The development, validation, and evaluation of quantitative assays for determining adherence of heart failure patients to carvedilol, enalapril and perindopril.

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Plagiarism Declaration

I, André Joubert, hereby declare that the work on which this thesis, " The development validation, and evaluation of quantitative assays for determining adherence of heart failure patients to carvedilol, enalapril, and perindopril ", is based on my original work, except where acknowledgements indicate otherwise, and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for research the whole or any portion of the contents in any manner whatsoever.

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Publications

Published

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Joubert A, Joubert A, Van der Merwe M, et al. Validation of a quantitative multiplex LC-MS/MS assay of carvedilol, enalaprilat, and perindoprilat in dried blood spots from heart failure patients and its cross validation with a plasma assay. *J Mass Spectrom Adv Clin Lab*. Published online 2022. doi: 10.1016/j.jmsacl.2022.12.003

Publications in preparation (at the time of submission)

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Abstract

Background: Heart failure (HF) is a global pandemic with a rising prevalence rate in low- and middle-income countries (LMICs). Poor medication adherence contributes to the impact of chronic diseases such as HF. However, there are sparse adherence data on HF patients in sub-Saharan Africa (SSA). This is problematic as African HF patients have a high mortality rate, which is poorly understood. Poor medication adherence could contribute to the high mortality rate of African HF patients. Objective adherence measures are better than subjective measures (for example, patient recall) at predicting outcomes. In addition, the adherence method should be applicable to resource-scarce settings. Novel multiplex assays were developed to quantify carvedilol, enalaprilat and perindoprilat in dried blood spots (DBS) and correlated with plasma. Carvedilol, enalapril and perindopril are medications commonly used to treat HF, with enalaprilat and perindoprilat being the active metabolites of enalapril and perindopril, respectively. The developed assays were then evaluated in terms of their ability to discern between non-adherent and adherent patients and their suitability for use in resource-scarce settings.

Method: The DBS and plasma assays were validated per the United States Food and Drug Administration (FDA) guidelines. The plasma assay was validated over a calibration range of 0.2–200 ng/mL for carvedilol, enalaprilat and perindoprilat. The DBS assay was validated over a range of 1.00–200 ng/mL for the three analytes. The DBS assays were correlated with plasma concentrations in a pilot intensive pharmacokinetic study of six patients. The correlation was determined using Deming regression, with Bland–Altman analysis used to establish agreement between observed and calculated plasma concentrations. Calculated plasma concentrations were obtained using the Deming regression equations describing the relationship between DBS and plasma concentrations.

Results: Accuracy, precision, selectivity and sensitivity were proven with complete and reproducible extraction recovery at all concentrations tested for both assays. Stability of the analytes in the matrix and throughout sample processing was proven for both assays. The full range of plasma pharmacokinetic samples could be quantified for all analytes, with the lower limit of quantification (LLOQ) of 0.2 ng/mL proving to be sufficient. The pharmacokinetic pilot study's full range of DBS concentrations could be quantified for enalaprilat but not for carvedilol and perindoprilat. The LLOQ of 1.00 ng/mL was not sensitive enough to quantify

the lowest concentrations of some patients for these two analytes. Good correlations were observed between DBS and plasma pharmacokinetic samples, with the Pearson's correlation coefficient (r) greater than 0.94 for all analytes. The difference between the observed and calculated plasma concentrations was less than 20% of their mean for > 67% of samples for all analytes, indicating good agreement between observed and calculated plasma concentrations for all analytes.

Conclusions: The plasma assay is suited for evaluating patient adherence to carvedilol, enalapril and perindopril medication. The assay is robust and sensitive enough to discern between those who are adherent and non-adherent. Due to the wealth of pharmacokinetic data available for the analytes in plasma, through pharmacokinetic modelling, it is possible to determine the most appropriate dose and weight-specific adherence interpretation for that patient rather than relying on a general cut-off value. In other words, adherence interpretation can be individualised based on a patient's own dose and weight. Plasma as a matrix, however, is not very amenable to resource-scarce settings. The matrix requires strict storage and transport conditions, so creating additional logistic difficulties and expenses in resource-scarce and remote locations. These are difficulties that would have to be accommodated to use the assay.

It was found that the DBS assay is more suitable as a screening assay for carvedilol and perindoprilat than as an assay to gauge adherence. The assay is suitable as an adherence-determining assay for enalaprilat, however. The prolonged terminal half-life of enalaprilat allows sufficient DBS concentrations to track adherence. The DBS assay's higher LLOQ and the higher concentration of the analytes in plasma versus that of whole blood places the assay at a stark disadvantage in terms of sensitivity relative to the plasma assay. DBS samples have a significant advantage over plasma samples in their less stringent storage and transport requirements. As a matrix, DBS is far more conducive to remote and resource-scarce areas when compared to plasma.

The robustness of both assays was proven with cross-validation using actual clinical samples. Good agreement between observed and calculated plasma concentrations means that DBS concentrations, once normalised, can be used interchangeably with plasma samples. DBS samples can be collected at the sampling sites, taking advantage of the DBS matrix's less stringent storage and transportation requirements. Once the samples are analysed, the concentrations can be converted to plasma concentrations, which can be interpreted more

efficiently in terms of adherence. However, this would only be feasible for enalaprilat, as the DBS assay for the carvedilol and perindoprilat analytes lacked sensitivity to reflect ingestion within the last 24 hours.

Contents

List of Abbreviations

ACCF	American College of Cardiology Foundation
ACE	Angiotensin-converting enzyme
AHA	American Heart Association
$AUC_{0\text{-}inf}$	Area under the concentration-time curve extrapolated to infinity
$AUC_{0\text{-}t}$	Area under the concentration-time curve from zero to t
BMI	Body mass index
B:P ratio	Blood to-plasma ratio
CE	Capillary electrophoresis
CL	Clearance
CL/F	Apparent clearance
CLu/F	Apparent unbound clearance
C_{max}	Maximum (peak) concentration
C_{trough}	Lowest concentration of medication before next dose
CV	Coefficient of variation
DBS	Dried blood spots
EMA	European Medicines Agency
ESI	Electrospray ionisation
EU	European Union
F	Bioavailibility
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
GFR	Glomerular filtration rate
HF	Heart failure
HF*p*EF	Heart failure with preserved ejection fraction
HF*r*EF	Heart failure with reduced ejection fraction
HILIC-MS/MS	Hydrophilic interaction liquid chromatography with tandem mass spectrometry
HPLC-ECD	High-performance liquid chromatography with electrochemical detection
ISTD	Internal standard
K_{el}	Elimination rate constant
K_3EDTA	Tripotassium ethylenediaminetetraacetic acid
LC	Liquid chromatography
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLOQ	Lower limit of quantification
LMIC	Low- and middle-income countries
MEMS	Medication event monitoring systems
MIC	Middle-income countries
MRM	Multiple reaction monitoring
MS	Mass spectrometry

NCA	Non-compartmental analysis
NCD	Non-communicable disease
NMR	Nuclear magnetic resonance mass spectroscopy
NYHA	New York Heart Association
OD	Once daily
PPT	Protein precipitation
QC	Quality control
QC DIL	Quality control dilution
QC H	Quality control high
QC L	Quality control low
QC M	Quality control medium
RAAS	Renin-Angiotensin-Aldosterone System
RBC	Red blood cells
RT	Retention time/Room temperature
SES	Socioeconomic status
SOP	Standard operating procedure
S/N	Signal to noise ratio
SPE	Solid-phase extraction
SSA	Sub-Saharan Africa
$T_{1/2}$	Half-life
TOFMS	Time-of-flight mass spectrometers
UIC	Upper-income countries
ULOQ	Upper limit of quantification
USA	United States of America
VAMS	Volumetric absorptive microsampling
Vd	Volume of distribution
Vd/F	Apparent volume of distribution
WB	Whole blood
WHO	World Health Organization
YLD	Years lost due to disability

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1 Background and Literature Review

This chapter gives background information on HF's impact globally and in SSA. It outlines the medications used to treat HF and discusses their effectiveness. The pharmacokinetics and modes of action of carvedilol, enalapril and perindopril are described as well as those of the metabolites enalaprilat and perindoprilat. The role of medication adherence in chronic disease, specifically HF, is described, and the factors influencing adherence are discussed. The advantages and disadvantages of available adherence-determining methods are explained, and reasoning is given for the selected matrices (DBS and plasma) evaluated in this study. A literature summary of published analytical methods for quantifying the analytes of interest (carvedilol, enalaprilat, and perindoprilat) in plasma and DBS is provided. Technical background on liquid chromatography-tandem mass spectrometry (LC-MS/MS), as well as sample preparation and extraction of plasma and DBS samples, are discussed. The methods used to establish agreement between the concentrations of different analytical methods are explained. This concludes with an outline of the project aims and objectives.

1.1 Prevalence and impact of heart failure

Heart failure (HF) can be defined as "a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood".[1,2] The disease is an epidemic and is a significant clinical and public health problem linked with high morbidity, mortality and healthcare expenditures.[3] The worldwide prevalence of HF is currently 64.63 million cases, equating to 8.52 cases per 1000 inhabitants, resulting in 9.91 million years lost due to disability (YLD). The most significant burden for HF is after 60 years of age, with the prevalence and YLD having increased by 3.9% and 4.5%, respectively, in the elderly over the last three decades.[4] The worldwide prevalence of heart failure in developed nations is estimated to be 1-3%.[5] In the European Union (EU) it is estimated that approximately 10 million people could be affected.[6] In the United States of America (USA), it is estimated that 85.6 million adults have at least one form of cardiovascular disease, with approximately 6.2 million adults living with HF.[5,7]

Although progress has been made in reducing HF-related mortality, HF-related hospitalisations remain frequent, with admissions continuing to rise.[3] The high hospital admissions and extensive use of healthcare resources result in significant costs. In Europe and the USA, the expenditure on HF is approximately 1–2% of their annual healthcare budgets. Globally, the economic burden of HF is approximately $108 billion per annum, with $65 billion and $43 billion of that due to direct and indirect costs, respectively.[8] This is a considerable burden on healthcare systems and related costs resulting from ageing populations and improvements in

how HF is managed with treatments, including beta-blockers, angiotensin-converting-enzyme (ACE) inhibitors and aldosterone inhibitors.[9]

1.1.1 Heart failure in sub-Saharan Africa

Africa is in the difficult position of facing a dual burden of infectious and chronic diseases. Although infectious diseases in Africa account for about two-thirds of deaths on the continent, the age-specific mortality rates of chronic diseases such as HF are higher in SSA for both men and women compared to virtually all other world regions.[10] The case fatality rate of HF in Africa is 34%, about double the world average of 16.5%.[11,12] In Africa, there has been an increase in non-communicable diseases (NCDs), such as cancers, diabetes mellitus and hypertension. This transition from communicable to non-communicable diseases is because of rapid urban development, an increase in unhealthy diets containing high quantities of fats and salts, the adoption of a Western lifestyle, and sedentary living.[11]

In SSA, cardiovascular disease is a significant health and socioeconomic burden because of its high prevalence and mortality rates. The peak in mortality rates of HF patients in Africa at an economically productive age is particularly burdensome.[11,13] This contrasts with the pattern that HF follows in high-income regions/countries such as North America, Europe and Japan, where HF is largely a disease of the eldery.[11] Insured and financially stable patients have access to resources which allow their condition to be better managed and controlled.[12]

1.2 Pharmacological treatment of heart failure

The heart's ejection fraction is used to help gauge its pumping efficiency. The ejection fraction refers to the quantity of blood pumped out of the heart's lower chambers, with a healthy heart having an ejection fraction of 50–70%.[14] Some patients with a normal ejection fraction can still suffer from HF. These patients suffer from HF with preserved ejection fraction (HF*p*EF).[14] Patients with an ejection fraction less than 40% suffer from HF with reduced ejection fraction (HF*r*EF).[15] Great strides have been made in reducing the mortality of HF*r*EF, with clear recommendations in Australian, European and American guidelines for its treatment.[16,17] However, the guidance on the treatment of HF*p*EF is limited because of its complexity and the lack of evidence for interventions that improve prognosis.[16,17]

The first-line drug therapy for patients with HF*r*EF includes an ACE inhibitor and a beta-blocker.[18] ACE inhibitors function by positively altering the haemodynamics in patients with systolic dysfunction. ACE inhibitors lessen systolic wall stress and reduce afterload and preload, allowing cardiac output to increase without an increase in heart rate.[19] The excretion

of salt through the augmentation of renal blood flow and the lowering of the quantity of aldosterone and antidiuretic hormone that is produced are also promoted.[19] The efficacy of ACE inhibitors has been demonstrated over several decades.[19] The CONSENSUS trial showed that mortality risk was reduced by 27% when patients were given enalapril, and the number of patients experiencing HF*r*EF progression decreased significantly.[18,19] It was demonstrated through the SOLVD trial that compared to a placebo, the treatment with enalapril over three years prevented the premature deaths of 50 patients and 350 hospitalisations per 1000 patients.[18,20] In the PEP-CHF study, patients older than 70 with chronic HF were randomised to placebo or 4 mg/day of perindopril. An increase in exercise capacity and a reduction in hospitalisation were observed in the first year on perindopril.[21,22] These trials have shown that when ACE inhibitors are taken concurrently with other medications, there is a significant reduction in both mortality and morbidity, with the benefits remaining clinically significant throughout extended courses of therapy.[18]

The benefits of beta-blockade in patients with HF*r*EF have also been documented for several decades.[18,23] Since the 1970s, data have indicated that using bisoprolol, carvedilol or sustained-release metoprolol succinate reduce both the morbidity and mortality of patients with Hf*r*EF.[18] These agents block the β_1 receptor, which prevents ventricular remodelling promoted by the Renin-Angiotensin-Aldosterone System (RAAS). Carvedilol also blocks the β_2 and α_1 receptors, so widening blood vessels.[18,24] In the COPERNICUS study, all-cause mortality was 11.4% (carvedilol group) versus 18.5% (placebo group). The CIBIS-II trial evaluated bisoprolol, which led to all-cause mortality of 8.8% (bisoprolol group) versus 13.2% (placebo group). The MERIT-HF trial evaluated patients on metoprolol succinate who were on a baseline ACE-inhibitor and diuretic therapy and found that all-cause mortality was 7.2% (metoprolol succinate group) versus 11% (placebo group). These three beta-blockers are the only ones involved in extensive clinical studies to have shown a reduction in mortality, which subsequently led to them being included in the HF guidelines as first-line treatment medications in all patients with Hf*r*EF.[18]

ACE inhibitors and beta-blockers are an essential part of appropriate drug therapy for HF patients.[25] Decades of successful use and published studies have shown that these medications result in lower hospitalisation rates and death rates for patients suffering from HF.[14,22]

1.3 Description and pharmacokinetics of carvedilol, enalapril, perindopril and the metabolites enalaprilat and perindoprilat

1.3.1 Carvedilol

Carvedilol is a racemic (S(-) and R(+) enantiomers), lipophilic, highly protein bound, non-selective β-blocker that also has α_1-adrenergic blocking, antioxidant and calcium antagonist properties.[27] By blocking both the β_1- and β_2-adrenergic receptors, carvedilol improves myocardial function, attenuation and adverse myocardial remodelling in HF.[27] Being part of the third generation of β-receptor blockers, carvedilol has significant clinical efficacy in treating both mild and severe congestive HF. It is often prescribed with an ACE inhibitor such as enalapril and perindopril to treat HF.[28]

The carvedilol molecule consists of an asymmetric carbon atom, which forms the basis for a pair of enantiomers.[29] The S-(—)-enantiomer is responsible for the β-receptor blockade, while both of the enantiomers are responsible for α1-adrenoceptor blocking activity.[27] The structure of carvedilol is shown below in Figure 1.1.

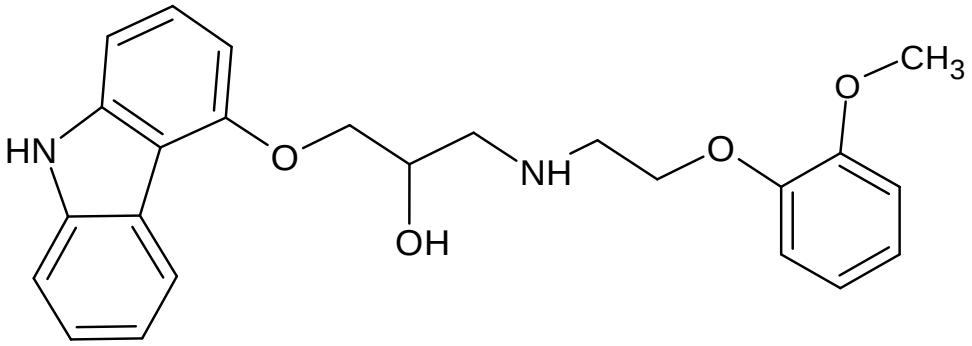


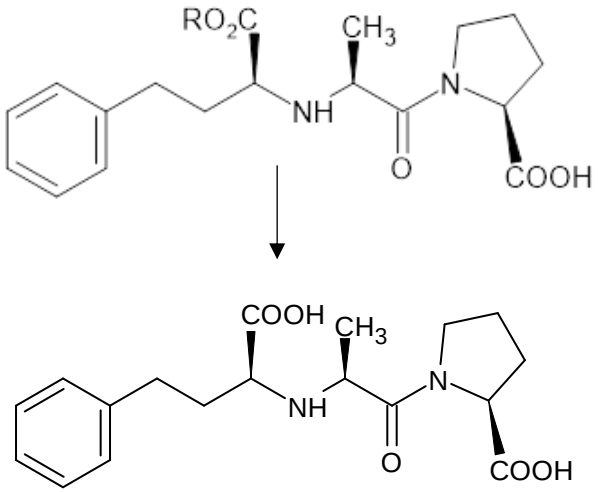
Figure 1.1 Chemical structure of carvedilol (created with ACD/Chemsketch 2020.2.1)

After oral administration, carvedilol is rapidly and extensively absorbed.[26] Because of first-pass metabolism, its oral bioavailability is 25–35%, with peak serum levels attained 1.0–1.5 hours after oral administration.[30] The plasma concentrations of the drug are proportional to the oral dose, while food decreases the absorption rate. The elimination half-life of the drug is four to seven hours. Carvedilol is eliminated mainly via hepatic metabolism, with most metabolites excreted into the bile and eliminated via the faeces.[31]

1.3.2 Enalapril and enalaprilat

Enalapril is a lipid-soluble, dicarbonyl-containing peptide.[32] It is an inactive prodrug, which is commonly prescribed for treating HF.[32–34] After oral administration, enalapril is bioactivated

by hydrolysis to enalaprilat, with enalaprilat being the active ACE inhibitor.[32] ACE, a peptidyl dipeptidase, catalyses angiotensin I to angiotensin II, a vasoconstrictor substance. Angiosten II is also known to stimulate the secretion of aldosterone by the adrenal cortex. The positive effects of enalapril (through its active metabolite enalaprilat) in the treatment of HF, primarily result from the suppression of the Renin-Angiotensin-Aldosterone System.[32] Enalaprilat's absorption across the gastrointestinal tract is limited as a result of its high polarity. This causes low bioavailability of enalaprilat when orally administrated.[35] Therefore, enalapril is given orally as a prodrug, with the phenylethyl group making the enalapril molecule more hydrophobic and allowing for better absorption across the gut wall.[36] Figure 1.2 below shows the structure of enalapril and enalaprilat.



Enalapril
R = C_2H_5

Enalaprilat

Figure 1.2 Structure of enalapril and enalaprilat. (Adapted from Aoki et al.[37] and created with ACD/Chemsketch 2020.2.1)

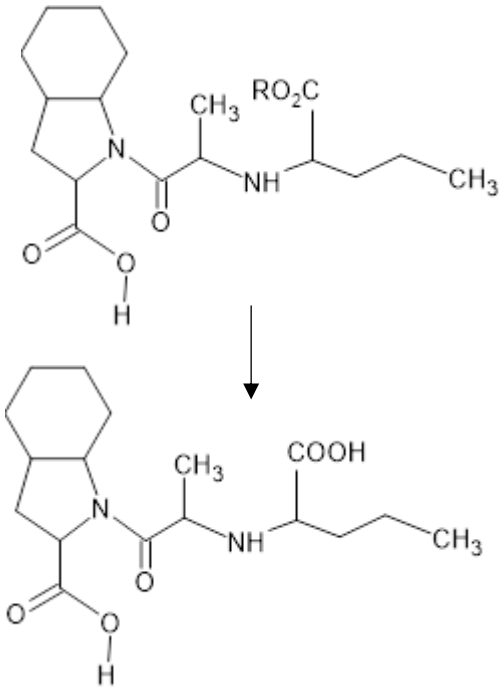
Enalapril has good oral absorption (60–70%), and its metabolism is not affected by food intake.[38] Peak serum concentrations of enalapril occur within one hour of oral administration and are then rapidly cleared with a half-life of less than one hour.[32,33,39] Most of the enalapril is excreted renally as enalaprilat, with some untransformed enalapril excreted in bile.[40]

As indicated before, enalapril's pharmacological activity is limited until it is metabolised in the liver (through de-esterification) to enalaprilat (Figure 1.2). Enalaprilat is its only metabolite and is responsible for suppressing the Renin-Angiotensin-Aldosterone System.[32,33] Peak enalaprilat concentrations occur 3–6 hours after enalapril is administered orally.[33,41] Enalaprilat displays a biphasic elimination; the initial elimination half-life is short, followed by a lengthy

second phase with a half-life greater than 30 hours.[28,33,40] Enalaprilat is excreted renally, and the protein binding is approximately 50%.[40]

1.3.3 Perindopril and perindoprilat

Perindopril is a lipophilic, long-acting ACE inibitor.[42] Like enalapril, it is a prodrug ester converted to the active metabolite, perindoprilat, after oral administration through hydrolysis in the liver and plasma.[42] Perindopril is one of the best studied ACE inhibitors in preclinical and clinical settings and is commonly used in treating HF.[22] It reduces (through its active metabolite perindoprilat) plasma angiotensin II, resulting in decreased vasoconstriction and aldosterone secretion.[22,42] The structure of perindopril and perindoprilat is shown in Figure 1.3.



Perindopril
R = C_2H_5

Perindoprilat

Figure 1.3 Chemical structure of perindopril and perindoprilat (Adapted from Remko et al.[43] and created with ACD/Chemsketch 2020.2.1)

Perindopril has similar pharmacokinetics to enalapril in that it is rapidly absorbed, with peak serum concentrations reached within 1 hour and then rapidly cleared (half-life ~1 hour).[42,44] Perindopril has six metabolites: perindoprilat, perindopril glucuronide, perindoprilat glucuronide, one perindopril lactam, and two perindoprilat lactams. The two primary circulating metabolites are perindoprilat and perindoprilat glucuronide.[45] The only pharmacologically active metabolite is perindoprilat.[45,46] As a result of its extensive metabolism, only 4–12% of perindopril is recovered in urine following oral administration, with the mean bioavailability of perindopril being approximately 95%.[45,46]

Perindoprilat is detectable 30 minutes after oral administration of perindopril, with peak plasma concentrations occurring 3–7 hours after administration.[42] The elimination profile of perindoprilat is also biphasic; the free fraction is subjected to rapid renal excretion (half-life 3–5 hours), with a long terminal half-life (~30 hours) of the ACE-bound fraction.[47–49] Perindoprilat is 10–20% bound to plasma proteins.[50]

1.4 Medication adherence

1.4.1 Consequences of poor medication adherence

Pharmacotherapy forms a critical part of the treatment plan for HF. Drug therapies are crucial in controlling symptoms and increasing survival rates.[51] Extensive research has allowed for the development of medications with proven efficacy and positive benefit-to-risk profiles.[52] However, between treatment and outcomes there is a critical intermediate step: medication adherence.[52]

Treatment➔ **Adherence**➔ Outcomes

Medication adherence refers to the ability of a patient to take their prescribed medications in the manner recommended by their healthcare provider.[53,54] Poor adherence can occur at any time in a patient's treatment cycle. It could occur at the beginning of the treatment process when the patient fails to fill their initial prescriptions, which is known as primary non-adherence. If a patient fails to correctly follow instructions or refill the prescription after therapy has started, it is referred to as secondary non-adherence.[55]

Poor medication adherence is associated with both higher rates of hospitalisations and deaths in HF patients.[56–59] A meta-analysis performed by Wu et al.[51] found that medication non-adherence rates in HF patients, specifically, are typically only between 40% and 60%.[56–59,59,60] Ruppar et al.[57] performed a systematic review of studies introducing strategies to improve adherence to HF medications. The study found that adherence-improving interventions among those suffering from HF reduced readmissions and mortality, with the final study recommendation being that medication adherence be part of the patient and clinician's discussion during follow-up visits.

Patients who do not fully adhere to prescribed medications also significantly affect healthcare systems due to the additional use of limited healthcare resources. Additional doctor visits, treatments, laboratory tests and hospital and nursing home admissions as a result of poor

adherence result in high costs.[53] In the United States, poor adherence is estimated to be the cause of 33–69% of medication-related hospital admissions. This results in an additional annual cost of $100 billion to $300 billion, which is equivalent to 3–10% of their total healthcare cost.[53,62] In Europe, the cost of poor adherence is €125 billion annually, while Australia loses $7 billion per annum.

1.4.2 Factors affecting medication adherence

The World Health Organization (WHO) classifies the factors which can contribute to poor adherence into five categories: disease-related factors, socioeconomic factors, therapy-related factors, patient-related factors, and factors that are associated with the healthcare team and systems in place. These can be grouped into patient-related, physician-related, and the factors related to the healthcare team/system.[52]

Several patient-related factors contribute to medication non-adherence, including the lack of understanding of the specific illness, lack of medical literacy, and minimal involvement in the treatment decision-making process.[52,63,64] Previous experience with pharmacological therapies, the patient's health beliefs, and attitudes towards the effectiveness of treatment can also affect the degree of medication adherence.[52,65] Socioeconomic status (SES) also influences adherence behaviour, with some studies indicating that patients of lower SES are less adherent than those of higher SES.[66,67]

Physicians can contribute to poor medication adherence by failing to explain adequately the medication's benefits and side effects and can generate barriers to medication adherence by stifling healthcare coordination and the patient’s access to adequate care.[52,68] Overburdened healthcare systems in which clinicians see an immense number of patients, and without the required resources to meet the individual needs of patients, could result in insufficient time for physicians to assess adherence-taking behaviours properly.[52]

1.4.3 Methods for determining medication adherence

Several methods have been developed and validated to be used in several different settings to determine adherence.[69–71] WHO classifies adherence methods into objective or subjective methods.[69] Objective methods of determining adherence include measurements of clinical outcomes, pharmacy records and the electronic monitoring of medication dosing. Subjective methods are susceptible to bias as they require the patient's self-assessment of their medication-taking habits. This usually involves using a questionnaire to reveal the patient’s behaviour. Objective methods are viewed as more effective in revealing actual adherence behaviour, with

discordance being found between patients' subjective and objective evaluated adherence data.[72–74] Subjective and objective methods of measuring adherence can be further classified into direct and indirect methods.[74] The section below expands on the available direct and indirect methods, with Table 1.1 summarising the methods.[74]

Table 1.1 Summary of direct and indirect methods of determining medication adherence (adapted from Osterberg and Blaschke)[74]

Test	Advantages	Disadvantages
Direct methods		
Directly observed therapy	Most accurate	Patients can hide pills in their mouths and then discard them; impractical for routine use.
Measurement of the concentration of medicine or metabolite in blood	Objective and accurate	Variations in metabolism and "white coat adherence" can give a false impression of adherence. It requires expensive analytical equipment and biological samples from the patient.
Measurement of a biological marker in the blood	Objective: in clinical trials, It can also be used to measure placebo	Requires expensive quantitative assays and collection of body fluids.
Indirect methods		
Patient questionnaires, patient self-reports	Simple; inexpensive; the most useful method in the clinical setting	Susceptible to error with increases in time between visits; the patient easily distorts results.
Pill counts	Objective, quantifiable, and easy to perform	Data are easily altered by the patient (e.g., pill dumping). A pill count is not equivalent to the ingestion of medication.
Rates of prescription refills	Objective; easy to obtain	A prescription refill is not equivalent to ingestion of medication; requires a closed pharmacy system.
Assessment of patient's clinical response	Simple; generally easy to perform	Factors other than medication adherence can affect clinical response.
Electronic medication monitors	Precise; results are easily quantified; tracks patterns of taking medication	Expensive; requires return visits and Downloads of data from medication vials.
Measurement of physiological markers (e.g., heart rate in patients taking beta-blockers)	Often easy to perform	The marker may be absent for other reasons (e.g., increased metabolism, poor absorption, and lack of response).
Patient diaries	Help to correct poor recall	Easily altered by the patient.

1.4.3.1 Indirect methods of determining adherence

Indirect methods for determining objective adherence include pill counts, medication event monitoring systems (MEMS), determining the rates at which prescriptions are refilled, and

evaluating clinical outcomes (Table 1.1.). Subjective indirect methods of evaluating adherence include patient questionnaires and patient diaries[73,74] (Table 1.1). Indirect methods are less expensive and resource-intensive, and are easy to implement, with most adherence clinical trials using indirect methods.[70]

Questionnaires are popular because of their low participant and provider burden. However, they are prone to survey bias and adherence is often overestimated.[73,74] During self-reporting, patients may feel pressurised to give answers that are deemed socially acceptable, and the skill of the interviewer and the quality of questions asked play a significant role in the validity of the answers that are obtained.[70]

The advantages of pill counts include a low burden and low costs.[73] However, the drawback of using pill counts is that one can only assume the degree of adherence as actual adherence cannot be confirmed.[70] It is also crucial that the correct data, such as refill dates or quantity that was dispensed, are obtained in order to be able to draw reliable conclusions. MEMS are a prevalent form of measuring participant adherence and is widely regarded as the gold standard, being both user-friendly and generally well accepted by patients.[73,74] Drawbacks include that one cannot confirm if pills were ingested as the system only registers the number of times that the container is opened. Product components and software required for data retrieval can also be expensive.[73]

1.4.3.2 Direct methods of determining adherence

Direct methods include observing the patient ingesting medication or quantifying a drug or metabolite in a biological matrix[74] (Table 1.1). These methods are all objective and more accurate than indirect methods. However, they can be costly and place a higher burden on the patient or healthcare provider.[74] Directly observing patients take their medication is very accurate but only practical if patients come into clinics and if healthcare providers are available to observe them.

Technological advances have made it possible to quantify accurately many analytes in several biological matrices.[53] Hyphenated mass spectrometry (MS) techniques that combine chromatography and MS have transformed the capability to analyse analytes in biological matrices.[74] These hyphenated MS techniques include liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), liquid chromatography-high resolution mass spectrometry (LC-HRMS) and gas chromatography-mass spectrometry (GC-MS). These techniques provide the required

specificity towards the target analyte by separating the analytes prior to MS, MS/MS or HRMS detection.[75,76] The mass spectrometer is an ideal detector for medication adherence assessment, providing sensitivity and data characteristics of the analyte.[75] The most commonly used liquid chromatography for liquid samples is high-performance liquid chromatography (HPLC).[75]

The sample collection methods most frequently used for determining adherence are liquid blood (plasma or serum) and urine. Plasma is considered the gold standard.[53,75] The cost of shipping these standard biological samples is often high, with the samples requiring cold storage. A phlebotomist is required to collect blood samples, which could prevent widespread acceptance of routine adherence monitoring. The collection of urine samples is not as invasive as blood samples. However, some patient groups might be disinclined to provide urine samples due to cultural, religious, or ethical issues.[53] It might also be difficult to collect or produce a sufficient sample – especially for the elderly.[53] In addition, light-sensitive compounds are more prone to photodegradation in urine when compared to that of whole blood due to lower turbidity and possible longer exposure to daylight.[75,77]

Dried blood spots (DBS) are produced by directly depositing capillary or venous blood onto filter paper. Capillary samples are drawn from an infant's heel or the digital pulp in adults and children.[78] Venous drawn blood is deposited onto filter paper using a pipette. DBS cards typically consist of pre-printed circles,12 mm in diameter, which can receive 10–70 µL of blood. The improvement in the detection capabilities of MS instruments has made the use of microsampling techniques such as DBS more feasible. The increased sophistication of MS instruments and the ease of use, transport and storage of DBS has increased the use of this method for determining adherence.[75,79–81] Clinical trials and cohort studies that require the collection, shipment, storage and analysis of thousands of samples could benefit from this advantage.[82] However, a challenge of DBS sampling is the variation in the sampling quality, which depends on the sampling collection and spotting.[83] The effect of the haematocrit on DBS samples is also an essential factor to consider when using this sampling method. A variation in haematocrit can lead to a variation in viscosity which affects the size of the spot.[84]

A microsampling technique that has been developed and recently introduced to the market is volumetric absorptive microsampling (VAMS). It consists of a plastic handle and a globous tip with a diameter of about 4 mm. The device's tip is "wicked" into the desired fluid specimen, and a fixed sample volume is absorbed into its pores. The device is left to dry before it is used

for analytical purposes. It promises to bring several advantages over DBS sampling in terms of accuracy of sampling volume, haematocrit dependence, and pre-treatment. Some aspects of the VAMS sampling system need to be further investigated but are increasingly recognised as a viable alternative to DBS.[85]

Hair has several advantages in determining adherence compared to other biological matrices.[53] One of the advantages is its non-invasive sampling which does not require specialised personnel. Transporting hair samples is also straightforward and is not as costly as with blood. However, the most critical advantage is that hair allows for the accumulation of medications over a long period. Through segmental analysis of hair strands, a history of an individual patient's exposure to medications can be built, making it possible to track adherence over weeks or even months.[86,87] However, a significant drawback of hair as a biological sample is that it is a very complex matrix resulting in its analytical preparation being time-consuming and complicated. The significant variation of hair from one person to another also makes it difficult to develop robust analytical methods.[86] Furthermore, not all patients have hair available for sampling.[88]

Saliva as a biological matrix has not been frequently used to determine adherence because of several limitations.[75] However, the increased capabilities of MS instruments have increased attention on this matrix. One study that determined adherence to antihypertensive drugs showed comparable saliva and plasma results except for the acidic drug compounds.[75,89] Table 1.2 summarises typical volumes and masses of biological samples.

Table 1.2 Typical volumes and masses of biological samples (adapted from Tanna et al.)[74]

Sample	Size	Drug Mass
Urine	100–200 mL	0.1–10 µg
Liquid blood	5–10 mL	10–100 ng
Saliva	0.5–2 mL	0.1–10 ng
DBS and VAMS	10–50 µL	1–300 pg
Hair	20–100 mg	1–300 pg

1.5 Determining the medication adherence of African HF patients prescribed carvedilol, enalapril and perindopril

1.5.1 The reasoning for determining adherence of HF patients from Africa

Most studies report 40-60% adherence to HF medication.[60] Data showing treatment adherence from Africa, however, are exceptionally limited.[90] In order to understand the high mortality rate of HF patients from this region, obtaining reliable adherence data is essential. HF patients from LMICs, including many African regions, are often exposed to socioeconomic factors and healthcare systems that differ from upper-income countries (UICs).[91] These contrasting conditions of LMICs could affect medication adherence behaviour in a manner not seen in UICs. With objective adherence data, the limited understanding of the high mortality rate of HF patients from Africa can be reduced.

1.5.2 Selecting appropriate methods for determining adherence to carvedilol, enalapril and perindopril of HF patients from Africa

Quantifying analytes directly in a biological matrix is the only direct and practical method that does not use data which are susceptible to patient manipulation or malfunction (i.e., pill counts, MEMS). These advantages make quantifying the analyte in a biological matrix preferable when trying to assess the adherence of HF patients, as the data need to be as explicit as possible. The next important question is what biological matrix to select for African HF patients. Tanna and Lawson[53] indicate that the following questions are important to consider when deciding on which sample type would be the most appropriate:

- Is it straightforward to collect a sample from a patient?
- Is the target drug found in the matrix?
- Does the size of the biosample ensure that adequate quantities of the target drug or metabolite can be detected?
- Are there validated analytical methods available, or is it possible to develop one?

As indicated before, liquid blood (plasma or serum) and urine are the most frequently used matrices for determining adherence, with DBS also gaining increasing attention due to the advantages of this sample type.[53] Patient collaboration (willing interaction) is crucial in selecting the appropriate sample type.[53] There should also be enough sample available for collection. Not all patients have hair, and urine can be challenging to collect, especially for the elderly, and some patient groups also have religious, cultural or ethical objections to its collection.[53,75] As a result, hair and urine are eliminated in terms of potential practical biological sample types in the context of this study.

The question of whether the drug is present in the matrix is crucial to consider when evaluating the appropriateness of the matrix. It is also important that this question be evaluated in the correct context. Carvedilol, enalapril and perindopril are all found in whole blood and plasma. Methods have been developed to quantify these analytes in both matrices.[28,92,93] Since these analytes are present in whole blood, by extension these analytes are also present in DBS, as DBS is essentially whole blood which has been placed onto a card. In the context of evaluating adherence, however, both enalapril and perindopril would not be appropriate, as their pharmacokinetics are such that they are both rapidly cleared after oral administration.[32,33,39,42,44] Analytes that are rapidly cleared are challenging to evaluate adherence for, as analytes need to be present in the matrix for at least several hours (preferably days) in order to be a helpful adherence marker. This problem can fortunately be circumnavigated with the metabolites of enalapril and perindopril, enalaprilat and perindoprilat, respectively. Enalaprilat and perindoprilat both have long terminal half-lives, allowing them to be quantifiable for extended periods after oral administration, thus making them suitable adherence markers.

The biosample should allow for a sufficient sample to be available to detect the analyte of interest. It is also useful if there are previously validated methods for the analytes of interest. Fortunately, as has been indicated, hyphenated mass spectrometry techniques have transformed the capability to analyse analytes in biological matrices due to their sensitivity and specificity.[53] Methods have been validated for quantifying carvedilol, enalaprilat and perindoprilat in plasma and whole blood. Analytical methods for quantifying enalaprilat and perindoprilat in DBS have also been developed.[94] With previous methods having been developed for all the analytes in most of the matrices, although more complex, simultaneously analysing the three analytes in whole blood, DBS or plasma would be feasible.

Using the questions outlined by Tanna and Lawson,[53] the matrices considered for analysis are filtered down to:

- Plasma
- DBS
- Whole blood

The questions help evaluate available matrices by considering analytical problems and constraints that might arise from patients. A limitation of the questions is that they lack a question that forces one to consider the collected samples' transport and storage stages. In the context of this study, it is a critical consideration as the directive is to develop analytical

methods for collecting samples of patients from African countries. Samples could come from remote and resource-scarce areas, which could mean lengthy transportation times and storage facilities that might not be able to cater for samples requiring storage at ~-80°C. DBS are advantageous in this case as they are usually shipped in sealed bags containing desiccant and can be transported at room temperature due to its improved stability relative to other matrices, which helps to reduce the cost of shipping.[83,95] In addition, because of the anti-microbial properties of DBS, samples do not require any additional biohazard arrangements.[83,95] Given the advantages that DBS as a sampling method provides with respect to resource-scarce areas, it was selected as a matrix for developing a method to simultaneously quantify the concentrations of carvedilol, enalaprilat and perindoprilat in African HF patients.

Although plasma does not have the same storage and stability advantages as DBS, it is still considered the gold standard in biosample analysis, with most pharmacokinetic studies being done using plasma.[53,75] Therefore, it was decided to also develop a plasma method quantifying carvedilol, enalaprilat and perindoprilat simultaneously. Being able to correlate plasma and DBS sample concentrations and convert between plasma and DBS concentrations is also helpful and will be discussed later. A method to determine the concentration of the three analytes in whole blood was not deemed necessary as it did not have any advantage compared to plasma and DBS analysis. Figure 1.4 is a summary of the decision-making process in terms of selecting the matrices.

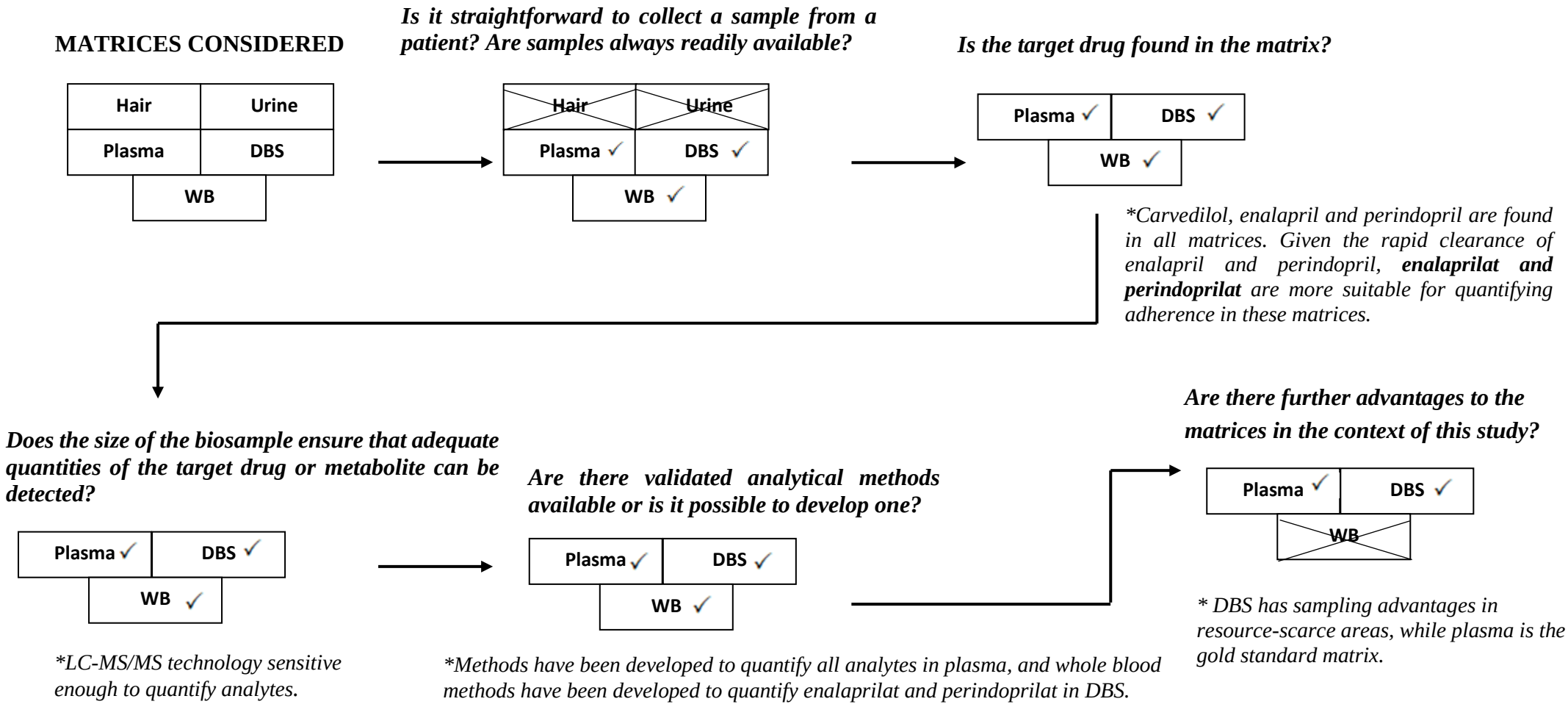


Figure 1.4 Summary of the decision-making process for selecting matrices for analytical development

1.5.3 Interpreting adherence from drug concentrations

Using drug concentration data to determine if the most recent dose has been taken is straightforward, as drug concentrations tend to increase shortly after ingestion. A very low concentration would indicate that the most recent dose had been missed. A limitation of this is the possibility of white coat adherence, where patients take their medication just before their clinic visit to give the impression of adherence[96]. A patient can therefore not necessarily be assumed to be adherent based on the most recent dose only. Analysing drug concentration data to infer adherence over a longer time frame, however, is complex depending on various factors, such as pharmacokinetic accumulation, drug half-lives, and the sensitivity of the assay used for quantification. This places quantifying drug concentrations to gauge historical adherence at a disadvantage when compared to patient self-reports or diaries (assuming the patients are reporting honestly). However, it is possible to evaluate extreme non-adherence using drug concentrations if the patient is not partaking in white coat adherence. Concentrations below the LLOQ of a sensitive assay quantifying a drug with a long half-life, for example, would suggest extreme non-adherence, as the patient would not have taken the medication for an extended period.

1.6 Quantifying carvedilol, enalaprilat and perindoprilat in human plasma

1.6.1 Collection, storage, and transportation of plasma

Whole blood is collected by inserting a needle into a vein and drawing blood directly into a collection tube containing the appropriate anticoagulant. The tube is filled to the indicated volume, and the sample is gently inverted 8-10 times to ensure proper mixing of the anticoagulant and blood. Samples must be processed within 2 hours of collection to separate the plasma from the blood cells. This is achieved by centrifugation at a speed and time appropriate for the type of tube used. After blood is centrifuged to separate the plasma, the resulting plasma should be stored in a freezer at a temperature of at least -20°C.[97] Samples should be shipped on dry ice or with other appropriate cooling methods to ensure that the samples remain frozen during transportation.

1.6.2 Hyphenated techniques

The last 20 years have seen a remarkable improvement in hyphenated analytical methods, which has allowed for the significant broadening of their applications in the analysis of biomaterials. In hyphenated techniques, a separation technique is coupled with an online spectroscopic detection technology.[98] To identify the compounds in crude samples, gas

chromatography (GC), capillary electrophoresis (CE) or liquid chromatography (typically high-performance liquid chromatography) are linked to a spectroscopic detection technique such as UV-vis absorbance, photodiode array or Fourier-transform infrared spectroscopy (FTIR), fluorescence emission, nuclear magnetic resonance mass spectroscopy (NMR) or mass spectroscopy (MS).[53,98,99] MS is the preferred detection method, with single- and triple-quadrupole, ion trap and time-of-flight mass spectrometers (TOFMS) being the most frequently used. Table 1.3 summarises the literature's hyphenated techniques, sample preparation and corresponding quantification range for the quantification of carvedilol, enalaprilat and perindoprilat in human plasma. The most frequently used technique for quantifying carvedilol, enalaprilat and perindoprilat in plasma is LC-MS or, more specifically, LC-MS/MS (Table 1.3).

Table 1.3 Literature survey of analytical methods, quantification range and sample preparation of carvedilol, enalaprilat and perindoprilat in human plasma

Analytical method	Analyte(s)	Quantification range	Sample preparation	Reference
GC-MS	Carvedilol	15–500 ng/mL	Carvedilol extracted with a mixture of diethyl ether and ethyl acetate at basic pH via liquid liquid extraction (LLE). Extracts derivatised with n-Methyl-n-(trimethylsilyl) trifluoroacetamide (MSTFA).	100
HPLC-MS/MS	Carvedilol	0.1–200 ng/mL	Extraction from plasma by LLE using a diethyl-ether solvent.	101
Hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-MS/MS)	Carvedilol	0.1–200 ng/mL	LLE extraction from plasma with methyl tert-butyl ether at basic pH.	102
HPLC-MS/MS	Carvedilol	2–100 ng/mL	Protein precipitation using acetonitrile.	103
HPLC-MS/MS	Carvedilol	0.5–200 ng/mL	Solid phase extraction with Phenomenex Strata-X 30 mg/1 cc extraction cartridge.	104
High-performance liquid chromatography with electrochemical detection (HPLC–ECD)	Carvedilol	0.1–150 ng/mL	LLE with 100 µL of 0.1N NaOH and 900 µL of saturated NaCl and 0.6 mg of ascorbic acid were added to plasma and then extracted with 5 mL of diethyl ether.	105
HPLC with fluorescence detection	Carvedilol	1–80 ng/mL	Protein precipitation using acetonitrile.	106
LC-MS/MS	Carvedilol	2–400 ng/mL	Protein precipitation using acetonitrile.	107
UPLC-MS/MS	Carvedilol and enalaprilat	0.024–50 ng/mL (both analytes)	A combination of protein precipitation prior to mixed-mode strong cation exchange solid-phase extraction was used for the sample preparation.	108
HPLC-MS/MS	Enalaprilat	0.638–255 ng/mL	One-step protein precipitation with methanol.	109
HPLC-MS/MS	Enalaprilat	0.506–162 ng/mL	Solid phase extraction (SPE) using Orpheus C18 (100 mg/mL) cartridges.	110
HPLC-MS/MS	Enalaprilat	0.1–20 ng/mL	One-step protein precipitation with perchloric acid.	111
HPLC-MS/MS	Enalaprilat	1–200 ng/mL	One-step protein precipitation with acetonitrile.	112

Table 1.3 Continued

Analytical Method	Analyte(s)	Quantification range	Sample preparation	Reference
HPLC-MS/MS	Enalaprilat	1–100 ng/mL	One-step protein precipitation with acetonitrile.	113
GC-MS	Enalaprilat	5–160 ng/mL	Solid-phase extraction with C18 cartridges and derivatisation with methyl iodide	114
HPLC-MS/MS	Enalaprilat	1–500 ng/mL	One-step protein precipitation with acetonitrile.	115
HPLC-MS/MS	Enalaprilat	1–100 ng/mL	Protein precipitation using 50:50 acetonitrile and methanol.	116
HPLC-MS/MS	Perindoprilat	0.3–40 ng/mL	Plasma treated with phosphoric acid followed by SPE using hydrophilic–lipophilic balance HLB cartridge.	117
UPLC-MS/MS	Perindoprilat	0.2–20 ng/mL	Protein precipitation using perchloric acid.	93
LC-MS/MS	Perindoprilat	0.1–200 ng/mL	Protein precipitation using acetonitrile.	118
UPLC-MS/MS	Perindoprilat and enalaprilat	Perindoprilat: 1–500 ng/mL Enalaprilat: 1–450 ng/mL	Protein precipitation using 50:50 acetonitrile and methanol.	119

1.6.3 LC-MS/MS

There are several distinct stages during LC-MS analysis. Separation of the sample components occurs via an HPLC column where the analytes are differentially partitioned between the mobile phase (eluent) and the stationary phase (fixed onto a support material and packed into the column). The mode of the chromatography dictates the mechanism of retention and separation and may include mechanisms such as hydrophobic interaction, ion exchange or ion pair. Once the sample species are separated, they are sprayed into an atmospheric pressure ion source. Here they are converted to ions in the gas phase, with the bulk of the eluent pumped to waste. Electrospray ionisation (ESI) is one of the principal ionisation techniques. ESI is primarily used to analyse charged species. ESI is a "soft" or very low energy process and usually produces the intact parent molecule with single or multiple charges.[120] A mass analyser is used to sort ions according to their specific mass-to-charge ratio.[121] As mentioned earlier, single- and triple-quadrupole, ion trap and TOFMS are the most frequently used. The ions emerging from the mass analyser are counted via a detector. Detectors that are widely used include dynode, electron multiplier and photodiode, as well as the multi-channel plate. All detection and mass analysis is performed under a high vacuum with a combination of foreline (roughing) and turbomolecular pumps.[121]

The ionisation from LC-MS mainly displays the molecular ion species with only a few fragment ions, since soft ionisation is typically used. The information obtained from a single LC-MS run is therefore reasonably poor. This problem is mitigated with the use of tandem mass spectrometry.[98] A tandem mass spectrometer is a single instrument that uses two (or more) mass analysers. The simplest is two mass analysers (MS/MS) connected in series by a chamber known as a collision cell.[122] In the first mass analyser, the sample is sorted and weighed. It is then fragmented in the collision cell, with the fragment ions being sorted and weighed in the second mass analyser.[122] Better selectivity and sensitivity for quantitative analysis are achieved, as both the first and second analysers are used to select representative ion transitions.[121] Figure 1.5 is a illustration of LC-MS/MS operation[123].

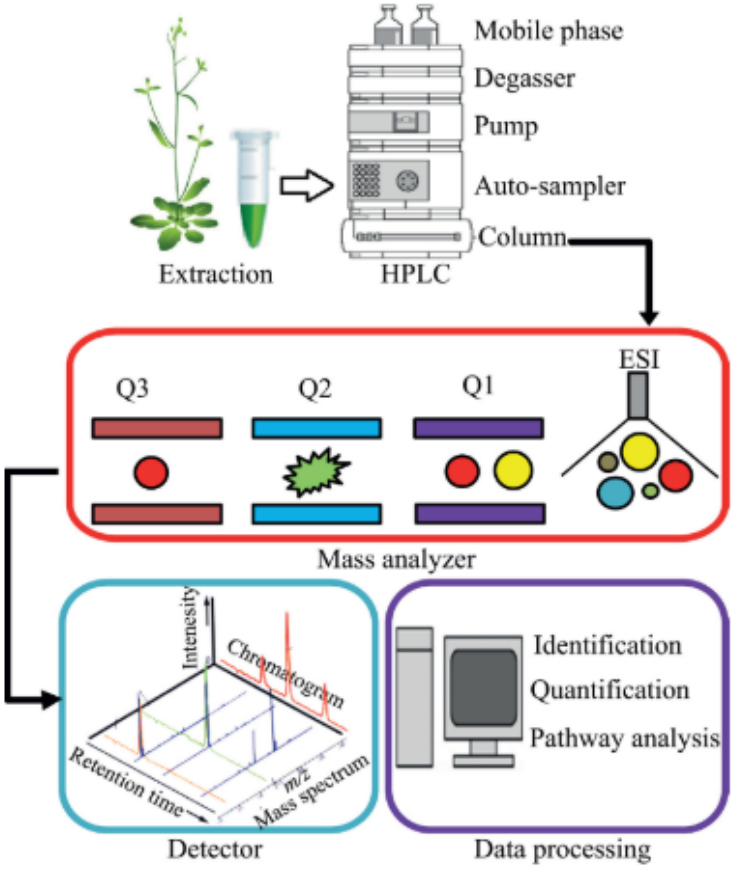


Figure 1.5 Illustration of LC-MS/MS operation[123]. (Permission obtained from publisher to use image)

The specificity, flexibility and abundance of information are some of the inherent strengths of the LC-MS/MS system.[120] The selection of the precursor ion using the first quadrupole and the product fragment ion with the second quadrupole allows for precise detection of a given molecule. LC-MS/MS allows for new assays to be developed in-house with a high degree of flexibility and within a short period, so long as a comprehensive validation is performed. Because of the fast ion-selection electronics, multi-parametric and quasi-parallel analyses with the mass spectrometer, many quantitative and qualitative results can be produced with a single LC-MS/MS analytical run.[120]

1.6.4 Sample preparation

Samples could need processing before LC-MS/MS analysis for several reasons. Often sample preparation is required to remove proteins or constituents that could precipitate when injected into the mobile phase and thus clog the chromatography column. Removing these components is vital in order to avoid damaging the column and causing excessive pressure build-up within the LC system.[124] Sample preparation can also improve chromatographic performance. The organic solvent, pH, volume, buffer and the injection solvent's aqueous composition can

influence peak shapes, peak separation and retention times (RT). These can in turn influence the limits of quantification and the selectivity and robustness of the assay. Biofluids often must be exchanged with an injection solution compatible with the LC method before injection. Finally, the long-term stability of the LC-MS/MS instrument response and the precision and accuracy of the method, can almost always be improved by increasing the analyte-to-matrix ratio by selectively depleting the biological matrix.[124] Table 1.4 summarises available sample preparation techniques for LC-MS/MS.

Table 1.4 An overview of LC-MS/MS sample preparation protocols (adapted from Stone)[124]

Protocol	Relative cost	Relative complexity
Dilution	Low	Simple
Protein precipitation	Low	Simple
Liquid–liquid extraction	High	Complex
Solid-phase extraction	High	Complex
Online SPE	High	Complex

Dilution methods involve the addition of purified water or the addition of the LC mobile phase to the given patient sample before LC-MS/MS analysis. The technique is often used for matrices low in protein, such as urine, as it is inexpensive, simple and fast.[124]

Protein precipitation (PPT) is similar to dilution methods but is intended for matrices with high protein content, such as whole blood or plasma. PPT is fast, simple, and cheap. The operating principle is based on adding an acid, salt or organic solvent to the sample. For example, adding an organic solvent to the sample reduces the dielectric constant in the protein sample containing the proteins. This results in water displacement from the protein surface's hydrophobic region, which disrupts the hydrophobic interactions between the proteins in the sample, so forcing the proteins to precipitate out of the solution. The sample, internal standard (ISTD) and a precipitating agent are mixed and filtered or centrifuged to separate the precipitated proteins before the resulting supernatant is injected into the LC-MS/MS system.[124,125]

Liquid–liquid extraction (LLE) involves partitioning the analytes from an aqueous biofluid into a water-immiscible organic solvent based on polarity. Its benefits include concentrating the assays, enhancing sensitivity, increasing selectivity, and depletion of matrix components. This process requires several steps and can be time-consuming. It requires the separation of the analytes into an organic solvent, separation of aqueous and organic layers, evaporation of the

organic solvent, and the reconstitution of the analytes in a solvent mixture miscible with the LC mobile phase.[124]

Solid-phase extraction (SPE) uses a selective stationary phase that binds or partitions the analytes. Often pre-treatment is required to allow for optimal extraction, followed by the diluted sample flowing through a stationary phase, capturing the analytes, and allowing the other matrix components to flow to waste. Following several wash steps, the analytes are recovered via an elution solvent. The samples may then need eluate evaporation and reconstitution with an LC-MS/MS-compatible solvent before analysis.[124]

In recent years, filtration plates have been developed that can remove phospholipids. Post-precipitation supernatant flows through a bed packed with moieties (i.e., zirconia-coated silica) that retain phospholipids. Although more costly, it allows for better selectivity while maintaining the simplicity of PPT protocols.[124,126]

Online SPE uses an LC "trap" column, which is analogous to an SPE cartridge or plate, so allowing the analyte to be captured while the matrix components flow to waste. Reversing the flow then elutes the target analytes directly onto the analytical LC columns. This reduces preparation time before LC-MS/MS injection but requires a more sophisticated LC setup with significant experience from the operator to ensure that performance is consistent.[124]

The bulk of published analytical methods quantifying enalaprilat and perindoprilat in plasma use protein precipitation in sample preparation (Table 1.3). Acetonitrile, methanol or a mixture of the two is generally used as the organic solvent. LLE or PPT is used in published analytical methods quantifying carvedilol in plasma (Table 1.3), with acetonitrile being preferred as the PPT organic solvent.

1.7 Quantifying carvedilol, enalaprilat and perindoprilat in DBS

Some challenges unique to the DBS matrix in sample analysis are discussed in this section. Mass spectrometry is the most common technique reported in the literature for DBS analysis, with LC-MS/MS being particularly popular.[83] The operation and inherent advantages of LC-MS/MS are not discussed again in this section. However, any method development or analytical challenges discussed are primarily discussed with LC-MS/MS in mind as the analytical method used for quantification.

1.7.1 Collection, processing, storage, and transportation of DBS

1.7.1.1 DBS card

It is important to do an in-depth check of the physicochemical properties of the target analyte before considering the use of DBS sampling. Air-sensitive or volatile samples should not be collected on DBS cards or paper.[127] Essential parameters of DBS cards include particle retention, pore size, absorption characteristics, and thickness. These characteristics determine the loading capacity and the spreadability of the blood sample onto the DBS card. Uniformity of the cards from lot to lot is also critical.

A popular DBS card used for sample collection is the Schleicher & Scheull 903 (S&S 903; Whatman 903 or 903®) card. It is listed as an FDA class II medical device and is made from 100% pure cotton without any wet strength additives. It has been used extensively in newborn screening and other applications worldwide.[127]

1.7.1.2 Sample collection

The collection of DBS samples is often done by pricking the toe, finger or heel with a lancet. The capillary blood is then directly applied onto the sampling paper within a pre-marked circle and ideally there should be one drop per spot. Capillary blood has many advantages over venous blood sampling in that it is less invasive, requires a smaller amount of blood and can be performed quickly and easily.[128] Venous blood can also be used for DBS collection, although it is more invasive than collecting capillary blood. An advantage of venous blood sampling is that spots can be created more accurately when compared to capillary blood sampling. This is because spots of a set volume are created from whole blood using calibrated pipettes. The emergence of VAMS, however, has the potential to combine the ease of capillary blood collection with the accuracy of venous blood collection.

When creating a DBS, the circular area should not be touched, especially before the blood that has been applied has been dried completely. Care should be taken to avoid clotting, supersaturating and layering. The pre-defined circular area should be filled homogenously and symmetrically, and both sides of the paper should display the same red colour. Samples that have been contaminated or have insufficient volume are not suitable for further analysis.[127] For samples created using a pipette, the tip should be a few millimetres above the card, with the blood dispelled with a single motion onto the card.[127]

1.7.1.3 Drying, storage and transportation

DBS must be completely dry before any storage or transportation occurs, with a minimum of 2–3 hours of drying required in an open space at room temperature (15–22°C).[127,129] Heating and stacking of samples should be avoided. Samples should not come into contact with other surfaces or be exposed to direct sunlight. Any moisture that remains on the spot could affect the integrity of the blood samples by stimulating bacterial growth, influencing extraction efficiency, and facilitating the breakdown of unstable analytes. Standard precautions against humidity and moisture include packing samples in zip-closure bags that contain desiccant and which are not gas-permeable. Depending on analyte stability, correctly packed DBS may be stored for months or even years at room temperature. Samples can also be transported by mail in an envelope, with no concerns of possible exposure to blood or infectious materials by those handling it.[127,129,130]

1.7.2 Effect of blood properties on DBS analysis

Unlike whole blood or plasma, for which the homogeneity of the sample can be readily ensured by thawing and thoroughly vortex-mixing in tubes or vials, DBS is a unique matrix in which the analyte of interest distributes after spotting. Although individually punched DBS spots should theoretically represent a specific homogenous blood volume, the quality of the DBS sample might vary from spot to spot. Unfortunately, remixing the blood samples prior to analysis is not possible. As a result, during the development of an LC-MS/MS assay method, a substantial amount of effort is required to assess the possible impact of various blood sample properties, which leads to unique challenges in DBS sample analysis.[127,129] This is discussed further below.

1.7.2.1 Effect of haematocrit

The haematocrit is the fraction of blood volume occupied by red blood cells and is independent of body size. Haematocrit usually is 0.41–0.51 for men and 0.37–0.47 for women.[127,131] Blood viscosity is directly proportional to the haematocrit and impacts the flux and diffusion properties of the blood spotted onto the card. A high haematocrit value may result in a poor distribution of the blood sample through the card. This means that for a partial punch (i.e., 3 mm) from a spot of fixed diameter, a larger blood volume would be contained in the punch from the high haematocrit sample compared to that of a low haematocrit sample. The influence

of the haematocrit can be mitigated by using the entire DBS sample instead of just a partially punched spot.[84,127]

1.7.2.2 Influence of blood spot volume

Calibration standards and QCs are normally prepared by adding a set blood volume using a calibrated pipette. Study sample volumes might not be controlled for, however. Different blood volumes on a card can result in different concentrations, even for a fixed sub-punch size and haematocrit. One study showed that the mean measured phenylalanine concentrations of 35 µL spots (85.1 ± 4.7 mg/L blood) are less than that of 100 µL spots (95.5 ± 9.1 mg/L blood).[127,132] If precise sample pipetting is not being used, different sample volumes will have to be evaluated during validation (e.g., 10, 20 and 60 µL).[127]

1.7.2.3 Distribution effect

The possible interaction of the blood or analytes with the materials of the DBS card is an additional factor that could create a difference in analyte concentrations between a spot's central and peripheral areas. The plasma component of the blood tends to occupy a greater fractional volume of the interior of the filter paper, with the erythrocytes concentrating towards the edge of the blood spot.[127,133,134] This results in increased concentrations of analytes with a greater affinity for the erythrocyte component in the peripheral sub-punches. Therefore, during the assay method development, it should be assessed whether the same analyte concentration is measured from different punched areas of the same DBS.[127]

In conclusion, haematocrit, blood volume and blood distribution could strongly influence the measured concentration of analytes found in DBS. Unless these potential influences have been carefully evaluated, it is suggested that accurate pipetting be done with a calibrated pipette, followed by cutting the entire blood spot from the card.[127]

1.7.3 DBS sample extraction

For quantitative analysis, one or more DBS discs are punched from the DBS card and then subjected to extraction. The extraction procedure is usually conducted by adding a certain quantity of extraction solvent (methanol, acetonitrile, or a mixture of water/organic) with the extraction solvent containing the ISTDs. The extraction solvent must be strong enough to break the binding of the analyte to the protein in the matrix and the paper material. Analytes are then extracted by gentle shaking or vortex-mixing, with sonication used to improve extraction efficiency.[127,135] Once centrifuged, the extracts are transferred to new tubes or microtiter plates.

Extracts can be injected directly into the LC-MS/MS system or be dried for reconstitution using an MS-friendly solvent before analysis. Derivatisation is a common approach that is used to enhance MS/MS detection sensitivity for trace analytes.[127,136,137] However, derivatisation could result in assay error as a result of unwanted reactions such as hydrolysis.[138]

1.7.4 Literature survey of quantification of carvedilol, enalaprilat or perindoprilat in DBS

Given the novelty of the matrix, the literature on the quantification of enalaprilat and perindoprilat in DBS is minimal. There are no published methods for quantifying carvedilol in DBS.[139] Peeters et al.[93] published an assay describing the quantification of enalaprilat and perindoprilat in DBS. In this assay, eight antihypertensive drugs and four active metabolites (which included both enalaprilat and perindoprilat) were quantified in DBS. Sampling was performed using Whatman protein saver 903 cards (Cardiff, United Kingdom). An acetonitrile and methanol mixture (1:1) containing ISTD was used to extract 6 mm punched samples. Once extracted, samples were sonicated, centrifuged, and diluted with eluent. Quantification took place via UHPLC-MS/MS, with the calibration ranges being 4.54–454 ng/mL and 5–500 ng/mL for enalaprilat and perindoprilat, respectively.

The feasibility of quantifying carvedilol and other antihypertensive drugs using 10 µL of Mitra® VAMS has been evaluated, but not in DBS. The lower limit of quantification (LLOQ) was 4 ng/mL. However, the method was not sensitive enough to quantify expected trough concentrations of patients involved in a proof-of-concept study.[139]

1.8 Method comparison of plasma and DBS assays

Comparison studies of measurement procedures are often used to assess the agreement between or detect bias between different analytical methods.[140] One method is usually the comparative method (x-method), and the other is the test method (y-method). The comparative method should ideally be a reference method, although it can also be a standardised procedure which the laboratory is familiar with.[127,140]

Methods have often been developed to quantify analyte concentrations in plasma during the early activities of drug discovery and development or at a particular stage of clinical diagnosis. A question will often arise regarding the correlation between the DBS and plasma concentrations.[127] Therefore, a comparison is often recommended as part of method validation. A strong correlation between plasma and DBS methods provides further confidence in quantitative analysis.[140–143]

1.8.1 Agreement and correlation

When comparing two analytical methods, one can evaluate both the correlation and agreement of the two methods. Agreement and correlation are two concepts that are widely used; both signal the degree of association between variables. Conceptually, however they are distinct, and therefore require different statistics. Correlation concentrates on the association of changes between two variables. The most popular measure of the association between two variables is the Pearson correlation coefficient (r),[144] which is only applicable when measuring the linear relationship and is described further below:[145]

- r = ± 1: Relationship is perfectly linear between two variables
- r = 0: no linear relationship
- r < 0: negative, inverse relationship
- r > 0: positive relationship

Agreement also assesses the relationships between outcomes of interest. The emphasis is also on the level of concordance between two or more assessments of the variable of interest. However, the agreement between variables of different constructs cannot be assessed. For example, one can assess the correlation between height and weight – but not agreement.[144]

1.8.1.1 Regression analysis (correlation)

Correlation is typically evaluated on a scatter plot of which the fitting line can be calculated using several approaches, including ordinary (vertical) linear regression, orthogonal regression, Deming regression or the Passing–Bablok method. Most linear regressions between DBS and other assays in the literature are done through either Passing–Bablok[146–149] or Deming regression,[94,150–153] and these two methods will be discussed further.

Deming regression

In simple linear regression, only the dependent variable is measured with error; in Deming regression, dependent and independent variables are measured with error. The technique is often used for comparative studies in clinical chemistry to evaluate the systematic differences between two measurement methods.[154]

In Deming regression, paired measurements (x_i, y_i) are measured with errors, ε_i and δ_i where:[154]

$$x_i = X_i + \varepsilon_i$$

$$y_i = Y_i + \delta_i$$

The intercept, β_o, and the slope, β_1, are calculated with the formula:

$$\hat{Y}_i = \beta_o + \beta_1 \hat{X}_i$$

X_i and Y_i, respectively represent the "true" values or the expected values. $\hat{X}_i$ and $\hat{Y}_i$ are the estimates of X_i and Y_i, respectively.

Deming regression assumes that the measurement error ratio, $\lambda = V(\varepsilon_i)/V(\delta_i)$, is constant. The regression coefficients and predicted values are determined with calculations described in Linnet.[154,155] The Pearson correlation coefficient of the Deming regression indicates the strength of the relationship between the two variables for which the regression was performed.

Passing–Bablok regression

Dependent and independent variables are also measured with error in Passing–Bablok regression. The two methods are compared by calculating the linear regression line and evaluating whether the intercept is zero and the slope is one. The intercept (β_o) and the slope (β_1) are fitted in Passing–Blok regression using the linear equation:[156]

$$Y = \beta_o + \beta_1 X$$

The intercept of the equation indicates the systematic bias (difference) between methods. The slope evaluates the proportional bias (difference).[156] The Pearson correlation coefficient of the Passing–Bablok regression indicates the strength of the relationship between the two variables for which the regression was performed.

1.8.1.2 Bland–Altman analysis (agreement)

Bland and Altman[157] introduced a plot (now called the the Bland–Altman plot) to describe the agreement between two quantitative measurements. The graph is a scatter XY plot, with the Y axis showing the difference between the two paired measurements (A-B) and the X axis representing the average of the two measurements ((A+B)/2). In summary, the difference between the two measurements is plotted against the mean of the two measurements.[158]

The Bland–Altman plot does not indicate if the extent of agreement is adequate to use a method. It can only quantify a bias and a range of agreement. Only the specific biological, analytical or clinical goals can define whether the agreement interval is too wide or sufficiently narrow. Allowable limits of acceptable differences should be defined *a priori* based on the relevant

biological or analytical criteria.[158] For example, the European Medicines Agency (EMA) indicates that for the cross-validation of two methods, the difference between two values should be less than 20% of the mean for the difference between the two values to be acceptable. This needs to be the case for at least 67% of the paired samples to pass the criteria of cross-validation.[159]

1.9 Conclusions

HF generates a significant clinical, societal and economic burden, with particularly significant ramifications in Africa due to the high mortality rate and high prevalence among the working-age population.[7,160] HF can often be treated with the appropriate medications; however, as with most chronic diseases, poor medication adherence exacerbates the impact of the disease. Concrete adherence data will increase the understanding of the high mortality rate of African HF patients. Carvedilol, enalapril and perindopril are HF medications that are commonly used to treat HF. Developing methods to determine adherence to these medications would therefore be beneficial.

To generate objective adherence data of HF patients from Africa, adherence-determining techniques need to be used that are practical in execution and which will also generate reliable data. Quantifying analytes directly in a biological matrix is an adherence-determining method that generates direct, objective adherence data. Of the biological matrices available to determine adherence, the use of DBS as a sampling method is well suited to remote, resource-scarce locations, such as many African areas. This is because of its less complex transportation, ease of storage, and the inherent stability of analytes in DBS relative to other matrices. The literature describing methods quantifying carvedilol, enalaprilat and perindoprilat in DBS is sparse.[94] There is also no method describing the simultaneous analysis of all three analytes in DBS. Simultaneously quantifying all three analytes will allow for a more practical assay.

Although DBS analysis has some advantages over plasma analysis, plasma is still considered the gold standard of biosample analysis. Therefore, developing both plasma and DBS analytical methods for quantifying analytes in African HF patient samples is useful. This allows for the cross-validation and comparison of the assays using appropriate statistical analysis such as linear regression and Bland–Altman plots. Much literature describes the quantification of carvedilol, enalaprilat and perindoprilat in plasma (Table 1.3). The bulk of these methods use HPLC-MS/MS with protein precipitation as sample preparation. However, no method in the literature describes the quantification of all three analytes simultaneously. Quantifying all three

analytes together will allow for a more functional assay, especially when quantifying many samples.

1.10 Project aims and objectives

1.10.1 Aim

To develop assays suitable for determining the adherence of African HF patients taking carvedilol, enalapril and/or perindopril.

1.10.2 Objectives

- Develop and validate a quantitative LC-MS/MS method to simultaneously determine carvedilol, enalaprilat and perindoprilat concentrations in plasma.
- Develop and validate a quantitative LC-MS/MS method to simultaneously determine carvedilol, enalaprilat and perindoprilat concentrations in DBS samples prepared from venous blood. Venous blood is to be accurately pipetted onto the DBS cards to create a controlled environment for the validation conditions.[150]
- Perform a pharmacokinetic pilot study of patients on carvedilol, enalapril and perindopril using the two assays developed. Furthermore, evaluate the robustness of the assays using actual patient samples. Evaluate pharmacokinetic parameters of carvedilol, enalaprilat and perindoprilat in DBS and plasma.
- Using the pharmacokinetic concentrations, evaluate the feasibility of using the assays for determining adherence to carvedilol, enalapril and perindopril.
- Compare and cross-validate the two assays through correlation and agreement using DBS and plasma concentrations from the pilot study. Evaluate the feasibility of using DBS and plasma concentrations interchangeably.

Figure 1.6 shows which chapters in the thesis address the above objectives. In addition, it shows objectives linked to the PhD but which are outside the scope of the PhD project. Data from the pharmacokinetic study were used to develop a pharmacokinetic model for evaluating adherence. This model is to be used at study sites in Africa to gauge the adherence of HF patients.

Chapter layout of objectives

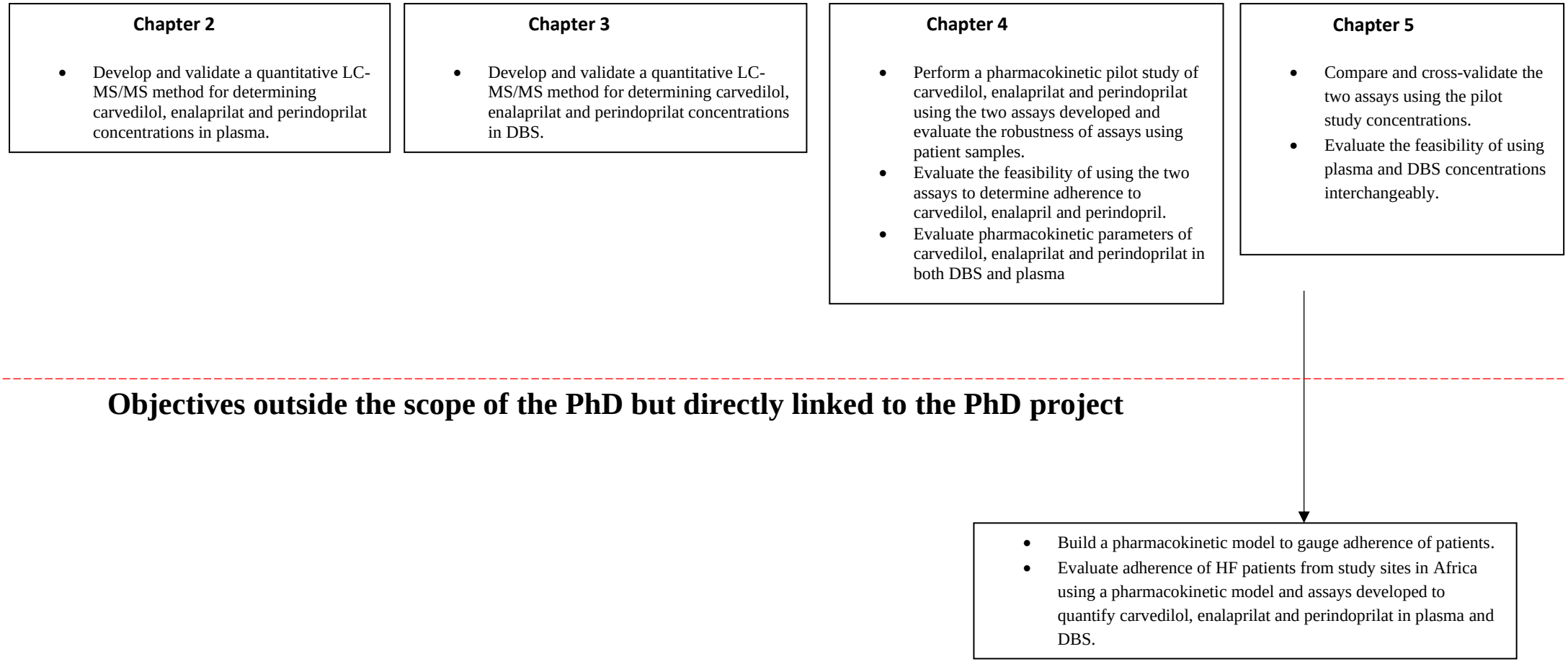


Figure 1.6 Layout of thesis objectives and objectives linked to the PhD project but outside the scope of the PhD project (shown below the red dashed line).

2 Simultaneous Quantification of Carvedilol, Enalaprilat and Perindoprilat in Plasma

2.1 Introduction

Carvedilol, enalapril and perindopril are medications that are often prescribed for the treatment of HF.[28,161] Carvedilol is a non-selective β-blocking agent, while enalapril and perindopril are prodrug ACE inhibitors, with their active metabolites being enalaprilat and perindoprilat, respectively.[101,161] Beta-blockers such as carvedilol are often prescribed with ACE inhibitors such as enalapril or perindopril for HF treatment.[161]

Studies have found that the mortality of African heart failure patients is exceptionally high, with no understanding of why this is the case.[162] One potential reason for the high mortality could be poor medication adherence. A direct, objective approach to evaluating HF patient adherence is determining the concentrations of carvedilol and the active metabolites of enalapril and perindopril, enalaprilat and perindoprilat, respectively, in plasma.[28] Plasma is still considered the gold standard of biosample analysis, with most pharmacokinetic data generated from plasma.[53,163]

Several analytical methods have been developed for quantifying carvedilol, enalaprilat and perindoprilat in plasma, with most published methods using LC-MS/MS.[93,101,104,110–113,115,119,164] Protein precipitation is frequently used in published analytical methods for all three analytes before LC-MS/MS quantification.[92,93,101,104,113] However, no published method quantifies all three analytes simultaneously in plasma. From an analytical perspective, having one method which can quantify all three analytes allows for a more functional assay. Samples containing different analytes do not have to be separated and can be quantified together. This is important when considering upscaling to large quantities of samples, such as with a clinical study. Analysing samples together means less time on the instrument and savings in chemical costs.

This chapter describes the development and validation of a method for extracting and simultaneously quantifying carvedilol, enalaprilat and perindoprilat in human plasma using LC-MS/MS. The objectives addressed in this chapter include:

- Developing a simple, sensitive and reproducible extraction and detection method for the simultaneous quantification of carvedilol, enalaprilat and perindoprilat in human plasma.
- Validation of the bioanalytical method according to the FDA guidelines.[165]

2.2 Materials and methods

2.2.1 Collection and storage of plasma samples

Validation experiments were performed using donated plasma not containing carvedilol, enalaprilat or perindoprilat. Quality controls and calibration standards were also prepared with donated analyte-free plasma.

2.2.2 Chemicals and reagents

Reference standards (carvedilol, enalaprilat dihydrate and perindoprilat powder) and ISTDs (carvedilol-d5, enalaprilat-d5 sodium salt, and perindoprilat-13C3) were sourced from Toronto Research Chemicals Inc. (Toronto, Canada). Ammonium acetate (≥ 99.99%) and acetic acid (Proanalyis grade) were supplied by Sigma-Aldrich (Modderfontein, South Africa) and Labchem (Johannesburg, South Africa), respectively. Methanol and acetonitrile, both LC-MS grade, were purchased from Honeywell (B&J) and Anatech (Bellville, South Africa), respectively. A Merck Millipore (Billerica, MA, USA) purification system was used to prepare deionised water.

2.2.3 Sample extraction

Plasma samples, including calibration standards, QC samples, blanks and unknown samples, and stored at ~-80°C, were thawed at room temperature. Samples were then briefly vortexed. In microcentrifuge tubes, 200 µL methanol: acetonitrile (1:1, v/v) precipitation solution was added to aliquoted plasma (50 µL). The precipitation solution contained ISTD (2.41 ng/mL of carvedilol-d5, 9.56 ng/mL of enalaprilat-d5, and 4.78 ng/mL of perindoprilat-$^{13}C_3$). After being vortex mixed for 30s, samples were equilibrated on bench for 5 minutes. Samples were centrifuged (5 minutes at 20238 g), and the entire supernatant was transferred via pipetting to glass tubes to evaporate under a nitrogen stream at 40°C for ~15 minutes. Dried samples were reconstituted with a 200 µL methanol:water:formic acid (40:60:0.2, v/v/v) solution, vortexed for 30 s, and transferred to 96-well plates. The plates were placed in the LC-MS/MS autosampler at ~8°C, and 20 µL of samples were injected.

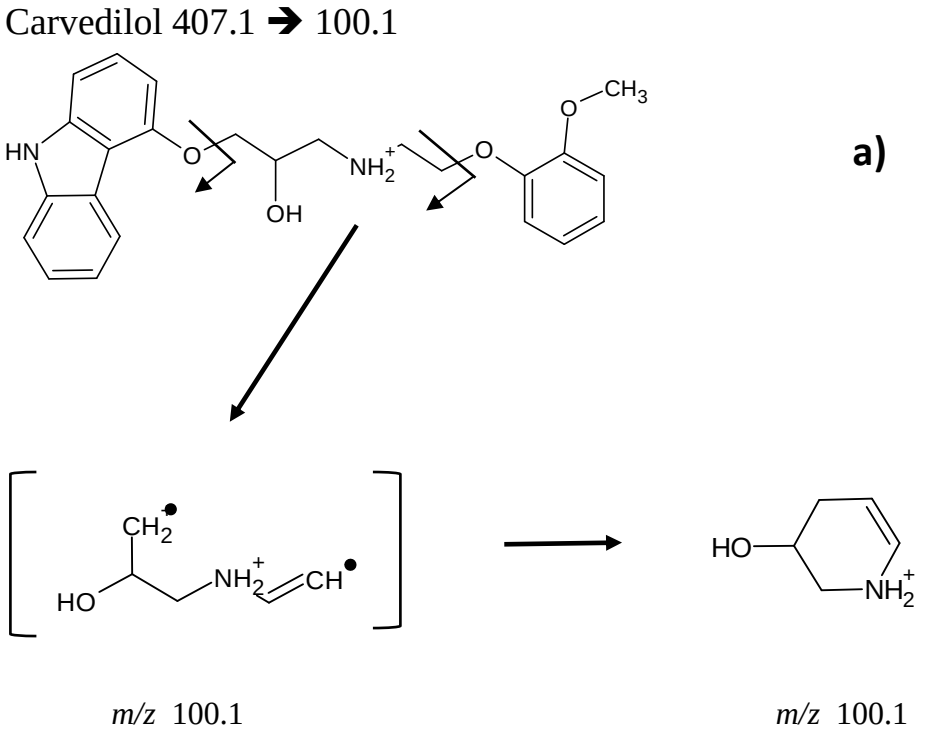
2.2.4 LC-MS/MS conditions and equipment

An AB Sciex API 5500 (AB Sciex™, Germany) Qtrap mass spectrometer in the positive electrospray ionisation mode was used. The optimum parameters were obtained via product ion scans of analytes and ISTDs. The collision gas was set at the "medium" level. The nebuliser, turbo, and curtain gas settings were 55, 55, and 30 psi, respectively, with source temperature and ion spray voltage set to 500°C and 5500V, respectively. An Agilent 1200 autosampler (Agilent, CA, USA) and an Infinity II binary pump was used. Table 2.1 further summarises the

multiple reaction monitoring (MRM) transitions and mass spectrometer conditions. Data collection and analysis were done using Analyst Version 1.7.1 (AB Sciex™, Germany). The proposed fragmentations are depicted in Figure 2.1 to Figure 2.6. The product ion mass spectra are shown in Figure 2.7 to Figure 2.12.

Table 2.1 Summary of MRM transitions and mass spectrometer conditions of analytes and ISTD

	MRM transition						
Analyte	**Quantifier, m/z**	**Qualifier, m/z**	**Dwell time (ms)**	**Declustering potential (V)**	**Entrance potential (V)**	**Collision energy (V)**	**Cell exit potential (V)**
Carvedilol	407.1 → 100.1	407.1 → 224.1	125	96	10	37	10
Carvedilol-d5	412.2 → 105.1	412.2 → 229.1	105	126	10	39	12
Enalaprilat	349.1 → 206.1	349.1 → 91.1	35	101	10	27	20
Enalaprilat-d5	354.2 → 211.1	354.2 → 96.0	50	91	10	27	20
Perindoprilat	341.2 → 98.1	341.2 → 170.1	65	96	10	45	10
Perindoprilat-$^{13}C_3$	344.2 → 100.1	344.2 → 170.1	60	81	10	47	10



Carvedilol 407.1 ➔ 224.1

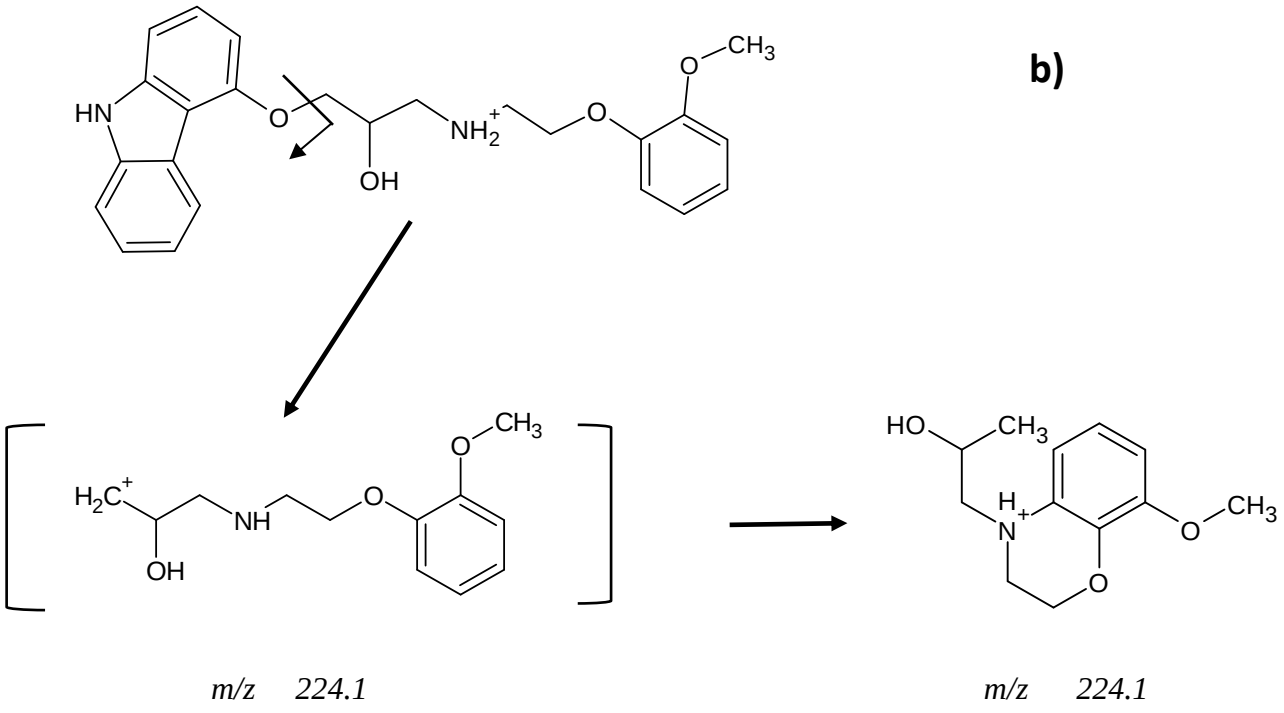
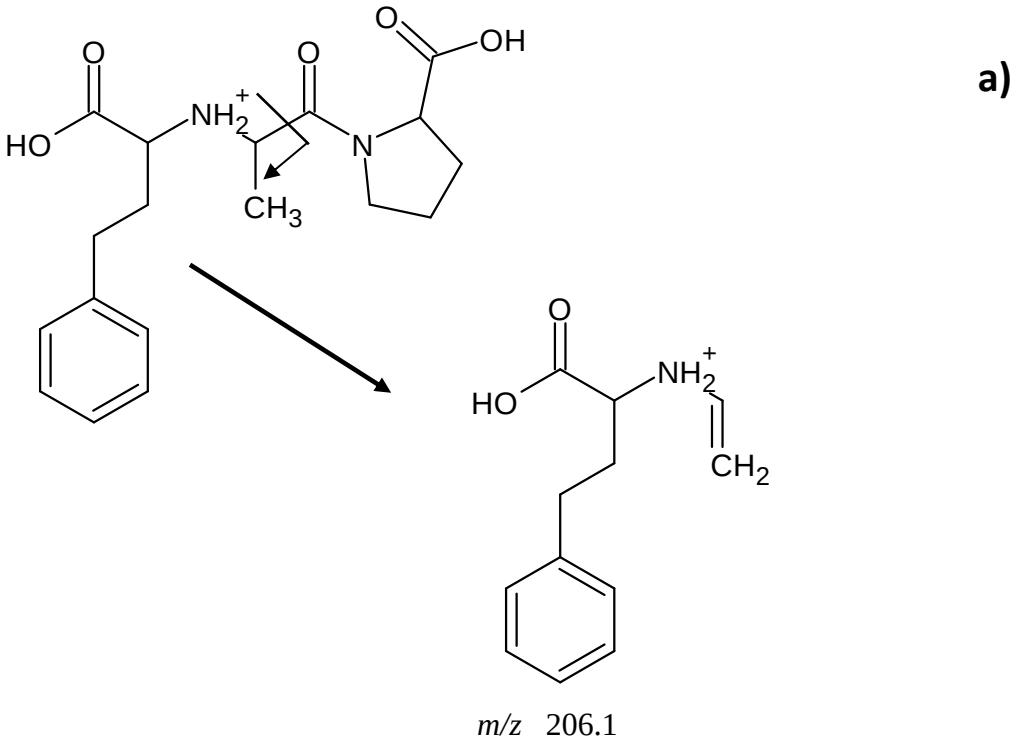


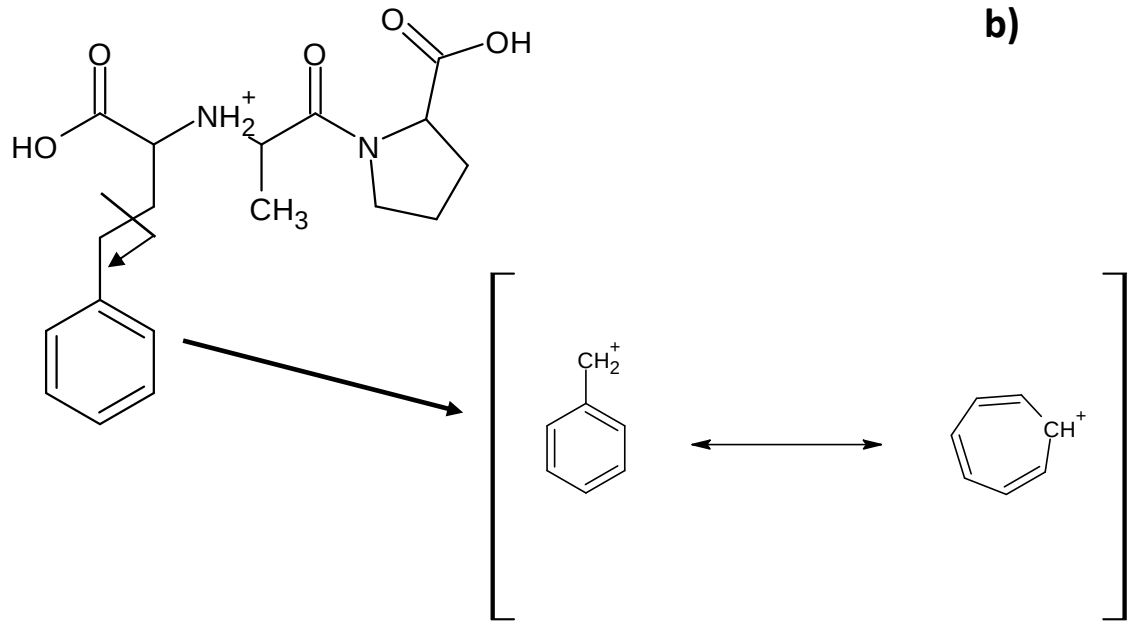
Figure 2.1 Proposed fragment ions for carvedilol: a) 407.1 ➔ 100.1 b) 407.1 ➔ 224.1

Enalaprilat 349.1 ➔ 206.1



m/z 206.1

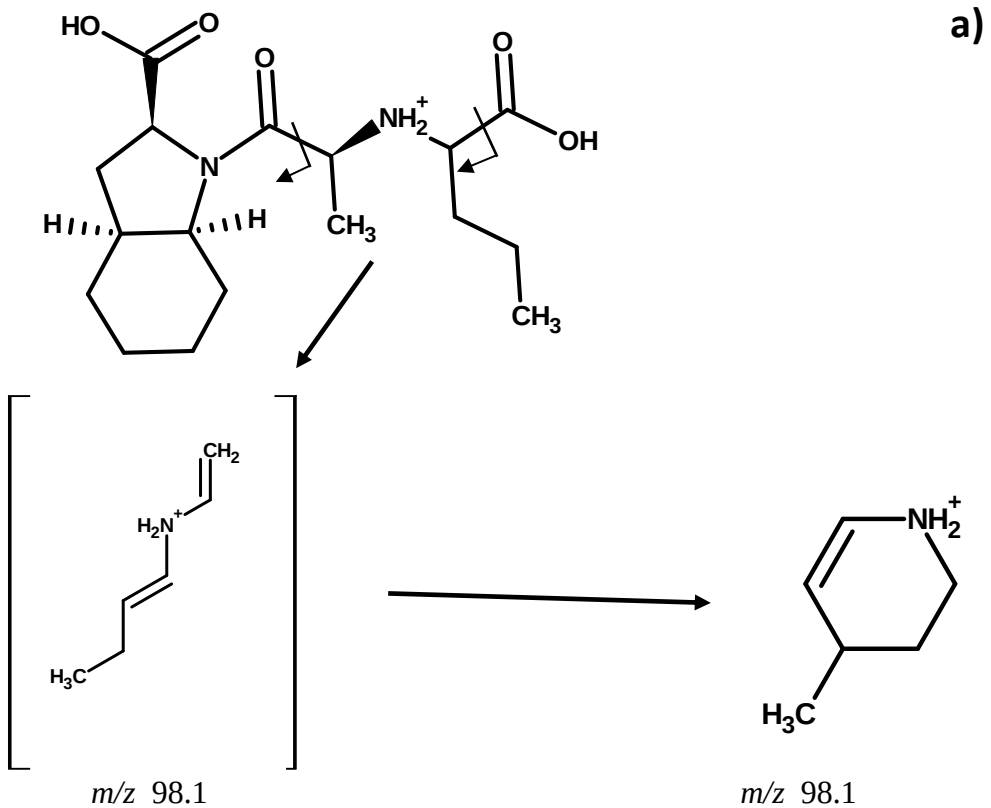
Enalaprilat 349.1 ➔ 91.1



m/z 91.1

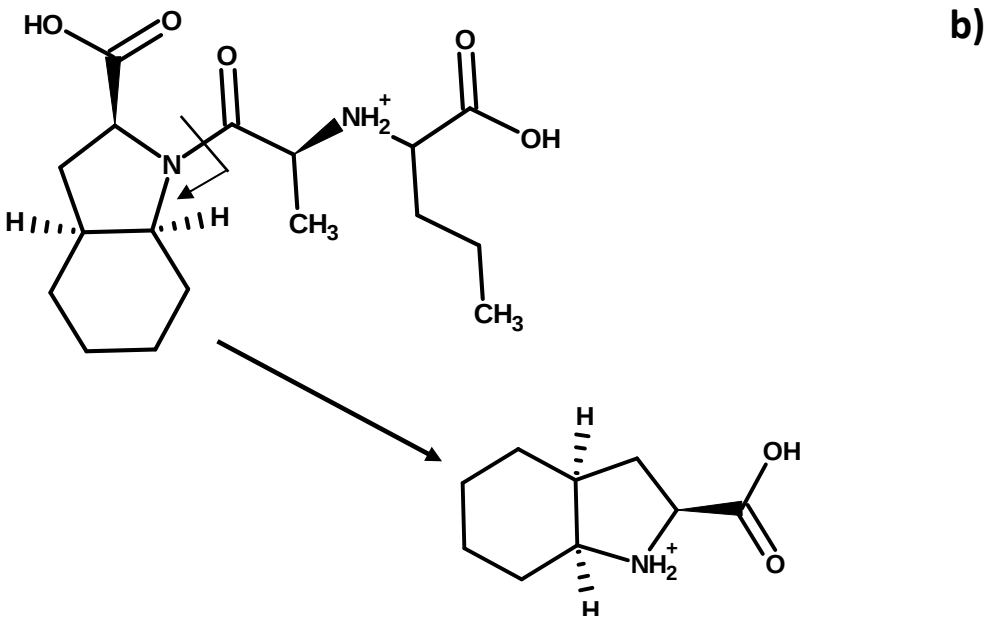
Figure 2.2 Proposed fragment ions for enalaprilat: a) 349.1 ➔ 206.1 b) 349.1 ➔ 91.1

Perindoprilat 341.2 ➔ 98.1



m/z 98.1 *m/z* 98.1

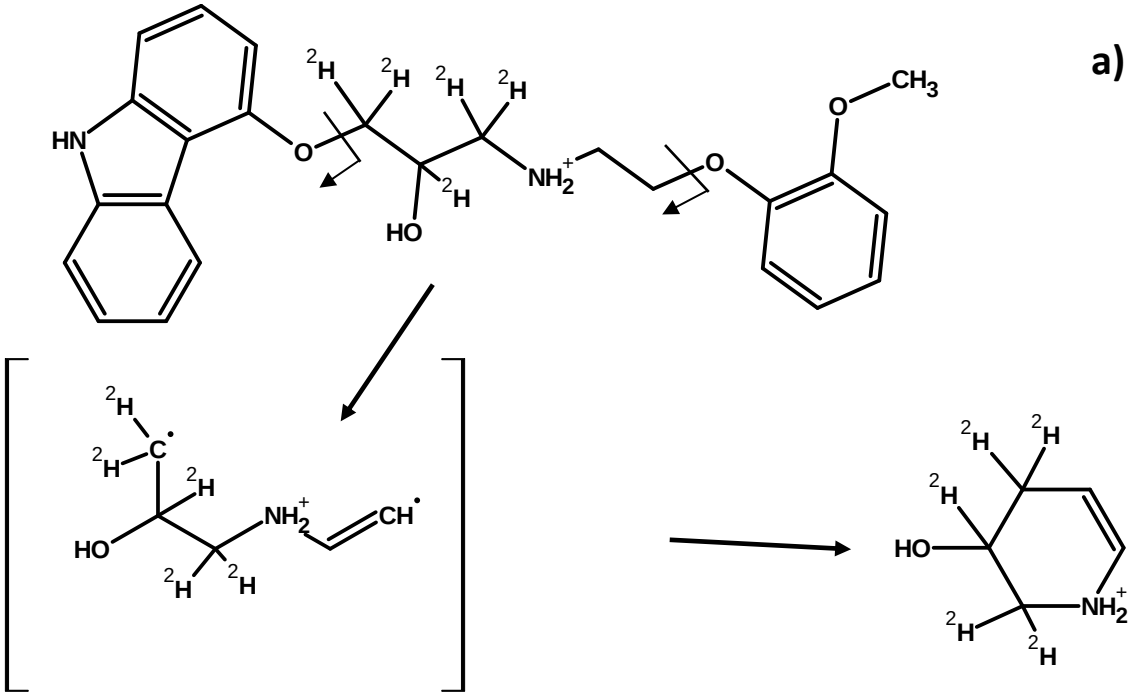
Perindoprilat 341.2 ➔ 170.1



m/z 170.1

Figure 2.3 Proposed fragment ions for perindoprilat: a) 341.2 ➔ 98.1 b) 341.2 ➔ 170.1

Carvedilol-d5 412.2 ➔ 105.1

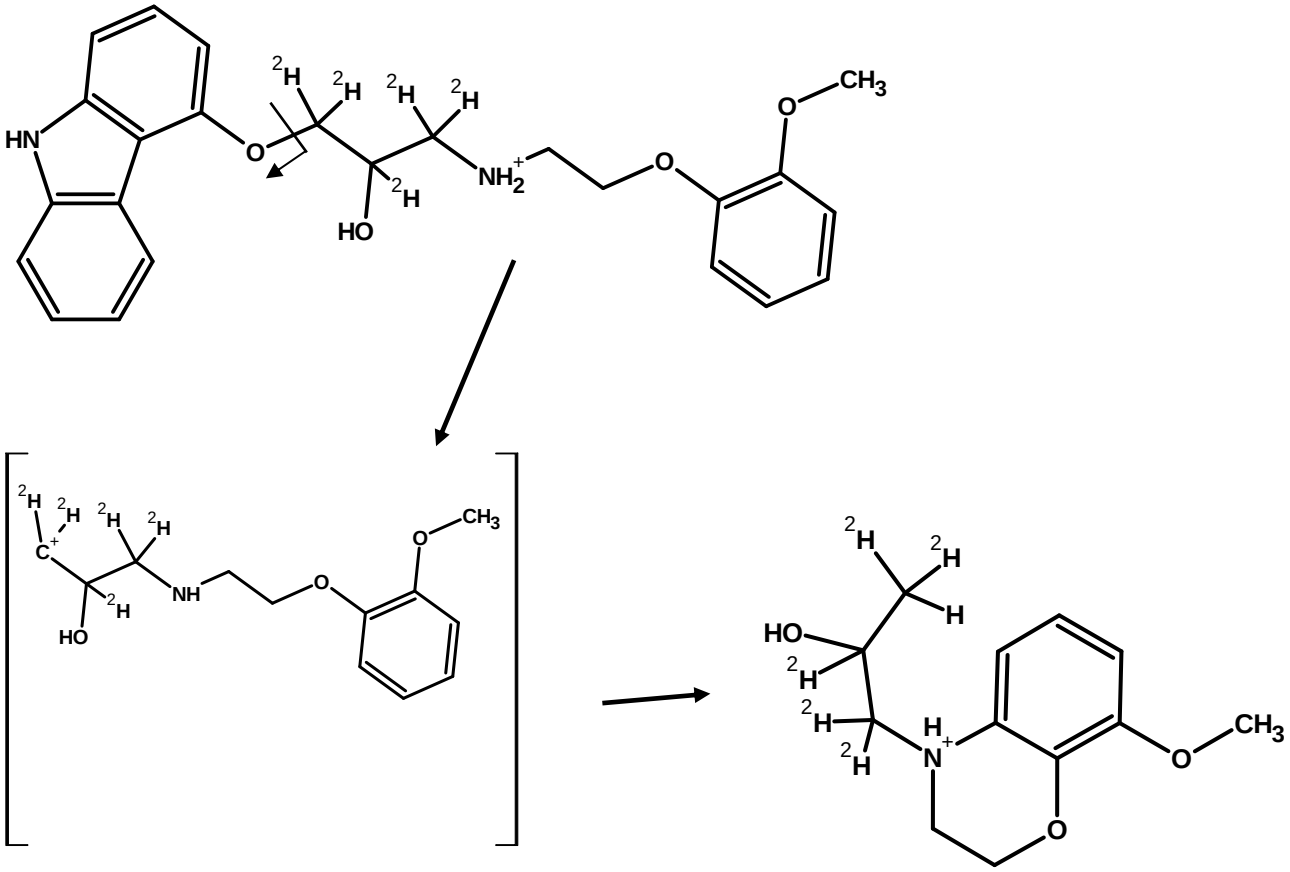


m/z 105.1

m/z 105.1

Carvedilol-d5 412.2 ➔ 229.1

b)



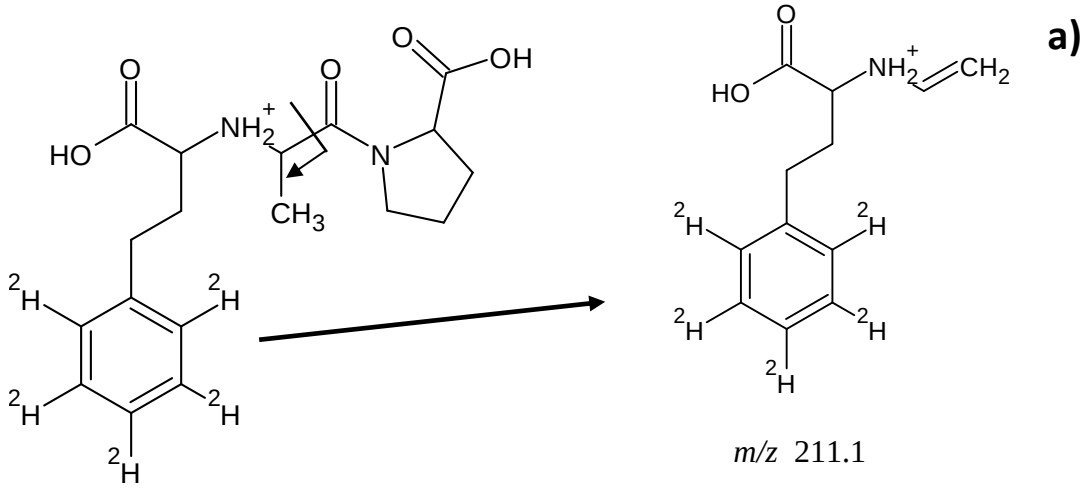
m/z 229.1

m/z 229.1

Figure 2.4 Proposed fragment ions for carvedilol-d5: a) 412.2 ➔ 105.1 b) 412.2 ➔ 229.1

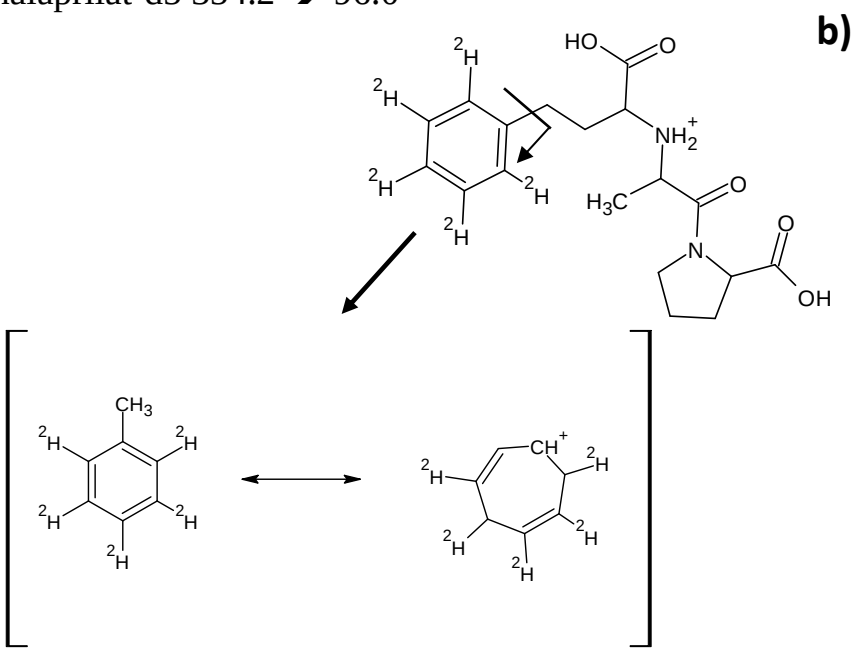
Enalaprilat-d5 354.2 ➔ 211.1

a)



m/z 211.1

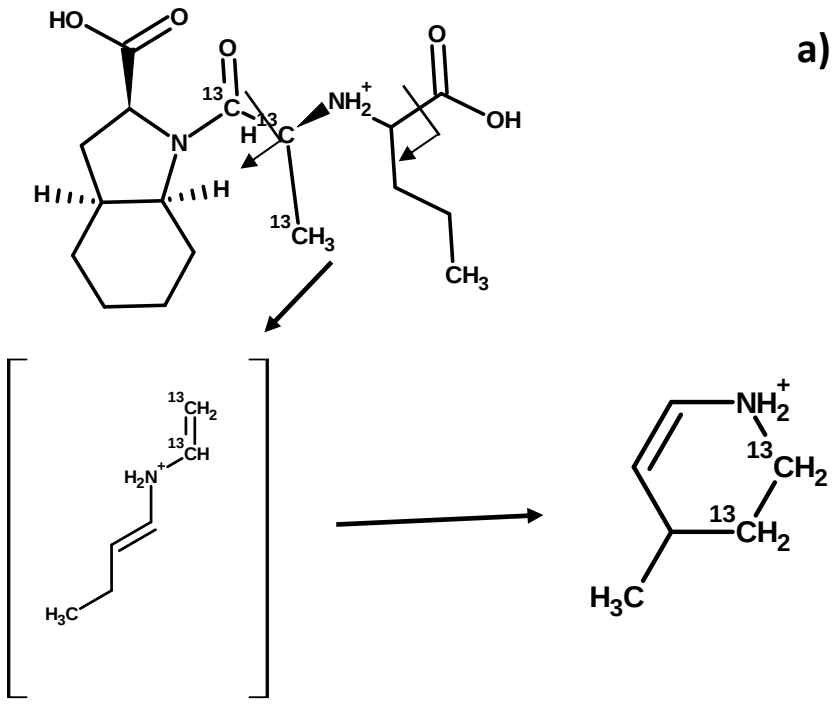
Enalaprilat-d5 354.2 ➔ 96.0



m/z 96.0

Figure 2.5 Proposed fragment ions for enalaprilat-d5: a) 354.2 ➔ 211.1 b) 354.2 ➔ 96.0

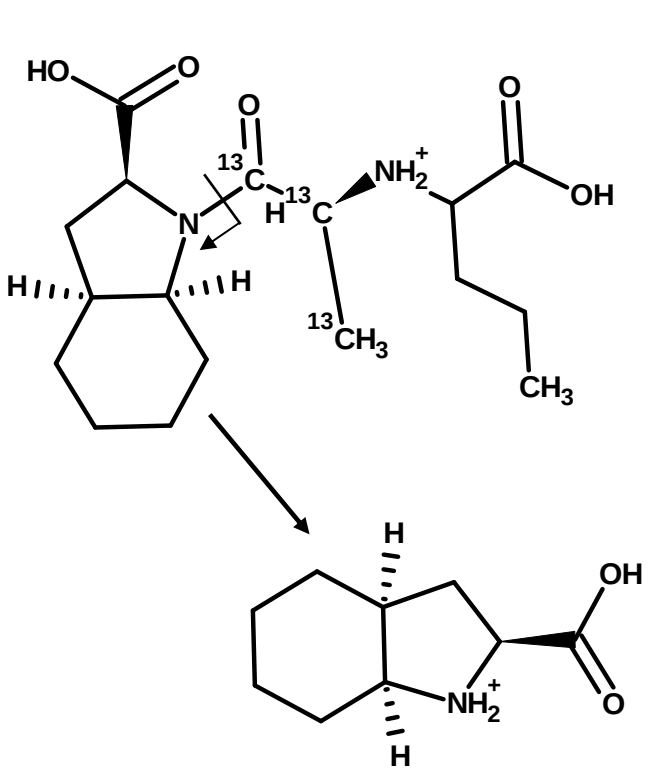
Perindoprilat-$^{13}C_3$ 344.2 ➔ 100.1



m/z 100.1

m/z 100.1

Perindoprilat-$^{13}C_3$ 344.2 ➔ 170.1



m/z 170.1

Figure 2.6 Proposed fragment ions for Perindoprilat-$^{13}C_3$: a) 344.2 ➔ 100.1 b) 344.2 ➔ 170.1

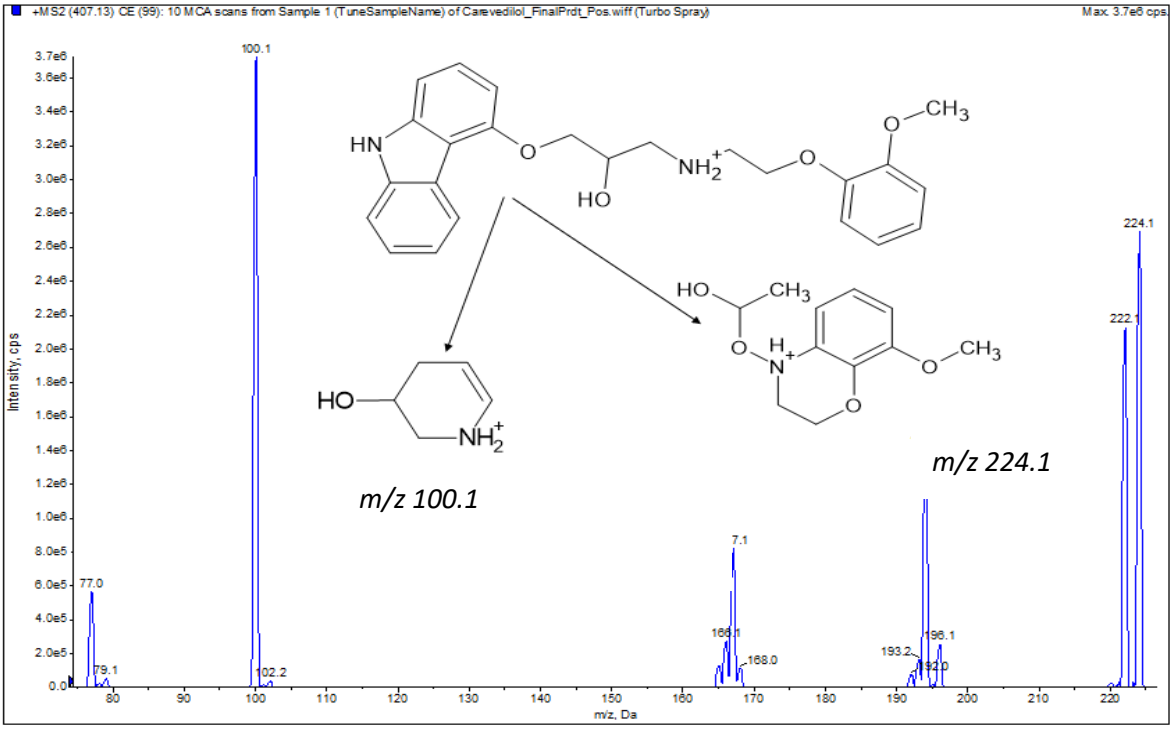


Figure 2.7 Final product ion mass spectra for carvedilol[28]

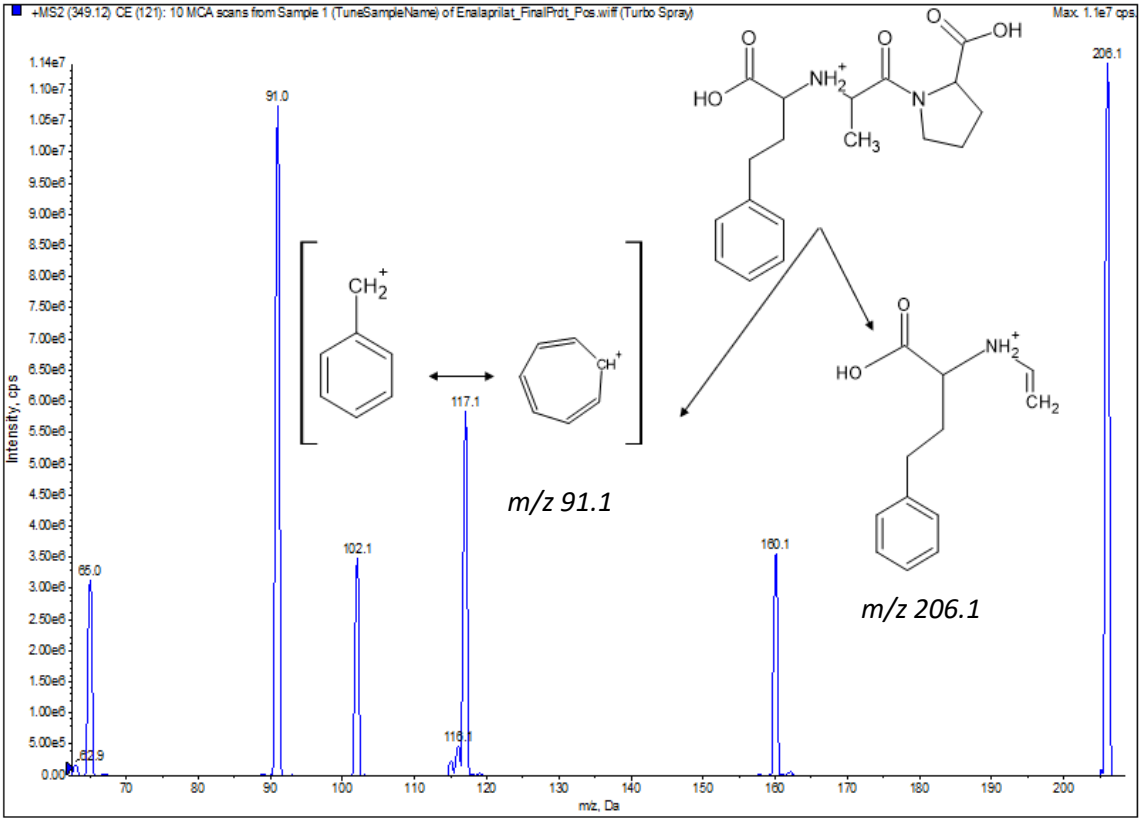


Figure 2.8 Final product ion mass spectra for enalaprilat[28]

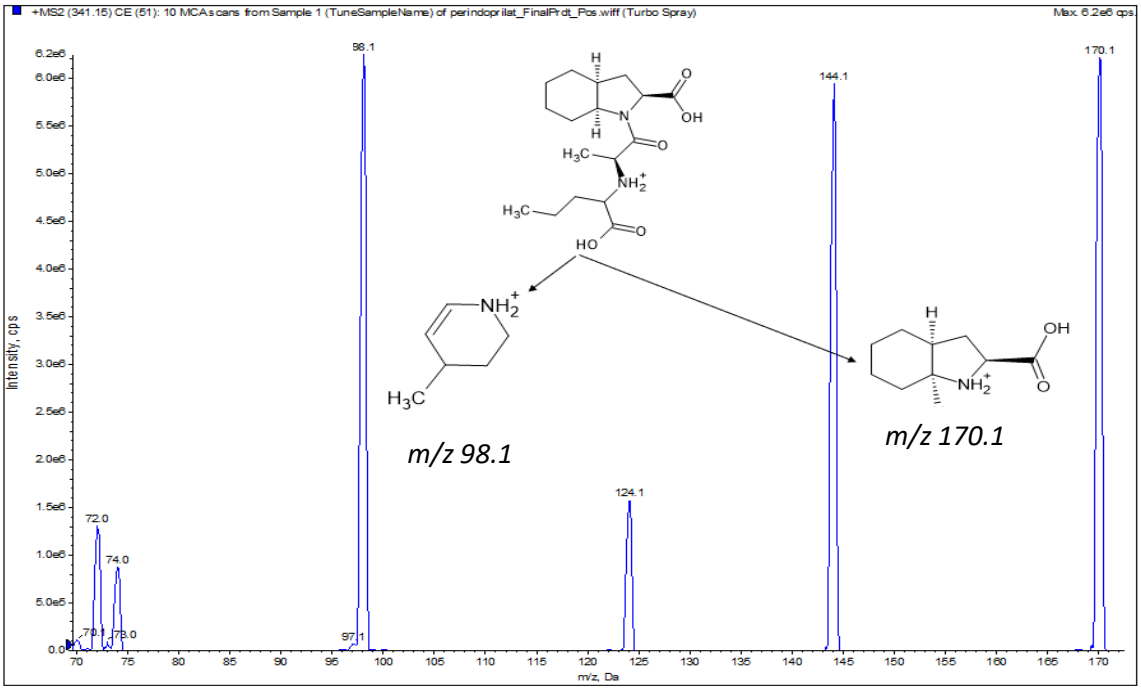


Figure 2.9 Final product ion mass spectra for perindoprilat[28]

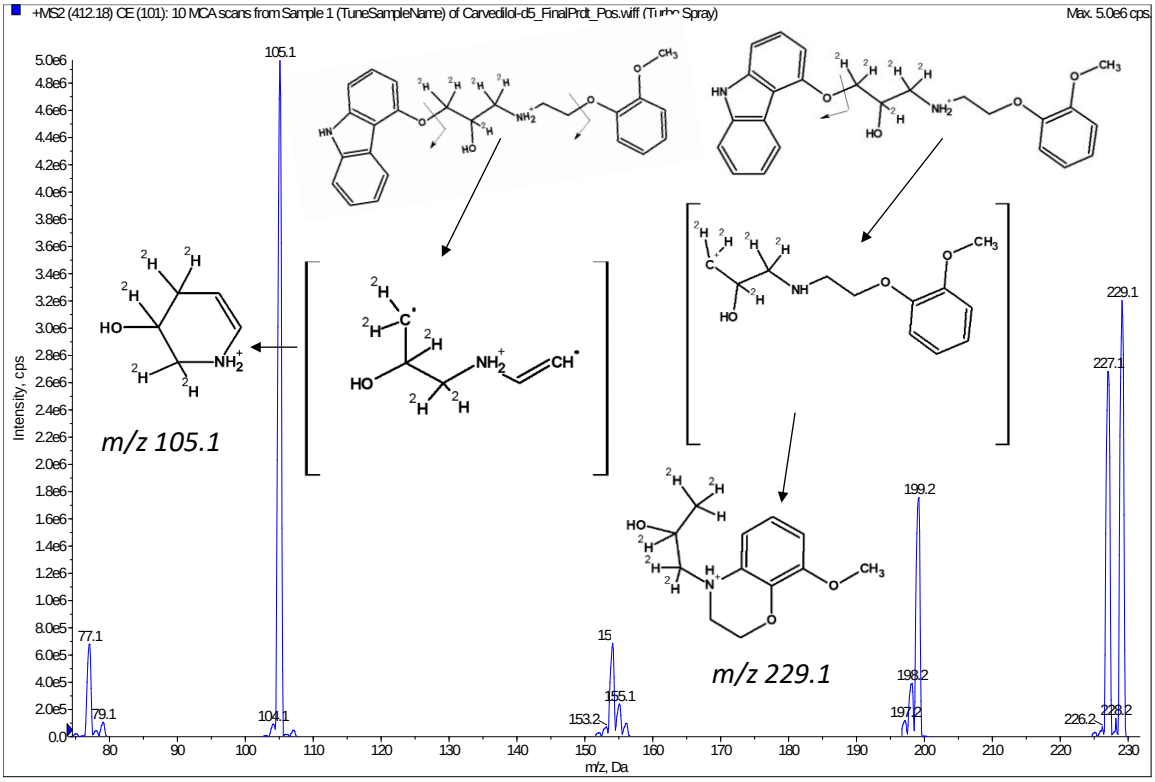


Figure 2.10 Final product ion mass spectra for carvedilol-d5[28]

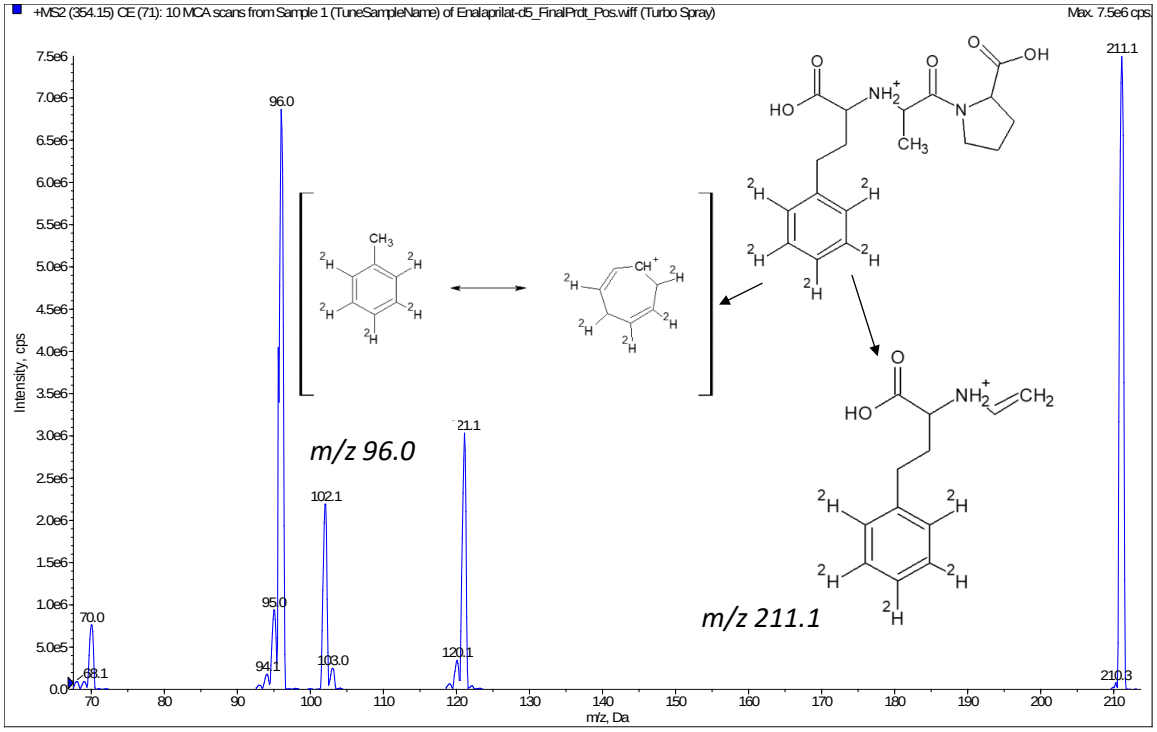


Figure 2.11 Final product ion mass spectra for enalaprilat-d5[28]

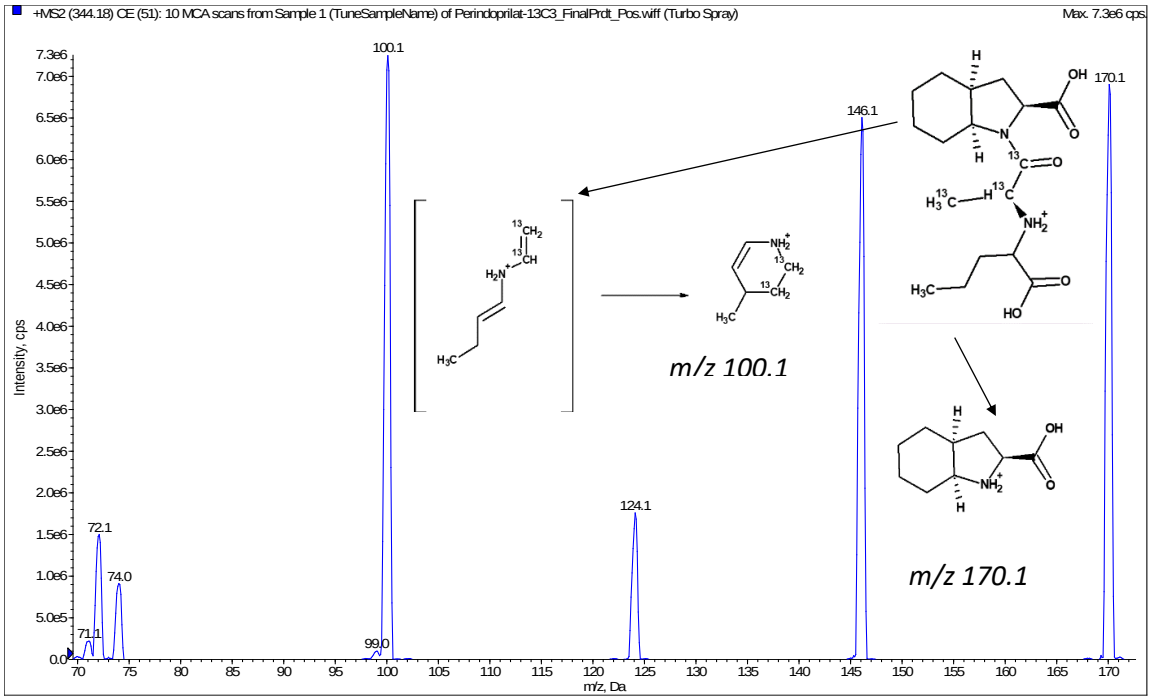


Figure 2.12 Final product ion mass spectra for perindoprilat-$^{13}C_3$[28]

The autosampler temperature was set at ~8°C and the mobile phase was introduced to the system at a flow rate of 300 µL/minute. A Restek Ultra Biphenyl column (100 mm × 2.1 mm, 3 µm) was used for chromatographic separation by employing gradient elution. Mobile phase A (aqueous) was made up of 5 mM ammonium acetate and 0.1% acetic acid in the water.

Mobile phase B (organic) consisted of a mixture of water, methanol and acetonitrile (10:20:70, v/v/v). The buffer of Mobile phase B also consisted of ammonium acetate (5 mM) paired with acetic acid (0.1% (v/v)). The initial mobile phase composition (5% B) was increased to 90% B (linearly) over a 30-second interval, held there for 2.5 minutes, and reduced back to 5% B in 0.1 minutes. The run was concluded with a 3.4-minute equilibration period.

2.2.5 UV–vis Spectrophotometer

The UV absorbances of stored stock solutions were compared with freshly prepared solutions on a standalone Cary 60 UV–vis Spectrophotometer (Agilent, CA, USA). Stock solutions were prepared for testing via dilution with methanol. UV absorbances at 206 nm for enalaprilat and perindoprilat and 242 for carvedilol were assessed as an expression of the concentrations of the analytes.

2.2.6 Method Validation

2.2.6.1 Preparation of calibration standards and quality controls

For the preparation of standards, a working solution containing carvedilol, enalaprilat and perindoprilat was prepared by spiking 20 µL of 1 mg/mL of each analyte into 4.940 mL methanol (WS1). From this solution, a series of working solutions was then prepared volumetrically in methanol. These working solutions were used to spike 1.90 mL K_3EDTA plasma for each respective calibration standard. Multiple 130 µL aliquots of each calibration standard were stored in individual 1.5 mL polypropylene tubes at ~-80°C in order to allow duplicate 50 µL extractions from each tube. Table 2.2 shows the preparation of working solutions and corresponding calibration standards.

Table 2.2 Preparation of working solutions and calibration standards for carvedilol, enalaprilat and perindoprilat

Working solutions (WS)	Blank solvent (methanol) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS solution (µg/mL)	Volume (µL) into 1.90 mL plasma	STD	Plasma concentration (ng/mL)
WS1	4 940	SS x 3	20 + 20 + 20	4.00	100	STD 1-ULOQ	200
WS2	250	WS1	750	3.00	100	STD 2	150
WS3	500	WS2	500	1.50	100	STD 3	75.0
WS4	500	WS3	250	0.500	100	STD 4	25.0
WS5	800	WS4	200	0.100	100	STD 5	5.00
WS6	600	WS5	400	0.0400	100	STD 6	2.00
WS7	600	WS6	200	0.0100	100	STD 7	0.500
WS8	600	WS7	400	0.0040	100	STD 8 - LLOQ	0.200

A volume of 20 µL of 1 mg/mL stock solutions of carvedilol, enalaprilat and perindoprilat was spiked into 2.440 mL methanol for QC preparation. This solution (WSQ1) prepared a series of working solutions (WSQ2–WSQ7) volumetrically in methanol. These working solutions were used to spike 1.90 mL K_3EDTA plasma for each respective QC. Multiple 130 µL aliquots of each QC were stored in individual 1.5 mL polypropylene tubes at ~-80°C to allow duplicate 50 µL extractions from each tube.

Table 2.3 shows the preparation of working solutions and corresponding quality controls.

Table 2.3 Preparation of working solutions and QCs for carvedilol, enalaprilat and perindoprilat

Working Solutions (WS)	**Blank solvent (methanol) volume (µL)**	**Spiking solution**	**Spiking solution volume (µL)**	**WS concentration (µg/mL)**	**Volume (µL) WS spiked into 1.90 mL plasma**	**QC**	**Plasma concentration (ng/mL)**
WSQ1	2440	SS x 3	20 + 20 + 20	8.00	100	QC DIL	400
WSQ2	450	WSQ1	300	3.20	100	QC H	160
WSQ3	400	WSQ2	400	1.60	100	QC M	80.0
WSQ4	1000	WSQ3	200	0.267	100	SYS 1	13.3
WSQ5	1000	WSQ4	200	0.0440	100	SYS 2	2.22
WSQ6	602	WSQ5	198	0.0110	100	QC L	0.550
WSQ7	1000	WSQ5	99.0	0.0040	100	LLOQ	0.200

2.2.7 Validation experiments

The method was validated as per 2018 US FDA, specific for industry bioanalytical method validation guidelines.[165] The following validation experiments were performed: accuracy and precision, stock solution and working solution stability, on-instrument stability and reinjection reproducibility, stability in the matrix, freeze-thaw stability, bench-top stability, recovery, process efficiency, matrix effects, haemolysis, whole blood stability, specificity, carry over, sensitivy, haemolysis and crosstalk.

2.3 Results and Discussion

2.3.1 Method development and optimisation

2.3.1.1 Extraction

Extraction was performed using an extraction solvent of methanol:acetonitrile (1:1, v/v). Both chemicals, either individually or in combination, are common extraction chemicals used in the literature for carvedilol, enalaprilat and perindoprilat assays.[103,106,109,112,113,115,116,119,164] The main challenge during the optimisation of the extraction method in this study was extract cleanliness.Cleanliness refers to the degree to which the extracted sample is free from unwanted or interfering compounds, such as matrix components, salts, proteins, lipids, or other impurities that may co-extract during the sample preparation process. Four varying extraction methods were investigated to determine which method allowed for sufficient extract cleanliness:

1) Precipitation ➔ Equilibration ➔ Centrifugation ➔ Drying ➔ Reconstitution ➔ Plate
2) Precipitation ➔ Equilibration ➔ Centrifugation ➔ Drying ➔ Reconstitution ➔ Centrifugation ➔ Plate
3) Precipitation (On Ice) ➔ Equilibration (at 4 °C) ➔ Centrifugation ➔ Drying ➔ Reconstitution ➔ Plate
4) Precipitation (On Ice) ➔ Equilibration (at 4 °C) ➔ Centrifugation ➔ Drying ➔ Reconstitution ➔ Centrifugation ➔ Plate

The principal variable investigated was adding a second centrifugation step after reconstitution (methods 2 and 4). The effect of carrying out precipitation and equilibration on ice and at 4ºC, respectively (methods 3 and 4), was also investigated. Both methods 3 and 4 appeared to have a cleaner supernatant relative to method 1 (reference method). The cleanliness of the sample was evaluated through visual inspection by examining the extent of particulate matter formed after centrifugation. Colder preparation conditions allowed for a cleaner extract. Methods 2 and 4, however, created a significant pellet at the bottom of the Eppendorf tube, with the second centrifugation step allowing for significant extraction cleanup relative to that of only one centrifugation step. No difference was observed in supernatant cleanliness between methods 4 and 2. Extraction cleanliness according to the method was ranked as follows (from most to least clean supernatant):

1) Methods 2 and 4
2) Method 3
3) Method 1

Method 2 was selected out of the four methods as the final extraction method, as it allowed for a significantly cleaner extraction than methods 1 and 3 while being a more straightforward extraction method than method 4.

2.3.1.2 Chromatography

Much of the liquid chromatography methods that have been published for the analysis of carvedilol in plasma use isocratic chromatography with a mobile phase consisting of a mixture of water and acetonitrile.[101,103,104] The bulk of the published assays for the quantification of enalaprilat were developed for the simultaneous quantification of its prodrug, enalapril, as well, with most of these assays also using isocratic chromatography to quantify these two analytes.[107,108,110,161] The mobile phases for these assays are either a mixture of acetonitrile and water or methanol and water. Assays developed for perindoprilat are also primarily developed to quantify its prodrug, perindopril, simultaneously.[93,117,164] Published assays indicate a preference for gradient chromatography to quantify perindopril and perindoprilat, with mobile phases consisting of mixtures of water and methanol. Consequently, gradient elution was used for this study to simultaneously quantify carvedilol, enalaprilat and perindoprilat in plasma. This was to cater for the analytes' different physiochemical properties. Carvedilol is the most hydrophobic of the three analytes, with enalaprilat and perindoprilat being hydrophilic.[167–169] The similarity in physiochemical properties resulted in similar elution times for enalaprilat and perindoprilat, with both preferring to partition into the mobile phase at a lower organic composition compared to carvedilol. Using the solvent gradient profile that appear in Figure 2.13, all three analytes could be separated from each other. Enalaprilat and perindoprilat elute within the gradient with carvedilol eluting at the maximum organic composition. The time shown in Figure 2.13 does not directly translate into retention time as the gradient front that is formed in the pump is delayed in reaching the column due to system dead volume.[170]

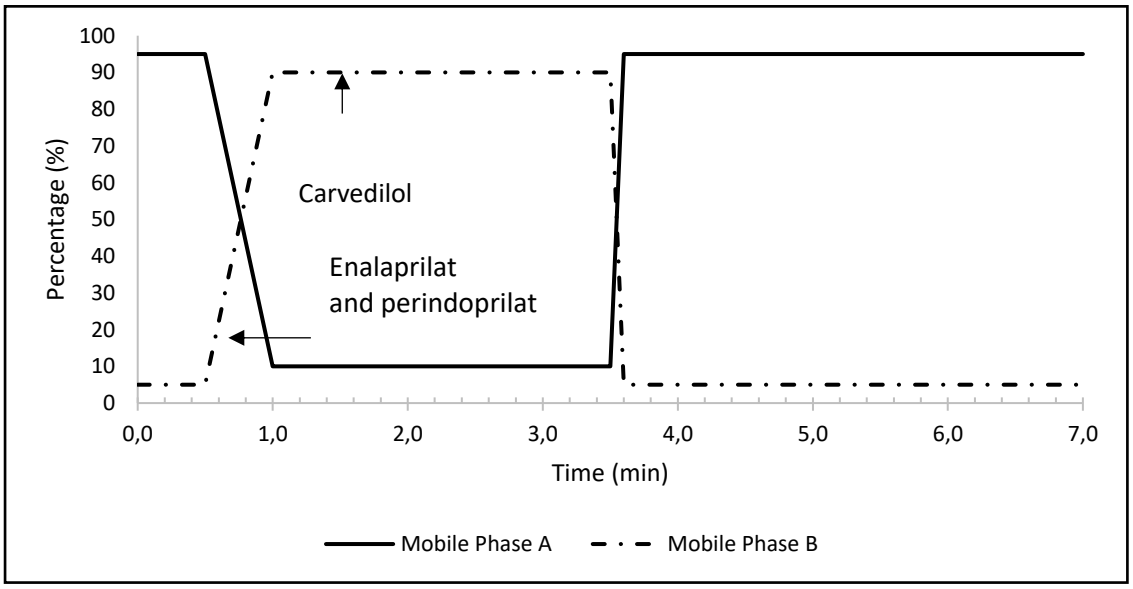


Figure 2.13 Solvent gradient profile. (Enalaprilat and perindoprilat are expected to elute in the gradient and carvedilol in the organic purge. This diagram illustrates only where they elute relative to the mobile phase composition. The times shown are not actual retention times of the analytes.)

Chromatographic separation was obtained on a Restek Ultra Biphenyl column (100 mm × 2.1 mm, 3 µm). Most assays developed for carvedilol, enalaprilat or perindoprilat use C18 columns for chromatographic separation.[93,101,103,104,109,110,112,115,164,166] Compounds are retained on a biphenyl stationary phase through the same dispersive forces as a C18. However, the biphenyl column allows more polarisable substances to be retained.[171] Figure 2.14 provides representative chromatograms of carvedilol, enalaprilat, and perindoprilat.

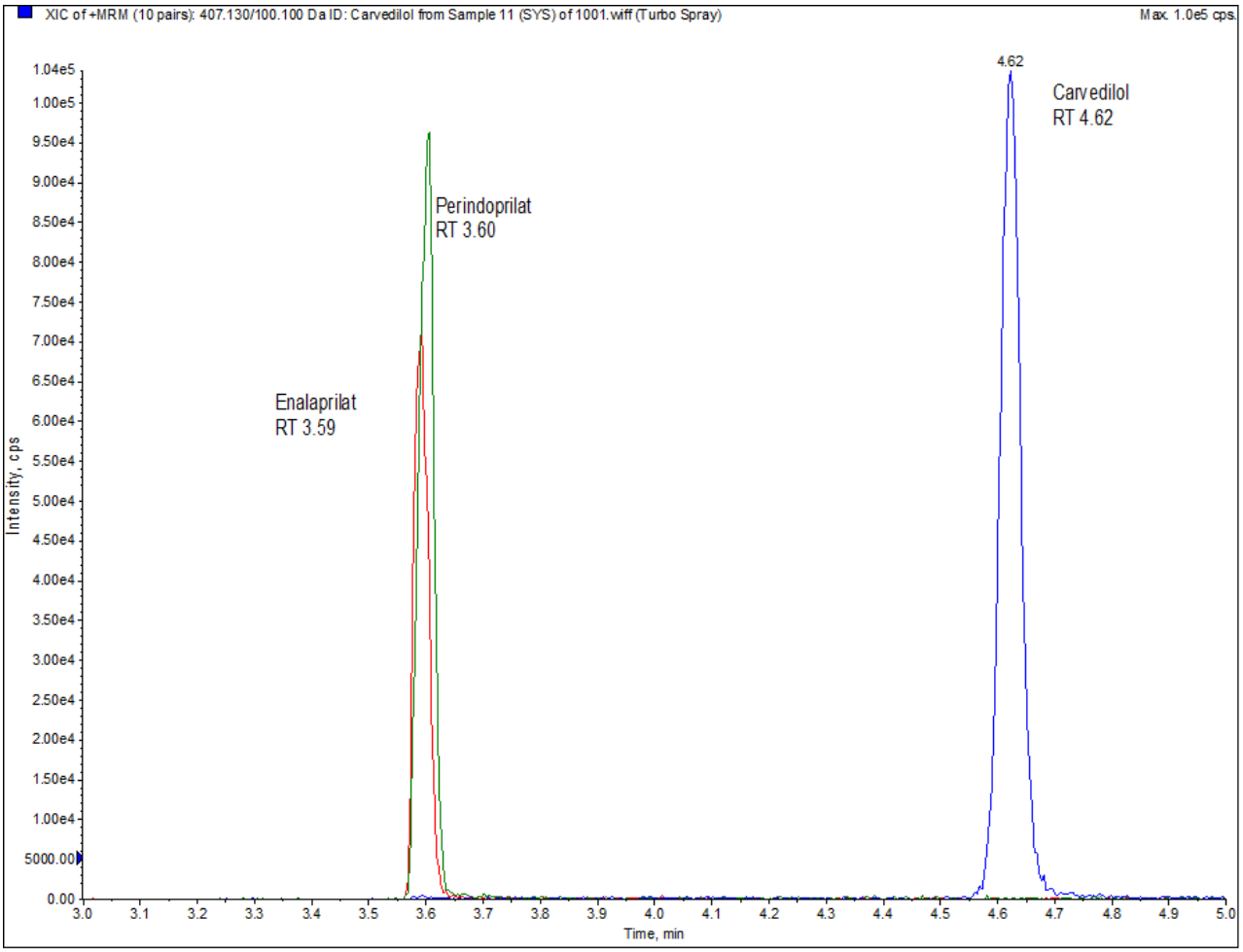


Figure 2.14 Overlaid chromatograms of carvedilol (blue), enalaprilat (red), and perindoprilat (green).

2.3.2 Method validation

2.3.2.1 Stock solution stability

For long-term stock solution stability determination, stock solution aliquots were stored at ~-80°C for ~62 days and tested on a UV spectrophotometer. Stock solution stability was also determined at ~-20°C and ~4°C for 24 hours. In addition, room temperature stability was assessed initially for 24 hours, for which only enalaprilat passed. Carvedilol and perindoprilat were subsequently assessed again for 6 hours. The UV spectrophotometer measured the

absorbance in triplicate at 242 nm, 208 nm and 206 nm for carvedilol, enalaprilat and perindoprilat, respectively. Results for stock solution stabilities are shown in Table 2.4.

The percentage difference between the reference and test solutions was less than 10% across all stability experiments. The CV(%) was also less than 10% for all analytes across all the stability experiments. Instability is indicated by a CV(%) greater than 15% or a difference in concentration of more than 15% from the reference.[165] The results shown indicate that carvedilol, enalaprilat and perindoprilat are stable in methanol at 1 mg/mL for at least 62 days at ~-80°C and for 24 hours at ~-20°C and ~4°C. In addition, carvedilol and perindoprilat are stable for 6 hours at room temperature, with enalaprilat stable for 24 hours at room temperature.

Table 2.4 Summary of working solution stability based on precision and %Difference

			Carvedilol		Enalaprilat		Perindoprilat	
		N	Precision CV(%)	%Difference	Precision CV(%)	%Difference	Precision CV(%)	%Difference
Short-term stability	6 hours at room temperature (carvedilol + perindoprilat) 24 hours at room temperature (enalaprilat)	3	4.5	2.9	0.2	-2.6	3.4	7.3
Long-term stability	~62 days at ~-80°C	3	1.1	1.4	2.8	-0.6	2.2	0.6

2.3.2.2 Working Solution Stability

Working solution stability was evaluated over seven days at ~-80°C and for ~4 hours at room temperature. Test and reference working solutions were diluted in an injection solution containing ISTD. Peak area ratios were compared using the developed LC-MS/MS method. Table 2.5 is a summary of the working solution stabilities.

Table 2.5 Summary of working solution stabilities of plasma assay

		Carvedilol		Enalaprilat		Perindoprilat	
Storage Conditions	**N**	**Precision CV(%)**	**%Difference**	**Precision CV(%)**	**%Difference**	**Precision CV(%)**	**%Difference**
**4.00 µg/mL at ~-80°C for ~7 days	6	1.4	7	3.4	6.4	1.3	4.4
*0.0040 µg/mL at ~-80°C for ~7 days	6	2.6	-1.2	2.1	-7.2	3.3	-8
**4.00 µg/mL at RT for ~4 hours	6	2.2	5.4	2.4	5.9	2.6	5.3
*0.0040 µg/mL at RT for ~4 hours	6	4.1	1.2	5.6	5.2	3.7	2.4

*Lowest working solution concentration: 0.0040 µg/mL; **Highest working solution concentration: 4.00 µg/mL

2.3.2.3 Calibration range

The method was validated over a calibration range of 0.2–200 ng/mL for carvedilol, enalaprilat and perindoprilat. The lowest LLOQs published in the literature are 0.024 ng/mL for both enalaprilat and carvedilol and 0.1 ng/mL for perindoprilat.[92,164] This method, however, is a novel multiplex method. Careful consideration of the LLOQ was required to ensure that time spent on method development was not excessive while ensuring the clinical goals attached to the assay could be achieved. An LLOQ of 0.2 ng/mL was a sufficient compromise. Based on available pharmacokinetic data in literature, the likelihood of adherent patient plasma concentrations below 0.2 ng/mL for the analytes is remote. The LLOQ therefore allows the assay's goals to be met, which is to discern between adherent and non-adherent patients.[172–177] The sufficiency of the LLOQ is further investigated in Chapter 4.

Calibration curves were generated for each of the three validation batches that were run. Quadratic regressions weighted by 1/x (carvedilol) and $1/x^2$ (enalaprilat and perindoprilat) were used. Due to the saturation of the detector response at high concentrations, particularly that of carvedilol, quadratic regressions were used. Each calibration curve showed a good fit. Figure 2.15 to Figure 2.17 show representative calibration curves of carvedilol, enalaprilat and perindoprilat, respectively.

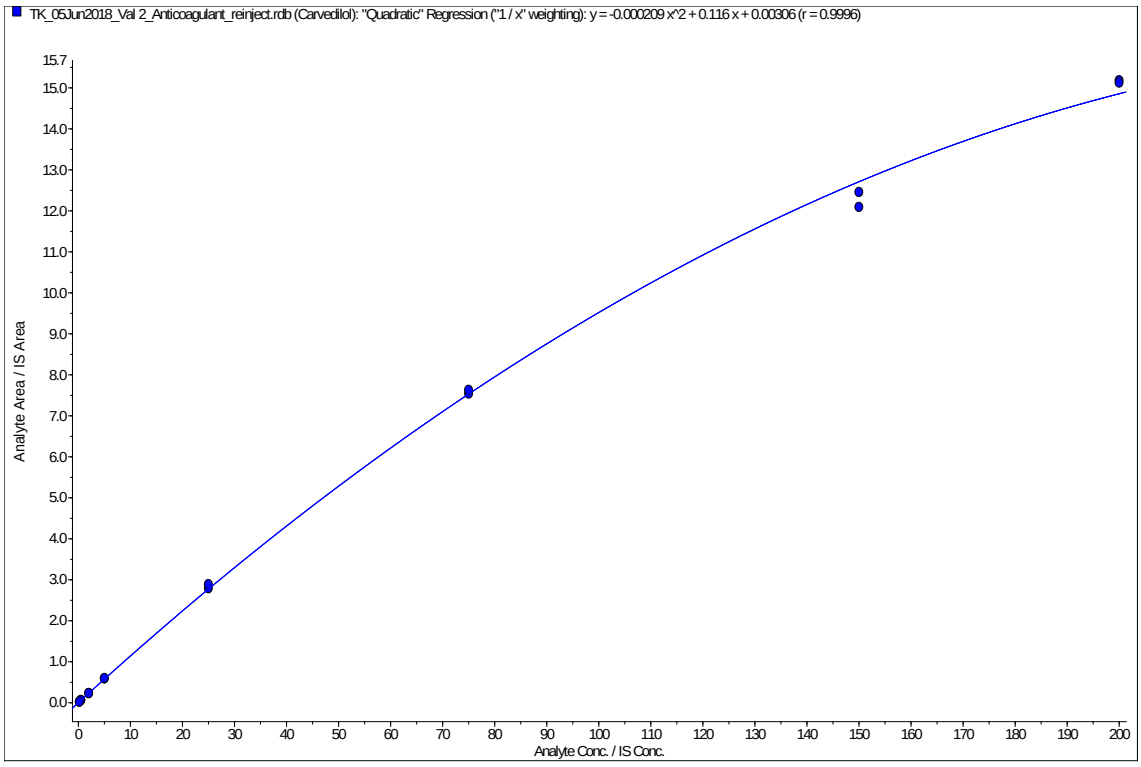


Figure 2.15 A representative calibration curve for carvedilol (r = 0.9996)

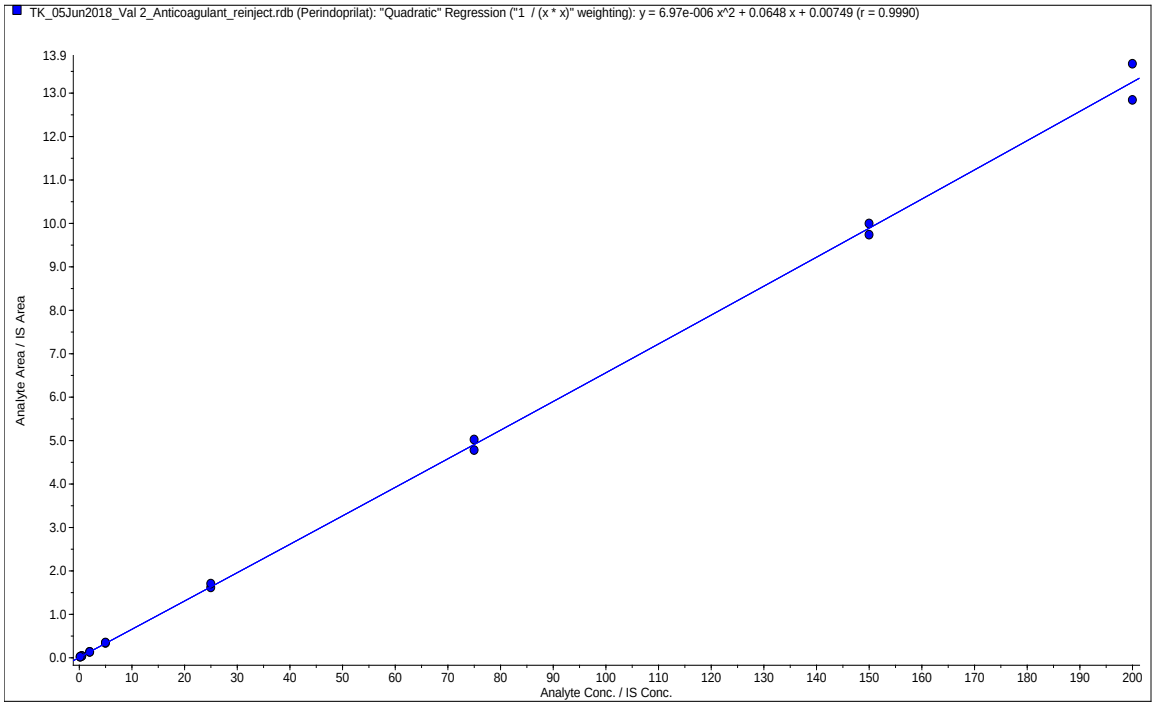


Figure 2.16 A representative calibration curve for enalaprilat (r = 0.999**1)**

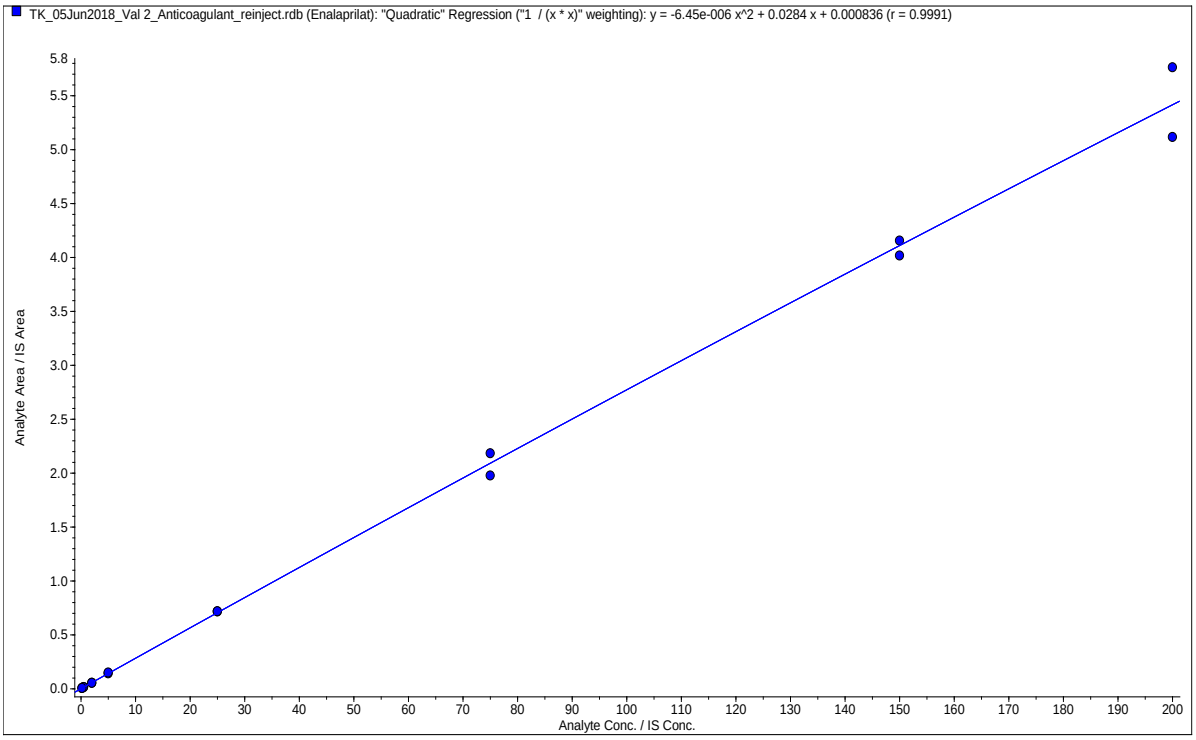


Figure 2.17 A representative calibration curve for perindoprilat (r = 0.9990)

2.3.2.4 Accuracy and precision (intra-day and inter-day; within-batch and between-batch)

Accuracy and precision were assessed by calculating the accuracy and precision statistics over the within and between-batch validation batches (three in total). Freshly spiked STDs were assayed in each analytical run by spiking blank K_3EDTA plasma (1.9 mL) with working

solutions to cover the calibration range of 0.200–200 ng/mL. Similarly, QCs were prepared by spiking blank K_3EDTA plasma (1.9 mL) with working solutions to obtain final concentrations of 0.200 (LLOQ), 0.550 (low), 80.0 (medium) and 160 (high) ng/mL. The calibration range was validated by analysing the QC samples in six-fold at the four concentration levels over a 3-day period to determine the intra- and inter-day accuracy and precision. Accuracy is expressed as the concentration of the analyte found as a percentage of the nominal concentration (%accuracy), while precision is defined as the coefficient of variation (CV(%)). For a valid method, the intra-day and inter-day accuracy are required to be within 15% over the entire calibration range and within 20% of the nominal concentration at the LLOQ. In addition, the intra-day and inter-day precision must be less than 15% over the entire calibration range and less than 20% at the LLOQ.[165,178] Table 2.6 to Table 2.11 summarise the accuracy and precision of standards and QCs for carvedilol, enalaprilat and perindoprilat over the three validation batches. The criteria were met for all analytes as required by the FDA.[165]

Table 2.6 Overall summary of calibration standard accuracy and precision: validation 1-3 (carvedilol)

Validation Batch	**Sample ID**	**STD 1 - ULOQ**	**STD 2**	**STD 3**	**STD 4**	**STD 5**	**STD 6**	**STD 7**	**STD 8 – LLOQ**
	Nominal conc.	**200 (ng/mL)**	**150 (ng/mL)**	**75.0 (ng/mL)**	**25.0 (ng/mL)**	**5.00 (ng/mL)**	**2.00 (ng/mL)**	**0.500 (ng/mL)**	**0.200 (ng/mL)**
	Replicates	**Observed conc.**	**Observed conc.**	**Observed conc.**	**Observed conc.**	**Observed conc.**	**Observed conc.**	**Observed conc.**	**Observed conc.**
Validation 1	1	197	157	75.1	23.4	4.75	1.93	0.517	0.213
	2	193	156	74.7	24.3	4.98	1.94	0.527	0.206
Validation 2	1	211	139	75.1	25.2	5.11	1.98	0.515	0.176
	2	209	145	76.2	26.1	5.20	2.01	0.534	0.187
Validation 3	1	*[164]*	131	76.4	24.2	4.72	2.04	0.517	0.198
	2	205	163	76.9	25.3	5.04	1.89	0.534	0.197
	N	5	6	6	6	6	6	6	6
	Average	203	149	75.7	24.8	4.97	1.97	0.524	0.196
	STDEV	7.75	12.2	0.88	0.965	0.194	0.0554	0.00881	0.0132
	CV(%)	3.8	8.2	1.2	3.9	3.9	2.8	1.7	6.7
	%Accuracy	101.5	99.0	101.0	99.0	99.3	98.3	104.8	98.1

[ ] failed standard excluded from the curve

Table 2.7 Overall quality control accuracy and precision estimation: carvedilol

Validation Batch	Sample ID Nominal conc. Replicates	LLOQ 0.200 (ng/mL) Observed conc.	QC - L 0.550 (ng/mL) Observed conc.	QC - Med 80.0 (ng/mL) Observed conc.	QC - High 160 (ng/mL) Observed conc.
Validation 1	1	0.230	0.530	82.3	171
	2	0.208	0.522	81.8	170
	3	0.228	0.534	81.6	167
	4	0.225	0.530	81.6	161
	5	0.227	0.522	76.7	158
	6	0.222	0.546	78.8	159
Validation 2	1	0.196	0.521	80.2	155
	2	0.204	0.508	82.6	156
	3	0.184	0.532	83.5	156
	4	0.212	0.524	86.3	158
	5	0.206	0.541	83.7	162
	6	0.207	0.517	80.7	159
Validation 3	1	0.206	0.532	85.6	136
	2	0.212	0.582	81.3	142
	3	0.206	0.536	80.3	159
	4	0.200	0.549	79.2	178
	5	0.195	0.599	76.9	181
	6	0.198	0.574	80.1	157
	N	18	18	18	18
	Average	0.209	0.539	81.2	160
	STDEV	0.0129	0.0239	2.60	10.9
	CV(%)	6.2	4.4	3.2	6.8
	%Accuracy	104.6	98.0	101.6	100.2

Table 2.8 Overall summary of calibration standard accuracy and precision: validation 1–3 (enalaprilat)

Validation batch	Sample ID Nominal conc. Replicates	STD 1 - ULOQ 200 (ng/mL) Observed conc.	STD 2 150 (ng/mL) Observed conc.	STD 3 75.0 (ng/mL) Observed conc.	STD 4 25.0 (ng/mL) Observed conc.	STD 5 5.00 (ng/mL) Observed conc.	STD 6 2.00 (ng/mL) Observed conc.	STD 7 0.500 (ng/mL) Observed conc.	STD 8 – LLOQ 0.200 (ng/mL) Observed conc.
Validation 1	1	177	142	76.3	21.8	4.52	1.88	0.497	0.181
	2	205	169	80.6	27.2	5.03	2.07	0.563	0.210
Validation 2	1	213	152	78.3	25.4	4.99	1.92	0.514	0.197
	2	188	146	70.8	25.4	5.28	1.89	0.500	0.202
Validation 3	1	200	155	81.0	24.9	4.94	1.97	0.472	0.184
	2	185	153	75.6	24.4	5.22	1.96	0.495	0.222
	N	6	6	6	6	6	6	6	6
	Average	195	153	77.1	24.9	5.00	1.95	0.507	0.199
	STDEV	13.6	9.28	3.78	1.77	0.269	0.0697	0.0307	0.0156
	CV(%)	7.0	6.1	4.9	7.1	5.4	3.6	6.1	7.8
	%Accuracy	97.3	101.9	102.8	99.4	99.9	97.4	101.4	99.7

Table 2.9 Overall quality control accuracy and precision estimation: enalaprilat

Validation batch	Sample ID Nominal conc. Replicates	LLOQ 0.200 (ng/mL) Observed conc.	QC - L 0.550 (ng/mL) Observed conc.	QC - Med 80.0 (ng/mL) Observed conc.	QC - High 160 (ng/mL) Observed conc.
Validation 1	1	0.192	0.490	84.4	181
	2	0.208	0.494	80.6	177
	3	0.189	0.493	78.7	173
	4	*0.249*	0.534	82.9	174
	5	0.221	0.543	82.8	164
	6	0.212	0.564	89.8	177
Validation 2	1	0.216	0.500	83.0	169
	2	0.198	0.529	83.7	164
	3	0.202	0.546	80.2	165
	4	0.192	0.524	78.2	151
	5	0.213	0.521	73.9	151
	6	0.191	0.531	75.3	147
Validation 3	1	0.205	0.527	82.5	158
	2	0.198	0.555	81.4	161
	3	0.193	0.512	85.4	161
	4	0.197	0.555	82.7	154
	5	0.211	0.556	77.0	157
	6	0.199	0.546	83.1	155
	N	18	18	18	18
	Average	0.205	0.529	81.4	163
	STDEV	0.0146	0.0235	3.80	10.1
	CV(%)	7.1	4.4	4.7	6.2
	%Accuracy	102.4	96.2	101.8	102.0

Table 2.10 Overall summary of calibration standard accuracy and precision: validation 1–3 (perindoprilat)

Validation batch	Sample ID Nominal conc. Replicates	STD 1 - ULOQ 200 (ng/mL) Observed conc.	STD 2 150 (ng/mL) Observed conc.	STD 3 75.0 (ng/mL) Observed conc.	STD 4 25.0 (ng/mL) Observed conc.	STD 5 5.00 (ng/mL) Observed conc.	STD 6 2.00 (ng/mL) Observed conc.	STD 7 0.500 (ng/mL) Observed conc.	STD 8 - LLOQ 0.200 (ng/mL) Observed conc.
Validation 1	1	175	148	76.7	23.6	4.72	1.92	0.503	0.203
	2	206	165	78.7	26.6	5.26	2.06	0.449	0.205
Validation 2	1	206	152	76.7	24.7	5.09	1.95	0.502	0.188
	2	194	148	73.0	26.1	5.25	1.91	0.474	0.217
Validation 3	1	197	151	80.2	25.4	4.91	2.00	0.478	0.197
	2	195	151	76.3	24.5	5.29	1.97	0.460	0.213
	N	6	6	6	6	6	6	6	6
	Average	196	153	76.9	25.2	5.09	1.97	0.478	0.204
	STDEV	11.4	6.35	2.44	1.10	0.230	0.0556	0.0218	0.0106
	CV(%)	5.8	4.2	3.2	4.4	4.5	2.8	4.6	5.2
	%Accuracy	97.8	101.7	102.6	100.6	101.7	98.4	95.5	101.9

Table 2.11 Overall quality control accuracy and precision estimation: perindoprilat

Validation Batch	Sample ID / Nominal Conc. / Replicates	LLOQ 0.200 (ng/mL) Observed conc.	QC - L 0.550 (ng/mL) Observed conc.	QC - Med 80.0 (ng/mL) Observed conc.	QC - High 160 (ng/mL) Observed conc.
Validation 1	1	0.180	0.489	84.8	176
	2	0.193	0.516	79.6	174
	3	0.160	0.457	81.8	170
	4	0.214	0.499	84.5	168
	5	0.200	0.625	81.6	164
	6	0.178	0.546	85.1	169
Validation 2	1	0.176	0.470	83.9	169
	2	0.175	0.524	82.8	170
	3	0.207	0.507	81.2	165
	4	0.180	0.526	80.1	154
	5	0.229	0.550	76.4	154
	6	0.205	0.542	78.4	154
Validation 3	1	0.198	0.535	86.2	162
	2	0.198	0.546	85.7	165
	3	0.224	0.524	86.6	170
	4	0.201	0.591	82.8	157
	5	0.211	0.542	78.7	161
	6	0.230	0.554	85.8	159
	N	18	18	18	18
	Average	0.198	0.530	82.6	165
	STDEV	0.0199	0.0398	3.023	6.87
	CV(%)	10.1	7.5	3.7	4.2
	%Accuracy	98.9	96.4	103.2	102.8

2.3.2.5 Matrix effects

Matrix effects were evaluated according to the methodology set out by Matuszewski et al.[179] Six blank sources of the appropriate biological matrix were extracted (without ISTD). Each matrix sample was spiked at low, medium and high concentration levels (taking into account any calculations for dilutions in the analytical method) and at one concentration of the ISTD. Analyte/ISTD peak area ratios for each concentration level in each matrix source and the area ratio vs concentration regression slopes are presented in Table 2.12.[165] The area ratio vs concentration regression slope measures the linearity of the response of the analytical method to the analyte in the presence of the biological matrix. This is calculated using the "SLOPE" function in Microsoft Excel®.

Table 2.12 Regression results from six different matrix sources for carvedilol, enalaprilat and perindoprilat

N		High conc. (160 ng/mL) Peak area ratio	Medium conc. (80 ng/mL) Peak area ratio	Low conc. (0.550 ng/mL) Peak area ratio	Area ratio vs conc. Regression slope
			Carvedilol		
6	Average	16.5	8.39	0.0653	0.103
	STDEV	0.615	0.165	0.00207	0.00386
	CV(%)	3.7	2	3.2	3.7
			Enalaprilat		
6	Average	4.65	2.33	0.0163	0.0291
	STDEV	0.18	0.0704	0.00142	0.00114
	CV(%)	3.9	3	8.7	3.9
			Perindoprilat		
6	Average	10.8	5.42	0.0418	0.0677
	STDEV	0.336	0.085	0.00058	0.00211
	CV(%)	3.1	1.6	1.4	3.1

The slope variability CV(%) for six different plasma samples is < 5.0% for all three analytes, which indicates that matrix effects do not adversely influence the precision of the assay.

2.3.2.6 Recovery

The extraction recovery pertains to the extraction efficiency of the analytical process within the limits of variability. It was determined by comparing the analytical response of the blank matrix spiked with the analyte and extracted with the response of the blank matrix first

extracted and then spiked with the analyte (theoretical, represents 100% recovery). No recovery of the ISTD was calculated. Test and reference samples were prepared as follows:

a. Extracted (test) samples: A minimum of six QCs at each concentration level (high, medium, and low) in six different matrices were extracted.

b. Theoretical samples: Samples were spiked at each concentration level (relative to the final concentration of the corresponding extracted QC level) in six-fold using extracted blank matrix from six different lots of matrices.

The analyte peak areas found after extraction compared to the theoretical peak area are expressed as a percentage recovery. Table 2.13 summarises the recovery results.[160]

Table 2.13 Summary of recovery results based on average precision and average %recovery between three concentration levels

Analyte	N	Average %Recovery	Average precision CV(%)
Carvedilol	6	72.9	7.8
Enalaprilat	6	77.1	2.9
Perindoprilat	6	77.0	0.9

The mean recovery of a quantitative drug assay method should be consistent, and the precision of the measured recovery expressed as a CV(%) should not exceed 15% for any concentration of the analyte at which it is determined.[165] Recovery reproducibility between concentration levels should not be > 15%. The mean recovery for carvedilol, enalaprilat and perindoprilat is 72.9%, 77.1% and 77.0%, with the corresponding CV(%) within acceptable limits.

2.3.2.7 Process efficiency

Process efficiency was determined by preparing six different lots of matrix at the low, medium and high QC levels and then extracting them per the standard operating procedure (SOP), which is described in section 2.2.3. The ISTD was spiked at the working concentration of the method. The neat, un-extracted samples were prepared in mobile phase B (water:methanol:acetonitrile; 10:20:70, with 5 mM ammonium acetate and 0.1% acetic acid) at the low, medium and high QC levels (considering any calculations for dilutions in the analytical method), in triplicate (no matrix present). The ISTD was spiked into the samples at the working concentration of the method. A volume of 200 µL of these samples was then added to 96-well plates reflective of the extraction procedure described in the Method SOP. The analyte/ISTD peak area ratios observed after extraction were compared to the neat samples' peak area ratios and expressed as percentage process efficiency. The results are shown in Table 2.14.

Table 2.14 Summary of the process efficiency results based on average precision and average %process efficiency between three concentration levels

Analyte	N	Average %Process efficiency	Average precision CV(%)
Carvedilol	6	68.2	9.0
Enalaprilat	6	77.0	5.1
Perindoprilat	6	75.2	1.3

The process efficiency of the assay must be reproducible, with the reproducibility not exceeding 15% between the concentration levels. The CV(%) of the process efficiency must not be greater than 15% at any concentration level.[165] Analyte process efficiency is all within the required acceptance criteria.

2.3.2.8 Specificity, sensitivity and carryover

Specificity for carvedilol, enalaprilat and perindoprilat was evaluated by analysing blank plasma collected from six volunteers. The observed responses (accepted criteria: response < 20% of LLOQ) in the extracted blank matrix samples at the retention time/mass transition of the analyte were evaluated. Similarly, the observed responses (accepted criteria: response < 5% of ISTD) in the extracted blank matrix samples at the retention time/mass transition of the ISTD were evaluated for carvedilol-d5, enalaprilat-d5 and perindoprilat-$^{13}C_3$.[165] Representative double blank and blank chromatograms are shown in Figure 2.18 and Figure 2.19, respectively.

No peaks were found at the retention time/mass transition of carvedilol, enalaprilat and perindoprilat in any of the lots of plasma used during this validation when double blank or blank samples were assessed. No peaks were observed for the ISTD in any of the double blank samples when no ISTD was spiked to the samples. This indicates sufficient selectivity and specificity of the method.

During the validation, carryover problems were observed for all three analytes, with perindoprilat being the most problematic. Carryover was mitigated by performing double blank injections (i.e. injecting a blank sample twice). A partial validation was also done at an LLOQ of 0.5 ng/mL. If the need arises for the LLOQ of an analyte to be raised to 0.5 ng/mL as an additional carryover mitigation, it can be done (see appendix A). Figure 2.18 and Figure 2.19 show the chromatograms of double and blank plasma samples for carvedilol, enalaprilat and perindoprilat, with double blank injections preceding them.

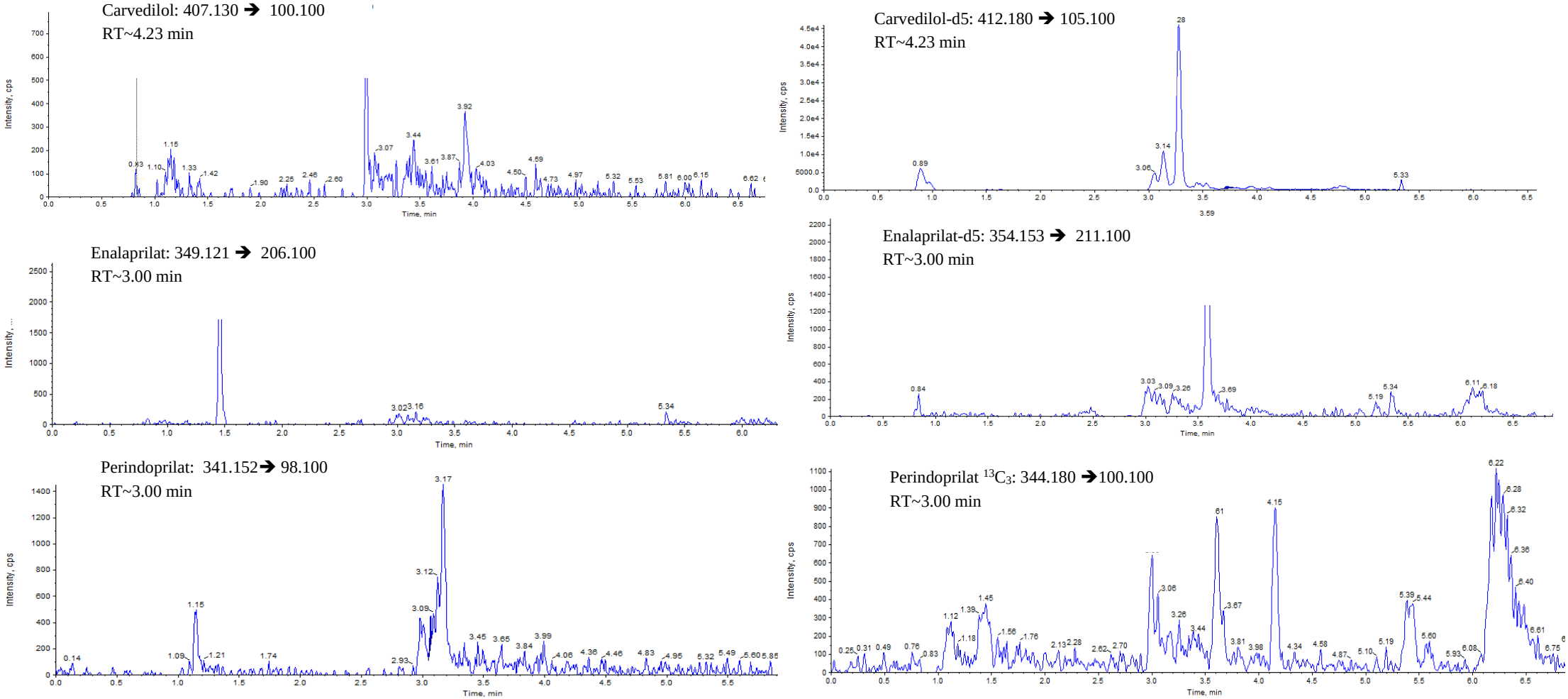


Figure 2.18 MRM chromatograms of double blank plasma samples for carvedilol, carvedilol-d5, enalaprilat, enalaprilat-d5, perindoprilat, and perindoprilat $^{13}C_3$

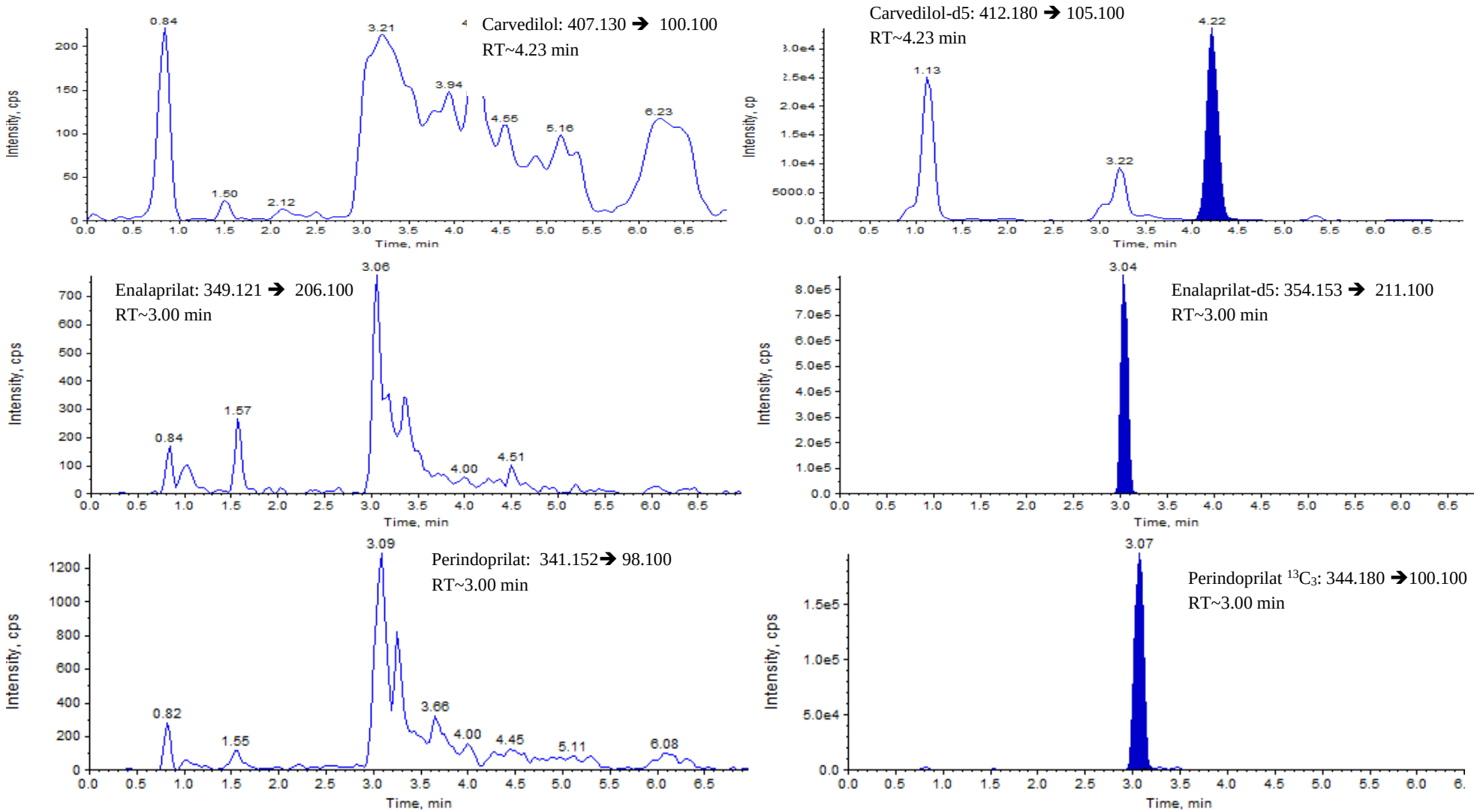


Figure 2.19 MRM chromatograms of blank plasma samples for carvedilol, carvedilol-d5, enalaprilat, enalaprilat-d5, perindoprilat, and perindoprilat $^{13}C_3$

The response of the analytes at LLOQ was evaluated by calculating the LLOQ signal-to-noise ratio (S/N). A mean response S/N of greater than 5 is acceptable.[165] The raw LLOQ sample chromatograms showed adequate intensities for the analytes with a mean S/N ratio of 80.6, 72.7 and 68.7 for carvedilol, enalaprilat and perindoprilat, respectively (determined from LLOQ samples spiked into blank plasma obtained from six different sources). Representative chromatograms of blank extracted samples overlaid with LLOQ extracted samples appear in Figure 2.20 to Figure 2.22.

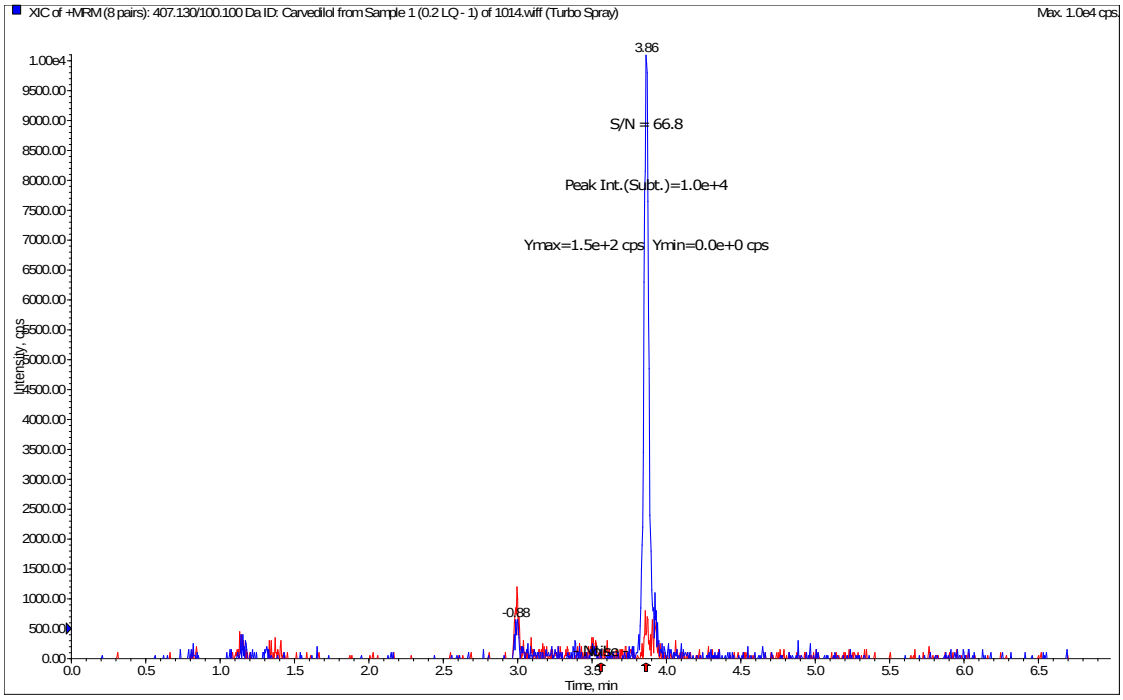


Figure 2.20 Overlay of LLOQ and blank carvedilol chromatograms. The LLOQ is shown in blue, and the blank is in red

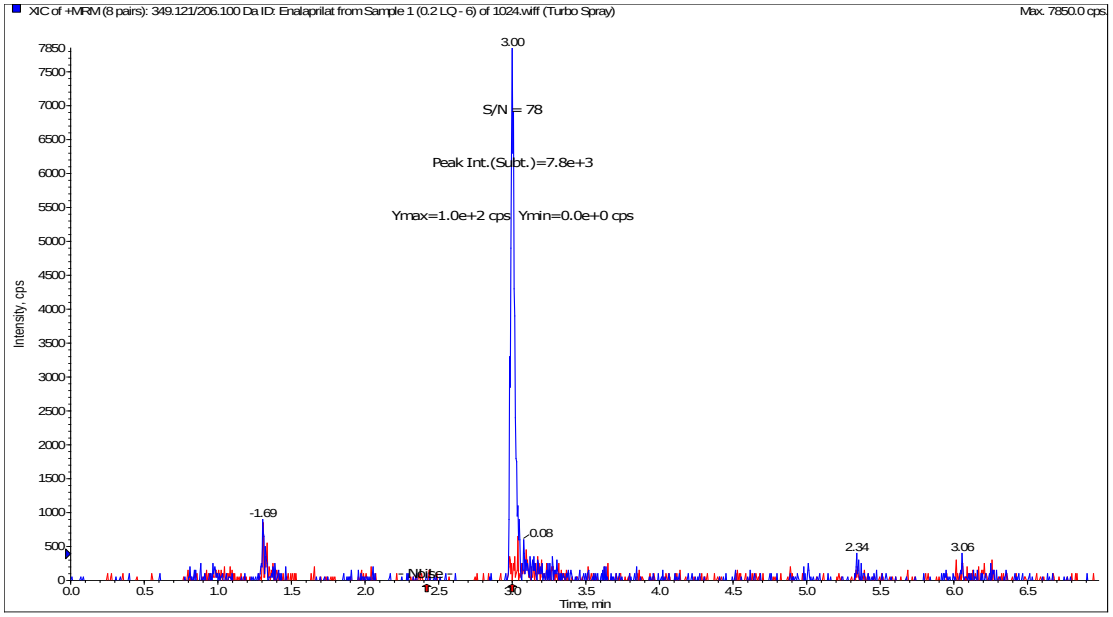


Figure 2.21 Overlay of LLOQ and blank enalaprilat chromatograms. The LLOQ is shown in blue, and the blank is in red

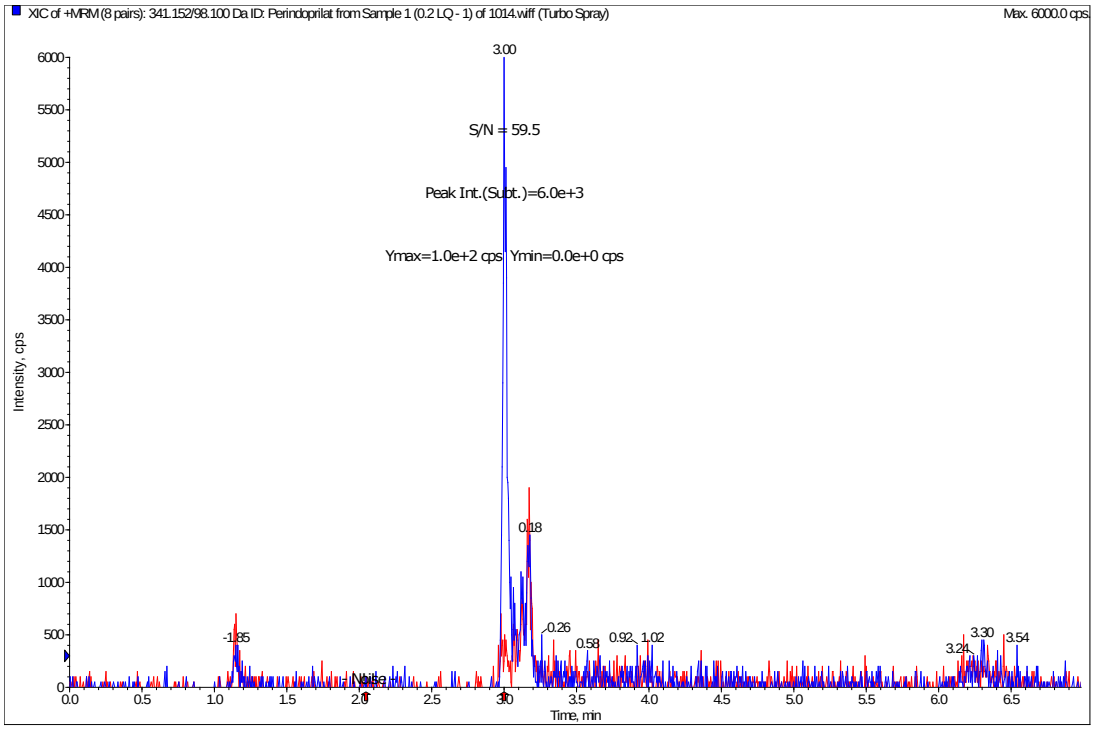


Figure 2.22 Overlay of LLOQ and blank perindoprilat chromatograms. The LLOQ is shown in blue, and the blank is in red

2.3.2.9 Reinjection reproducibility and on-instrument stability

Carvedilol, enalaprilat, and perindoprilat reinjection reproducibility and on-instrument stability were evaluated to cover stability during instrument interruption. To assess reinjection reproducibility, the extracted samples of the first validation run remained in the autosampler at the method-defined temperature (~8°C) for a further ~24 and ~48 hours. The analytical run was entirely reinjected after ~24 hours and then again after ~48 hours. Reinjection reproducibility was demonstrated for up to 48 hours, as both ~24 hour and ~48 hour reinjection met all criteria.[165] On-instrument stability over a ~24- and ~48-hour period was assessed by comparing reinjected high and low QC peak area ratios to those generated from the initial injection. Carvedilol and enalaprilat extracts demonstrated ~24 hour on-instrument stability, with perindoprilat extracts exhibiting stability for ~48 hours. In the event of instrument failure, enalaprilat and carvedilol extracts must be reinjected within 24 hours. Perindoprilat extracts must be reinjected within 48 hours. Table 2.15 is a summary of the autosampler stability results.

Table 2.15 Summary of autosampler stability based on precision and %Difference

		Autosampler stability ~8°C			
		Precision CV(%)		%Difference	
Analyte	**N**	**QCH**	**QCL**	**QCH**	**QCL**
*Carvedilol	6	1.7	3.1	-7.5	3.7
*Enalaprilat	6	7.6	2.9	-3.0	5.6
**Perindoprilat	6	2.7	5.3	-5.2	-5.7

*24-hour stability results **48-hour stability results
Concentrations (ng/mL): QCH = 160, QCL= 0.550

2.3.2.10 Matrix, freeze-thaw, bench-top, and whole blood stability

To evaluate matrix stability, high- and low-quality control samples for carvedilol, enalaprilat and perindoprilat were prepared and stored at ~-80ºC. These stored QCs were analysed against a freshly prepared calibration curve and compared to the nominal concentration to determine analyte stability in the matrix at approximately -80ºC for 1085 days. In addition, stored QCs were analysed against a freshly prepared calibration curve and compared to the nominal concentration to determine analyte stability in the matrix at approximately -20°C. Perindoprilat stability was re-evaluated for 71 days at approximately ~-80ºC, as stability did not pass for 1085 days.

To ascertain freeze–thaw stability, low and high QCs were frozen at ~-80ºC and subjected to three consecutive freeze and thaw cycles (~2 hours thaw and 24 hours freeze duration per

cycle). These samples were then analysed against a freshly prepared calibration curve and assessed for accuracy against the nominal QC concentration.

Low and high QCs were frozen at ~-80ºC and left on bench at room temperature for approximately six hours (maximum anticipated time for future study samples to be left thawed until extracted). These samples were analysed against a valid fresh calibration curve to evaluate bench-top stability.

Whole blood room temperature stability was assessed to evaluate the longest allowable time samples can be left at ambient conditions before centrifugation (to ensure stability during sample collection). Test samples were prepared by spiking whole blood at low and high concentrations and leaving it on bench for two hours before centrifugation. The peak area ratios of the reference (samples that were centrifuged immediately to obtain plasma) and test samples were compared. Table 2.16 summarises the matrix, freeze–thaw, bench-top, and whole blood stability results.

Table 2.16 Summary of matrix, freeze–thaw, bench-top, and whole blood stability results

			Carvedilol		Enalaprilat		Perindoprilat	
Validation experiment	**Sample tested**	**N**	**Precision CV(%)**	**Accuracy (%Nom)**	**Precision CV(%)**	**Accuracy (%Nom)**	**Precision CV(%)**	**Accuracy (%Nom)**
Matrix stability	QCL at ~-20°C for 15 days	6	1.7	100.5	10.2	100.1	0.9	107.3
	QCH at ~-20°C for 15 days	6	3.1	95.5	1.9	97.9	3.5	107.2
	QCL at ~-80°C for 71 days	6	-	-	-	-	9.0	105.2
	QCH at ~-80°C for 71 days	6	-	-	-	-	4.4	103.2
	*S7 at ~-80°C for 1085 days	6	4.7	93.2	8.6	87.5	-	-
	*S2 at ~-80°C for 1085 days	6	9.3	100.0	4.6	86.3	-	-
Freeze and thaw stability	QCL	6	3.7	102.7	2.8	99.6	4.4	108.6
	QCH	6	8.4	96.4	3.5	101.7	3.8	105.8
Bench-top stability	QCL	6	3.3	100.8	3.8	99.5	4.5	104.5
	QCH	6	2.0	108.5	5.4	94.4	2.2	110.0
Whole blood stability	QCL	6	1.5	98.1	7.4	105.2	7.3	103.8
	QCH	6	5.1	97.7	8.6	101.5	5.4	98.7

Concentrations (ng/mL): QCH = 160, QCL= 0.550, S2 = 150, S7 = 0.500
*S7 and S2 were used instead of QCH and QCL, as stored QCH and QCL samples had been finished.

Matrix stability results indicate the stability of carvedilol, enalaprilat and perindoprilat in plasma for 15 days when stored at ~-20°C and for 1085 days for carvedilol and enalaprilat at ~-80ºC. Perindoprilat has 71 days stability at ~-80ºC. Freeze–thaw stability was demonstrated following three thaw cycles at room temperature and storage at ~-80°C. Tested freeze–thaw QC concentrations were within 9% of the nominal for all analytes. Bench-top plasma stability is indicated for ~6 hours (high and low QC concentrations were all within 10% of the nominal concentrations). Whole blood stability for all analytes was proven for at least two hours.

2.3.2.11 Haemolysis

Normal plasma and haemolysed blood samples at high and low concentrations were compared (six-fold) to evaluate the impact of haemolysed blood on the assay. The ability of the ISTD to provide sufficient compensation for analyte determination was assessed by comparing normal and haemolysed plasma response ratios at high and low concentrations. Table 2.17 is a summary of the haemolysis results.

Table 2.17 Effect of 2% haemolysis

		2% Haemolysis			
		Precision CV(%)		%Difference	
Analyte	**N**	**QCH**	**QCL**	**QCH**	**QCL**
Carvedilol	6	5.0	-1.5	4.3	-0.9
Enalaprilat	6	5.7	4.2	2.3	-0.4
Perindoprilat	6	4.2	6.2	3.6	-2.3

Concentrations (ng/mL): QCH = 160, QCL= 0.550

The results reported above show that the %Difference and CV(%) are within 15% for carvedilol, enalaprilat, and perindoprilat in 2% haemolysed plasma samples, indicating no significant effect on the assay for the three analytes.

2.3.2.12 Crosstalk

Blank matrix was extracted and then individually spiked with each of the ISTDs. ULOQ and LLOQ samples for each of the three analytes were spiked separately into blank matrix without ISTDs to evaluate possible crosstalk between the ISTDs and the analytes. Each extracted sample was submitted for analysis for three injections on the LC-MS/MS system.

Enalapril and perindopril are prodrugs metabolised to the pharmacologically active enalaprilat and perindoprilat, respectively. However, these two analytes are not quantified in the assay. Enalapril and perindopril samples were still included as part of the crosstalk validation experiment to assess their crosstalk contribution should they be present. An ULOQ enalapril

sample was monitored in the enalaprilat MRM channel to assess any crosstalk contribution from the enalapril. Perindopril contribution was evaluated similarly by monitoring the perindoprilat channel for any contribution. Perindoprilat glucuronide, an important metabolite of perindopril, was also evaluated in terms of crosstalk during the experiment, although the assay does not quantify it. An ULOQ perindoprilat glucuronide sample was prepared and monitored in the perindoprilat channel for any potential contribution.

The response of any interfering peak in the blank sample at the retention time and mass transition of the analyte should be < 20% of the LLOQ peak response of the analyte. The mean response of the interfering peak at the retention time and mass transition of the ISTD must be < 5% of the mean response of the ISTD in the blank samples.[165] No significant interfering peaks were observed in the analyte channel for the blank sample from three injections of samples containing ISTD, indicating no crosstalk between the analytes and the ISTDs. Crosstalk was observed between enalaprilat and enalapril, perindoprilat and perindopril, as well as perindoprilat and perindoprilat glucuronide – all of which were greater than the 20% criteria. This was because of the in-source formation of perindoprilat, enalaprilat and perindoprilat-glucuronide, not technical crosstalk. The independent integration and quantification of analytes were made possible due to the separation of eluting peaks, rendering the contribution inconsequential. Chromatograms are shown in Figure 2.23 to Figure 2.25 for the three analyte pairs where crosstalk was observed. Although the two peaks are not baseline separated in Figure 2.25, the perindoprilat glucuronide peak forms a small fraction of perindoprilat peak, indicating that interference with perindoprilat quantification will be sufficiently insignificant.

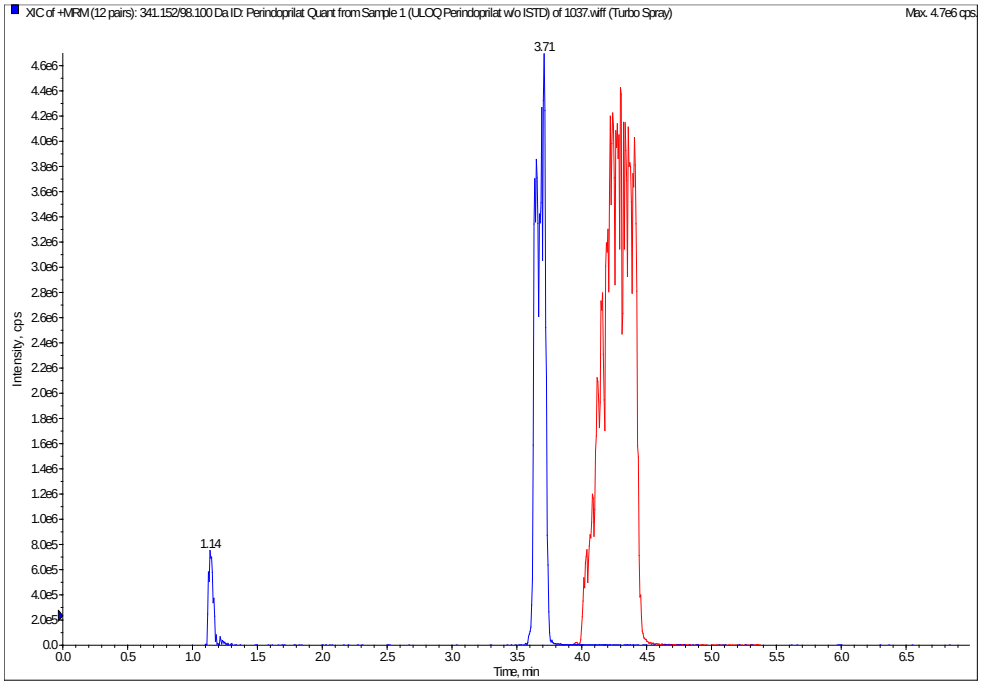


Figure 2.23 Overlay of enalaprilat (Blue) and enalapril (Red) chromatograms at ULOQ

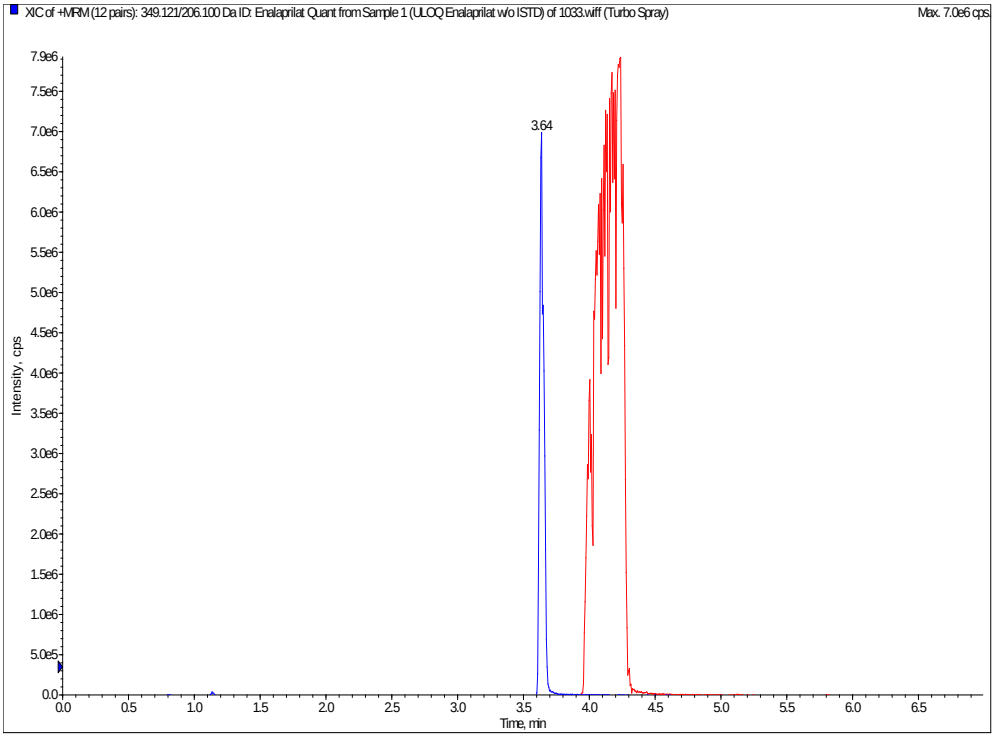


Figure 2.24 Overlay of perindoprilat (Blue) and perindopril (Red) chromatograms at ULOQ

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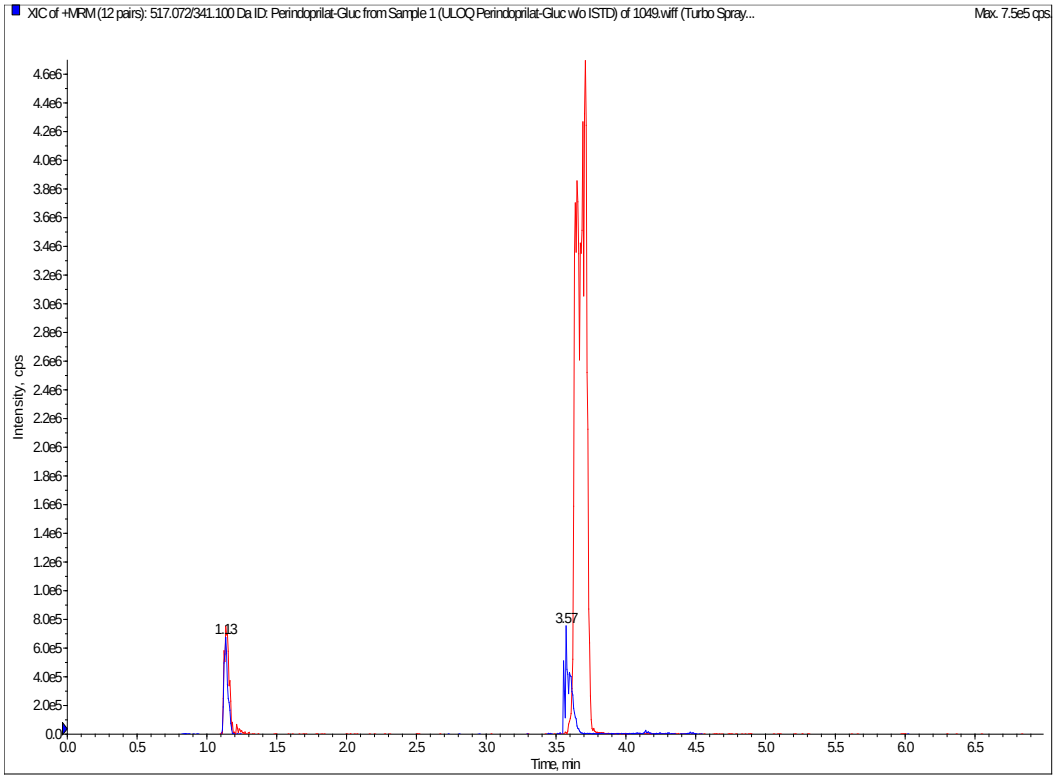


Figure 2.25 Overlay of perindoprilat glucuronide (Blue) and perindoprilat (Red) chromatograms at ULOQ

2.4 Summary and conclusions

This chapter described developing and validating a novel, sensitive and specific multiplex LC-MS/MS assay to determine carvedilol, enalaprilat and perindoprilat concentrations in human plasma. The method consists of protein precipitation using methanol:acetonitrile (1:1, v/v). A Restek Ultra II Biphenyl column was used with a corresponding flow rate of 300 µL/min. Gradient elution allowed for the timely seperation of the analytes, compensating for their differing physiochemical properties. Extract cleanliness proved to be an important obstacle during method development. It was found that centrifuging samples both before drying down and after reconstitution was necessary to allow for the required extract cleanliness.

The method was validated over the calibration range of 0.2–200 ng/mL for all three analytes. The intraday and interday accuracy and precision results for both the calibration standards and QCs fell within accepted criteria demonstrating that the method was accurate and precise for the validated calibration range. Carryover proved problematic for all analytes. A two-pronged approach was taken to mitigate carryover effects: double blanks were injected between samples to allow a system and column cleanse between each injection. In addition, a partial validation

was completed, allowing for the option to increase the LLOQ to 0.5 ng/mL in the unlikely event that the injected double blanks are insufficient.

The assay is the first to allow carvedilol, enalaprilat and perindoprilat to be quantified simultaneously, allowing the assay to be functional when a large sample throughput is required, such as a clinical trial. Moreover, the validation data presented prove that the method developed is suited for single-dose analysis and multiple-dose pharmacokinetic studies. Although published pharmacokinetic data indicate that the assay is sensitive enough to be used as an adherence-determining assay, its potential as an adherence measure is further evaluated through a pharmacokinetic pilot study in chapter 4.[172–177]

3 Simultaneous Quantification of Carvedilol, Enalaprilat and Perindoprilat in Dried Blood Spots

3.1 Introduction

The previous chapter described the development and validation of a method quantifying carvedilol, enalaprilat and perindoprilat in plasma. Plasma is considered the gold standard of biosample analysis.[53] However, it does have some limitations in determining adherence in resource-scarce areas. Well established sampling methods, such as plasma, can be resource-intensive during sample collection and storage.[95,180] The quantification of analytes in DBS has important advantages compared to conventional sampling methods, with it being particularly well suited to resource-scarce environments.[146,181,182] Untrained staff or patients can prepare DBS samples if capillary samples are being collected.[95,182] DBS cards can be transported at room temperature due to improved stability compared to other matrices, thus reducing the cost of shipping.[95,183] Moreover, the anti-microbial properties of the dried matrix mitigates the need for biohazard precautions.[95,183] DBS storage is often simplified as a result of the flat sample shape and relatively small size.

There are significantly fewer published methods quantifying carvedilol, enalaprilat and perindoprilat in DBS, with no method describing the simultaneous quantification of all three. One published method quantifies both enalaprilat and perindoprilat as part of an assay that quantifies eight antihypertensive drugs and four active metabolites.[94] The calibration ranges included 4.54–454 ng/mL and 5–500 ng/mL for enalaprilat and perindoprilat, respectively. No published DBS methods are available that quantify carvedilol. One publication describes the quantification of carvedilol and other antihypertensive drugs using VAMS,[139] validated for an LLOQ of 4 ng/mL.

A method for determining carvedilol, enalaprilat and perindoprilat in 50 µL whole-blood DBS using protein precipitation and LC-MS/MS is described in this chapter, the first to allow for the simultaneous quantification of carvedilol, enalaprilat and perindoprilat in the DBS matrix. The primary aim of this chapter is to describe the development and validation of a method for extracting and the simultaneous quantifying of carvedilol, enalaprilat and perindoprilat in whole-blood DBS using LC-MS/MS. The objectives addressed in this chapter include:

- To develop a simple, sensitive and reproducible extraction and detection method for the simultaneous quantification of carvedilol, enalaprilat and perindoprilat in whole-blood DBS.
- The validation of the bioanalytical method according to the FDA guidelines.

3.2 Materials and methods

3.2.1 Collection and storage of samples

Donated whole blood which is free of carvedilol, enalaprilat, and perindoprilat, was used during method development, validation, and for preparing calibration standards and quality control samples (QCs). Whole blood was drawn via venepuncture from consenting subjects not on carvedilol, enalapril or perindopril and DBS cards were prepared using Whatman 903 Protein Saver Cards (GE Healthcare, Chicago, USA). Fifty microlitres of blood were dispensed accurately by wet tip pipetting. The DBS cards were dried for two hours at room temperature out of direct sunlight and stored at ~-80°C in sealable plastic bags containing 3 x 1 g desiccant sachets.

3.2.2 Chemicals and reagents

Reference standards of carvedilol, enalaprilat dihydrate and perindoprilat in powder form were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Carvedilol-d5, enalaprilat-d5 sodium salt and perindoprilat-$^{13}C_3$ were purchased from Toronto Research Chemicals Inc. (Toronto, Canada) and were used as ISTDs. Acetic acid (Proanalysis grade) was obtained from Labchem (Johannesburg, South Africa). Acetonitrile and methanol of LC-MS grade, were supplied by Anatech (Bellville, South Africa). A Synergy Water Purification System obtained from Merck Millipore (Billerica, MA, USA) was used to prepare deionised water. Sigma-Aldrich (Modderfontein, South Africa) supplied ammonium acetate (≥ 99.99%).

3.2.3 Sample extraction

DBS samples were punched out (12 mm punch size) and placed into 2.0 mL microcentrifuge tubes. Spot-to-spot carryover was mitigated by punching unfiltered paper between punching of sample spots. Two hundred microlitres of water were pipetted onto the DBS discs and vortex mixed for 30 seconds. After water-soaking, 1 mL of precipitation solution (methanol:acetonitrile (1:1, v/v)) containing the ISTDs (0.500 ng/mL of carvedilol-d5, 2.00 ng/mL of enalaprilat-d5, and 1.00 ng/mL of perindoprilat-13C3) was added and the samples were vortex mixed for 30 seconds. Samples were centrifuged for 5 minutes (20238 g) with the supernatant transferred to glass tubes and evaporated under a gentle nitrogen stream (~40°C for ~15 minutes). Two hundred microlitres of reconstitution solution (methanol:water:formic acid (40:60:0.2, v/v/v)) was added after sample drying and samples were dissolved by vortex mixing for 30 seconds. After reconstitution, the extract was transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 minutes at 20238 g. The supernatants were

transferred to 96-well plates, and the plates were placed in the LC-MS/MS autosampler (~8ºC). Five microlitres of the sample were injected for analysis.

3.2.4 LC-MS/MS equipment and conditions

Taking advantage of the existing plasma method, much of the plasma method's LC-MS/MS and chromatographic conditions were transferred to the DBS assay. ESI in the positive ionisation mode was employed for detection on an AB Sciex API 5500 Qtrap mass spectrometer (AB Sciex^{TM}, Germany). The collision gas parameter was set at the "medium" level with 55, 55 and 30 psi settings for the nebuliser, turbo, and curtain gases, respectively. The ion spray voltage setting was 5500 V, and the source temperature was 500°C. Mass transitions included 407.1 > 100.1, 349.1 > 206.1, 341.2 > 170.1, 412.1 > 105.1, 354.1 > 211.0, and 344.2 > 100.1 for carvedilol, enalaprilat, perindoprilat, carvedilol-d5, enalaprilat-d5, and perindoprilat-^{13}C3, respectively. Analyst Version 1.7.1 (AB Sciex^{TM}, Germany) software was used to collect and analyse the data. Table 3.1 summarises the MRM transitions and the final mass spectrometric conditions. All transitions and mass spectrometric conditions are the same as for the plasma assay, except it was decided to use 341.2 > 170.1 as the quantifier transition instead of 341.2 > 98.1 for perindoprilat. The product ion mass spectra and proposed fragmentations are depicted in Figures 2.1 to 2.6 (section 2.2.4).

Table 3.1 Summary of MRM transitions and mass spectrometer conditions of analytes and ISTD (DBS)

	MRM transition						
Analyte	**Quantifier, m/z**	**Qualifier, m/z**	**Dwell time (ms)**	**Declustering potential (V)**	**Entrance potential (V)**	**Collision energy (V)**	**Cell exit potential (V)**
Carvedilol	407.1 → 100.1	407.1 → 224.1	125	96	10	37	10
Carvedilol-d5	412.2 → 105.1	412.2 → 229.1	105	126	10	39	12
Enalaprilat	349.1 → 206.1	349.1 → 91.1	35	101	10	27	20
Enalaprilat-d5	354.2 → 211.1	354.2 → 96.0	50	91	10	27	20
Perindoprilat	341.2 → 170.1	341.2 → 98.1	65	96	10	45	10
Perindoprilat-$^{13}C_3$	344.2 → 100.1	344.2 → 170.1	60	81	10	47	10

Gradient elution was employed with mobile phase A and B composition and a corresponding gradient profile identical to the plasma assay, so catering to the varying physiochemical properties of the analytes (section 2.2.4). A Restek Ultra Biphenyl column (100 mm × 2.1 mm, 3 µm) was used for chromatographic separation.

3.2.5 Method validation

3.2.5.1 Preparation of calibration standards and quality controls

A working solution containing the three analytes was prepared by spiking 20 µL of 1 mg/mL solutions of carvedilol, enalaprilat and perindoprilat into 39940 µL methanol. A series of working solutions was then prepared volumetrically in methanol. These working solutions were spiked directly onto blank 50 µL DBS for each calibration standard (Table 3.2). The storing of DBS-prepared standards was at ~-80°C.

Table 3.2 Preparation of working solutions and calibration standards for carvedilol, enalaprilat and perindoprilat

Working solutions (WS)	**Blank solvent (methanol) volume (µL)**	**Spiking solution**	**Spiking solution volume (µL)**	**WS solution concentration (ng/mL)**	**Volume pipetted onto 50 µL DBS (µL)**	**STD**	**DBS concentration (ng/mL)**
WS1	39940	SS x 3	20+20+20	500	20	STD 1-ULOQ	200
WS2	2000	WS1	6000	375	20	STD 2	150
WS3	4000	WS2	4000	188	20	STD 3	75.0
WS4	4800	WS3	2400	62.5	20	STD 4	25.0
WS5	4800	WS4	3200	25.0	20	STD 5	10.0
WS6	4800	WS5	3200	10.0	20	STD 6	4.00
WS7	3200	WS6	3200	5.00	20	STD 7	2.00
WS8	3200	WS7	3200	2.50	20	STD 8 - LLOQ	1.00

To prepare QCs, 20 µL of 1 mg/mL of carvedilol, enalaprilat and perindoprilat were spiked into 19940 µL methanol. This solution was used to prepare a series of QC working solutions volumetrically in methanol (Table 3.3). These working solutions were spiked directly onto 50 µL DBS for each QC. The QCs were stored at ~-80°C.

Table 3.3 Preparation of working solutions and QCs for carvedilol, enalaprilat and perindoprilat

Working solutions (WS)	Blank solvent (methanol) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS solution concentration (ng/mL)	Volume pipetted onto 50 µL DBS (µL)	STD	DBS concentration (ng/mL)
WSQ1	19940	SS x 3	20 + 20 + 20	1000	20	QC Dil	400
WSQ2	3600	WSQ1	2400	400	20	QCH	160
WSQ3	3000	WSQ2	3000	200	20	QCM	80.0
WSQ4	3000	WSQ3	3000	100	20	SYS 1	40.0
WSQ5	3000	WSQ4	2000	40.0	20	SYS 2	16.0
WSQ6	9000	WSQ5	3000	10.0	20	SYS 3	4.00
WSQ7	3000	WSQ6	3000	5.00	20	QCL	2.00
WSQ8	3000	WSQ7	3000	2.50	20	QCLLOQ	1.00

3.2.5.2 Validation experiments

The method was validated as required by the 2018 US FDA specific for industry bioanalytical method validation guidelines.[165] The following validation experiments were performed: accuracy and precision, working solution stability, on-instrument stability and reinjection reproducibility, stability in the matrix, freeze–thaw stability, bench-top stability, recovery, process efficiency, matrix effects, specificity, carryover, sensitivity, and crosstalk. Validation experiments that were already completed for the plasma assay and which are not matrix-specific were not repeated (stock stability, whole blood stability and haemolysis).

3.2.6 Additional experiments

3.2.6.1 Comparison of Whatman 903® protein saver cards and Capitainer®qDBS cards

Capitainer®qDBS (Stockholm, Sweden) cards are like VAMS devices in that they are designed to absorb a fixed volume of the sample but consist of a sample collection disc closer in resemblance to that of a DBS sample (Figure 3.1). It uses paper and polymer microfluidics to absorb a fixed volume from an undefined volume of finger-prick blood. A valve consisting of a thin dissolvable membrane opens and removes any excess blood from the inlet. A second membrane then opens and allows for the transfer of the metered blood volume onto the collection disc.[184] The advantage of the technology is that the haematocrit effect is overcome since the full volume defined spot is used for analysis. Furthermore, it makes patient self-sampling more practical as only a fixed volume is absorbed into the disc. All the study DBS samples quantified using the DBS assay developed in this study were generated from patient blood samples using pipettes, and so patient self-sampling is avoided. Cards such as

Capitainer®qDBS could make the shift to patient self-sampling more feasible. However, the Capitainer®qDBS cards were evaluated using the assay developed for the Whatman 903® protein saver cards.

Whole blood was spiked with working solutions to prepare QH, QM and QL whole blood quality controls. Working solutions could not be spiked directly onto the Capitainer cards to create standards or QCs as microchannels in the cards force a set volume to be absorbed. This made spiking onto the card after whole blood had already been absorbed impractical. Therefore, although difficult and time-consuming, QCs prepared in whole blood were used for both the Whatman and Capitainer cards. Six 50 µL DBS were created on the Whatman cards for each QH, QM and QL by pipetting the whole blood onto the cards. Similarly, six different Capitainer spots were made at each QC level by pipetting 25 µL (absorbable volume is 13.5 µL) of the whole blood onto the Capitainer input area, allowing the microchannel to be filled up. The cards were only compared in terms of repeatability (CV(%)).

Steps that were added when preparing the Capitainer spots included:

- Before spotting, the whole blood was heated for 5 minutes (40°C) in 2 mL Eppendorf tubes to reduce viscosity. This step was helpful as the whole blood samples were at room temperature, and the Capitainer Cards were designed for blood at body temperature.
- Placing the cards at a 45° angle helped fill the microchannel once blood had been spotted onto the Capitainer cards.

After spotting, the Whatman and Capitainer spots were submitted to the extraction using the method developed for analysing enalaprilat, perindoprilat and carvedilol in DBS.

Figure 3.1 Capitainer®qDBS card [184] on the left and a Whatman card[185] on the right.

3.3 Results and discussion

3.3.1 Method development and optimisation

3.3.1.1 Extraction and chromatography

The technique proposed by Lee et al.[186] was used where the DBS was soaked in water prior to the addition of the organic solvent to ensure that the diffusion of the small molecules from the DBS card was sufficient and to return the DBS sample to a fresh blood-like state. Unlike blood, the DBS matrix's constituents are dry and densely packed on the DBS card. As a result of this distinctive physical state of the matrix, applying small molecule extraction techniques that are typically used in blood or plasma, such as direct incubation of the sample in an organic solvent, may be less compatible with DBS extraction. The molecules are less likely to be effectively diffused from the DBS card.[186] Blood is mainly composed of water (> 90%), with the formation of DBS occurring when the water is evaporated. Therefore, water is the most appropriate reconstitution solvent for DBS.[186] Two hundred microlitres of water were added prior to the addition of the organic solvent. This allowed for sufficient soaking of the spot while ensuring that time spent drying down was not excessive.

Like the plasma method (section 2.2.3), an acetonitrile and methanol mixture (1:1, v/v) was added after water soaking the spot. The extraction solvent volume increased five-fold compared to the plasma assay, from 0.2 to 1.00 mL, to allow for the complete submersion of the punched-out spot. The concentration of the ISTD in the extraction solvent was reduced by approximately five-fold to compensate for the increased extraction solvent volume used. As with the plasma method, centrifugation before drying and reconstitution allowed for sufficient sample clean-up. The mobile phase's solvent gradient profile and corresponding aqueous and organic compositions were transferred from the plasma assay to the DBS assay.[187]

3.3.1.2 Preparation of standards and QCs

Published assays describing the quantification of drugs and metabolites in DBS typically elect to prepare DBS standards and QCs by spiking working solutions into whole blood first to prepare whole blood standards and QCs. DBS standards and QCs are then prepared by spiking the whole blood standards and QCs onto the DBS cards.[188–193] During method development, standards and QCs were initially made using the same procedure. The organic working solution, however, caused coagulation of the whole blood upon contact, reducing the practicality of standard and QC preparation. To make the assay more practical, standards and QCs were prepared by spiking the working solution directly onto blank DBS spots[194,195]. DBS were punched and placed in 2 mL Eppendorf tubes, and the working solution was then spiked

onto the spots in the Eppendorf tubes. Placing the spots into the Eppendorf tubes before spiking the working solution ensured that the spot was the only area the solution could diffuse across. Spiking the working solution onto the spot before punching allows the working solution opportunity to diffuse outside the radius of the spot.

Extractions were performed on DBS samples that were prepared with whole blood that was spiked with working solution before being spotted on the cards. Extractions were performed of samples at 150 ng/mL and at 2.00 ng/mL in six-fold to evaluate if the precision of the assay is severely affected when DBS samples prepared from pre-spiked whole blood were extracted (Table 3.4).

Table 3.4 Precision (CV (%)) results of DBS samples that have been prepared by pre-spiked whole blood.

	150 ng/mL (S2)		2.00 ng/mL (QL)	
Analyte	**Number of samples evaluated**	**Precision CV (%)**	**Number of samples evaluated**	**Precision CV (%)**
Carvedilol	6	8.7	6	4.8
Enalaprilat	*5	7.2	*5	4.4
Perindoprilat	*5	5.0	*5	3.6

Concentrations (ng/mL): S2 = Standard 2, QCL= Quality control low
*One sample removed because of bench/experimental error

The CV(%) was < 10% for all samples. The precision is not affected when DBS is extracted, which was prepared from pre-spiked whole blood. Moreover, the advantage of this DBS assay is that it has a corresponding plasma assay. The robustness of the assay can be further confirmed by cross-validating it using the plasma assay. If the method chosen to prepare the standards and QCs did cause a deficiency with the assay, the deficiency would manifest during the cross-validation. The cross-validation using patient samples is discussed in Chapter 5.

3.3.2 Method validation

3.3.2.1 Working solution stability

Working solution stabilities were evaluated using the method outlined in section 2.3.2.2. Table 3.5 summarises the working solution stabilities. The highest (0.5 µg/mL) and lowest (0.0025 µg/mL) working solution concentrations for all analytes are stable at room temperature for ~4 hours, with long-term working solution stability demonstrated for up to ~125 days in methanol at ~-80°C.

Table 3.5 Summary of working solution stabilities of DBS assay

Storage conditions	N	Carvedilol		Enalaprilat		Perindoprilat	
		Precision CV(%)	%Difference	Precision CV(%)	%Difference	Precision CV(%)	%Difference
**0.500 µg/mL at ~-80°C for ~125 days	6	1.5	7.2	3.0	4.3	2.9	-8.6
*0.0025 µg/mL at ~-80°C for ~125 days	6	1.5	3.6	4.0	6.8	3.6	-8.9
**0.500 µg/mL at RT for ~4 hours	6	2.2	5.4	2.4	5.9	2.6	5.3
*0.0025 µg/mL at RT for ~4 hours	6	1.7	-6.3	11.1	-4.3	3.2	-5.9

*Lowest working solution concentration: 0.0025 µg/mL; **Highest working solution concentration: 0.500 µg/mL

3.3.2.2 Calibration range

The method was validated over the calibration range of 1.00–200 ng/mL for carvedilol, enalaprilat and perindoprilat. The selection of an appropriate calibration range for the DBS assay was more complicated than the plasma assay due to the lack of available pharmacokinetic data. The LLOQ should be lower or at least match the plasma assay due to the higher concentration of the analytes in plasma than in whole blood.[92,93] During method development, it became apparent that to create an assay that would meet the validation criteria, the LLOQ for the DBS assay would need to be set at a minimum of 1.00 ng/mL. This was due to the additional complexity of using paper as a collection medium for DBS samples, which contains various components, including cellulose. Therefore, there would be some limitations regarding the DBS LLOQ, especially when one wants to convert from the DBS concentration to plasma concentrations. The limitation could not be fully understood without a pharmacokinetic study being done. The pharmacokinetic study discussed in Chapter 4 was therefore critical for further understanding the assay and its limitations. Although the LLOQs of enalaprilat and perindoprilat are lower for the plasma assay than the DBS assay, they are substantially lower than published DBS LLOQs (4.54 ng/mL for enalaprilat and 5.00 ng/mL for perindoprilat).[94]

Calibration curves were generated for each of the three validation batches. A quadratic regression weighted by $1/x^2$ for all three analytes was used. Each calibration curve showed a good fit, with all R^2 values above 0.99. Figure 3.2 to Figure 3.4 are representative calibration curves of carvedilol, enalaprilat and perindoprilat, respectively.

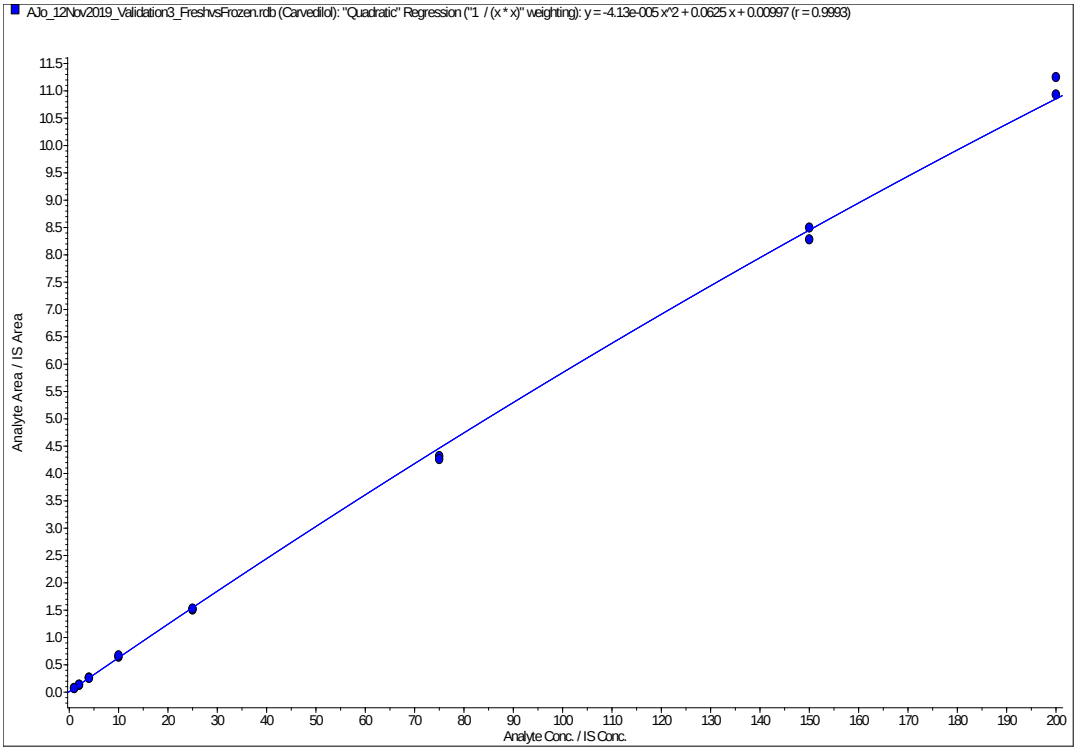


Figure 3.2 A representative calibration curve for carvedilol (r = 0.9993)

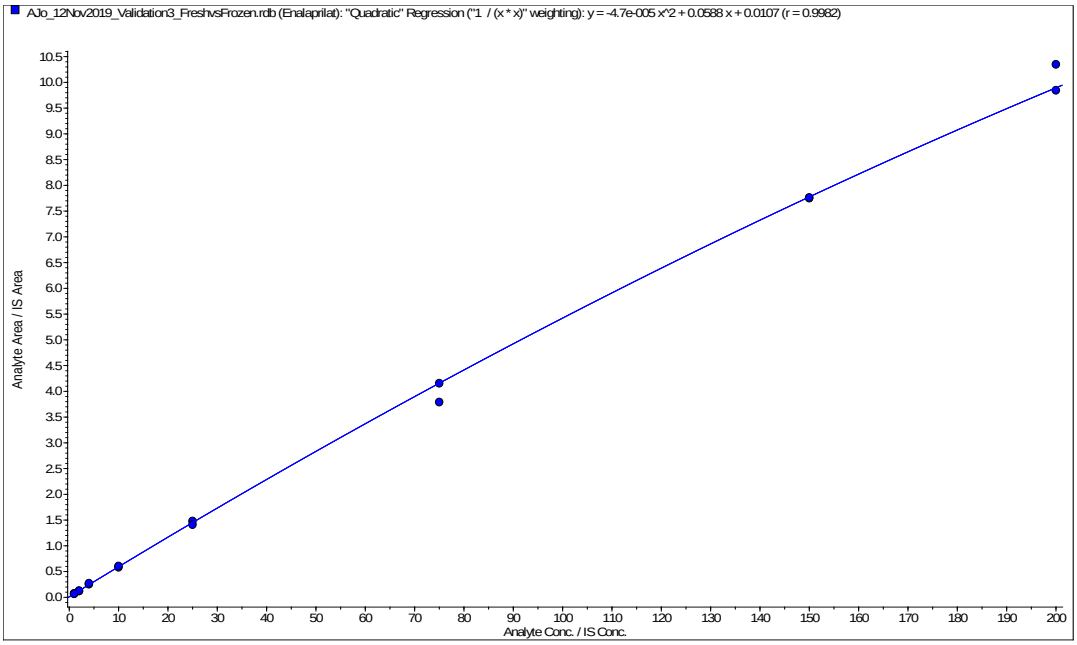


Figure 3.3 A representative calibration curve for enalaprilat (r = 0.9982)

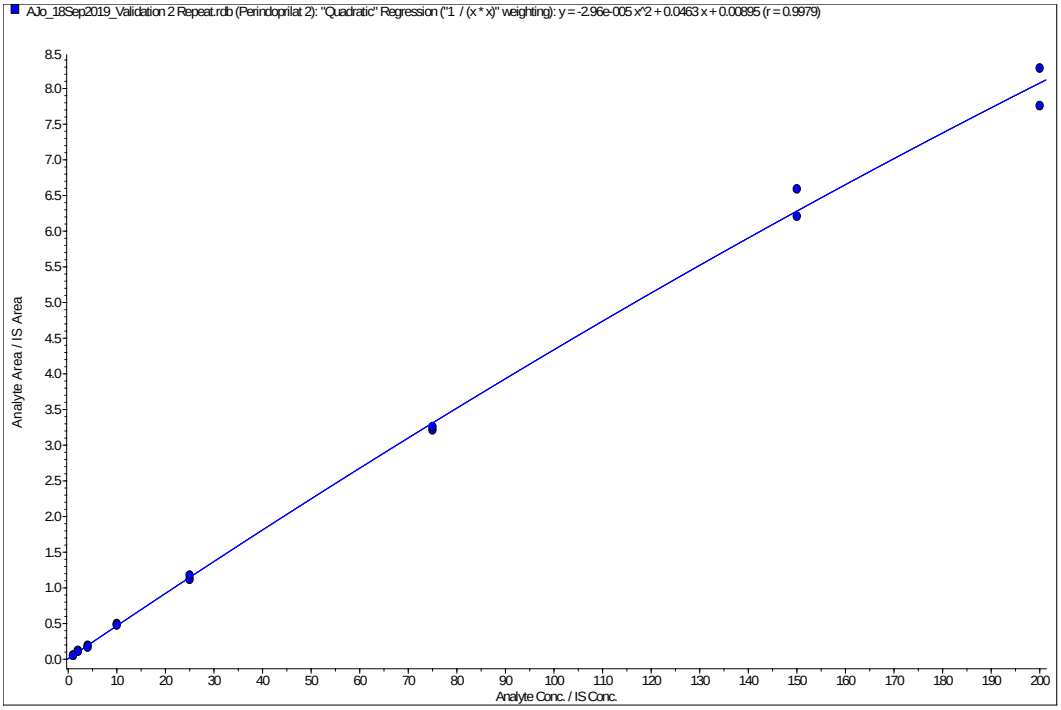


Figure 3.4 A representative calibration curve for perindoprilat (r = 0.9979)

3.3.2.3 Accuracy and precision (intra-day and inter-day; within-batch and between-batch)

The accuracy and precision of the assay were assessed as described in section 2.3.2.4, with precision and accuracy statistics calculated over the within and between-batch validation batches (three batches in total). Table 3.6 to Table 3.11 show the accuracy and precision of standards and QCs for carvedilol, enalaprilat and perindoprilat over the three validation batches. The criteria are met for all analytes as required by the FDA.[165]

Table 3.6 Overall summary of calibration standard accuracy and precision: validation 1–3: carvedilol

Validation Batch	**Sample ID** **Nominal conc.** **Replicates**	**STD 1 - ULOQ** **200 (ng/mL)** **Observed conc.**	**STD 2** **150 (ng/mL)** **Observed conc.**	**STD 3** **75.0 (ng/mL)** **Observed conc.**	**STD 4** **25.0 (ng/mL)** **Observed conc.**	**STD 5** **10.0 (ng/mL)** **Observed conc.**	**STD 6** **4.00 (ng/mL)** **Observed conc.**	**STD 7** **2.00 (ng/mL)** **Observed conc.**	**STD 8 – LLOQ** **1.00 (ng/mL)** **Observed conc.**
Validation 1	1	220	145	65.8	24.3	10.2	4.21	2.00	0.949
	2	201	151	72.7	24.4	10.5	4.17	2.01	1.02
Validation 2	1	208	162	71.1	24.2	10.6	3.88	2.26	0.908
	2	187	151	72.1	25.2	10.3	3.50	2.09	1.04
Validation 3	1	202	147	72.4	24.4	10.2	4.11	1.92	1.02
	2	209	151	71.4	24.7	10.7	3.97	2.03	0.977
	N	6	6	6	6	6	6	6	6
	Average	205	151	70.9	24.5	10.4	3.97	2.05	0.986
	STDEV	10.93	5.88	2.58	0.367	0.214	0.263	0.116	0.0505
	CV(%)	5.3	3.9	3.6	1.5	2.1	6.6	5.6	5.1
	%Accuracy	102.3	100.8	94.6	98.1	104.2	99.3	102.6	98.6

Table 3.7 Overall quality control accuracy and precision estimation: carvedilol

Validation batch	Sample ID Nominal conc. Replicates	LLOQ 1.00 (ng/mL) Observed conc.	QC - L 2.00 (ng/mL) Observed conc.	QC - Med 80.0 (ng/mL) Observed conc.	QC - High 160 (ng/mL) Observed conc.
Validation 1	1	0.853	1.74	76.0	170
	2	1.02	1.95	80.3	175
	3	0.848	1.75	77.4	172
	4	1.02	1.95	81.1	172
	5	0.908	1.95	81.6	178
	6	0.812	1.81	80.1	179
Validation 2	1	0.864	2.34	84.5	171
	2	0.930	2.03	84.1	178
	3	0.920	1.94	82.0	173
	4	0.803	1.76	84.0	176
	5	0.788	2.11	83.9	171
	6	0.918	1.97	86.0	182
Validation 3	1	0.846	2.20	83.0	186
	2	0.830	2.07	81.1	173
	3	0.797	2.05	80.7	182
	4	0.737	2.22	84.2	159
	5	*[1.74]*	2.16	81.9	184
	6	0.810	2.20	81.8	169
	N	17	18	18	18
	Average	0.865	2.01	81.9	175
	STDEV	0.0785	0.1755	2.5	6.5
	CV(%)	9.1	8.7	3.1	3.7
	%Accuracy	86.5	100.6	102.3	109.4

[] - Failed acceptance criteria due to technical error not included in the calculation

Table 3.8 Overall summary of calibration standard accuracy and precision: validation 1–3: enalaprilat

Validation Batch	**Sample ID** **Nominal conc.** **Replicates**	**STD 1 - ULOQ** **200 (ng/mL)** **Observed conc.**	**STD 2** **150 (ng/mL)** **Observed conc.**	**STD 3** **75.0 (ng/mL)** **Observed conc.**	**STD 4** **25.0 (ng/mL)** **Observed conc.**	**STD 5** **10.0 (ng/mL)** **Observed conc.**	**STD 6** **4.00 (ng/mL)** **Observed conc.**	**STD 7** **2.00 (ng/mL)** **Observed conc.**	**STD 8 – LLOQ** **1.00 (ng/mL)** **Observed conc.**
Validation 1	1	223	145	65.3	26.4	9.85	4.00	1.97	0.941
	2	201	150	72.1	24.0	10.5	4.33	2.00	1.04
Validation 2	1	221	156	68.8	24.0	10.4	4.06	2.20	0.988
	2	192	147	69.7	24.8	9.97	*[3.27]*	2.09	0.935
Validation 3	1	211	149	75.0	25.5	9.78	4.13	1.86	1.05
	2	199	150	67.9	24.3	10.2	4.44	1.97	0.960
	N	6	6	6	6	6	5	6	6
	Average	208	150	69.8	24.8	10.12	4.19	2.02	0.986
	STDEV	12.5	3.73	3.38	0.956	0.297	0.186	0.117	0.0496
	CV(%)	6.0	2.5	4.8	3.9	2.9	4.4	5.8	5.0
	%Accuracy	103.9	99.7	93.1	99.3	101.2	104.8	100.8	98.6

[ ] failed standard excluded from the curve

Table 3.9 Overall quality control accuracy and precision estimation: enalaprilat

Validation batch	Sample ID Nominal conc. Replicates	LLOQ 1.00 (ng/mL) Observed conc.	QC - L 2.00 (ng/mL) Observed conc.	QC - Med 80.0 (ng/mL) Observed conc.	QC - High 160 (ng/mL) Observed conc.
Validation 1	1	0.890	1.74	71.3	173
	2	0.890	1.70	77.0	168
	3	0.978	1.85	75.8	174
	4	0.850	1.72	73.8	173
	5	0.914	1.82	75.6	173
	6	0.869	1.77	74.2	168
Validation 2	1	0.773	1.87	80.2	168
	2	0.727	1.91	81.4	170
	3	0.821	1.74	78.2	172
	4	0.748	1.63	81.2	172
	5	0.887	1.71	77.0	166
	6	0.868	1.71	86.4	182
Validation 3	1	0.725	2.28	85.6	176
	2	0.951	2.12	82.6	164
	3	0.817	2.11	80.6	172
	4	0.792	2.13	82.0	168
	5	*[1.67]*	2.54	80.6	188
	6	0.973	2.17	81.9	176
	N	17	18	18	18
	Average	0.851	1.92	79.2	172
	STDEV	0.0804	0.250	4.09	5.7
	CV(%)	9.4	13.0	5.2	3.3
	%Accuracy	85.1	95.9	99.0	107.7

[] - Failed acceptance criteria due to technical error not included in the calculation

Table 3.10 Overall summary of calibration standard accuracy and precision: validation 1–3: perindoprilat

Validation batch	Sample ID	STD 1 - ULOQ	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8 - LLOQ
	Nominal conc.	200 (ng/mL)	150 (ng/mL)	75.0 (ng/mL)	25.0 (ng/mL)	10.0 (ng/mL)	4.00 (ng/mL)	2.00 (ng/mL)	1.00 (ng/mL)
	Replicates	Observed conc.	Observed conc.	Observed conc.	Observed conc.	Observed conc.	Observed conc.	Observed conc.	Observed conc.
Validation 1	1	222	144	62.4	24.3	10.1	4.11	1.93	0.99
	2	204	143	69.7	23.6	10.7	4.31	1.96	1.01
Validation 2	1	206	158	72.7	24.4	10.6	4.00	*[2.46]*	0.985
	2	191	148	73.7	25.7	10.2	3.45	2.18	1.00
Validation 3	1	211	156	73.6	23.7	10.1	4.09	2.11	0.96
	2	195	142	74.7	25.2	10.2	3.95	1.96	1.02
	N	6	6	6	6	6	6	5	6
	Average	205	149	71.1	24.5	10.3	3.99	2.03	0.994
	STDEV	11.2	6.92	4.61	0.828	0.264	0.290	0.110	0.0215
	CV(%)	5.4	4.7	6.5	3.4	2.6	7.3	5.4	2.2
	%Accuracy	102.4	99.0	94.8	97.9	103.2	99.6	101.4	99.4

[ ] failed standard excluded from the curve

Table 3.11 Overall quality control accuracy and precision estimation: perindoprilat

Validation batch	**Sample ID nominal conc.** / **Replicates**	**LLOQ** **1.00 (ng/mL)** **Observed conc.**	**QC - L** **2.00 (ng/mL)** **Observed conc.**	**QC - Med** **80.0 (ng/mL)** **Observed conc.**	**QC - High** **160 (ng/mL)** **Observed conc.**
Validation 1	1	0.939	1.79	73.0	172
	2	0.969	1.76	77.7	177
	3	1.020	1.82	78.1	172
	4	0.988	1.82	78.5	172
	5	0.982	1.80	75.1	173
	6	0.841	1.70	78.4	178
Validation 2	1	0.887	2.11	82.8	174
	2	0.920	1.96	84.9	174
	3	0.936	2.03	83.9	169
	4	0.886	1.82	85.9	188
	5	0.896	1.86	81.7	174
	6	0.908	1.86	88.4	183
Validation 3	1	0.803	2.51	80.3	168
	2	0.967	2.26	81.2	162
	3	0.965	2.15	77.3	170
	4	0.815	2.09	83.1	164
	5	*[1.96]*	2.58	75.0	185
	6	0.967	2.21	79.6	167
	N	17	18	18	18
	Average	0.923	2.01	80.3	173
	STDEV	0.0621	0.257	4.1	6.9
	CV(%)	6.7	12.8	5.1	4.0
	%Accuracy	92.3	100.4	100.3	108.4

[] - Failed acceptance criteria due to technical error not included in the calculation

3.3.2.4 Matrix effects

The influence of matrix components on the analyte and ISTD was evaluated as set out by Matuszewski et al.[179] Blank DBS were spiked from six different sources of whole blood. The individual matrix samples were spiked at low, medium and high concentrations and a single ISTD concentration. Simple linear regressions were generated using the peak area ratios of the analyte/ISTD at every concentration level (Table 3.12). Matrix effects were minimal, with only carvedilol displaying a slope variability of slightly greater than 5% across the six different matrix sources.

Table 3.12 Regression results from six different matrix sources for carvedilol, enalaprilat and perindoprilat

N		High conc. (160 ng/mL) Peak area ratio	Medium conc. (80 ng/mL) Peak area ratio	Low conc. (2.00 ng/mL) Peak area ratio	Area ratio v conc. Regression slope
			Carvedilol		
6	Average	7.67	3.42	0.213	0.0472
	STDEV	0.409	0.242	0.0117	0.00260
	CV(%)	5.3	7.1	5.5	5.5
			Enalaprilat		
6	Average	8.07	3.78	0.297	0.0490
	STDEV	0.322	0.293	0.0204	0.00212
	CV(%)	4.0	7.8	6.9	4.3
			Perindoprilat		
6	Average	5.69	2.68	0.171	0.0349
	STDEV	0.216	0.246	0.0130	0.00138
	CV(%)	3.8	9.2	7.6	4.0

Using the criteria outlined by Matuszewski et al.,[179] matrix effects were observed for carvedilol (5.5%) greater than the maximum criteria of 5%. Carvedilol's deviation outside the maximum criteria was accepted because of the small size of the deviation, the CV(%) being < 8.0% for all concentrations, and the complexity of the DBS matrix. No significant endogenous matrix effects were observed for detecting enalaprilat and perindoprilat.

3.3.2.5 Recovery

Low (2.00 ng/mL), medium (80.0 ng/mL) and high (160 ng/mL) QCs were each extracted in six-fold, serving as the test samples. The reference samples were prepared by spiking into the extracted blank DBS matrix at each concentration level in six-fold. By comparing the peak area

ratios of the test and reference samples, the recovery of the analytes could be established. Recovery results are shown in Table 3.13.

Table 3.13 Summary of recovery results based on average precision and average %recovery between three concentration levels

Analyte	N	Average %Recovery	Average precision CV(%)
Carvedilol	6	120.6	5.5
Enalaprilat	6	114.5	6.8
Perindoprilat	6	109.7	5.4

Average extraction recoveries across the high, medium and low concentrations were 120.6%, 114.5%, and 109.7% for carvedilol, enalaprilat, and perindoprilat, respectively. The recovery reproducibility between concentration levels was all < 15%. The mean recovery for carvedilol, enalaprilat and perindoprilat in plasma is 72.9%, 77.1% and 77.0% (Table 2.13). Therefore, DBS samples had complete recovery of all three analytes from the matrix, while plasma samples did not achieve complete recovery.

3.3.2.6 Process efficiency

The process efficiency of the assay was evaluated by comparing the instrument response of extracted and neat unextracted samples. QCs at low (2.00 ng/mL), medium (80.0 ng/mL) and high (160 ng/mL) concentrations were prepared in six different lots of matrix and extracted as per the SOP as discussed in section 3.2.3. Low, medium and high concentrations of the neat (unextracted) samples were prepared in injection solution (methanol:water:formic acid (40:60:0.2, v/v/v)), in triplicate. By comparing the peak area ratios of the analytes after extraction to those of the analytes in the neat samples (Table 3.14), the percentage process efficiency could be quantified. The reproducibility of the process efficiency between concentration levels must be < 15%. Observed process efficiency precision (CV(%)) must also be < 15%.[165] All analytes met the required criteria. The process efficiency of the analytes in plasma was lower (Table 2.14). The formula for process efficiency (PE) in terms of matrix effects (ME) and recovery (RE) is[179]:

PE (%) = (ME x RE) / 100

Since DBS samples had higher recovery rates than plasma samples, the recovery value in the formula would be higher for DBS samples, which would result in a higher process efficiency value.

Table 3.14 Summary of the process efficiency results based on average precision and average %process efficiency between three concentration levels

Analyte	N	Average %Process efficiency	Average precision CV(%)
Carvedilol	6	102.4	4.2
Enalaprilat	6	119.5	7.3
Perindoprilat	6	109.3	3.3

3.3.2.7 Specificity, sensitivity and carryover

The chromatograms of blank DBS spots (spots generated from six different whole blood sources) were evaluated to ascertain the influence of endogenous matrix components in quantifying and differentiating between the three analytes. The response must be < 20% of the LLOQ and < 5% of the ISTD at the retention times and mass transitions of the analytes and ISTD, respectively. Figure 3.6 shows double blank and blank chromatograms of the three analytes, respectively, with the criteria required for specificity fulfilled for all analytes.

The DBS assay carryover also proved problematic for all three analytes, which was mitigated by injecting double blanks between each sample to allow for a column and system cleanse before the injection of samples for quantification.

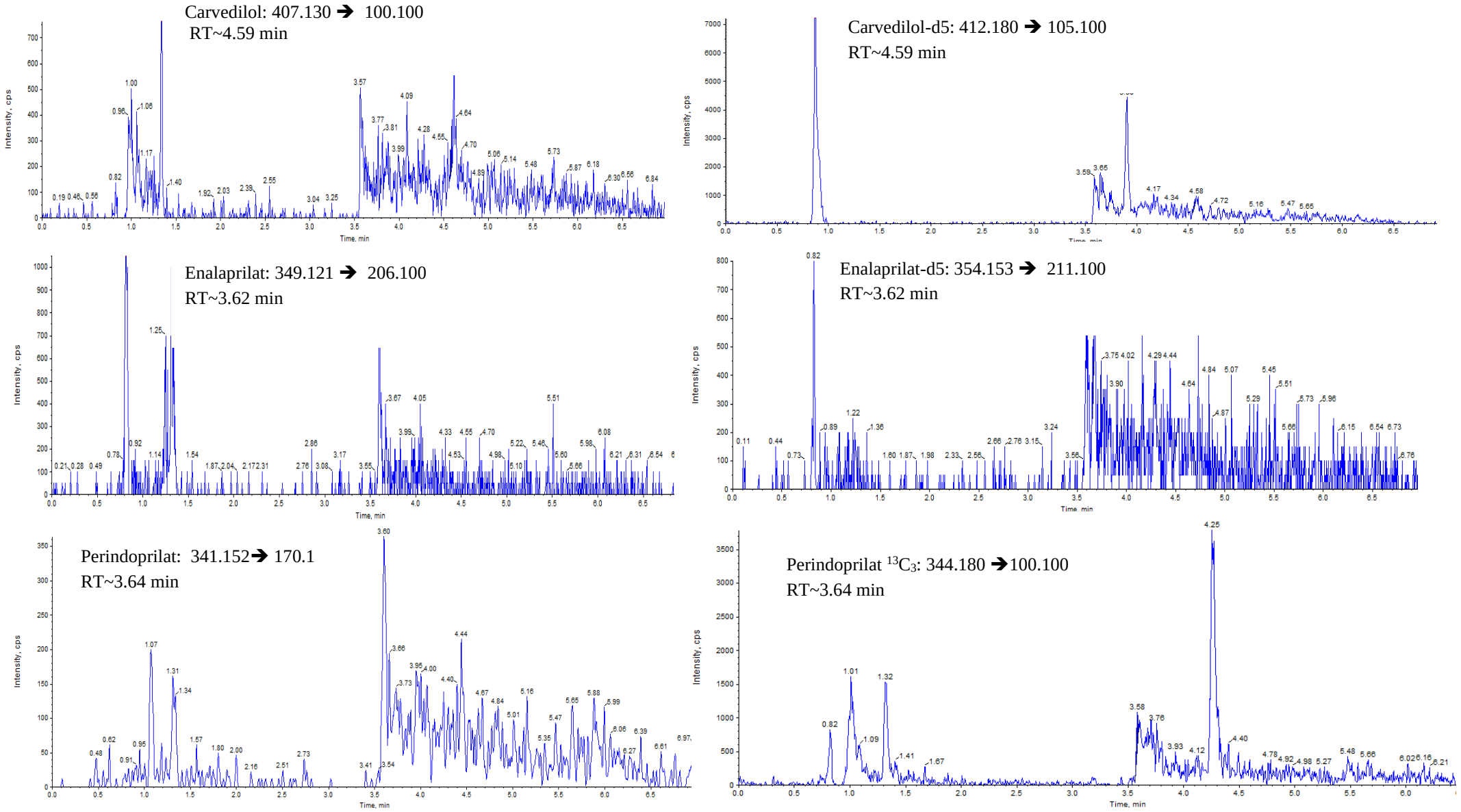


Figure 3.5 MRM chromatograms of double blank DBS samples for carvedilol, carvedilol-d5, enalaprilat, enalaprilat-d5, perindoprilat, and perindoprilat $^{13}C_3$

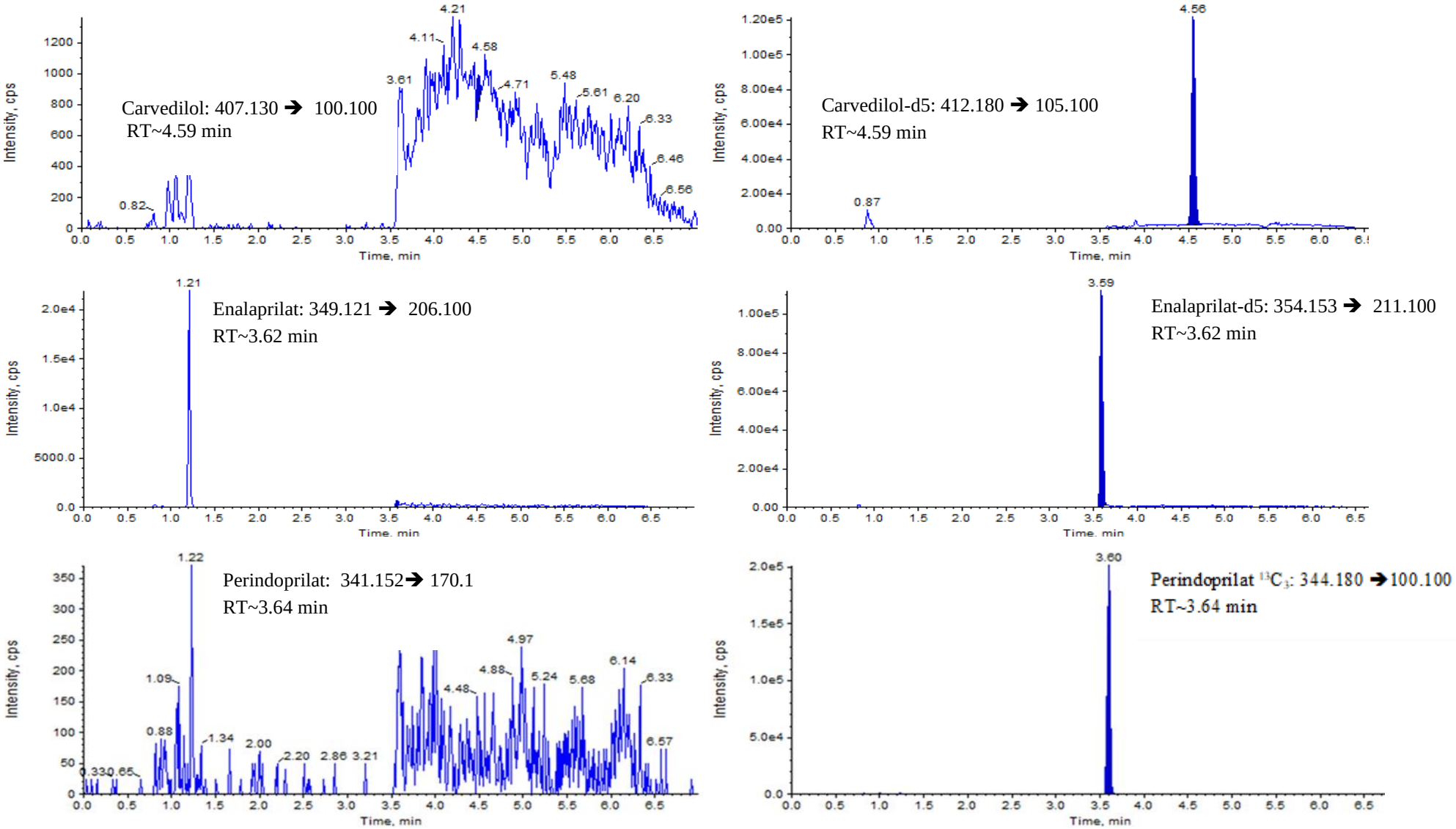


Figure 3.6 MRM chromatograms of blank DBS samples for carvedilol, carvedilol-d5, enalaprilat, enalaprilat-d5, perindoprilat, and perindoprilat $^{13}C_3$

Six different lots of DBS were spiked at the LLOQ (1.00 ng/mL) concentration and extracted to determine the S/N ratio at this level. The same six lots of matrix were also extracted as double blanks without any analyte. The raw LLOQ sample chromatograms showed acceptable intensities for the analytes with an average signal/noise ratio of 37.3, 41.2 and 48.3 for carvedilol, enalaprilat and perindoprilat, respectively, determined from LLOQ samples spiked into blank DBS obtained from six different sources. Figure 3.7 to Figure 3.9 are representative chromatograms of the three analytes at LLOQ.

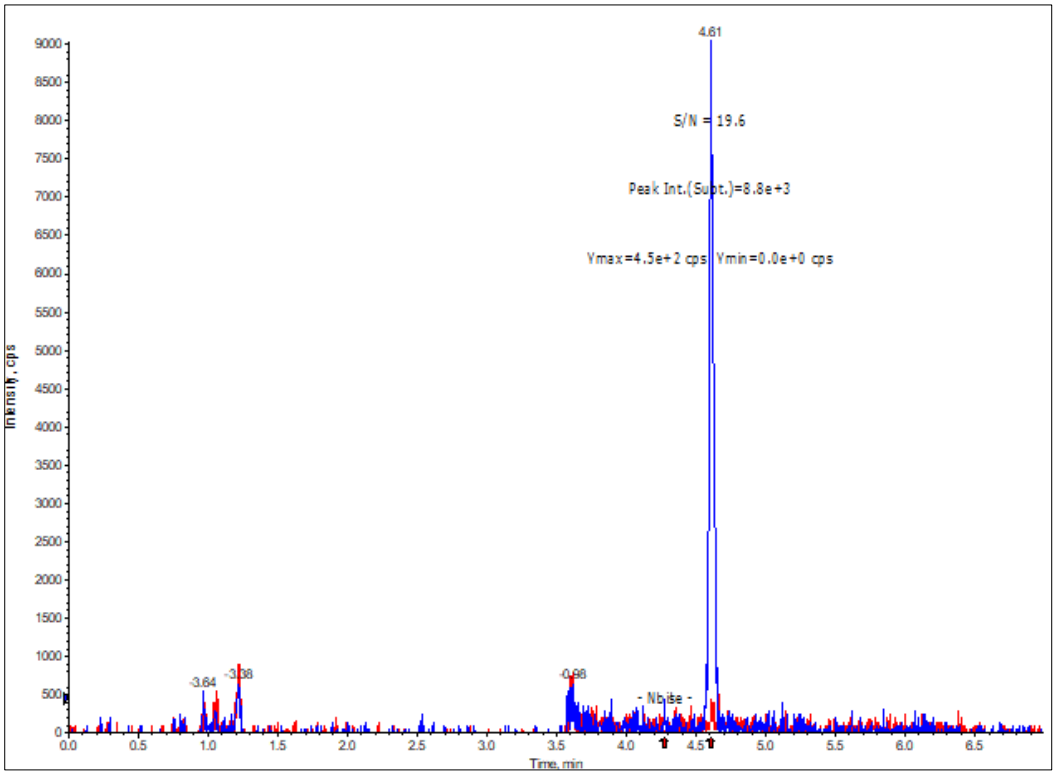


Figure 3.7 Overlay of LLOQ and blank carvedilol chromatograms. The LLOQ is shown in blue, and the blank is in red

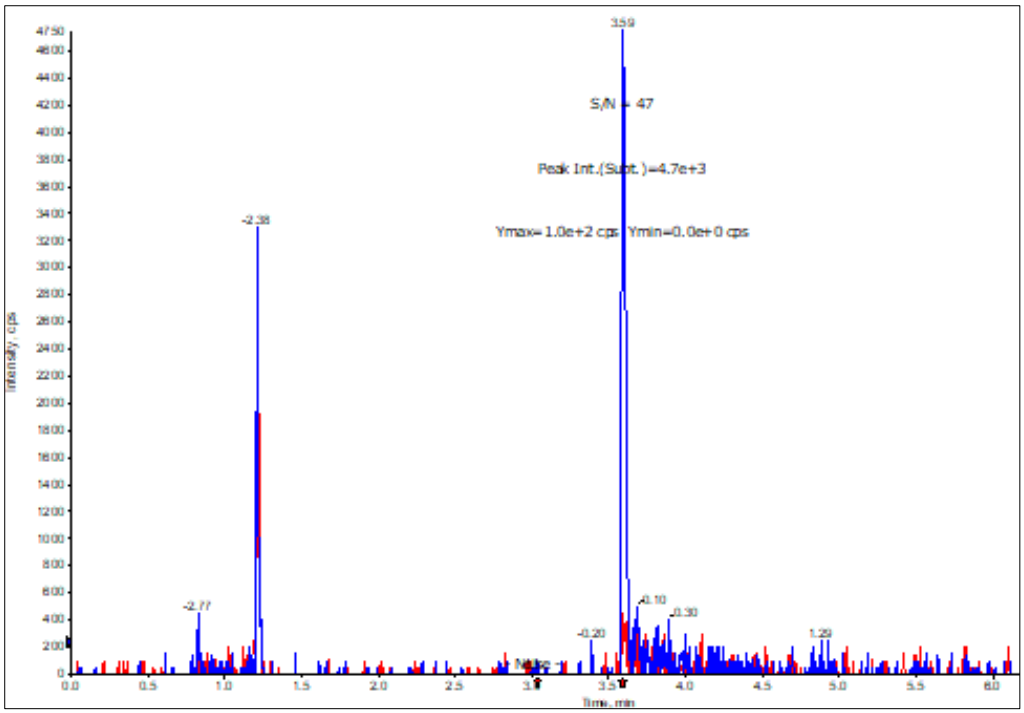


Figure 3.8 Overlay of LLOQ and blank enalaprilat chromatograms. The LLOQ is shown in blue, and the blank is in red

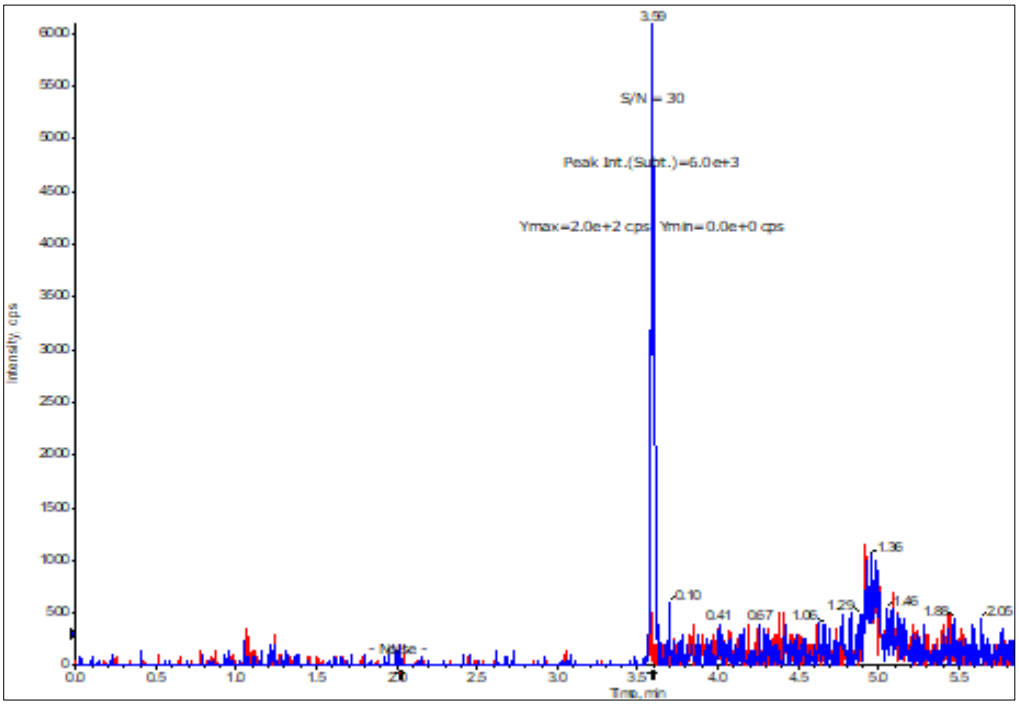


Figure 3.9 Overlay of LLOQ and blank perindoprilat chromatograms. The LLOQ is shown in blue, and the blank is in red

3.3.2.8 Reinjection reproducibility and on-instrument stability

Reinjection reproducibility was determined through the reinjection of extracted samples which had remained in the autosampler for 140 hours at ~8°C. Autosampler stability (140 hours) was assessed via the comparison of low (2.00 ng/mL) and high (160 ng/mL) QC peak area ratios (six-fold) to those obtained during the first injection sequence (Table 3.15).

Table 3.15 Summary of autosampler stability (140 hours) based on precision and %Difference

		Autosampler stability ~8°C			
		Precision CV(%)		%Difference	
Analyte	**N**	**QCH**	**QCL**	**QCH**	**QCL**
Carvedilol	6	4.8	3.5	8.3	10.9
Enalaprilat	6	4.5	11.0	11.7	9.6
Perindoprilat	6	8.5	7.2	9.9	11.6

Concentrations (ng/mL): QCH = 160, QCL= 2.00

The required criteria for 140 hours stability (CV(%) < 15% and %Difference < 15%) were fulfilled for all analytes. Samples can be reinjected within 140 hours provided they have been stored at the method-defined temperature of ~8°C.

3.3.2.9 Matrix, freeze–thaw, and bench-top stability

Long-term matrix stability was assessed by analysing high- and low-quality control DBS spots stored at -80ºC for 124 days against a freshly prepared calibration curve. Freeze–thaw stability was evaluated by subjecting low and high QCs frozen at ~-80ºC through three consecutive freeze and thaw cycles. Before the experiment, samples were frozen for 24 hours, with each cycle consisting of a four-hour thawing period at room temperature followed by ~16 hours of freezing time. Samples subjected to the freeze/thaw cycles were then analysed against a freshly prepared calibration curve. To evaluate bench-top stability of DBS samples, high- and low-quality control DBS spots were frozen at ~-80ºC, left on the bench at room temperature for 12 hours, and evaluated against a fresh calibration curve.

Carvedilol, enalaprilat and perindoprilat were stable in DBS for three freeze–thaw cycles with freeze–thaw QCs all within 10% of the nominal concentrations. Bench-top stability percentage differences were all within 11% (across both high and low concentrations) of the nominal concentrations, indicating analyte stability in DBS for ~12 hours at room temperature. Carvedilol, enalaprilat and perindoprilat when stored at ~-80°C in DBS were stable for a period of at least ~124 days (Table 3.16).

Table 3.16 Matrix stability, freeze–thaw stability and bench-top stability of carvedilol, enalaprilat and perindoprilat in DBS

			Carvedilol		Enalaprilat		Perindoprilat	
Validation experiment	**Sample tested**	**N**	**Precision CV(%)**	**Accuracy (%Nom)**	**Precision CV(%)**	**Accuracy (%Nom)**	**Precision CV(%)**	**Accuracy (%Nom)**
Matrix stability	QCL at ~-80°C for 124 days	6	2.7	99.2	5.9	104.2	3.0	95.1
	QCH at ~-80°C for 124 days	6	3.3	99.4	3.8	99.8	3.0	96.9
Freeze and thaw stability	QCL-3 F/T cycles	6	3.4	90.6	3.7	92.2	1.7	90.6
	QCH-3 F/T cycles	*5	4.4	111.0	2.5	104.0	2.4	111.6
Bench-top stability	QCL-RT for 12 hours	6	9.2	94.2	8.1	89.7	10.3	93.3
	QCH-RT for 12 hours	6	5.2	110.8	3.4	102.5	5.2	109.3

Concentrations (ng/mL): QCH = 160, QCL= 2.00
*QC removed because of bench/experimental error

3.3.2.10 Crosstalk

An investigation was carried out to evaluate potential crosstalk between MRM channels for analytes and ISTD in the DBS matrix. Analytes were evaluated separately by spiking (without ISTD) blank DBS samples at the ULOQ for each analyte. Similarly, LLOQ samples were prepared for each analyte which served as the reference to calculate percentage crosstalk. Like the plasma assay (2.3.2.12), any contribution from the pro-drugs, enalapril and perindopril, and the metabolite, perindoprilat-glucuronide, was evaluated. The interfering peak response in the blank sample must be < 20% of the response of the same analyte at LLOQ in the DBS matrix. ISTDs were evaluated separately by spiking blank DBS with individual ISTDs (carvedilol-d5, enalaprilat-d5, and perindoprilat-13C3). The mean response of the interfering peak at the retention time and mass transition of the ISTD must be < 5% of the mean response of the ISTD in the blank samples.[165]

The only crosstalk observed was in the perindoprilat channel of the perindoprilat glucuronide samples (response > 20% of LLOQ). However, as with the plasma assay, this was the in-source formation, not technical crosstalk. Separate eluting peaks allowed for acceptable integration and quantification, so rendering the contribution immaterial.(Refer to Figure 2.23 to Figure 2.25)

3.3.3 Additional experiments

3.3.3.1 Comparison of Whatman 903® protein saver cards and Capitainer®qDBS cards

Figure 3.10 show the CV(%) results of the Capitainer and Whatman cards at QM and QH. The QL results are not shown, as peak areas were either not present or highly variable for the Capitainer cards. The Capitainer cards only absorb 13.5 µL, while 50 µL was used for the Whatman cards. The lower Capitainer card volume meant that the concentration of the analytes was too low to quantify the analytes accurately at QL, with the experiment highlighting a limitation of the low absorbable volume of the Capitainer card.

The CV(%) of the Capitainer cards was higher for all analytes at all concentrations (QM and QH) barring perindoprilat at the QM concentration. The advantage of the Capitainer cards lies in patient self-sampling. However, the Whatman and Capitainer samples were prepared via pipetting. This allowed a fixed volume of 50 µL to be absorbed onto the Whatman cards, with the Capitainer cards absorbing a fixed volume of 13.5 µL. If samples were used from patients who prepared their own samples through finger pricking onto the Whatman and Capitainer cards, the CV(%) would likely be much more favourable for the Capitainer cards than the Whatman cards. This is because of the microfluidic technology used by the Capitainer cards,

which allows only a fixed volume to be absorbed.[197] The Capitainer cards evaluated were a single iteration in the product's development cycle. A newer product has subsequently been developed consisting of 2 x 10µL collection discs.[184]

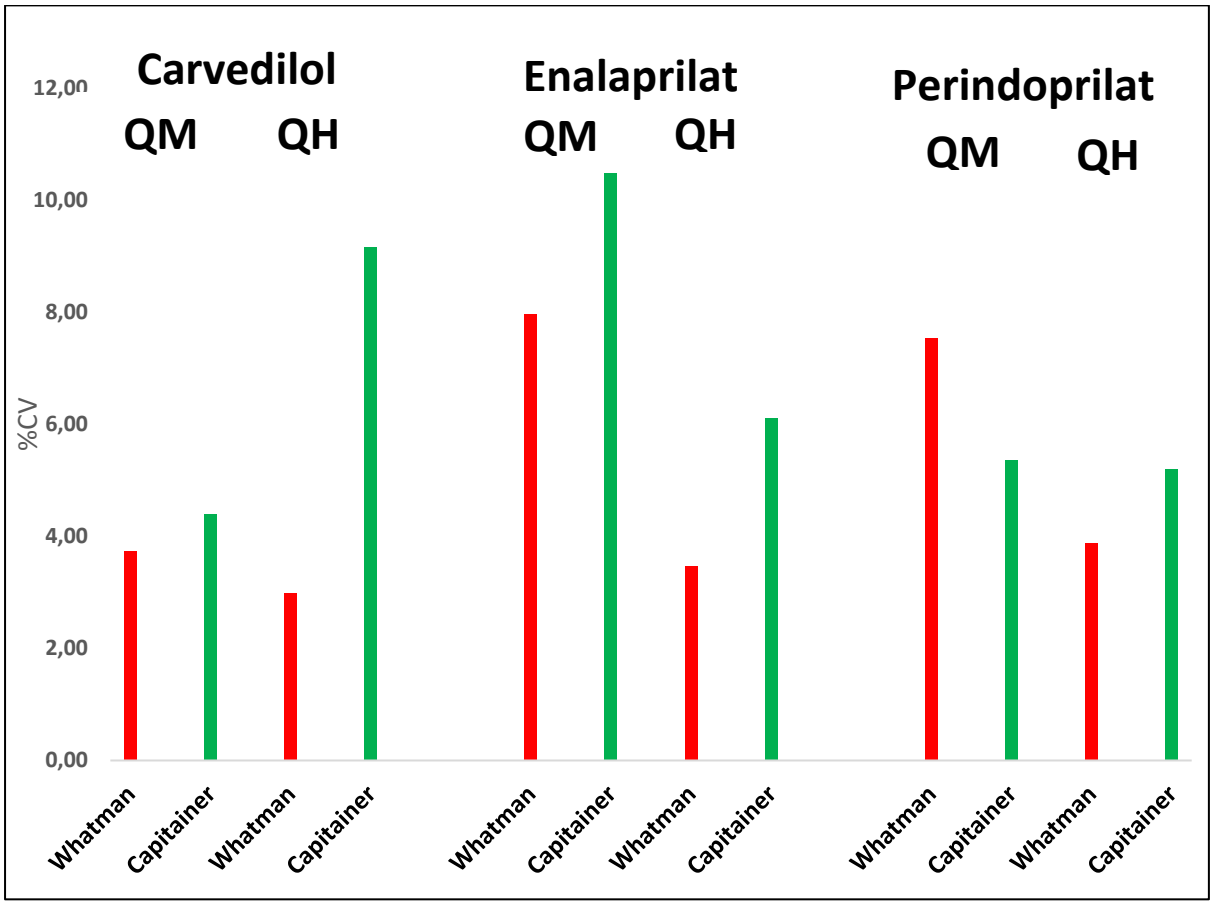


Figure 3.10 Precision comparison at QM and QH of Capitainer and Whatman cards for carvedilol, enalaprilat, and perindoprilat.

3.4 Summary and conclusions

The method development and validation of a novel multiplex LC-MS/MS assay for the quantification of carvedilol, enalaprilat and perindoprilat in DBS were presented in this chapter. Sample extraction included pre-soaking the spots with water to assist analyte diffusion and adding organic solvent (methanol:acetonitrile (1:1, v/v)). The entire DBS spot is punched for extraction to avoid the haematocrit effect. Standard and QC preparation involved spiking the working solution directly onto the DBS, so circumventing any problems that could arise from coagulation when spiking the working solution into whole blood. Most of the plasma assay's LC-MS/MS and chromatographic conditions were transferred to the DBS assay.

The method was validated over the calibration range of 1.00–200 ng/mL for all three analytes. The LLOQs of enalaprilat and perindoprilat are lower than that reported in literature (4.54

ng/mL and 5.00 ng/mL for enalaprilat and perindorpilat, respectively).[94] No other assays quantifying carvedilol in DBS have been published. The intraday and interday accuracy and precision results for both the calibration standards and QCs fell within accepted criteria demonstrating that the method was accurate and precise for the validated calibration range. Similar to the plasma assay, carryover proved problematic. This was mitigated by injecting double blank samples before each sample injection.

Whatman 903® protein saver cards (used during method validation) and Capitainer®qDBS cards were compared in terms of precision (CV(%)). The Capitainer cards are designed to absorb a fixed volume of the blood sample, removing sample variation that might occur during self-sampling. Because of the low absorbable volume of the Capitainer cards, analyte concentrations were too low for quantification at QL (2.00 ng/mL), thus highlighting a limitation of the cards. The CV(%) of the Whatman cards was overall lower than that of the Capitainer cards at QM (80.0 ng/mL) and QH (160 ng/mL), likely because of the Whatman samples being prepared via pipette; allowing exact volumes to be displaced onto the Whatman cards. In the case of patient self-sampling, the Capitainer cards will likely allow for a better CV(%) due to absorbing a fixed volume. Further experimentation with devices that absorb a metered volume of blood, such as Capitainer cards or VAMS devices, is critical if patient self-sampling is to be routinely implemented.

Although the method has been validated analytically according to the required FDA criteria,[198] its feasibility as an adherence-determining assay is uncertain because of sparse pharmacokinetic whole blood/DBS data for the analytes. Its evaluation through a pharmacokinetic study is critical for understanding its potential and further limitations. This is investigated in Chapter 4.

4 Application of Plasma and DBS Assays to a Pharmacokinetic Pilot Study

4.1 Introduction

The development and validation of plasma and DBS assays for quantifying carvedilol, enalaprilat and perindoprilat have been discussed in the previous two chapters. This chapter shows the results of the two assays applied to a pharmacokinetic pilot study.

Many pharmacokinetic studies of carvedilol, enalaprilat and perindoprilat have been done in plasma.[33,40,201–207,44,46,172,174,175,177,199,200] However, this pharmacokinetic pilot study's primary aim was not to generate novel pharmacokinetic data of carvedilol, enalaprilat and perindoprilat in plasma but to further evaluate the robustness of the analytical methods developed using patient samples. A pharmacokinetic study is a valuable way to observe the effectiveness of assays developed as it allows for a wide range of concentrations to be quantified. It was also an important objective to gauge the ability of the assays to track adherence to carvedilol, enalapril and perindopril. Because of the availability of significant pharmacokinetic data in plasma for all three analytes, the calibration range of 0.2–200 ng/mL was selected as it was expected that analytes would be quantifiable (> LLOQ) for the entire concentration range of adherent patients.[103,113–115,177,208,209] A pharmacokinetic study would help to confirm this.

No pharmacokinetic data have been published using DBS as a matrix for carvedilol, enalaprilat and perindoprilat, with only published whole blood perindoprilat pharmacokinetic data available.[93] As with the plasma assay, the primary aim of the pharmacokinetic pilot study was to evaluate the DBS assay's robustness further using patient samples and to assess its ability to act as an adherence-determining assay. Generating pharmacokinetic data was critical in further evaluating this assay's ability to determine adherence given that pharmacokinetic data are sparse.

The objectives of this chapter include:

- To generate concentration-time profiles of carvedilol, enalaprilat and perindoprilat of plasma and DBS samples from a pilot pharmacokinetic study using the assays developed in Chapters 2 and 3.
- Evaluate pharmacokinetic parameters of plasma and DBS samples using non-compartmental analysis (NCA).
- Evaluate the robustness of the assays using the pharmacokinetic profiles and evaluate the feasibility of the assays developed to determine adherence.

4.2 Methods

4.2.1 Study population

Patients admitted with the diagnosis of heart failure to Groote Schuur Hospital were screened for inclusion in the study. Patients had to be:

1. ≥ 18 years of age.
2. Clinically stable – New York Heart Association Functional Class (NYHA-FC) II-III and on background therapy with enalapril, perindopril and carvedilol.
3. At steady state on the current dose of enalapril or perindopril and carvedilol for 28 days.
4. Able to provide written informed consent.

Patients were excluded from the study if:

1. They were haemodynamically unstable, in the opinion of the investigator.
2. They had renal failure (patients on dialysis or creatinine > 220 µmol/L), nephrotic syndrome, hepatic failure or other causes of hypoalbuminemia.
3. Had anaemia (haemoglobin < 8 g/dL).
4. Pregnant or within three months postpartum.

4.2.2 Study design and sample size

The University of Cape Town, Faculty of Health Science Research Ethics Committee, approved to conduct of this study (HREC/REF: 480/2018). The investigation started with ten participants. Four withdrew during the study. During the study's first phase, participants were on combined enalapril and carvedilol treatment. Study participants were on 20 mg/day or 10 mg/day enalapril and on 25 mg/day or 50 mg/day carvedilol. Both enalapril and carvedilol were taken twice daily. Blood samples were obtained through venepuncture once steady-state concentrations were attained. Sampling was performed pre-dose and at 1.5, 3, 5, 8, and 12 hours after the dose was administered. After the first sampling set was concluded, participants were switched to perindopril instead of enalapril. Participants on 20 mg/day enalapril were switched to 8 mg/day perindopril, and participants on 10 mg/day enalapril were switched to 4 mg/day perindopril. The second set of samples was taken four weeks after changing the medication. Samples were taken pre-dose and at 1.5, 3, 5, 8, 12, and 24 hours after the dose was administered.[28,187]

The numbers of paired DBS and plasma samples were 78, 35 and 42 for carvedilol, enalaprilat and perindoprilat, respectively. Carvedilol had a total of 78 paired samples, as it was present in patients for both sampling occasions. Six samples were taken for each patient for the first sampling occasion, while seven were taken for the second. Six samples were taken for the first occasion, as enalapril is taken twice a day and samples were taken up to 12 hours post-dose to cover the half-day period. For the second occasion, seven samples were taken to cover a 24-hour sampling period, as perindopril is taken once a day. Therefore, carvedilol had 36 paired samples for the first occasion and 42 paired samples for the second occasion. Enalaprilat had 36 paired samples, but one DBS sample was not viable for analysis, resulting in 35 paired plasma and DBS samples. Perindoprilat had its complete set of 42 paired samples.

4.2.3 Pharmacokinetic analysis

The pharmacokinetic parameters evaluated for the DBS and plasma matrices were the area under the concentration-time curve from zero to t (AUC_{0-t}), the area under the concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), peak concentration (C_{max}), the time to C_{max} (T_{max}), lowest concentration of medication before next dose (C_{trough}), elimination rate constant (K_{el}), apparent volume of distribution (Vd/F), apparent clearance (CL/F), and elimination half-life ($t_{1/2}$). These parameters were calculated using noncompartmental analysis with Pkanalix version 2019R1 (Lixsoft, Anthony, France).

4.3 Results and discussion

4.3.1 Patient demographics and baseline characteristics

The baseline characteristics of the participants are listed in Table 4.1. The average age and body mass index (BMI) of the six participants (three male, three female) was 41.0 ± 6.3 years and 35.2 ± 7.6 kg/m^2 respectively. Five of the six participants were obese (BMI ≥ 30 kg/m^2).[210,211] The NYHA-FC of the participants ranged from I–III, with most patients having a NYHA-FC of II. Five participants were on the 10 mg bidaily enalapril dose regimen, and one on the 5 mg bidaily dose regimen. When patients were switched to perindopril, five participants were on the 8 mg/day regimen, with one on the 4 mg/day regimen.[187]

Table 4.1 Summary of patient demographics, baseline characteristics and dosages.[28,187]

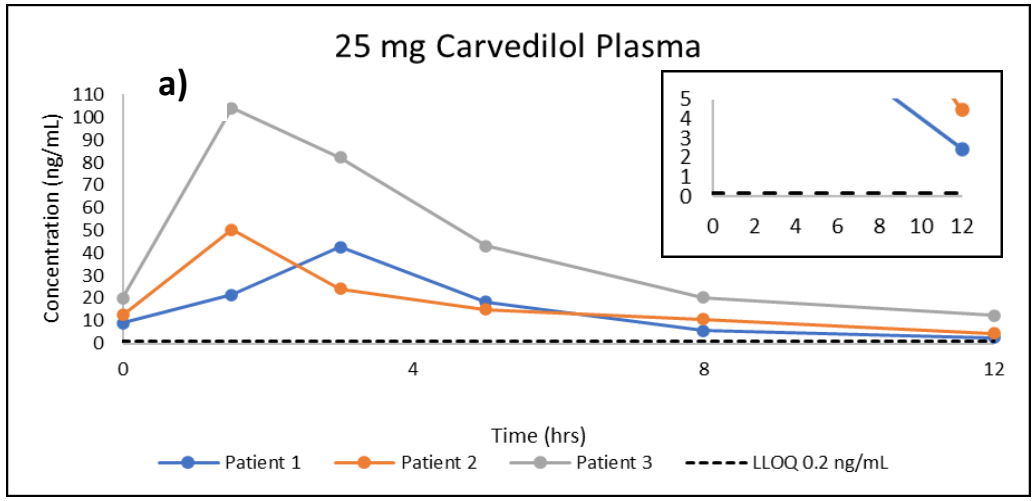
Patient	Sex	Age (Years)	BMI (kg/m^2)	NYHA FC	Carvedilol dose (mg)	Enalapril dose (mg)	Perindopril dose (mg)
1	F	30	41.4	1	25 BD	10 BD	8 OD
2	F	49	33.8	2	25 BD	5 BD	4 OD
3	F	40	37.3	2	12.5 BD	10 BD	8 OD
4	M	37	45.5	2	25 BD	10 BD	8 OD
5	M	47	21.6	2	12.5 BD	10 BD	8 OD
6	M	43	31.4	3	12.5 BD	10 BD	8 OD

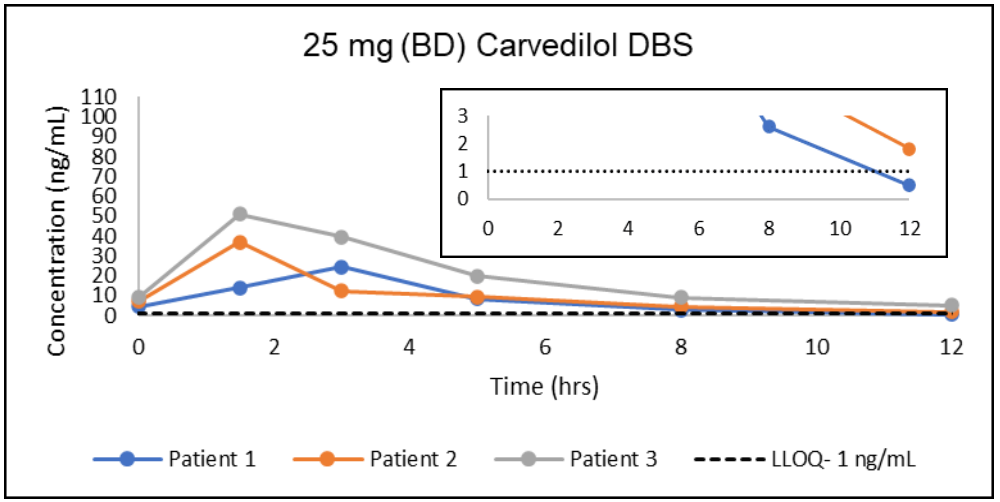
BMI – Body mass index, NYHA FC – New York Heart Association Functional Classification, BD – Bidaily, OD – Once daily

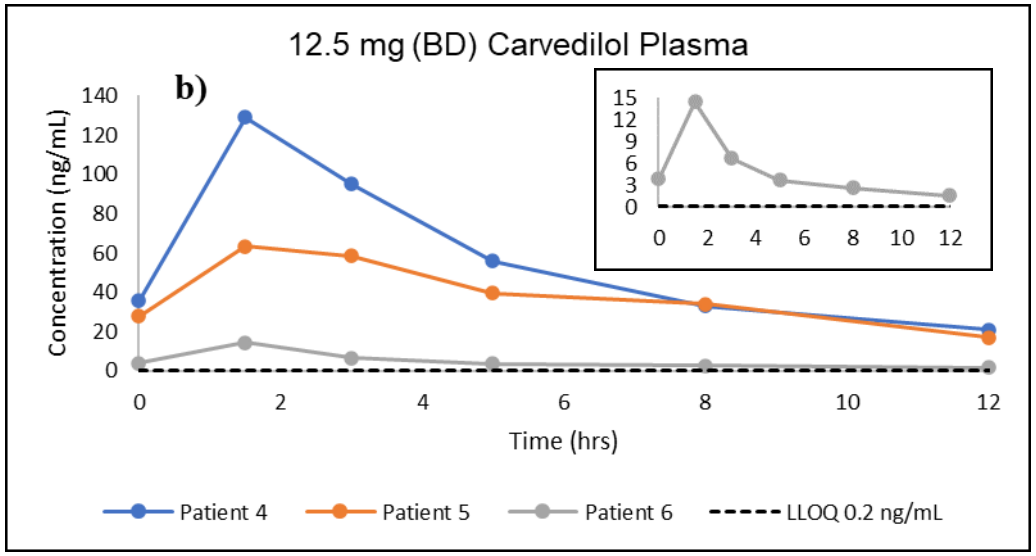
Note: For the first phase of the study, carvedilol and enalapril were taken in combination at the dosages shown in the table. During the second phase, carvedilol and perindopril were taken in combination at the dosages shown in the table.[187]

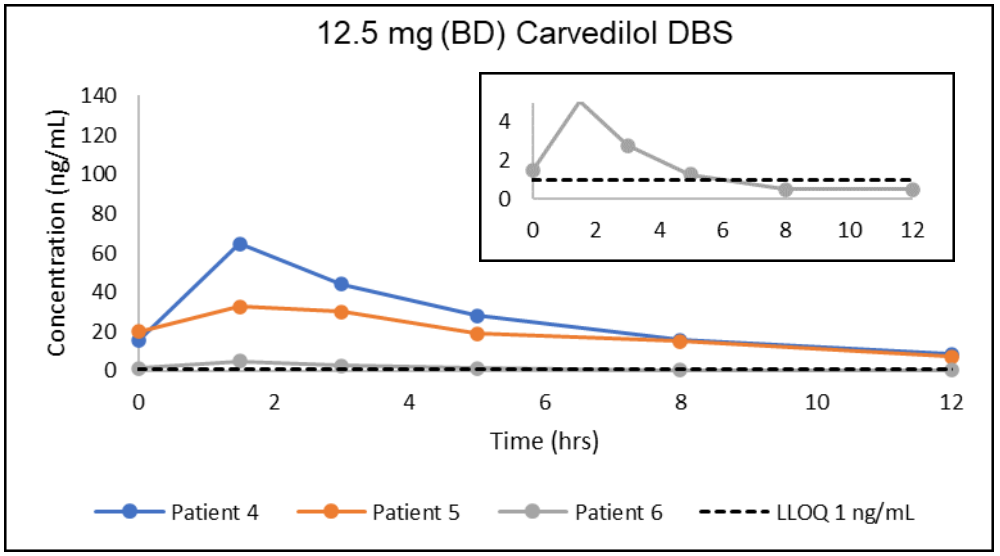
4.3.2 Pharmacokinetic plots and pharmacokinetic parameters

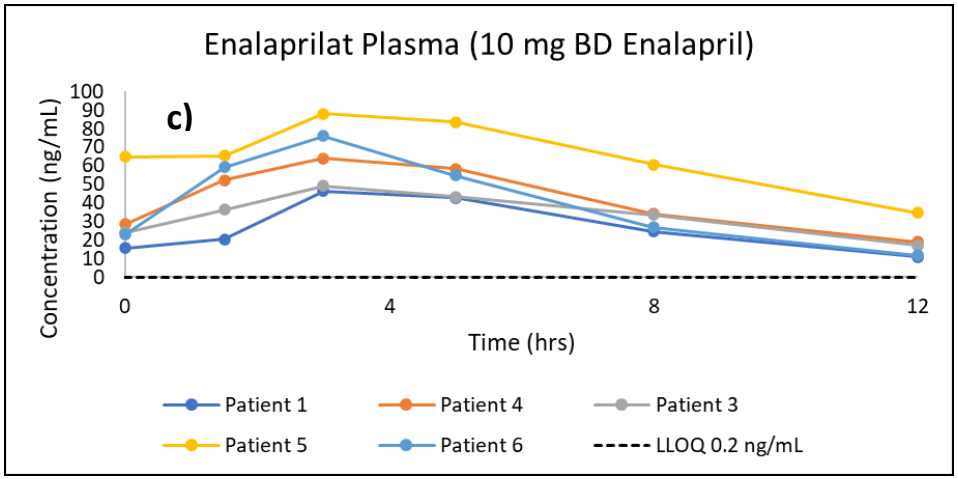
Figure 4.1 shows the individual plasma and DBS concentrations versus time profiles for the six patients. The same sample volume was used during both plasma and DBS extractions (50 µL plasma and 50 µL whole blood), and therefore normalisation to equivalent volumes was unnecessary. Table 4.2 and Table 4.3 are a summary of the plasma and DBS pharmacokinetic parameters, respectively. The C_{trough} concentrations for the plasma and DBS samples are summarised in Table 4.4 and Table 4.5, respectively. The semi-logarithmic profiles of the paired DBS and plasma concentrations (geometric mean) versus time profiles are shown in Figure 4.2.

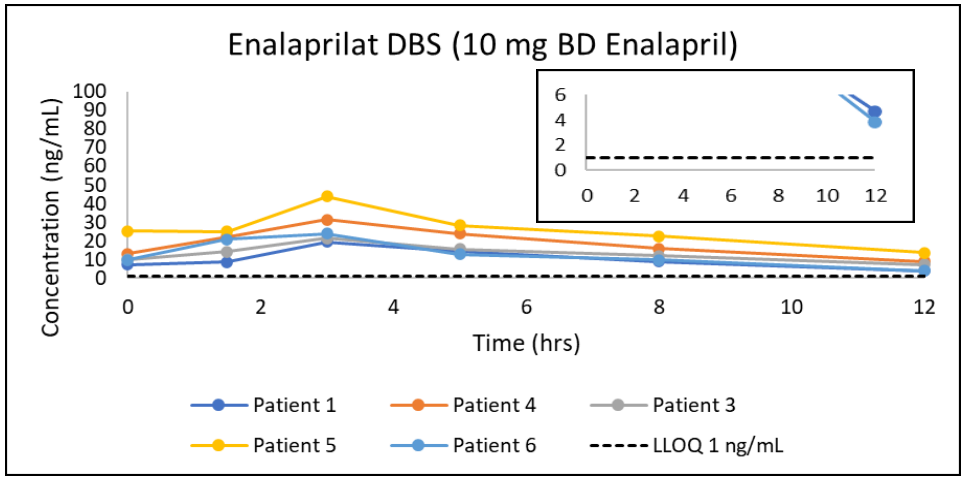

25 mg Carvedilol Plasma
a)
Concentration (ng/mL)
Time (hrs)
Patient 1
Patient 2
Patient 3
LLOQ 0.2 ng/mL

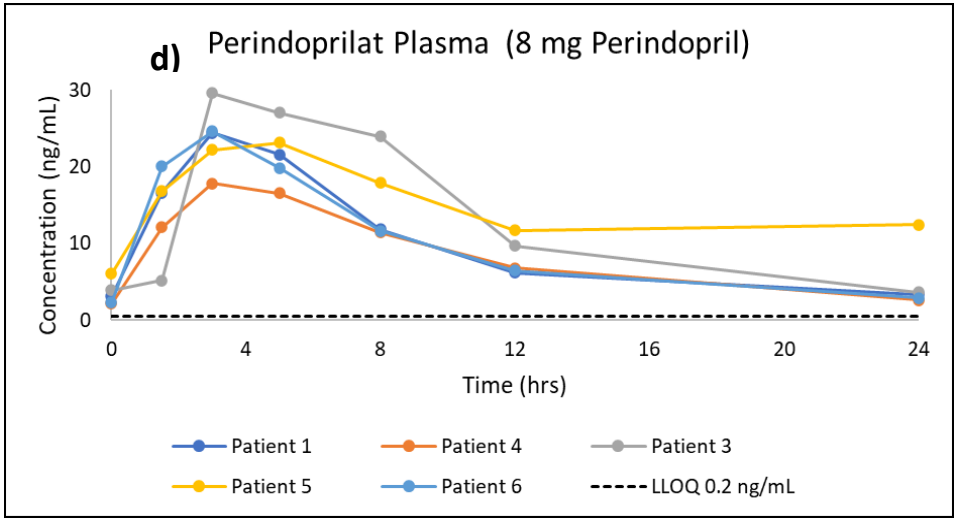

25 mg (BD) Carvedilol DBS
Concentration (ng/mL)
Time (hrs)
Patient 1
Patient 2
Patient 3
LLOQ- 1 ng/mL

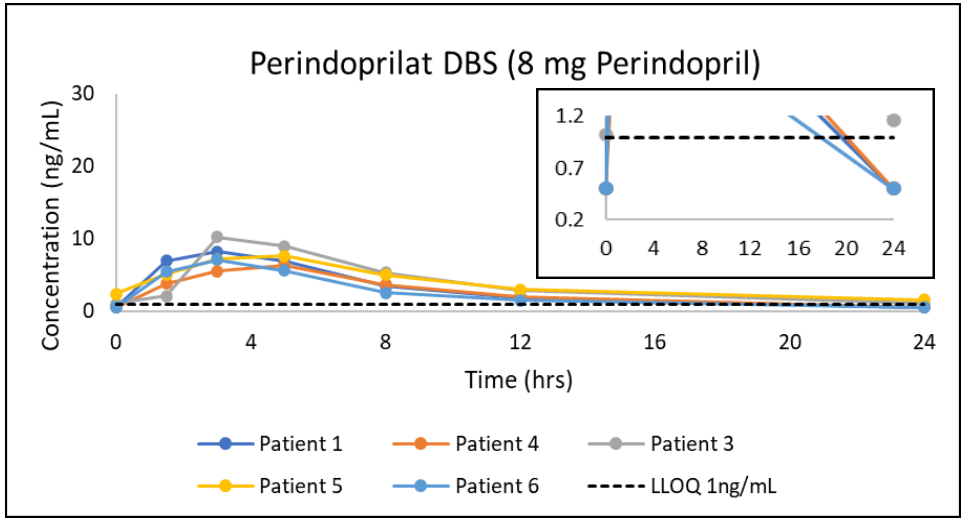

12.5 mg (BD) Carvedilol Plasma
b)
Concentration (ng/mL)
Time (hrs)
Patient 4
Patient 5
Patient 6
LLOQ 0.2 ng/mL

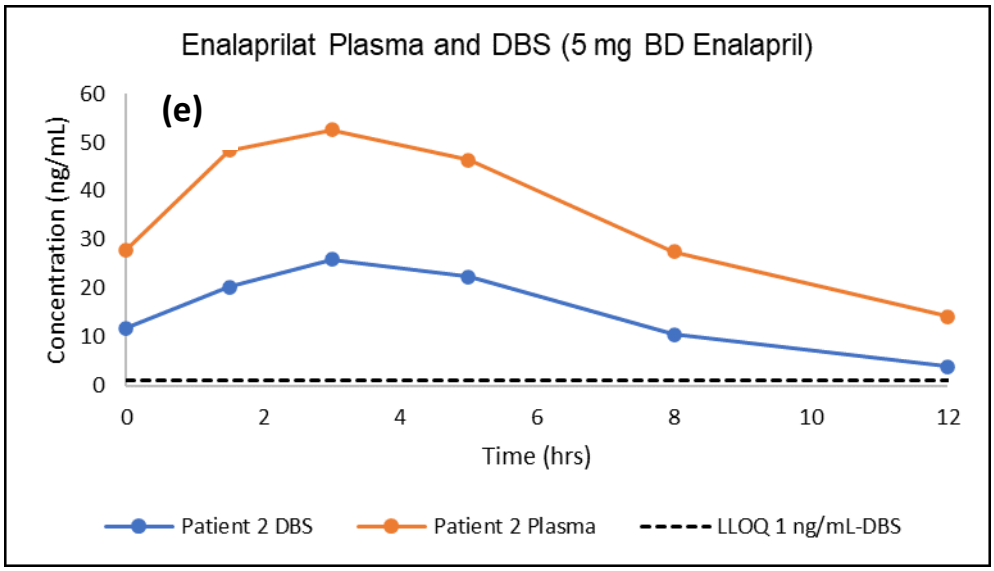

12.5 mg (BD) Carvedilol DBS
Concentration (ng/mL)
Time (hrs)
Patient 4
Patient 5
Patient 6
LLOQ 1 ng/mL


Enalaprilat Plasma (10 mg BD Enalapril)
c)
Concentration (ng/mL)
Time (hrs)
Patient 1
Patient 4
Patient 3
Patient 5
Patient 6
LLOQ 0.2 ng/mL


Enalaprilat DBS (10 mg BD Enalapril)
Concentration (ng/mL)
Time (hrs)
Patient 1
Patient 4
Patient 3
Patient 5
Patient 6
LLOQ 1 ng/mL


d)
Perindoprilat Plasma (8 mg Perindopril)
Concentration (ng/mL)
Time (hrs)
Patient 1
Patient 4
Patient 3
Patient 5
Patient 6
LLOQ 0.2 ng/mL


Perindoprilat DBS (8 mg Perindopril)
Concentration (ng/mL)
Time (hrs)
Patient 1
Patient 4
Patient 3
Patient 5
Patient 6
LLOQ 1ng/mL



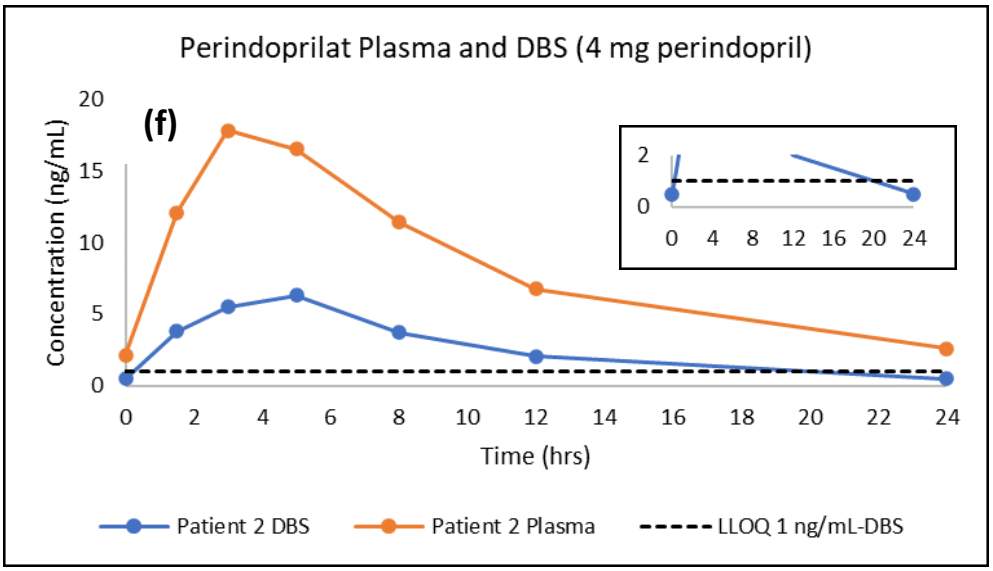


Figure 4.1 Individual profiles of (a) Carvedilol plasma (left) and DBS (right) samples (25 mg BD), (b) Carvedilol plasma (left) and DBS (right) samples (12.5 mg BD), (c) Enalaprilat plasma (left) and DBS (right) samples (10 mg Enalapril BD), (d) Perindoprilat plasma (left) and DBS (right) samples (8 mg perindopril BD), € Enalaprilat plasma and DBS plasma samples (5 mg BD enalapril), and (f) Perindoprilat plasma and DBS samples (4 mg perindopril).

Table 4.2. Summary of plasma pharmacokinetic parameters

	Carvedilol		**Enalaprilat**		**Perindoprilat**	
	N = 3	**N = 3**	**N = 5**	**N = 1**	**N = 5**	**N = 1**
	25 mg BD	**12.5 mg BD**	**10 mg BD**	**5 mg BD**	**8 mg**	**4 mg**
C_{max} (ng/ml)	65.7 ± 27.3	68.9 ± 46.9	64.8 ± 15.8	52.6	24.1 ± 4.19	11.4
AUC_{0-t} (h.ng/ml)	296 ± 150	399 ± 257	505 ± 152	421	238 ± 36.9	172
AUC_{0-inf} (h.ng/ml)	327 ± 170	499 ± 317	631 ± 227	505	271 ± 39.0	255
Tmax (h)	2.00 ± 0.707	1.5 ± 0.00	3.00	3.00	3.00 ± 0.00	5.00
Kel (1/h)	0.224 ± 0.0434	0.130 ± 0.00655	0.167 ± 0.0351	0.169	0.0947 ± 0.00933	0.0507
$T_{1/2}$ (h)	3.20 ± 0.573	5.36 ± 0.262	4.34 ± 0.881	4.1	7.39 ± 0.709	13.67
Vd/F (L)	471 ± 185	711 ± 764	130 ± 31.7	70.3	368 ± 72.7	459
CL/F (ml/min)	1740 ± 664	1469 ± 1543	357 ± 94.4	98.1	571 ± 66.67	388

C_{max} (ng/mL) – Peak concentration
AUC_{0-t} (h.ng/ml) – Area under the concentration-time curve from zero to t; for carvedilol/enalaprilat t = 12 hr and perindoprilat t = 24 hr
AUC_{0-inf} (h.ng/ml) – AUC_{0-t} extrapolated to infinity
T_{max} (h) – The time to peak concentration
Kel (1/h) – Elimination rate constant
$T_{1/2}$ (h) – Half-life
Vd/F (L) – Apparent volume of distribution
CL/F (ml/min) – Apparent clearance

Table 4.3. Summary of DBS pharmacokinetic parameters

	Carvedilol		**Enalaprilat**		**Perindoprilat**	
	N = 3	**N = 3**	**N = 5**	**N =1**	**N = 5**	**N = 1**
	25 mg BD	**12.5 mg BD**	**10 mg BD**	**5 mg BD**	**8 mg**	**4 mg**
C_{max} (ng/ml)	37.4 ± 10.8	34.2 ± 24.4	28.0 ± 8.93	26.0	7.9 ± 1.31	5.09
AUC_{0-t} (h.ng/ml)	150 ± 63.2	190 ± 127	195 ± 66.0	186	74.8 ± 15.7	60.2
AUC_{0-inf} (h.ng/ml)	161 ± 72.2	223 ± 149	255 ± 103	215	84.9 ± 22.4	86.2
Tmax (h)	2.00 ± 0.707	1.50 ± 0.00	3.00 ± 0.00	3.00	3.80 ± 0.980	5.00
Kel (1/h)	0.287 ± 0.0820	0.17 ± 0.0159	0.143 ± 0.0320	0.195	0.0998 ± 0.0205	0.0497
$T_{1/2}$ (h)	2.59 ± 0.615	4.11 ± 0.388	5.12 ± 1.16	3.55	7.29 ± 1.72	13.9
Vd/F (L)	674 ± 170	1350 ± 1493	402 ± 99.5	138	1137 ± 194	1340
CL/F (ml/min)	3233 ± 1134	4175 ± 4800	949 ± 280	448	1850 ± 378	1106

C_{max} (ng/mL) – Peak concentration
AUC_{0-t} (h.ng/ml) – Area under the concentration-time curve from zero to t; for carvedilol/enalaprilat t = 12 hr and perindoprilat t = 24 hr
AUC_{0-inf} (h.ng/ml) – AUC_{0-t} extrapolated to infinity
T_{max}(h) – The time to peak concentration
Kel (1/h) – Elimination rate constant
$T_{1/2}$ (h) – Half-life
Vd/F (L) – Apparent volume of distribution
CL/F (ml/min) – Apparent clearance

Table 4.4 C_{trough} plasma concentrations (ng/mL) observed for carvedilol, enalaprilat, and perindoprilat at different dosages of carvedilol, enalapril and perindopril, respectively.

Carvedilol C_{trough} (ng/mL)		**Enalaprilat C_{trough} (ng/mL)**		**Perindoprilat C_{trough} (ng/mL)**	
25 mg	**12.5 mg**	**20 mg**	**10 mg**	**8 mg**	**4 mg**
2.46	21.1	11.0	14.2	3.37	2.59
4.47	17.1	19.0		2.59	
12.3	1.59	17.1		3.63	
		34.6		12.4	
		11.8		2.84	

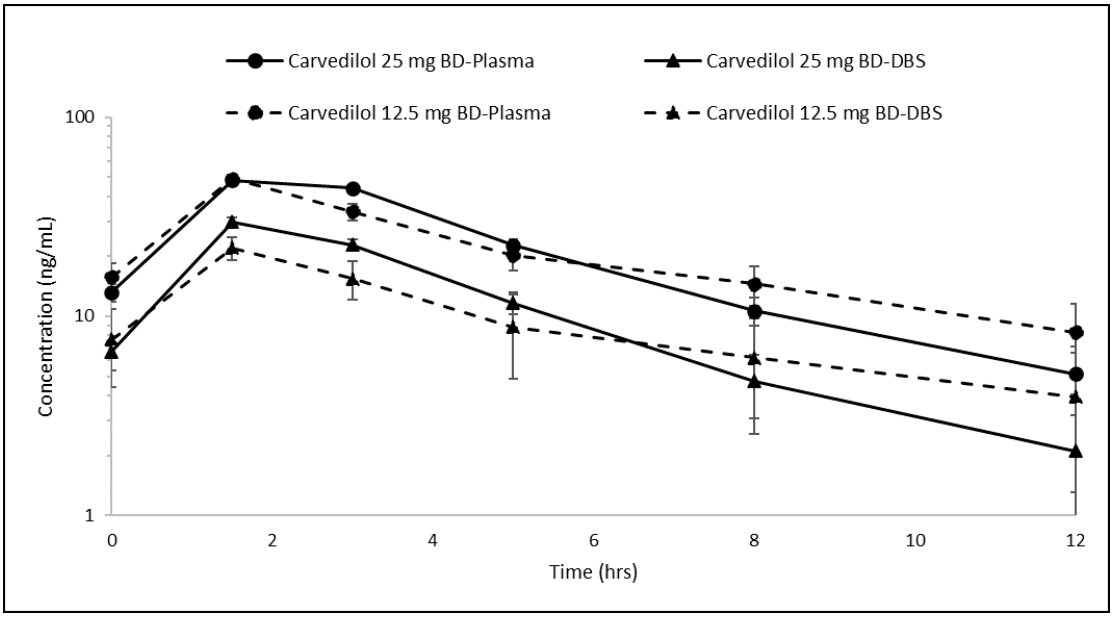
* LLOQ of plasma assay is 0.2 ng/mL

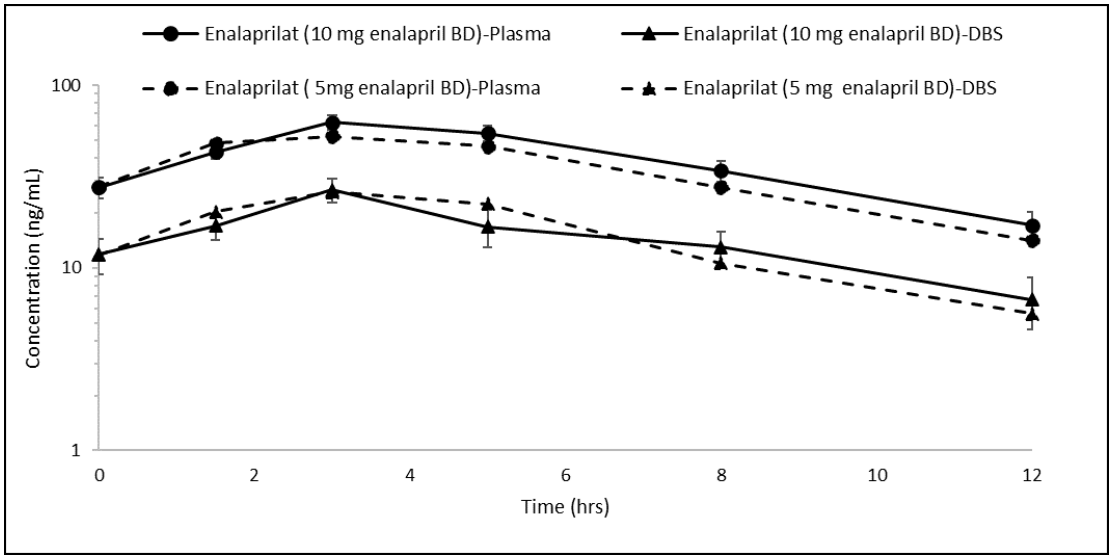
Table 4.5 C_{trough} DBS concentrations (ng/mL) observed for carvedilol, enalaprilat, and perindoprilat at different dosages of carvedilol, enalapril and perindopril, respectively.

Carvedilol C_{trough} (ng/mL)		**Enalaprilat C_{trough} (ng/mL)**		**Perindoprilat C_{trough} (ng/mL)**	
25 mg	**12.5 mg**	**20 mg**	**10 mg**	**8 mg**	**4 mg**
0.500	8.60	3.99	5.63	0.500	0.500
1.82	7.15	8.98		0.500	
5.14	0.500	7.28		1.16	
		13.6		1.61	
		3.87		0.500	

*LLOQ of DBS assay is 1.00 ng/mL

Note: All C_{trough} concentrations < LLOQ are represented as LLOQ/2 (0.500 ng/mL)





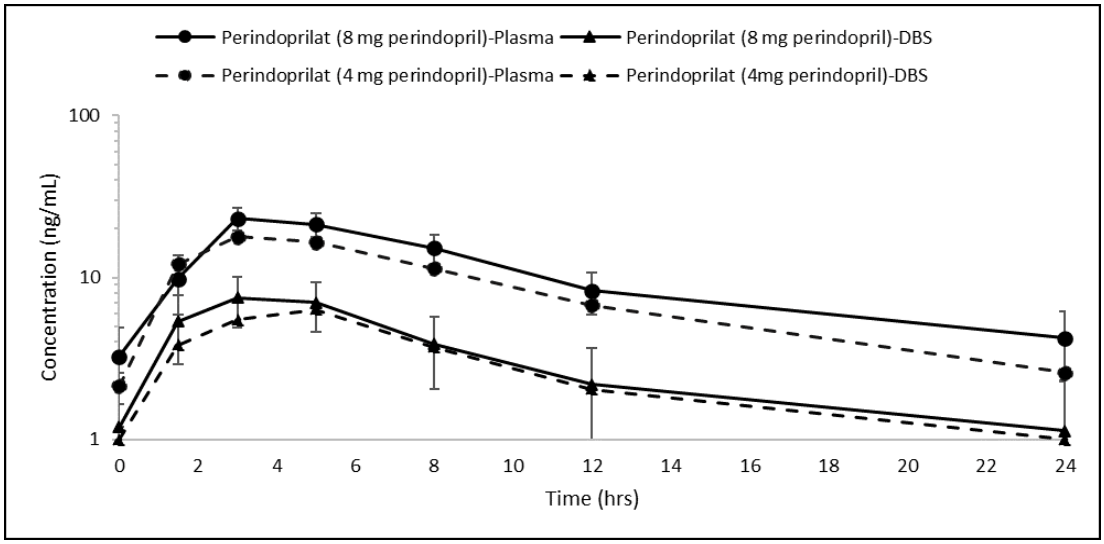


Figure 4.2 Semi-logarithmic profiles of the geometric mean of (a) 25 mg BD carvedilol plasma and DBS samples (N = 3) and 12.5 mg BD carvedilol plasma and DBS samples (N = 3), (b) enalaprilat

(10 mg BD enalapril) plasma and DBS samples (N = 5) and enalaprilat (5 mg BD enalapril) plasma and DBS samples (N = 1) and (c) perindoprilat (8 mg perindopril) plasma and DBS samples (N = 5) and perindoprilat (4 mg perindopril) plasma and DBS samples (N = 1) (error bars represent geometric standard deviation). For plotting purposes, DBS concentrations quantified below LLOQ were set equal to LLOQ.

Figure 4.1 shows that the sensitivity of the DBS assay is limited compared to the plasma assay, as some of the participant sample concentrations were below the DBS assay's LLOQ (1.00 ng/mL). None of the plasma sample concentrations were below the LLOQ of 0.2 ng/mL. The lowest plasma concentrations observed were 1.3 ng/mL, 11.0 ng/mL and 2.13 ng/mL for carvedilol, enalaprilat and perindoprilat, respectively. Of the plasma samples, carvedilol plasma concentrations were closest to the LLOQ, but the lowest concentration was still more than six times greater than the LLOQ. For the DBS samples, eight of the carvedilol and six of the perindoprilat samples were below the LLOQ. The lower DBS concentrations result from how the analytes are distributed in whole blood, with a sequestration of the analytes in the plasma fraction rather than the RBC.[92,93] In terms of pharmacokinetic evaluation for these analytes, plasma should be the preferred matrix. The preferred matrix choice for pharmacokinetic evaluation must be tied to the blood-to-plasma ratio.[163] If the analyte is more concentrated in the plasma fraction, plasma should be the preferred matrix of choice. If the analyte is more concentrated in RBC, whole blood should ideally be used. Either whole blood or plasma can be used if the analyte is evenly separated in concentration between RBC and plasma. However, plasma is still preferred if this is the case.[163] This is further elucidated by Figure 4.3.

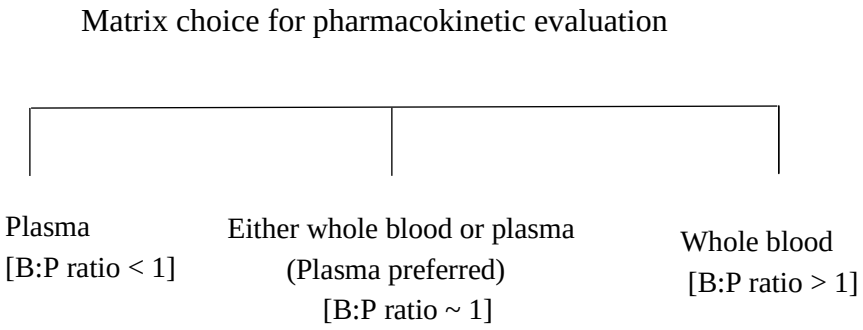


Figure 4.3 Matrix selection for analyte pharmacokinetic evaluation based on the blood-to-plasma ratio of analyte concentration. Adapted from Dash et al.[163]

Table 4.2 and Table 4.3 show the plasma and DBS pharmacokinetic parameters, respectively. The carvedilol T_{max} for both the plasma and DBS samples is similar to that observed for previous pharmacokinetic studies done in plasma.[101,212] The observed C_{max} plasma

concentrations of carvedilol were 65.7 ± 27.3 and 68.9 ± 46.9 ng/mL for the 25 mg and 12.5 mg dosages, respectively. Literature C_{max} concentrations are higher for the 25 mg dose of carvedilol and similar for the 12.5 mg dose of this study.[212] The C_{max} concentrations observed in DBS were 37.4 ± 10.8 and 34.2 ± 24.2 ng/mL for the 25 mg and 12.5 mg dosages, respectively. The $T_{1/2}$ values of the 25 mg carvedilol dosage in both plasma and DBS samples were slightly lower than that in the literature for plasma studies, while $T_{1/2}$ values of the 12.5 mg dose for both plasma and DBS agree with published values.[200] The plasma CL/F was 1740 ± 664 mL/min and 1469 ± 1543 mL/min for the 25 mg and 12.5 mg carvedilol dose, respectively, with the small sample size likely contributing to the high variability.[213,214] The Vd/F was 471 ± 185 L and 711 ± 764 L for the 25 mg and 12.5 mg doses, respectively, with high variability also observed. Reported carvedilol plasma CL and Vd in the literature are 500–700 mL/min and 115 L, respectively.[215] The carvedilol CL/F and Vd/F calculated for the DBS pharmacokinetic samples were greater than calculated plasma values due to the sequestration of carvedilol into the plasma fraction rather than the RBC.[216]

Enalaprilat plasma and DBS C_{max} concentrations were obtained three hours after oral administration of enalapril, corresponding with literature plasma studies.[33,40] Five participants were on the 10 mg bidaily enalapril dose regimen, and one was on the 5 mg bidaily dose regimen. The C_{max} enalaprilat plasma and DBS concentrations observed for the 10 mg bidaily group were 64.8 ± 15.8 ng/mL and 28 ± 8.93 ng/mL, respectively. The 5 mg bidaily dose participant had C_{max} concentrations of 52.6 and 26.0 ng/mL for their plasma and DBS enalaprilat samples, respectively. Steady-state plasma concentrations for enalaprilat at the indicated enalapril dosages and dosage times could not be found for comparison in the literature. No pharmacokinetic studies using DBS/whole blood could be found for enalaprilat. Enalaprilat elimination is biphasic, with the initial elimination half-life being 2–6 hours, after which follows a prolonged terminal half-life (>30 hours).[200] The half-lives observed for the DBS and plasma samples fall within the former range, with the bulk of pharmacokinetic sampling done within the initial elimination phase.[33] The enalaprilat plasma CL/F was calculated to be 357 ± 94.4 mL/min and 98.1 mL/min for the 10 mg BD and 5 mg BD dose, respectively. Enalaprilat clearance reported in the literature is 158–173 mL/min.[40,217] Calculated plasma Vd/F was 130 ± 31.7 and 70.3 for the 10 mg BD and 5 mg BD dose, respectively. As with carvedilol, DBS CL/F and Vd/F were calculated to be greater compared to plasma due to lower whole blood concentration of enalaprilat.

For perindopril, five participants were on the 8 mg/day dose, with one on the 4 mg/day dose. C_{max} perindoprilat concentrations in DBS and plasma were observed ~3-5 hours after the oral administration of perindopril, which is in line with published data on plasma T_{max} values.[40] For the 8 mg dose, C_{max} concentrations observed were 24.1 ± 4.19 ng/mL and 7.9 ± 15.7 ng/mL for the plasma and DBS, respectively. The 4 mg bidaily dose participant had C_{max} concentrations of 11.4 and 5.09 ng/mL for plasma and DBS, respectively. Both doses' observed plasma C_{max} concentrations agree with a previous plasma pharmacokinetic study.[177] Perindoprilat also has a biphasic elimination profile, with the free fraction being excreted rapidly, followed by a prolonged terminal half-life (30–120 hours) of the fraction bound to the ACE.[169] The DBS and plasma half-lives ranged from 7 to 14 hours, indicating that the bulk of the pharmacokinetic sampling was still within the initial phase of the biphasic elimination process. Perindoprilat plasma CL/F was calculated to be 571 ± 66.67 mL/min and 388 mL/min for the 8 mg and 4 mg dose, respectively. The plasma Vd/F was calculated to be 368 ± 72.7 L and 459 L for the 8 mg and 4 mg dose, respectively. A study by Parker et al.[218] found the Vd/F of perindoprilat to be dose-dependent. The Vd/F values in the study ranged from 59 L (after multiple 8 mg perindopril doses) to 480 L (after multiple 4 mg perindopril doses). The apparent unbound clearance (CLu/F) was independent of dose and ranged from 984 to 1833 mL/min. As with the previous analytes, the CL/F and Vd/F calculated for the DBS pharmacokinetic samples were greater than calculated plasma due to higher concentrations in the plasma fraction rather than the RBC.[216]

Figure 4.2 which shows the geometric mean's semi-logarithmic profiles, does not show a linear increase in concentration with the dose, as one would expect.[177,209] The small patient sample size is potentially a reason for this. The profiles should therefore not be viewed as the complete pharmacokinetic picture of carvedilol, enalaprilat and perindoprilat, but are still a useful guide to assess the feasibility of using the assays for adherence-determination. This is especially true for the DBS assay, as very limited pharmacokinetic data are available for carvedilol, enalaprilat and perindoprilat in the literature for DBS/whole blood. Moreover, obesity affects the pharmacokinetics of medications.[219] There is a difference in the distribution of adipose weight in obese patients compared to patients of normal weight. A person of normal weight (BMI 25 kg/m^2) typically has 80% lean tissue and 20% adipose tissue. An obese individual's total body weight (BMI 30 kg/m^2) consists of 60% lean and 40% adipose tissue.[220] Lipophilic drugs, such as carvedilol, distribute more readily into adipose tissue.[220] This increases Vd of the drug and reduces plasma concentration. In obese patients, drug clearance can also be altered because of

a change in some physiological processes. Drug clearance is less dependent on the drug's physiochemical properties when compared to Vd and is mainly influenced by physiology.[219,221] The kidneys (renal clearance) and liver (hepatic clearance) are the organs primarily involved in the clearance of drugs. Fat accumulation in the liver of obese patients may alter liver blood flow, which could affect hepatic drug clearance. The processes involved in drug elimination through the kidneys include glomerular filtration, tubular secretion and tubular reabsorption. An increase in absolute glomerular filtration rate (GFR) seen in obese patients could influence the efficacy of the drug due to increased clearance.[222] Obesity can also influence the t½ of patients. Clearance and Vd are parameters that are biologically independent, with Vd mainly influenced by the physiochemical properties of a drug and clearance by the physiology of the liver and kidneys, as mentioned before. Both Vd and clearance influence the t½. As a result, a change in t½ could be a result of a change in CL, Vd, or both parameters.[219] Drug absorption may be affected by obesity as obesity influences gastric emptying and gut permeability.[223]

4.3.3 Feasibility of using assays for determining adherence

Carvedilol, enalaprilat and perindoprilat were quantifiable for all the patient samples in the pharmacokinetic study. This indicates that the assay's LLOQ (0.2 ng/mL) is sufficient to quantify the analytes for a period after ingestion equivalent to at least the dose interval of carvedilol, enalapril and perindopril. This interval is 12 hours for carvedilol and enalapril and 24 hours for perindopril and is the sampling interval for the first and second phases of the pharmacokinetic study, respectively. Plasma samples below LLOQ would strongly indicate that, at the very least, the patient had missed their last dose. None of the patient C_{trough} concentrations were below the LLOQ (Table 4.4). The lowest plasma concentrations observed were 1.3 ng/mL, 11.0 ng/mL and 2.13 ng/mL for carvedilol, enalaprilat and perindoprilat, respectively, indicating the analytes would also be quantifiable past the dosing interval. The plasma assay can therefore be used to evaluate adherence to carvedilol, enalapril and perindopril.

The limitation of the DBS assay due to the sequestration of the analytes in plasma rather than RBC is compounded by a restriction in the DBS analytical method: the higher DBS assay LLOQ relative to the plasma assay. The plasma assay is five times more sensitive compared to the DBS assay (1.00 ng/mL for DBS vs 0.2 ng/mL for plasma). The pharmacokinetic profiles have shown that concentrations of less than 1.00 ng/mL of adherent patients can be expected, with some of the C_{trough} concentrations observed being less than 1.00 ng/mL (Table 4.5). If a DBS concentration of less than 1.00 ng/mL is observed for either carvedilol or perindoprilat,

one cannot comment on the patient's adherence with any certainty as the assay cannot quantify the analytes for a period after ingestion equivalent to the dosing interval of carvedilol and perindopril. On the other hand, if a plasma concentration of below 0.2 ng/mL is observed for either carvedilol or perindoprilat, the certainty that a patient has not been adherent is much greater. The pharmacokinetics also played a role in the DBS assay's inability to quantify the entire concentration range of carvedilol and perindoprilat in the pharmacokinetic study. The pharmacokinetics of carvedilol, in particular, are limiting as the drug is present in the matrix for a shorter time compared to enalaprilat and perindoprilat. Carvedilol does not have a long terminal half-life like enalaprilat or perindoprilat, with its half-life being 7–10 hours.[224] The DBS assay is suited as an initial screening measure for carvedilol and perindoprilat but not as an adherence measure.

In contrast to carvedilol and perindoprilat, the lowest enalaprilat concentration observed in the DBS pharmacokinetic samples was 3.87 ng/mL, almost four times that of the LLOQ of 1.00 ng/mL. The assay can therefore quantify enalaprilat for enalapril's dosing interval (12 hours) and beyond. The DBS assay is therefore suitable as an adherence-determining assay for enalapril. Enalaprilat's long terminal half-life results in elevated concentrations for extended periods. Perindoprilat also has a long terminal half-life. However, the difference in the dosing protocol between enalapril and perindopril likely played a role. Perindoprilat was sampled for 24 hours compared to enalaprilat's 12 hours as perindopril is taken daily and enalapril bidaily. The extended sampling period of perindoprilat would have given the analyte an additional 12-hour window to fall below the LLOQ before sampling.

4.4 Summary and conclusions

The pharmacokinetic profiles indicate a greater distribution of the analytes in the plasma fraction than the RBC fraction for all three analytes. This corresponds with published data for whole blood and plasma matrices for these analytes.[92,93] The greater concentration of the analytes in the plasma fraction makes the plasma the preferable matrix if a pharmacokinetic evaluation is the primary goal.[163] The analytes' affinity for the plasma fraction also makes the plasma assay's LLOQ of 0.2 ng/mL sufficient to track patient adherence to carvedilol, enalapril and perindopril. No plasma samples fell below the LLOQ for any pharmacokinetic samples, indicating that carvedilol, enalaprilat and perindoprilat can be quantified for at least the dose interval of carvedilol (12 hours), enalapril (12 hours) and perindopril (24 hours), respectively. The long terminal half-lives of enalaprilat and perindoprilat make the analytes quantifiable for

an extensive period past the dosing interval, making the plasma assay especially well-suited for those analytes as an adherence measure.

The lower concentration of the analytes in the DBS, coupled with the higher LLOQ of the DBS method (1.00 ng/mL for DBS vs 0.2 ng/mL for plasma), places the DBS assay at a disadvantage compared to the plasma assay in terms of its adherence-tracking ability. This is particularly true for carvedilol and perindoprilat. Even though the LLOQs of the DBS assay developed are lower than other published methods, some samples for carvedilol and perindoprilat still fell below the LLOQ.[94] Therefore, the assay cannot quantify carvedilol and perindoprilat for a period after ingestion equal to the dosing interval of carvedilol (12 hours) and perindopril (24 hours). The DBS assay is suited for screening purposes for carvedilol and perindoprilat instead of as an adherence-determining assay. Although enalaprilat concentrations were lower than those in the DBS matrix compared to plasma, no enalaprilat DBS samples fell below the LLOQ. The assay can therefore quantify enalaprilat for the 12-hour dose period of enalapril. Furthermore, the lowest concentration observed for the enalaprilat DBS pharmacokinetic data was almost four times that of the LLOQ, indicating that the assay can quantify enalaprilat for an extensive period after the last ingestion of enalapril, well past the 12-hour dosing period. The DBS assay is therefore suited to track adherence to enalapril.

The pharmacokinetic data do have some limitations. There were only six participating patients, five of whom had obesity. However, the study gives valuable insight into the feasibility of using the assays in tracking adherence. This is especially so for the DBS assay as there is very limited whole blood/DBS pharmacokinetic data available for carvedilol, enalaprilat and perindoprilat. Chapter 5 evaluates the correlation and agreement of the pharmacokinetic plasma and DBS samples.

5 Evaluation of Correlation and Agreement Between Plasma and DBS Samples

5.1 Introduction

Both the DBS and plasma assays' capability to discern adherence was evaluated by applying the assays to a pharmacokinetic pilot study. Assays can evaluate adherence if they can quantify analytes for a period after medication ingestion equal to or greater than the dosing interval. The dose intervals are 12 hours for carvedilol and enalapril and 24 hours for perindopril. The plasma assay has clear advantages over the DBS assay as an adherence-determining assay. DBS concentrations of the analytes are inherently lower than plasma concentrations, a disadvantage which is compounded by the higher LLOQ of the DBS assay. The plasma assay can be used to determine adherence to carvedilol, enalapril and perindopril. The DBS assay is only suitable as a screening measure for carvedilol and perindoprilat. It is still useful, however, as an adherence-determining measure for enalapril. However, the higher LLOQ of the DBS assay means that the timeframe as an adherence measure is shorter than the plasma assay.

To further evaluate the DBS assay's robustness and whether it can be implemented into routine clinical practice, it is essential to cross-validate the plasma and DBS methods.[225] Furthermore, as a quantitative adherence-determining assay, the DBS concentrations are more useful when converted to plasma concentrations. Most pharmacokinetic data for carvedilol, enalaprilat and perindoprilat is from plasma. Plasma concentrations are therefore simpler to interpret or evaluate in terms of adherence, especially when adherence interpretations become weight- or dose-specific. Using the plasma pharmacokinetic data available, UCT's pharmacometrics research group was able to develop an adherence model tracking adherence to enalapril using enalaprilat concentrations as a proxy for adherence.[226] The model takes a patient's weight and dose into consideration for adherence evaluation. If DBS concentrations and plasma concentrations correlate well, DBS concentrations can be converted to plasma concentrations and interpreted using the model developed.

The objectives addressed in this chapter include:

- Further evaluate the robustness of the assays by cross-validation; evaluate the correlation and agreement between the DBS and plasma pharmacokinetic samples.
- Evaluate the feasibility of using DBS and plasma concentrations interchangeably.
- Evaluate what the limitations are when converting from DBS concentrations to plasma concentrations.

5.2 Methods

5.2.1 Samples excluded

Samples below LLOQ and outliers were excluded from further analysis.[94] Box and whisker plots of paired plasma and DBS concentration ratios were used to determine outliers. Ratios smaller than 1.5 times the interquartile range of ratios or larger than 1.5 times the interquartile range of the ratios were considered outliers[227].

5.2.2 Evaluation of Correlation

Deming regression was used to evaluate the relationship between plasma and DBS concentrations.[228] The CV(%) of the inter-assay variation generated during validation was inputted into the Deming regression as the measurement error for each assay. The regression slope was calculated with standard error (SE) and a 95% confidence interval (95% CI). The degree of correlation between plasma and DBS concentrations was expressed using the Pearson correlation coefficient (r). Medcalc® statistical software version 20.013 (MedCalc Software, Ostend, Belgium) provided statistical analysis.

Equation 5.1 predicted plasma concentrations from the observed DBS concentrations. The equation was obtained from the observed plasma and observed DBS concentrations' Deming regression.[48,149]

$$\text{PredPlasma} = mC_{DBS}+b \qquad \textbf{5.1}$$

The predicted plasma and observed DBS concentrations are represented by PredPlasma and C_{DBS}, respectively. The constant and proportional bias is represented by b and m, respectively, which was generated by each analyte's Deming regression.[151]

5.2.3 Evaluation of agreement

Bland–Altman plots expressed the extent of agreement between calculated and observed plasma concentrations. The plots were generated using Medcalc® statistical software version 20.013 (Ostend, Belgium). Agreement acceptance criteria were based on the guideline given by the EMA; the difference between the observed and calculated plasma concentrations should be less than 20% of their mean for at least 67% of the samples.[229] Figure 5.1 gives a workflow of the exclusion, correlation and evaluation of agreement between paired samples.

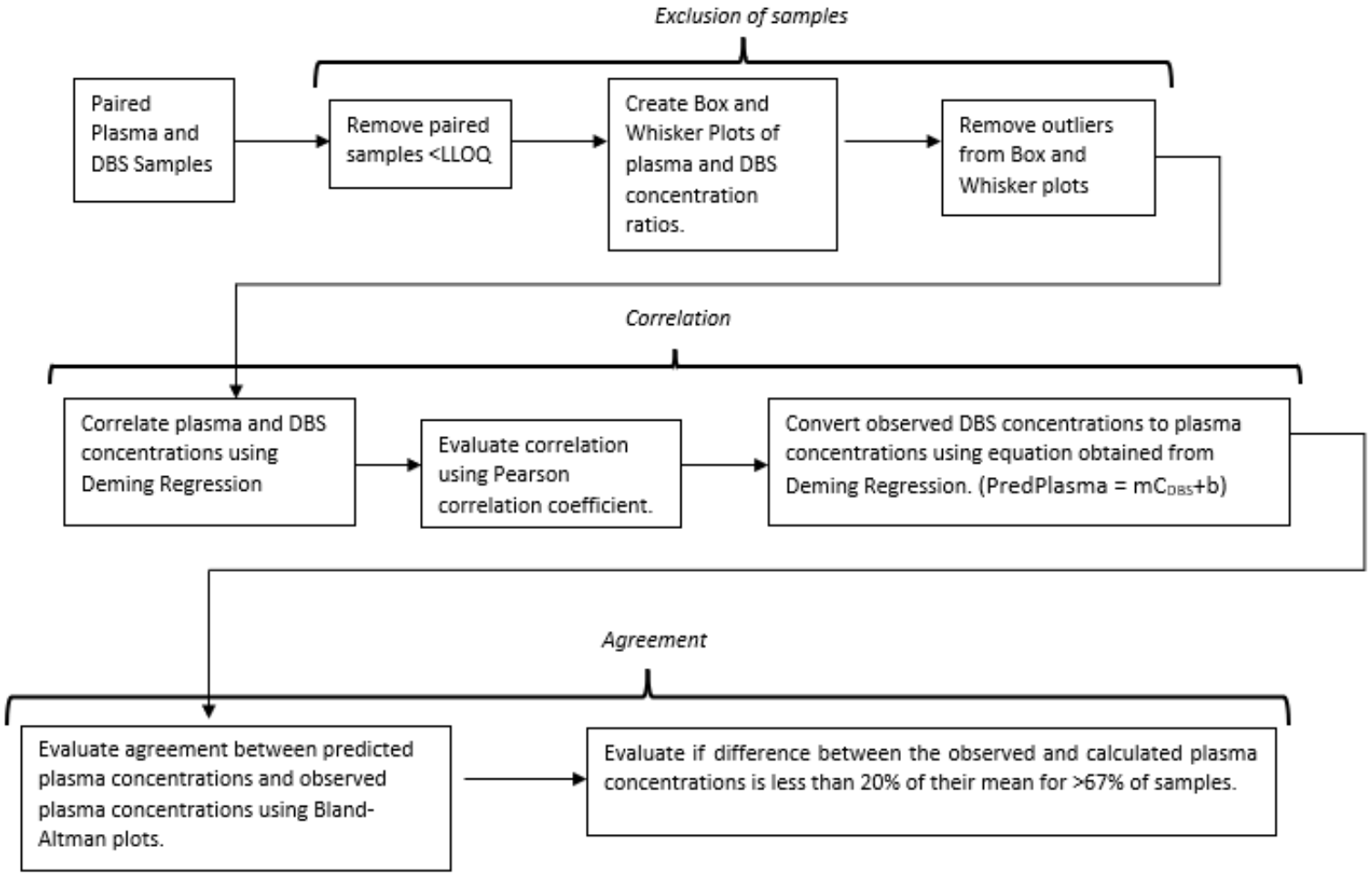


Figure 5.1 Workflow for exclusion, correlation and evaluation of agreement between paired samples

5.3 Results and discussion

5.3.1 Box and whisker plots of concentration ratios

The paired DBS and plasma concentration ratios are shown in the box and whisker plots of Figure 5.2. Before further analysis, there were three, one and four outliers excluded for carvedilol, enalaprilat and perindoprilat, respectively.

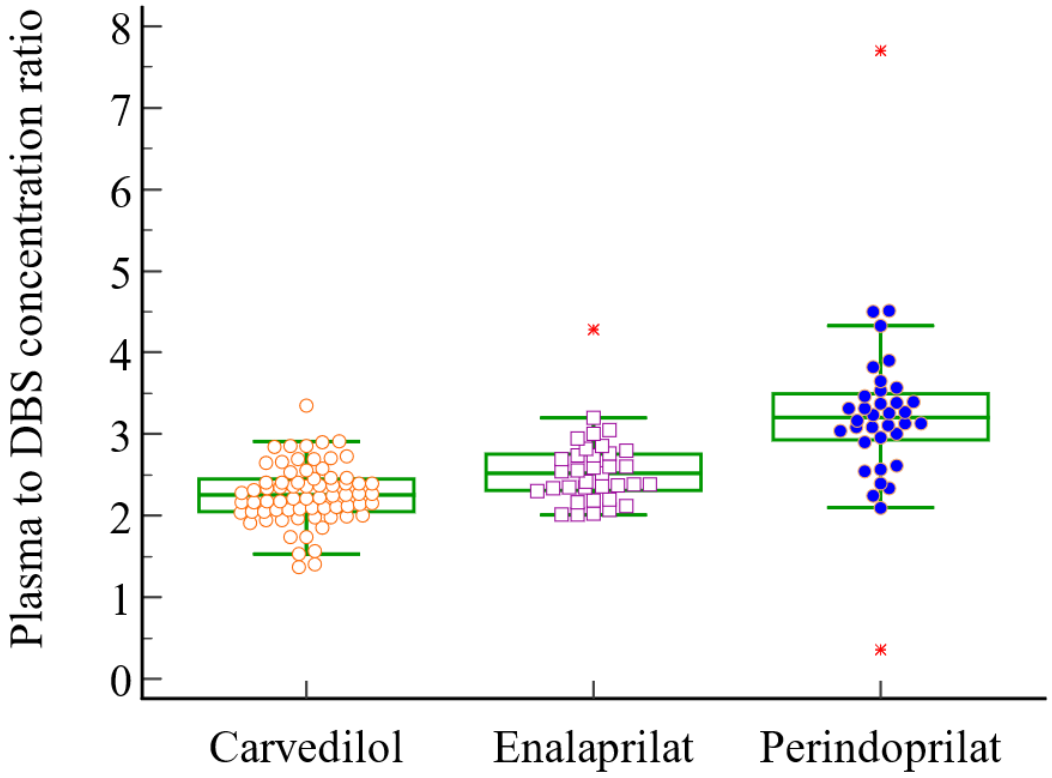


Figure 5.2 Box and whisker plots of plasma to DBS concentration ratios for carvedilol, enalaprilat, and perindoprilat. Outliers are outside the whiskers of the plot. Extreme outliers are designated with the red stars.

5.3.2 Correlation of paired plasma and DBS samples

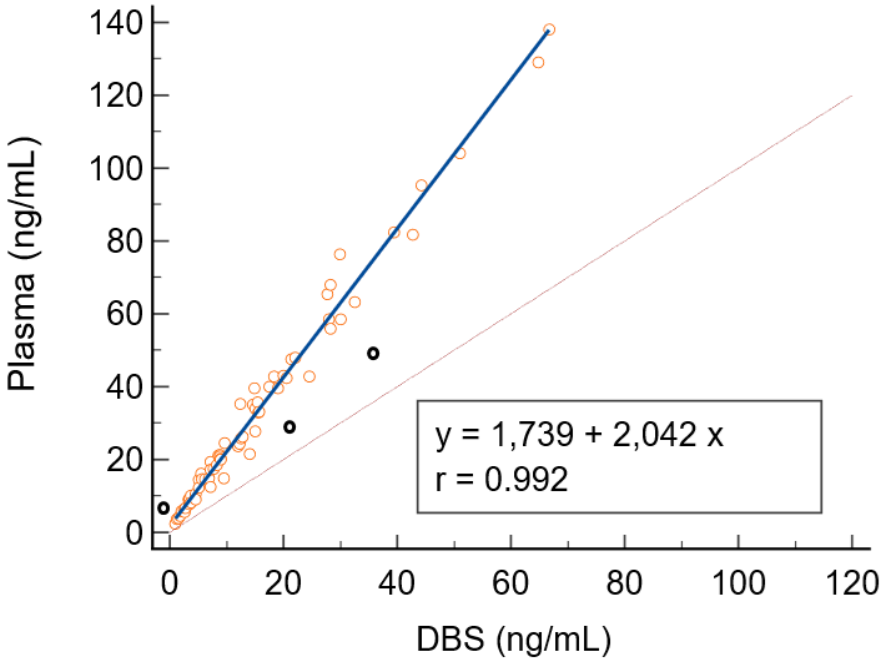
Table 5.1 summarises the Deming regression parameters for carvedilol, enalaprilat and perindoprilat. For all analytes, more than 30 paired samples were used for correlation, which is the minimum number required by the EMA.[229] Figure 5.3 shows the Deming regressions for the three analytes, with the Pearson correlation coefficient (r) >0.94 for all analytes indicating good correlation.

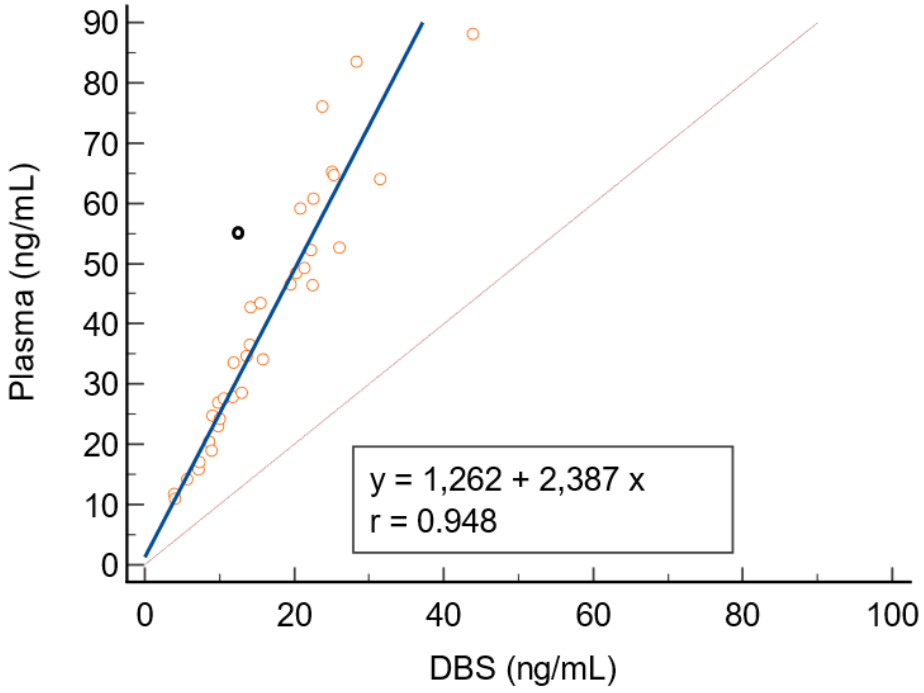
When converting from DBS to plasma concentrations, the limiting assay is the DBS assay, as it has a higher LLOQ than the plasma assay. DBS concentrations < 1.00 ng/mL cannot be converted to plasma concentrations even though the LLOQ of the plasma assay is 0.2 ng/mL. The lowest plasma concentrations that can be derived from DBS samples can be calculated by substituting the DBS LLOQ (1.00 ng/mL) into the Deming regression equation. The lowest calculable plasma concentrations are 3.78, 3.65 and 3.28 ng/mL for carvedilol, enalaprilat and perindoprilat, respectively (Table 5.2). The lowest plasma concentrations observed from the pharmacokinetic pilot study were 1.3 ng/mL and 2.12 ng/mL for carvedilol and perindoprilat, respectively, below the limit of calculable plasma concentrations. This further illustrates that DBS can be used as a screening measure for carvedilol and perindoprilat – but not to determine adherence. From the pharmacokinetic study, enalaprilat's lowest plasma concentration was 11.0 ng/mL, well above the 3.65 ng/mL limit, which can be calculated from the DBS LLOQ.

Table 5.1 Summary of Deming regression analysis of observed plasma and observed DBS concentrations

Analyte	Number of total paired samples	Number of outliers	N < LLOQ DBS	N < LLOQ Plasma	*Number of paired samples used for regression	Slope (95% CI)	Y-Intercept (95% CI)	Pearson correlation coefficient-r (95% CI)
Carvedilol	78	3	8	0	67	2.04 (1.98 - 2.12)	1.74 (0.765 - 2.71)	0.992 (0.987 - 0.995)
Enalaprilat	35	1	0	0	34	2.39 (1.81 - 2.97)	1.26 (-6.03 - 8.56)	0.948 (0.897 - 0.974)
Perindoprilat	42	4	6	0	32	3.01 (2.79 - 3.24)	0.267 (-0.735 - 1.27)	0.968 (0.935 - 0.984)

*Number of paired DBS and plasma samples used for Deming regression after removal of outliers and samples below LLOQ


Plasma (ng/mL)
DBS (ng/mL)
y = 1,739 + 2,042 x
r = 0.992
0
20
40
60
80
100
120
140


Plasma (ng/mL)
DBS (ng/mL)
y = 1,262 + 2,387 x
r = 0.948
0
10
20
30
40
50
60
70
80
90
100

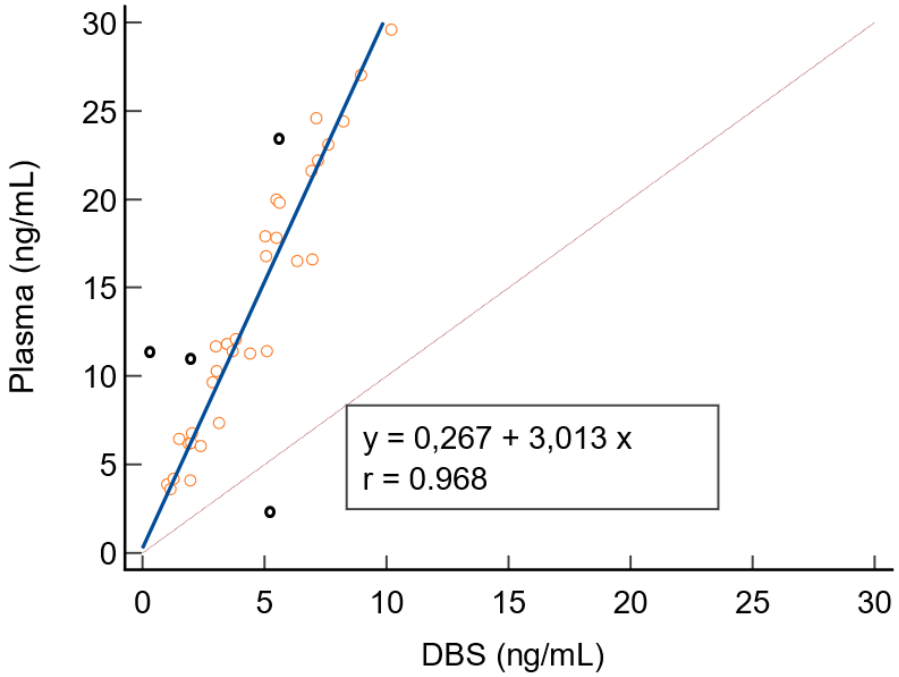


Figure 5.3 Deming regression for (a) carvedilol (N = 67), (b) enalaprilat (N = 34) and (c) perindoprilat (N = 32). Pearson's coefficient represented as r.

Table 5.2 Corresponding plasma concentration at DBS LLOQ when derived from Deming regression.

DBS LLOQ (ng/mL)	Derived plasma concentration at DBS LLOQ (ng/mL)
1.00	3.78
1.00	3.65
1.00	3.28

Previous modelling further validates that the lowest calculable plasma concentration of 3.65 ng/mL is sufficient for tracking adherence to enalapril. Modelling has been performed by Gebreyesus et al.[226] (unpublished) for 5 mg, 10 mg and 20 mg enalapril taken twice daily for a patient weight range of 40–140 kg (Table 5.3). The lowest predicted concentration simulated is for patients taking 5 mg enalapril BD who weigh 140 kg. According to the simulation, more than 95% of patient concentrations should be above 3.75 ng/mL within 12 hours of the last enalapril dose taken for patients weighing 140 kg (on 5 mg BD). At this extreme weight, 3.65 ng/mL is still sufficient to discern adherence, even if only for the last 12-hour period. At the other extreme of simulated concentrations, 95% of patients who weigh 40 kg and who are on 20 mg BD should have a plasma concentration greater than 32.6 ng/mL (13.1 ng/mL DBS equivalent concentration) within 12 hours of the last enalapril dose.

Table 5.3 Fifth percentile of predicted plasma concentrations 12 hours after last dose of enalapril was taken for various dosages and weights. The time taken to reach LLOQ after medication has been stopped is also shown (adapted from Gebreyesus et al.[226](unpublished))

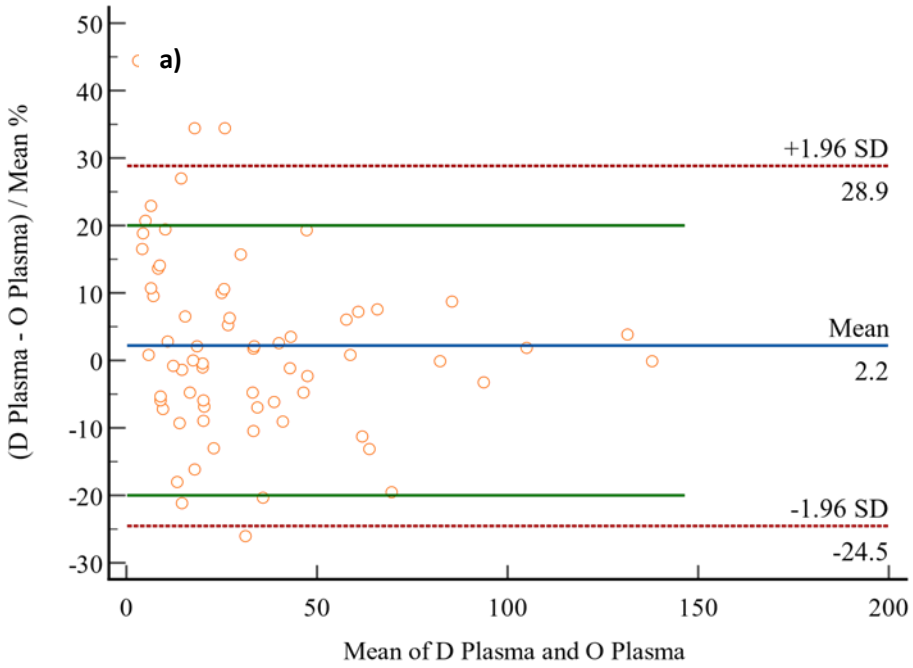
Dose: 5 mg twice daily		
Weight (kg)	**Fifth percentile of predicted concentrations at 12 hours[1] (mg/L)**	**Time to below the LLOQ[2] (days)**
40	7.35	3.5
60	6.60	3.5
80	5.09	3
100	4.72	2.5
120	4.08	2
140	3.75	2
Dose: 10 mg twice daily		
40	16.9	5.5
60	12.9	5
80	10.9	5
100	9.51	4.5
120	8.37	4.5
140	8.03	4
Dose: 20 mg twice daily		
40	32.6	7
60	25.1	7
80	22.4	7
100	18.4	7
120	17.0	6.5
140	16.3	6.5

[1]Predicted concentration obtained from 5000 stochastic simulations per weight and dose group
[1]Predicted concentrations are at time = 12 hours (last dose was given at time = 0)
[2]Lower limit of quantification (LLOQ) = 0.2 mg/L

Weight bands of 20 kg
≤ 40 kg = results for 40 kg
> 40 kg – 60 kg = results for 60 kg
> 60 kg – 80 kg = results for 80 kg
> 80 kg – 100 kg = results for 100 kg
> 100 kg – 120 kg = results for 120 kg
> 120 kg – 140 kg = results for 140 kg

5.3.3 Agreement of observed and calculated plasma concentrations

Figure 5.4 shows the Bland–Altman plots of the observed and calculated plasma concentrations. The difference between the observed and calculated plasma concentrations was less than 20% of their mean for 88.1%, 97.1%, and 78.1% of the paired carvedilol, enalaprilat, and perindoprilat samples, respectively. The analytes fall within the accepted criteria for cross validation (the difference between paired samples is within ± 20% of their mean for > 67% of samples).[159] The Deming regression formulae are adequate to allow for good agreement between observed and calculated plasma concentrations. Therefore, DBS and plasma concentrations can be used interchangeably once DBS concentrations have been converted to plasma concentrations using the Deming regression formulae. Peeters et al.[94] evaluated the agreement of concentrations between eight antihypertensive drugs and four metabolites (including enalaprilat and perindoprilat) in plasma and capillary-derived whole-blood DBS. The agreement observed was 66.7% and 31.6 % for enalaprilat and perindoprilat, respectively. The LLOQ of perindoprilat (5 ng/mL) proved to be a significant limitation in the study as 22 of the DBS samples from the 41 paired samples were below the LLOQ concentration and had to be removed before agreement could be evaluated.



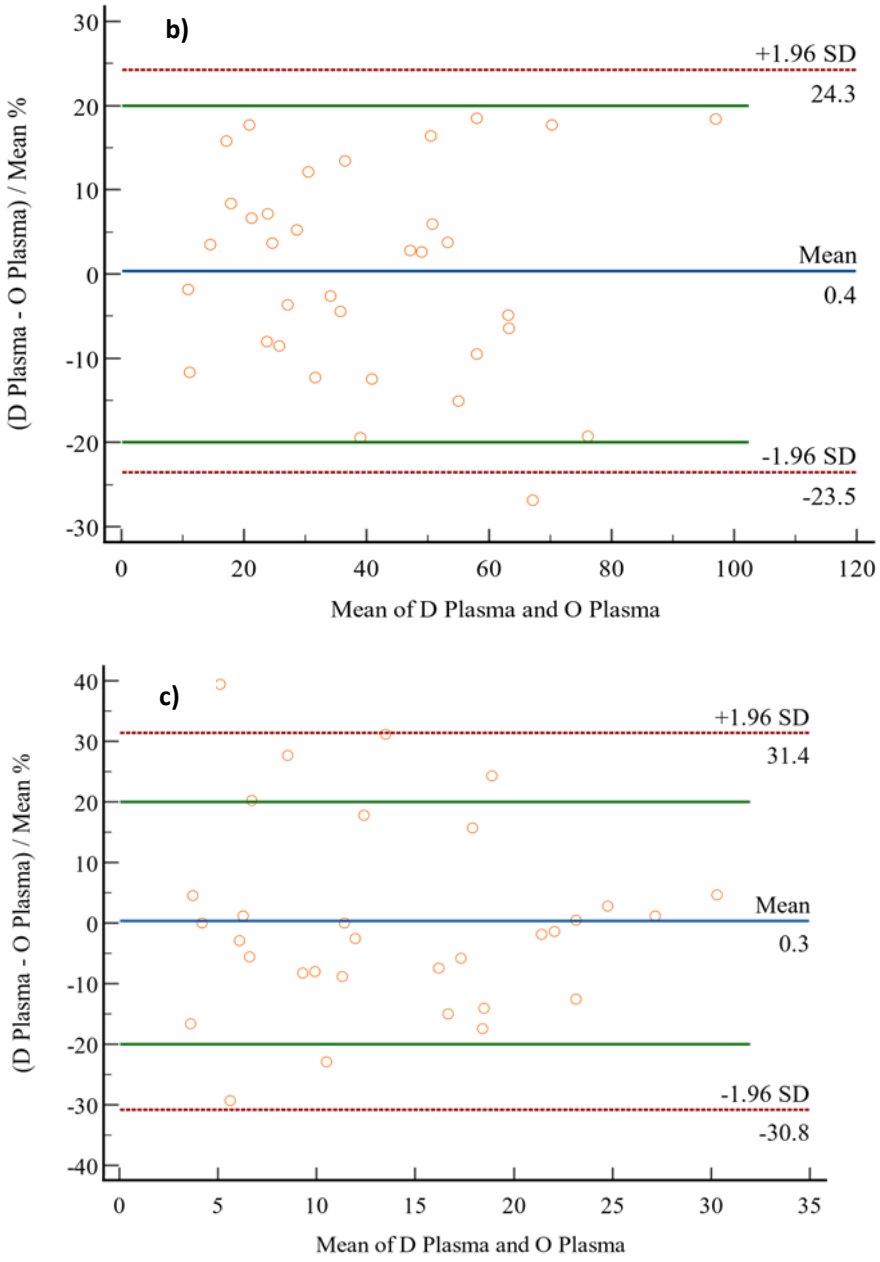


Figure 5.4 Bland–Altman plots of derived plasma (D plasma) and observed plasma (O plasma) concentrations, respectively, for (a) carvedilol, (b) enalaprilat and (c) perindoprilat. The solid green lines on either side of the solid blue line (mean % error) represent the 20% acceptable bias.

Table 5.4 Bland–Altman results of the analyses of observed plasma vs DBS-derived plasma concentrations

Analyte	N	Mean % Bias	*Δ Within 20% of average (%)
Carvedilol	67	2.2	88.1
Enalaprilat	34	0.3	97.1
Perindoprilat	32	0.4	78.1

*Acceptance limit > 67%.

5.4 Summary and conclusions

Deming regression showed a good correlation between plasma and DBS concentrations for carvedilol, enalaprilat and perindoprilat, with r values all >0.94. Substituting the DBS LLOQ into the equations obtained from the Deming regressions indicates that calculated plasma concentrations obtained from DBS concentrations are limited to a minimum of 3.78, 3.65, and 3.28 ng/mL for carvedilol, enalaprilat, and perindoprilat, respectively. The lowest plasma concentrations obtained from the pharmacokinetic pilot study were 1.3 ng/mL, 11.0 ng/mL, and 2.13 ng/mL for carvedilol, enalaprilat, and perindoprilat, respectively. This further corroborates the conclusions drawn in the previous chapter that the DBS assay would be suited as a screening measure for carvedilol and perindoprilat rather than an assay used for tracking adherence. The DBS assay is, however, suited to track adherence to enalapril as the lowest enalaprilat plasma concentration obtained from the pharmacokinetic study is substantially higher than the minimum quantifiable plasma concentrations obtained from DBS concentrations. Previous modelling of plasma enalaprilat concentrations further confirms that a concentration of 3.65 ng/mL is sufficient to discern adherence at relatively low doses and for an extensive weight range[226].

Good agreement was shown between calculated plasma concentrations and observed plasma concentrations from the pharmacokinetic pilot study. The difference between the observed and calculated plasma concentrations was less than 20% of their mean for >67% of samples for all analytes. The good agreement between samples indicates that DBS and plasma samples can be used interchangeably. This further validates the assays clinically and confirms the robustness of the assays.

The simplicity of sampling, storage and transportation of DBS samples can be used synergistically with the substantial pharmacokinetic data available for plasma to track enalapril adherence. Enalaprilat patient DBS concentrations can be normalised to plasma concentrations using linear regression, and a patient's adherence to enalapril can be interpreted. Carvedilol and perindoprilat DBS concentrations can be used interchangeably with plasma concentrations once normalised, but the DBS assay's ability to track adherence for these two analytes is limited.

6 Conclusions

6.1 Research summary

Non-adherence to prescribed treatment regimens is a pervasive problem in chronic diseases such as HF.[66] However, a concrete understanding of HF patients' adherence behaviour in African countries is limited because of a lack of data. This is a critical gap in understanding as the mortality rate of African HF patients is high.[13] Two novel assays were developed and validated to help close this gap in understanding. Assays were developed and validated to quantify carvedilol, enalaprilat and perindoprilat in plasma and DBS simultaneously. The assays developed are direct quantitative methods for determining adherence, thus removing bias prevalent in self-reporting methods.

The validated calibration range of the plasma assay (0.2–200 ng/mL) is sufficient to determine adherence to carvedilol, enalapril and perindopril (through their metabolites enalaprilat and perindoprilat, respectively). The assay can quantify carvedilol, enalaprilat and perindoprilat for a period greater than the dosing interval of carvedilol (12 hours), enalapril (12 hours) and perindopril (24 hours), respectively, after medication ingestion. The plasma pharmacokinetic data available for carvedilol, enalaprilat and perindoprilat are adequate to create pharmacokinetic models that will allow for dose- or weight-specific adherence interpretation of concentrations. Such a model for enalaprilat already exists.[226] However, the plasma matrix's cumbersome sampling, storage and transportation requirements are a drawback in remote and resource-scarce areas. Specialised laboratory equipment is sometimes limited in remote areas, and the matrix's stringent storage and transportation requirements complicate logistics. The assay, however, is suitable to generate adherence data if the transportation and storage requirements of the matrix are catered for.

Limited whole blood and DBS pharmacokinetic data made the selection of a suitable calibration range for the DBS assay challenging. The matrix's complexity also hindered the development of a method that allowed for an LLOQ as low as the plasma assay. A literature review revealed only one developed assay quantifying enalaprilat and perindoprilat in DBS with LLOQs substantially higher than the LLOQ developed for this assay.[94] No fully validated DBS quantitative method was found for carvedilol. Evaluation of the assay through a pharmacokinetic study indicated that the LLOQ of 1.00 ng/mL was insufficient to use the assay as an adherence measure for carvedilol and perindopril. However, the assay is sufficiently

sensitive to track enalaprilat concentrations for enalapril adherence evaluation (enalaprilat is quantifiable > 12 hours after enalapril ingestion). Sampling, storage and transportation of DBS samples are more suited to resource-scarce and isolated areas than plasma samples. However, pharmacokinetic data in whole blood and DBS of adherent participants as reference is sparse. This limits adherence classification to a single cut-off value, regardless of dose or patient weight.

Deming regression of paired plasma and DBS pharmacokinetic samples showed a strong correlation for all three analytes. Observed plasma concentrations and plasma concentrations calculated from DBS concentrations using the Deming regression formulae showed good agreement. The difference between the observed and calculated plasma concentrations was less than 20% of their mean for > 67% of samples for all analytes. The criteria for cross-validation were therefore fulfilled for all analytes.[159] The cross-validation of the DBS and plasma assay is important as it helps to bring additional confidence to both assays. Spiking working solutions directly onto blank DBS did not influence the integrity of the assay as was proven by cross-validating it with a plasma assay using clinical samples.

The synergy between DBS and plasma assays

Cross-validation showed that DBS concentrations could be accurately converted to plasma concentrations using the Deming regression formulae. Therefore, synergistic use of the assays can exploit each assay's advantages. Although DBS have sampling and storage advantages, pharmacokinetic data to interpret DBS concentrations in terms of adherence is limited. The DBS matrix's less stringent transport and storage requirements can be taken advantage of, while the rich pharmacokinetic data of the plasma matrix can be taken advantage of by converting DBS concentrations to plasma concentrations after quantification. Less importance is therefore placed on absolute DBS concentrations and more importance on strong correlation and agreement between DBS and plasma concentrations, as it is not absolute DBS concentrations that are interpreted. Such a synergistic use of the assays can only be fully realised for adherence evaluation to enalapril (through enalaprilat) as the DBS LLOQ of 1.00 ng/mL for carvedilol and perindoprilat is not sufficiently low to discern between adherent and non-adherent patients.

6.2 Study limitations and recommendations

The DBS assay's LLOQ of 1 ng/mL is a limitation in assessing adherence to carvedilol and perindopril. This limitation only became apparent after the assay was evaluated using a pharmacokinetic study. Developing the assay further to reduce carvedilol and perindoprilat LLOQs will allow the assay to be used as an adherence measure for carvedilol and perindopril. This is especially likely in the case of perindoprilat due to the analyte's long terminal half-life, which could make it possible to evaluate if the patient had not taken their medication for several days.

Standards and QCs for the DBS assay were not prepared by spiking working solutions into whole blood first. Instead, they were prepared by spiking working solutions onto blank DBS spots. This is not a limitation because the robustness of the assay was proven by cross-validation with the plasma assay. However, to simulate authentic matrix conditions closely, it is recommended to perform further stability experiments through incurred sample reanalysis of patient samples, especially for extreme temperatures (> 30ºC), which could be expected in locations that do not have refrigeration.

Evaluation of the assays using the pharmacokinetic study gave insight into the assays' capabilities as adherence measures. More participants are needed, however, to give a clearer picture of the pharmacokinetics of carvedilol, enalaprilat and perindoprilat in DBS. It is recommended that further pharmacokinetic studies are done in DBS.

The DBS assay was validated using venous blood pipetted onto DBS cards to allow for controlled validation conditions. It is recommended that the correlation between analyte concentrations of capillary blood and plasma is evaluated. Capillary blood is collected by pricking the heel or finger with a lancet and is different to venous blood composition.[230] Because of its simplicity, patients can create capillary samples themselves. This gives capillary samples an advantage in remote locations. Moreover, accurate sampling devices such as Capitainer®qDBS or VAMS use capillary blood.

An important caveat to consider for both assays regarding their adherence determining ability for all analytes is the potential for white coat adherence. White coat adherence can lead to artificially high drug concentrations, which may not accurately reflect a patient's typical medication-taking behaviour outside of a clinical setting. This is a limitation inherent to the adherence determining method of quantifying analytes directly in a biological matrix. Using multiple measures of adherence, such as self-report, pill counts, or electronic monitoring

devices, can provide a more comprehensive understanding of a patient's medication-taking behaviour over time. Combining drug concentration measurements with other adherence measures, if practical, can help identify patterns of adherence and non-adherence that may not be apparent from drug concentration measurements alone.

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8 Appendix: Plasma Assay Partial Validations

Accuracy and precision, as well as matrix effects, were done at a 5µL injection volume for all analytes to allow the option of the lower injection volume should it be needed for the plasma assay (sensitivity variation of the instrument). In addition, accuracy and precision were done for a raised LLOQ of 0.5 ng/mL, specifically if perindoprilat carryover should become a problem. This was done at 5µL and 20 µL.

8.1 Preparation of calibration standards and quality controls

(Refer to section 2.2.6.1 for standard and quality control preparation description)

Table 8.1 Preparation of working solutions and calibration standards for carvedilol, enalaprilat and perindoprilat with LLOQ raised to 0.5 ng/mL

Working solutions (WS)	Blank solvent (methanol) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS Solution (µg/mL)	Volume (µl) WS spiked into 1.90ml plasma	STD	Plasma Concentration (ng/mL)
WS1	4940	SS x 3	20 + 20 + 20	4.00	100	STD 1-ULOQ	200
WS2	250	WS1	750	3.00	100	STD 2	150
WS3	500	WS2	500	1.50	100	STD 3	75.0
WS4	500	WS3	250	0.500	100	STD 4	25.0
WS5	800	WS4	200	0.100	100	STD 5	5.00
WS6	600	WS5	400	0.0400	100	STD 6	2.00
WS7	600	WS6	200	0.0100	100	STD 7	0.500

Table 8.2 Preparation of working solutions and quality controls for carvedilol, enalaprilat and perindoprilat with LLOQ raised to 0.5 ng/mL

Working solutions (WS)	Blank solvent (methanol) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS concentration (µg/mL)	Volume (µL) WS spiked into 1.90ml plasma	QC	Plasma concentration (ng/mL)
WSQ1	2440	SS x 3	20 + 20 + 20	8.00	100	QC DIL	400
WSQ2	450	WSQ1	300	3.20	100	QC H	160
WSQ3	400	WSQ2	400	1.60	100	QC M	80.0
WSQ4	1000	WSQ3	200	0.267	100	SYS 1	13.3
WSQ5	1000	WSQ4	200	0.0440	100	SYS 2	2.22
WSQ6	602	WSQ5	198	0.0110	100	QC L	0.550
WSQ7	1000	WSQ5	290	0.0040	100	LLOQ	0.500

8.2 Accuracy & precision – 5 µL injection

Carvedilol

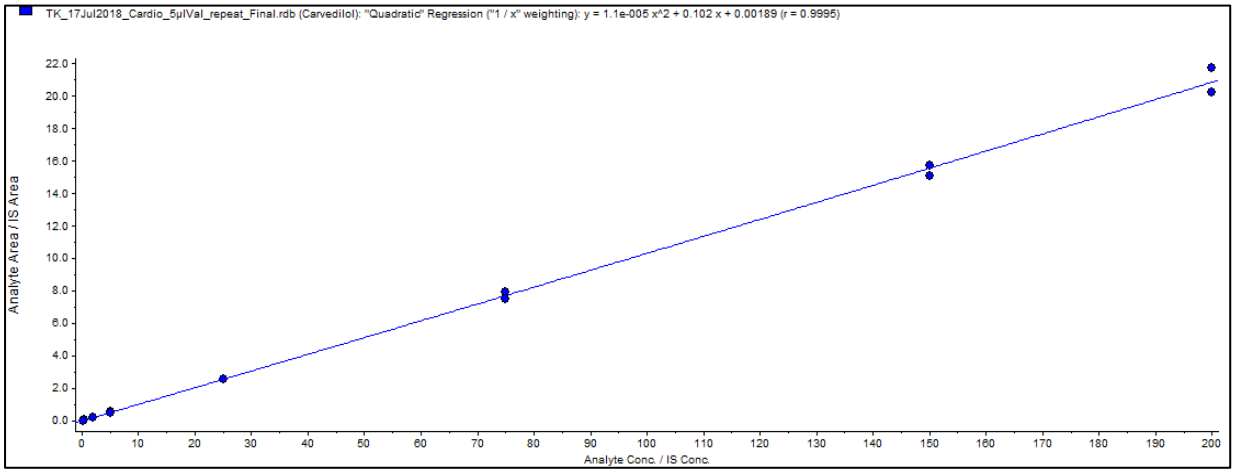

Figure 8.1 Representative calibration curve for carvedilol: 5µl injection

Table 8.3 Carvedilol calibration standard accuracy and precision: 5µL injection

Sample ID	Nominal conc. ng/ml	Mean observed conc. ng/ml	STDEV	CV(%)	%Accuracy	n
S8	0.200	0.171	0.0123	7.5	85.7	2 of 2
S7	0.500	0.553	0.0266	4.8	110.7	2 of 2
S6	2.00	1.98	0.0170	0.8	98.9	2 of 2
S5	5.00	5.20	0.163	3.1	103.9	2 of 2
S4	25.0	25.4	0.0390	0.2	101.5	2 of 2
S3	75.0	74.7	3.05	4.1	99.7	2 of 2
S2	150	149	4.20	2.8	99.1	2 of 2
S1	200	201	10.0	4.8	100.5	1 of 2

Table 8.4 Summary of carvedilol intra-validation quality controls: 5µL injection

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ	0.200	0.194	0.0140	7.3	97.2	6 of 6
QC L	0.550	0.524	0.0200	3.2	95.2	6 of 6
QC M	80.0	80.8	2.50	3.2	101.0	6 of 6
QC H	160	158	2.70	1.7	98.7	6 of 6

Enalaprilat

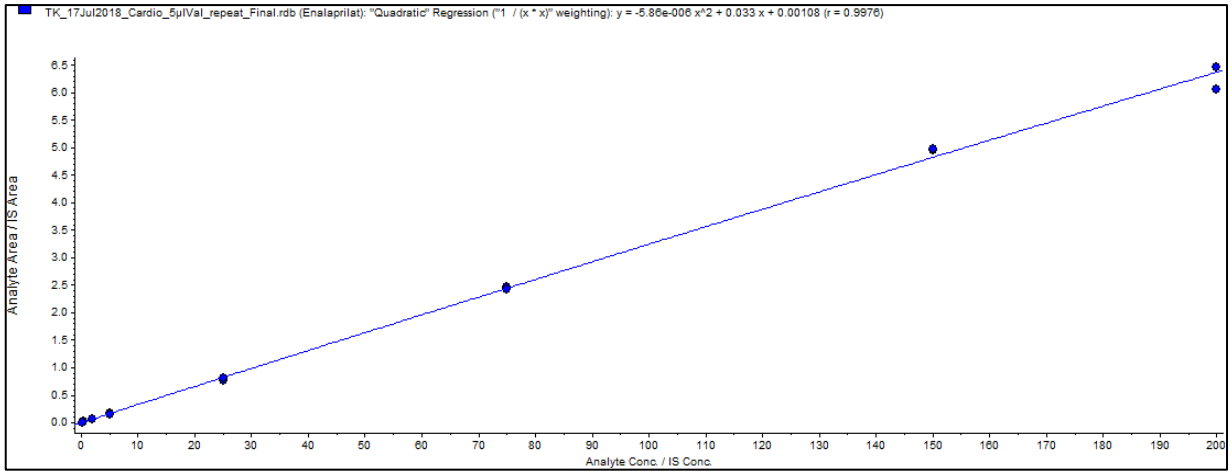

Figure 8.2 Representative calibration curve for enalaprilat: 5µL injection.

Table 8.5 Enalaprilat calibration standard accuracy and precision: 5µL injection

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/ml	STDEV	CV(%)	%Accuracy	n
S8	0.200	0.198	0.0224	11.3	98.9	2 of 2
S7	0.500	0.512	0.0786	15.3	102.5	2 of 2
S6	2.00	2.02	0.0448	2.2	101.1	2 of 2
S5	5.00	5.04	0.332	6.6	100.8	2 of 2
S4	25.0	23.9	1.15	4.8	95.4	2 of 2
S3	75.0	75.0	1.35	1.8	100.0	2 of 2
S2	150	155	0.480	0.3	103.2	2 of 2
S1	200	196	9.00	4.6	98.1	2 of 2

Table 8.6 Summary of enalaprilat intra-validation quality controls: 5µl injection

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ	0.200	0.198	0.0371	18.8	98.8	6 of 6
QC L	0.550	0.519	0.0168	3.2	94.3	6 of 6
QC M	80.0	79.1	2.68	3.4	98.9	6 of 6
QC H	160	155	3.65	2.4	96.6	6 of 6

Perindoprilat

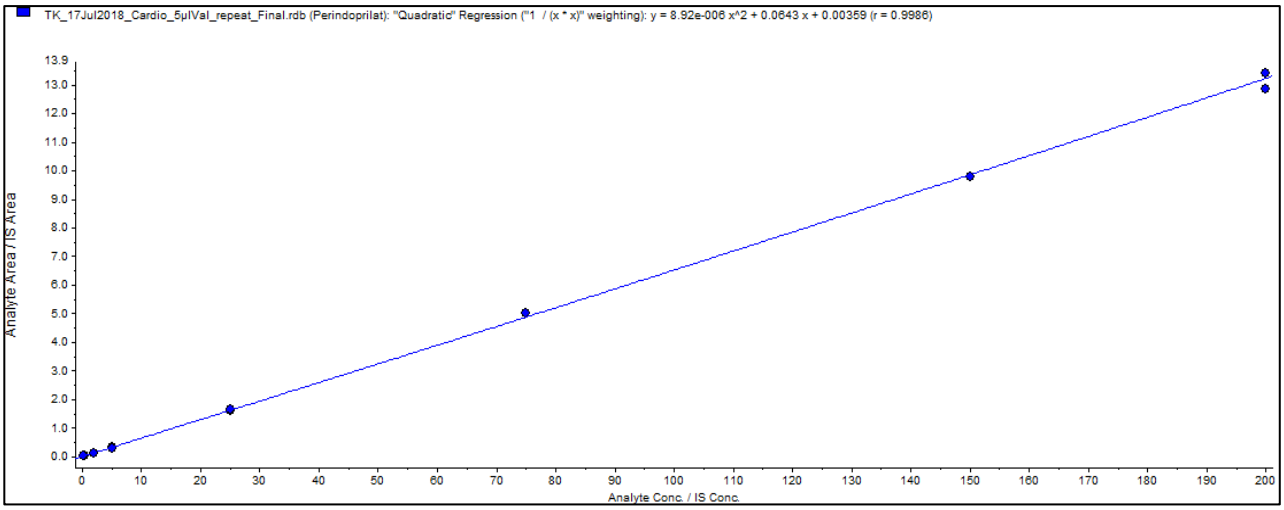

Figure 8.3 Representative calibration curve for perindoprilat: 5µL injection.

Table 8.7 Perindoprilat calibration standard accuracy and precision: 5 µL injection

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
S8	0.200	0.196	0.0108	5.5	97.9	2 of 2
S7	0.500	0.534	0.0211	3.9	106.7	2 of 2
S6	2.00	1.87	0.0657	3.5	93.6	2 of 2
S5	5.00	4.98	0.425	8.5	99.7	2 of 2
S4	25.0	25.1	0.619	2.5	100.4	2 of 2
S3	75.0	77.1	0.340	0.4	102.8	2 of 2
S2	150	149	0.019	0.0	99.5	2 of 2
S1	200	199	5.66	2.8	99.4	2 of 2

Table 8.8 Summary of perindoprilat intra-validation quality controls: 5µL injection

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ	0.200	0.226	0.0164	7.3	113.1	6 of 6
QC L	0.550	0.517	0.0343	6.6	94.0	6 of 6
QC M	80.0	80.2	0.790	1.0	100.2	6 of 6
QC H	160	158	6.25	4.0	98.6	6 of 6

8.3 Accuracy & precision – 0.5 ng/mL LLOQ

Carvedilol

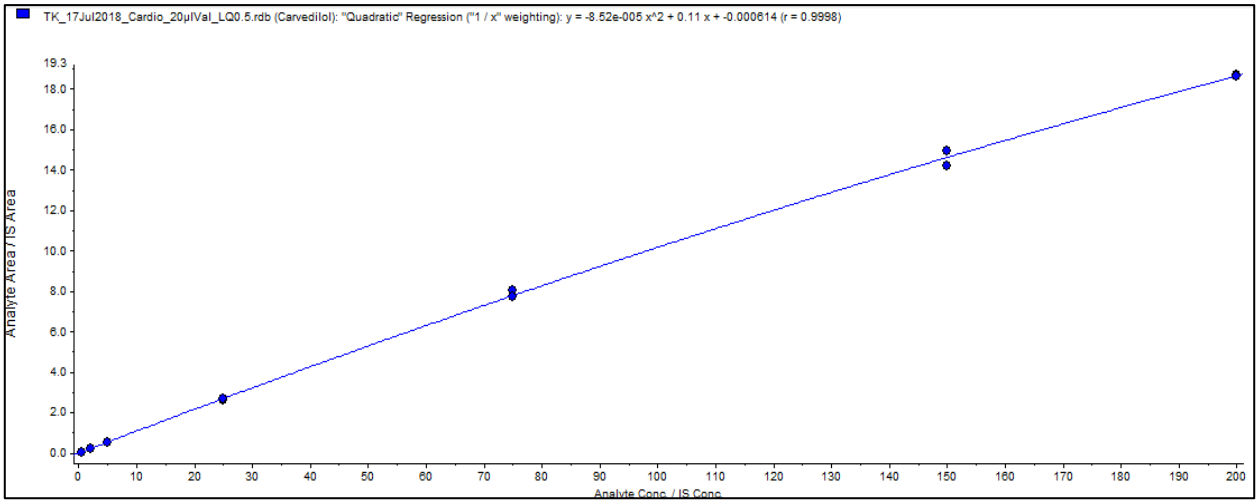

Figure 8.4 Representative calibration curve for carvedilol: LQ set to 0.5 ng/mL

Table 8.9 Carvedilol calibration standard accuracy and precision: LQ set to 0.5 ng/mL

Sample ID	Nominal conc. ng/ml	Mean observed conc. ng/ml	STDEV	CV(%)	%Accuracy	n
S7	0.500	0.530	0.0264	5.0	105.9	2 of 2
S6	2.00	1.92	0.0590	3.1	95.8	2 of 2
S5	5.00	4.95	0.111	2.2	99.0	2 of 2
S4	25.0	24.6	0.353	1.4	98.2	2 of 2
S3	75.0	76.1	1.92	2.5	101.4	2 of 2
S2	150	149	6.60	4.4	99.6	2 of 2
S1	200	200	0.200	0.2	100.1	2 of 2

Table 8.10 Summary of carvedilol intra-validation quality controls: LQ set to 0.5 ng/mL

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ	0.500	0.466	0.0250	5.3	93.1	6 of 6
QC L	0.550	0.525	0.0300	4.9	95.4	6 of 6
QC M	80.0	80.3	1.80	2.3	100.4	6 of 6
QC H	160	162	2.80	1.7	101.0	6 of 6

Enalaprilat

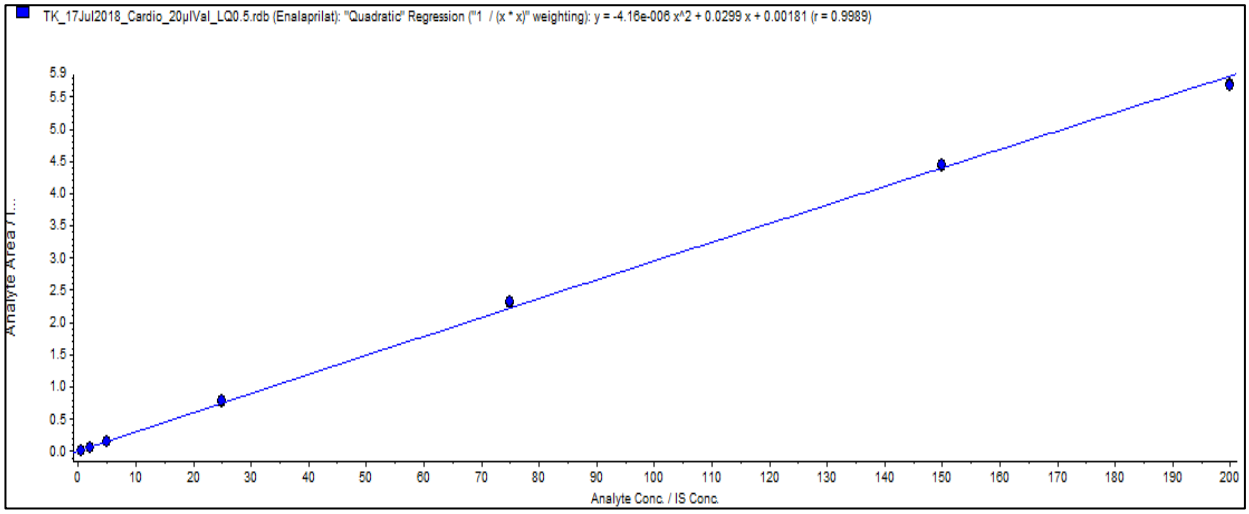

Figure 8.5 Representative calibration curve for enalaprilat: LQ set to 0.5 ng/mL

Table 8.11 Enalaprilat calibration standard accuracy and precision: LQ set to 0.5 ng/mL

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/ml	STDEV	CV(%)	%Accuracy	n
S7	0.500	0.508	0.0211	4.2	101.6	2 of 2
S6	2.00	1.86	0.108	5.8	93.2	2 of 2
S5	5.00	5.00	0.196	3.9	99.9	2 of 2
S4	25.0	25.7	0.409	1.6	102.9	2 of 2
S3	75.0	78.0	0.200	0.3	104.0	2 of 2
S2	150	151	0.850	0.6	100.9	2 of 2
S1	200	195	0.500	0.2	97.5	2 of 2

Table 8.12 Summary of enalaprilat intra-validation quality controls: LQ set to 0.5 ng/mL

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	% Accuracy	n
QC LLOQ	0.500	0.426	0.0219	5.1	85.3	6 of 6
QC L	0.550	0.502	0.0128	2.5	91.2	6 of 6
QC M	80.0	80.5	1.41	1.8	100.6	6 of 6
QC H	160	158	3.43	2.2	98.6	6 of 6

Perindoprilat

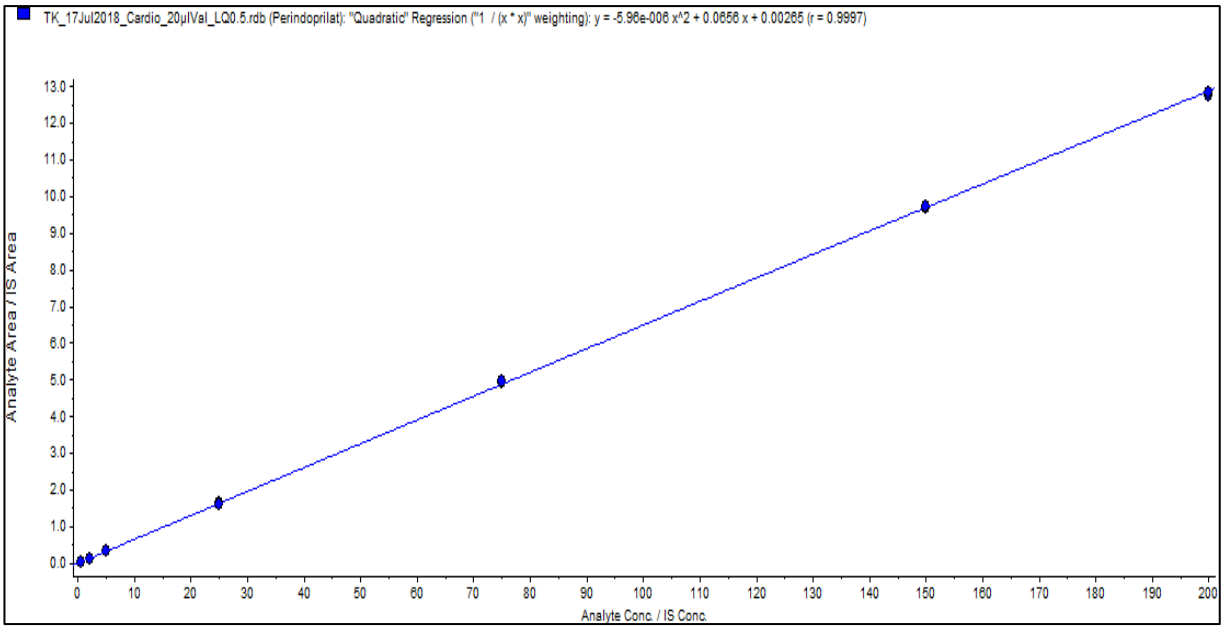

Figure 8.6 Representative calibration curve for perindoprilat: LQ set to 0.5 ng/mL

Table 8.13 Perindoprilat calibration standard accuracy and precision: LQ set to 0.5 ng/mL

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
S7	0.500	0.504	0.0203	4.0	100.7	2 of 2
S6	2.00	1.93	0.0502	2.6	96.4	2 of 2
S5	5.00	5.08	0.00600	0.1	101.6	2 of 2
S4	25.0	25.1	0.336	1.3	100.4	2 of 2
S3	75.0	76.1	0.500	0.7	101.5	2 of 2
S2	150	150	0.194	0.1	100.1	2 of 2
S1	200	199	0.930	0.5	99.3	2 of 2

Table 8.14 Perindoprilat summary of quality control accuracy and precision: LQ set to 0.5 ng/mL

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ	0.500	0.491	0.0440	9.0	98.3	6 of 6
QC L	0.550	0.533	0.0388	7.3	96.8	6 of 6
QC M	80.0	81.3	1.28	1.6	101.6	6 of 6
QC H	160	158	3.05	1.9	98.6	6 of 6

8.4 Accuracy & precision – 0.5 ng/mL and 5 µL injection volume

Carvedilol

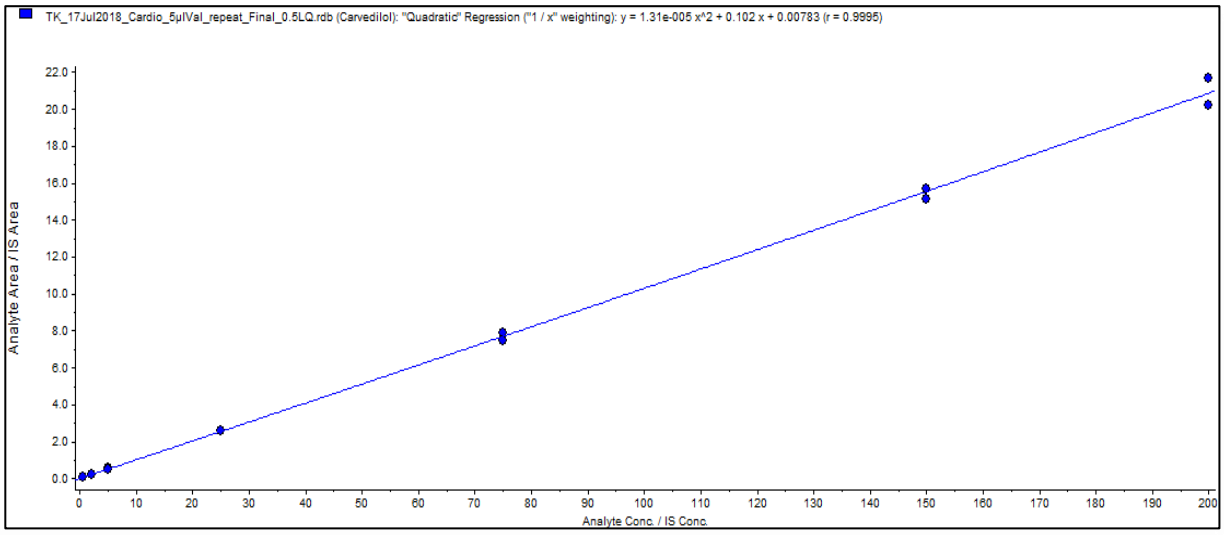

Figure 8.7 Representative calibration curve for carvedilol: 5uL injection with the LLOQ at 0.500 ng/ml.

Table 8.15 Carvedilol calibration standard accuracy and precision: 5ul injection with the LLOQ at 0.500 ng/mL

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
S7	0.500	0.497	0.0267	5.4	100.7	2 of 2
S6	2.00	1.93	0.0170	0.9	96.4	2 of 2
S5	5.00	5.16	0.163	3.2	101.6	2 of 2
S4	25.0	25.4	0.0390	0.2	100.4	2 of 2
S3	75.0	74.9	3.05	4.1	101.5	2 of 2
S2	150	149	4.10	2.8	100.1	2 of 2
S1	200	201	10.0	4.8	99.3	2 of 2

Table 8.16 Summary of carvedilol intra-validation quality controls: 5uL injection with the LLOQ at 0.500 ng/mL

Sample ID	Nominal conc. ng/ml	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ2	0.500	0.421	0.0270	6.4	84.1	6 of 6
QC L	0.550	0.467	0.0200	3.6	85.0	6 of 6
QC M	80.0	80.9	2.50	3.1	101.2	6 of 6
QC H	160	158	2.60	1.7	98.7	6 of 6

Enalaprilat

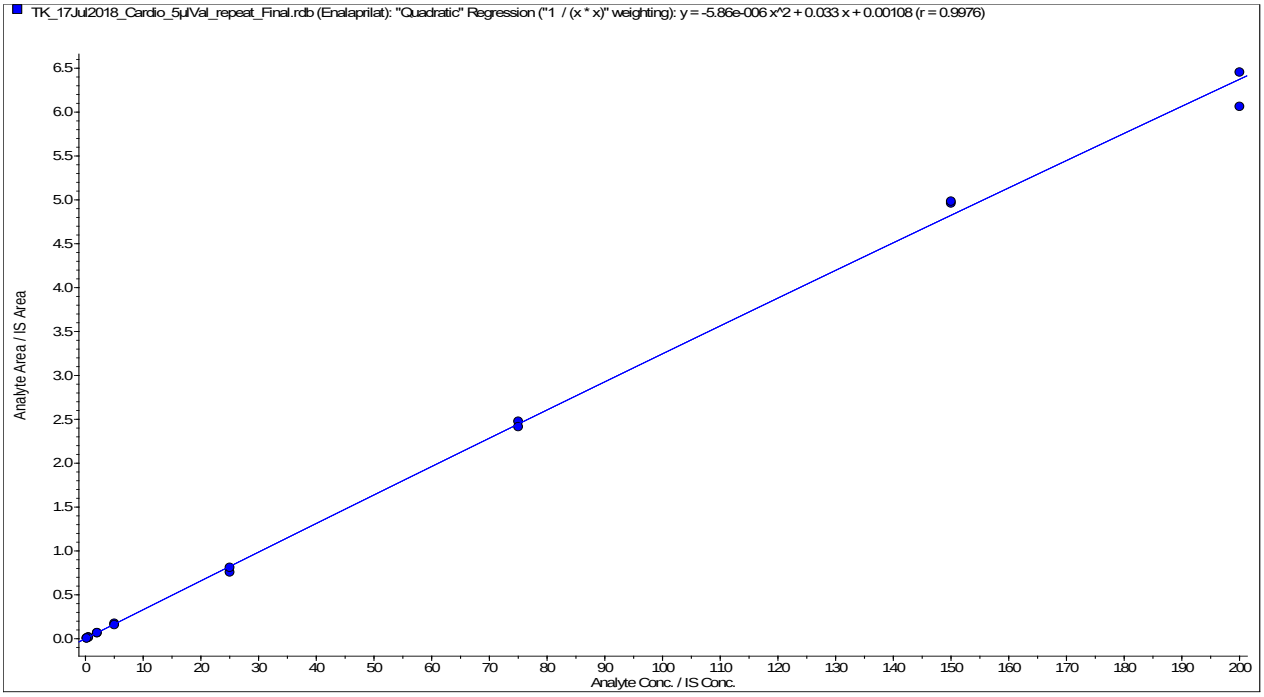

Figure 8.8 Representative calibration curve for carvedilol: 5uL injection with the LLOQ at 0.500 ng/mL

Table 8.17 Enalaprilat calibration standard accuracy and precision: 5uL injection with the LLOQ at 0.500 ng/ml

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	% Accuracy	n
S7	0.500	0.498	0.0794	15.9	99.6	2 of 2
S6	2.00	2.02	0.0452	2.2	101.2	2 of 2
S5	5.00	5.07	0.335	6.6	101.4	2 of 2
S4	25.0	24.1	1.16	4.8	96.2	2 of 2
S3	75.0	75.4	1.35	1.8	100.6	2 of 2
S2	150	155	0.480	0.3	103.2	2 of 2
S1	200	196	8.80	4.5	97.8	2 of 2

Table 8.18 Summary of enalaprilat intra-validation quality controls: 5uL injection with the LLOQ at 0.500 ng/ml

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
QC LLOQ2	0.500	0.464	0.0293	6.3	92.7	6 of 6
QC L	0.550	0.504	0.0170	3.4	91.7	6 of 6
QC M	80.0	79.5	2.68	3.4	99.4	6 of 6
QC H	160	155	3.61	2.3	96.6	6 of 6

Perindoprilat

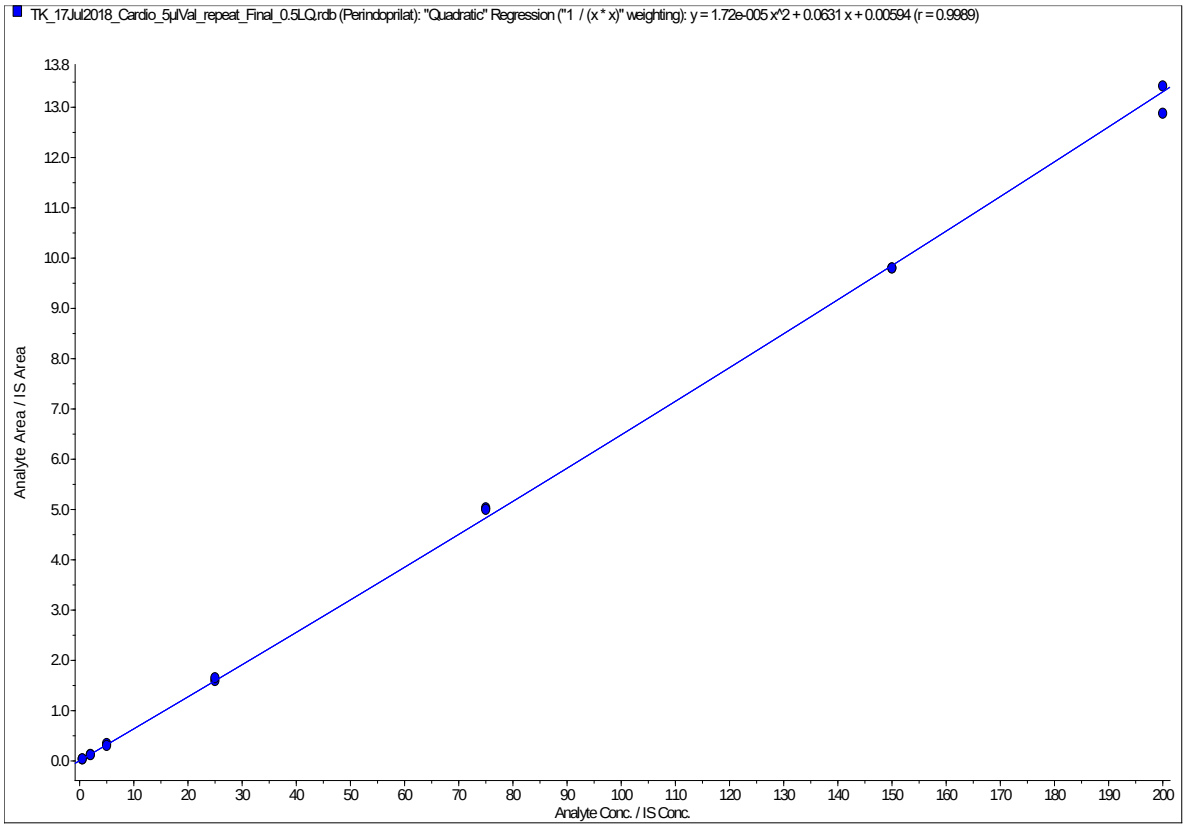

Figure 8.9 Representative calibration curve for perindoprilat: 5uL injection with the LLOQ at 0.500 ng/ml

Table 8.19 Perindoprilat calibration standard accuracy and precision: 5uL injection with the LLOQ at 0.500 ng/ml

Sample ID	Nominal conc. ng/mL	Mean observed conc. ng/mL	STDEV	CV(%)	%Accuracy	n
S7	0.500	0.507	0.0215	4.2	101.4	2 of 2
S6	2.00	1.87	0.0670	3.6	93.7	2 of 2
S5	5.00	5.05	0.433	8.6	100.9	2 of 2
S4	25.0	25.5	0.627	2.5	101.9	2 of 2
S3	75.0	77.8	0.340	0.4	103.7	2 of 2
S2	150	149	0.0180	0.0	99.5	2 of 2
S1	200	198	5.50	2.8	98.9	2 of 2

Table 8.20 Summary of perindoprilat intra-validation quality controls: 5uL injection with the LLOQ at 0.500 ng/ml

Sample ID	Nominal conc. ng/ml	Mean observed conc. ng/ml	STDEV	CV(%)	%Accuracy	n
QC LLOQ	0.500	0.474	0.0339	7.2	94.7	6 of 6
QC L	0.550	0.490	0.0349	7.1	89.2	6 of 6
QC M	80.0	80.9	0.780	1.0	101.1	6 of 6
QC H	160	158	6.13	3.9	98.6	6 of 6

8.5 Matrix effects in plasma – 5µL injection volume

Table 8.21 Peak areas for carvedilol and internal standard at high, medium and low concentration levels

	High concentration		Medium concentration		Low concentration	
	Analyte peak area	**ISTD peak area**	**Analyte peak area**	**ISTD peak area**	**Analyte peak area**	**ISTD peak area**
Sample 1	4540000	251000	2390000	273000	20900	316000
Sample 2	4660000	261000	2490000	285000	21500	291000
Sample 3	4280000	243000	2460000	281000	20400	305000
Sample 4	4650000	263000	2500000	288000	21300	310000
Sample 5	4450000	240000	2390000	274000	21000	309000
Sample 6	4560000	244000	2330000	259000	20800	331000

Table 8.22 Regression results from six different matrix sources spiked with carvedilol

	High conc. 160 ng/mL Peak area ratio	**Medium conc. 80.0 ng/mL Peak area ratio**	**Low conc. 0.550 ng/mL Peak area ratio**	**Area ratio v Conc. Regression slope**
Sample 1	18.09	8.75	0.0661	0.113
Sample 2	17.9	8.74	0.0739	0.112
Sample 3	17.6	8.75	0.0669	0.110
Sample 4	17.7	8.68	0.0687	0.110
Sample 5	18.5	8.72	0.0680	0.116
Sample 6	18.7	9.00	0.0628	0.117
Average	18.1	8.77	0.0677	0.113
STDEV	0.450	0.112	0.00363	0.00284
CV(%)	2.5	1.3	5.4	2.5

Table 8.23 Peak areas for enalaprilat and internal standard at high, medium and low concentration levels

	High concentration		Medium concentration		Low concentration	
	Analyte peak area	**ISTD peak area**	**Analyte peak area**	**ISTD peak area**	**Analyte peak area**	**ISTD peak area**
Sample 1	1940000	399000	944000	383000	6410	383000
Sample 2	3420000	662000	1610000	638000	12600	640000
Sample 3	2900000	545000	1460000	570000	9620	547000
Sample 4	2410000	454000	1080000	446000	7030	404000
Sample 5	3210000	631000	1580000	622000	10600	551000
Sample 6	3710000	702000	1760000	683000	11900	676000

Table 8.24 Regression results from six different matrix sources spiked with enalaprilat

	High conc. 160 ng/mL Peak area ratio	Medium conc. 80.0 ng/mL Peak area ratio	Low conc. 0.550 ng/mL Peak area ratio	Area ratio v Conc. Regression slope
Sample 1	4.86	2.46	0.017	0.030
Sample 2	5.17	2.52	0.020	0.032
Sample 3	5.32	2.56	0.018	0.033
Sample 4	5.31	2.42	0.017	0.033
Sample 5	5.09	2.54	0.019	0.032
Sample 6	5.28	2.58	0.018	0.033
Average	5.17	2.51	0.018	0.032
STDEV	0.177	0.0599	0.00115	0.00111
CV(%)	3.4	2.4	6.4	3.4

Table 8.25 Peak areas for perindoprilat and internal standard at high, medium and low concentration levels

	High concentration		Medium concentration		Low concentration	
	Analyte peak area	ISTD peak area	Analyte peak area	ISTD peak area	Analyte peak area	ISTD peak area
Sample 1	1910000	171000	938000	166000	8340	163000
Sample 2	3380000	298000	1570000	292000	11100	281000
Sample 3	2770000	248000	1410000	256000	10100	240000
Sample 4	2340000	207000	1080000	198000	7030	184000
Sample 5	3070000	258000	1430000	260000	10000	233000
Sample 6	3000000	275000	1450000	275000	11100	268000

Table 8.26 Regression results from six different matrix sources spiked with perindoprilat

	High conc. 160 ng/mL Peak area ratio	Medium conc. 80.0 ng/mL Peak area ratio	Low conc. 0.550 ng/mL Peak area ratio	Area ratio v Conc. Regression slope
Sample 1	11.2	5.65	0.0512	0.0697
Sample 2	11.3	5.38	0.0395	0.0709
Sample 3	11.2	5.51	0.0421	0.0698
Sample 4	11.3	5.45	0.0382	0.0707
Sample 5	11.9	5.50	0.0429	0.0744
Sample 6	10.9	5.27	0.0414	0.0682
Average	11.30	5.46	0.0425	0.0706
STDEV	0.331	0.128	0.00456	0.00208
CV(%)	2.9	2.3	10.7	2.9