mL*	3190.3 pg/mL*	3404.8 pg/mL*
Dilution factor	**Back-calculated concentration [pg/mL] (deviation from calculated value of spiked sample [%])**		
2	n/a	n/a	n/a
4	2778.5 (+4.3)	2954.1 (-7.4)	3404.1 (±0.0)
8	2943.5 (+10.5)	3360.7 (+5.3)	3425.6 (+0.6)
16	2860.0 (+7.4)	3377.2 (+5.9)	3592.5 (+5.5)
CV [%]	2.9	7.4	3.0

*theoretically calculated values; n/a: not applicable (value not reported because the obtained optical density was outside the assay range); CV: coefficient of variation

Matrix effect

Ten sources were assessed to investigate a possible ME. Because native samples differ regarding inherent aldosterone levels, the aldosterone concentrations of the assessed ten human sources varied after treatment with spiking solutions. Samples that had been spiked with the low-concentration aldosterone solution ranged from 85.4 pg/mL to 212.1 pg/mL, while samples spiked with the high-concentration solution showed concentrations between 516.6 pg/mL and 844.7 pg/mL. For nine samples spiked at lower concentrations, the aldosterone concentration ranged from -8.7 % to +12.5 % of the nominal values. One haemolysed sample showed a deviation of +32.7 %. For all ten samples spiked at higher concentrations, the aldosterone concentration was between -9.9 % and +8.8 % of the nominal values. For 90 % of the matrices tested, the concentration

was within the required limit of ±20 % of the nominal values. This complies with EMA requirements, which specify that at least 80 % of all matrices assessed have to be within ±20 %. Results are shown in **Table 4.7**.

Table 4.7 Results for matrix effect (aldosterone assay). The percentage deviations of the values obtained by the spiked samples from the nominal values are shown for ten sources. Each source was spiked at a low- and a high-concentration level.

Sex	Source	Percentage deviation [%]	
		Concentration level	
		low	high
♀	**1**	+1.0	-0.7
♂	**2**	-1.2	+8.8
♀	**3**	+0.6	+1.4
♂	**4**	+2.3	+0.1
♀	**5**	-8.7	-8.9
♀	**6**	+1.5	-9.9
♀	**7 (haemolysed)**	+32.7	-2.0
♂	**8**	+12.5	+5.3
♂	**9**	-5.0*	-6.1*
♀	**10**	+3.9	-8.7

*Calculations of native values were based on one instead of three replicates because the optical densities of the other replicates were outside the assay range

Stability

The mean values (± standard deviations (SD)) that had been determined on the day of blood collection (day 0) were 85.1 pg/mL (±9.9 pg/mL) for the lower concentration and 391.0 pg/mL (±13.1 pg/mL) for the higher concentration sample. These values were used as reference values further on. At each concentration level, the mean values were calculated on the basis of six replicates.

After one day on the laboratory bench, the mean value for the short-term stability sample at a lower concentration level showed a deviation of -10.7 % from the reference value, while the mean value for the serum sample at a higher concentration level deviated by +7.3 %. Because the results did not exceed the

accuracy limit of ±20 % compared to reference values, short-term stability over one day was proven.

For freeze-thaw stability, the same reference values as in the case of short-term stability were applied. After storage of the samples at -80 °C for 44 days and three freeze and thaw cycles over the final three days of this period, the percentage deviations of the mean values obtained by stressed samples from the reference values were -16.3 % for the lower concentration sample and -9.1 % for the higher concentration level. As these values did not deviate by more than the limit of ±20 % from the reference values, it was demonstrated that three freeze and thaw cycles do not affect accurate sample analysis.

Results of assay runs evaluating long-term stability are shown in **Table 4.8**. All mean values determined over a period of 70 days were within ±20 % of the values determined on the day of blood collection (day 0). Thus, long-term stability for aldosterone over a time period of ten weeks was proven.

Regarding processed sample stability, all mean values obtained for calibration standards as well as QC levels and unknown samples were within the respective limits over the time period of 120 minutes after the termination of the enzyme reaction. However, it became obvious based on changes in the slope of the regression curve that the assay's discrimination ability decreased over the time period. Therefore, a maximum period of 30 minutes between the termination of the enzyme reaction and measurement should not be exceeded.

Table 4.8 Long-term stability of aldosterone. Values were determined at different times after the first analysis on day 0. The percentage deviations from the reference values (day 0) were calculated (reported in brackets).

	Long-term stability		
Days after blood collection	**Mean concentration [pg/mL] (deviation from reference value [%])**		
	1st conc. level	**2nd conc. level**	**3rd conc. level**
0	85.1	391.0	468.5
7	82.3 (-3.3)	400.7 (+2.5)	507.6 (+8.3)
14	100.9 (+18.6)	377.6 (-3.4)	513.6 (+9.6)
21	75.9 (-10.8)	368.7 (-5.7)	511.9 (+9.3)
28	79.4 (-6.7)	390.9 (±0.0)	519.4 (+10.9)
35	93.6 (+10.0)	385.1 (-1.5)	499.0 (+6.5)
42	83.2 (-2.2)	389.2 (-0.5)	489.7 (+4.5)
49	78.5 (-7.8)	397.2 (+1.6)	482.7 (+3.0)
56	87.2 (+2.5)	387.9 (-0.8)	501.9 (+7.1)
70	79.7 (-6.3)	376.7 (-3.7)	520.8 (+11.2)

Conc. level: concentration level

Impact of haemolysed blood samples on accurate sample measurement

The effect was investigated in three human sources. No impact of haemolysis on accurate sample measurements was observed. Percentage deviations of haemolysed samples compared to non-haemolysed samples were -1.6 %, -6.8 %, and -13.6 %. However, one haemolysed sample of a different source showed results out of specification when assessed for ME (see section ‘Matrix effect’).

4.2.2.2 Renin assay

Calibration curve and Quality Control levels

On the basis of the regression model 4-parameter Marquardt (extrapolation 1.5, **Formula 4.1**), the best curve fitting within the assay range from 4 pg/mL to 128 pg/mL was obtained in 37 assay runs.

Fig. 4.10 shows an example of a typical standard curve. Details on the corresponding regression parameters are given in **Table 4.9**. Six independent assay runs were performed on four different days. Regarding the between-run accuracy, the relative error of the calibration standards ranged from -1.5 % to +1.0 % across the whole calibration range, while the relative error of the QC levels was between -10.4 % and +0.4 %. With regard to the within-run accuracy of the calibration standards, the relative error varied between -7.5 % and +7.5 %, while the relative error of the QC levels ranged between -18.3 % and +6.7 %. As all measurements of calibration standards and QC levels were within the required limits of ±20 % of the nominal values (±25 % at LLOQ), the suitability of the regression model of the calibration curve and of the QC levels was proven.

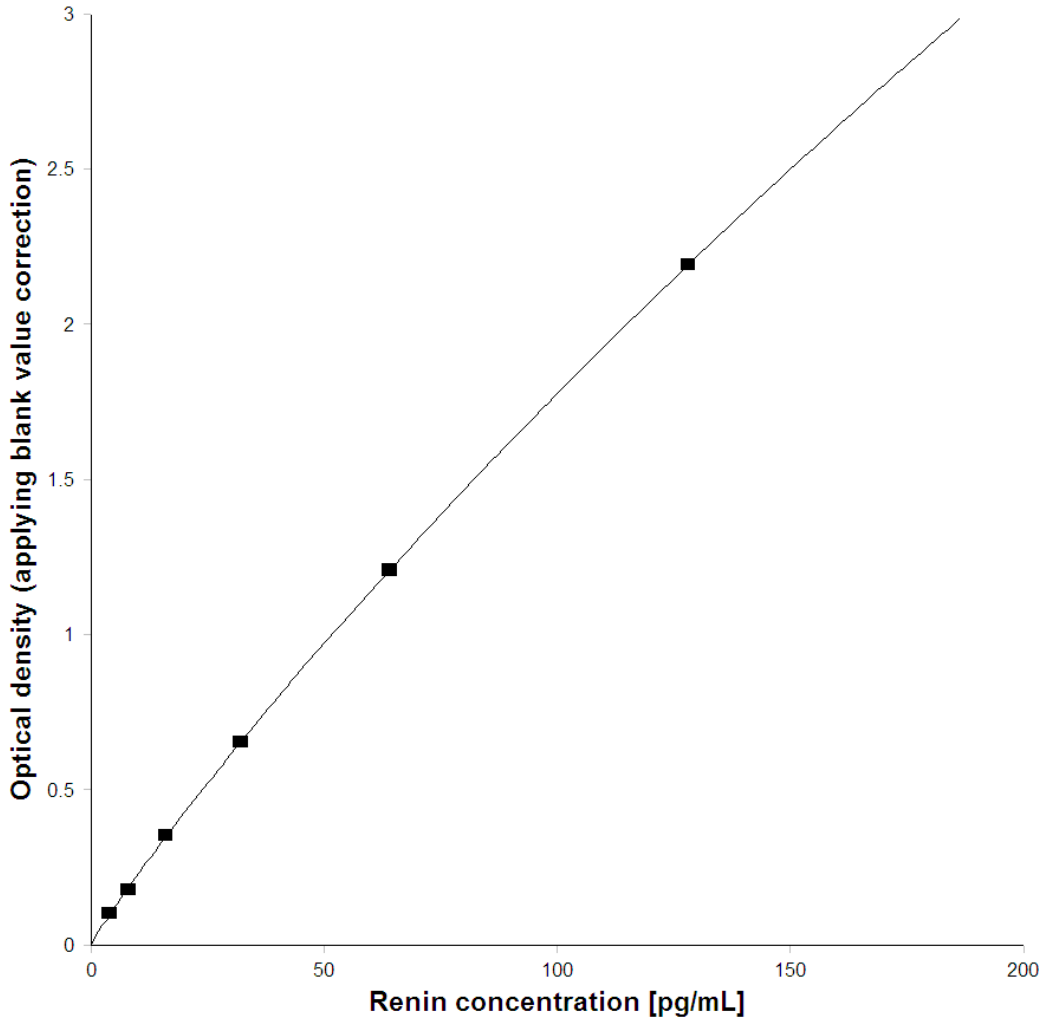

Fig. 4.10 Example of a renin standard curve. The calibration curve consists of six non-zero calibration standards and covers the assay range from 4 pg/mL to 128 pg/mL.

Table 4.9 Regression parameters of six assay runs (renin assay). For each run, all regression parameters as well as the mean squared error and the correlation coefficient are given.

	Regression model 4-parameter Marquardt (extrapolation 1.0)					
Assay run	**A**	**B**	**C**	**D**	**d**	**r**
1st	0.0025421	1.0389	263.1	6.812	0.0020764	1.00
2nd	-0.023711	0.94134	345.69	7.5849	0.013426	0.99982
3rd	0.02638	1.1271	192.7	5.8437	0.0081582	0.99994
4th	0.051491	1.4174	78.54	3.3883	0.0072318	0.99996
5th	0.038244	1.2988	98.827	3.4548	0.010187	0.99989
6th	0.00097537	0.92202	1554.7	24.1	0.0034462	0.99999

d: mean squared error; r: correlation coefficient

Accuracy and precision

The relative error of the within-run accuracy was between -15.0 % and +9.7 % across the five different concentration levels tested. For between-run accuracy, the relative error varied from -3.3 % to +3.0 %. All results were within the required guideline limit of ±20 % of the nominal values (±25 % at LLOQ). Within-run precision across all five concentration levels was between 0.7 % and 10.2 % (CV), and between-run precision (one-way ANOVA) ranged from 4.9 % to 11.3 % (CV). All results did not exceed the guideline limit of 20 % (25 % at LLOQ). The accuracy results of the five replicates per concentration level in six assay runs are shown in **Fig. 4.11**, and detailed results for within-run and between-run accuracy and precision are given in **Table 4.10** and **Table 4.11**.

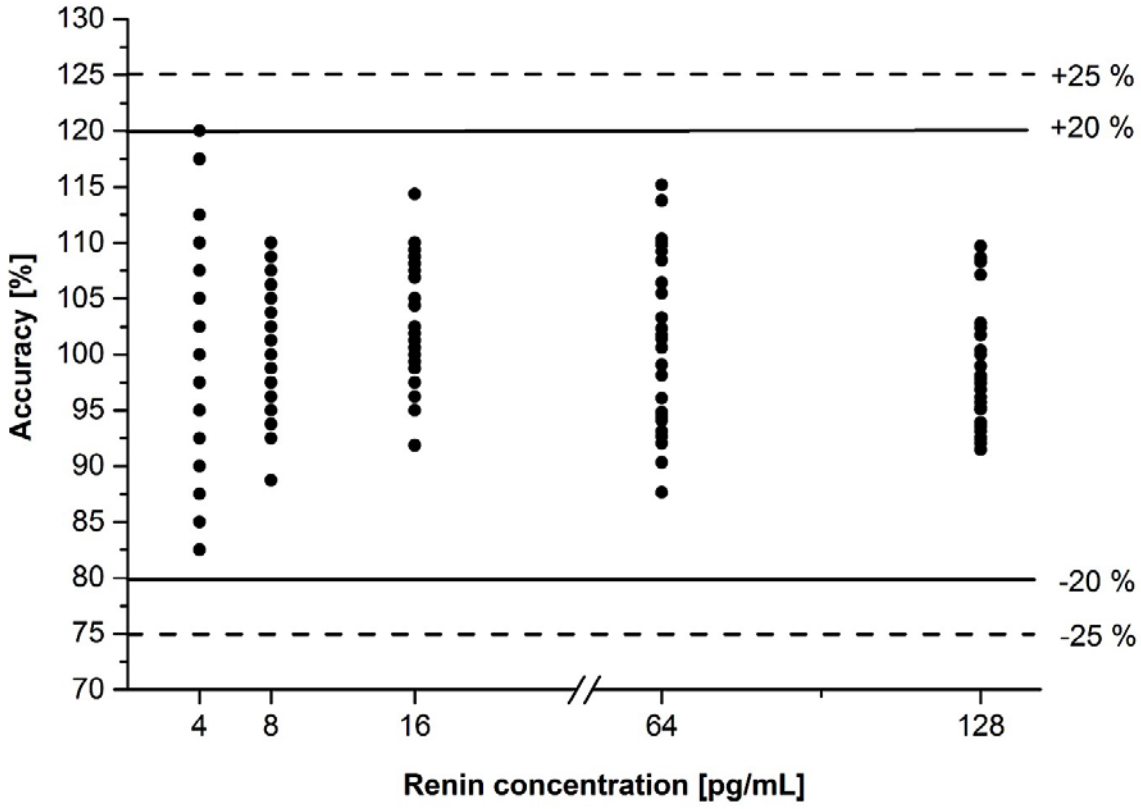

Fig. 4.11 Accuracy of the renin assay. Five concentration levels (4 pg/mL, 8 pg/mL, 16 pg/mL, 64 pg/mL, and 128 pg/mL) were determined in six independent assay runs. For each concentration level, single determinations of all five replicates in six assay runs are shown (30 determinations per concentration level). Dashed black lines indicate the ±25 % limit for the lower limit of quantification, while the continuous black lines highlight the accuracy limits for all other concentration levels.

Regarding the LLOQ (4 pg/mL), the relative error of the within-run accuracy ranged from -15.0 % to +7.5 %, while between-run accuracy across the six assay runs performed was -3.3 %. Within-run precision was between 5.6 % and 10.2 % (CV), while between-run precision (one-way ANOVA) was 11.3 % (CV). In terms of the ULOQ (128 pg/mL), the relative error of the within-run accuracy was between -6.3 % and +3.9 %, while between-run accuracy was -1.4 %. Within-run precision varied between 1.7 % and 7.1 % and between-run precision (on-way ANOVA) was 5.8 % (CV). For the different concentration levels tested, the total error of the method was between 5.3 % and 14.2 %, which did not exceed the guideline limit of 30 %.

Table 4.10 Within-run accuracy (relative error) and within-run precision (CV) of the renin assay.

Nominal concentration level [pg/mL]	Assay run	Mean concentration found ± SD [pg/mL]	Relative error (within-run) [%]	CV (within-run) [%]*
4 (LLOQ)	1st	4.2 ± 0.3	+5.0	6.4
	2nd	4.3 ± 0.4	+7.5	8.6
	3rd	3.6 ± 0.2	-10.0	6.1
	4th	3.4 ± 0.2	-15.0	6.4
	5th	3.9 ± 0.4	-2.5	10.2
	6th	3.8 ± 0.2	-5.0	5.6
8	1st	8.4 ± 0.3	+5.0	3.4
	2nd	7.9 ± 0.1	-1.3	0.7
	3rd	8.1 ± 0.3	+1.3	3.6
	4th	8.0 ± 0.5	±0.0	6.6
	5th	7.8 ± 0.5	-2.5	6.8
	6th	7.8 ± 0.5	-2.5	6.2
16	1st	17.0 ± 0.5	+6.3	3.0
	2nd	16.1 ± 0.9	+0.6	5.7
	3rd	16.8 ± 0.6	+5.0	3.7
	4th	16.9 ± 0.9	+5.6	5.6
	5th	16.3 ± 0.6	+1.9	3.7
	6th	15.8 ± 0.5	-1.3	3.4
64	1st	70.2 ± 1.9	+9.7	2.8
	2nd	70.0 ± 2.3	+9.4	3.3
	3rd	66.1 ± 4.0	+3.3	6.1
	4th	59.4 ± 1.9	-7.2	3.2
	5th	60.4 ± 3.0	-5.6	4.9
	6th	64.8 ± 1.0	+1.3	1.5
128 (ULOQ)	1st	122.2 ± 2.6	-4.5	2.1
	2nd	130.8 ± 7.6	+2.2	5.8
	3rd	126.7 ± 3.4	-1.0	2.7
	4th	133.0 ± 9.5	+3.9	7.1
	5th	124.7 ± 6.1	-2.6	4.9
	6th	119.9 ± 2.0	-6.3	1.7

CV: coefficient of variation; SD: standard deviation; *calculated with **Formula 3.1** (section 3.2.5.3)

Table 4.11 Between-run accuracy (relative error) and between-run precision (CV) of the renin assay.

Nominal concentration level [pg/mL]	Mean concentration found ± SD [pg/mL]	Relative error (between-run) [%]	CV (between-run) [%]*
4 (LLOQ)	3.9 ± 0.4	-3.3	11.3
8	8.0 ± 0.4	±0.0	5.4
16	16.5 ± 0.8	+3.0	4.9
64	65.2 ± 4.8	+1.8	7.9
128 (ULOQ)	126.2 ± 7.1	-1.4	5.8

CV: coefficient of variation; SD: standard deviation; *calculated by one-way ANOVA (**Formula 3.2**, 3.2.5.3)

Parallelism

Renin concentrations of the three spiked sources ranged from 50.4 pg/mL to 61.6 pg/mL. Back-calculated concentrations of diluted samples (dilution factors 1:2, 1:4, and 1:8) were between -11.5 % and +16.5 % of the values obtained for the corresponding spiked samples and were therefore within the required limit of ±20 %. The effect of the 1:16 dilutions was not evaluable because the obtained optical densities were below the LLOQ. The precision of the serially diluted samples ranged from 2.4 % to 5.6 % (CV) across the three sources and therefore did not exceed the limit of 30 %. The results of parallelism are shown in **Table 4.12**.

Table 4.12. Parallelism of the renin assay. The values of the spiked samples and the back-calculated values of the diluted samples are given. For the back-calculated values, the percentage deviations from the spiked samples without dilution are given.

	1st source	2nd source	3rd source
Spiked sample	53.1 pg/mL	50.4 pg/mL	61.6 pg/mL
Dilution factor	**Back-calculated concentration [pg/mL] (deviation from spiked sample [%])**		
2	54.8 (+3.2)	54.7 (+8.5)	55.8 (-9.4)
4	53.8 (+1.3)	55.4 (+9.9)	57.2 (-7.1)
8	59.7 (+12.4)	58.7 (+16.5)	54.5 (-11.5)
16	n/a	n/a	n/a
CV [%]	5.6	3.8	2.4

n/a: not applicable (value not reported because the mean optical density of the three replicates was outside the assay range); CV: coefficient of variation

Matrix effect

The ME was evaluated in ten human sources. Final renin concentrations obtained by the spiking of native samples were dependent on the renin concentration inherently present in native samples. Samples that had been spiked with the low-concentration renin spiking solution ranged from 11.6 pg/mL to 44.5 pg/mL, while samples spiked with the high-concentration spiking solution showed values ranging from 39.5 pg/mL to 75.8 pg/mL. Nine out of ten samples that had been spiked at lower concentrations showed renin concentrations deviating between -2.9 % and +14.7 % from the nominal values, which was within the required limits (±20 %) (**Table 4.13**). One sample showed a deviation of +20.5 % from the nominal value. For nine out of ten samples spiked at higher concentrations, renin concentrations ranged from -1.0 % to +17.7 % of the nominal values, which was within ±20 %. However, one sample deviated by +38.0 % from the nominal value, exceeding the upper limit (nominal value ±20 %). In summary, 90 % of the matrices tested, including one haemolysed source, were within the required limit of ±20 % of the nominal values. The results thereby comply with EMA

requirements, which specify that at least 80 % of all matrices assessed have to be within in the limit of ±20 %.

Table 4.13 Results for matrix effect (renin assay). The percentage deviations of the values obtained by the spiked samples from the nominal values are shown for ten sources. Each source was spiked at a low- and a high-concentration level.

Sex	Source	Percentage deviation [%]	
		Concentration level	
		low	high
♀	**1 (haemolysed)**	+7.6	+11.0
♀	**2**	+9.9	-1.0
♂	**3**	-2.9	+5.2
♂	**4**	+0.9	+8.5
♀	**5**	+8.4	+17.7
♂	**6**	-2.0*	+15.4*
♂	**7**	+14.7	+16.7
♂	**8**	+4.7*	+15.7
♀	**9**	+20.5	+38.0
♀	**10**	+8.6	+4.7

*Calculations of native values were based on two instead of three replicates, as the optical density of the third replicate was outside the assay range

Stability

Values determined for fresh samples on day 0 were used as reference values for stability investigations. The mean values (± standard deviations (SD)) were 9.4 pg/mL (±0.5 pg/mL) for the lower and 26.6 pg/mL (±1.1 pg/mL) for the higher concentration sample. Mean values were calculated on the basis of six replicate determinations.

Regarding short-term stability, the mean value of the six replicates determined for the low-concentration plasma sample deviated by +9.6 % from the reference value, while the mean value determined for the high-concentration plasma sample deviated by -4.1 %. In the case of both concentration levels, values did not deviate

by more than ±20 %, so that short-term stability under these conditions was shown for plasma samples.

After passing four freeze and thaw cycles, the percentage deviations of the mean values determined for stressed plasma samples from the reference values (9.4 pg/mL, 26.6 pg/mL) were +5.3 % for the plasma sample with a lower renin concentration and -8.6 % for the sample with a higher concentration. Because deviations from the reference values were less than 20 %, four freeze and thaw cycles do not impair accurate sample analysis.

Mean values for long-term stability plasma samples stored at -80 °C are shown in **Table 4.14**. All values determined over a period of 112 days were within ±20 % of the values determined on the day of blood collection (day 0), so that a stability of renin over 16 weeks was confirmed.

Table 4.14 Long-term stability of renin. Values were determined over an extended time period after the first analysis on day 0 (blood collection). Values are given as the mean of six replicate determinations per stability sample. Percentage deviations from the reference value determined on day 0 were calculated (reported in brackets).

	Long-term stability		
Days after blood collection	**Mean concentration [pg/mL] (deviation from reference value [%])**		
	1st conc. level	**2nd conc. level**	**3rd conc. level**
0	9.4	26.6	52.8
7	10.7 (+13.8)	25.3 (-4.9)	54.0 (+2.3)
14	10.1 (+7.4)	25.3 (-4.9)	55.7 (+5.5)
21	9.9 (+5.3)	26.7 (+0.4)	53.5 (+1.3)
28	9.8 (+4.3)	26.4 (-0.8)	55.1 (+4.4)
35	10.2 (+8.5)	25.8 (-3.0)	53.1 (+0.6)
42	9.5 (+1.1)	23.8 (-10.5)	54.0 (+2.3)
49	10.7 (+13.8)	25.6 (-3.8)	51.1 (-3.2)
56	10.4 (+10.6)	25.8 (-3.0)	49.2 (-6.8)
70	10.3 (+9.6)	27.6 (+3.8)	57.4 (+8.7)
84	10.7 (+13.8)	27.1 (+1.9)	51.7 (-2.1)
98	n/a	n/a	50.4 (-4.5)
112	10.5 (+11.7)	25.2 (-5.3)	n/a

Conc. level: concentration level; n/a: not applicable

Regarding processed sample stability, the mean values obtained for calibration standards, QC levels, and unknown samples were within the required specifications in all readings performed within 90 minutes after having stopped the enzyme reaction. However, because it became obvious that the assay's discrimination ability decreased over that time period, a maximum time of 30 minutes should not be exceeded between stopping the enzyme reaction and measurement.

Impact of haemolysed blood samples on accurate sample measurement

The impact of haemolysis on accurate sample measurement was assessed in three human sources. Percentage deviations of haemolysed samples from non-haemolysed samples were +1.5 %, +4.1 %, and +17.4 % respectively. Because the values of the haemolysed samples were within ±20 % of those obtained by non-haemolysed samples, the impact of haemolysis on accurate sample measurement was regarded as negligible.

4.2.3 External verification

The subsequent sections contain parts of the manuscripts 'Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes' by Schaefer et al. (35) and 'Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations' by Schaefer et al. (36).

4.2.3.1 Aldosterone assay

The Reference Institute for Bioanalytics (Bonn, Germany) certified that accuracy requirements were met in the ring test. Both unknown samples that had been received from the Reference Institute for Bioanalytics were compared to the reference method value obtained by LC-MS/MS. This comparison demonstrated that the concentrations of both samples had been accurately determined utilizing the low-volume assay. Concentration levels determined using the in-house validated assay are located in the middle of concentration levels that were reported by other participants of the ring test applying the same method. The test results of all participants of the ring test are shown in **Fig. 4.12**.

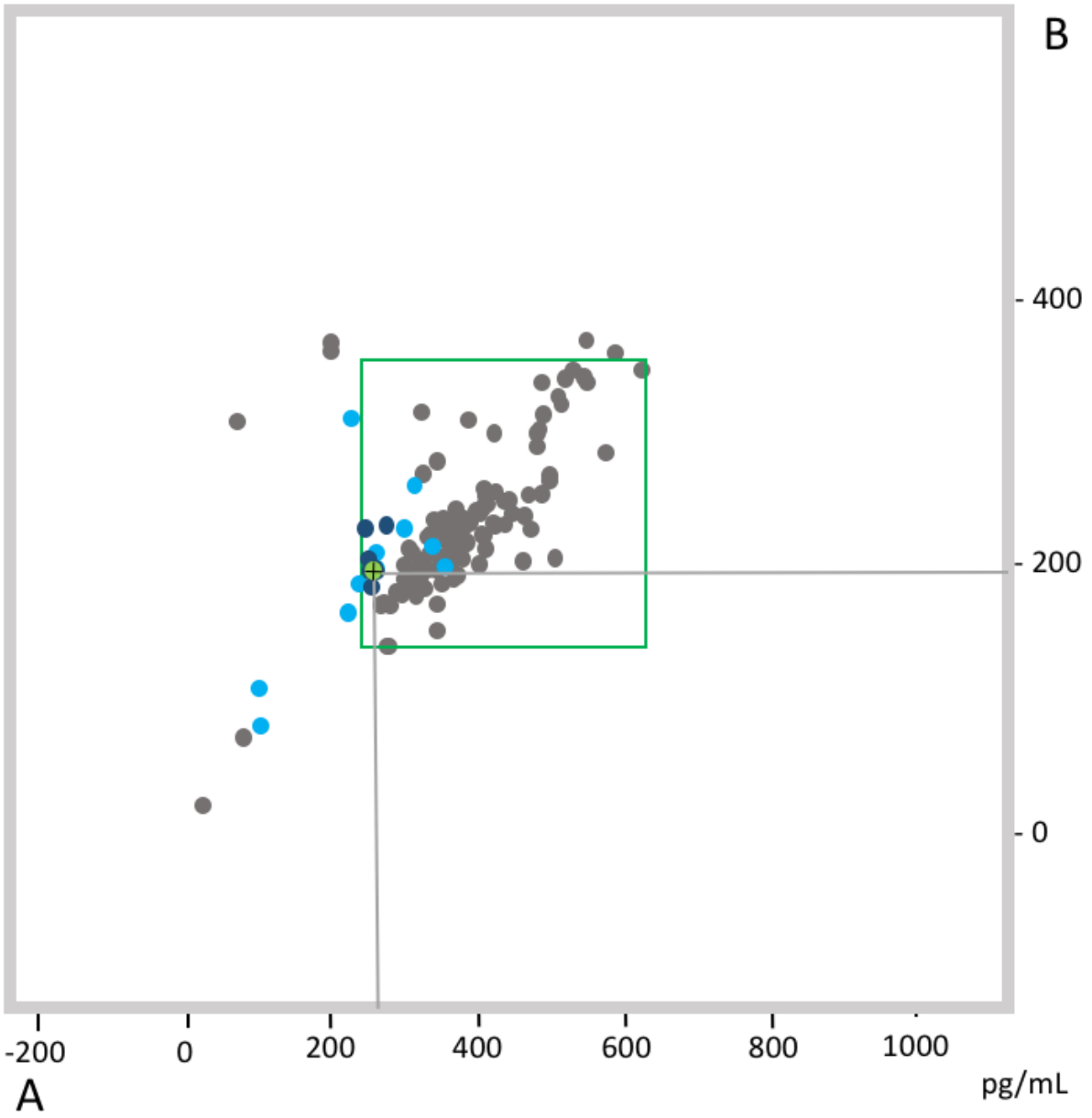

Fig. 4.12 Youden plot showing the results of the aldosterone ring test conducted by the Reference Institute for Bioanalytics (Bonn, Germany) in April 2016 (adapted from (68)). The marked circle coloured in green shows the location of the pair of values for sample A (target value 440 pg/mL) and sample B (target value 243 pg/mL) determined in the ring test. Values reported by other participants of the same collective (same method and manufacturer combination) are coloured in dark blue, and those of participants using the same method (methods with photometric measurement) are shown in light blue. Grey circles indicate other determination methods (e.g., radioimmunoassays or liquid chromatography-mass spectrometry). Most of the methods applied in the ring test were immunoassays. The green rectangle highlights the valid limits for the applied method.

4.2.3.2 Renin assay

The developed renin assay also successfully passed the ring test. The Reference Institute for Bioanalytics (Bonn, Germany) certified that the values that had been reported for both test samples with different concentration levels were within the required accuracy limits of the ring test. Therefore, the assay provides reliable data and can be applied to laboratory medicine. The detailed results of the ring test are given in **Fig. 4.13**.

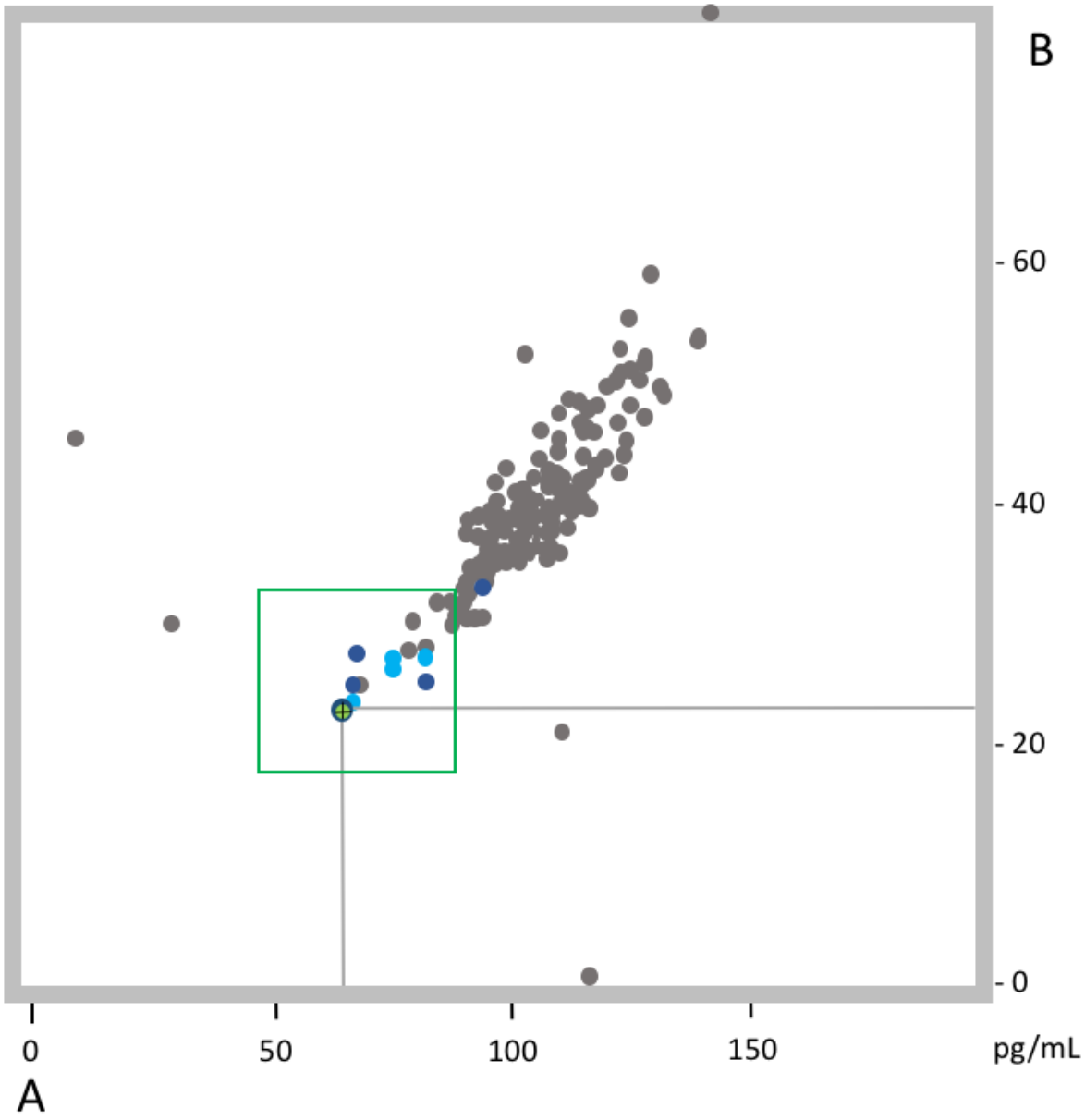

Fig. 4.13 Youden plot showing the results of the renin ring test conducted by the Reference Institute for Bioanalytics (Bonn, Germany) in April 2016 (adapted from (69)). The marked circle coloured in green shows the location of the pair of values for sample A (target value 67.1 pg/mL) and sample B (target value 25.2 pg/mL) determined in the ring test. Values reported by other participants of the same collective (same method and manufacturer combination) are coloured in dark blue, and those of participants using the same method (methods with photometric measurement) are shown in light blue. Grey circles indicate results obtained by other participants using assays with different kinds of measurement. The green rectangle highlights the valid limits for the applied method.

4.3 Proof of concept

All parts of the validation were based on either human samples or an artificial matrix made of treated human samples with preservatives. Therefore, the assays' applicability to human samples was already shown during the validation process. This result was supported by the time-concentration profiles of a healthy adult that were obtained over twelve hours for aldosterone and renin.

The subsequent sections contain parts of the manuscripts 'Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes' by Schaefer et al. (35) and 'Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations' by Schaefer et al. (36).

4.3.1 Twelve-hour profiles

The aldosterone and renin profiles of a 30-year-old healthy female obtained during a period of twelve hours are presented below.

4.3.1.1 Aldosterone assay

All aldosterone levels determined by use of the low-volume ELISA were within the reference range of a healthy adult in a supine position, varying from 17.6 pg/mL to 232 pg/mL (70). Values varied between 40.0 pg/mL and 95.5 pg/mL (**Fig. 4.14**) and showed a circadian rhythm.

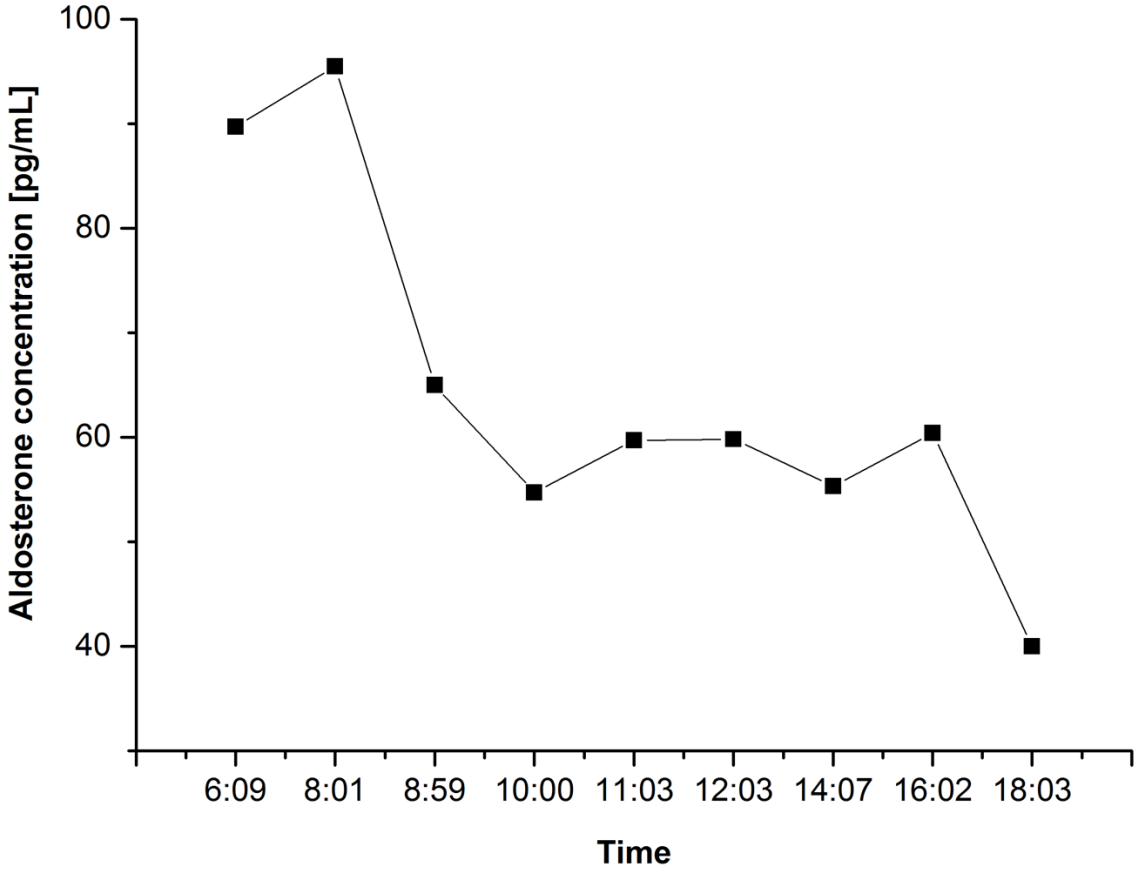

Fig. 4.14 Twelve-hour aldosterone profile. Aldosterone concentrations of a supine healthy female (30 years of age) over twelve hours. The first sampling at 6:09 a.m. was performed after night's rest, while the subject had been in a supine position for at least 30 minutes prior to all other sampling points.

4.3.1.2 Renin assay

The renin values obtained during the period of twelve hours were within the expected reference range from 1.7 pg/mL to 23.9 pg/mL for healthy adults in a supine position (70). Values ranged between 5.6 pg/mL and 8.1 pg/mL (**Fig. 4.15**).

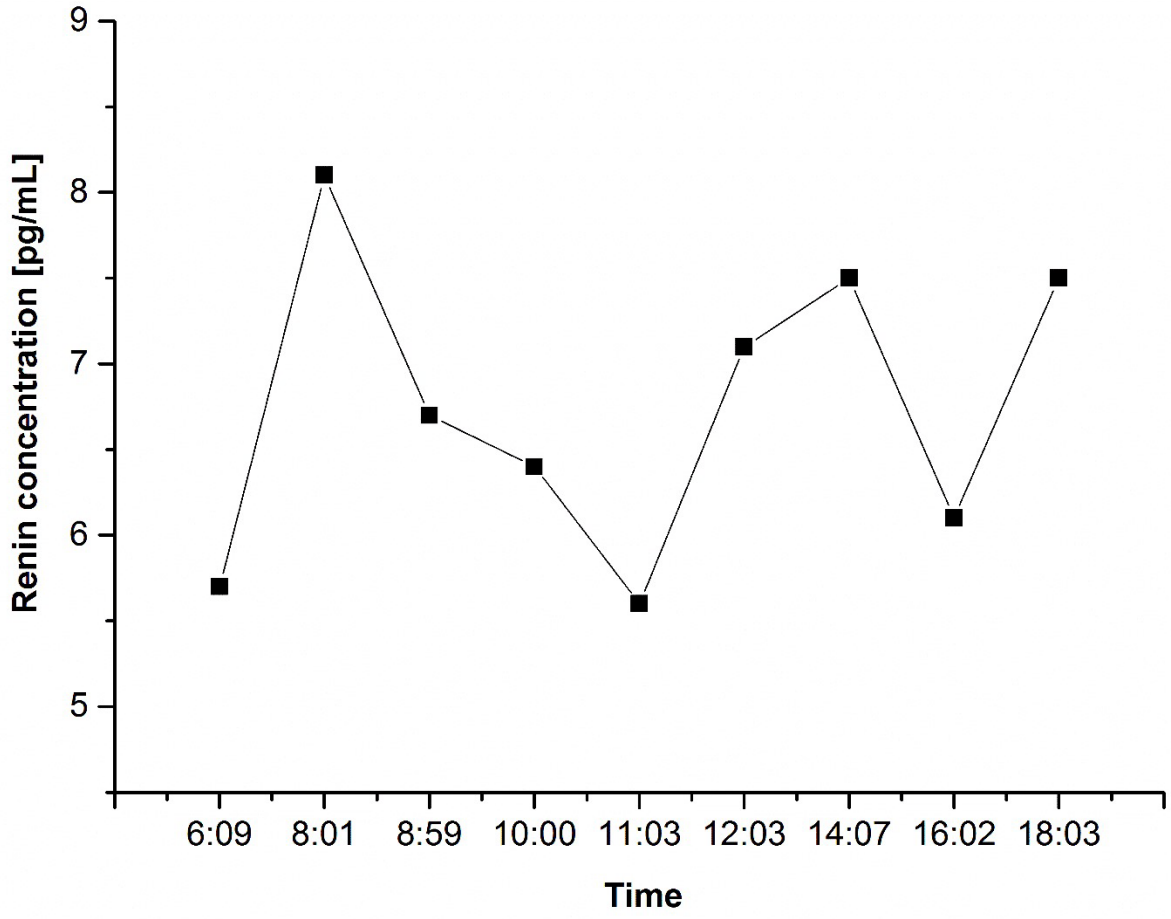

Fig. 4.15 Twelve-hour renin profile. Time-dependent renin concentrations of a healthy 30-year-old female volunteer over a period of twelve hours. The first sampling at 6:09 a.m. was performed after night's rest, while the subject had been in a supine position for at least 30 minutes prior to all other sampling points.

4.3.2 Application to paediatric population

As the clinical studies of the LENA project are still ongoing, an assessment of the assays' applicability to a clinical setting under GCLP conditions is not possible at this stage. At the end of the project in October 2018, an assessment of the assays' performance in a GCLP-conform environment will be feasible.

4.4 Development of a performance qualification test for a microplate reader

For the assessment of the microplate reader's functionality in regular test runs, a simple and rapid PQ test was developed:

The test is based on the dye Alizarin Yellow (dye content 50 %) by Sigma-Aldrich® with an absorption maximum at 362 nm (37). An aqueous stock solution showing an OD of about 3 is prepared by dissolving 0.050 g Alizarin Yellow in double-distilled water (final volume 50.0 mL) and treating in an ultrasonic bath for 10 minutes. Starting from the prepared stock solution, five serial dilutions with double-distilled water are prepared according to **Table 4.15**.

Table 4.15 Preparation of serial dilutions of the Alizarin Yellow stock solution for the performance qualification test. At each dilution step, 3.0 mL double-distilled water are added to 3.0 mL Alizarin Yellow stock solution or the corresponding aqueous dilution.

	Volume 1	Volume 2	Dilution factor
Dilution 1	3.0 mL AY **stock solution**	3.0 mL double-distilled water	2
Dilution 2	3.0 mL **dilution 1**	3.0 mL double-distilled water	4
Dilution 3	3.0 mL **dilution 2**	3.0 mL double-distilled water	8
Dilution 4	3.0 mL **dilution 3**	3.0 mL double-distilled water	16
Dilution 5	3.0 mL **dilution 4**	3.0 mL double-distilled water	32

OD: optical density; AY: Alizarin Yellow

Five replicates of all solutions (stock solution and five dilutions) are pipetted into the wells of an uncoated microplate, with 200 µL each. The OD is determined at 450 nm, and the mean values of the five replicates per solution are determined. The OD limits for considering the reader to be in good working order have to be in the range of ±10 % of the OD of values obtained during the last performed test run.

The stability of the aqueous stock solution in the dark at 2 - 8 °C was demonstrated over a period of 34 days, as the OD of the stock solution and the corresponding dilutions did not deviate by more than 10 % from the reference values, that had been obtained by the freshly prepared solutions on day 0 (**Table 4.16**).

Table 4.16 Stability of the Alizarin Yellow stock solution. The optical density of the Alizarin yellow stock solution and the serial dilutions at day 0 and at day 34 with the corresponding percentage deviations.

	Mean OD at day 0	**Mean OD at day 34**	**Percentage deviation of OD at day 34 from day 0 [%]**
AY stock solution	3.0221	2.9636	-1.9
Dilution 1	1.4453	1.5124	+4.6
Dilution 2	0.75322	0.76486	+1.5
Dilution 3	0.39368	0.40268	+2.3
Dilution 4	0.21386	0.21942	+2.6
Dilution 5	0.12228	0.12736	+4.2

OD: optical density; AY: Alizarin Yellow

5 Discussion

The preceding literature search confirmed the apparent lack of reliable pharmacodynamic data in the paediatric population. The available data was obtained by measurements that used various analytical methods of uncertain reliability. This could be one reason for the heterogeneous data sets and the partly high variation. Due to the lack of data especially with regard to the paediatric population and the high variation among the values found, no meaningful trend of concentration values could be observed between the different age groups or with regard to the impact of ACEi.

To support future research and the collection of reliable data on both children and adults, two low-volume immunoassays for the determination of aldosterone and renin concentrations have been developed in the course of this work. The reliability of the obtained data is assured by a successful validation according to current bioanalytical guidelines provided by the EMA (26) and FDA (27). Due to their compliance with regulatory recommendations, the assays are suitable for an implementation in clinical studies under conditions of GCLP.

As external quality assurance in addition to the performed validation, both assays successfully passed a ring test, which is the external quality assurance for laboratory medicine required by the German Medical Association (28).

To confirm the preceding results and to assess the applicability of both low-volume assays to human samples, profiles of aldosterone and renin in a human volunteer were compiled over a period of twelve hours. Because all data was within the expected reference values and showed a circadian rhythm, the applicability of both low-volume assays to human samples was further substantiated.

The use of paediatric samples during the validation process has not been viable. According to ethical recommendations, the trial-related blood loss is restricted in paediatric patients, which is a significant challenge not only to routine analytics but

also to validation processes. According to the EMA, the trial-related blood loss (including any losses in the manoeuvre) in neonates should not exceed 3 % of the total blood volume during a period of four weeks and should not exceed 1 % at any single time (17). Based on a blood volume estimated at 80 - 90 mL/kg body weight (17), this corresponds to approximately 1.2 mL - 1.4 mL and 0.4 mL - 0.45 mL serum and plasma respectively. Furthermore, it is questionable if the limited blood volume in this vulnerable population should be used for validation purposes. This is probably the most important issue to be considered with regard to ethics.

Both assays are currently used in the paediatric studies of the EU-funded project ‘Labeling of Enalapril from Neonates up to Adolescents’ which investigates a new child-appropriate drug formulation of the ACE inhibitor enalapril. This will facilitate an evaluation of the assays’ performance within a clinical setting under conditions of GCLP.

To support this implementation in a GCLP-compliant environment, a PQ test for the corresponding microplate reader has been developed and - due to its practicable and time-saving characteristics - has been successfully implemented in the clinical studies of the LENA project. This test is performed once a day prior to measurements, and it assures correct readings by the microplate reader at the corresponding wavelength (450 nm).

Low-volume immunoassay for the determination of aldosterone concentrations in human serum samples

The developed aldosterone ELISA described in this work provides reliable data within the concentration range from 31.3 pg/mL to 1000 pg/mL aldosterone in serum. In the following, the assay's characteristics in comparison to already available methods will be discussed.

Recently, the automated platforms LIAISON® by DiaSorin Inc. and ISYS by IDS Ltd. became available, which are cleared by the FDA and can be used for the determination of aldosterone concentrations on the basis of a CLIA. Fortunato et al. (71) reported on the comparison of these platforms to the usually applied EIA and RIA methods and revealed some advantages of these new variants, such as a better reproducibility, a shorter laboratory turnaround time, and a reduced requirement of 'hands-on labour'. In contrast to these automated platforms, the required sample volume is reduced by 84 to 87 % for the aldosterone ELISA presented in this work. While these automated CLIAs require serum/plasma volumes varying between 250 µL and 300 µL (sample volume plus dead volume) (72, 73), the immunoassay developed in this work runs with a sample volume of 40 µL serum. As stated above, the trial-related blood loss is limited in paediatric patients, according to ethical recommendations (17). In parallel, several parameters have to be measured in this young population of which reliable data is sparse and meaningful data can only be obtained by several measurements at various times. Furthermore, a certain amount of sample volume will also be required for the measurement of safety parameters. Consequently, the use of the aforementioned automated assays would exceed the limited blood volume available. Reducing the required sample volume by sample dilution does not seem to be a reasonable approach. A minimum 6-fold dilution would be necessary to achieve a sample volume of approximately 40 µL. With a LLOQ of 30 pg/mL, this would require an aldosterone concentration of at least 180 pg/mL to remain within

the assay range after sample dilution. However, this cannot be assumed for all paediatric patients. Angiotensin-converting enzyme inhibitors, for example, which are a standard medication in heart failure, influence the RAAS and might lower the aldosterone concentration. Moreover, dilution always affects the matrix and therefore should not be the standard procedure or must be verified by a proper validation. In conclusion, the automated assays do not seem to be appropriate for bioanalytics in systematic paediatric investigations.

In spite of its low sample volume, the immunoassay presented in this work still shows a measuring range (from 31.3 pg/mL to 1000 pg/mL) comparable to the automated platforms, which cover ranges from 30 pg/mL to 1000.0 pg/mL (LIAISON® by DiaSorin Inc., (72)) and from 37 pg/mL to 1320 pg/mL (ISYS by IDS Ltd., (73)) respectively.

An alternative method for the measurement of aldosterone is liquid chromatography-mass spectrometry, which is characterized by a high specificity and sensitivity. Although challenges have been described due to the low concentration, interferences with endogenous steroids, or the relatively poor ionization efficiency (74), there are chromatographic methods available for the reliable determination of aldosterone in small sample volumes. An LC/MS method for the determination of aldosterone in plasma, which was presented by Waters Corp., simplifies sample analysis by coupling to an online solid phase extraction system, covers the range from approximately 8 pg/mL to 2000 pg/mL (converted from pmol/L), and runs with 25 µL of a pre-diluted plasma sample (75). Shimadzu Corp. recently introduced an LC-MS/MS method with fully automated sample preparation that can be used for the analysis of ten steroid hormones, including aldosterone (76). For a reliable sample analysis in the concentration range from 30 pg/mL to 1140 pg/mL, 30 µL serum are required.

Although these methods seem to be a promising approach for the determination of aldosterone concentrations in small sample volumes, gas chromatography or high-performance liquid chromatography in combination with mass spectrometry are not used by most of the routine clinical services (77). Possible reasons for this are the lower costs and the higher practicability of immunoassays, as they usually perform without sample extraction steps and show less complexity.

In addition to the aforementioned advantages of immunoassays in general, commercially available ELISAs often require low sample volumes of approximately 50 - 100 µL serum or plasma (e.g., 78, 79), which is beneficial with regard to the limited blood volume available in paediatric patients. Consequently, the ELISA method was chosen as a basis for the development of a low-volume assay that is applicable to paediatric samples and routine analysis. However, the commercially available assays do not show themselves to be compliant with the current bioanalytical guidelines of EMA and the FDA and therefore do not meet regulatory recommendations for bioanalytical methods used in clinical trials.

The aldosterone immunoassay introduced by this work has been successfully validated according to EMA and FDA terms. The assay provides accurate and precise data within the calibration range from 31.3 pg/mL to 1000 pg/mL. For within-run accuracy, the relative error varied between -11.1 % and +9.0 %, while the relative error of the between-run accuracy ranged from -3.8 % to -0.8 %. These results were confirmed by a successful participation in an external ring test, which compared the reported values to the results of the reference method (LC-MS/MS). Within-run precision was between 1.2 % and 11.8 % (CV), while between-run precision (one-way ANOVA) varied from 4.9 % to 8.9 % (CV).

Parallelism was demonstrated: All diluted samples after correction for dilution led to results within ±20 % of the corresponding undiluted samples. This shows that the artificial blank matrix sufficiently mimics the concentration-response relationship of aldosterone in the sample matrix. This finding might be of clinical

relevance, given that a pre-dilution step with a proper matrix reduces the original sample volume required and might allow for a measurement in cases where otherwise no determination could be performed at all.

Dilutional linearity was tested for concentrations up to 3404.8 pg/mL and showed a relative error varying between -7.4 % and +10.5 %. As these results were within the accuracy limits of ±20 %, a dilution of samples exceeding the ULOQ is justified. Considering the possible occurrence of aldosterone breakthrough (an increase of aldosterone levels following an initial decrease in up to 40 % of symptomatic heart failure patients on RAAS blockers (24)) this might be of practical relevance.

The results of tests for a possible ME were within the required specification, with one haemolysed source showing a deviation of +32.7 % from the expected value, exceeding the limit of ±20 %. In tests performed to investigate the impact of haemolysed samples on accurate sample analysis, all haemolysed samples were within the required specification (±20 % of the expected value). Yet, the observed deviation of +32.7 % obtained by a haemolysed sample in tests for ME should be taken into account when haemolysed samples are present. Burns et Yoshikawa (2002) stated that “Hemolysis may result in spuriously elevated serum. . . bilirubin. . .” (82) and may thereby exceed the concentration limit for bilirubin concentrations in samples provided by the vendor (0.125 mg/mL: see **Appendix 9.4**). The results for ME substantiated the previous findings for accuracy, as native samples spiked with an external aldosterone source were in accordance with theoretically calculated values.

All stability tests performed during the validation process were of high relevance in determining the terms of sample handling. The short-term stability of serum samples has been confirmed for the duration of one day at room temperature, whereby it must be considered that all test results refer to the stability of serum samples: the corresponding stability of whole blood samples remains to be

investigated. The stability of aldosterone was not affected by three freeze and thaw cycles. A long-term storage at -80 °C was proven over a period of ten weeks. Both findings are of interest in cases of required sample reanalysis. All tests on sample stability performed during the validation process were adjusted to conditions of clinical routine analysis.

The specificity of the assay had already been evaluated by the vendor and was not investigated further in the present work. In renal impairment, elevated concentration levels of polar aldosterone metabolites, such as aldosterone-18-glucuronide and tetrahydroaldosterone have to be considered. This might lead to an overestimation of aldosterone and require prior solvent extraction steps (81).

Low-volume immunoassay for the determination of renin concentrations in human plasma samples

The renin immunoassay presented in this work requires a sample volume of 40 µL plasma and covers a concentration range from 4 pg/mL to 128 pg/mL renin in plasma.

When compared to the automated platform LIAISON® by DiaSorin Inc. mentioned above, the required sample volume for the determination of direct renin concentrations was reduced by 89 %. While the developed immunoassay requires 40 µL plasma, the automated platform performs with a minimum plasma volume of 350 µL (sample volume plus dead volume) (80). In comparison to the automated platform ISYS by IDS Ltd., which runs with 290 µL plasma (83), the required sample volume is reduced by 86 %. As mentioned above, the sample volume available in paediatric patients is exceeded by the automated assays when one considers the measurement of several parameters at various times in addition to the determination of safety parameter levels.

Available ELISAs are able to measure samples with lower sample volumes but do not prove to be in line with current regulatory recommendations (e.g., 84, 85).

The presented immunoassay for the determination of direct renin concentrations has been successfully validated according to the recommendations of the EMA and the FDA. The low-volume assay facilitates the accurate and precise measurement of direct renin in human plasma samples. The relative error of the within-run accuracy varied from -15.0 % to +9.7 %, while between-run accuracy ranged from -3.3 % to +3.0 % (relative error). All results were within the required guideline limits of ±20 % of the nominal values. These results were confirmed by an external ring test. Within-run precision showed values between 0.7 % and 10.2 % (CV), while between-run precision (one-way ANOVA) ranged from 4.9 % to 11.3 % (CV). The guideline requirements were successfully met.

For parallelism, all back-calculated concentrations for diluted samples were within ±20 % of the expected values, which confirms that the diluted samples lie in parallel with the calibration curve and that the substitute matrix is sufficient.

The vendor observed no high-dose hook effect up to 8200 pg/mL (**Appendix 9.7**). However, dilutional linearity could not be validated in this work because renin stock solutions with appropriate concentrations were missing. A high-dose hook effect may lead to a decreased signal in the case of very high analyte concentrations. This phenomenon, which is confined to one-step sandwich immunoassays, can be attributed to the fact that high analyte concentrations saturate the capture and detection antibodies. This impedes the formation of complexes (capture antibody/analyte/detection antibody) that lead to the detected signal (67).

Results for a possible ME of the assay were within the required specification of the EMA, including results for haemolysed samples. This finding was supported by further tests, as no impact of haemolysis on accurate sample analysis was found. The results obtained for ME additionally confirmed the previous accuracy results: Values obtained by spiked native samples were within ±20 % of the expected values.

Short-term stability has been proven over one day at room temperature. It must be noted that all stability tests were carried out after the supernatant had been separated from solid blood components: results therefore refer to plasma samples. Four freeze and thaw cycles did not affect the sample stability, and long-term stability at -80 °C was demonstrated for a period of 4 months. All aforementioned stability results are consistent with conditions under routine analysis.

Due to the low sample volume required, both assays are especially relevant to paediatric investigations. Additionally, these tests can be beneficial to bioanalytics in adults in cases where only low sample volumes are available. Furthermore, a low sample volume allows for the measurement of more parameters out of the

same sample volume, or for a more frequent sampling, which increases the validity of obtained data.

6 Conclusion

The developed low-volume immunoassays contribute to the collection of reliable pharmacodynamic data on paediatrics. This is the necessary prerequisite for an understanding of the characteristics of the pathophysiology in this young population and subsequently a child-appropriate pharmacotherapy. In this way, the long-term objective of the availability of medicines, that are optimised for paediatric needs on the basis of actual clinical data, can be achieved.

Still, many clinical investigations in paediatric patients are required in order to acquire a sufficient data background for scientific and therapeutic evaluations. Corresponding bioanalytical methods have to meet the special requirements of this young population and of clinical settings to facilitate the collection of reliable data. This approach presents several challenges but is inevitable for better medicines in children.

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85. Cusabio Biotech Co., Ltd, Human Renin (REN) ELISA Kit (Cat. No. CSB-E08701h), http://www.cusabio.com/uploadfile/Ins/2016-08-02/CSB-E08701h.pdf (last access on January 5th, 2017).

9 Appendix

6.2 Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the frame holder.
2. Dispense **50 µL** of each ***Standard, Control*** and **samples** with new disposable tips into appropriate wells.
3. Dispense **150 µL *Enzyme Conjugate*** into each well.
   Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
4. Incubate for **60 minutes** at room temperature.
5. Briskly shake out the contents of the wells. Rinse the wells
   5 x with **400 µL** diluted *Wash Solution* per well (if an plate washer is used) - or.
   5 x with **300 µL**/well for manual washing.
   Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6. Add **200 µL** of ***Substrate Solution*** to each well.
7. Incubate for **30 minutes** at room temperature.
8. Stop the enzymatic reaction by adding **100 µL** of ***Stop Solution*** to each well.
9. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader.
   It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

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Appendix 9.1 Recommendations on the test procedure for the unmodified assay provided by the vendor (04/2014) (aldosterone assay). Excerpt from the corresponding manual (1).

6.2 Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the frame holder.
2. Dispense **50 µL** of each ***Standard, Control*** and **samples** with new disposable tips into appropriate wells.
   For urine samples dispense 50 µL of the pre-treated and diluted urine samples (see chapter 5.2.2 Protocol for Urine Sample Pre-treatment, step 7).
3. Incubate for 30 minutes at room temperature.
4. Dispense **150 µL *Enzyme Conjugate*** into each well.
   Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
5. Incubate for **60 minutes** at room temperature.
6. Briskly shake out the contents of the wells. Rinse the wells
   5 x with **400 µL** diluted *Wash Solution* per well (if a plate washer is used) - or.
   5 x with **300 µL**/well for manual washing.
   Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
7. Add **200 µL** of ***Substrate Solution*** to each well.
8. Incubate for **30 minutes** at room temperature.
9. Stop the enzymatic reaction by adding **100 µL** of ***Stop Solution*** to each well.
10. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader.
    It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

Appendix 9.2 Updated recommendations on the test procedure for the unmodified assay provided by the vendor (02/2017) (aldosterone assay). Excerpt from the updated manual.

9 PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range

The range of the assay is between 5.7 pg/mL – 1000 pg/mL.

9.2 Specificity of Antibodies (Cross Reactivity)

The following substances were tested for cross reactivity of the assay:

3 β, 5 α Tetrahydroaldosterone:	17.2 %
3 β, 5 β Tetrahydroaldosterone:	0.12 %
Prednisolone:	0.017 %
Cortisol:	< 0.003 %
11-Deoxycortisol:	< 0.003 %
Progesterone:	< 0.003 %
Testosterone:	< 0.002 %
Androstenedione:	< 0.002 %

9.3 Sensitivity

The analytical sensitivity of the DRG ELISA was calculated by subtracting 2 standard deviations from the mean of 20 replicate analyses of the *Standard 0* (S0) and was found to be < 5.7 pg/mL.

9.4 Reproducibility

9.4.1 Intra Assay

The within assay variability is shown below:

Sample	n	Mean (pg/mL)	CV (%)
Serum 1	20	85.1	9.7
Serum 2	20	210.3	7.4
Serum 3	20	532.2	3.9
Urine 1	20	191.8	5.0
Urine 2	20	391.3	5.6
Urine 3	20	936.8	3.8

9.4.2 Inter Assay

The between assay variability is shown below:

Sample	n	Mean (pg/mL)	CV (%)
1	40	101.0	9.9
2	40	315.1	8.6
3	40	656.8	9.4
Urine 1	32	386.7	11.5
Urine 2	32	444.0	11.1
Urine 3	32	876.7	10.4

9.5 Recovery

Samples have been spiked by adding aldosterone solutions with known concentrations in a 1:1 ratio.
The % recovery has been calculated by multiplication of the ratio of the measurements and the expected values with 100 (expected value = (endogenous aldosterone + added aldosterone) / 2; because of a 1:2 dilution of serum with spike material).

		Serum 1	Serum 2	Serum 3
Concentration [pg/mL]		82.7	96.1	167.9
Average Recovery		112.5	111.0	106.8
Range of Recovery [%]	from	108.2	108.9	92.4
	to	114.6	114.5	114.8

Appendix 9.3 Performance characteristics of the unmodified commercially available aldosterone assay provided by the vendor (Part 1). Excerpt from the corresponding manual (1).

9.6 Linearity

		Serum 1	Serum 2	Serum 3	Urine 1	Urine 2	Urine 3
Concentration [pg/mL]		600.5	546.2	672.0	559.0	645.0	464.0
Average Recovery		98.4	95.5	96.4	106.8	98.2	98.0
Range of Recovery [%]	from	95.5	87.8	86.0	104.5	87.8	86.2
	to	103.0	103.6	102.5	111.6	107.9	105.6

10 LIMITATIONS OF USE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

Haemoglobin (up to 4 mg/mL), Bilirubin (up to 0.125 mg/mL) and Triglyceride (up to 30 mg/mL) have no influence on the assay results.

10.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of aldosterone in a sample.

10.3 High-Dose-Hook Effect

No hook effect was observed in this test.

11 LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact DRG.

11.2 Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient. Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived.
The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2. are also invalid. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

Appendix 9.4 Performance characteristics of the unmodified commercially available aldosterone assay provided by the vendor (Part 2). Excerpt from the corresponding manual (1).

6.2 Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the frame holder.
2. Dispense **150 µL** of ***Assay Buffer*** in all wells.
3. Dispense **50 µL** of each ***Standard, Control*** and **samples** with new disposable tips into appropriate wells.
4. Incubate for **90 minutes** at room temperature on a plate shaker with 300 - 700 rpm.
5. Briskly shake out the contents of the wells.
   Rinse the wells **4 times** with **300 µL** diluted ***Wash Solution***. Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6. Dispense **100 µL** ***Enzyme Conjugate*** in all wells.
7. Incubate for **90 minutes** at room temperature on a plate shaker with 300 - 700 rpm.
8. Briskly shake out the contents of the wells.
   Rinse the wells **4 times** with **300 µL** diluted ***Wash Solution***. Strike the wells sharply on absorbent paper to remove residual droplets.
9. Add **100 µL** of ***Substrate Solution*** to each well.
10. Incubate for **15 minutes** at room temperature.
11. Stop the enzymatic reaction by adding **100 µL** of ***Stop Solution*** to each well.
12. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader.
    It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

Appendix 9.5 Recommendations on the test procedure for the unmodified assay provided by the vendor (10/2012) (renin assay). Excerpt from the corresponding manual (33).

9 PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range

The range of the assay is between 0.81 – 128 pg/mL.

9.2 Specificity of Antibodies (Cross-Reactivity)

The following substances were tested for cross-reactivity of the assay:
Mean cross reactivity with Prorenin was 0.71% (mean value when prorenin was spiked in a concentration range from 256 – 4096 pg/mL). However, the observed cross reactivity may only represent a contamination of the recombinant prorenin preparation with active renin due to auto-activation.

Cross-reactivity was not detectable against human serum albumin, human gamma globulin, human hepcidine, and pepsin.

9.3 Sensitivity

The analytical sensitivity of the DRG ELISA was calculated by adding 2 standard deviations to the mean of 20 replicate analyses of the Zero Standard (S0) and was found to be 0.81 pg/mL.

9.4 Reproducibility

9.4.1 Intra Assay

The within assay variability is shown below:

Sample	1	2	3
Mean (pg/mL)	9.12	26.98	43.99
CV (%)	8.73	3.88	4.24
n =	20	20	20

9.4.2 Inter Assay

The between assay variability is shown below:

Sample	1	2	3
Mean (pg/mL)	19.28	36.20	66.72
CV (%)	8.88	6.27	5.19
n =	12	12	12

9.5 Recovery

Samples have been spiked by adding Renin solutions with known concentrations in a 1:1 ratio.
The % recovery has been calculated by multiplication of the ratio of the measurements and the expected values with 100 (expected value = (endogenous Renin + added Renin) / 2; because of a 1:2 dilution of plasma with spike material).

		Sample 1	Sample 2	Sample 3
Concentration [pg/mL]		16.71	40.21	15.97
Average Recovery		92.92	95.09	96.00
Range of Recovery [%]	from	85.99	87.93	86.83
	to	105.47	101.37	105.25

Appendix 9.6 Performance characteristics of the unmodified commercially available renin assay provided by the vendor (Part 1). Excerpt from the corresponding manual (33).

9.6 Linearity

		Sample 1	Sample 2	Sample 3
Concentration [pg/mL]		45.16	53.20	126.0
Average Recovery		101.7	102.8	98.5
Range of Recovery [%]	from	96.7	95.6	94.9
	to	108.6	114.6	100.8

10 LIMITATIONS OF USE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.

Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

Haemoglobin (up to 1 mg/mL), Bilirubin (up to 0.5 mg/mL) and Triglyceride (up to 30 mg/mL) have no influence on the assay results.

10.2 Drug Interferences

The renin inhibitor aliskiren will increase active renin immunoreactivity in a dose-dependant manner, from 0.54 µM (+ 121%) up to 540 µM (+151%).
In addition, the level of active renin in plasma may be affected by antihypertensive medication (e.g. diuretics, ACE inhibitors, beta adrenergic blocking agents, or vasodilators)

10.3 High-Dose-Hook Effect

No hook effect was observed in this test up to 8,200 pg/mL of Renin.

Appendix 9.7 Performance characteristics of the unmodified commercially available renin assay provided by the vendor (Part 2). Excerpt from the corresponding manual (33).

Parts of this thesis have already been published in international journals or were previously presented at conferences:

Publications

1) Laven A, Schaefer J, Laeer S. PHARMAGRIPS: Strukturierte PHARMAzeutische Beratung in der Selbstmedikation des GRIPpalen InfekteS.: Eine randomisierte kontrollierte Studie (RCT). MMP 2014; 37.(6):209–20.

2) Schaefer J, Burckhardt BB, Tins J, Bartel A, Laeer S. Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes. (revised manuscript submitted to Practical Laboratory Medicine, June 2017).

3) Schaefer J, Burckhardt BB, Tins J, Bartel A, Laeer S. Validated low-volume immunoassay for the reliable determination of direct renin especially valuable for pediatric investigations. (revised manuscript submitted to Journal of Immunoassay and Immunochemistry, June 2017).

Regarding publications 2) and 3) listed above, the first author Julia Schäfer was responsible for and substantially contributed to the conception and design as well as the analysis and interpretation of data, the drafting and the critical revision of the articles, and the final approval of the versions to be published.

Poster presentations

1) 15th Congress of the European Society for Developmental, Perinatal and Paediatric Pharmacology (ESDPPP) in Belgrad, Serbien
   (23rd - 26th June 2015)
   'Evaluation of the applicability of a child-appropriate high throughput HPLC-MS/MS method for the determination of enalapril and enalaprilat in small sample volumes of serum and urine within a GCLP-compliant environment' by Julia Schaefer, Bjoern B. Burckhardt, Jutta Tins, and Stephanie Laeer (Arch Dis Child 2016 101:e1 doi:10.1136/archdischild-2015-310148.71)

2) 'Deutsche Pharmazeutische Gesellschaft' (DPhG), annual meeting 2015 in Duesseldorf (23rd - 25th September 2015)
   'Child-appropriate high throughput HPLC-MS/MS for enalapril and enalaprilat in small sample volumes of serum and urine within a GCLP-compliant environment' by Julia Schaefer, Bjoern B. Burckhardt, Jutta Tins, and Stephanie Laeer

3) 'Deutsche Pharmazeutische Gesellschaft' (DPhG), annual meeting 2016 in Munich (5th - 7th October 2016)
   'Development and validation of enzyme-linked immunosorbent assays for the determination of aldosterone and renin concentrations in small sample volumes - a paediatric-tailored approach' by Julia Schaefer, Bjoern B. Burckhardt, Jutta Tins, Anke Bartel, and Stephanie Laeer