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Research Article

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

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ABSTRACT

Introduction: Adherence to medication is an important determinant of outcomes in chronic diseases like heart failure. Drug assays provide objective adherence biomarkers. Dried blood spots (DBS) are appealing samples for drug assays due to less demanding transportation and storage requirements.
Objectives: To analytically validate a LC-MS/MS method for the simultaneous quantification of carvedilol, enalaprilat, and perindoprilat in DBS and evaluate the feasibility of using the method as an adherence determining assay. To validate the assay further clinically by establishing correlation and agreement between plasma and DBS samples from a pharmacokinetic pilot study.
Methods: The method was validated over a concentration range of 1.00–200 ng/mL according to FDA guidelines. Adherence tracking ability of the assay was evaluated using a pharmacokinetic pilot study. Correlation and agreement were evaluated through Deming regression and Bland-Altman analysis, respectively.
Results: Accuracy, precision, selectivity, and sensitivity were proven with complete and reproducible extraction recovery at all concentrations tested. Stability of the analytes in the matrix and throughout sample processing was proven. The full range of concentrations of the pharmacokinetic pilot study could be quantified for enalaprilat, but not for carvedilol and perindoprilat. The difference between the observed and calculated plasma concentrations was less than 20 % of their mean for >67 % of samples for all analytes.
Conclusions: The assay is suitable as a screening tool for carvedilol and perindoprilat, while suitable as an adherence determining assay for enalaprilat. Equivalence between observed and predicted plasma concentrations proves DBS and plasma concentrations can be used interchangeably.

1. Introduction

The worldwide prevalence of heart failure (HF) in 2020 was estimated to be 64.34 million cases [1]. The highest case fatality rates of chronic heart failure (CHF) are exhibited by African patients [2]. Both health outcomes and health care costs are negatively affected by poor treatment adherence to HF medicines [3,4]. Medication adherence forms a vital part of patient self-care, with studies indicating a reduction

Abbreviations: ALQ, Above the Limit of Quantitation; ACE-I, Angiotensin-converting enzyme inhibitors; BD, Bidaily; BMI, Body mass index; CHF, Chronic Heart Failure; CID, Collision-induced dissociation; CV, Co-efficient of variation; DBS, Dried Blood Spots; ESI, Electrospray ionization; EMA, European Medicines Agency; HF, Heart Failure; ISTD, Internal standard; ITP, Initial testing procedure; LOD, Limit of detection; LC-MS/MS, Liquid Chromatography with tandem mass spectrometry; LLOQ, lower limit of quantitation; NYHA FC, New York Heart Association Functional Classification; MRM, Multiple reaction monitoring; QC, Quality Control; QC DIL, Quality control dilution; QC LLOQ, Quality control lowest level of quantification; QCH, Quality control high; QCL, Quality control low; QCM, Quality control medium; S/N, signal-to-noise ratio; SOP, Standard operating procedure; OD, Once Daily; ULOQ, upper limit of quantification; VAMS, volumetric absorptive micro sampling.

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in mortality and readmissions of patients when adherence-increasing strategies are introduced [4–6]. The bulk of available data describing adherence to HF medications indicate 40–60 % adherence [6]. However, the data currently available describing the adherence patterns of African HF patients is not sufficient [3]. The dire statistics of patients suffering from HF in Africa, coupled with a paucity of adherence data from this region, testify to the necessity to obtain concrete adherence data of African HF patients.

Carvedilol is a third generation β-blocker, which improves myocardial function by blocking both β_1- and β_2-andrenergic receptors [7,8]. Medications that are commonly prescribed in conjunction with β-blockers, such as carvedilol, are angiotensin converting enzyme inhibitors (ACE-I). ACE-I are medications that block the production of angiotensin II. Angiotensin II is a hormone with multiple effects on the cardiovascular system, including the restriction of blood vessels, which, in turn, can increase the work load on the heart [9]. Enalapril and perindopril are both ACE-I medications, with their active metabolites being enalaprilat and perindoprilat, respectively. A direct and objective method to evaluate the adherence of patients suffering from HF is to quantify the concentration of carvedilol as well as the metabolites enalaprilat and perindoprilat [10].

Using sampling methods that allow for large scale sample analysis while minimizing cost and ensuring practicality is important. Traditional sampling methods are often resource-intensive at both the collection and storage chain stages [11,12]. Quantifying analytes in dried blood spots (DBS) has several advantages when compared to traditional sampling methods, being particularly useful in settings of resource scarcity [13–15]. DBS sample preparation can be performed without the use of specialized laboratory apparatus, such as centrifuges, and, therefore, untrained staff, or even the patients themselves, can prepare the samples [11,14]. Dried blood spot cards are generally shipped in sealed bags containing desiccant and can be transported at room temperature due to improved stability relative to other matrices, helping to reduce shipping costs [11,16]. Moreover, due to the antimicrobial properties of the dried-out DBS cards, they do not require any additional biohazard arrangements [11,16]. Storage of DBS samples is often simplified due to the shape and size of the samples, with room temperature storage suitable for most samples [17].

The only published method available for the quantification of enalaprilat and perindoprilat in DBS is that of Peeters et al. [18], who quantified eight antihypertensive medications and four active metabolites (which included both enalaprilat and perindoprilat) in DBS. Whatman protein saver 903 cards (Cardiff, United Kingdom) were used for DBS sampling, with a 6-mm diameter sample punched from the DBS sample. An acetonitrile and methanol mixture (1:1) containing internal standard (ISTD) was used for extraction. Quantification took place via UHPLC-MS/MS with the calibration ranges being 4.54–454 μg/L and 5–500 μg/L for enalaprilat and perindoprilat, respectively. Mazzarino et al. [19] developed an Initial Testing Procedure (ITP) that allows for the screening of 235 analytes, including carvedilol, in DBS. The limit of detection (LOD) for carvedilol is 0.5 ng/mL for this method. A method describing quantifying carvedilol using volumetric absorptive microsampling (VAMS) has also been published [20]. The feasibility of quantifying carvedilol and other antihypertensive drugs using 10 μL of Mitra® VAMS was evaluated. The LLOQ was validated at 4 ng/mL. However, the method was not sensitive enough to quantify expected trough concentrations of patients involved in a proof of concept study [20].

The method described here is, as far as we know, the first to allow for the simultaneous quantification of carvedilol, enalaprilat, and perindoprilat in DBS. Paired plasma and DBS samples were analysed from a pharmacokinetic study using the validated DBS method and our previously validated plasma method [10]. Plasma sample concentrations were predicted from DBS sample concentrations using Deming regression and the differences between the derived and observed plasma samples were evaluated using Bland-Altman plots. The goal is to help generate adherence data of African HF patients using the assay developed.

2. Materials and methods

2.1. Sample collection and storage

Whole blood was drawn via venepuncture from consenting subjects not on carvedilol, enalapril or perindopril. DBS cards were prepared by using Whatman 903 Protein Saver Cards (GE Healthcare, Chicago, USA); 50 μL of blood was dispensed accurately via the wet tip pipetting technique. The DBS cards were then allowed to dry completely for 2 h at room temperature out of direct sunlight, and then stored at approximately −80 °C with 3 × 1 g desiccant sachets in a sealable plastic bag.

2.2. Chemicals

All reference standards and ISTDs were sourced from Toronto Research Chemicals Inc. (Toronto, Canada) in powder form. Methanol and acetonitrile (LC-MS grade) was supplied by Anatech (Bellville, South Africa), with proanalysis grade acetic acid purchased from Labchem (Johannesburg, South Africa). Sigma-Aldrich (Modderfontein, South Africa) supplied ammonium acetate (≥99.99 %).

2.3. Extraction procedure

Single 50 μL spotted DBS samples were punched out (12 mm punch size) and transferred into 2.0 mL microcentrifuge tubes after which 200 μL water was added to the DBS discs and vortex mixed for 30 s. One millilitre of precipitation solution (methanol:acetonitrile (1:1, v/v)) containing the ISTD (0.500 ng/mL of carvedilol-d5, 2.00 ng/mL of enalaprilat-d5, and 1.00 ng/mL of perindoprilat-$^{13}C_3$) was then added. Samples were vortex mixed for a further 30 s and centrifuged for 5 min at 20,238 g. The resulting supernatant was transferred to glass tubes and evaporated under a gentle nitrogen stream for approximately 15 min at 40ºC. Reconstitution solution (200 μL) was added to the samples after drying. The reconstitution solution consisted of methanol:water:formic acid (40:60:0.2, v/v/v). The samples were then dissolved by vortex mixing for 30 s. The total volume of the reconstituted extracts was transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 min at 20,238 g. The supernatants were placed in the LC-MS/MS autosampler after being pipetted into 96 well plates. The autosampler temperature was approximately 8ºC and 5 μL of sample was injected.

LC-MS/MS equipment and conditions

LC-MS/MS and chromatographic conditions similar to our previously developed plasma assay was used [10]. Electrospray ionization (ESI) in the positive ionisation mode was employed for detection on an AB Sciex API 5500 Qtrap mass spectrometer (AB Sciex™, Germany). Product ion scans of each analyte and ISTD were executed to fine-tune mass spectrometer parameters. The collision gas parameter was adjusted to the "medium" level with the nebuliser gas setting at 55 psi. Turbo and curtain gases were set at 55 psi and 30 psi, respectively. The ion spray voltage was adjusted to 5500 V and the source temperature to 500 °C. Mass transitions included 407.1 > 100.1, 349.1 > 206.1, 341.2 > 170.1, 412.2 > 105.1, 354.2 > 211.0, and 344.2 > 100.1 for carvedilol, enalaprilat, perindoprilat, carvedilol-d5, enalaprilat-d5, and perindoprilat-^{13}C3, respectively. The collision energies were 37, 27, 45, 39, 27, and 47 eV for carvedilol, enalaprilat, perindoprilat, carvedilol-d5, enalaprilat-d5, and perindoprilat-^{13}C3, respectively. Data was collected and interpreted using Analyst Version 1.7.1 (AB Sciex™, Germany).

A Restek Ultra Biphenyl column (100 mm × 2.1 mm, 3 μm) was used for chromatographic separation. The column temperature was set at 40 °C. The mobile phase (300 μL/min) was introduced with an Agilent

Table 1
Accuracy and precision results for carvedilol, enalaprilat, and perindoprilat QCs.

Validation experiment	Sample tested	N	Carvedilol		Enalaprilat		Perindoprilat	
			Precision CV(%)	Accuracy (%Nom)	Precision CV(%)	Accuracy (%Nom)	Precision CV(%)	Accuracy (%Nom)
Day 1, 2, and 3	QC LLOQ	*17	9.1	86.5	9.4	85.1	6.7	92.3
	QCL	18	8.7	100.6	13.0	95.9	12.8	100.4
	QCM	18	3.1	102.3	5.2	99.0	5.1	100.3
	QCH	18	3.7	109.4	4.3	106.1	4.0	108.4
	QC DIL	6	11.5	93.8	13.5	85.6	13.6	88.1

Concentrations (ng/mL): QC DIL = 400, QCH = 160, QCM = 80, QCL = 2.00, QC LLOQ = 1.00.
QCL- Quality control low.
QCH- Quality control high.
QCM- Quality control medium.
QC DIL- Quality control dilution.
QC LLOQ- Quality control lowest level of quantification.
*One QC removed due to bench/experimental error.

Table 2
Stability summary of carvedilol, enalaprilat, and perindoprilat.

Validation experiment	Sample tested	N	Carvedilol		Enalaprilat		Perindoprilat	
			Precision CV(%)	%Difference	Precision CV(%)	%Difference	Precision CV(%)	%Difference
Stock solution stability	Room Temperature[a]	3	4.5	2.9	0.2	−2.6	3.4	7.3
	~62 days at ~−80 °C	3	1.1	1.4	2.8	−0.6	2.2	0.6
Working solution stability	**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 h	6	2.2	5.4	2.4	5.9	2.6	5.3
	*0.0025 μg/mL at RT for ~4 h	6	1.7	−6.3	11.1	−4.3	3.2	−5.9
Matrix stability	QCL at ~−80 °C for 124 days	6	10.7	1.7	5.3	14.3	6.0	−5.2
	QCH at ~−80 °C for 124 days	6	5.3	4.5	2.8	5.6	3.2	−6.5
Freeze and thaw stability	QCL-3 F/T Cycles	6	3.4	9.4	3.7	7.8	1.7	9.4
	QCH- 3 F/T Cycles	***5	4.4	11.0	2.5	4.0	2.4	11.6
Benchtop stability	QCL-RT for 12 hrs	6	9.2	5.8	8.1	10.3	10.3	6.7
	QCH-RT for 12 hrs	6	5.2	10.8	3.4	2.5	5.2	9.3

[a]Carvedilol and perindoprilat 6 h stability at room temperature. Enalaprilat 24 h stability at room temperature.
*Lowest working solution concentration: 0.0025 μg/mL; **Highest working solution concentration: 0.500 μg/mL.
***One QC removed due to bench/experimental error.

Table 3
Carvedilol, enalaprilat, and perindoprilat matrix effects summary.

Analyte	Peak Area Ratio (%CV)			*Area ratio vs concentration regression slope (%CV)
	QCH	QCM	QCL	
Carvedilol	5.3	7.1	5.5	5.5
Enalaprilat	4.0	7.8	6.9	4.3
Perindoprilat	3.8	9.2	7.6	4.0

Concentrations (ng/mL): QCH = 160, QCM = 80, QCL = 2.00.
*Regressions are generated using the area ratios at each concentration level.

1260 Infinity II binary pump and an Agilent 1200 Autosampler (Agilent, CA, USA) was used for sample injection (autosampler temperature ~8 °C). Mobile phase A (aqueous) was made up of 5 mM ammonium acetate and 0.1 % acetic acid in water, with mobile phase B (organic) consisting of a mixture of water, methanol, and acetonitrile (10:20:70, v/v/v). Mobile phase B buffer also consisted of ammonium acetate (5 mM) paired with acetic acid (0.1 % (v/v)). Gradient elution was employed with the initial mobile phase composition (5 % B) increased to 90 % B linearly over a 30 s interval. The mobile phase was held at 90 % B for 2.5 min, after which it was reduced back to 5 % B in 0.1 min. A 3.4-minute equilibration period preceded the next injection [10].

2.5. Analytical validation

2.5.1. Preparation of quality controls, standards, and ISTDs

Published assays quantifying analyte concentrations in DBS show a preference for preparing DBS standards and QCs by first spiking working solutions into whole blood. These whole blood standards and QCs are then spotted onto the DBS cards to create the DBS standards and QCs [21–26]. In the initial attempts in developing the method, DBS standards and QCs were made by spiking working solutions directly into whole blood to prepare whole blood standards and QCs, which were then spiked onto DBS cards to prepare the DBS standards and QCs. The coagulation of fresh blood upon contact with the organic working solution made preparing standards and QCs impractical. To make the assay more practical, standards and QCs were prepared by spiking the working solutions directly onto blank DBS spots [27,28]. For this study, spots were first punched and placed in 2 mL Eppendorf tubes. The working solution was then spiked onto the spots after being placed in the Eppendorf tube, as this then allowed for the spot to be the only area that the working solution could diffuse across. Initially, spiking was performed prior to punching, but this allowed opportunity for the working solution to diffuse outside the radius of the spot [29]. The preparation of the standards and QCs is discussed in detail below:

Stock and working solutions were prepared in methanol and stored at ~−80 °C. All reference and ISTD stock solutions had a concentration of 1000 μg/mL, except enalaprilat-d5, which was 5000 μg/mL. Twenty μL of carvedilol, enalaprilat, and perindoprilat were spiked into 39.94 mL and 19.94 mL of methanol for the preparation of calibration

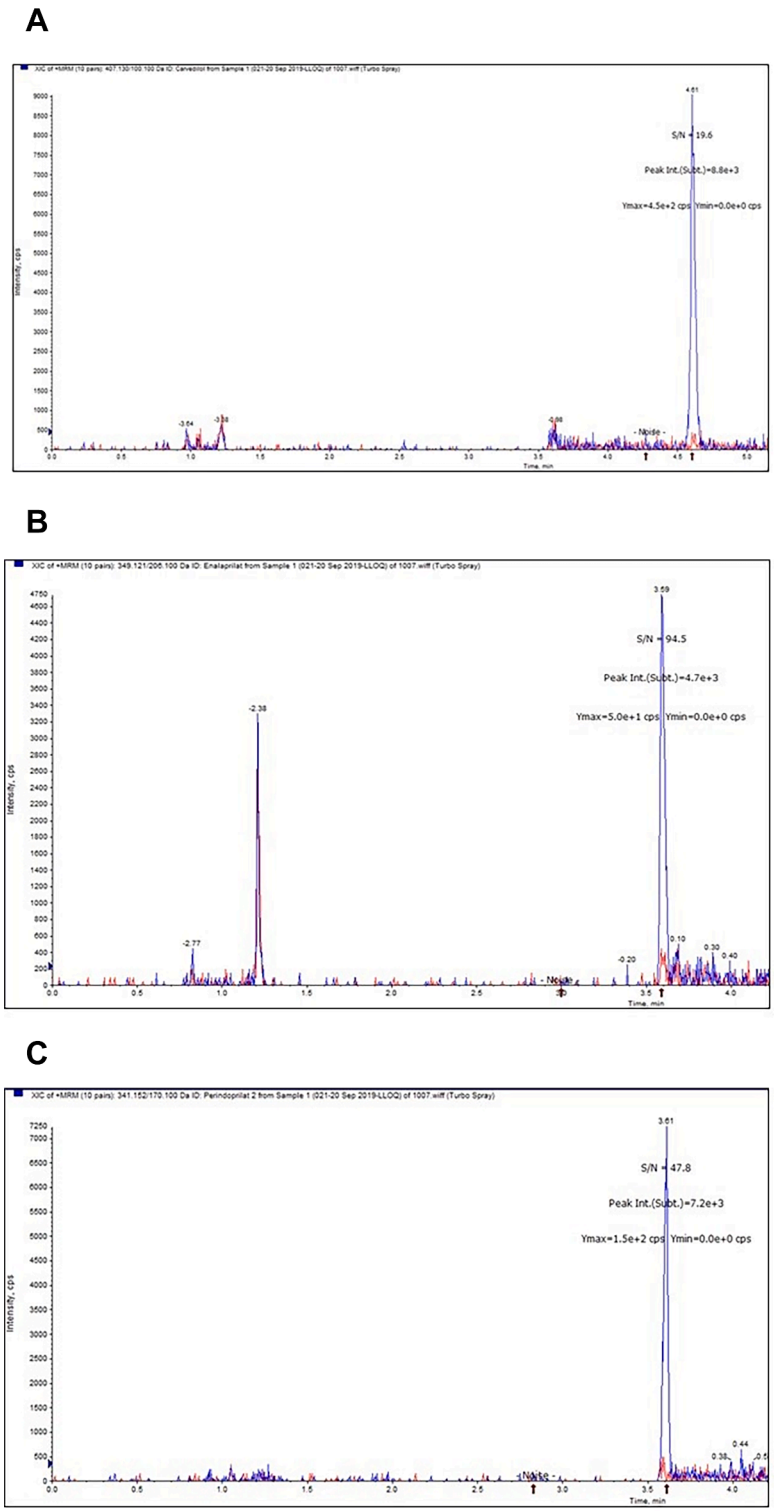


Fig. 1. Chromatograms of (A) carvedilol (S/N = 19.6), (B) enalaprilat (S/N = 94.5), and (C) perindoprilat (S/N = 47.8) at LLOQ (blue) overlaid with corresponding blank chromatograms (red).

standards and QC working solutions, respectively. From these solutions, working solutions were volumetrically prepared.

Blank DBS (50 μL whole blood) were punched out and placed in 2 mL Eppendorf tubes. Standards were prepared for each analytical run by spiking the blank DBS with 20 μL working solution covering the range from 1.00 ng/mL to 200 ng/mL. The DBS spots were allowed to dry after spiking. QCs were prepared by spiking punched out blank DBS (50 μL whole blood) in 2 mL Eppendorf tubes with 20 μL working solution to obtain the concentrations of 1.00 (LLOQ), 2.00 (QL), 80.0 (QM), and 160 (QH) ng/mL, with the spots allowed to dry after spiking.

2.5.2. *Stability of stock and working solutions*

The stability of carvedilol, enalaprilat, and perindoprilat stock solutions was assessed at ~−80 °C and at room temperature. The UV absorbances of stored solutions were compared with freshly prepared solutions on a standalone Cary 60 UV–vis Spectrophotometer (Agilent,

Table 4
Characteristics of patients and their corresponding dosages of carvedilol, enalapril and perindopril (Adapted from Joubert et al. [10] CC BY 4.0).

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: Carvedilol and enalapril were taken in combination at the dosages shown in the table for the study's first phase. For the second phase, patients were switched to a carvedilol and perindopril combination at the dosages shown in the table.

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 [10].

To evaluate the working solution stability of carvedilol, enalaprilat, and perindoprilat, both freshly prepared and stored working solutions were diluted with reconstitution solution containing carvedilol-d5, enalaprilat-d5, and perindoprilat-^{13}C3. Working solution stability was assessed at both ~−80 °C and room temperature on the LC-MS/MS.

2.5.3. Reinjection reproducibility and on-instrument stability

Samples from a validation batch remained in the autosampler at 8 °C for a period of a 140 h, after which the analytical run was reinjected in its entirety so that reinjection reproducibility could be assessed. Absolute autosampler stability was evaluated by comparing the peak area ratios of the reinjected low (2.00 ng/mL) and high (160 ng/mL) QCs against that of the initial low (2.00 ng/mL) and high (160 ng/mL) QC peak area ratios. This was repeated six times.

2.5.4. Matrix stability

To evaluate the stability of carvedilol, enalaprilat, and perindoprilat in the DBS matrix, low (2.00 ng/mL) and high (160 ng/mL) QCs were stored for 124 days at ~−80 °C. The stored QCs were analysed in six replicates against a fresh calibration curve.

2.5.5. Freeze-thaw stability

Low (2.00 ng/mL) and high (160 ng/mL) QCs were frozen at ~−80 °C and subsequently exposed to three consecutive freeze–thaw cycles. Each cycle consisted of a 4-hour room temperature thawing period, followed by a 16-hour freezing period. The QCs subjected to the freeze–thaw cycles were evaluated against a fresh calibration curve.

2.5.6. Bench-top stability

Bench-top stability was evaluated by placing previously frozen low (2.00 ng/mL) and high (160 ng/mL) QCs on bench for a period of 12 h. These test samples were analysed against a freshly prepared calibration curve. The mean observed concentration at each QC level was compared to the nominal QC concentration.

2.5.7. Recovery

Low (2.00 ng/mL), medium (80.0 ng/mL), and high (160 ng/mL) QCs were each extracted in six replicates. These served as the test samples. Reference samples were prepared by spiking into the extracted blank matrix at each concentration level in six replicates. Each analyte's recovery was then evaluated by comparing the peak area ratios of the test and reference samples.

2.5.8. Process efficiency

To determine process efficiency, a comparison was made between the instrument response of extracted samples and that of unextracted neat samples. QC samples were prepared in six different lots of matrix at low (2.00 ng/mL), medium (80.0 ng/mL), and high (160 ng/mL) concentrations and extracted according to the method standard operating procedure (SOP). 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. The percentage process efficiency was calculated by comparing peak area ratios of the analytes after extraction to those of the analytes in the neat samples.

2.5.9. Matrix effects

Matrix effects were quantified across the calibration range of the assay [30]. Blank DBS spots from six different sources were extracted and spiked at the high (160 ng/mL), medium (80 ng/mL), and low (2.00 ng/mL) concentrations. Regressions were generated for each individual matrix using the peak area ratios of the analyte / ISTD for each level.

2.5.10. Selectivity and carry-over

Blank DBS spots from six different sources were analysed (without ISTD) to evaluate the analytical method's ability to discern between the analytes of interest and other components inherent to the matrix. Carry over was monitored by positioning a double blank sample immediately after the highest calibration standard in the injection sequence. Double blank samples were inspected for the presence of any analyte peaks.

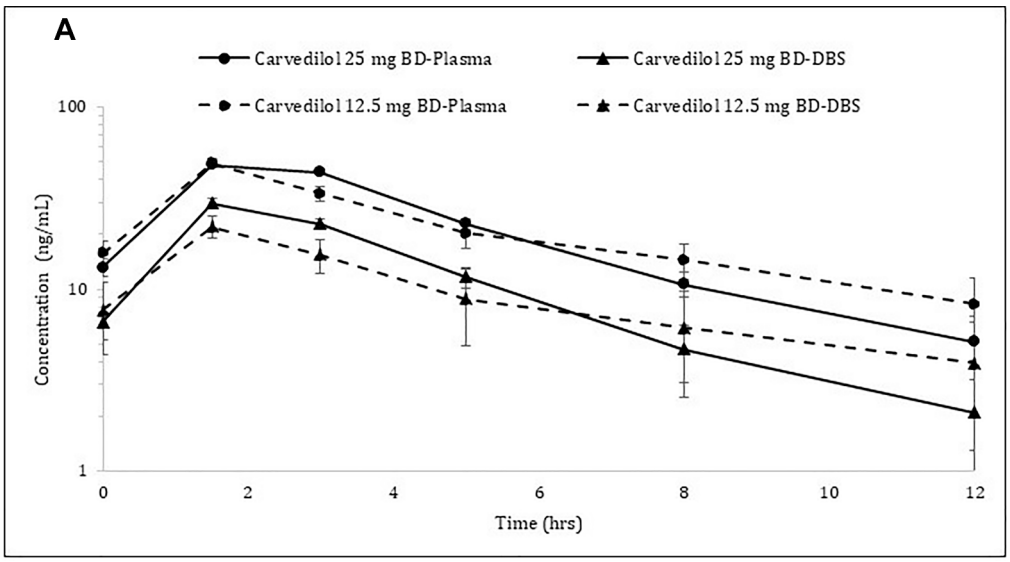
2.5.11. Cross-talk

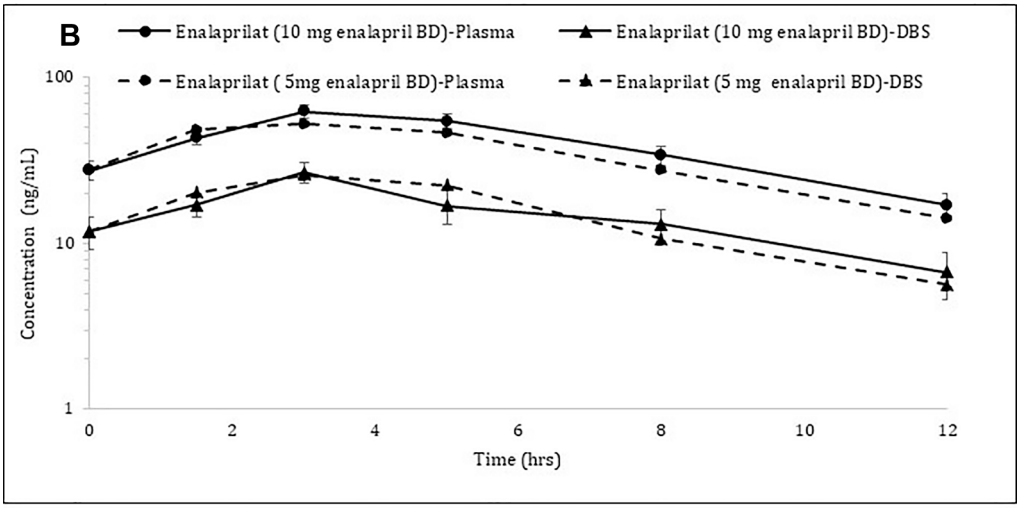
Cross-talk between the multiple reaction monitoring (MRM) channels of analytes and ISTDs were assessed. Additionally, due to the assay being multiplexed, each of the analytes was evaluated at the upper limit of quantification (ULOQ) to confirm that it did not appear in other analyte channels. Although they are not quantified in the assay, any contribution that could occur from enalapril, perindopril, and perindoprilat glucuronide (perindopril metabolite) was also determined.

2.6. Clinical validation of assay

2.6.1. Pharmacokinetic study design and size

The DBS assay described in this manuscript, as well as the plasma assay published by our research group, were used to analyse paired plasma and DBS clinical samples. The paired samples were collected from participants who were part of a pharmacokinetic pilot study [10]. Approval to conduct the study (HREC/REF: 480/2018) was given by the Faculty of Health Science's Research Ethics Committee (University of Cape Town). Participants were recruited from the Groote Schuur hospital HF clinic. All participants were stable on HF medication consisting of enalapril (5 mg or 10 mg taken twice daily) and were also taking carvedilol (12.5 mg or 25 mg twice daily). Participants were admitted overnight. Blood samples were collected via venepuncture before the administered dose (pre-dose) and at 1.5, 3, 5, 8, and 12 h after the dose





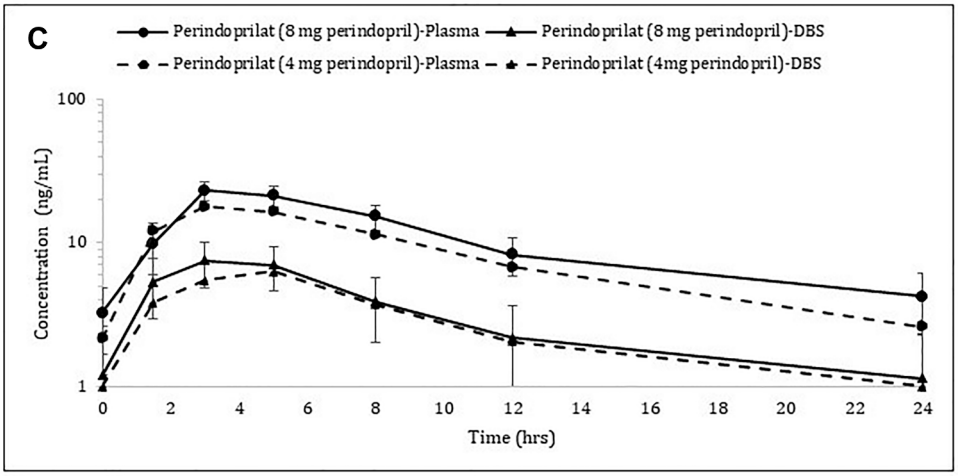


Fig. 2. Semi-logarithmic plots of geometric mean of: (A) 25 mg BD carvedilol plasma and DBS samples (N = 3) and 12.5 mg BD carvedilol plasma and DBS samples (N = 3), (B) enalaprilat (10 mg BD enalapril) plasma and DBS samples (N = 5) and enalaprilat (5 mg BD enalapril) plasma and DBS samples (N = 1) 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 that were quantified to be below LLOQ were made equal to LLOQ.

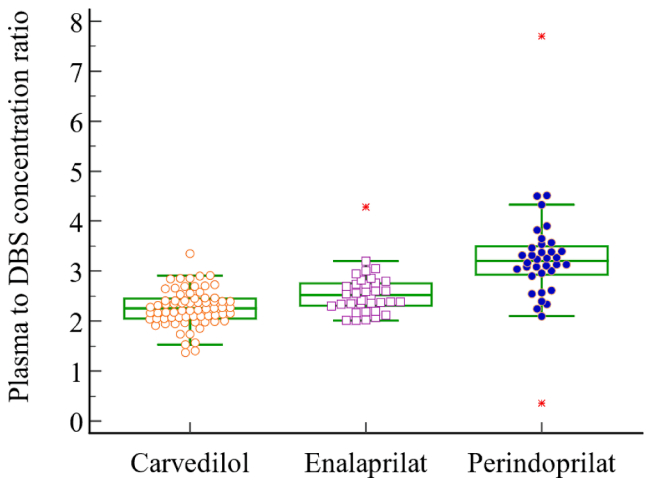


Fig. 3. Box-and-whisker plots of plasma to DBS concentration ratios for carvedilol, enalaprilat, and perindoprilat.

was administered. Once sampling was completed, participants were switched from enalapril to perindopril for the next phase of the study. Participants who were on 10 mg/day enalapril were switched to a 4 mg/day perindopril, while those who were on 20 mg/day enalapril were switched to 8 mg/day perindopril. After participants were switched to the carvedilol and perindopril regimen, blood samples were taken again after 4 weeks. Blood samples were drawn at the same time intervals as when patients were on enalapril, except an additional time point was added at 24 h since perindopril was taken once, not twice, a day [10].

2.6.2. Statistical analysis

Outliers and samples below the LLOQ from the study were excluded from the correlation analysis [18]. Outliers were determined by evaluating the ratios of each of the paired plasma and DBS samples for each analyte. Ratios smaller than 1.5 times the interquartile range of the ratios or >1.5 times the interquartile range of the ratios were considered outliers [31].The relationship between the analyte concentrations in the DBS and plasma was evaluated using Deming regression [32]. The known measurement errors in y and x, which were entered into the Deming regression, were the co-efficient of variation from the inter-assay variations during validation for the plasma and DBS assays, respectively. Regression slopes were calculated with a standard error (SE) and a 95 % confidence interval (95 % CI), with the Pearson correlation coefficient (r) used to describe the correlation between sampling methods. Statistical analyses were done using Medcalc® statistical software version 20.013 (MedCalc Software, Ostend, Belgium). Plasma concentrations were predicted from observed DBS concentrations using the calculated Deming fit between the observed plasma and observed DBS concentrations with the following equation [33,34]: $PredPlasma = m \bullet C_{DBS} + b$.

Where PredPlasma and C_{DBS} represent the predicted plasma concentrations and observed DBS concentrations, respectively, with b and m being the constant and proportional bias, respectively, obtained from the Deming regression for each analyte [34]. The acceptance criteria for the agreement between the observed and derived plasma concentrations were based on the European Medicines Agency (EMA) guidelines; the difference between the observed and calculated plasma concentrations should be within 20 % of the mean for at least 67 % of the samples [35]. The difference between observed and derived plasma concentrations was expressed using Bland-Altman plots, which were drawn using Medcalc® statistical software version 20.013 (Ostend, Belgium).

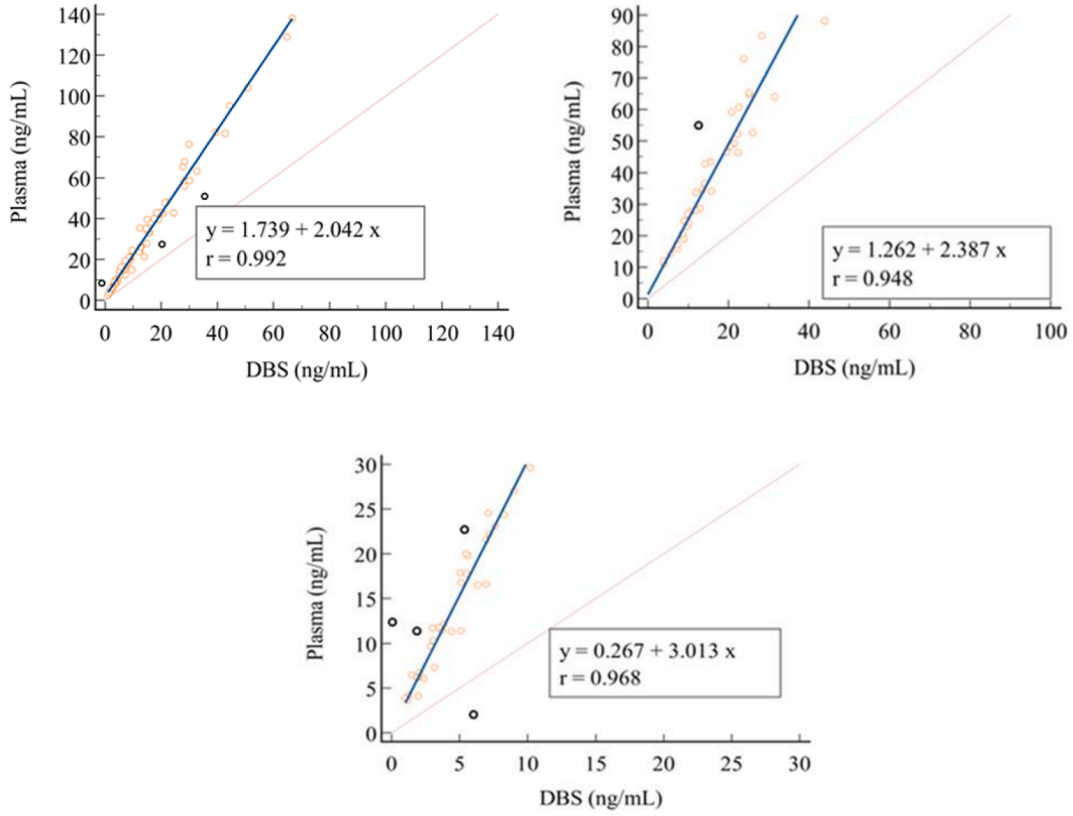


Fig. 4. Deming regression for (A) carvedilol (N = 67), (B) enalaprilat (N = 34), and (C) perindoprilat (N = 32). Pearson's coefficient represented as r. Data points designated as outliers are shown as black circles. These data points do not contribute to the Deming regression as they were removed from the data set prior to analysis.

Table 5
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.

Table 6
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

3.Results and discussion

3.1. Analytical validation

A method was developed and validated for the quantification of carvedilol, enalaprilat, and perindoprilat in DBS using protein precipitation. A previous method was developed and validated quantifying the same analytes in plasma [10], which was subsequently adapted for DBS quantification. During method development it was found that, as with the plasma method, an acetonitrile and methanol mixture (1:1, v/v) gave satisfactory extraction, provided that the extraction volume is increased from 0.2 to 1.00 mL to allow for the complete submersion of the punched-out spot.

The same mobile phase composition and reverse phase gradient method that was used for the plasma method was implemented for the DBS method [10], with the organic mobile phase increased linearly to 90 % (from an initial composition of 5 %) over a 30 s interval. The effect of hyperlipidaemia was not tested for during validation, but the chromatographic method does mitigate any effect it might have. Triglycerides and cholesterol are both non-polar lipid substances [36]. Very non-polar compounds such as triglycerides and cholesterol are removed during the equilibration period of 3.4 min.

Protonated molecular ions were shown by full scan positive mass spectra to be at *m/z* 412.2, *m/z* 354.2, and 344.2 for carvedilol-d5, enalaprilat-d5, and perindoprilat-$^{13}C_3$ respectively, with that of carvedilol, enalaprilat, and perindoprilat being at *m/z* 407.1, *m/z* 349.1, and *m/z* 341.2, respectively. Upon collision induced dissociation, the most abundant product ions were at *m/z* 100.2, *m/z* 91.0, *m/z* 98.1, *m/z* 105.2, *m/z* 96.0, and *m/z* 100.2 for carvedilol, enalaprilat, perindoprilat, carvedilol-d5, enalaprilat-d5, and perindoprilat-$^{13}C_3$, respectively [10].

Quadratic regressions (weighted by $1/x^2$ for all analytes, x = concentration) fit the calibration curves over the range of 1.00–200 ng/mL for all three analytes. Table 1 provides a summary of both the accuracy and precision data generated from three validation batches for all QCs. Accurate concentrations were produced over the three successive runs. The three analytes had percentage accuracies (%Nom) ranging from 93.1 % to 104.8 % and 85.1 to 109.4 for standards and QCs, respectively, while precision (CV%) statistics for the three analytes were revealed to all be less than 15 %.

Methanol stock solutions of carvedilol, enalaprilat, and perindoprilat were shown to be stable for ~62 days when stored at ~−80 °C (Table 2). Stock solutions stored at room temperature showed 6 h carvedilol and perindoprilat stability in methanol, while enalaprilat proved to be stable for a 24 h period [10]. Working solution stability in methanol was demonstrated for all three analytes for a period of 125 days at ~−80 °C. This was demonstrated at both the lowest (0.0025 μg/mL) and highest (0.5 μg/mL) working solution concentrations. Working solutions proved to be stable for a period of four hours at room temperature for all analytes, at both the lowest and highest working solution concentrations.

Both reinjection reproducibility and on-instrument stability were evaluated for carvedilol, enalaprilat, and perindoprilat to assess if an analytical run can be reinjected, should an instrument interruption occur. It was shown that all analytes can be reinjected within approximately 140 h. Stability was proven in the DBS matrix for three freeze–thaw cycles for all three analytes (Table 2). The refrozen duration consisted of 16 h with a corresponding thaw period of 4 h. All three analytes showed long term matrix stability of 124 days when stored at ~−80 °C. Analytes were shown to be stable in the DBS matrix for 12 h when stored on-bench, with the difference from that of the nominal concentration across the high and low concentrations all <15 %. During the clinical study, both the DBS and plasma samples of a patient were taken, with DBS and plasma samples stored and transported together for logistical efficiency. Hence, the DBS would have been transported at temperatures required for plasma transportation, not ambient temperatures. Therefore, the stability conditions evaluated in DBS, for the three analytes, during validation are sufficient to cover the study transportation conditions. The 12-hour on-bench stability indicated for the analytes in the DBS is also sufficient to cover the period that samples might spend on bench after they have been created. However, the eventual aim when determining adherence of HF patients is to limit plasma sampling, as much as possible, due to the resource and logistical burden of generating and transporting plasma samples in isolated and resource-scarce settings. Therefore, further stability experiments must be carried out at ambient and higher (>30 °C) temperatures to simulate conditions when transport and storage temperatures are not tightly controlled. In the future, this testing will be done through incurred sample reanalysis of patient DBS samples from the clinical study to allow for authentic matrix composition.

Matrix effects were minimal, with only carvedilol displaying a slope variability of slightly >5 % across the six different matrix sources (Table 3) [30]. Matrix effects using QCs that were prepared from pre-spiked whole blood were not evaluated. However, no matrix effects were observed during the validation of our plasma assay, where QCs were prepared by spiking directly into the plasma [10].Average extraction recoveries across the high, medium, and low concentrations were calculated to be 120.6 %, 114.5 %, and 109.7 % for carvedilol, enalaprilat, and perindoprilat, respectively, indicating that the analytes are completely extracted from the matrix. The corresponding average process efficiencies of the method were calculated to be 102.4 %, 119.5 %, and 109.3 % for carvedilol, enalaprilat, and perindoprilat, respectively. Fig. 1 shows blank chromatograms overlaid with the chromatograms at LLOQ for each analyte. The signal-to-noise ratio was above that of the accepted criteria (S/N > 5) for all of the analytes [37].

Carryover was problematic for all three analytes. Blank extracted DBS was injected between each sample to address this issue. Cross-talk

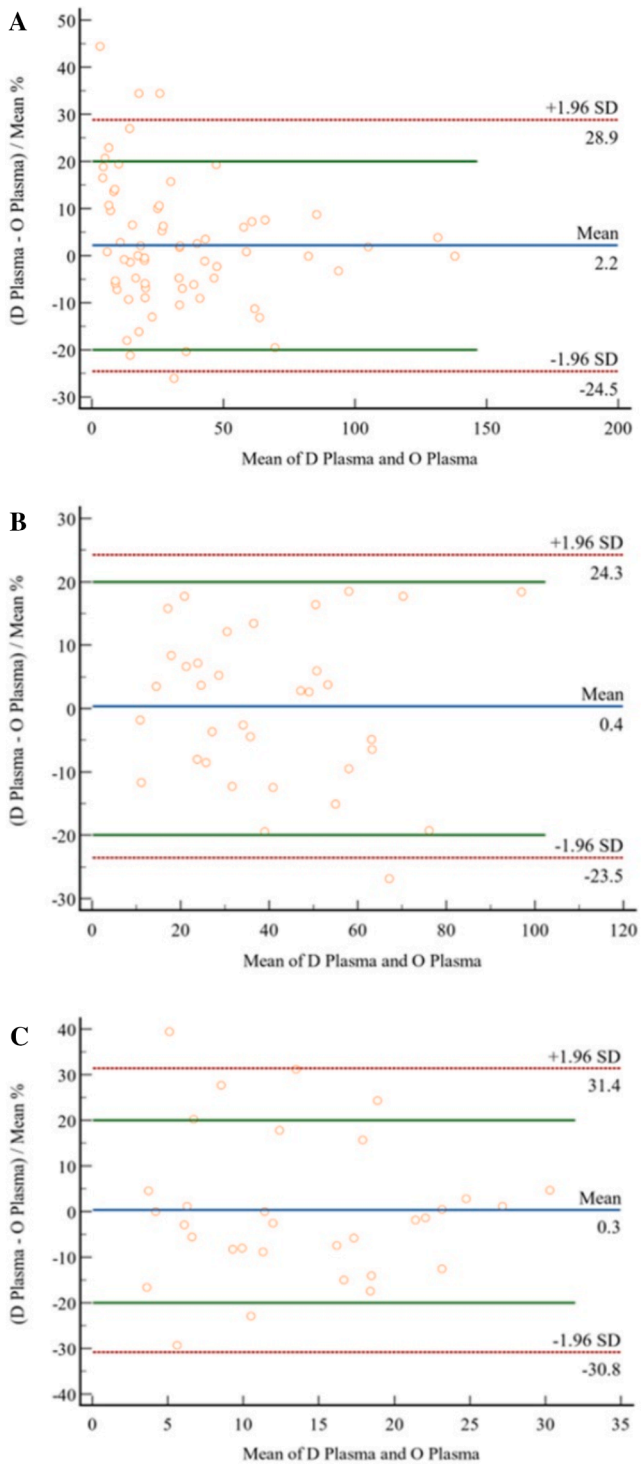


Fig. 5. Bland-Altman plots of derived plasma (D plasma) and observed plasma (O plasma) concentrations respectively for (A) carvedilol, (B) enalaprilat, and (C) perindoprilat. The solid green lines on either side of the solid blue line (mean % error) represents the 20% acceptable bias.

experiments revealed the formation of perindoprilat from perindoprilat-glucuronide sample injection. This was found to occur due to in-source formation of perindoprilat, as opposed to technical cross-talk. The ability to separate the perindoprilat and perindoprilat-glucuronide peaks allowed for the interference of perindoprilat to be controlled.

Table 7
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 %.

3.2. Clinical validation

The validated method was applied to a pharmacokinetic study to assess the correlation between DBS and plasma concentrations and evaluate the feasibility of using the DBS assay as a measure of adherence.

Table 4, adapted from Joubert *et al.* [10], gives a summary of the baseline characteristics and dosages of the study participants. Six participants (three Male, three Female) with an average age of 41.0 ± 6.3 years completed the study. The New York Heart Association Functional Classification (NYHA-FC) of the participants ranged from 1 to 3, with the bulk of the patients (4), having a NYHA-FC of 2. The average body mass index (BMI) of the participants was 35.2 ± 7.6 kg/m^2. Three patients were obese (BMI ≥ 30 kg/m^2) and two were morbidly obese (BMI ≥ 40 kg/m^2) [38,39]. Semi-logarithmic plots of the paired DBS and plasma concentrations vs time profiles are shown in Fig. 2. Fig. 2 does not show a proportional increase in concentration with dose, as expected. The majority of patients who participated in the study were clinically obese. Obesity can have important effects on key pharmacokinetic parameters [40]. Both obesity and the small number of participating patients, are factors that could have resulted in a non-linear dose and analyte concentration relationship.

The paired plasma and DBS concentration ratios with corresponding outliers are shown in Fig. 3. Fig. 4 shows the Deming regressions for carvedilol, enalaprilat, and perindoprilat. Table 5 is a summary of the Deming regression parameters. The Pearson correlation coefficient (r) was 0.992, 0.948, and 0.968 for carvedilol, enalaprilat, and perindoprilat, respectively, showing acceptable correlations between DBS and plasma concentrations for all analytes. The lowest plasma concentrations that can be derived from DBS concentrations can be calculated by substituting the DBS LLOQ (1.00 ng/mL) into the Deming regression equations obtained. From these, the lowest plasma concentrations that can be calculated from observed DBS concentrations are 3.78, 3.65, and 3.28 ng/mL for carvedilol, enalaprilat, and perindoprilat, respectively (Table 6).

Observed DBS concentrations were substituted into the Deming regression equations for each analyte, thus correcting for bias, to obtain the corresponding derived plasma concentrations. The extent of agreement between derived plasma concentrations from DBS concentrations and the actual observed plasma concentrations were evaluated with the use of Bland-Altman plots. Fig. 5 and Table 7 show the results of the Bland-Altman analysis. The difference between the observed and calculated plasma concentrations was less than 20 % of the mean, for 88.1 %, 97.1 %, and 78.1 % for paired carvedilol, enalaprilat, and perindoprilat plasma and DBS samples, respectively. All analytes thus fall within the accepted criteria (i.e., the difference between paired samples with ±20 % of mean of paired samples for >67 % of samples) [35]. Peeters et al. [18] compared correlations between DBS and plasma samples for both enalaprilat and perindoprilat. The difference between paired DBS and plasma samples was within ±20 % of the mean for 67 % and 31.6 % of their enalaprilat and perindoprilat samples, respectively. The LLOQs of the assay developed by Peeters et al. [18] were higher compared to this study (i.e., 4.54 ng/mL and 5.00 ng/mL for enalaprilat and perindoprilat, respectively), with many of their DBS samples having to be removed before statistical analysis due to lower than LLOQ concentrations.

Some difficulties associated with DBS analysis include the variation

in the physio-pathological state of the patients, which affects the haematocrit [15]. Predicting plasma concentrations from DBS samples is complicated by the variation in haematocrit that can lead to varying spot sizes, which could cause bias [41]. Having to extract the analyte from a paper sample also adds variability and complexity when compared to that of plasma samples. A well-known effect of a varying haematocrit is that it can affect the blood viscosity, which in turn influences the manner in which blood is distributed on filter paper [41]. An increased haematocrit leads to lower viscosity, forcing a reduced distribution of blood across the paper and a smaller DBS spot, and can also cause differential analyte distribution across the spot. Therefore, partial punching could result in poor accuracy. The assay reported here uses the entire spot, therefore, this effect is mitigated [41].

Table 5 indicates the number of DBS patient samples that were below the LLOQ. Both perindoprilat and carvedilol had participant samples below the LLOQ during the pharmacokinetic sampling period, notably at the end of the dosing intervals. Although DBS and plasma samples correlate well for carvedilol and perindoprilat, the LLOQ for the DBS assay of 1.00 ng/mL is a limiting factor to track patient adherence for carvedilol and perindopril. This is true even though the LLOQs of the DBS assay developed is lower than that of previously published assays [18,20]. None of the DBS enalaprilat samples had concentrations below the LLOQ and because enalaprilat's terminal half-life is >30 h, it should be possible to accurately track enalaprilat concentrations in DBS several days after enalapril medication has been terminated [42].

Venous blood pipetted accurately onto the DBS cards was used during validation to allow for controlled validation conditions. An aspect of the study that needs further investigation is the correlation between plasma and capillary blood for carvedilol, enalaprilat and perindoprilat. Capillary samples are created by pricking the finger or heel with a lancet and collecting the blood on the DBS card. The ease of capillary blood sample collection allows patients to collect the samples themselves. Patient self-sampling is critical to realise the full benefits of DBS sampling in remote areas, as this would eliminate the need for trained personnel to take samples. The correlation between plasma and capillary blood could be different than venous blood and plasma due to variation in capillary and venous blood composition [43]. Moreover, the precision of capillary blood DBS samples is likely to be lower than venous DBS samples that have been created by accurate pipetting, making evaluating the accuracy of capillary samples created in a clinical setting important.

3.3. Synergy between plasma and DBS assays

The DBS assay is not stand-alone as it has a corresponding plasma assay [10]. In terms of determining adherence, the DBS concentrations can only be interpreted once converted to plasma concentrations. Pharmacokinetic data for carvedilol and enalaprilat are only available as plasma concentrations, whereas only one study is available with published whole blood pharmacokinetic data for perindoprilat [44]. Plasma concentrations are, therefore, significantly easier to interpret or evaluate in terms of adherence when compared to that of DBS concentrations, especially when modelling is implemented to interpret adherence according to weight and dose.

The fact that the DBS samples, once normalised, can be used interchangeably with the plasma samples, means that some advantages of each assay can be maximised, while some of the disadvantages can be mitigated. DBS samples have the advantage of being more practical in terms of sampling and storage when compared to the plasma samples. The simpler sampling, ease of storage, and less cumbersome transport requirements of the DBS matrix can be taken advantage off by using it as the onsite sampling method. By converting the DBS concentrations to plasma concentrations, concentrations can be interpreted at a deeper level by making use of models based on the large quantity of available pharmacokinetic data. This will only be viable for enalaprilat, however, as the DBS assay is sensitive enough to track adherence for this analyte only.

The cross-validation of the DBS and plasma assay is critical as it helps to bring additional confidence in the DBS assay. The fact that the calibrators were prepared by spiking the working solutions onto blank DBS is, therefore, not a limitation, as the robustness of the DBS assay has been proven by cross validating the assay with a plasma assay using actual clinical samples. Moreover, the absolute concentration of DBS samples is not interpreted. Only once they are converted to plasma concentrations can they be interpreted in terms of adherence. This places less importance on the absolute DBS concentrations and more importance on correlation with the plasma concentrations.

4. Conclusions

A novel multiplex LC-MS/MS assay for the quantification of carvedilol, enalaprilat, and perindoprilat in DBS was developed and validated both analytically and clinically. Correlation of concentrations between paired DBS and plasma samples from a pharmacokinetic study using Deming regression was investigated, with acceptable correlations observed for all three analytes over the concentration range of the pharmacokinetic study. Bland-Altman analysis revealed acceptable agreement between observed plasma and derived plasma concentrations obtained from the Deming regression equations.

Some of the carvedilol and perindoprilat samples fell below the LLOQ of the DBS method at the end of the dosing intervals, indicating that the method's ability to track HF patient adherence to carvedilol and perindopril will be limited. The DBS assay will still be useful as an initial screening tool for carvedilol and perindoprilat, while plasma samples will have to be quantified to further investigate adherence using these analytes if needed. The lowest patient enalaprilat concentrations were substantially greater than the LLOQ, indicating that the DBS method could track enalaprilat concentrations for several days after enalapril has been stopped by the patient.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] G. Lippi, F. Sanchis-Gomar, Global epidemiology and future trends of heart failure, AME Med. J. 5 (2020) 15–15. https://doi.org/10.21037/amj.2020.03.03.
[2] A.A. Ajayi, G.G. Sofowora, G.O. Ladipo, Explaining heart failure hyper-mortality in Sub Saharan Africa: global genomic and environmental contribution review, J. Natl. Med. Assoc. 112 (2020) 141–157, https://doi.org/10.1016/j.jnma.2020.02.003.
[3] V. Ruf, S. Stewart, S. Pretorius, M. Kubheka, C. Lautenschläger, P. Presek, K. Sliwa, Medication adherence, self-care behaviour and knowledge on heart failure in urban South Africa: the Heart of Soweto study, Cardiovasc J. Afr. 21 (2010) 86–92.
[4] S.R. Hood, A.J. Giazzon, G. Seamon, K.A. Lane, J. Wang, G.J. Eckert, W. Tu, M. D. Murray, Association between medication adherence and the outcomes of heart failure, Pharmacother. J. Hum. Pharmacol. Drug Ther. 38 (5) (2018) 539–545.
[5] T.M. Ruppar, P.S. Cooper, D.R. Mehr, J.M. Delgado, J.M. Dunbar-Jacob, Medication adherence interventions improve heart failure mortality and readmission rates: systematic review and meta-analysis of controlled trials, J. Amercian Hear. Assoc. 10 (2016) 1–18, https://doi.org/10.1161/JAHA.115.002606.
[6] D. Shah, K. Simms, D. Barksdale, J.-R. Wu, Improving medication adherence of patients with chronic heart failure: challenges and solutions, Res. Reports Clin. Cardiol. (2015) 87, https://doi.org/10.2147/rrcc.s50658.
[7] N.C. do Carmo Borges, G. Duarte Mendes, D. de Oliveira Silva, V. Marcondes Rezende, R.E. Barrientos-Astigarraga, G. De Nucci, Quantification of carvedilol in human plasma by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry: Application to bioequivalence study, J. Chromatogr. B Anal. Technol. Biomed Life Sci. 822 (1-2) (2005) 253–262.

[8] L.S. Henderson, D.M. Tenero, C.A. Baidoo, A.M. Campanile, A.H. Harter, D. Boyle, T.M. Danoff, Pharmacokinetic and pharmacodynamic comparison of controlled-release carvedilol and immediate-release carvedilol at steady state in patients with hypertension, Am. J. Cardiol. 98 (2006) 17–26, https://doi.org/10.1016/j.amjcard.2006.07.015.
[9] N.K. Sweitzer, What is an angiotensin converting enzyme inhibitor? Circulation. 108 (2003) 1–3, https://doi.org/10.1161/01.cir.0000075957.16003.07.
[10] A. Joubert, T. Kellermann, A. Joubert, M. van der Merwe, J. Norman, S. Castel, K. Sliwa, G. Maartens, P. Sinxadi, L. Wiesner, Simultaneous Determination of Carvedilol, Enalaprilat, and Perindoprilat in Human Plasma Using LC – MS / MS and Its Application to a Pharmacokinetic Pilot Study, Chromatographia. 85 (5) (2022) 455–468.
[11] P. Van Amsterdam, C. Waldrop, The application of dried blood spot sampling in global clinical trials, Bioanalysis. 2 (2010) 1783–1786, https://doi.org/10.4155/bio.10.158.
[12] Neoteryx, neotryx website, (n.d.). https://www.neoteryx.com/microsampling-blog/why-use-dried-blood-tests-vs-wet-blood-tests- (accessed October 14, 2021).
[13] C. Orrell, K. Cohen, R. Leisegang, D.R. Bangsberg, R. Wood, G. Maartens, Comparison of six methods to estimate adherence in an ART-naïve cohort in a resource-poor setting: Which best predicts virological and resistance outcomes? AIDS Res. Ther. 14 (2017) 1–11, https://doi.org/10.1186/s12981-017-0138-y.
[14] K.I. Foerster, A. Huppertz, A.D. Meid, O.J. Müller, T. Rizos, L. Tilemann, W. E. Haefeli, J. Burhenne, Dried-blood-spot technique to monitor direct oral anticoagulants: clinical validation of a UPLC-MS/MS-Based Assay, Anal. Chem. 90 (2018) 9395–9402, https://doi.org/10.1021/acs.analchem.8b02046.
[15] L. Scribel, A.P. Zavascki, D. Matos, F. Silveira, T. Peralta, N. Gonçalves Landgraf, P. Lamb Wink, A.C. Cezimbra da Silva, N. Bordin Andriguetti, L. Loss Lisboa, M. Venzon Antunes, R. Linden, Vancomycin and creatinine determination in dried blood spots: Analytical validation and clinical assessment, J. Chromatogr. B Anal. Technol. Biomed Life Sci. 1137 (2020), 121897, https://doi.org/10.1016/j.jchromb.2019.121897.
[16] M.D. Lim, Review article: dried blood spots for global health diagnostics and surveillance: opportunities and challenges, Am. J. Trop. Med. Hyg. 99 (2018) 256–265, https://doi.org/10.4269/ajtmh.17-0889.
[17] R. Zakaria, K.J. Allen, J.J. Koplin, P. Roche, R.F. Greaves, Advantages and challenges of dried blood spot analysis by mass spectrometry across the total testing process, J. Int. Fed. Clin. Chem. Lab. Med. 27 (2016) 288–317.
[18] L.E.J. Peeters, L. Feyz, E. Hameli, T. Zwart, S. Bahmany, J. Daemen, T. van Gelder, J. Versmissen, B.C.P. Koch, Clinical validation of a dried blood spot assay for 8 antihypertensive drugs and 4 active metabolites, Ther. Drug Monit. 42 (2020) 460–467, https://doi.org/10.1097/FTD.0000000000000703.
[19] M. Mazzarino, L. Di Costanzo, F. Comunita, C. Stacchini, X. De Torre, F. Botre, UHPLC – HRMS Method for the Simultaneous Screening of 235 Drugs in Capillary Blood for Doping Control Purpose : Comparative Evaluation of Volumetric and Non-volumetric Dried Blood Spotting Devices, (2022). https://doi.org/10.1021/acsomega.2c01417.
[20] C.M. Jacobs, M. Kunz, F. Mahfoud, L. Wagmann, M.R. Meyer, Evaluation and analytical applicability of a novel volumetric absorptive microsampling strategy for adherence monitoring of antihypertensive drugs by means of LC-HRMS/MS, Anal. Chim. Acta. 1187 (2021), 339137, https://doi.org/10.1016/j.aca.2021.339137.
[21] D.C.G. Bedor, N.C.T.C. Bedor, J.W.V. Da Silva, G.D. Sousa, D.P. De Santana, F. Garcia-Bournissen, J. Altcheh, B. Blum, F. Alves, I. Ribeiro, Dried blood spot technique-based liquid chromatography-tandem mass spectrometry method as a simple alternative for benznidazole pharmacokinetic assessment, Antimicrob. Agents Chemother. 62 (2018) 1–12, https://doi.org/10.1128/AAC.00845-18.
[22] J. Zheng, L.A. Guida, C. Rower, J. Castillo-mancilla, A. Meditz, B. Klein, B.J. Kerr, J. Langness, L. Bushman, J. Kiser, P.L. Anderson, NIH Public Access (2015) 1–23, https://doi.org/10.1016/j.jpba.2013.08.033.Quantitation.
[23] G. Lawson, P. Patel, H. Mulla, S. Tanna, Dried blood spot sampling with LC-MS analysis for routine therapeutic caffeine monitoring in neonates dried blood spot sampling with LC-MS analysis for, ISRN Chromatogr. 2012 (2012) 1–7.
[24] H.M. Kim, J.H. Park, N.P. Long, D.D. Kim, S.W. Kwon, Simultaneous determination of cardiovascular drugs in dried blood spot by liquid chromatography-tandem mass spectrometry, J. Food Drug Anal. 27 (2019) 906–914, https://doi.org/10.1016/j.jfda.2019.06.001.
[25] Y. Harahap, G.A. Amalia, B.P. Maggadani, Research article analysis of rifampicin in dried blood spots using high performance liquid chromatography, Asian J. Sci. Res. 11 (2018) 232–239, https://doi.org/10.3923/ajsr.2018.232.239.
[26] M.F. Suyagh, G. Iheagwaram, P.L. Kole, J. Millership, P. Collier, H. Halliday, J. C. McElnay, Development and validation of a dried blood spot – HPLC assay for the determination of metronidazole in neonatal whole blood samples, Anal Bioanal Chem. 397 (2) (2010) 687–693.
[27] A.P. Schauer, C. Sykes, M.L. Cottrell, H. Prince, A.D.M. Kashuba, Validation of an LC–MS/MS assay to simultaneously monitor the intracellular active metabolites of tenofovir, emtricitabine, and lamivudine in dried blood spots, J. Pharm. Biomed. Anal. 149 (2018) 40–45, https://doi.org/10.1016/j.jpba.2017.10.030.
[28] B.T. Alquadeib, N.M. Aloudah, A.S. Almurshedi, I.M. Alfagih, B.N. Aldosari, A. S. Almeleky, N.M. Almubyedh, Development and validation of a simple and sensitive LC-MS/MS method for quantification of metformin in dried blood spot its application as an indicator for medication adherence, Int. J. Gen. Med. 14 (2021) 3225–3233, https://doi.org/10.2147/IJGM.S312633.
[29] A.A. Provatas, C.A. King, S.L. Kolakowski, J.D. Stuart, C.R. Perkins, Quantification of polycyclic aromatic hydrocarbons in avian dried blood spots by ultra-performance liquid chromatography with simple liquid extraction and phospholipid solid-phase extraction preparation, Anal. Lett. 50 (2017) 2649–2660, https://doi.org/10.1080/00032719.2017.1302461.
[30] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030, https://doi.org/10.1021/ac020361s.
[31] J.W. Tukey, Exploratory Data Analysis, Addison-Wesley, Reading, Massachusetts, 1977.
[32] R.F. Martin, General deming regression for estimating systematic bias and its confidence interval in method-comparison studies, Clin. Chem. 46 (2000) 100–104, https://doi.org/10.1093/clinchem/46.1.100.
[33] A.E. Kip, H. Rosing, M.J.X. Hillebrand, S. Blesson, B. Mengesha, E. Diro, A. Hailu, J. H.M. Schellens, J.H. Beijnen, T.P.C. Dorlo, Validation and clinical evaluation of a novel method to measure miltefosine in leishmaniasis patients using dried blood spot sample collection, Antimicrob. Agents Chemother. 60 (2016) 2081–2089, https://doi.org/10.1128/AAC.02976-15.
[34] C.C.L.M. Boons, A. Chahbouni, A.M. Schimmel, A.J. Wilhelm, Y.M. den Hartog, J.J. W.M. Janssen, N.H. Hendrikse, J.G. Hugtenburg, E.L. Swart, Dried blood spot sampling of nilotinib in patients with chronic myeloid leukaemia: a comparison with venous blood sampling, J. Pharm. Pharmacol. 69 (2017) 1265–1274, https://doi.org/10.1111/jphp.12757.
[35] EMA, (n.d.). https://www.ema.europa.eu/en (accessed June 11, 2020).
[36] D.S. Robinson, Plasma triglyceride metabolism, J. Clin. Pathol. (1973) 5–10, https://doi.org/10.1136/jcp.s1-5.1.5.
[37] FDA, Bioanalytical Method Validation Guidance, (n.d.). https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry.
[38] N. Yazdani, N. Elahi, F. Sharif, S.V. Hosseini, The comparison of morbid obesity quality of life and body image between surgery and other treatments: A case – control study, J. Educ Health Promot. (2020) 1–7, https://doi.org/10.4103/jehp.jehp.
[39] S.M. Fruh, Obesity: Risk factors, complications, and strategies for sustainable long-term weight management, J. Am. Assoc. Nurse Pract. 29 (2017) 3–14, https://doi.org/10.1002/2327-6924.12510.
[40] L.E.C. De Baerdemaeker, E.P. Mortier, M.M.R.F. Struys, Pharmacokinetics in obese patients, Contin. Educ. Anaesthesia, Crit. Care Pain. 4 (2004) 152–155, https://doi.org/10.1093/bjaceaccp/mkh042.
[41] C. Daousani, V. Karalis, A. Malenović, Y. Dotsikas, Hematocrit effect on dried blood spots in adults: a computational study and theoretical considerations, Scand. J. Clin Lab. Invest. 79 (2019) 325–333, https://doi.org/10.1080/00365513.2019.1622033.
[42] T. Arafat, R. Awad, M. Hamad, R. Azzam, A. Al-Nasan, A. Jehanli, K. Matalka, Pharmacokinetics and pharmacodynamics profiles of enalapril maleate in healthy volunteers following determination of enalapril and enalaprilat by two specific enzyme immunoassays, J. Clin. Pharm. Ther. 30 (2005) 319–328, https://doi.org/10.1111/j.1365-2710.2005.00646.x.
[43] S.M. Kayiran, N. Özbek, M. Turan, B. Gürakan, Significant differences between capillary and venous complete blood counts in the neonatal period, Clin. Lab. Haematol. 25 (2003) 9–16, https://doi.org/10.1046/j.1365-2257.2003.00484.x.
[44] Y. Tao, S. Wang, L. Wang, M. Song, T. Hang, Simultaneous determination of indapamide, perindopril and perindoprilat in human plasma or whole blood by UPLC-MS/MS and its pharmacokinetic application, J. Pharm. Anal. 8 (2018) 333–340, https://doi.org/10.1016/j.jpha.2018.05.004.