Bioanalytical method performance verification concept for cardiovascular research in pediatrics: From development to application in clinical trials

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I. Erklärung zur Dissertation

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

Bioanalytical method performance verification concept for cardiovascular research in pediatrics: From development to application in clinical trials.

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 Mohsin Ali

II. Acknowledgement

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III. Summary

Angiotensin-converting enzyme inhibitors (ACEIs) have been engaged for treatment of various cardiovascular diseases including heart failure, hypertension and congenital heart disease. The therapeutic effectiveness of these cardiovascular agents is closely related to medication adherence and has become a concern for the clinicians. On the other hand, the increased availability of these medicinal agents outstretched the likelihood of intoxication events either intentionally or unintentionally. Additionally, the monitoring of long term method performance of qualified method is necessary for quality of the bioanalytical data.

In the first section of the thesis, a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) screening method for commonly used ACEIs was developed and fit-for-purpose method validation was performed to ensure method applicability for its intended purpose. The developed screening method can be implemented for fast and reliable evaluation of intentional/unintentional intoxication as well as medication adherence of commonly used ACEIs. Owing to the utilisation of residual blood volume and lower detection limit, the method is also applicable even in small children where the sampling volume is an ethical restriction. The customised validation underlined the suitability of the developed bioanalytical method for its intended purpose.

In contrary to adults where sufficient data about the use of ACEIs is existing, there is a deficiency of evidence-based pharmacotherapy requiring clinical trials in pediatrics. For reliable data generation within the clinical trials, it is not only the method development and validation but also monitoring the long term method performance is crucial to ensure data reliability. Therefore, multi-parameter quality control system to monitor the continuous performance of the bioanalytical method was proposed to assess the reliability and quality of bioanalytical data in academia environment. This quality control system lies within the study sample analysis and evaluates the post validation analysis which in turn ensures the data quality of generated clinical data sets.

The results showed that such a quality ensuring approach is worthwhile and achievable. It ensured the optimal monitoring and evaluation of bioanalytical data.

Invalid data, which otherwise would not be detected if only the validity of the particular batch would have been monitored, was identified and contributed to the increase in quality. So, based upon regulatory recommendations internationally and current scientific discussions, such quality control system is meaningful to monitor the long term performance of bioanalytical method measuring drug concentration of study samples ensuring the data quality to achieve more patient safety.

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V. List of abbreviations

AAPS	American Association of Pharmaceutical Scientists
ACE	Angiotensin-converting enzyme
ACEIs	Angiotensin-converting enzyme inhibitors
ACN	Acetonitrile
ADH	Anti-diuretic hormone
AHA	American Heart Association
Ang-I	Angiotensin I
Ang-II	Angiotensin II
ANOVA	Analysis of variance
ARBs	Angiotensin receptor blockers
AT1-R	Angiotensin type 1 receptors
AT2-R	Angiotensin type 2 receptors
a.u	arbitrary units
BBs	Beta blockers
BMV	Bioanalytical method validation
CAD	Coronary artery disease
CC	Calibration curve
CE	Collision energy
CEP	Cell entrance potential
CHD	Congenital heart disease
CP	Chemically pure
CXP	Collision cell exit potential
DP	Declustering potential
EMA	European Medicines Agency
EP	Entrance potential
ESC	European Society of Cardiology
ESI	Electrospray ionisation
FDA	US Food and Drug Administration
FIA	Flow injection analysis

FP	Focusing potential
GCLP	Good Clinical Laboratory Practice
HF	Heart failure
HFrEF	Heart failure with reduced ejection fraction
HPLC	High-performance liquid chromatography
HQC	High quality control
IS	Internal standard
ISR	Incurred sample reanalysis
JCs	Juxtaglomerular cells
JG	Juxtaglomerular
LC-MS/MS	Liquid chromatography tandem mass spectrometery
LVEF	Left ventricle ejection fraction
LENA	Labelling of Enalapril from Neonates up to Adolescents
LIMS	Laboratory information management system
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Lower quality control
LSL	Lower specification limit
$[M+H]^+$	Protonated molecular mass ion
$[M-H]^-$	Deprotonated molecular mass ion
MAX	Mix mode anion exchange
MRAs	Mineralocorticoid receptor antagonists
MQC	Middle quality control
MRM	Multiple reaction monitoring
m/z	Mass to charge ratio
NCD	Non communicable diseases
NYHA	New York Heart Association
p.a	per analysis
PD	Pharmacodynamic

pH	Negative logarithm of hydrogen ion concentration
PK	Pharmacokinetic
pKa	Negative logarithm of acid dissociation constant
Q1	First quadruple
Q2	Second quadruple
Q3	Third quadruple
QC	Quality control
RAAS	Renin-angiotensin-aldosterone system
RSD	Relative standard deviation
SOP	Standard operating procedure
SPE	Solid phase extraction
USL	Upper specification limit
USP	United States Pharmacopoeia
ULOQ	Upper limit of quality control
WHO	World Health Organisation

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Chapter 1

1. General introduction

1.1 Renin-angiotensin-aldosterone system

The circulating renin-angiotensin-aldosterone system (RAAS) in humans plays a vibrant role in the regulation of the blood pressure, fluid volume and sodium potassium balance, thus maintains the haemodynamic stability (1, 2). Also, in many tissues including kidney, heart, nervous system and adrenal glands, the local renin-angiotensin system is expressed where it mediates many cellular functions including cell proliferation, growth and metabolism (3).

As the name expresses, the critical effector components involved in this system are renin, angiotensin and aldosterone. The presence of the renin in rabbit renal cortex was first identified by Tigerstedt and Bergmann in 1898. They demonstrated that a thermo-labile substance termed "renin" leading to an increase in arterial pressure. Later it was found that renin exerts its pressor activity indirectly by acting on plasma substrate known as "angiotensinogen" and releasing a direct-acting pressor peptide initially known as "angiotonin" and later the "angiotensin". After that Skeggs and colleagues discovered that this peptide exists in two forms "angiotensin-I" and "angiotensin-II" in early 1950 during their attempt aimed at purification of the angiotensin (4).

The biosynthesis of the renin by the juxtaglomerular cells (JCs) that line afferent arterioles of the renal glomerulus is the initial step in the activation of the RAAS cascade**.** The precursor of the renin named pro-renin is 406 amino acid long protein, which is cleaved into active renin by the proteolytic (pro-protein convertase 1) removal of the 46 amino acid pro-segment peptide from the N-terminus of the pro-renin in kidney and 20 amino acid pre-segment. Renin is a 340 amino acid protein in active form (4, 5). The mature renin is stored in the granules of JC and sympathetic nervous system activity (beta-1-adrenoceptor activation), low arterial blood pressure and low sodium chloride lead to its release (6). Renin causes the hydrolysis of liver secreted α-2-globulin (angiotensinogen) by acting on a bond

between leucine and valine to form biologically inert decapeptide angiotensin I (Ang-I).

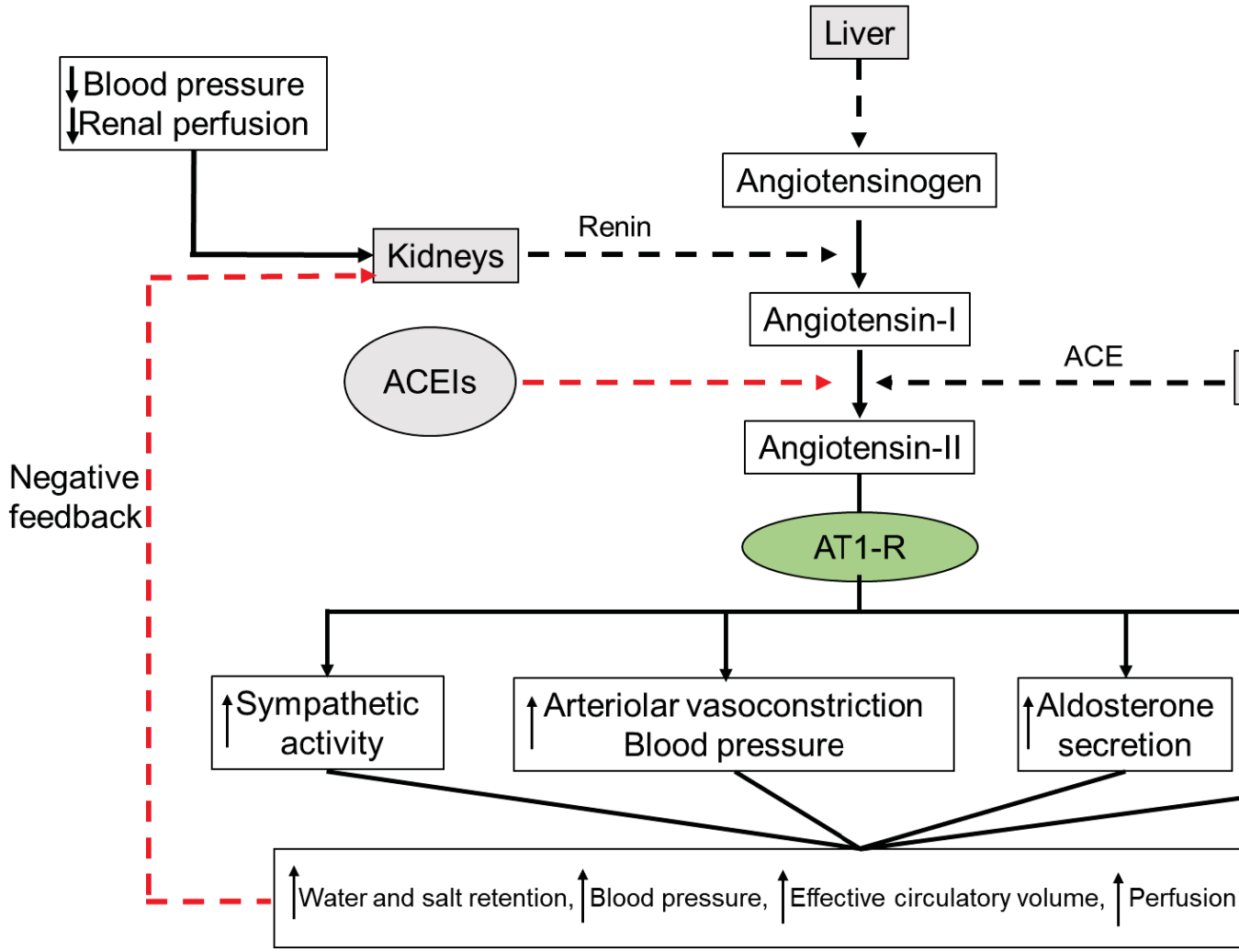
Angiotensin-converting enzyme (ACE) converts the inactive Ang-I to the main effector of the RAAS, angiotensin II (Ang-II), a biologically active potent vasoconstrictor. Also, the Ang-I or Ang-II can be cleaved by neutral endopeptidase into angiotensin (1-7) another active peptide with opposite effect to Ang-II or angiotensin (1-7) can also be produced from Ang-II by the action of another carboxypeptidase angiotensin converting enzyme 2, structurally homologous to ACE (7). Angiotensin (1-7) acts via angiotensin type 1 receptor (AT1-R) exerting vasodilator effect acting as a natural inhibitor of the ACE (4). Until now four (4) different types of angiotensin receptor subtypes have been found.

Most of the known responses triggered by the Ang-II are through the angiotensin type 1 receptors (AT1-R). These responses include the effect on the cardiovascular system (increased vasoconstriction leading to increased blood pressure), adrenal cortex (stimulation of aldosterone synthesis), kidney (tubular sodium reabsorption by stimulating the Na+/H+ channels located in apical membrane cells) (8). The cell proliferation, inflammatory effect and oxidative stress effects of Ang-II are also mediated by the AT1-R (9). The angiotensin type 2 receptors (AT2-R) are generally abundant during fetal life and considered to be reduced in postnatal life. However, these receptors mediate the antiproliferative, vasodilator and apoptotic effect in vascular smooth muscle and prevent remodelling of the heart. The function of the angiotensin type 3 receptors is still unknown and angiotensin type 4 receptors are thought to mediate the release of the plasminogen activator inhibitor 1 by the action of the Ang-II (4).

Another effector molecule of the RAAS is aldosterone. The aldosterone is synthesised in zona glomerulosa of the adrenal cortex by the Ang-II via AT1-R. Aldosterone modulates the haemostasis by promoting sodium reabsorption, water retention and potassium and magnesium loss by acting on the distal tubule and collecting ducts (1, 4).

Initially the activation of RAAS acts as compensatory mechanism for renal hypoperfusion and sympathetic activation, however, the continuous activation has

consequences towards heart failure (10). **Figure 1-1** represents the RAAS cascade and site of action of the ACEIs.



***Abbreviations**: ACEIs = Angiotensin-converting enzyme inhibitors, JG = Juxtaglomerular, ADH = Anti-diuretic hormone, ACE = R = Angiotensin type 1 receptors*

Figure 1-1 *Renin-angiotensin-aldosterone system and site of action of the angiotensin-converting enz*

1.2 Heart failure

A clinical syndrome characterised by the low cardiac output was first described in the 1950s as heart failure (HF), and later it included the circulatory, neurohormonal and molecular abnormalities. In 2016 the European Society of Cardiology (ESC) defined the HF as follows “HF is a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral oedema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress” (11).

There are differences in aetiology and pathogenesis of the HF between children and adults. The first is related to ischemia (60-70%) and coronary artery disease (CAD) and high blood pressure. However, in children, the associated causes of HF included congenital heart disease (CHD) or cardiomyopathies. Hsu and Pearson have defined the HF in children as a syndrome consisted of cardiovascular and non-cardiovascular abnormalities resulting in various sign and symptoms including volume overload, pressure overload, both ventricles defects and metabolic abnormalities (12-14).

About 37.5 million people are living with HF globally. Increasing prevalence of heart failure is affecting 1-2% of the adult population in developed countries (15). Depending upon the studied population and the diagnostic criteria, the global incidence of HF is from 100 to 900 cases per 100,000 persons. In 2012, a report from the American Heart Association (AHA) stated the estimated 915,000 cases of HF in the USA (16). A population-based perspective cohort study revealed that the lifetime risk of HF at age 55 years is 33% for men and 28% for women (17). According to the Centre for Disease Control and Prevention, the CHD are the most common birth defects affecting 1% or about 40,000 birth in US per year. In children, the incidence of HF is 0.97 to 7.4 per 100,000. In pediatrics, 25–75% HF patients are with the underlying cause of CHD (18). In 2010, more than 2 million infants, children (about 1 million), adolescents and adults (1.4 million) were living with CHD in US (19).

In adults, New York Heart Association (NYHA) is used to classify the different stages of the HF based upon functional ability. According to NYHA class I patients experience no undue fatigue and dyspnoea (shortness of breath) upon physical activity. While class II patients are with slight limitation of physical activity resulting in fatigue, palpitation and shortness of breath. The class III and IV patients are characterised with moderate and severe indications of fatigue and discomfort respectively (20).

1.3 Treatment of heart failure

The pathophysiology of HF is centrally associated with the RAAS. The keystone for the therapeutic management of the HF and its main risk factor hypertension is targeting of the RAAS. Neuro-hormonal antagonists including ACEIs, beta-blockers (BBs), angiotensin receptor blockers (ARBs) and mineralocorticoid receptor antagonists (MRAs) are associated with decreased mortality and morbidity based upon large randomised clinical trials for treatment of patients with HFrEF (21).

According to the European Society of Cardiology, the ACEIs are recommended for both the symptomatic heart failure with reduced ejection fraction (HFrEF) patients and also patients with asymptomatic left ventricle systolic dysfunction up to the maximum tolerated dose (22). The ACEIs and BBs are recommended in combination and are considered to be complementary to each other in symptomatic patients with HFrEF to reduce the risk of HF hospitalization and death. MRAs (spironolactone and eplerenone) are recommended despite the treatment with ACEIs and BBs in a patient with symptomatic HFrEF ≤ 35%.

Recently PARADIGM-HF study was conducted in patients with symptomatic HFrEF with left ventricle ejection fraction (LVEF) ≤40% to investigate the dual inhibition of the angiotensin receptor neprilysin inhibitor (ARNI) (sacubitril/valsartan) against the ACEI (enalapril) (23). Despite the superiority of this combination against the enalapril in reducing the worsening to hospitalisation, the symptomatic hypotension associated with sacubitril/valsartan (in patient age ≥ 75%) was 18% against the enalapril group, in which it was 12%. Still, long term safety in this aspect is missing. Aliskiren is another direct-acting renin inhibitor, however, is not recommended as an alternative to ACEIs and ARBs because of increased renal dysfunction and hyperkalaemia (11).

The treatment goals for HF in adults and in pediatrics are similar to decrease the mortality and morbidity. Mostly the pharmacotherapy for HF is well-investigated in adults, which cannot be directly translated to pediatrics due to differences in

disease aetiology in both populations. There is a lack of evidence-based treatment of pediatric HF as compared to adults due to non-conductance of clinical trials in pediatrics requiring further investigation.

ACEIs and BBs are used for the treatment of pediatric HF as an initial therapy despite the lack of the evidence-based randomised perspective studies. The aldosterone antagonist is also used along with some diuretics to maintain fluid retention during ventricular dysfunction (24). Among ACEIs, the captopril and enalapril are most studied in pediatrics with clinical betterment from left to right shunt in HF (25, 26). However, there are some contradictory opinions found in literature about the benefit of the enalapril in children with single ventricular failure during the first year of life (27). Such type of findings reinforces the conduction of pediatric studies using appropriate analytical techniques for the generation of reliable clinical data to achieve patient safety by evidence-based efficacious treatment for cardiac diseases.

1.4 Angiotensin-converting enzyme inhibitors

Angiotensin-converting enzymes inhibitors are being in use since 1981, when the first drug of this class came into the market, captopril (28). ACEIs have been proved to be the drug class that showed a reduction in mortality and hospitalisation (29, 30). There are following classes of ACEIs available in literature based upon their chemical structure (31, 32):

a. Sulfhydryl containing group include captopril and zofinopril.

b. Carboxyalkyldipeptides include benazepril, enalapril, lisinopril, perindopril, quinapril, ramipril, trandolapril.

c. Phosphorus-containing group e.g. fosinopril.

Within this thesis, carboxyalkylpeptides were included pertinent to screening LC-MS/MS method development and fit-for-purpose validation.

1.4.1 Benazepril

Benazepril hydrochloride is [(3S)-3-[[(1S)-1-(ethoxy carbonyl)-3-phenylpropyl]amino]-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl] acetic acid hydrochloride. It is not orally active and requires conversion into its active metabolite benazeprilat, in the liver by cleavage of ester linkage through hydrolysis. Benazepril hydrochloride is absorbed rapidly after oral administration of a single dose of 20 mg with peak plasma concentration reaching in 0.5 to 1.0 hour (t_{max}) and converted to its active metabolite (33).

1.4.2 Enalapril

Enalapril maleate is chemically described as (S)-1- [N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline, (Z)-2-butenedioate (2S)-1-[(2S)-2-[[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]pyrrolidine-2-carboxylic acid. The enalapril is inactive prodrug with 60 to 70% good oral absorption reaching at peak plasma concentration time in 1 hour. It is rapidly metabolised after de-esterification to enalaprilat, an active diacid metabolite in liver. Enalaprilat peak plasma concentration reaches in 2 - 4 hours (34).

1.4.3 Perindopril

Perindopril is another dicarboxylic group containing angiotensin-converting enzymes inhibitor. Perindopril is also prodrug like most of the ACEIs and it is converted into perindoprilat. The hepatic esterases in human are responsible for biotransformation of perindopril to its active metabolite, perindoprilat (35). The oral bioavailability of perindopril is 95% with its rapid absorption after 8 mg oral dose. Only 20% of the parent drug is converted into its active metabolite, perindoprilat through hepatic metabolism (36).

1.4.4 Quinapril

Quinapril is another non-sulfhydryl monoethyl ester prodrug converting to its active diacid metabolite, quinaprilat, once it is hydrolysed after absorption by a liver esterases which is a more potent ACEI than the parent drug. With potent binding affinity and short elimination half-life of quinaprilat to angiotensin converting enzyme enables single daily dose. The absorption is 60% in a healthy individual after an oral dose. The peak plasma concentration of quinapril reaches to its peak in 1 hour after administration and quinaprilat in 2 hours following an oral dose 2 – 80 mg respectively (37).

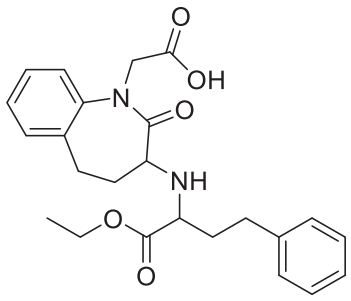
1.4.5 Ramipril

The chemical name of ramipril is (2S,3aS,6aS)-1-[(2S)- 2-[[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl] amino]propanoyl]- 3,3a,4,5,6,6a-hexahydro-2H-cyclopenta[d]pyrrole-2-carboxylic acid. Like other ACEIs, the ramipril is a prodrug with its metabolite ramiprilat, which is a competitive inhibitor of angiotensin converting enzyme. It is rapidly hydrolysing to ramiprilat after gastrointestinal tract absorption. After a single oral dose of 2.5 to 10 mg, peak plasma concentration reaches in 1 hour for ramipril and 2 – 4 hours for ramiprilat (38). Ramipril and its metabolite is 73% and 56% bound to plasma proteins respectively. Most of the drug is eliminated as ramiprilat its glucuronide conjugates. Initial elimination from body takes place in half-life of 7 hours and 102 hours for the late phase (39).

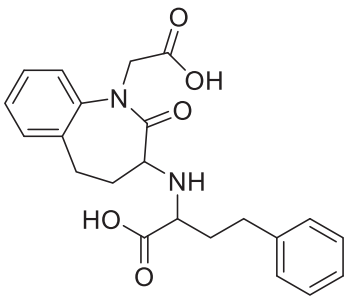
1.4.6 Trandolapril

Trandolapril is also non-sulfhydryl prodrug which is converted to its diacid metabolite trandolaprilat in the liver through hepatic esterases and exerts its antihypertensive effect. In healthy individual at therapeutic dose range, the peak plasma concentration of trandolapril occurs in 1 hour and for trandolaprilat in approximately 2 – 4 hours after 2 mg single dose. The mean elimination half-life of trandolapril is 0.7 – 1.3 hours (40, 41).

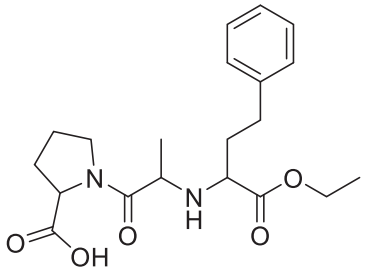
The structures of the investigated ACEIs are represented in **Figure 1-2**.



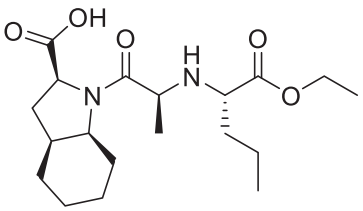
Benazepril



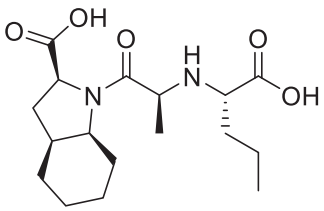
Benazeprilat



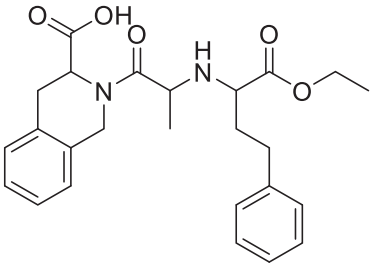
Enalapril



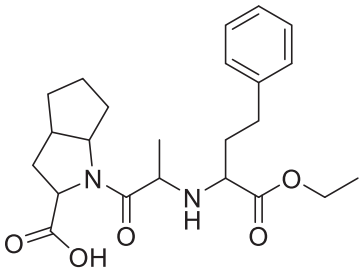
Perindopril



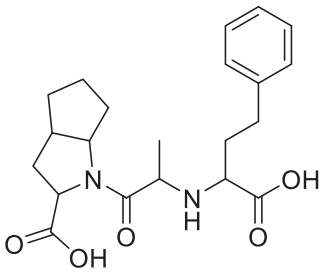
Perindoprilat



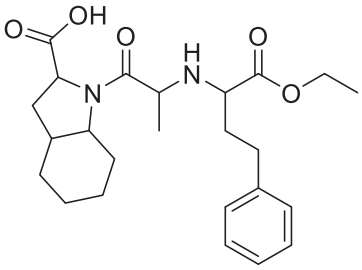
Quinapril



Ramipril



Ramiprilat



Trandolapril

Figure 1-2 *Structure of investigated angiotensin-converting enzyme inhbitors.*

1.5 Medication adherence and intoxication to angiotensin-converting enzyme inhibitors

Medication adherence refers to the extent to which the patient follows the prescribed dose regimen by practitioners. The adherence to cardiovascular medication is a concern for the clinicians leading to increased risk of morbidity and mortality (42). According to the World Health Organisation (WHO), 50-70% of the patient do not take their prescribed medication leading to the most significant cause of the uncontrolled blood pressure. Therefore, the worsening of the disease and overprescribing lead to the adherence problems (43).

Medication adherence can be evaluated directly or indirectly. Indirect methods for evaluating the medication adherence are pill counts, electronic database and self-reported questionnaires. Direct methods comprised of measuring the level of drugs in various matrices including serum, urine and blood. Being more reliable, the bioanalytical methods for assessing the medication adherence have got attention (44, 45). In a study including 149 hypertensive patients monitored by using an electronic pillbox, 42% were found to be non-adherent against defined criteria of taking less than 80% of the prescribed medication (46). The study using HPLC-MS/MS for monitoring the drug levels among 1348 patient in urine and serum exhibited rates of 41.6% and 31.5% in the United Kingdom (UK) and Czech respectively (47).

In addition to monitoring the adherence of ACEIs, it is also important to prevent harm to patients or people who are in close contact with the patients treated, e.g. children. The increased availability of ACEIs escalated the likelihood of intoxication events due to ACEIs either intentionally or unintentionally (48, 49). ACEIs were the most commonly used drugs in the United States of America (USA) with 162.8 million prescriptions in 2009 (50). In the Netherlands, there were 9 million prescriptions of ACEIs in 2013. In Netherland perindopril, enalapril and lisinopril are among top fifty drugs with 3.18, 2.86 and 2.76 million users in 2017 respectively (51). In 2013, the first antihypertensive drug was ramipril with more than 24 million prescriptions in the UK (52).

Intentional intoxication for suicidal attempts using antihypertensive drugs including ACEIs were reported in the investigation by Chodorowski in 201 adults (mean age 36 years) (53). Data published in 2014 by Mainz Poison Centre, Germany, reported in a retrospective analysis about 241 unintentional exposures in children with ACEIs out of 1489 cases with antihypertensive drugs (54).

Due to the occurrence of events of intentional and unintentional poisoning in adults and children, and for monitoring medication adherence, screening methods for rational treatment especially in the vulnerable pediatric population are required. Although the screening methods for cardiovascular medication are available in the literature, however, they are meant to be applied in adults owing to high sample volume and high limit of detection making their application difficult in pediatrics. The problems associated with pediatric sample analysis are the small sample volume in combination with a lack of sensitive analytical technique detecting at lower concentrations and, therefore, requiring new methods.

1.6 Pediatric clinical trials

The transformations in the physiology from preterm neonates to adolescents bring about the different pharmacokinetic and pharmacodynamic behaviour of the drug. For example, at birth the gastric pH is neutral (6-8) till the 10^{th} day of the birth, and it returns to the adult level pH (2-3) by the age of three years affecting dissolution and absorption of the drugs (55). Another difference is the total body water contents that account 85% of the total body weight in the preterm infant, in a term infant it is 75% and it is accounted 60% in 6 months old and older having implications on dosing interval (56). The differences in the disease aetiology also do not allow to transform adult pharmacotherapy to pediatrics. Therefore, at the postnatal period the adjustments of adult's dose to the pediatric is unsafe and unreliable (57). Undoubtedly, to understand the pharmacokinetic behaviour of the drugs in children, the high quality studies are required in this vulnerable population.

However, the study design implemented in adults is difficult to directly apply in children regarding the sampling volume and sampling points without endangering patient safety. Guidance related to blood sampling volume directs that the blood loss should not exceed 3% of the total blood volume in neonates during the period of one month and not exceed 1% at any single time (58). Therefore, considering the ethical constraints of limited blood volume and limited pool of the pediatric patients present for the disease of interest along with robust analytical techniques make the clinical trials conductance in this population highly challenging. Regardless of above all encountered challenges, the evidence-based therapy is of utmost importance to achieve more patient safety in pediatrics.

The clinical data sets in this vulnerable population are rare and serve as a basis for future research. Using a validated method is one of the steps towards obtaining a reliable bioanalytical data. However, during clinical trials where the methods are repeatedly applied, the continuous monitoring of method performance brings about more confidence about the reliable data generation. Therefore, the reliability of these data sets should be established by incorporating quality control system ensuring high data quality.

1.7 Bioanalytical data quality

Recently there is guidance initiative from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (Q14) and USP (Chapter 1220) about the analytical method life cycle management. The purpose of this guidance is to address the future trends regarding the analytical method development, qualification and continuous monitoring in a holistic manner (59, 60). It consisted of mainly three steps: 1) Design and development, 2) procedure performance qualification and 3) continued performance verification. The routine use of qualified or validated method over a long duration may lead to the drift in its application causing variation in the interday reported results. The goal of continuous method performance is to reduce the risk associated with the analytical method during its life cycle.

However, the same paradigm of method life cycle management may be implemented for the bioanalysis where the bioanalytical method is applied for quantification of the drugs in biological fluids (plasma, serum, urine) once validated or qualified. Bioanalytical methods take a long journey from its development to application in pre-clinical or clinical trials for their defined purpose. After the method is developed, it is submitted to the set of well-established parameters for their validity to be fit for its intended purpose as per regulatory guidance (61, 62). Once validated, the bioanalytical methods are repeatedly applied to create pharmacokinetic (PK) and pharmacodynamic (PD) data for making decisions during drug development.

There are generally four parameters for establishing the quality of bioanalytical data as presented in United States Pharmacopoeia (USP): analytical instrument qualification (1058) (63). Among these parameters (1) analytical instrument qualification and, (2) method validation, are restricted to the prior of the study sample analysis fulfilling the criteria whether the instrument is fit for the purpose and the method is properly validated respectively. The third (3) is system suitability testing generally performed on the day of the analysis before submitting the study samples and, (4) quality control samples which are associated with each particular analytical run.

Despite the above described parameters and availability of the well-guided method validation criteria by the regulatory agencies as one of the components towards bioanalytical data quality, the continuous method performance verification or analytical life procedure in terms of inter-run variability and long term reproducibility still needs consideration. The aim of this evolving concept of life cycle management or continuous method performance is to reduce the bioanalytical method variation and the measurement uncertainty for the reported values. Therefore, quality control system monitoring the continuous performance of bioanalytical method was proposed with the aim of obtaining more reliable data for PK interpretation to enhance the patient safety. Further such method performance monitoring becomes more meaningful with the involvement of the pediatric population during clinical trials, in which the recurrent measurement cannot be conducted due to ethical constraints.

1.8 High-performance liquid chromatography

The high-performance liquid chromatography (HPLC) is a fast separation technique commonly employed in reverse phase mode applying polar mobile phases (hydrophilic) and non-polar stationary phases (hydrophobic). The most important aspect of the HPLC is to bring about the resolution of the closely related components from baseline separation making an accurate measurement of its peak area and height. Most of the ACEIs are the prodrug (hydrophobic) and undergo hydrolysis to convert into their active metabolites (hydrophilic). The metabolites of the drugs are more polar as compared to their parent drug substance, therefore, they tend to elute earlier leading to less retention. To increase the retention of the polar substances on the reverse phase column, modifiers are used. In the case of chromatographic detection using methods other than mass spectrometer, these modifiers may be non-volatile such as phosphate buffers and other inorganic additives. However, in LC-MS inorganic modifiers may lead to the contamination of the ion source and ion suppression effects. Therefore, organic modifiers including formic acid and acetic acid may be used at concentration 0.1% or higher. Additionally, the formate and acetate salts of ammonium are used in the range from 2 to 10 mmol/L (64).

The chromatographic methods involving the drug and its metabolite in single run generally require gradient elution methods including 1) isocratic hold by initial higher hydrophilic concentration causing retention of the more polar substance; 2) gradient time from low organic to high organic concentration (eluent composition changing leading to separation on to the column); 3) final high concentration of the organic to cause the elution); 4) purging with high concentration of organic to elute strongly retained analytes and isocratic hold to ensure the elution of the all possible analytes; 5) conditioning (quickly returning to the initial organic concentration) and equilibration (time to equilibrate the column to achieve reliable quantification in subsequent injection and to reduce carryover (65).

1.9 Mass spectrometry

It was 1897 when J.J Thomson first time discovered mass spectrometer. However, small molecules started routinely analysed by 1980. In 1988 J. B. Fenn discovered electrospray ionisation (ESI), a soft ionisation technique by making use of early experiments of the Malcolm Dole and received a noble prize in 2002 for his work. The invention of ESI has revolutionised the use of mass spectrometry in the biological field and it is promisingly employed for the identification, sensitive detection and quantification of the analytes in complex biological matrices (66, 67). The HPLC is interfaced with the mass spectrometer using ESI source which can be operated at atmospheric pressure. The already solution protonated $[M+H]^+$ or deprotonated $[M-H]^-$ ions reach at the charged capillary (2.5 – 6.0 kV). The charged ions exiting at the tip of the charged capillary are pushed towards the mass analyser because of the potential and the pressure gradient

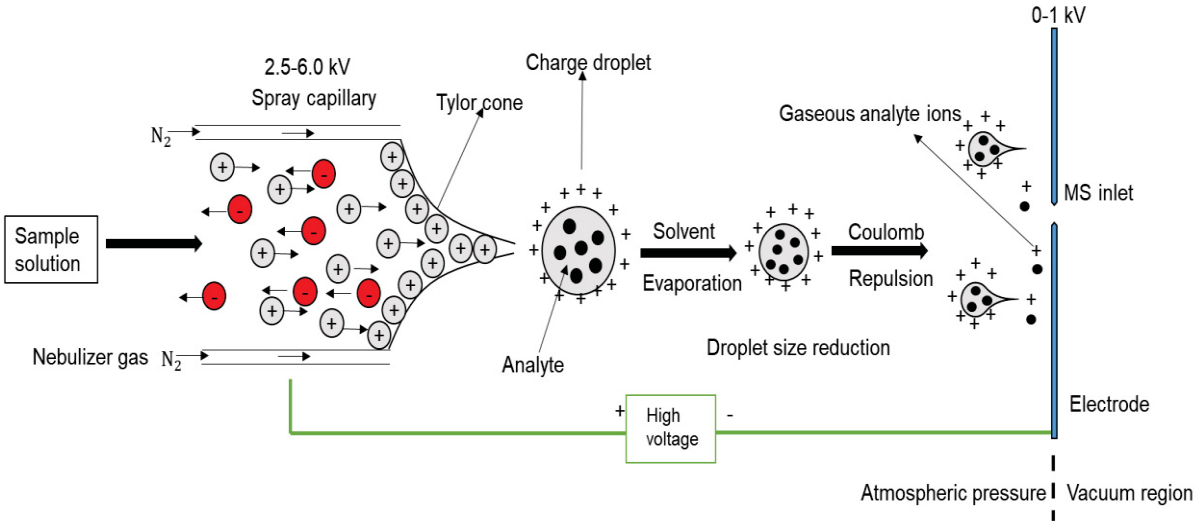


Figure 1-3 *Tylor cone formation at the end of the capillary, coulomb repulsion and subsequent gaseous ion formation (68).*

inside the source environment. The conversion of these solution charged ions into gas-phase ions is accompanied by the elevated source temperature in addition to another dry gas, which causes the solvent evaporation of the charged droplet and causing the reduction in its size and increases surface charge density and decreases droplet size eventually leading to increased coulomb repulsion. At this point, there occurs an ejection of the gaseous phase ion from the droplet

surface and passes from skimmer cone on to the analyser for further measurement of the signal intensity **(Figure 1-3)** (69).

The triple quadruple (QqQ) mass analyser is commonly employed in clinical measurements and consisted of three sets of equidistance diagonally place four metal rods making instrument triple quadruple **(Figure 1-4)**. To achieve the more selectivity and specificity for the under investigation analyte, the multiple reaction monitoring (MRM) mode is utilised. In this operation mode, both the first quadruple (Q1) and the third quadruple (Q3) act as a static filter for the selected precursor ion and the product ion respectively. However, the second quadruple (Q2) acts as a collision cell leading to the fragmentation of the selected precursor ion of specific mass. For clinical bioanalysis generally the Q1 and Q3 scan are used to identify the precursor ion and the product ion respectively and used for quantitative analysis of the various drugs in different matrices including blood, plasma, serum and urine (68).

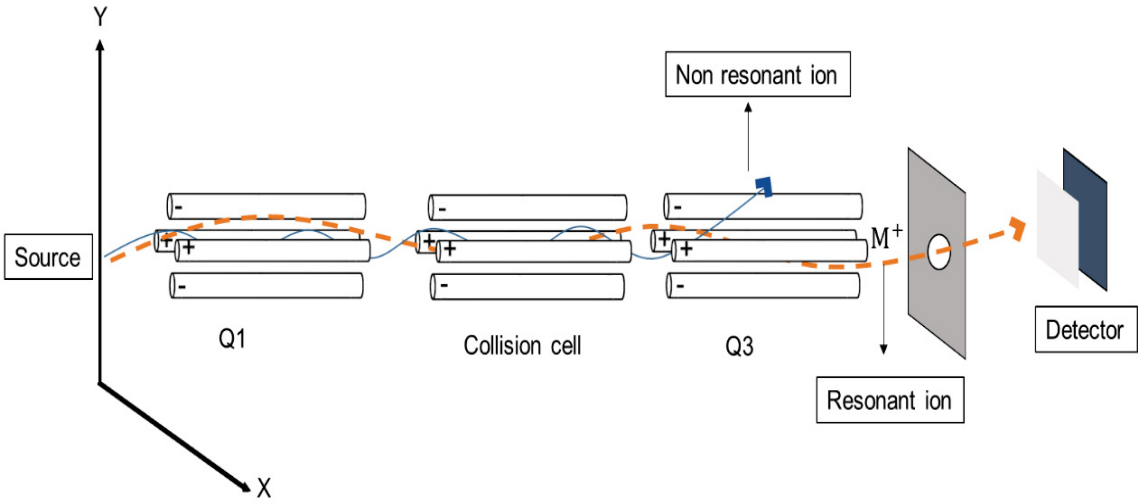


Figure 1-4 *Schematic diagram of the triple quadruple showing filtering of the ions (69).*

1.10 Aims of the thesis

The sensitive and robust bioanalytical methods are the pillar for qualitative and quantitative measurement of the analytes of interest. However, monitoring its performance facilitates substantially its reliability. Initially the method validation is conducted against a set of regulatory parameters verifying method performance to enter in clinical or non-clinical application. Owing to repeated application, the long term method performance verification is also essential to establish the quality of the bioanalytical data.

The thesis will comprise of mainly the two parts: first part is related to LC-MS/MS screening micro-assay development for angiotensin-converting enzyme inhibitors for its application in medication adherence and toxicology utilizing 50 µL plasma volume. Fit-for-purpose validation was performed to make developed method eligible for its intended purpose.

The second part of the thesis will focus on the establishment of the comprehensive quality control system to monitor the long term performance to ensure the quality of bioanalytical data to obtain reliable pharmacokinetics in Good Clinical Laboratory Practice compliant settings during clinical trials in an academia environment. The method development and its validation make the method fit to be used for its purpose. However, monitoring the long term method performance make certain of reliable data generation.

Each section will comprise of the short introduction at hand, applied methodology as well as results and discussion. Finally, the thesis will present the overall conclusion with its perspective application.

Chapter 2

LC-MS/MS Method for Screening of Intoxication and Medication Adherence of Angiotensin-Converting Enzyme Inhibitors in Plasma.

2.1 Introduction

Hyphenating liquid chromatography with tandem mass spectrometry (LC-MS/MS) provides a reliable and powerful technique for qualitative and quantitative evaluation of the analyte of interest. There are three main steps involved during the LC-MS/MS method development (68); 1) sophisticated sample preparation method enabling the detection or quantification at lower possible concentration; 2) despite the intrinsic ability of the sensitive analyte detection mass spectrometer requires method development for finding the appropriate fragment ion and optimisation of the mass parameters involving low sample volume); 3) fast separation liquid chromatographic method development assisting MS/MS measurement by reducing the matrix effect.

Until now, only a few bioanalytical methods are available for simultaneous screening or quantification of ACEIs (70-74). Although they were published recently, some of them require inappropriate high blood volumes (500 µL) that cannot be collected as an add-on to routine investigations in children. The other methods are characterized by a high limit of detection, without proper separation of prodrugs and their metabolite. Further, most of ACEIs exist as a prodrug and undergo hydrolysis after absorption into their active metabolite. They are often determined simultaneously in biological fluids, which is also important for pharmacokinetic studies.

Elsebaei *et al*. developed an assay comprising of seven angiotensin-converting enzyme inhibitors using 500 µL of plasma or urine by HPLC-UV with a limit of detection 17-64 ng/mL and a run time of 14.00 min (72). Another method was reported by Dias *et al*, who substantially reduced the required blood volume, but their LC-MS assay for the screening of cardiovascular drugs included five ACEIs only (74). Gonzalez *et al*. developed a quantification method in 100 µL plasma

including six ACEIs with a recovery of ≤ 68.5% and a limit of detection 18 ng/mL (73). Thus, there is still need of a suitable method, which comprehensively detects ACEIs with the benefit of utilising residual sample volume and sophisticated sample preparation technique for high recoveries ensuring detection even at lower limits. Therefore, a sensitive and stable screening method for commonly used ACEIs and their active metabolites utilising residual blood using LC-MS/MS that allows for the evaluation of medication adherence and toxicity for its application in adults and pediatrics was developed and validated for its intended application.

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2.2 Material and methods

2.2.1 Reference standards and biological sample

Benazepril hydrochloride, enalapril maleate, enalaprilat dihydrate, perindopril, quinapril hydrochloride, ramipril, trandolapril, trandolaprilat all were certified reference standards supplied by European Directorate for the Quality of Medicines and Healthcare (Strasbourg, France). Perindoprilat (USP reference standard), quinaprilat hydrate (98%, HPLC) and internal standard enalapril (phenyl d_5) maleate (98%, chemically pure (CP)) were procured from Sigma Aldrich (Steinheim, Germany). Plasma samples were voluntarily donated by consent from a member of the Institute of Clinical Pharmacy and Pharmacotherapy (Dusseldorf, Germany).

2.2.2 Chemicals and solvents

Methanol (HPLC grade) and ammonia solution (25%, per analysis (p.a.)) were obtained from VWR chemicals (France). Water (HPLC grade), formic acid (98%, p.a.) and phosphoric acid (85%, p.a.) were purchased from AppliChem (Darmstadt, Germany). Acetonitrile, acetone (HPLC grade) and ammonium format (mass spectrometry grade) were supplied by Sigma Aldrich (Steinheim, Germany).

2.2.3 Solid phase extraction apparatus and analytical column

Oasis® mix mode anion exchange (MAX) 96 well plate 30 µm (10 mg) with in-housed constructed positive pressure manifold was utilised. The analytical column as well as guard column, X-select CSH™ C18 (3.0 x 150 mm; 3.5 µm) and (3.0 x 20 mm) respectively were used. Both the solid phase extraction (SPE) 96 well plates and columns were obtained from Waters (Eschborn, Germany).

2.2.4 Instruments and softwares

HPLC, Shimadzu Deutschland GmbH (Duisburg, Germany), two separate pumps LC-10ADvp with SCL-10Avp controller, three-channel online degasser DGU-20A, column oven (L-2300, VWR/Hitachi), autosampler, mass spectrometer API 2000 (Applied Biosystems/MDS SCIEX, Concord, Canada), pipettes 10-100µL, 100-

1000 µL, 10000 µL, multichannel pipette (100-1000 µL) (Eppendorf, Hamburg Germany), analytical balance Shimadzu (Duisburg, Germany), Analyst ® software version 1.5.1, MultiQuant™ 3.0.2 software, OriginPro for graphing were used.

2.2.5 Standard solutions

2.2.5.1 Stock solution and working solution

Stoke solution of each analyte including internal standard (IS) was prepared separately at concentration 100 µg/mL in methanol (calculated for salt) and stored at -20 °C. This was achieved by accurately weighing the 5 mg of the reference standards and dissolving it into the small amount of HPLC grade methanol and then making the final volume up to 50 mL using volumetric flask with methanol. Further 1:10 dilution was performed by diluting the 100 µL of stock solution in HPLC grade water and making final volume up to 500 µL. This resulted into final working solution of 20 µg/mL.

2.2.5.2 Calibration standards and quality controls

The finally prepared working solution (20 µg/mL) in water for each analyte was utilized to arrange the final standards/quality controls. The highest calibration standard was obtained by spiking drug-free plasma to a concentration of 200 ng/mL of each analyte (base). Further serial dilution (1:2) with blank plasma was performed to establish the required calibration standard (0.78 ng/mL to 100 ng/mL). Quality controls were prepared separately at three different levels: low [LQC, 3.13 ng/mL], medium [MQC, 25 ng/mL] and high [HQC, 100 ng/mL].

2.2.6 Sample preparation and extraction protocol

Biological specimen including serum, plasma, urine constitute mainly the lipids, protein, metabolite degradation products as the leading cause of the matrix effect leading to ionisation enhancement and or suppression and also the clogging of the column. This effect becomes more pronounced when the ESI source is used in LC-MS/MS (75). SPE for sample preparation provides the clearer extract thereby reducing the matrix effect and leading to the methods ability to detect the

lowest possible concentration. Liquid-liquid extraction (LLE) is also comparable to the SPE but it has the disadvantage of utilising the costly organic solvents.

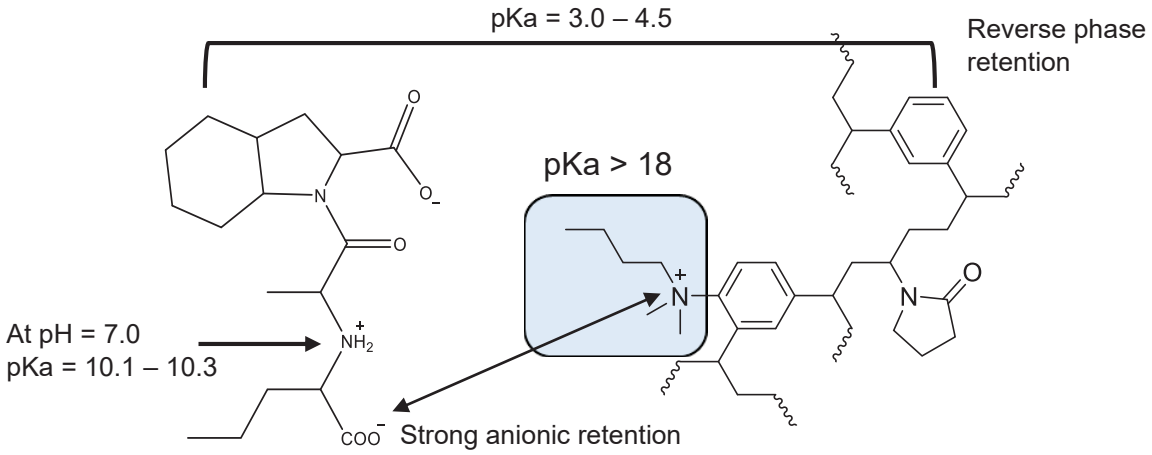


Figure 2-1 *Retention of angiotensin-converting enzyme inhibitor (perindopril) on mixed-mode anion exchange material showing reverse-phase interaction between two cyclic rings and strong anionic interaction between highly basic tertiary amine (pKa > 18) and highly acidic carboxylic group (pKa = 1.9 - 2.1) (76).*

Additionally, phospholipids constitute 80% of the matrix effect, there might be some organic solvents which also extract the undesired matrix. The SPE is considered as a gold standard for sample preparation for different matrices. It makes use of different types of stationary phase composed of modified silica and polymeric sorbents as in HPLC column to purify the analyte. Mix mode ion exchange sorbent in SPE provides the most sophisticated sorbent for sample preparation based upon the polarity of the analyte of interests. SPE is also advantageous owing to its high thorough output due to the use of 96 well plate. The analytes included in current screening method belong to the ethyl ester group of the ACEIs ranging from pKa 1.64 to 4.6 rendering them acidic to slightly acidic. To achieve the maximum recovery and to remove the matrix effect, mix mode anion (MAX) exchange sorbent was implemented. The extraction of the analyte of interest depends on retention mechanism following two pathways: first is the strong anion retention mechanism at high pH and second is the reverse phase mechanism when the pH is lowered and acidic analytes are in the neutral form **(Figure 2-1; Figure 2-2).**

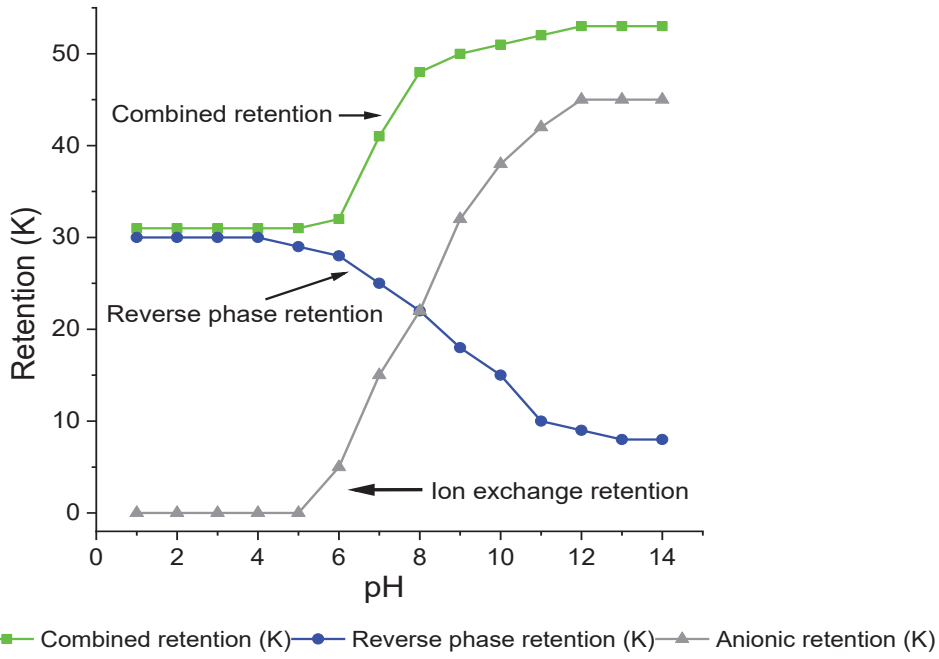


Figure 2-2 *Effect of pH on reverse phase and ionic retention of acidic compounds on mixed-mode anion exchange material (76).*

Oasis® mix mode anion exchange (MAX) 96 well plate was used for solid-phase extraction (SPE) in positive pressure mode. **Figure 2-3** represents the different steps involved during sample preparation. Conditioning and equilibration of SPE 96 well plate was performed by 1 mL of 2% formic acid in acetonitrile and 1 mL of water respectively. Before loading the sample to SPE well, 50 µL of already spiked calibration standards and quality controls were diluted with 1100 µL water and 5 µL of internal standard (20 µg/mL). Then diluted plasma samples (1155 µL) were transferred to SPE well. Washing involved 1 mL of water, 1 mL of methanol: acetone (60:40) and 1 mL of methanol respectively. Eluate (800 µL) was obtained by using 2% formic acid in acetonitrile and subjected to dryness using nitrogen by placing the SPE plate on thermomixer (Eppendorf Hamburg, Germany), previously set at a temperature of 40 °C at 550 rpm for 50 min. Dried samples were reconstituted in 100 µL mobile phase (water: ACN, 60:40) and 20 µL was injected into LC-MS/MS.

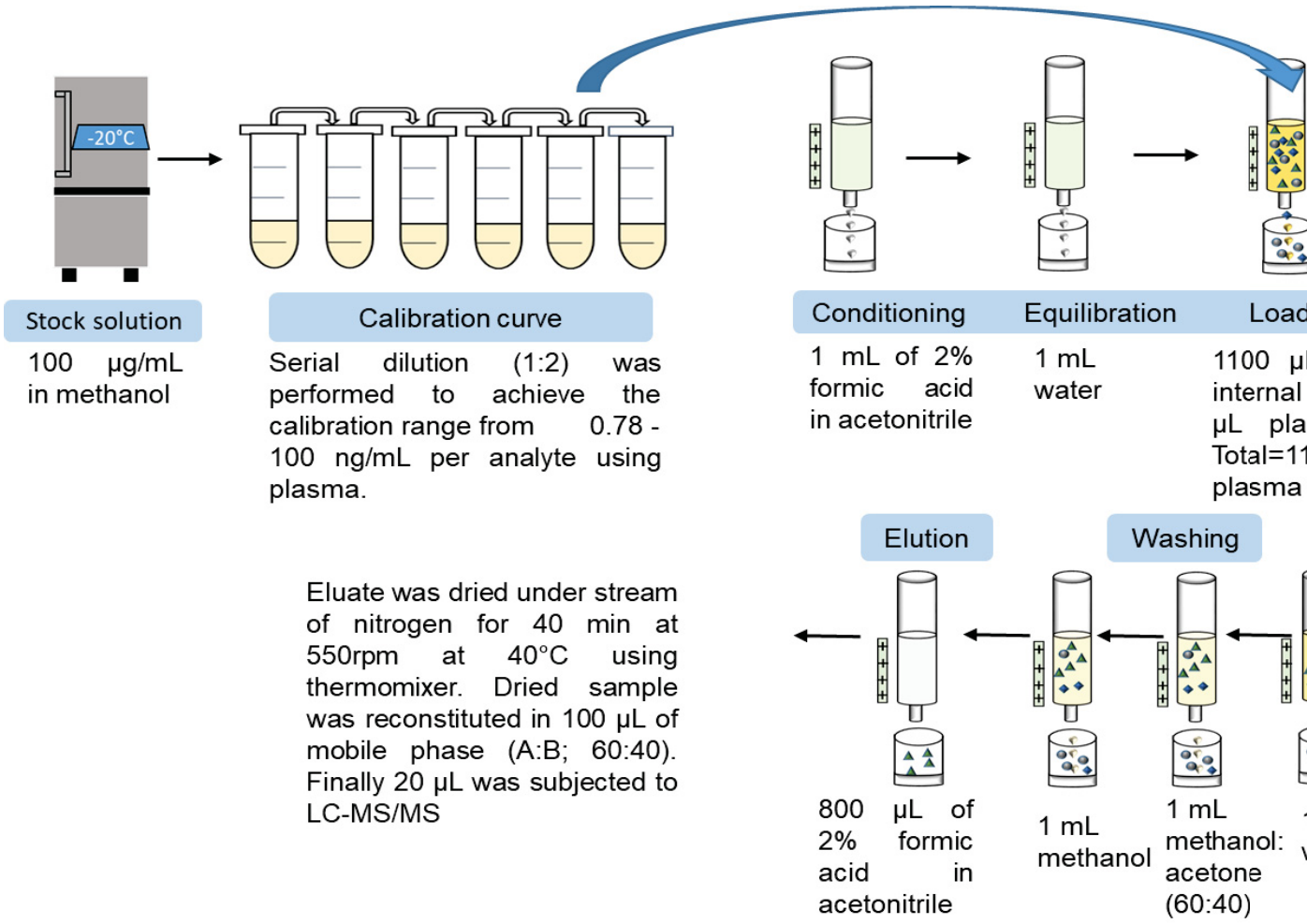


Figure 2-3 *Preparation of calibration curve using serial dilution (1:2) from 200 ng/mL to 1.56 ng/mL a preparation using solid phase extraction. Conditioning and equilibration involved 1 mL of 2% formic acid in me 1155 µL sample was loaded (1100 µL water + 5 µL internal standard + 50 µL plasma sample). Washing invo acetone (60:40) and 1 mL methanol. Finally, 2% formic acid in acetonitrile was used for elution and 20 µL wa*

2.2.7 Chromatographic and mass spectrometric conditions

2.2.7.1 Chromatographic conditions

The isocratic flow brought the elution of all investigated analytes without proper resolution. Therefore, the gradient elution was implemented to attain the better resolution due to involvement of the pro-drug and its metabolite. The analysis was carried out using water (mobile phase A) and acetonitrile (mobile phase B) acidified with 1% formic acid and 2mmol ammonium formate. Following gradient was applied: 0 - 0.99 min 3% B, 1.20 min 10% B, 1.21 – 5.50 min from 35 - 55% B, 5.51 – 7.50 min at 95% B, 8.00 min 3% B. Total run time was 10 minutes with two minutes for re-equilibration. The flow rate was kept at 0.4 mL/min, while the column temperature was thermostatically controlled at 60 °C.

2.2.7.2 Mass spectrometric conditions

Mass spectrometer API 2000 (Applied Biosystems/MDS SCIEX, Concord, Canada) was used for mass spectrometric detection in positive mode with electrospray ionization (ESI). **Table 2-1** represents a summary of chromatographic conditions and mass-dependent parameters. Ionisation source temperature was kept at 375 °C with ion spray voltage 4500 V. Other mass parameters included curtain gas 18 arbitrary units (a.u), gas 1 (30 a.u), gas 2 (90 a.u) and collision gas (8 a.u). Initially, the mass spectrum was obtained for all analytes using Q1 scan **(Figure 2-4)**. Further, the Q3 scan was performed for product ion using syringe infusion at a flow rate of 10 µL/mL respectively. This provides the initial estimation of the compound dependent parameters mainly including the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP) and collision energy (CE).

Table 2-1 *Summary of chromatographic and mass dependent parameters.*

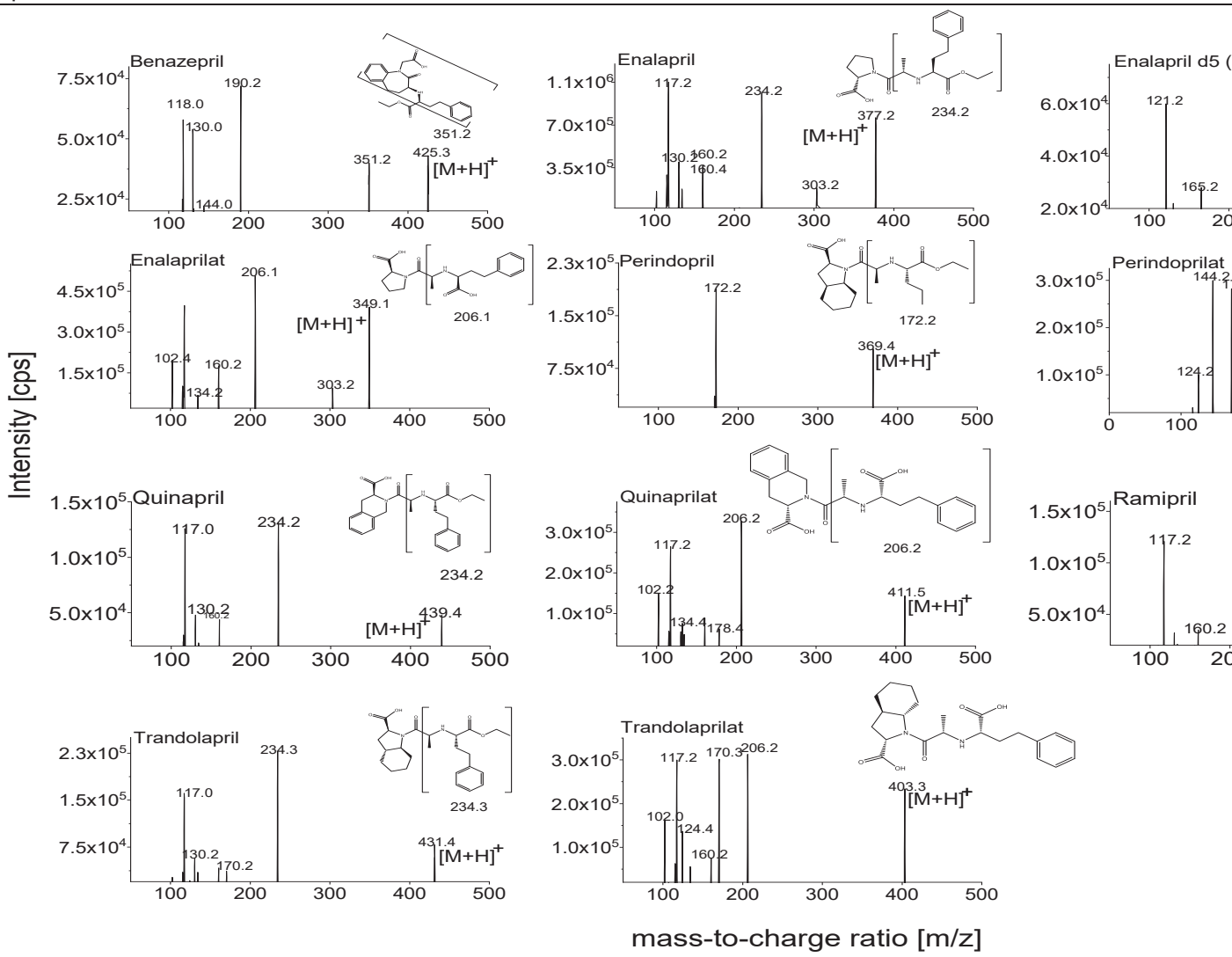
Parameter	Information
Sample preparation	1100 µL water + 5 µL IS (benazepril) + 50 µL plasma containing all analytes
Mobile phase	Solvent A = Water with 1% formic acid and 2mmol ammonium formate Solvent B = Acetonitrile with 1% formic acid and 2 mmol ammonium formate
Column	X-select CSH™ C18 3.5 µm analytical column and guard column
Column temperature	60 °C
Flow rate	0.4 mL/min
Ionisation source temperature	375 °C
Ion spray voltage	4500 V
Curtain gas	18 a.u
Collison gas	8 a.u

Collision energy is an important aspect during the Q2 scan to optimise the fragmentation pattern for the most abundant product ion. However, the optimal source dependent parameters are depending upon the LC conditions. To observe the flow rate effect on the optimised mass and compound dependent parameters, flow injection analysis (FIA) was performed near or at the time when the liquid chromatography method was developed. FIA involved the introduction of the analytes using multiple injections at constant higher flow required for further optimisation of the compound and instrument dependent parameters including nebuliser gas, curtain gas, ion source temperature and ion spray voltage. Finally, the precursor ion and product ion were used to perform analysis in multiple reaction monitoring mode (MRM) for all analytes keeping dwell time at 125 msec. All compound dependent parameters along with precursor ion and product ion are presented in **Table 2-2**. Analyst ® software version 1.5.1 was used for evaluation.

Table 2-2 *Precursor ion and product ion with compound dependent parameters for all analytes.*

Analyte name	Mass		Poten		
	Precursor ion	Product ion	DP	FP	EP
Benazepril	425.3	351.2	26	150	12
Enalapril	377.2	234.2	56	300	11.5
Enalapril d5 (IS)	382.2	239.1	31	370	10
Enalaprilat	349.1	206.1	56	370	11.5
Perindopril	369.4	172.2	56	340	10.5
Perindoprilat	341.3	170.3	46	340	10.5
Quinapril	439.4	234.2	46	370	10
Quinaprilat	411.5	206.2	61	350	10.5
Ramipril	417.5	234.2	51	370	10
Trandolapril	431.4	234.3	51	340	9.50
Trandolaprilat	403.3	170.3	51	340	10.5

Abbreviations: *DP (Declustering potential), FP (Focusing potential), EP (Entrance potential), CEP (Coll (Collision energy), CXP (Collision cell exit potential), V (Volt), IS= Internal standard*.



Abbreviations: *cps = Cycles per second, [M+H]⁺ = Molecular ion mass*

Figure 2-4 *Mass spectrum (Q1 scan) of all analytes including internal standard obtained in positive ion mod*

2.2.8 Qualitative method validation

Method validation is a set of parameters usually applied to the developed method to assess the applicability of the method for the intended purpose. The international guidelines including the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) outlined the different parameters for the validation (61, 62). For validation of the screening method, the following parameters were investigated: limit of detection (LOD), linearity range, recovery, matrix effect repeatability and carry over. Validation of the aforementioned parameters allowed the reliable and robust semi-quantitative analysis of all analytes. Additionally, accuracy and precision were also checked. Since the method was intended to apply for medication adherence and intoxication, therefore, limits for accuracy and precision were less strict as these are necessary for therapeutic drug monitoring or for quantification.

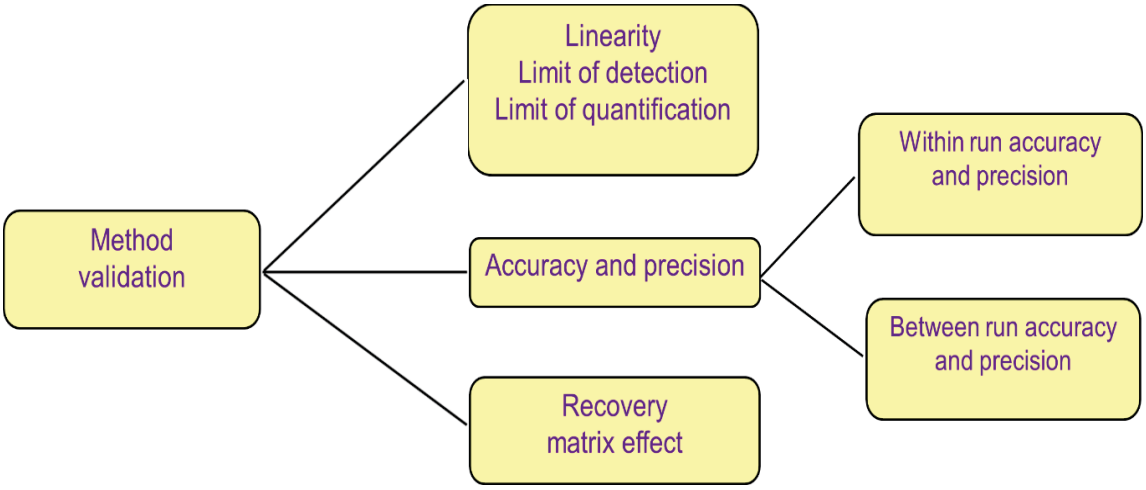


Figure 2-5 *Scheme showing parameters used for fit-for-purpose qualitative method validation.*

2.2.8.1 Recovery, matrix effect and carry over

Recovery and matrix effect was determined for each analyte including internal standard at 100 ng/mL concentration by following an already proposed method (77). Following samples were processed: pre-spiked plasma sample before SPE (A), post-spiked plasma sample after SPE (B) and neat solution in solvent (C).

Recovery was calculated by dividing the detector response of A to the detector response of B using the following equation:

$$\text{Recovery } (\%) = \frac{\text{Area}_\text{A}}{\text{Area}_\text{B}} * 100 \qquad \textbf{Equation (2-1)}$$

The acceptable recovery rates were defined as ≥ 85% to enable the detection of analyte at the lowest concentration levels.

Similarly, the absolute matrix effect was calculated by dividing the detector response of B to detector response of C as under:

$$\text{Matrix effect } (\%) = \frac{\text{Area}_\text{B}}{\text{Area}_\text{C}} * 100 \qquad \textbf{Equation (2-2)}$$

Since it is known that ESI is more sensitive to matrix effect, maximum alteration of ±15% was regarded as acceptable. A value of matrix effect less than 100% intimate ion suppression, whereas a higher one indicates ion enhancement.

Instrument carryover was checked by the response of detector for blank solution sample after injection of the high-quality control sample (100 ng/mL, n=5) of calibration standards and IS. It must not be greater than 20% of the lower limit of quantification and 5% for the internal standard.

2.2.8.2 Limit of detection, quantification and calibration curve

A calibration curve based on internal standard normalised peak areas (analyte area/IS area) against nominal concentrations was used to estimate the LOD and limit of quantification (LOQ) according to criteria of International Conference on Technical Requirements for Registrations of Pharmaceuticals for Human Use (ICH) (64). The value of LOD and LOQ was calculated by using standard error and slope of the regression line of a specific calibration curve using the following expressions:

$$\text{Limit of detection (LOD)} = 3.3 * \frac{\sigma}{s} \qquad \textbf{Equation (2-3)}$$

$$\text{Limit of quantification (LOQ)} = 10 * \frac{\sigma}{s} \qquad \textbf{Equation (2-4)}$$

Where σ is the standard error of the y-intercept, used as standard deviation and s is the slope of the regression line, respectively. The calibration curve was constructed using eight calibration standards along with zero and blank.

2.2.8.3 Accuracy and precision

Intraday and inter-day accuracy and precision were evaluated by using three concentration levels: low, medium and high in sets of five replicates on three different days as per FDA guideline (62). The intraday and inter-day accuracy were expressed as a percentage from the mean of calculated concentration of single-day replicates and from the mean of three days to nominal concentrations, respectively. The relative standard deviation (%RSD) was used to express precision in the same way. Since here presented assay was intended for screening purposes only, maximum acceptable deviation to nominal concentration and variability were predefined as ±30% being suitable for semi-quantitative analysis.

2.2.8.4 Repeatability, reproducibility and specificity

Following the definition given in the FDA guideline, reproducibility/repeatability "represents the precision of the method under the same operating conditions over a short period" (62). Apart from the recently published method for repeatability and reproducibility (78), five quality control samples at three different concentration level (LQC, MQC, HQC) were being measured at three different days. The calculation was conducted applying a single factor analysis of variance (ANOVA) and obtained the mean sum of square incorporated into following equation:

$$\text{Repeatability}\ (\%) = \frac{\sqrt{\text{MS}_{\text{wg}}}}{\bar{\mu}} \text{X}\ 100 \qquad \textbf{Equation (2-5)}$$

Where (MS_{wg}) denotes mean sum of squares within the group and $\bar{\mu}$ represents the mean of all measured concentration.

To evaluate the specificity of the method, blank plasma samples were purified as described and check for the interference from other analytes and endogenous matrix compounds at a retention time of analyte.

2.3 Results

2.3.1 Sample preparation and extraction protocol

The complex nature of the biological matrix and simultaneous detection of different analytes having different physicochemical properties necessitates the use of a sophisticated sample preparation approach. While using mix mode anion exchange SPE, pH plays an important role in the extraction of the analyte. Dilution of plasma sample by a suitable solvent is required to maintain proper pH throughout the SPE process for separation of the analyte of interest and to remove the interfering substances. The optimal volume for dilution was based on former investigations, showing that a ratio of 1:20 is useful in terms of the flow rate through sorbent material and interaction with ion exchanger (79). Dilution of spiked plasma samples with the 4% phosphoric acid lowered the pH and kept all analytes in unionised form leading no retention on anionic exchange material. However, dilution with 5% ammonia performed better in terms of recovery (91 to 98%)

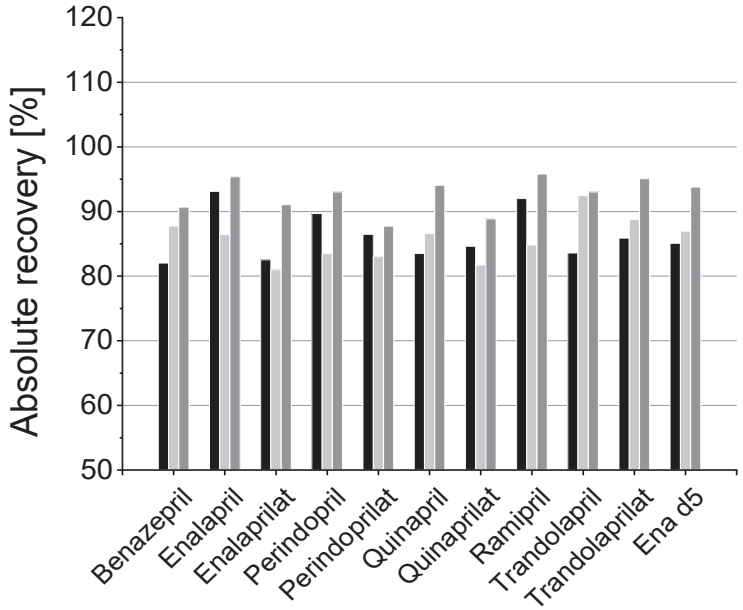


Figure 2-6 *Effect of different conditioning, washing and elution solution on absolute recovery of all analytes and internal standard enalapril D5 (Ena D5),* ***Black*** *= Water and methanol: acetone (60:40) for washing and 2% formic acid in methanol for conditioning and elution,* ***Light grey*** *= Water, methanol: acetone (60:40) and methanol for washing and 2% formic acid in methanol for conditioning and elution,* ***Dark grey*** *= Water, methanol: acetone (60:40) and methanol for washing and 2% formic acid in acetonitrile for conditioning and elution.*

for all analytes except fosinopril and fosinoprilat (14%), which were excluded from final method due to different physicochemical properties (highly polar). Finally, the dilution with water yielded optimum recoveries ranging from 87 to 95% for all of the ten analytes **(Figure 2-6)**.

Additionally, the washing and elution conditions were also optimised. A wider range of matrix effect (86 to 125%) was observed while using only water and methanol: acetone mixture (60:40) as washing steps. However, it was assumed that washing with an additional 1 mL of pure methanol further removed interfering residues, and analytes experienced less matrix effect variation (100 to 113%) during chromatographic separation making it final approach **(Figure 2-7)**. Regarding elution, the use of 2% formic acid in acetonitrile vs. 2% formic acid in methanol did not substantially alter the recovery (87 to 95% vs 81 to 92%, respectively), while matrix effect with acetonitrile and methanol solution was from 100 to 113% and 100 to 116% respectively.

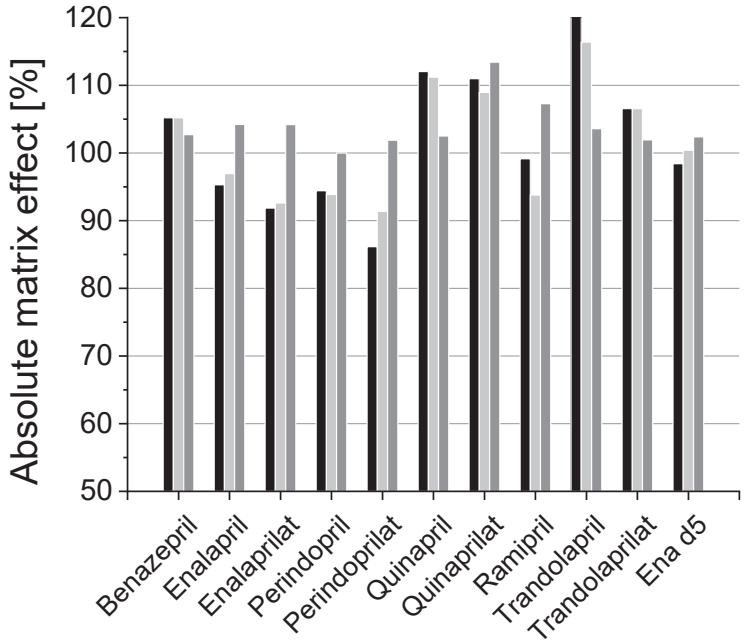


Figure 2-7 *Effect of different conditioning, washing and elution solution on absolute matrix effect of all analytes and internal standard enalapril D5 (Ena D5),* ***Black*** *= Water and methanol: acetone (60:40) for washing and 2% formic acid in methanol for conditioning and elution,* ***Light grey*** *= Water, methanol: acetone (60:40) and methanol for washing and 2% formic acid in methanol for conditioning and elution,* ***Dark grey*** *= Water, methanol: acetone (60:40) and methanol for washing and 2% formic acid in acetonitrile for conditioning and elution.*

Nevertheless, it has already been reported that using acetonitrile for elution during SPE is capable of retaining the matrices on the SPE cartridges in comparison to methanol and thus minimizing matrix effect (80). Combining all efforts, SPE protocol optimization resulted in better recoveries and less matrix effect as compared to other published screening methods for ACEIs (72, 73).

2.3.2 Chromatographic and mass spectrometric conditions

By applying the gradient system in combination with reverse phase chromatography acetonitrile caused earlier elution and produced sharp peaks for most of the analytes as compared to methanol **(Figure 2-8)**. During chromatographic separation, the splitting of analyte peak (perindoprilat) was observed at a column temperature 40 °C which was rectified by increasing the column temperature to 60 °C. The possible reason for the splitting was reported to be caused by cis-trans isomerism of ACEIs at a lower temperature (81, 82). For mass spectrometric evaluation, the prominent fragment was selected during the product ion scan **(Figure 2-4).**

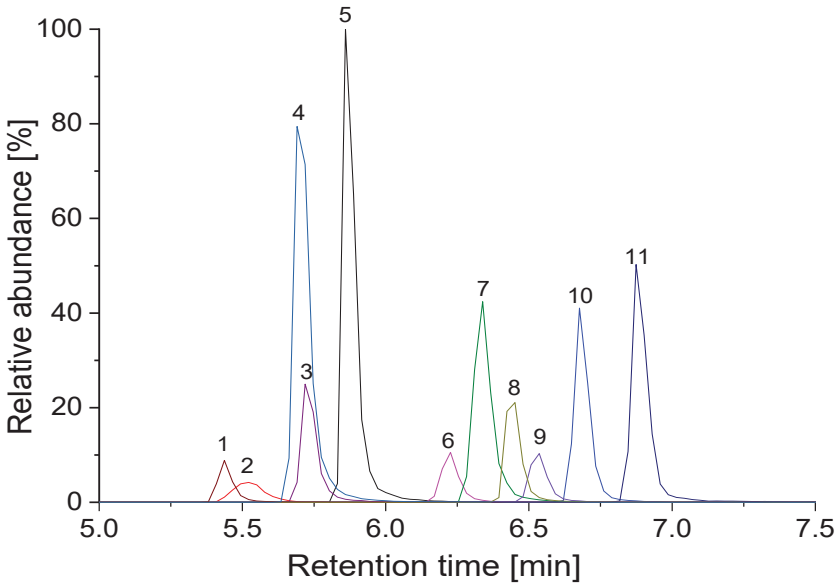


Figure 2-8 *Representative chromatogram showing peak and retention time for all analytes at 100 ng/mL. 1. Enalaprilat (RT=5.40 min), 2. Perindoprilat (RT=5.48 min), 3. Enalapril D5 (RT=5.56 min), (IS) 4. Enalapril (RT=5.69 min), 5. Perindopril (RT=5.83 min), 6. Quinaprilat (RT=6.17 min), 7. Ramipril (RT=6.29 min), 8. Benazepril (RT= 6.40 min), 9. Trandolaprilat (RT=6.47 min), 10. Quinapril (RT=6.62 min), 11. Trandolapril (RT=6.82 min).*

Due to closely related chemical structure, mass fragmentation resulted in cross-talk producing the same product ion for some analytes. However, keeping dwell time 125 ms and performing analysis in MRM has an advantage of no interference of analytes with similar product ions (66). Total gradient run time was 10 min, which was shorter than already published assays for simultaneous screening with run times of 13.5 and 14.0 min (72, 74).

2.3.3 Qualitative method validation

2.3.3.1 Recovery, matrix effect and carry over

Elaborated tailored SPE protocol resulted in high recoveries for all analytes. The calculated absolute recovery for all analytes were ≥ 87%, while the absolute matrix effect ranged from 100 to 113%. The latter is within international guideline limits of ±15%. Details on the extent of recovery and matrix effect for all analytes including IS are provided in **Table 2-3**. The sample preparation method using SPE resulted in better recoveries and less matrix effect as compared to other published methods.

Table 2-3 *Absolute recovery (%) and matrix effect (%) for all analytes at 100 ng/mL.*

Analyte name	**Mean peak area pre-spiked plasma samples**	**Mean peak area post-spiked plasma samples**	**Mean peak area in neat solution**	**Recovery** [%]	**Matrix effect** [%]
Benazepril	224000	247000	240500	90.69	102.70
Enalapril	423500	444000	426000	95.38	104.23
Enalapril d5	121000	129000	126000	93.80	102.38
Enalaprilat	102000	112000	107500	91.07	104.26
Perindopril	674000	724500	724500	93.03	100.00
Perindoprilat	76250	86900	85300	87.74	101.88
Quinapril	404500	430000	419500	94.07	102.50
Quinaprilat	124000	139500	123000	88.89	113.41
Ramipril	480000	501000	467000	95.81	107.28
Trandolapril	468000	503000	485500	93.04	103.60
Trandolaprilat	125500	132000	129500	95.08	101.93

No matrix effect = 100, Ion enhancement > 100, Ion suppression < 100

2.3.3.2 Limit of detection, quantification and calibration curve

The chromatograms at blank and LOQ are represented in **Figure 2-9**. The LOD and LOQ ranged from 0.41 to 0.65 ng/mL and 1.26 to 1.99 ng/mL, respectively, summarised in **Table 2-4**. Three calibration curves were constructed and mean of their internal standard normalised area was plotted against nominal concentration. A simple linear regression method with weighting $1/x^2$ was the best fit for all analytes. Regression parameters including intercepts, slope and coefficient of determination values ($r \geq 0.993$) are presented in **Table 2-4**. The mean back calculated concentration (n = 3) of calibration standards for all analytes are represented in **Appendix-I**.

Table 2-4 *Regression equation and coefficient of correlation (r- values) from the mean of three calibration curve for all analytes (n=3) along with limit of detection (LOD) and limit of quantification (LOQ).*

Analyte name	Linearity [ng/mL]	Regression equation	r-value	LOD [ng/mL]	LOQ [ng/mL]
Benazepril	0.78 – 100	y = 0.0026x + 7.47E-04	0.997	0.56	1.72
Enalapril		y = 0.0032x + 8.09E-04	0.999	0.56	1.70
Enalaprilat		y = 0.0010x + 2.37E-04	0.998	0.60	1.82
Perindopril		y = 0.0089x + 0.0119	0.993	0.41	1.26
Perindoprilat		y = 9.84E-04x + 2.33E-04	0.998	0.54	1.65
Quinapril		y = 0.0039x + 0.0010	0.998	0.58	1.56
Quinaprilat		y = 0.0012x + 6.47E-04	0.998	0.60	1.83
Ramipril		y = 0.0048x + 0.0019	0.996	0.65	1.99
Trandolapril		y = 0.0049x - 0.0016	0.999	0.59	1.79
Trandolaprilat		y = 0.0013x + 3.28E-04	0.999	0.50	1.76

2.3.3.3 Accuracy and precision

2.3.3.3.1 Intraday and interday accuracy and precision

Outcomes for accuracy (intraday and interday) and %RSD (inter-day) of quality control samples (n=5) spiked at three different concentrations (LQC = 3.13, MQC

= 25, HQC = 100 ng/mL) from three different days that are represented in **Table 2-5** and **Appendix-II**. Interday accuracy at LQC was within ± 20% of international guideline limits (80.98 to 108.71%), while at MQC and HQC, it was within ± 15% (90.06 to 109.64%) for all analytes (67). Intraday accuracies at LQC were within the acceptable limit of ± 20% for all analytes except benazepril (21.3% at LQC), quinapril (21.1% at LQC), quinaprilat (29.1% at LQC), and trandolapril (23.1% at LQC). For perindopril (16.1% at HQC) and ramipril (17.3% at HQC) deviations to specifications for quantitative methods were found at the highest quality control level (guideline limit: ± 15%).

The %RSD for interday precision was within the range of ±20% (1.98 to 11.79%) for all analytes at LQC except perindopril and trandolapril (21.59 and 22.84%), while %RSD at MQC and HQC was within ± 15% (2.55 to 14.93) for all analytes. For intraday precision, the %RSD at all levels was also within the acceptable limits of ± 20% (at LOQ) and ± 15% (at MQC and HQC) except for one run of quinaprilat (29.5% at LQC and 25.1% at MQC). During multianalyte analysis, it is hard to establish optimised chromatographic and mass spectrometric conditions for all analytes, therefore some deviation for certain analyte (s) may be observed (74), anyhow the developed method was fit for the purpose.

Table 2-5 *Inter-day accuracy and precision for all analytes.*

Analyte name	Concentration				
	Day 1 [ng/mL (%accuracy)]	**Day 2** [ng/mL (%accuracy)]	**Day 3** [ng/mL (%accuracy)]	**Mean ± SD** [ng/mL]	**Mean int accurac**
Benazepril	2.64 (84.28)	3.09 (98.59)	2.46 (78.65)	2.73 ± 0.32	87.1
	22.14 (88.56)	24.54 (98.16)	25.12 (100.48)	23.93 ± 1.58	95.7
	84.30 (84.30)	97.32 (97.32)	105.09 (105.09)	95.57 ± 10.99	95.5
Enalapril	3.09 (98.66)	3.09 (98.72)	2.75 (87.82)	2.98 ± 0.20	95.0
	23.54 (94.16)	24.76 (99.04)	24.96 (99.84)	24.42 ± 0.77	97.6
	86.90 (86.90)	95.86 (95.86)	102.91 (102.91)	95.22 ± 8.02	95.2
Enalaprilat	2.83 (90.42)	2.72 (87.03)	2.88 (92.01)	2.81 ± 0.08	89.8
	22.72 (90.88)	23.50 (94.00)	25.99 (103.96)	24.07 ± 1.71	96.2
	87.28 (87.82)	96.84 (96.84)	108.15 (108.15)	97.42 ± 10.45	97.4
Perindopril	4.21 (134.50)	3.22 (103)	2.77 (88.62)	3.40 ± 0.73	108.7
	28.90 (115.60)	24.76 (99)	25.84 (103.36)	26.50 ± 2.15	106.0
	83.90 (83.90)	96.50 (96.50)	102.63 (102.63)	94.34 ± 9.55	94.3
Perindoprilat	2.92 (93.42)	2.92 (93.16)	2.82 (90.12)	2.89 ± 0.06	92.2
	23.24 (92.96)	22.68 (90.72)	25.00 (100)	23.64 ± 1.21	94.5
	89.16 (89.16)	92.40 (92.40)	106.02 (106.02)	95.86 ± 8.95	95.8
Quinapril	2.85 (91.12)	2.90 (92.59)	2.47 (78.88)	2.74 ± 0.24	87.5
	23.16 (92.64)	23.50 (94)	24.92 (99.68)	23.86 ± 0.93	95.4
	87.84 (87.84)	96.82 (96.82)	104.61 (104.61)	96.42 ± 8.39	96.4
Quinaprilat	2.67 (85.18)	2.72 (86.90)	2.2 (70.86)	2.5 ± 0.3	80.9
	22.56 (90.24)	24.52 (98.08)	22.3 (89.32)	23.1 ± 1.2	92.5
	87.58 (87.58)	99.44 (99.44)	109.5 (109.5)	98.8 ± 11.0	98.8
Ramipril	3.18 (101.73)	2.80 (89.46)	2.62 (83.83)	2.87 ± 0.29	91.6
	27.34 (109.36)	22.30 (89.20)	25.78 (103.12)	25.14 ± 2.58	100.5
	91.86 (91.86)	82.76 (82.67)	95.56 (95.96)	90.06 ± 6.59	90.0
Trandolapril	3.82 (122.11)	3.06 (97.89)	2.41 (76.93)	3.10 ± 0.71	98.9
	32.12 (128.48)	24.74 (98.96)	25.37 (101.48)	27.41 ± 4.09	109.6
	117.60 (117.60)	97.84 (97.84)	105.12 (105.12)	106.85 ± 9.99	106.8
Trandolaprilat	2.84 (90.61)	3.25 (103.77)	2.70 (86.13)	2.93 ± 0.29	93.5
	22.96 (91.84)	23.64 (94.56)	24.16 (96.64)	23.59 ± 0.60	93.5
	88.78 (88.74)	95.94 (95.94)	106.80 (106.8)	97.17 ± 9.07	94.3

SD = Standard deviation, RSD = Relative standard deviation

2.3.3.4 Repeatability, reproducibility and specificity

Repeatability of the screening method was determined on three runs on three different days. The repeatability was evaluated using ANOVA for calculation of the repeatability. Obtained variability in the method during three different days ranged between 2.5 to 8.4% for all analytes of interest at HQC (100 ng/mL). At MQC (25 ng/mL) the repeatability varied between 1.9 to 14.4%, while at the LQC (3.13 ng/mL) it varied from 1.3 to 15.8%. The repeatability results are shown in **Table 2-6**.

Table 2-6 *Repeatability of method expressed as a percentage by applying single factor ANOVA at all level of quality controls.*

Analyte name	LQC (3.13 ng/mL)	MQC (25 ng/mL)	HQC (100 ng/mL)
		%	
Benazepril	6.19	7.38	2.71
Enalapril	6.8	1.93	3.15
Enalaprilat	9.18	4.81	4.24
Perindopril	8.22	6.32	3.43
Perindoprilat	6.01	4.63	6.88
Quinapril	1.31	6.09	3.62
Quinaprilat	15.76	14.43	8.36
Ramipril	8.26	3.81	4.43
Trandolapril	7.82	3.43	2.47
Trandolaprilat	7.24	5.22	2.78

Lower quality control (LQC = 3.13 ng/mL), Middle quality control (MQC = 25 ng/mL), High quality control (HQC = 100 ng/mL)

The specificity of the method was assessed by observing the blank chromatogram for any interference by other analytes or endogenous matrix compounds. No interference was detected. The data of blank and LOQ are presented as an overlay in **Figure 2-9.** Stabilities of all analytes of interest were investigated comprehensively in previous publications. Rudzki *et al*. summarized stability data of available ACEIs showing that these drugs have to be considered stable up to 7 months at -20 °C in the matrix. Stock solutions were even stable for one year if stored at -40 °C (81). Due to these findings, it was concluded that no additional stability investigations were required.

2.4 Discussion

ACEIs are amongst the widely used medication for cardiovascular diseases including HF, hypertension and myocardial infarction both in pediatrics and adults. The therapeutic effectiveness of these drugs is closely related to medication adherence. Additionally, the increased availability of these drug substances has increased the likelihood of their utilisation for intentional and unintentional intoxication. A sensitive screening micro-assay was developed and fit-for-purpose validation was performed for a mixture of commonly used ACEIs in plasma utilising residual blood volume. The customised validation underlined the suitability of the developed bioanalytical method for its intended purpose.

Initially, the effort was made to develop a distinct sample preparation method for analytes belonging to three classes of ACEIs using SPE. However, the captopril and lisinopril (only active agents) were highly hydrophilic owing to following log P values (captopril, log P = 0.84; lisinopril, log P = -0.94). Therefore, these analytes were unable to retain on the mixed-mode ion exchange material due to their water solubility during the first washing step in SPE process involving water. In contrast to captopril and lisinopril, the fosinopril and its metabolite fosinoprilat were hydrophobic with a log P value of 6.61 and 5.20 respectively (83). The fosinopril and fosinoprilat were not able to resolute, possibly due to their affinity on the X-select CSH™ C18 column during the chromatography separation. Therefore, in the final method the analytes belonging to carboxyalkyldipeptides were included, still comprising the more numbers of ACEIs as compared to previously published qualitative or quantitative methods.

During sample preparation using SPE, the pre-treatment of plasma with water was optimised at ratio 1:20 (79). Along with various optimised washing solutions, the elution with 2% formic acid in acetonitrile was found to be more effective than 2% formic acid in methanol in terms of recovery (≥ 87%) and matrix effect (100 – 113.41%). Gonzalez *et al* showed recoveries from 29.9 to 68.5% with matrix effect ranging 112.3 to 129.3% using protein precipitation by LC-MS/MS (73). In another method for simultaneous quantification of seven ACEIs by HPLC/UV, up

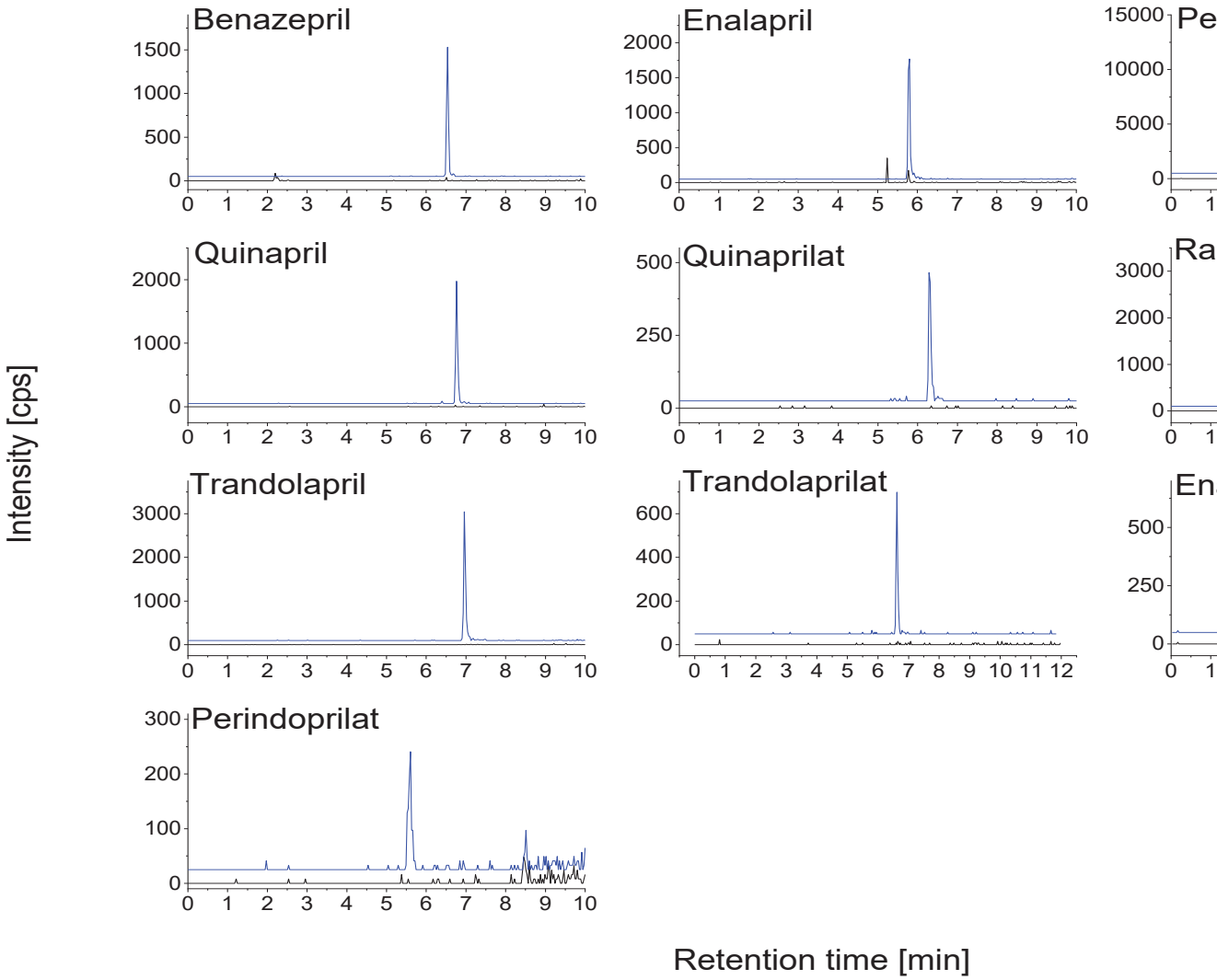


Figure 2-9 *Overlaid chromatograms representing blank plasma sample (black) and limit of detection (*

to 90% yielded recovery by using SPE was mentioned by the authors (72). The needle wash solution was 1% formic acid in 80% acetonitrile which accounted for a reduction of the carryover. The gradient revealed that analytes started eluting when the concentration of organic was approximately (70 to 80%). Using the same concentration of needle wash solution prevented the system carry over. This was confirmed by observing the chromatogram of the blank solution after five consecutive HQC samples **(Figure 2-9)**.

An isocratic system provides fixed polarity of the mobile phase composition throughout the chromatographic procedure, thereby causing elution of closely related compounds (carboxylic group ACEIs) with bad resolution. In contrast to an isocratic system, gradient system accounts for changing the polarity by changing mobile phase composition and causes the elution of compounds based on the compound's hydrophobicity resulting in better separation. Much effort was utilised to achieve the proper gradient for the high peak area for all analytes and baseline separation specifically between the pro-drug and its metabolite. The starting gradient (organic concentration) was monitored from 3% to 25%, the former behaved better in terms of the analyte peak area and resolution. This outcome was also supported by the solvophobic theory which states that the water brings about the retention the analyte on to the reverse phase column C18 hydrocarbons leading to increase concentration on to the column and more peak intensity upon elution (84).

A broad linearity range from 0.78 to 100 ng/mL was established using small plasma volume. Compared to the published screening methods on simultaneous ACEIs, the requirement of plasma volume as low as 50 µL represents the lowest reported so far (72-74). Although a broad linear range (0.78 to 100 ng/mL) was successfully established, the lowest point of this range (0.78 ng/mL) did not always represent the LOQ for the specific analyte. The latter is justified by the fact that LOD and LOQ values were calculated based upon the standard error of y-intercept and the slope of the calibration curve.

The pediatric patients, who are at risk of severe side effects even with very low drug concentration due to inappropriate administration or unintentional intoxication, screening methods must detect low levels. Published methods for simultaneous detection of ACE inhibitors are characterized by 17 to 64 ng/mL and 18 to 60 ng/mL respectively (72, 73). The aforementioned levels are suitable to detect common drug levels in adults but inappropriate for children. Thus, these methods may be inappropriate for the detection of intoxications in small children. Therefore, the established lower levels of the here presented method are of advantage in pediatric care ranging from 0.41 to 0.65 ng/mL as LOD and 1.26 to 1.99 ng/mL as LOQ. Additionally, the accuracy and precision outcomes also showed that the developed method was fit-for-purpose.

The intention of the here presented screening method was its application for medication adherence and intoxication, which is ensured by above-listed validation results. Reproducibility and consistency of measurements by applying the screening method were proven by fit-for-purpose method validation. Its maximum deviation to nominal concentration (accuracy) and variability (precision) for all analytes ensures reliable semi-quantitative detection of the analyte of interest in 50 µL plasma.

Chapter 3

Fit-for-Purpose Quality Control System in Continuous Bioanalysis during Long-Term Pediatric Clinical Trials

3.1 Introduction

In 1990, the first bioanalytical workshop was held by the American Association of Pharmaceutical Scientists (AAPS) / FDA (85). Later in 2001, the first edition of Bioanalytical Method Validation (BMV) guidance was issued by the FDA. During the last decade, a consensus has been reached on numerous specifications and recommendations given by the guidelines on the conduct of method validation and its routine clinical application. Once validated, the bioanalytical methods are repeatedly applied to create pharmacokinetic (PK) and pharmacodynamics (PD) data for making decisions during drug development. Therefore, ensuring the high quality of the bioanalytical method is of utmost importance.

The structured guidance for assessing the data quality of a single analytical run is available in terms of quality control (QC) checks. However, monitoring of associated analytical runs in clinical trials is important to ensure the comparability of study data, even if it comes from different analytical runs over different time spans. This would also facilitate the evaluation of the least-attended aspect of between-run applicability of the bioanalytical method. Additionally, the guidelines suggested incurred sample reanalysis (ISR) as one of the tools to determine the long-term reproducibility of the validated bioanalytical method, however, this area is still under discussion among experts (86).

There is no direct method to assess the integrity of the determined concentrations from unknown samples except to establish the reliability and reproducibility of the accompanying bioanalytical data from the calibration curve (CC), QC checks, ISR and internal standard (IS) (87-89). In the pediatric population, comprehensive PK and PD data sets are lacking. Subsequently, clinical trials are highly necessary for evidence-based pharmacotherapy in children (90). Based on, e.g., ethical

constraints, comprehensive studies in vulnerable populations are often only performed once. These data sets will form the basis for evidence-based pharmacotherapy. It is therefore of utmost importance that rigorous clinical data generated during pediatric clinical trials must be reliable. Appropriate reliability of clinical data can be ensured by the implementation of a suitable analytical quality control system. While international guideline suggestions for quality control of essential parameters (e.g., CC, QC checks, etc.) are limited to single batches, a reliable clinical data set can only be generated if the quality is monitored across batches or studies. Moreover, the peculiarity of pediatric studies must be reflected within such a quality control system.

An institutionalized multi-parameter quality control system utilizing descriptive statistical and graphical techniques for evaluating long term method performance in terms of accuracy and reproducibility of accompanied bioanalytical data was applied. The validity of the individual runs was determined immediately, while the between run performance was performed as a post-hoc analysis. The applicability of the established quality control system was investigated by monitoring bioanalysis within the EU-funded, academically-driven “Labelling of Enalapril from Neonates up to Adolescents” project (LENA). This project aimed to assess a novel child-appropriate dosage form in children suffering from heart failure comprised of PK and PD bioanalysis of three parallel clinical studies across all pediatric age groups (0-12 years). Besides method validation and system suitability tests, this approach served as an additional tool for making certain the reliable and comparable results of unknown pediatric samples over a whole 31 months' duration in three closely related pediatric studies.

3.2 Methods

3.2.1 In study sample analysis

Bioanalysis of clinical study samples was performed by using a fully validated bioanalytical method according to the EMA and FDA bioanalytical method validation guidelines (61, 62). Calibration curves and quality controls were obtained by spiking blank human serum with European Pharmacopoeia Reference Standards of the drug enalapril and its active metabolite enalaprilat (European Directorate for the Quality of Medicines, France). Benazepril (Sigma-Aldrich, Germany) acted as an internal standard. The stability of the stock solution was up to six months as previously established during validation. Freshly prepared calibration standards, quality controls and unknown samples were purified by solid-phase extraction (Oasis MAX 96-well plates, Waters, USA). The measurements were conducted with high-performance liquid chromatography (Modular 10-series, Shimadzu, Germany) coupled with triple quadrupole tandem mass spectrometry (LC-MS/MS) in positive ionization mode (API 2000, AB Sciex, Canada). The gradient elution was used, consisting of methanol and water (both containing 0.1% formic acid and 2 mM ammonium formate) as mobile phase with the total run time of 7 minutes. An XBridge® BEH C18 3.5 µm column (3.0 mm x 150 mm) was used for chromatographic separation at column temperature 40°C. Following multiple reaction monitoring transitions (MRM) were monitored for quantification of enalapril and enalaprilat: 377.2 m/z → 234.2 m/z and 349.1 m/z → 206.1 m/z respectively.

The initial full validation proved accuracy (determined by relative error) within guideline criteria ±15% (±20% at LLOQ) for both analytes. The between run precision ranged from 2.2 to 5.0% for enalapril and from 4.9 to 18.0% for enalaprilat. Additionally, a confirmatory partial validation was performed for accuracy and precision using four different runs in 2017 while making no change to assay. The obtained mean accuracy values of enalapril ranged from -3.9% to 8.4% and for enalaprilat from -12.0% to 6.4% (relative error) at all levels. Within run, precision varied from 4.7 to 7.5% for enalapril and from 2.6 to 10.3% for enalaprilat. Between run precision was as followed: 5.0 to 9.5% for enalapril and 4.3 to 13.4%

for enalaprilat. Moreover, internal standard normalized relative matrix effect was evaluated using seven donors (age = 29-86 years) of both gender at lower (enalapril: 0.39 ng/mL, enalaprilat: 0.35 ng/mL) and high concentration (enalapril: 200 ng/mL, enalaprilat: 180 ng/mL). The coefficient of variation (%CV) between donors ranged from 1.87 to 12.56% for both analytes at both levels. The absolute matrix effect for IS (benazepril) varied by -7.2 %CV. Other parameters including selectivity, recovery and stability were also evaluated (79).

3.2.2 Good clinical laboratory practice

Good clinical laboratory practice (GCLP) compliant environment ensures the generation of the quality data from the clinical trials. In the academia driven LENA project, such a GCLP compliant environment was established. This included GCLP training for laboratory as well as medical personnel (91), establishment of a standard operating procedure (SOP) system, traceable raw data generation, computerized system validation, guideline compliant reporting to make clinical outcomes reliable, reproducible and auditable. A tailored laboratory information management system (LIMS) was also implemented as part of GCLP for receiving, processing, storage, specimen detail and traceability of the data. The whole GCLP system was successfully audited by an external auditor.

At a glance, the conduct of sample collection and analysis was conducted as follows: At first ready-to-use pouches with unique pre-labelled consumables (collection tubes etc.) were sent to the respective clinical sites for sample collection. After sample collection and on-ward sample preparation by trained staff, samples were shipped back to the bioanalytical laboratory under tracked temperature conditions (-80°C). The received samples were kept at -80°C before and after the analysis. All bioanalytical determinations were conducted and documented using the four-eye principle. Once a measured batch was declared valid by the analysts, an additional double-check was performed by using a validated excel tool that confirmed the compliance with all quality specifications on CC, QCs, blank etc. The overall final release of the data was the responsibility of the head of the bioanalytical team.

3.2.3 Established quality control system for bioanalysis

An owned and customized institutionalized quality control system was enrolled. This established internal quality control system was comprised of evaluating CC data, QC samples and IS response data from each analytical batch. Moreover, the ISR evaluation of randomly selected study samples to further establish the quality of the data reported from unknown concentrations was also included. Bracketing the specifications from the EMA and FDA bioanalytical guidelines approaches within the established bioanalytical quality control systems and addressing the specific demands in pediatric research constituted the fit-for-purpose control system. The system suitability and performance qualification utilized before the study analysis is conducted to assure whether the system is suitable for the purpose required. This quality control system lies within the study sample analysis and evaluates the post validation analysis which in turn ensures the data quality. The control system was applied within the bioanalysis of the three pediatric studies of the LENA-project ("Labelling enalapril from neonates up to adolescents") with the trial registration numbers: EudraCT 2015-002335-17, EudraCT 2015-002396-18, EudraCT 2015-002397-21. Written informed consent from the parent(s)/legal representative and assent from the patient according to national legislation and as far as achievable from the child were obtained. The subsequent sections deal with the quality control of the individual parameter part of the quality control system.

3.2.3.1 Calibration curve

Calibration curve consistency and accuracy was considered as the first component of the adapted quality control process.

For pediatric sample analysis within the LENA-project, calibration curves for both analytes (enalapril and enalaprilat) were prepared freshly for each run in serum using a stock solution of known concentration. All calibration standards were purified and determined as described in section 2.1. Based on previously conducted validation, a linearity range from 0.195 to 200 ng/mL and 0.175 to 188 ng/mL was

established for enalapril and enalaprilat, respectively. Eleven calibration standards were used to construct a calibration curve by plotting x_i vs y_i. Where x_i represented analyte concentration ratio (analyte concentration/internal standard concentration) and y_i value represented an instrumental response ratio (peak area analyte/ peak area internal standard). Three replicates (n=3) were measured for each calibration level. Linear regression ($y = b + mx$) with a weighting factor ($w_i = \frac{1}{x_i^2}$) was applied to determine the regression parameters. The model parameters [intercept (b), slope(m)] of the weighted regression line were calculated by using the following expression (92):

$$Slope(b) = \frac{\sum w_i . \sum w_i x_i y_i - \sum w_i x_i . \sum w_i y_i}{\sum w_i . \sum w_i x_i^2 - (\sum w_i x_i)^2} \qquad \textbf{Equation (3-1)}$$

$$Intercept(m) = \frac{\sum w_i x_i^2 . \sum w_i y_i - \sum w_i x_i . \sum w_i x_i y_i}{\sum w_i . \sum w_i x_i^2 - (\sum w_i x_i)^2} \qquad \textbf{Equation (3-2)}$$

The coefficient of correlation (r -value) was used to express the dependence of two variable having linear relationship. The calibration curves with r –value ≥ 0.995 were considered linear (93). The following expression was used (92):

$$r = \frac{\sum w_i . \sum w_i x_i y_i - \sum w_i x_i . \sum w_i y_i}{\sqrt{\sum w_i . \sum w_i x_i^2 - (\sum w_i x_i)^2} . \sqrt{\sum w_i . \sum w_i y_i^2 - (\sum w_i y_i)^2}} \qquad \textbf{Equation (3-3)}$$

where x_i, y_i is the ith data pair of the n observations and w_i is the weighting applied.

Validity of the calibration curves was defined as follows: A maximum deviation of ±15% was allowed for each calibration standard compared to its nominal concentration. This accuracy criterion was applied to all concentration levels except of the lower limit of quantification (LLOQ) where ±20% were accepted. This is in line with recommendations given in EMA and FDA bioanalytical guidelines (61, 62). The deviation was assessed by calculating the relative error (%RE) using following expression (94):

$$\%RE = \frac{Con_{found} - Con_{actual}}{Con_{actual}} X\ 100 \qquad \textbf{Equation (3-4)}$$

Where $Con_{actual}$is nominal concentration and Con_{found} is predicted concentration or back-calculated concentration from the calibration curve.

Moreover, a minimum of 75% calibrators had to fulfil the back calculated concentration specifications for accuracy. Further, the calibrator levels with less than 50% passed replicates were also excluded. In case where the upper and the lower calibration level did not meet the criteria, the next calibration level was taken as acceptable lower or upper standards with new regression analysis as suggested by the international guidelines (61, 62). This still retained the criteria for fulfilling the minimum number of six calibration standards for a valid calibration curve. The resulting regression equation derived from the established calibration curves is characterized by a coefficient of correlation with r –value ≥ 0.995. Additionally, the variability in terms of coefficient of variation (%CV) in regression parameters (slope and intercept) was calculated for enalapril and enalaprilat from each run over the whole bioanalytical period.

3.2.3.2 Quality control samples

The CC is commonly measured once during routine clinical sample analysis, however, QC samples with known concentration are distributed equally as a measure for assay performance within routine clinical sample analysis.

Freshly prepared quality controls at five different levels were implemented to cover whole expected concentration range of the unknown samples for both analytes of interest. EMA guidelines suggest to include QC samples at three levels in duplicate or 5% of the study sample, or whichever number is high. However stricter criteria were implemented by using more levels and amount of the QC samples to monitor accuracy and precision in equidistance manner across the whole calibration range. In the applied 96-well approach, at least 10 single QCs, whereby five different quality control levels (ULOQ [200 ng/mL enalapril, 188 ng/mL enalaprilat, QC1], high [100 ng/mL enalapril, 94 ng/mL enalaprilat, QC2],

medium [25 ng/mL enalapril, 23.5 ng/mL enalaprilat, QC4], low [3.13 ng/mL enalapril, 2.93 ng/mL enalaprilat, QC7], about three times the LLOQ [0.78 ng/mL enalapril, 0.73 ng/mL enalaprilat, QC9]) were determined in duplicates. Passed criteria for all quality controls levels was fixed at accuracy of ±15% of their nominal concentration. At least 67% of total quality control samples and 50% in case of replicates at each level must meet the criteria as per international bioanalytical guideline for single runs (61, 62). The accuracy of the quality controls was expressed as %RE of their back-calculated concentrations by using **equation 3-4** and plotted them using box plots. Further marginal histograms were used to analyse the distribution of the observed concentration for quality control samples at all levels during the whole duration of the analysis.

The long-term reproducibility was determined in terms of the %CV. The mean pooled standard deviation (S_{pooled}) was calculated for each QC level for both analytes to establish the %CV by using the following expression (95):

$$S_{pooled} = \sqrt{\frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2 + \cdots + (n_k - 1){s_k}^2}{n_1 + n_2 + \cdots + n_k - K}} \qquad \textbf{Equation (3-5)}$$

where $n_1 + n_2 \ldots\ldots n_k - K$ is the degree of freedom and s_k is the standard deviation of the measured concentration at each QC level for K_{th} time within six months for both analytes.

Additionally, modified Westgard rules as multiple statistical rules were combined with Shewhart control charts. Westgard rules can detect any systematic and random variation in contrast to the "4-6-X" rule used for batch-wise acceptance, which refers to at least 67% of the QC samples should be within ±X% (where X = ±15%). However, the strict application of Westgard rules may lead to the rejection of acceptable data as per current guideline recommendations. Therefore, the following decision rules derived from the Westgard rules were applied: 1) Consecutive two points exceeds the action limit (mean±3sd); 2) Consecutive four points are outside warning limit (mean±2sd); and 3) Consecutive 11 points are on the same side of the mean to evaluate the control charts (96, 97). The mean

value was obtained from the observed concentration over six-month intervals depending upon the established stability of the stock solution.

3.2.3.3 Incurred sample reanalysis

The third component of the established quality assessment system was the evaluation of the ISR. The non-pooled samples from the dosed subjects (incurred samples) were used to demonstrate the reproducibility of the bioanalytical method on a different occasion in addition to QC samples. The ISR represents an important measure of accuracy and comparability within pediatric studies as the pediatric serum was not considered during method validation owing to ethical constraints, e.g., matrix effect was investigated with the human serum of adults. In particular, the ongoing maturation in childhood would have necessitated many pediatric matrix samples of several pediatric age groups to assess the possible effect of the difference in protein-binding, concomitant medication, matrix composition and changing metabolic behavior.
Sample analysis was performed blinded and all samples were selected on a random basis. As such, the goal was to reanalyze 7.5% of the total samples within ISR. The %difference of the reanalyzed incurred samples was calculated by using expression given in regulatory guidelines (61, 62)

$$\%difference = \frac{Repeat\ value - Original\ value}{Mean\ value} X\ 100\ \leq 20\% \qquad \textbf{Equation (3-6)}$$

The %difference of at least 67% of the total reanalyzed incurred samples within ±20% was considered as acceptable to establish method reproducibility. The Following expression was employed to calculate the %cumulative ISR (98):

$$\%\ Cumulative\ ISR = \frac{Number\ of\ ISR\ pair\ with\ absolute\ \%\ difference \leq 20\%}{Total\ number\ of\ ISR\ pairs} X100 \qquad \textbf{Equation (3-7)}$$

3.2.3.4 Internal standard response

An IS is normally employed during the analysis to compensate any discrepancies that arise during sample preparation, actual injection volumes and instrument

performance owing to the matrix effect, specifically in LC-MS/MS (88). Both the FDA and EMA guidelines suggest monitoring the IS response variation (86).

The IS response check was adapted as an additional parameter in the quality assessment system to establish the reliability of the results of unknown samples from the LENA clinical trials. Benazepril hydrochloride was used as IS at a concentration of 80 ng/mL which is a structural analogue to enalapril and enalaprilat. As deuterated IS was not commercially available at a time point of method validation, the structurally related compound benazepril was applied. The IS was added to all known calibration standards, QCs and study samples at equal concentration prior to the sample extraction process. Based on the obtained mean IS response of the known standards (CC and QCs), the detected IS response of each samples should vary between ±3sd of the mean. Deviations from this rule were only acceptable in case of justified reasons (97). Additionally, %CV from the first to the last injection within each analytical run for IS response was calculated for information.

3.2.3.5 Integration of the studied parameters

Beside single observation per parameter, the data was monitored for any trend in observed outliers of CC, QCs, unmatched ISR and deviation in IS. Therefore, all invalid runs were plotted using scattered matrix plots to identify any impact of extreme values among studied parameters.

3.3 Results

3.3.1 Calibration curve

38 CCs were constructed per analyte of interest (enalapril and enalaprilat) during the whole duration of bioanalysis from February 2016 to August 2018. For all valid CCs, a total of 939 and 919 calibration standards were measured for enalapril and enalaprilat, respectively. Within those measured calibration standards, 30 and 67 outliers were detected against the outlined criteria for enalapril and enalaprilat, respectively, resulting in 97% and 93% of total calibration standards within the guideline specific criteria for both analytes. This low number of outliers confirmed the goodness of fit of the weighted ($w_i = \frac{1}{x_i^2}$) linear regression model. Further, only two (enalapril) and six CCs (enalaprilat) were reconstructed with a narrowed linearity range for enalapril and enalaprilat, respectively, owing to outliers at the LLOQ and ULOQ. Out of total 38 analytical runs for each analyte, four runs for enalapril and five runs for enalaprilat were considered invalid owing to the inaccuracy of more than 50% of calibration standards.

Amongst these invalid runs, different standard levels were effected that subsequently does not allow to identify any trend. The overall pass rate for valid analytical runs was 84% with not more than two consecutive invalid runs. The slope varied from 1.0841 to 4.3641 with 22.69 %CV for enalapril and 0.1035 to 0.7581 for enalaprilat with 39.93 %CV during 31 months. Shapiro Wilk test showed slope values were normally distributed for enalapril (p = 0.127) and enalaprilat (p = 0.156). More variation was observed in intercept value between the runs however this variation caused no impact on the linearity of the CC (89). Slope and intercept data was represented in **Table 3-1**.

Table 3-1 *Slope and intercept values of calibration curve for enalapril and enalaprilat from valid analytical runs.*

Number of valid analytical run (February 2016 to August 2018)	Enalapril		Enalaprilat	
	Slope	Intercept	Slope	Intercept
1	3.8620	0.0164	0.5405	0.0013
2	4.3758	0.0089	0.4184	0.0008
3	2.8060	0.0019	0.3409	0.0004
4	3.0682	0.0046	0.3911	0.0002
5	3.4733	0.0067	0.4367	0.0004
6	3.4346	0.0030	0.4574	0.0006
7	2.9138	0.0094	0.4139	0.0004
8	3.0281	0.0019	0.4282	0.0008
9	2.4828	0.0012	0.1587	0.0008
10	3.3020	0.0041	0.4360	0.0011
11	2.9920	0.0024	0.2741	0.0010
12	2.4958	0.0526	0.4540	0.0006
13	1.0841	0.0000	0.2735	0.0020
14	2.4374	0.0176	0.1923	0.0001
15	2.5350	0.0014	0.4147	0.0002
16	1.8817	0.0009	0.3044	0.0002
17	2.6824	0.0033	0.5265	0.0010
18	2.7958	0.0015	0.4689	0.0005
19	2.3532	0.0008	0.4290	0.0003
20	2.5349	0.0003	0.3969	0.0001
21	2.4509	-0.0002	0.5039	0.0007
22	2.6068	0.0020	0.7581	0.0007
23	2.5828	0.0069	0.6755	0.0004
24	2.1048	0.0017	0.5480	0.0007
25	2.5507	0.0046	0.4306	0.0007
26	2.7781	0.0012	0.3599	0.0002
27	2.7896	0.0011	0.4186	0.0006
28	4.1120	0.0014	0.4050	0.0006
29	3.2384	0.0213	0.7055	0.0007
30	2.5860	0.0064	0.1872	0.0001
31	2.4090	0.0031	0.1133	0.0003
32	3.4986	0.0051	0.1035	0.0003
Mean	**2.8202**	**0.0060**	**0.4011**	**0.0006**
SD	**0.6400**	**0.0100**	**0.1602**	**0.0004**
%CV	**22.6944**	**164.8058**	**39.9318**	**66.3023**

SD=Standard deviation, %CV=Co-efficient of variation

3.3.2 Quality control samples

Investigation of QC samples resulted in the exclusion of an additional two enalapril run. In both runs, 50% of lower QC (3xLLOQ = 0.76 ng/mL) failed to pass (±15%). One invalid run for enalaprilat was found to be associated with only 58% QC (±15%) passed (67% required according to the guideline). Here, also the QC level at 3xLLOQ caused the invalidity (less than 50% of standards within the accuracy limits). Within 32 valid analytical runs, based upon the CC and the QC sample acceptance criteria, on average, 94% of enalapril QC samples were within guideline limits [(ULOQ, 200 ng/mL = 94%), (high, 100 ng/mL = 90%), (medium, 25 ng/mL = 94%), (low, 3.13 ng/mL = 96%), (3 x LLOQ, 0.78 ng/mL = 95%)]. Similarly, 89% of all enalaprilat QC samples were within the limits with individual success rates: [(ULOQ, 180 ng/mL = 92%), (high, 90 ng/mL = 92%), (medium, 22.5 ng/mL = 92%), (low, 2.81 ng/mL = 91%), (3xLLOQ, 0.70 ng/mL = 78%)]. These results exhibited strong agreement with the guidelines recommended in terms of acceptance criteria of at least 67% passing QC checks. The distribution of the observed back-calculated concentrations for the QC samples at all levels for both analytes was observed using marginal histogram **(Figure 3-1)**. The upper specification limit (USL) and lower specification limit (LSL) correspond to the target range recommended by the guidelines (±15% of the nominal concentration). The grey shaded area represents the exact number of 67%+ QC control samples. At all levels, this grey area did not surpass the USL or LSL, thereby indicating guideline compliance. The box plots were employed to observe the variation in %RE for back-calculated concentration at all QC levels from the valid runs **(Figure 3-2)**. The box plot showed that the mean and median was cantered at all QC levels with equal variation in the upper and lower quantiles (except for the LLOQ of enalaprilat with a slightly higher variability). The %CV was employed to observe the long-term reproducibility and it ranged from 3.6% to 10.6% for enalapril **(Table 3-2)** and 5.7% to 10.4% for enalaprilat **(Table 3-3)**. As the new stock solution was regularly prepared every six months due to stability reasons. Also depending upon the total number of valid and invalid analytical runs within this period, the number of runs differed as only valid runs were considered

for analysis. **Figure 3-3** depicted the trend analysis charts for enalapril and enalaprilat. The plotted graphs were investigated against the pre-defined derived Westgard rules and it was observed that no violation of these rules was found, hence providing more confidence in terms of the method applicability over the entire duration of the bioanalysis.

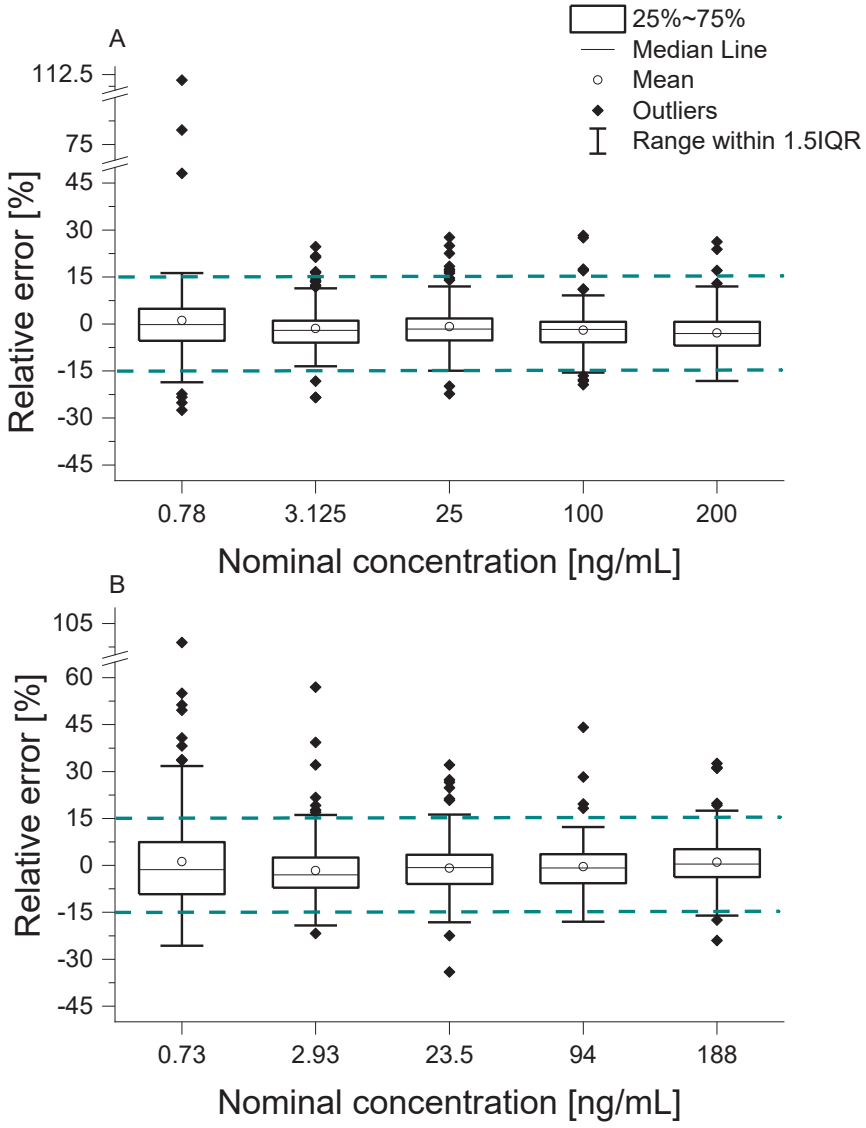


Figure 3-1 *Box plots for %relative error of the back-calculated concentration of the QC samples for enalpril and enalaprilat from all valid analytical runs. Any observation outside the range Q1–1.5 × IQR and Q3 + 1.5 × IQR was considered as an outlier;* ***A)*** *enalapril,* ***B)*** *enalaprilat. QC = Quality control*

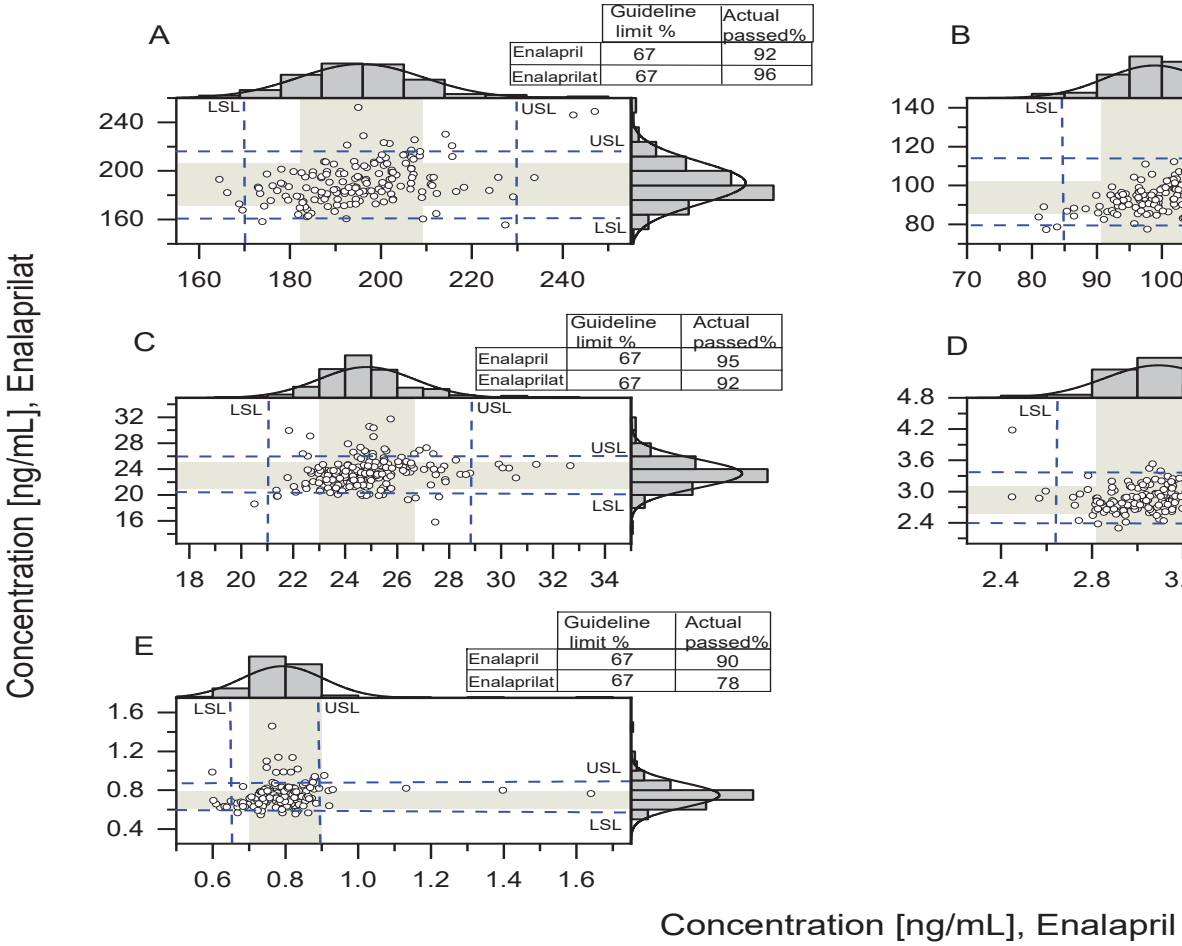


Figure 3-2 *Marginal histograms for all QC levels for enalapril and enalaprilat. LSL, lower specification limit (− 15% specification limit (+ 15% of the nominal concentration); gray-shaded area, one standard deviation (1SD) of the mean of the back calculated concentrations. Dotted lines represent the specification limits of ±15%.* ***A)*** *(ULOQ [200 ng/mL enalapril ng/mL enalapril, 94 ng/mL enalaprilat, QC2],* ***C)*** *medium [25 ng/mL enalapril, 23.5 ng/mL enalaprilat, QC4],* ***D)*** *low [3.13 ng/* ***E)*** *three times the LLOQ [0.78 ng/mL enalapril, 0.73 ng/mL enalaprilat, QC9]). ULOQ = Upper limit of quality control, LLOQ*

Table 3-2 *Long-term reproducibility of all conducted bioanalytical runs of enalapril.*

	Enalapril solution (ng/mL)				
	1	**2**	**3**	**4**	**5**
200	187.32	183.33	172.15	192.27	204.76
	194.38	195.18	188.68	199.79	202.53
	194.06	208.89	200.20	183.50	206.49
	199.30	189.27	187.96	205.92	196.11
	189.19	220.66	205.28	181.79	186.89
				197.99	188.40
				201.36	209.21
S_{pooled}	10.30				
Mean	195.46				
%CV	5.27				
100	104.36	86.92	95.08	99.65	
	99.29	92.87	97.18	100.91	
	97.95	99.74	98.68	101.73	
	95.41	96.60	103.47	100.65	
		97.70	96.59	99.87	
		101.32	97.74		
S_{pooled}	3.58				
Mean	98.43				
%CV	3.64				
25	25.14	24.13	24.03	23.40	28.12
	23.98	25.84	24.53	24.39	25.23
	23.88	25.16	25.01	25.56	25.46
	24.76	24.74	24.33	25.75	24.97
	25.23	24.57	24.60	25.52	23.20
	23.74		23.31	23.62	24.72
				23.61	26.60
					25.33
S_{pooled}	0.99				
Mean	24.81				
%CV	3.99				
3.13	3.44	2.98	3.07	2.86	3.22
	3.12	3.33	3.01	3.02	3.07
	2.94	3.12	3.09	3.20	3.20
	3.04	3.08	2.96	3.18	3.12
	3.35	2.96	3.19	2.82	3.02
	3.13		2.99	3.27	3.22
				3.02	3.25
					3.14
S_{pooled}	0.14				
Mean	3.10				
%CV	4.49				
0.78	0.84	1.15*	0.76	0.87	
	0.82	0.82	0.79	0.75	
	0.76	0.73	0.80	0.81	
	0.85	0.75	0.82	0.77	
		0.77	0.77	0.81	
		0.74	0.75	0.81	
			0.73	0.78	
				0.77	
S_{pooled}	0.09				
Mean	0.80				
%CV	10.63				

*SD, standard deviation; Spooled, pooled standard deviation. Number of enclosed runs differed within each 6-month period as only valid analytical run were enclosed. *Single values outside the acceptance limit (± 15%).*

Table 3-3 *Long-term reproducibility of all conducted bioanalytical runs of enalaprilat.*

	Enalaprilat solution (ng/mL)				
	1	**2**	**3**	**4**	**5**
188	167.63 177.19 180.11 183.84 181.76 171.46	182.24 179.18 197.66 219.11*	171.75 205.51 186.91 185.10 182.05 199.40	187.68 194.20 180.17 192.33 178.55 188.67 196.84	207.36 208.94 187.18 187.51 193.11 221.54 219.27
S_{pooled}	11.71				
Mean	190.57				
%CV	6.14				
94	87.83 108.24* 88.06	83.43 86.97 97.10 87.61 90.77 96.64 96.86	90.94 92.39 95.81 95.08 94.75 91.33 97.34	98.58 99.96 91.53 94.88 97.95	
S_{pooled}	5.49				
Mean	93.82				
%CV	5.86				
23.5	21.17 23.35 20.99 22.28 22.66 22.59	22.10 22.16 26.30 22.46	21.62 23.19 23.79 24.72 22.13 23.31 23.31	22.04 23.03 24.13 22.89 23.03 21.62 22.43	27.43 24.49 23.09 23.96 22.92 25.78 26.93 23.08
S_{pooled}	1.35				
Mean	23.27				
%CV	5.81				
2.87	2.67 3.00 2.73 2.72 2.88 2.77	2.74 2.72 2.85 2.81	2.87 2.68 3.00 2.95 2.63 2.96 2.77	2.66 2.83 2.91 2.80 2.62 3.14 2.85	3.08 2.99 2.99 3.27 2.81 3.17 3.10 3.55*
S_{pooled}	0.16				
Mean	2.89				
%CV	5.70				
0.73	0.75 0.69 0.92*	0.93* 0.80 0.74 0.82 0.68 0.73 0.66	0.80 0.72 0.73 0.66 0.70 0.78 0.62	0.78 0.75 0.66 0.77 0.83 0.79 0.79 0.84	
S_{pooled}	0.08				
Mean	0.75				
%CV	10.41				

*SD, standard deviation; Spooled, pooled standard deviation. Number of enclosed runs differed within each 6-month period as only valid analytical run were enclosed. *Single values outside the acceptance limit (± 15%).*

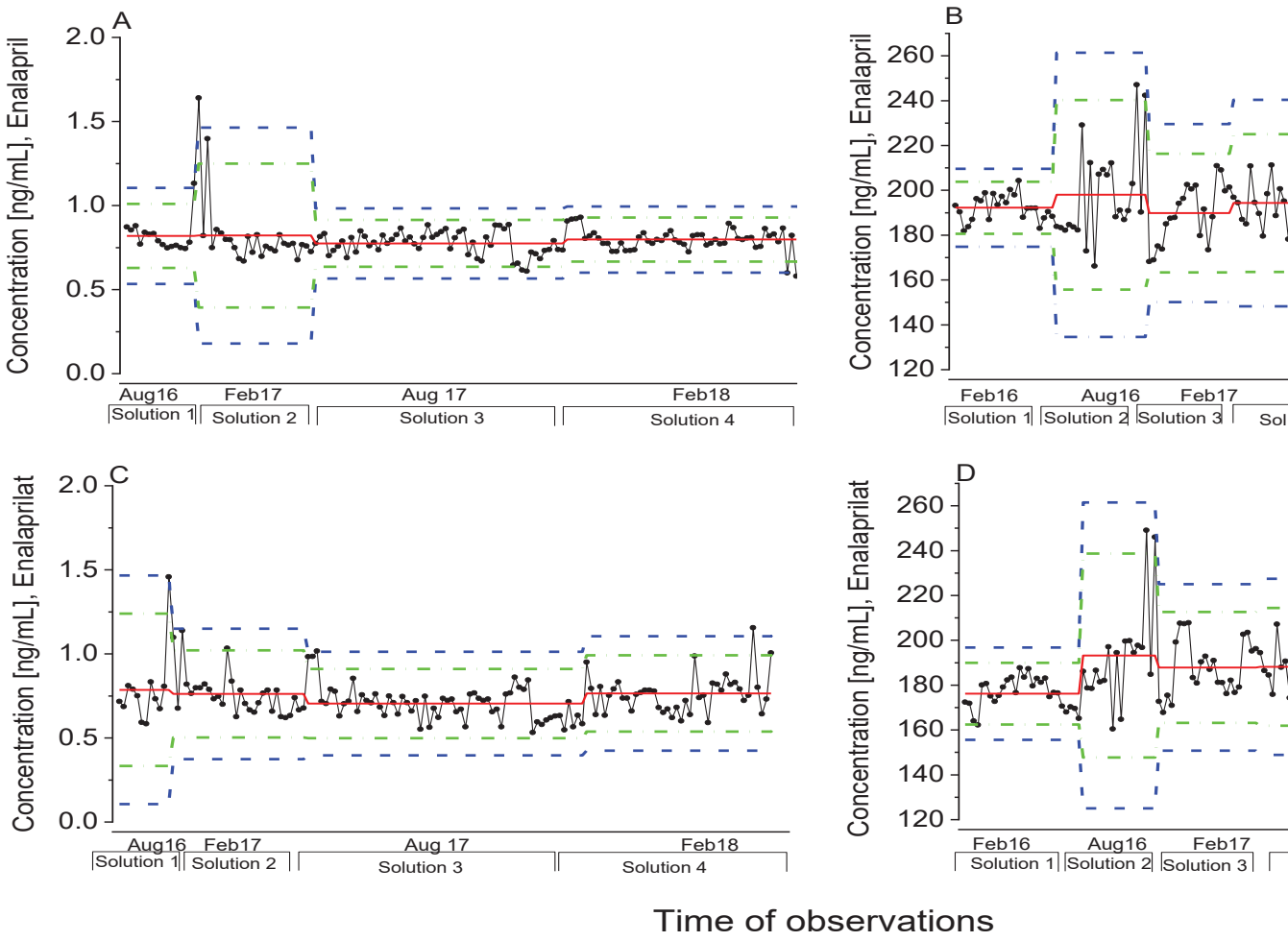


Figure 3-3 *Process control charts for quality control samples for enalpril and enalaprilat at 3 × LLOQ and ULO* ***B*** *enalapril (ULOQ): 200 ng/mL);* ***C*** *enalaprilat 3 × LLOQ = 0.70 ng/mL;* ***D*** *enalaprilat (ULOQ): 188 ng/mL. ULOQ=Upper limit of quality control. Solution = New stock solution prepared after every six months.*

3.3.3 Incurred sample reanalysis

Based upon the pre-calculated estimated sample size for the LENA clinical trials in accordance with the study protocols, almost 7.5% of incurred samples were reanalyzed for enalapril and enalaprilat. This calculation took into account that not all scheduled samples might become available because of the missing sample point and inappropriate sample volume. In total, 93 and 103 randomly selected incurred samples were reanalyzed for enalapril and enalaprilat, respectively. Enalapril (71%) and enalaprilat (67%) were within predefined guideline criteria of ±20% for at least 66.7% of the total reanalyzed samples showing the reproducibility of the applied method. The Bland-Altman plot revealed that randomly selected sample concentrations ranged between 0 ng/mL (below LLOQ values reported as zero) to 45 ng/mL for both enalapril and enalaprilat. The contribution of each ISR pair towards the overall performance of at least 67% ISR was shown by using %cumulative graphs **(Figure 3-4).**

3.3.4 Internal standard response

Internal standard (IS) response variation through the analytical run can be used to assess the validity of the results and hence the acceptability of the unknown results. High robustness of the internal standard response was observed during the whole study period of February 2016 to August 2018 indicated by acceptable %CV values of 3.0 to 20.9 for all analytical runs. Only for two runs, the %CV values of 63.5 and 44.1 were detected. However, as an adequate signal to noise ratio was maintained for all samples; therefore, a negative impact on reliable quantification of unknown samples was excluded (97). Subsequently, only a few individuals IS response per analytical run that exceeded the maximum limit of ± 3sd of the run-specific mean were found indicating random errors.

3.3.5 Integration of the studied parameters

The plotted data of six invalid runs exhibited that number of more outliers in CC was associated with decrease passed QC (%). Increased in slope was associated with increased peak area ratio. A large shift in IS response was found to be indirectly related to peak area ratio and slope. Any deviation in these parameters

showed relation to the unmatched ISR results between original and reanalysed analytical runs. However, the small number of invalid runs restricted any definite inferences while evaluating this data set.

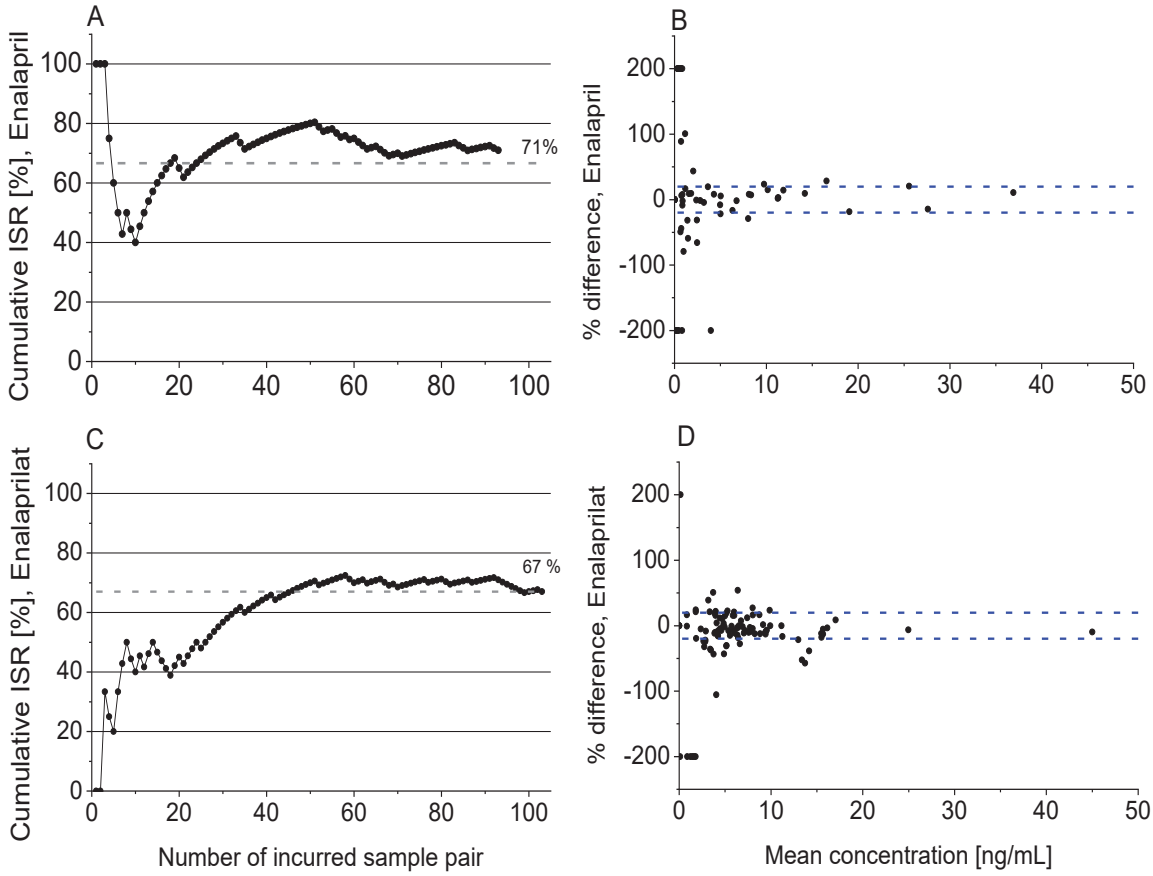


Figure 3-4 *%Cumulative plot and %difference plot for enalapril and enalaprilat incurred sample reanalysis (ISR).* ***A*** *%cumulative plot for enalapril;* ***B*** *%difference plot for enalapril;* ***C*** *%cumulative plot for enalaprilat;* ***D*** *%difference plot for enalapril. Blue-dashed upper and lower lines: ± 15% limit; gray-dashed line: 66.7%; calculation of %difference: Repeat − Original/Mean × 100. The different total number of valid enalapril and enalaprilat ISR pairs is due to the fact that the validity of the two substances per analytical run was independently determined. Thus, for each analytical run, a valid evaluation could possibly only be made for one substance, so that only these ISR pairs were included in the evaluation. The ISR pair are plotted according sequence of reanalysis. Additionally, each pair does not reflect the same sample for enalapril and enalaprilat.*

3.4 Discussion

A fit-for-purpose quality control system in pediatric research was successfully developed. It addresses current bioanalytical requirements advised in international guidelines (EMA, FDA) and also encompasses the specific situations in pediatric research current insufficiently reflected by guidelines. This quality control system analyzed a multi-parameter approach and their relationship to each other during three pediatric clinical trials within the EU-funded LENA project. The customized system monitored and ensured reliable assay performance over the whole period of bioanalysis. This developed in-house quality control system applies primarily for analysis using LC-MS/MS. For immunoassay or bioassay, different criteria are defined in current bioanalytical guidelines and should be implemented into separate quality control systems accordingly.

As PK data obtained in adults can only be very moderately extrapolated to the vulnerable pediatric population, it became obvious that clinical trials in the pediatric population are highly necessary for rational and safe drug therapy. However, these studies are often lacking or being discontinued (91). Unfortunately, clinical trials are commonly only conducted once in the pediatric population owing to, e.g., ethical constraints. It is therefore of utmost importance to generate high-quality data in the limited amount of conducted pediatric studies as this data forms the bases for evidence-based pharmacotherapy in this population. From a bioanalytical point of view, comprehensive method validation is a regulatory prerequisite for reliable data generation, and subsequently for method application in clinical trials. Nevertheless, such validations are often performed only once and do not automatically ensure reliability over the entire period of a clinical trials. Additionally, blood samples of healthy or diseased pediatrics, especially at very young ages, are often unavailable for validation based on ethical constraints and can be taken into consideration during the earliest analytical runs. Although EMA and FDA outlined the specifications for individual bioanalytical runs, monitoring comparability of study data in continuous bioanalysis is unattended. The developed control system was meant to overcome these current hurdles, especially in pediatric research where "en bloc" bioanalysis is often impossible because of the long recruitment periods.

Well-established regulatory requirements regarding the suitability of CC and within-run QCs were directly incorporated in the present control system. However, long-term comparability indicates reliability in the case of the bioanalytical data. Therefore, the control system was amended concerning between-run QCs as well as the long-term performance of IS and ISR. With regard to the monitoring of between-run quality control performance and comparability, multiple statistical rules, known as Westgard rules, were implemented. These have sought to assess any systematic and random variation between-run performance in contrast to the guideline approach by the “4-6-X rule” (at least 67% of the data should be within ±X%) focusing on single analytical runs. However, the strict application of all Westgard rules may lead to the rejection of the acceptable data as per current guideline recommendations. Therefore, modified rules based on suggestions from Bruijnsvoort *et al*. were applied (96). No violation of the modified Westgard rules was observed across any bioanalytical runs of the LENA project, thereby indicating no systemic pattern or random variation in the applied bioanalytical method.

In pediatric research, many clinical trials last for several years owing to, e.g., poor recruitment. Therefore, long-term reproducibility of the method is important as the stability of analytes of interest is often limited and needing continuous bioanalysis. The reliable long-term performance was assessed via the concept of pooled standard deviation, which provided a strong estimate of variation along with the whole duration of the analysis characterized by coefficients of variation (%CV) ranging between 3.6% to 10.6% for enalapril and 5.7% to 10.4% for enalaprilat. Moreover, no remarkable variation amongst the different QC levels was observed by comparing the relative error over time and level. The latter proved that no QC check differed substantially or level tended toward inaccuracy over time. Overall, evaluation of within and between-run QC checks supported reliable bioanalysis and confidence for the measured unknown concentrations over a long period of time. The actual matrix of unknown samples could substantially impact precise and accurate determination. Usually, the impact of the sample matrix is investigated during method validation. However, ethical constraints impede the investi-

gation of a pediatric sample matrix as it may potentially differ from adults. Nevertheless, the maturation of the pediatric organism, unknown metabolites, concomitant medications and changing protein-binding reflects certain reasons for possible imprecisions during bioanalysis within clinical trials (99, 100).

The use of ISR of pediatric samples was therefore implemented into the bioanalytical quality control system to evaluate the impact of the actual matrix on reproducibility and subsequently on accuracy and precision. Sample volume restrictions and preferable measurement of the PK primary endpoint and secondary PD endpoints using the same sample volume restricted the reanalysis of the incurred samples. Almost 7.5% of the incurred sample was reanalyzed for both analytes. Although the FDA asks for 10% of reanalyzed samples for the first 1000 samples, it reached 7.5% of ISR with a total of 1250 unknown samples within these pediatric trials, thereby appearing sufficient bearing in mind ethical constraints for the sample volume involving vulnerable pediatric population. Rudzki et al (101) have demonstrated that reproducibility of the assay is not exclusively dependent on ISR fixed-rate (e.g. 10% for 1000 samples and 5% for subsequent samples) as currently recommended in regulatory guidelines. They have proposed to use fixed numbers of ISR pairs (e.g. 30) instead of a fixed rate as it sufficiently allows to check the reproducibility and non-reproducibility of the assay.

Bridging both current suggestions together, here evaluated 100 ISR pairs (7.5%) sufficiently allowed for appropriate assessment of reproducibility. The FDA recommendations on ISR suggested using samples at/close to the maximum concentration (C_{max}) and near the end of the elimination phase (102). This condition was difficult to comply with during the pediatric LENA project for several reasons. First, bioanalysis of enalapril and enalaprilat was conducted blinded and randomized. Second, the pro-drug, enalapril, and its active metabolite, enalaprilat, are characterized by different PK parameters (in adults: t_{max} at roughly 1 hour vs. approximately 4 hours) and subsequently would necessitate more samples to be analyzed to address the regulatory conditions appropriately.

In added to the above restrictions, the bioanalysis was performed completely independent of PK evaluation. The latter was only started after the study samples

had been successfully analyzed. Thus, there was no feedback from the PK analysis to identify samples at the C_{max} or elimination phase of each patient. Therefore, recommendations to select only samples in the range of three times LLOQ and 80% of ULOQ could not be realized. Thus, performing this sample selection for reanalysis was a worst-case scenario. Furthermore, it should be kept in mind that the study population is very heterogeneous due to the maturation of the organism and that no uniform C_{max} and elimination concentrations, such as these might be expected from adult studies, could be derived. There is no detailed information available about the C_{max} and concentration at the elimination phase of enalapril and its active metabolite in pediatrics. Two studies revealed enalaprilat serum concentration 12.7 ng/mL at dose of 0.08 mg/kg in congestive heart failure patients (age < 12 months) and 2-25 ng/mL in children (age 2 months – 15 years) at a dose 0.07 to 0.14 mg/kg with hypertension respectively (103, 104). Within the here presented quality control system, concentrations between 0-37 ng/mL (enalapril) and 0-45 ng/mL (enalaprilat) were determined **(Figure 3-4)**. Therefore, based upon the age range of studied population from neonates to 12 years, it was anticipated that randomly selected incurred samples appropriately covered C_{max} and concentration values around the elimination phase for different age range. Moreover, it was assumed that reanalyzed incurred sample covered the lower, middle and the higher concentration ranges across pediatric age and assay range.

The possible reasons for close agreement of the ISR (enalapril = 71%; enalaprilat = 67%) to the guideline acceptance limit (66.7%) were found to be the variations associated with peak area ratio, slope and IS response. In case of enalapril, one run covering more number of incurred samples than commonly selected was reanalyzed in August 2018, which were first analyzed in October 2016. The observed high between run IS variation was assumed to had an impact on unmatched ISR (60% difference in IS). For enalaprilat between run slope variation (original run in March 2017: 0.457 vs ISR run in April 2017: 0.273) was a possible reason for unmatched ISR. Both run named were conducted with a higher amount of ISR if compared to other bioanalytical runs conducted within the LENA project. Therefore, it is suggested to distribute ISR sample equally across all analytical

runs to avoid biasing the ISR results by borderline runs (e.g. high variability in IS response) that include higher numbers of ISRs. However, there is still much consensus to be achieved in this area regarding the selection, number and control criteria of the ISR (86).

The international bioanalytical guidelines preferred to implement labelled IS like deuterated, C^{13} or N^{15} which may compensate more efficiently for matrix effect during LC-MS/MS analysis. The current method utilized non labelled IS because deuterated IS was unavailable commercially at the time of method validation. During validation IS-normalized matrix effect was within guidelines for both analytes. Nevertheless, application of labelled IS should be prioritized whenever applicable. If a labelled IS is not applicable for any reason, it is advisable to monitor in parallel for the specific mass transition of phospholipids (e.g. m/z: 524.0/184.0) (105). Mostly phospholipids (glycerophosphocholines and lysophospholipids) are associated with ion suppression or ion enhancement (106). Therefore, their monitoring allows observing possible matrix effect that unlabeled IS might not compensate sufficiently.

The LENA project collected unique data on the treatment of children aged 0-12 years with heart failure with angiotensin converting enzyme inhibitor therapy. The data collected should meet the high-quality requirements known for clinical trials in a regulated environment. Therefore, a GCLP-compliant environment was created for the collection of bioanalytical data that could be accomplished despite the limited personal and financial situation of the academic project. The results showed that such a quality ensuring approach like the here presented quality control system is worthwhile and achievable from an academic point of view. It ensured the optimal monitoring and evaluation of bioanalytical data. Invalid data, which otherwise would not be detected if only the validity of the particular batch would have been monitored, were identified and contributed to the increase in quality. This outcome showed that this undertaking was feasible and should encourage other research groups in e.g. academia to establish a comparable system to generate comparable high-quality data. A fit-for-purpose quality control system pertinent to pediatric research was successfully developed. It addresses current bioanalytical requirements of international guidelines (EMA, FDA) but

also encompasses specific situations in pediatric research. Descriptive statistical and graphical representations allowed for monitoring bioanalytical data quality of three pediatric studies.

4. Overall conclusion and perspective

The bioanalytical methods are the mainstay to determine the unknown concentrations of the drugs and their metabolites for the pharmacokinetic and pharmacodynamic interpretation during clinical trials. Monitoring the continuous performance of the bioanalytical method provides substantial reliability. The developed method undergoes validation as one of the steps to make the method applicable for its intended purpose. Once validated, the bioanalytical methods are repeatedly applied during the whole duration of the clinical trials. Therefore, monitoring the method performance for corrective or preventive actions over the whole life cycle is critical for maintaining the quality of the bioanalytical data.

The sensitive screening LC-MS/MS method was developed for a mixture of commonly prescribed angiotensin-converting enzyme inhibitors using residual blood volume to avoid additional sampling stress making its applicability for adults as well as in pediatrics. Fit-for-purpose method validation including the limit of detection, recovery and absolute matrix effect required for the screening method was also performed to make method qualified for its intended applicability. As a future perspective, the developed screening method can be implemented for fast and reliable evaluation of medication adherence as well as intentional/unintentional intoxication of commonly used ACEIs. Its short run time, the lower limit of detection and requirement of small plasma volume (50 µL) for reliable determination enables even its application in children. The fit-for-purpose method validation qualifies the developed method for its intended purpose.

Additionally, the multiparameter in-housed quality control system was proposed utilising the accompanied bioanalytical data to establish the reliability of the reported unknown concentrations. The developed system was applied to bioanalytical data generated during LENA clinical trials. Fulfilling the general recommendation from the EMA and FDA guidelines, the developed quality control system supported to monitor the long term method performance to establish data quality for reliable pharmacokinetic evaluation to enhance the patient safety.

The proposed quality control system is valuable because, in the absence of such quality control system, only the reanalysis of incurred samples is the indicator of

the method reproducibility and hence the data quality. But it is not feasible to include the incurred samples in each analytical run specifically in pediatric studies owing to limited sample volume. Therefore, applying such quality control system would be practical and achievable. It ensured the optimal long term monitoring and evaluation of bioanalytical data. Invalid data, which otherwise would not be detected if only the validity of the particular batch would have been monitored, were identified and contributed to the increase in quality.

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7. Publications and posters

The parts of this thesis have already been published in international peer reviewed journals and were previously presented at conferences as posters:

Publications

1**. Ali M**, Tins J, Burckhardt BB, on behalf of the LENA consortium. Fit-for-Purpose quality control system in continuous bioanalysis during long-term pediatric studies. The AAPS Journal (2019) 21: 104.

2. **Ali M**, Läer S, Burckhardt BB. LC-MS/MS method for screening of intoxication and drug adherence of angiotensin converting enzyme inhibitors in plasma. Bioanalysis. 2018.

3. Feickert M, Burdman I, Makowski N, **Ali M**, Bartel A, Burckhardt BB. A continued method performance monitoring approach for the determination of pediatric renin samples - application within a European clinical trial. Clinical Chemistry and Laboratory Medicine (CCLM). vol:58, iss:6. 2019.

4. Makowski N, Ciplea MA, **Ali M,** Burdman I, Bartel A, Burckhardt BB. A comprehensive quality control system suitable for academic research-Application in a paediatric clinical study. Submitted to Bioanalysis 2019, Under revision

Posters presentations

1. **Ali M**, Laeer S, Burckhardt BB. LC-MS/MS method for screening of intoxication and drug adherence of angiotensin converting enzyme inhibitors in plasma. Mass Spectrometry Application in Clinical Lab (MSACL) Salzburg, Austria (September 2019).

2. **Ali M,** Tins J, Burckhardt BB. Establishing the integrity of the continuous paediatric pharmacokinetic bioanalysis for clinical trial within an academia environment using quality assessment process. European Society for Developmental Perinatal and Paediatric Pharmacology (ESDPPP) Basal, Switzerland (May 2019).

3. Feickert M, Burdman I, Makowski N, **Ali M**, Farahani S, Majid H, Ciplea MA, Bartel A, Burckhardt BB. Reliable results in continuous bioanalysis of paediatric renin samples – Comprehensive quality assessment within clinical studies in children. European Society for Developmental Perinatal and Paediatric Pharmacology (ESDPPP) Basal, Switzerland (May 2019).

8. Appendices

Appendix I: *Mean back calculated concentrations of calibration curve for all analytes*.

Appendix II: *Within-run and between run accuracy and precision for all analytes*.

***Appendix I**: Mean back calculated concentrations of calibration curve for all analytes (n = 3).*

	Benazepril (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.78	1.50	2.92	6.50	12.73	24.37
SD	0.01	0.21	0.14	0.52	0.58	1.08
CV (%)	1.45	13.70	4.86	8.05	4.53	4.43
Accuracy (%)	100.47	96.37	93.29	99.64	101.87	97.47
	Enalapril (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.77	1.48	3.12	6.68	12.50	24.77
SD	0.01	0.25	0.06	0.51	0.85	0.42
CV (%)	1.58	16.97	1.82	7.70	6.84	1.68
Accuracy (%)	98.59	94.87	99.79	106.88	100.00	99.07
	Enalaprilat (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.77	1.56	2.94	6.67	12.50	24.33
SD	0.02	0.21	0.21	1.03	0.66	1.62
CV (%)	2.73	13.32	7.20	15.41	5.25	6.64
Accuracy (%)	99.27	100.21	93.93	106.77	100.00	97.33

	Perindopril (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.74	1.53	3.02	7.49	12.87	25.53
SD	0.06	0.45	0.08	0.38	0.31	3.10
CV (%)	7.62	29.53	2.75	5.10	2.37	12.14
Accuracy (%)	94.32	98.08	96.59	119.89	102.93	102.13
	Perindoprilat (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.80	1.41	3.04	6.41	12.47	24.53
SD	0.03	0.14	0.07	0.38	0.38	0.51
CV (%)	4.07	10.23	2.24	5.95	3.04	2.09
Accuracy (%)	102.31	90.38	97.23	102.56	99.73	98.13
	Quinapril (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.79	1.36	3.09	6.17	12.67	24.73
SD	0.03	0.29	0.19	0.14	0.50	0.51
CV (%)	3.25	21.20	5.99	2.25	3.97	2.07
Accuracy (%)	101.50	87.18	98.72	98.72	101.33	98.93

	Quinalprilat (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.78	1.41	3.23	6.56	12.40	24.67
SD	0.01	0.23	0.38	0.66	1.11	1.69
CV (%)	1.72	16.48	11.86	10.01	8.98	6.86
Accuracy (%)	100.17	90.17	103.19	105.01	99.20	98.67
	Ramipril (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.74	1.47	3.33	7.21	13.00	24.67
SD	0.04	0.42	0.17	0.60	0.53	0.60
CV (%)	4.73	28.54	5.11	8.37	4.07	2.44
Accuracy (%)	95.09	93.91	106.28	115.31	104.00	98.67
	Trandolapril (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.79	1.50	3.03	6.78	12.40	24.50
SD	0.01	0.32	0.13	0.70	0.10	0.40
CV (%)	0.70	21.18	4.29	10.40	0.81	1.63
Accuracy (%)	100.94	96.15	96.81	108.43	99.20	98.00

	Trandolaprilat (ng/mL)					
Nominal con.	**0.78**	**1.56**	**3.13**	**6.25**	**12.5**	**25**
Mean back calculated con.	0.76	1.55	3.14	6.16	12.60	23.87
SD	0.03	0.25	0.15	0.27	0.82	1.12
CV (%)	4.12	16.05	4.62	4.45	6.50	4.67
Accuracy (%)	97.86	99.15	100.21	98.56	100.80	95.47

SD = Standard deviation, CV (%) = % co-efficient of variation,

***Appendix II**: Within-run and between run accuracy and precision for all analytes.*

	Nominal concentra-tion [ng/mL]	**Calculated concentration [ng/mL]**			**Between-run accuracy and precision**		
Benazepril		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1		2.59	3.25	2.42			
Injection 2		2.78	3.02	2.38			
Injection 3	**3.13**	2.39	3.08	2.36	**2.73**	**-12.82**	**11.79**
Injection 4		2.69	3.05	2.28			
Injection 5		2.74	3.03	2.86			
Within-run mean		**2.64**	**3.09**	**2.46**			
Within-run RE% mean		**-15.71**	**-1.41**	**-21.34**			
Within-run RSD%		**5.91**	**3.06**	**9.28**			
Injection 1		23.4	23.5	25.90			
Injection 2		21.7	25	25.50			
Injection 3	**25**	21.8	24.3	26.30	**23.93**	**-4.26**	**6.60**
Injection 4		21.8	26.3	20.40			
Injection 5		22	23.6	27.50			
Within-run mean		**22.14**	**24.54**	**25.12**			
Within-run RE% mean		**-11.44**	**-1.84**	**0.48**			
Within-run RSD%		**3.22**	**4.70**	**10.91**			
Injection 1		79.90	97.70	105.00			
Injection 2		88.30	97.20	104.00			
Injection 3	**100**	83.50	96.90	101.45	**95.57**	**-4.43**	**10.99**
Injection 4		83.40	96.90	110.00			
Injection 5		86.40	97.90	105.00			
Within-run mean		**84.30**	**97.32**	**105.09**			
Within-run RE% mean		**-15.70**	**-2.68**	**5.09**			
Within-run RSD%		**3.81**	**0.47**	**2.95**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Enalapril		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD %**
Injection 1		2.96	2.83	2.49			
Injection 2		3.06	3.02	2.95			
Injection 3	**3.13**	3.11	3.16	2.48	**2.98**	**-4.93**	**6.60**
Injection 4		3.24	3.09	2.70			
Injection 5		3.07	3.35	3.10			
Within-run mean		**3.09**	**3.09**	**2.75**			
Within-run RE% mean		**-1.34**	**-1.27**	**-12.17**			
Within-run RSD%		**3.28**	**6.16**	**10.05**			
Injection 1		23.5	24.10	24.65			
Injection 2		23.2	24.80	25.10			
Injection 3	**25**	22.8	24.90	24.95	**24.42**	**-2.32**	**3.15**
Injection 4		23.7	25.30	25.35			
Injection 5		24.5	24.70	24.75			
Within-run mean		**23.54**	**24.76**	**24.96**			
Within-run RE% mean		**-5.84**	**-0.96**	**-0.16**			
Within-run RSD%		**2.70**	**1.75**	**1.11**			
Injection 1		84.50	94.40	102.10	**95.22**	**-4.77**	**8.43**
Injection 2		0	98.70	105.00			
Injection 3	**100**	85.70	98.40	107.50			
Injection 4		85.80	95.20	99.65			
Injection 5		86.30	92.60	100.30			
Within-run mean		**86.90**	**95.86**	**102.91**			
Within-run RE% mean		**-13.10**	**-4.14**	**2.91**			
Within-run RSD%		**3.49**	**2.75**	**3.20**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Enalaprilat		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1		2.96	2.89	2.88			
Injection 2		2.86	2.31	3.09			
Injection 3	**3.13**	2.71	2.67	2.33	**2.81**	**2.83**	**-10.18**
Injection 4		2.99	2.71	3.07			
Injection 5		2.63	3.04	3.02			
Within-run mean		**2.83**	**2.72**	**2.88**			
Within-run RE% mean		**-9.58**	**-12.97**	**-7.98**			
Within-run RSD%		**5.53**	**10.09**	**10.96**			
Injection 1		23.10	23.60	27.20			
Injection 2		21.50	23.20	26.05			
Injection 3	**25**	22.70	22.10	24.85	**24.07**	**-3.72**	**7.10**
Injection 4		24.50	25.30	24.85			
Injection 5		21.80	23.30	27.00			
Within-run mean		**22.72**	**23.50**	**25.99**			
Within-run RE% mean		**-9.12**	**-6.00**	**3.96**			
Within-run RSD%		**5.23**	**4.92**	**4.33**			
Injection 1		84.00	96.30	111.00			
Injection 2		92.70	101.00	108.00			
Injection 3	**100**	84.40	102.00	107.00	**97.42**	**-2.58**	**10.72**
Injection 4		86.60	96.60	103.75			
Injection 5		88.70	88.30	111.00			
Within-run mean		**87.28**	**96.84**	**108.15**			
Within-run RE% mean		**-12.72**	**-3.16**	**8.15**			
Within-run RSD%		**4.09**	**5.59**	**2.81**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Perindopril		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1		4.41	3.05	2.66			
Injection 2		3.66	3.03	3.07			
Injection 3	**3.13**	4.41	3.39	2.58	**3.40**	**8.71**	**21.59**
Injection 4		4.49	3.34	2.45			
Injection 5		4.08	3.31	3.09			
Within-run mean		**4.21**	**3.22**	**2.77**			
Within-run RE% mean		**34.50**	**3.00**	**-11.37**			
Within-run RSD%		**8.21**	**5.29**	**10.58**			
Injection 1		29.10	25.00	27.05			
Injection 2		30.00	24.10	27.75			
Injection 3	**25**	29.10	25.10	27.00	26.50	6.00	8.10
Injection 4		26.30	23.50	22.15			
Injection 5		30.00	26.10	25.25			
Within-run mean		28.90	24.76	25.84			
Within-run RE% mean		15.60	-0.96	3.36			
Within-run RSD%		5.26	4.03	8.74			
Injection 1		86.50	94.00	99.60			
Injection 2		77.80	100.00	101.30			
Injection 3	**100**	83.30	93.30	104.50	**94.34**	**10.12**	**-5.66**
Injection 4		85.90	101.00	103.25			
Injection 5		86.00	94.20	104.50			
Within-run mean		**83.90**	**96.50**	**102.63**			
Within-run RE% mean		**-16.10**	**-3.50**	**2.63**			
Within-run RSD%		**4.33**	**3.82**	**2.08**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Perindoprilat		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1		2.82	2.99	2.74			
Injection 2		2.80	2.77	2.70			
Injection 3	**3.13**	2.95	3.05	2.67	**2.89**	**-7.76**	**1.98**
Injection 4		2.84	2.68	3.01			
Injection 5		3.21	3.09	3.00			
Within-run mean		**2.92**	**2.92**	**2.82**			
Within-run RE% mean		**-6.58**	**-6.83**	**-9.87**			
Within-run RSD%		**5.82**	**6.20**	**6.02**			
Injection 1		24.10	21.50	24.25			
Injection 2		22.80	22.90	25.55			
Injection 3	**25**	23.80	23.20	25.85	**23.64**	**-5.44**	**5.12**
Injection 4		23.60	23.20	22.75			
Injection 5		21.90	22.60	26.60			
Within-run mean		**23.24**	**22.68**	**25.00**			
Within-run RE% mean		**-7.04**	**-9.28**	**0**			
Within-run RSD%		**3.83**	**3.11**	**6.06**			
Injection 1		86.00	91.60	106.00			
Injection 2		96.00	91.90	108.50			
Injection 3	**100**	86.80	95.70	96.70	**95.86**	**-4.14**	**9.33**
Injection 4		86.80	93.90	104.40			
Injection 5		90.20	88.90	114.50			
Within-run mean		**89.16**	**92.40**	**106.02**			
Within-run RE% mean		**-10.84**	**-7.60**	**6.02**			
Within-run RSD%		**4.66**	**2.77**	**6.10**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Quinapril		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1		2.93	3.01	2.44			
Injection 2		2.76	2.82	2.46			
Injection 3	**3.13**	2.76	2.95	2.41	**2.74**	**-12.47**	**8.60**
Injection 4		2.92	2.82	2.11			
Injection 5		2.89	2.89	2.91			
Within-run mean		**2.85**	**2.90**	**2.47**			
Within-run RE% mean		**-8.88**	**-7.41**	**-21.11**			
Within-run RSD%		**2.99**	**2.86**	**11.60**			
Injection 1		22.60	22.80	24.60			
Injection 2		23.30	23.40	25.55			
Injection 3	**25**	23.40	23.10	25.15	**23.86**	**-4.56**	**3.91**
Injection 4		23.80	24.70	21.40			
Injection 5		22.70	23.50	27.90			
Within-run mean		**23.16**	**23.50**	**24.92**			
Within-run RE% mean		**-7.36**	**-6.00**	**-0.32**			
Within-run RSD%		**2.17**	**3.08**	**9.37**			
Injection 1		82.70	98.00	105.50			
Injection 2		90.80	98.40	98.80			
Injection 3	**100**	89.90	98.90	102.00	**96.42**	**-3.58**	**8.70**
Injection 4		87.40	97.20	107.75			
Injection 5		88.40	91.60	109.00			
Within-run mean		**87.84**	**96.82**	**104.61**			
Within-run RE% mean		**-12.16**	**-3.18**	**4.61**			
Within-run RSD%		**3.60**	**3.08**	**4.01**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Quinaprilat		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1	**3.13**	2.93	2.74	1.73	**2.53**	**-19.02**	**10.87**
Injection 2		2.62	2.80	3.35			
Injection 3		2.37	2.73	2.17			
Injection 4		2.78	2.59	1.87			
Injection 5		2.63	2.74	1.95			
Within-run mean		**2.67**	**2.72**	**2.22**			
Within-run RE% mean		**-14.82**	**-13.09**	**-29.13**			
Within-run RSD%		**7.82**	**2.86**	**29.54**			
Injection 1	**25**	21.90	24.80	24.20	**23.14**	**5.20**	**-7.45**
Injection 2		22.20	24.50	24.80			
Injection 3		23.60	23.50	29.00			
Injection 4		21.90	26.30	14.45			
Injection 5		23.20	23.50	19.20			
Within-run mean		**22.56**	**24.52**	**22.33**			
Within-run RE% mean		**-9.76**	**-1.92**	**-10.68**			
Within-run RSD%		**3.50**	**4.71**	**25.13**			
Injection 1	**100**	85.20	94.70	90.80	**98.83**	**-1.17**	**11.08**
Injection 2		92.10	107.00	109.00			
Injection 3		86.80	101.00	119.00			
Injection 4		86.70	98.70	104.00			
Injection 5		87.10	95.80	124.50			
Within-run mean		**87.58**	**99.44**	**109.46**			
Within-run RE% mean		**-12.42**	**-0.56**	**9.46**			
Within-run RSD%		**3.01**	**4.92**	**12.04**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentration [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Ramipril		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1		3.06	2.83	2.61			
Injection 2		2.79	2.65	2.71			
Injection 3	**3.13**	3.13	2.86	2.51	**2.87**	**-8.33**	**9.98**
Injection 4		3.34	2.60	2.36			
Injection 5		3.60	3.06	2.91			
Within-run mean		**3.18**	**2.80**	**2.62**			
Within-run RE% mean		**1.72**	**-10.54**	**-16.16**			
Within-run RSD%		**9.56**	**6.55**	**7.83**			
Injection 1		26.30	22.10	25.70			
Injection 2		26.60	22.70	24.50			
Injection 3	**25**	27.60	22.60	25.70	**25.14**	**0.56**	**10.26**
Injection 4		26.80	21.70	25.65			
Injection 5		29.40	22.40	27.35			
Within-run mean		**27.34**	**22.30**	**25.78**			
Within-run RE% mean		**9.36**	**-10.80**	**3.12**			
Within-run RSD%		**4.57**	**1.82**	**3.94**			
Injection 1		89.00	82.00	99.85			
Injection 2		95.30	89.50	91.65			
Injection 3	**100**	93.80	81.80	92.30	**90.06**	**-9.94**	**7.31**
Injection 4		92.70	80.80	101.85			
Injection 5		88.50	79.70	92.15			
Within-run mean		**91.86**	**82.76**	**95.56**			
Within-run RE% mean		**-8.14**	**-17.24**	**-4.44**			
Within-run RSD%		**3.26**	**4.68**	**5.11**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentration [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Trandolapril		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1	**3.13**	4.08	3.15	2.17	**3.10**	**-1.02**	**22.84**
Injection 2		3.64	3.05	2.38			
Injection 3		3.44	3.17	2.41			
Injection 4		4.00	2.87	2.17			
Injection 5		3.95	3.08	2.90			
Within-run mean		**3.82**	**3.06**	**2.41**			
Within-run RE% mean		**22.10**	**-2.10**	**-23.06**			
Within-run RSD%		**7.09**	**3.89**	**12.35**			
Injection 1	**25**	31.70	24.70	25.05	**27.41**	**9.64**	**14.93**
Injection 2		32.40	24.40	25.05			
Injection 3		31.90	25.30	24.75			
Injection 4		32.30	24.60	23.95			
Injection 5		32.30	24.70	28.05			
Within-run mean		**32.12**	**24.74**	**25.37**			
Within-run RE% mean		**28.48**	**-1.04**	**1.48**			
Within-run RSD%		**0.94**	**1.36**	**6.16**			
Injection 1	**100**	116.00	94.60	104.60	**106.85**	**6.85**	**9.35**
Injection 2		118.00	99.90	102.50			
Injection 3		117.00	103.00	107.00			
Injection 4		121.00	94.30	106.50			
Injection 5		116.00	97.40	105.00			
Within-run mean		**117.60**	**97.84**	**105.12**			
Within-run RE% mean		**17.60**	**-2.16**	**5.12**			
Within-run RSD%		**1.76**	**3.76**	**1.68**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation

	Nominal concentra-tion [ng/mL]	Calculated concentration [ng/mL]			Between-run accuracy and precision		
Trandolaprilat		**Day 1**	**Day 2**	**Day 3**	**Mean**	**RE%**	**RSD%**
Injection 1	**3.13**	2.72	3.28	2.52	**2.93**	**-6.50**	**9.80**
Injection 2		2.76	3.35	2.62			
Injection 3		2.78	3.19	2.46			
Injection 4		2.72	2.94	2.89			
Injection 5		3.20	3.48	2.98			
Within-run mean		**2.84**	**3.25**	**2.70**			
Within-run RE% mean		**-9.39**	**3.76**	**-13.86**			
Within-run RSD%		**7.23**	**6.22**	**8.45**			
Injection 1	**25**	23.10	23.60	24.65	**23.59**	**-5.65**	**2.55**
Injection 2		22.50	23.50	24.90			
Injection 3		23.30	22.90	25.00			
Injection 4		23.00	24.80	20.65			
Injection 5		22.90	23.40	25.60			
Within-run mean		**22.96**	**23.64**	**24.16**			
Within-run RE% mean		**-8.16**	**-5.44**	**-3.36**			
Within-run RSD%		**1.29**	**2.97**	**8.24**			
Injection 1	**100**	83.60	96.70	106.00	**97.17**	**-2.83**	**9.34**
Injection 2		92.80	97.30	102.50			
Injection 3		87.30	96.20	108.00			
Injection 4		89.10	94.70	107.50			
Injection 5		91.10	94.80	110.00			
Within-run mean		**88.78**	**95.94**	**106.80**			
Within-run RE% mean		**-11.22**	**-4.06**	**6.8**			
Within-run RSD%		**4.01**	**1.20**	**2.61**			

RE% = Percentage relative error, RSD% = Percentage relative standard deviation