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"Look,
there are two kinds of people,
those who talk,
and those who act."
Peter 'Ouwe' Veenhof

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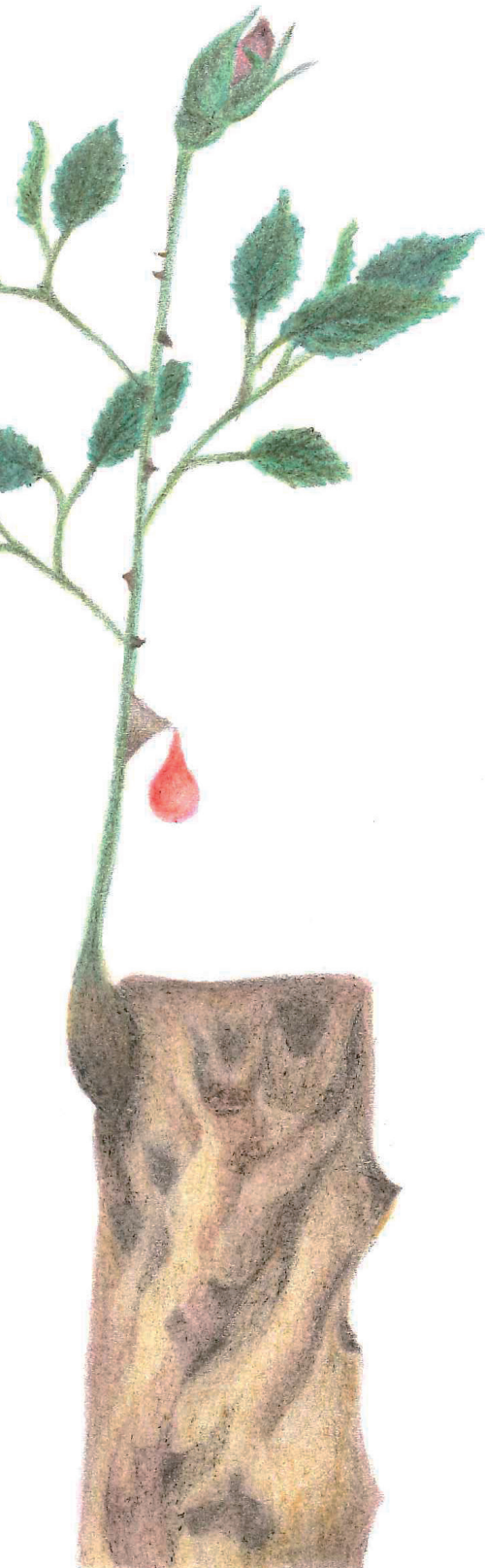

Chapter 1

Introduction and outline

Transplantation

In 1954, the first successful kidney transplantation was performed. Since then, there has been an exponential worldwide growth in numbers of solid organ transplantations, which include kidneys, pancreas, lungs, livers, small intestines and hearts, of which kidney transplantation is performed most frequently. In the UMCG for example, 166 kidney transplantations were performed in 2018 and the total number of solid organ transplantations performed was 293. For patients suffering from end stage renal disease, the risk of premature death for kidney transplant recipients is less than half compared to dialysis patients.[1] Apart from reduced risk of premature death, quality of life is drastically improved for kidney transplant patients. Post-transplantation, patients can be free from symptoms like chronic fatigue, the need of multiple hour dialysis sessions 3 times a week and social isolation due to a chronic condition.[2]

One of the major concerns for kidney transplant patients is rejection of the allograft. Since the cells of the donated kidney differ genetically from the cells from the recipient, the recipients' immune system will perceive the donated organ as foreign and this can trigger an immune response.[3] If this response is not controlled, it will usually lead to the destruction of the transplanted organ.

Immunosuppressive drugs

With the introduction of immunosuppressive drugs, a tool to manage this immune response became available, greatly improving clinical outcomes for transplant patients. Treatment protocols including combinations of several immunosuppressants have reduced the first-year incidence of biopsy-proven acute rejections in kidney transplant recipients to 15% or less.[4] The most widely used immunosuppressant in allograft rejection prevention today is tacrolimus. This drug prevents activation and proliferation of T-cells and thereby reduces the immune response.[5] Usually, tacrolimus combined with mycophenolic acid and sometimes prednisolone is the treatment protocol of choice after transplantation.[1] Other immunosuppressants that are used, either in combination with or instead of tacrolimus are cyclosporin A, sirolimus and everolimus.[5] Because rejection of the transplanted organ is always a threat, treatment with tacrolimus and most other immunosuppressants is lifelong or until reinstallation of dialysis treatment.

Immunosuppressive drugs have three effects: (1) a therapeutic effect (suppressing a potential rejection), (2) undesired consequences of immunosuppression (mostly infections and cancer) and (3) non-immune-related toxicity such as nephrotoxicity.[5] Some of these side-effects have detrimental consequences and greatly reduce the

quality of life of transplanted patients. In the past decades, maximizing therapeutic effects while minimizing unwanted side-effects and toxicity has been one of the main focuses in transplant patient care.[1,4]

Therapeutic Drug Monitoring

In basic pharmacology, the effect of a drug is determined by the concentration of the drug at the target site. Ideally, the concentration of the drug in the blood is proportional to the dose of the drug and correlates with the concentration at the target site.[6] If this were true, a fixed dose of a certain drug would result in a predictable effect in every patient. However, this 'one-dose-fits-all' approach has shown to fail in treating transplant patients with immunosuppressants.[1,7] Clinical effects of immunosuppressants are dependent on the pharmacokinetics (PK) and pharmacodynamics (PD) of the drug.[4] PK parameters such as absorption, distribution, metabolism and excretion of the drug can greatly differ between patients and have shown to be of major influence on clinical results.[1,4,6] Many PD parameters for tacrolimus have been described, such as the association of low trough concentrations with increased graft rejection.[6] Currently, the exposure of an individual patient to tacrolimus best predicts clinical outcomes for this patient.[4] This exposure can be measured by obtaining and analyzing multiple blood samples over a period of 12 or 24 hours, depending on the drug formulation. From these mulitiple blood samples, a PK curve can be derived.[6] The Area Under the Curve (AUC) is currently the best method available to describe the exposure. PK studies demonstrated that the trough concentration (C_0, concentration measured at the lowest point of the PK curve) correlates well with the AUC corresponding to that particular dose.[1] Therefore, in clinical care, dosing of tacrolimus is based on trough concentrations measured in whole blood obtained from a venipuncture.

In addition to varying PK and PD parameters of tacrolimus, target trough concentrations are different depending on time since transplantation. Early after transplantation, higher trough concentrations are targeted. Several months after transplantation, target trough concentrations are tapered. For all these targeted trough concentrations, the therapeutic window is narrow, which means that the difference between the lower and upper level of the window associated with optimal treatment is small. As a consequence, frequent measurement of trough concentrations of tacrolimus and other immunosuppressive drugs have been a cornerstone of transplant patient care for decades, to make sure that the dose results in a concentration in the therapeutic window. This process of repeated measurement of blood-drug concentrations and adjusting the dose accordingly is known as Therapeutic Drug Monitoring (TDM).[1,4]

Dried Blood Spot sampling

To perform TDM, patients frequently travel to the hospital for venous blood sampling. In general, TDM is performed weekly in the first month post-discharge after kidney transplantation. Over a period of approximately one year, the frequency is tapered to a 3-monthly visit which will last a lifetime in most cases. Given the time delay between blood sampling and availability of analytical results, the blood trough concentrations of immunosuppressants are usually not yet available when the physician sees the patient. This requires the patient to sample a few days earlier, or requires the physician to schedule another appointment (usually by telephone) to discuss the TDM results. For both patient and physician, this workflow is suboptimal.

Recently, a Dried Blood Spot (DBS) sampling method was developed that allows patients to sample at home.[8,9] In DBS sampling, 2 droplets of blood from a fingerprick are applied to a sampling card. After drying, the sample can be sent to the laboratory under ambient conditions using regular mail. From these blood spots, immunosuppressant blood drug concentrations can be measured.[10] Implementation of Dried Blood Spot home sampling can potentially lead to an improved workflow for physician and patient since immunosuppressant blood drug concentrations could be available when the patient is at the outpatient clinic. This could lead to improved patient quality of life as well as cost reduction.[11] In addition, the sampling method is minimally invasive and can be performed by patients at home.

The Dried Blood Spot analysis method was first introduced in 1963 by Guthrie to measure phenylalanine in neonates as part of phenylketonuria screening.[12] With the introduction of new, highly sensitive bioanalytical methods, mainly Liquid Chromatography combined with tandem Mass Spectrometry (LC-MS/MS), very small amounts (10-50 µL) of blood are needed to measure immunosuppressant blood drug concentrations.[8-10,13] Therefore, the use of DBS sampling and –analysis has increased in the field of TDM in the past 15 years.[9] Despite this increase, several challenges remain to be solved in the field of DBS sampling and –analysis.

Current challenges in Dried Blood Spot sampling

Analytical validation

Current DBS analytical methods are developed and analytically validated based on guidelines issued by the EMA and the FDA on bioanalytical method validation.[14,15] However, these guidelines are written for traditional matrices such as liquid

blood, plasma or serum and are not always easily translated to analyses of DBS. In addition, DBS specific parameters such as the effect of the hematocrit on spot formation are not discussed. Therefore, there is currently no optimal development and validation strategy for DBS analytical assays.

Clinical validation

Although many analytical DBS assays are described in literature, very few of them are tested in a clinical study.[16] For immunosuppressants, traditional venous whole blood sampling and analysis has been part of routine care for decades.[1,17] All PK/PD research, including establishment of relevant target trough concentrations is based on venous whole blood data. Therefore, results from a new analysis method (DBS) should be interchangeable with the reference method (venous whole blood).[18] Novel DBS methods should be tested in a clinical study comparing paired fingerprick DBS samples with conventional venous whole blood samples.[16,18] Although for some immunosuppressants, such as tacrolimus and cyclosporine A, these studies exist, they often have a small sample size and sometimes do not use fingerprick blood to produce DBS, but rather blood from a venously collected whole blood sample.[19-21] In addition, specific guidelines on sample size, appropriate statistical tests and study design are lacking.[16] Therefore, there is currently no optimal clinical validation strategy for TDM using DBS assays.

Implementation in clinical care

Because very few TDM DBS assays are used in clinical care, there are very limited data about the implementation of TDM DBS assays. Some studies have focused on the feasibility of DBS sampling regarding sample quality of DBS samples produced by patients.[22-26] Only one study focuses on feasibility and implementation of DBS home sampling for tacrolimus TDM, but this study lacked a control group.[22] Although DBS home sampling is perceived as a cost-saving tool, this has never been shown in a clinical study.[9,11] Therefore, there are currently no data on cost saving and implementation of TDM DBS assays.

Aim of this thesis

The aim of this thesis is to evaluate the implementation of Dried Blood Spot home sampling for immunosuppressant TDM in transplant patients. The evaluation consists of the analytical and clinical performance of the immunosuppressant DBS assay. Furthermore, costs, logistics, patient satisfaction and patient sampling performance are evaluated.

Outline of the thesis

In chapter 2, we plan to develop and analytically validate a multi-analyte DBS assay. This assay consist of the 5 small-molecule immunosuppressants that are currently most widely used in transplantation: tacrolimus, everolimus, sirolimus, cyclosporine A and mycophenolic acid.

In chapters 3 and 4, we will perform clinical validation studies, comparing paired fingerprick DBS samples and venous whole blood samples obtained from transplant patients for the drugs tacrolimus, cyclosporine A, everolimus and sirolimus. In addition, creatinine levels measured from DBS samples will be assessed.

In chapters 5 and 6, quality of DBS samples will be evaluated and discussed. In chapter 5 DBS quality criteria will be presented and applied to a large DBS sample set from four different countries. In chapter 6, a web-based application (app) capable of measuring DBS sample quality by means of taking a picture of the sampling card will be developed. The performance of this app will be tested on the DBS sample dataset from chapters 3 and 5.

In chapter 7 the effects, costs and implementation of DBS home sampling for tacrolimus TDM will be evaluated in a randomized controlled trial involving adult kidney transplant patients who will perform DBS sampling during the first 6 months post-transplantation. Patient satisfaction concerning DBS home sampling will be evaluated and discussed.

In chapter 8 a guideline is presented on the development, analytical and clinical validation and quality control of DBS methods for TDM. This guideline will discuss the DBS-specific parameters that are not discussed in general validation guidelines by the EMA and FDA.[14,15]

In chapters 9 and 10, a different micro-sampling device will be evaluated and discussed. The Mitra© tip is a Volumetric Absorptive Micro Sampling (VAMS) device designed to wick up an exact volume of blood (10 or 20 µL).[27] This approach could in theory mitigate hematocrit-related effects to volume as well as improve sample quality and result in an easier sampling procedure compared to DBS. The analytical validation of the VAMS assay will be presented in chapter 9. We will evaluate paired VAMS fingerprick samples, DBS fingerprick samples and conventional venous whole blood samples in a clinical validation study in chapter 10.

In chapter 11, a general discussion and the future perspectives of this thesis will be presented.

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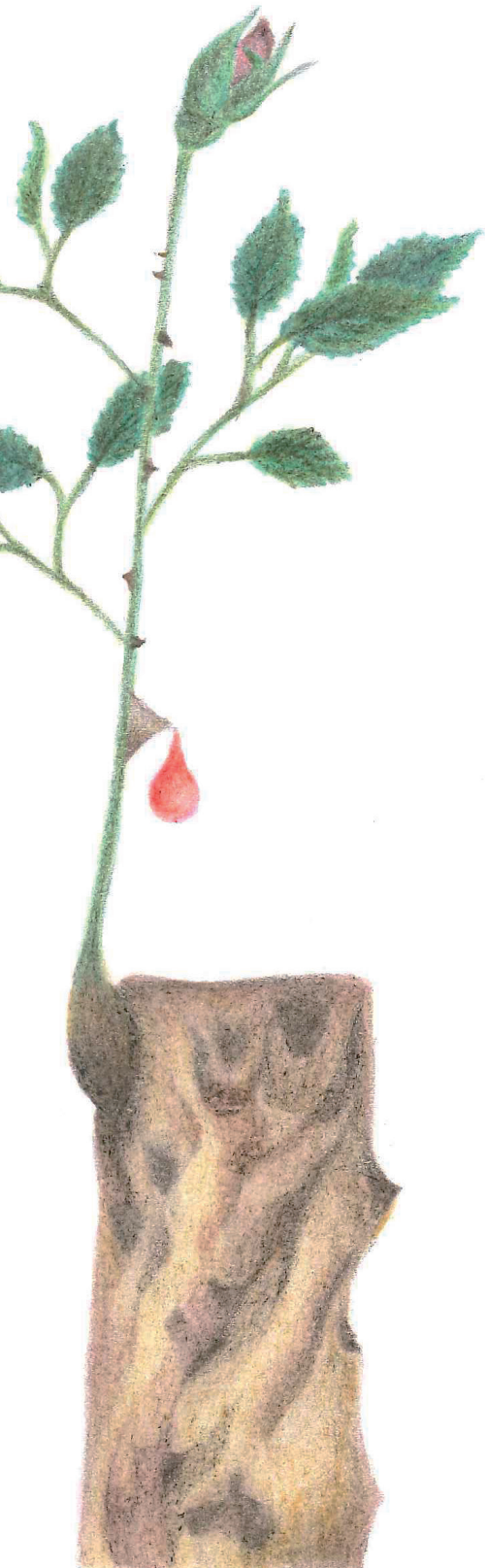

Chapter 2

Dried blood spot validation of five immunosuppressants, without hematocrit correction, on two LC–MS/MS systems

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Abstract

Aim: Hematocrit (Ht) effects remain a challenge in dried blood spot (DBS) sampling. The aim was to develop an immunosuppressant DBS assay on two LC–MS/MS systems covering a clinically relevant Ht range without Ht correction. Results: The method was partially validated for tacrolimus, sirolimus, everolimus, cyclosporin A and fully validated for mycophenolic acid on an Agilent and Thermo LC–MS/MS system. Bias caused by Ht effects were within 15% for all immunosuppressants between Ht levels of 0.23 and 0.48 l/l. Clinical validation of DBS versus whole blood samples for tacrolimus and cyclosporin A showed no differences between the two matrices. Conclusion: A multiple immunosuppressant DBS method without Ht correction, has been validated, including a clinical validation for tacrolimus and cyclosporin A, making this procedure suitable for home sampling.

Introduction

In the last years, dried blood spot (DBS) sampling has been applied as a therapeutic drug monitoring (TDM) tool that enables patients to sample at home.[1] Various analytical methods have been described and some are clinically validated for the quantitation of immunosuppressants, anticancer drugs and tuberculostatics.[1-5] For immunosuppressants, several DBS methods have been published, including multianalyte assays (e.g., for tacrolimus [TaC], sirolimus [SiR], everolimus [EvE], cyclosporin A [CsA] and mycophenolic acid [MPA]).[6-9] Although these methods were found suitable for determination of these immunosuppressants, several problems were observed, with the hematocrit (Ht) effect as the most important one. The Ht effect influenced the analytical results of some immunosuppressants and caused irreproducible recoveries for SiR and EvE if Ht values and substance concentrations varied. Extensive research showed that the varying recoveries for SiR and EvE could be attributed to interaction of the analytic substances with the filter paper matrix.[10,11] A higher number of hydrogen bond acceptors of the substance was related to lower recoveries at lower Ht and higher concentrations of analytic substances. This effect was consistent with different types of DBS cards.[11] Correction for Ht by measuring Ht of the blood in a DBS is very complicated for SiR and EvE, because of the mixed Ht effects due to interactions with the DBS card caused by the formation of the DBS and the lower extraction recoveries at low Ht and high concentration. Three methods have been described for the determination of the Ht of a DBS. The first is by measuring the potassium in the DBS by an auto-analyzer and uses an extra DBS for the Ht analysis.[12,13] The second is by measuring the Ht based on noncontact diffuse reflectance spectroscopy[14] and the third is by using near-infrared spectroscopy.[15] Although the three methods have good potential in future use, they have not yet been applied in routine analysis. Although immunosuppressant DBS assays were reported successful in small-scale studies, they lacked robustness for the routine processing of large series of samples.[6-9,16-19] Therefore, our aim was to develop a multianalyte assay covering a sufficiently wide Ht range without the need for Ht correction, which could easily run on different LC–MS/MS systems. The validated methods will be used for outpatient monitoring of transplant patients.

Experimental section

Chemicals & Materials

TaC was purchased from USP (MD, USA). EvE and MPA were purchased from Sigma-Aldrich, Inc. (MO, USA). SiR was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany) and CsA was purchased from EDQM (Strasbourg, France). The following

isotopically labeled internal standards (ISs) were purchased from Alsachim (Illkirch Graffenstaden, France): TaC [$^{13}C,^{2}H_2$], EvE [$^{13}C_2,^{2}H_4$], CsA [$^{2}H_{12}$] and MPA [$^{13}C,^{2}H_3$]. During previous method development it became clear that SiR [$^{13}C,^{2}H_3$] was 2.9% contaminated with SiR. For this reason it was decided to validate without SiR [$^{13}C,^{2}H_3$] and to use EvE [$^{13}C,^{2}H_4$] as the IS for SiR instead.[6] Analytical grade methanol was purchased from Merck (Darmstadt, Germany). Purified water was prepared by a Milli-Q Integral system (MA, USA). Ammonium formate was purchased from Acros (Geel, Belgium). Citrate whole blood was purchased from Sanquin (Amsterdam, The Netherlands). The whole blood was stored at 4°C and was used within two weeks after donation. The blood was checked for hemolysis prior to use. The Whatman FTA DMPK-C (Kent, UK) cards were used for validation. A Hettich centrifuge (Tuttlingen, Germany) model 460R was used to centrifuge the whole blood for Ht preparation and a XN9000 hematology analyzer from Sysmex (Hyogo, Japan) was used for all Ht analyses. The experiments were performed on two LC–MS/MS systems. An Agilent 6460A (CA, USA) triple quadrupole LC–MS/MS system, with an Agilent 1200 series combined LC system. The second LC–MS/MS system was a Thermo Fisher Quantiva (MA, USA) triple quadrupole LC–MS/MS with a Dionex Ultimate 3000 series UPLC system. All mass selective detectors operated in electrospray positive ionization mode and performed multiple reaction monitoring (MRM) with unit mass resolution. All precursor ions, product ions and collision energy values were tuned and optimized and are shown in Table 1. For Tac, SiR, EvE and CsA [NH4]+ adducts are selected in the first quadrupole.

Agilent LC-MS/MS settings

The Agilent optimum source parameters were a capillary voltage of 4500 V, gas temperature of 200°C, gas flow of 13 l/min, nebulizer gas pressure of 18 psi, sheath gas temperature of 200°C, sheath gas flow of 12 l/min and nozzle voltage of 0 V. The autosampler temperature was set at 10°C and the column oven temperature was set at 60°C. The Agilent mobile phase consisted of methanol and a 20 mM ammonium for- mate buffer pH 3.5, with a flow of 0.5 ml/min and a run time of 3.5 min. Analyses were performed with a 3 µm 50 × 2.1 mm Thermo HyPURITY C18 analytical column (MA, USA). The Agilent binary pump LC gradient was optimized for separation of the MPA glucuronide and only involved the first part of the gradient. The gradient started at 30% methanol and 70% 20 mM ammonium formate buffer pH 3.5 and changed to 73% methanol between 0.35 and 0.76 min, followed by an increase to 77% methanol in 1.52 min. From 2.28 to 2.48 min, the methanol concentration increased to 95% and was maintained at this level until 3.10 min. From 3.11 to 3.50 min, the gradient was maintained at 30% methanol to stabilize the column for the next injection. Peak area ratios of the substance and its IS were used to calculate concentrations. Agilent Masshunter (version B.04.00) was used for quantification of the analytes in DBS.

Thermo LC-MS/MS settings

The autosampler temperature was set at 10°C and the column oven temperature was set at 60°C. The Thermo quaternary pump LC method was optimized for UPLC analysis (including separation of the MPA glucuronide) with runtimes of 1.5 min using a Thermo Accucore C18 2.6 µm 50 × 2.1 mm analytical column equipped with a 5 µm Thermo inline frit filter. The Thermo LC gradient consisted of 0.2 M ammonium formate buffer pH 3.5, purified water and methanol. Chromatographic separation was performed by means of a gradient with a flow of 1.0 ml/min and a run time of 1.5 min. The gradient started at 30% methanol, 65% of purified water and 5% 0.2 M ammonium formate buffer pH 3.5 and changed to 78% methanol at 0.002 min and was maintained at 78% methanol until 0.835 min. From 0.835 to 0.840 min, the methanol increased to 95% and was maintained until 1.135 min. From 1.140 to 1.500 min, the gradient was maintained at 30% methanol to stabilize the column for the next injection. During the gradient, the percentage of ammonium formate buffer was maintained at 5%. Peak area ratios of the substance and its IS were used to calculate concentrations. Thermo Xcaliber software (version 3.0) was used for quantification of the analytes in DBS.

Table 1. Agilent 6460 A triple quad mass spectrometer settings for all substances.

Substance	Precursor ion (m/z)	Product ion (m/z)	Thermo RF lens (V)	Thermo collision energy (V)	Agilent fragmentor voltage (V)	Agilent collision energy (V)
Tacrolimus	821.5	768.4	82	20	190	11
Tacrolimus [$^{13}C,^{2}H_2$]	824.5	771.4	82	20	140	15
Sirolimus	931.5	864.4	83	15	140	6
Everolimus	975.6	908.5	88	16	121	10
Everolimus [$^{13}C_2,^{2}H_4$]	981.6	914.5	88	16	165	13
Cyclosporin A	1219.8	1202.8	93	15	200	30
Cyclosporin A [$^{2}H_{12}$]	1231.8	1214.8	93	15	170	16
Mycophenolic acid	321.1	207.0	58	22	118	16
Mycophenolic acid [$^{13}C,^{2}H_3$]	325.1	211.0	58	22	118	16

Sample preparation

The DBS extraction method was performed as described previously.[6,20] The extraction solution consisted of methanol:water (80:20 v/v%) and contained the isotopically labeled ISs TaC [$^{13}C,^{2}H_3$], EvE [$^{13}C_2,^{2}H_4$], CsA [$^{2}H_{12}$] and MPA [$^{13}C_2,^{2}H_3$] at concentrations of 2.5, 1.0, 10 and 250 ng/ml, respectively. EvE [$^{13}C_2,^{2}H_4$] was used as IS for EvE and SiR. In short, for the preparation of the DBS samples 50 µl of blood was pipetted on the DBS card, dried for 24 h. An 8 mm disk from the central part of the blood spot was punched into an eppendorf tube and 200 µl

extraction solution was added. The samples were vortex mixed for 60 s, sonicated for 15 min and then vortex mixed again for 60 s. The extract was transferred into a 200 µl glass insert and placed at -20°C for 10 min to improve protein precipitation. After centrifugation at 10,000 × g for 5 min, the extract was injected in the LC–MS/MS system. The autosampler needle height was set high enough in order to avoid injection of precipitated blood, which will cause blockage of the autosampler needle and injection loop. The preparation of the different target Ht values was performed as described previously by removing or add- ing plasma to achieve the different target Ht values. The prepared Ht values were confirmed by analysis.[21]

Analytical validation

An earlier described validation was performed with the use of Whatman 31 ET CHR paper which was available in large sheets.[6] This was not very practical for patient sampling, so Whatman FTA DMPK-C DBS cards were chosen for the current validation. The use of Whatman FTA DMPK-C DBS cards was validated on the Agilent LC–MS/MS system. In order to enhance the analysis speed and to have a back-up system for the DBS analysis, the method was also developed for the Thermo LC–MS/MS sys- tem. The current DBS analytical method validation was performed based on EMA and US FDA guide- lines and was extended with validation for spot volume and Ht effect.[22,23] The following parameters were previously successfully validated and described for the Agilent LC–MS/MS system: selectivity, carry-over, matrix effect and short-term stability in whole blood and DBS.[6,24] Selectivity, carry-over and matrix effects were also tested for the Thermo LC–MS/MS system. For MPA, stability in DBS was validated by assessing low and high concentrations in fivefold, which were compared with simultaneously prepared DBS which were stored at -20°C. Stability of MPA in DBS was assessed at 22, 37 and 50°C. Stability of MPA was assessed as processed sample in the auto-sampler at 10°C. Spot-to-spot carry-over was tested in each validation run by punching and extracting a blank DBS after the highest calibrator. Spot homogeneity testing was not applicable because the 8 mm-diameter punch covered the largest part of the spot area, eliminating possible spot inhomogeneity effects. The methods were validated with a two-point calibration curve, consisting of the lowest and highest concentrations of the linear range, according to Tan et al.[25] The main reason to use a two-point calibration curve was to minimize overhead sample analysis, which decreases patient sample turnaround time. The calibration curve and accuracy and precision samples were analyzed on three consecutive days. The validation was performed with a maximum tolerated bias and CV of 20% for the LLOQ and 15% for all other calibration and QC concentrations, including the stability evaluation. For the determination of the accuracy and precision, all QC concentrations were measured in fivefold in three separate runs on separate days. For each accuracy and precision concentration,

bias and CV were calculated per run. Within-run, between-run and overall CVs were calculated with the use of one-way ANOVA. The concentration range for TaC, SiR and EvE was 1.0–50, for CsA 20–1000 and 100–15,000 ng/ml for MPA. To assess the effect of the blood volume used to create a blood spot, blood was prepared with a Ht of 0.35 l/l. DBS were prepared at low and medium concentrations with volumes of 30, 50 and 70 µl. The 50 µl spots were considered the standard spot and the biases of the other volumes were calculated with a maximum acceptable bias of 15% and maximum CV of 15%.The following Ht values were prepared to test the influence of the Ht: 0.23, 0.28, 0.33, 0.38, 0.43, 0.48 and 0.53 l/l. These Ht values were all spiked at two concentrations per substance and contained all five substances in one Ht preparation. At low level: 3 ng/ml for TaC, SiR and EvE, 60 ng/ml for CsA and 300 ng/ml for MPA. At medium (therapeutic trough) level: 10 ng/ml for TaC, SiR and EvE, 200 ng/ml for CsA and 1200 ng/ml for MPA. From these blood samples, DBS was created using 50 µl of blood. The Ht of 0.38 l/l was considered as the standard Ht based on a previous study where the average Ht was 0.387 with a SD of 0.054 and a range of 0.252–0.514 in 199 kidney transplant patients.[6,19]

Clinical sample analysis on two LC–MS/MS systems

Paired patient whole blood and DBS samples were collected during routine visits of patients to the hospital using the home sampling technique available online.[19,26] The need to obtain written informed consent from subjects was waived by the ethics committee of the University Medical Center Groningen because the clinical validation was part of an approved implementation process of DBS sampling in routine care. Whole blood samples were analyzed for CsA and TaC, according to a previously described analysis method using a Thermo Quantum Access triple quadrupole mass spectrometer with a Surveyor LC system.[24] DBS patient samples were analyzed for CsA and TaC on the Agilent LC–MS/MS. For TaC and CsA, respectively, 85 and 57 patient samples were reinjected on the Thermo Quantiva LC–MS/MS and analyzed. Method comparison was done using Passing and Bablok regression analysis and Bland–Altman was used for bias calculation. All statistical analyses were done using Analyse-it® Method Validation Edition for Microsoft Excel version 2.30 (Leeds, UK).[27,28] Statistical significance was set at 0.05, results are presented with 95% CI.

Table 2. Dried blood spot validation results of the accuracy (bias) and precision (CV) calculated with a two-point calibration curve performed on an Agilent 6460 A triple quad MS.

Substance	Concentration (ng/ml)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Tacrolimus	LLOQ (1.0)	6.5	5.6	8.6	4.7
	Low (3.0)	4.0	5.0	6.4	1.5
	Med (10)	2.6	3.3	4.3	7.6
	High (40)	2.6	1.2	2.9	4.6
Sirolimus	LLOQ (1.0)	9.9	10.9	14.7	-0.9
	Low (3.0)	7.3	0.0	7.3	-4.7
	Med (10)	4.9	0.0	4.9	0.9
	High (40)	3.9	3.1	5.0	3.1
Everolimus	LLOQ (1.0)	7.5	1.1	7.5	7.3
	Low (3.0)	5.5	1.7	5.8	-3.7
	Med (10)	4.5	0.0	4.5	1.7
	High (40)	3.2	1.8	3.6	3.5
Cyclosporin A	LLOQ (20.0)	5.6	3.4	6.6	8.5
	Low (60.0)	2.7	3.1	4.2	-4.7
	Med (200)	4.8	1.9	5.2	-1.2
	High (800)	3.3	1.7	3.7	3.0
Mycophenolic acid	LLOQ (100)	1.4	5.7	5.9	3.0
	Low (300)	3.1	6.0	6.8	4.9
	Med (7500)	3.1	6.1	6.8	3.5
	High (12,000)	3.1	7.1	7.7	1.7

CV and bias should be within 15% (20% for the LLOQ) n = 15.

Table 3. Dried blood spot validation results of the accuracy (bias) and precision (CV) calculated with a two-point calibration curve performed on an Thermo Quantiva triple quad MS.

Substance	Concentration (ng/ml)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Tacrolimus	LLOQ (1.0)	7.4	0.0	7.4	10.2
	Low (3.0)	3.7	1.4	4.0	9.7
	Med (10)	2.7	3.4	4.3	10.1
	High (40)	2.5	2.9	3.8	6.3
Sirolimus	LLOQ (1.0)	8.8	7.1	11.3	7.6
	Low (3.0)	5.6	5.0	7.5	3.9
	Med (10)	2.5	3.8	4.6	1.1
	High (40)	4.2	2.8	5.0	1.2

Table 3. (Continued)

Substance	Concentration (ng/ml)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Everolimus	LLOQ (1.0)	9.5	7.0	11.7	1.7
	Low (3.0)	5.4	2.9	6.1	-2.5
	Med (10)	3.2	2.2	3.9	0.6
	High (40)	3.6	1.9	4.1	0.1
Cyclosporin A	LLOQ (20.0)	5.3	1.3	5.5	-3.6
	Low (60.0)	5.1	1.4	5.3	2.9
	Med (200)	2.0	4.7	5.1	-5.9
	High (800)	3.7	2.6	4.5	-4.1
Mycophenolic acid	LLOQ (100)	1.8	3.8	4.2	4.2
	Low (300)	3.2	4.5	5.5	6.7
	Med (7500)	2.9	5.0	5.7	1.8
	High (12,000)	3.1	5.7	6.5	0.0

CV and bias should be within 15% (20% for the LLOQ). n = 15.

Results and Discussion

Analytical validation

Despite difference in LC columns and gradient speeds between the Thermo and Agilent LC–MS/MS systems, the chromatographic performance was principally similar, as can be seen in Supplementary Figures 1–4 (only published online). The Thermo LC–MS/MS system showed to have good selectivity and no carry-over (no interfering peaks higher than 20% of the LLOQ in blank samples and after the highest calibrator) and no matrix effects. MPA showed to be stable in DBS for 2 months at -20, 22 and 37°C and for 14 days at 50°C. MPA showed to be stable for at least 2 days as processed sample in the auto-sampler at 10°C. The punching method showed to have no spot-to-spot carry-over. The accuracy and precision results on the Agilent 6460 A showed a maximum overall CV of 14.7% for SiR at 1.0 ng/ml, while the maximum overall bias was 8.5% for CsA at 20.0 ng/ml (Table 2). On the Thermo Quantiva, the maximum overall CV was 11.7% for EvE at 1.0 ng/ml, while the maximum overall bias was 10.2% for TaC at 1.0 ng/ml (Table 3). While the previously validated quadratic calibration curve for CsA had a concentration range of 20–2000 ng/ml, the currently validated range of 20–1000 ng/ml for CsA had a linear fit, which was suitable for a two-point calibration curve.[6] The blood spot volume and Ht effects are related to the interaction of the blood and substance with the DBS card and were assumed to be independent of the type of LC–MS/MS. Therefore, these validation tests

Table 4. Effect of the blood spot volume of 30 and 70 ml on the bias at two concentrations with the standard spot volume at 50 ml, performed on an Agilent 6460 A triple quad MS.

Spot volume	Tacrolimus				Sirolimus				Everolimus				Cyclosporin A				Mycophenolic acid			
	3.0 ng/ml		10 ng/ml		3.0 ng/ml		10 ng/ml		3.0 ng/ml		10 ng/ml		60 ng/ml		200 ng/ml		300 ng/ml		12,000 ng/ml	
	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)
30	2.9	-2.2	2.9	-4.3	6.1	-5.8	2.8	-8.9	5.0	0.7	3.6	-7.5	2.7	-3.8	1.3	-6.6	3.6	-2.9	3.2	1.7
70	5.0	-1.7	1.7	-0.1	3.7	1.1	2.7	-2.1	6.5	3.5	2.6	-0.5	2.7	-2.3	0.8	2.6	6.3	4.1	4.7	2.2

These data are independent of the used LC–MS/MS system.

Table 5. Effect of the hematocrit on the bias at two concentrations with the standard hematocrit set at 0.38 l/l, performed on an Agilent 6460 A triple quad MS.

Hematocrit (L/L)	Tacrolimus				Sirolimus				Everolimus				Cyclosporin A				Mycophenolic acid			
	3.0 ng/ml		10 ng/ml		3.0 ng/ml		10 ng/ml		3.0 ng/ml		10 ng/ml		60 ng/ml		200 ng/ml		300 ng/ml		12,000 ng/ml	
	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)	CV n = 5 (%)	Bias n = 5 (%)
0.23	3.3	-7.0	3.3	-9.8	7.9	-12.8	3.9	-5.7	3.0	-6.8	3.5	-5.1	2.2	0.4	2.5	1.7	4.0	1.8	4.8	-2.3
0.28	4.3	-4.9	1.6	-1.8	5.8	-15.1	3.9	-2.1	4.6	-10.0	3.6	-0.1	2.6	-4.6	1.6	5.3	4.0	3.3	1.4	-7.8
0.33	3.3	1.2	4.4	4.3	5.9	-3.8	3.3	-3.5	5.9	0.9	2.4	-0.9	1.6	2.1	1.9	1.2	10.4	14.8	7.3	-2.5
0.43	4.1	4.6	2.3	-3.6	6.4	-5.7	4.4	-3.9	4.0	-6.5	1.9	-1.8	4.8	-6.8	1.3	-7.6	3.1	-10.9	1.0	-3.8
0.48	3.8	1.3	3.3	-2.3	5.4	-6.1	6.2	-5.6	3.6	-0.7	3.1	-5.0	2.5	-8.3	8.3	-15.0	2.5	7.1	2.5	0.5
0.53	2.9	3.9	2.5	-1.2	5.7	-10.5	5.2	-8.9	1.8	-2.4	4.1	-8.2	1.3	-14.9	1.9	-17.8	4.4	32.6	1.7	-2.9

These data are independent of the used LC–MS/MS system.

were only performed on the Agilent LC–MS/MS system. The blood spot volume was validated for all substances and had minor influence on the analytical results with the largest bias found at -8.9% for SiR at 30 µl and 10 ng/ml (Table 4). Ht effects were currently validated at low and high trough levels expected for the intended patient population. At the Ht of 0.23 l/l, SiR showed a maximum bias of -12.8% at 3.0 ng/ml and -5.7% at 10 ng/ml (Table 5). While EvE showed a maximum bias of -6.8% at 3.0 ng/ml and -5.1% at 10 ng/ml at the Ht of 0.23 l/l. At the Ht of 0.28 l/l, the bias for SiR was -15.1% at 3.0 ng/ml and therefore exceeded the acceptance limit of 15% bias by 0.1%. However, the bias for SiR at the Ht level of 0.23 l/l was within the 15% bias limit, so the Ht range of 0.23–0.53 was accepted. The bias of CsA at 200 ng/ml at the Ht of 0.53 l/l was -17.8% and it was therefore concluded that the validated Ht range for CsA was 0.23–0.48 l/l. At the Ht of 0.53 l/l MPA showed a bias of 32.6% for the low level. Although this could be a preparation error, it is concluded that the Ht effect is acceptable form 0.23 to 0.48 l/l for the low level of MPA. All other biases due to Ht effects were within 15% bias (Table 5). In line with our current finding of relatively large bias due to Ht effects for EvE and particularly for SiR, it was previously reported that DBS assays of SiR and EvE are subject to relatively large Ht effects, which have been attributed to the combined effect of the Ht on the formation of the DBS and binding of the analytical substance to the cellulose of the card matrix.[6,10] At low Ht and high concentration of the analytical substance, this negatively influenced bias due to the DBS formation and the extraction recovery. In the previous validation for DBS assays that we performed, the assays for SiR and EvE showed to be subject to significant Ht effects, even after adjustment for Ht by multivariate regression, with biases of -20 and -28%, respectively at a relatively high concentration of 40 ng/ml of both analytic substances.[6] Testing the Ht effects at lower (more clinically relevant) concentrations (3.0 and 10 ng/ml), slightly higher Ht range (0.23–0.53 l/l instead of 0.20–0.50 l/l) and a better performing DBS card (Whatman DMPK-C instead of 31-ET- CHR), resulted in far less distinct Ht effects for SiR and EvE in the current validation.[11] The use of a different type of DBS card positively influenced the formation of the DBS and the extraction recoveries. Additionally, improved blood Ht preparation positively influenced part of the Ht effects.[21] However, the deteriorating recoveries of SiR and EvE at high concentrations and low Ht in combination with the used sampling matrix will not be completely resolved at this time. For the measurement of trough levels and incidental toxic concentrations, this analytical method is considered to be acceptable.

Clinical validation

Tacrolimus

Comparison of the DBS Thermo samples with whole blood samples for TaC (n = 85) yielded a Passing and Bablok fit of y = 1.04 × -0.25 (95% CI slope: 0.96–1.12; intercept: -0.73–0.16) showing no systematic difference as seen in Figure 1. Bland–Alt- man analysis showed a non-statistically significant bias of -0.01 ng/ml (95% CI: -0.17–0.15).

Cyclosporin A

Comparison of the Thermo DBS samples with whole blood samples for CsA (n = 57) yielded a Passing and Bablok fit of y = 1.05 × -3.64 (95% CI slope: 0.97–1.15; intercept: -10.17–2.23) showing no sys- tematic differences as seen in Figure 2. Bland–Alt- man analysis showed a non-statistically significant bias of 2.6 ng/ml (95% CI: -0.8–5.9).

As previously described, the analytical results for TaC and CsA of the DBS Agilent method are comparable with whole blood analytical results.[19] The results described above prove the same for the Thermo DBS samples for TaC and CsA. All patient samples for TaC showed to have Ht values within the validated range of 0.23–0.53 l/l. For CsA the validated Ht range was 0.23–0.48 l/l and one patient sample had a higher Ht value of 0.51 l/l. The DBS sample from the patient that exceeded the analytically validated Ht range of CsA still showed acceptable and minor differences compared with the whole blood results. For SiR and EvE it is expected that the validated Ht range of 0.23–0.53 l/l will be sufficient for the patient population based on an earlier study.[19] A direct comparison of the DBS sample results from the Thermo LC–MS/MS versus the DBS sample results from the Agilent LC–MS/MS showed good correlation and can be found in the supplementary data (published online). Results from DBS analysis are interchangeable with results from whole blood analysis. This makes both the Agilent and Thermo DBS analysis method feasible for TDM in routine analysis of patient immunosuppressant blood concentrations. For SiR, EvE and MPA not enough paired samples were collected. Currently samples are being collected and in the future a clinical validation will follow.

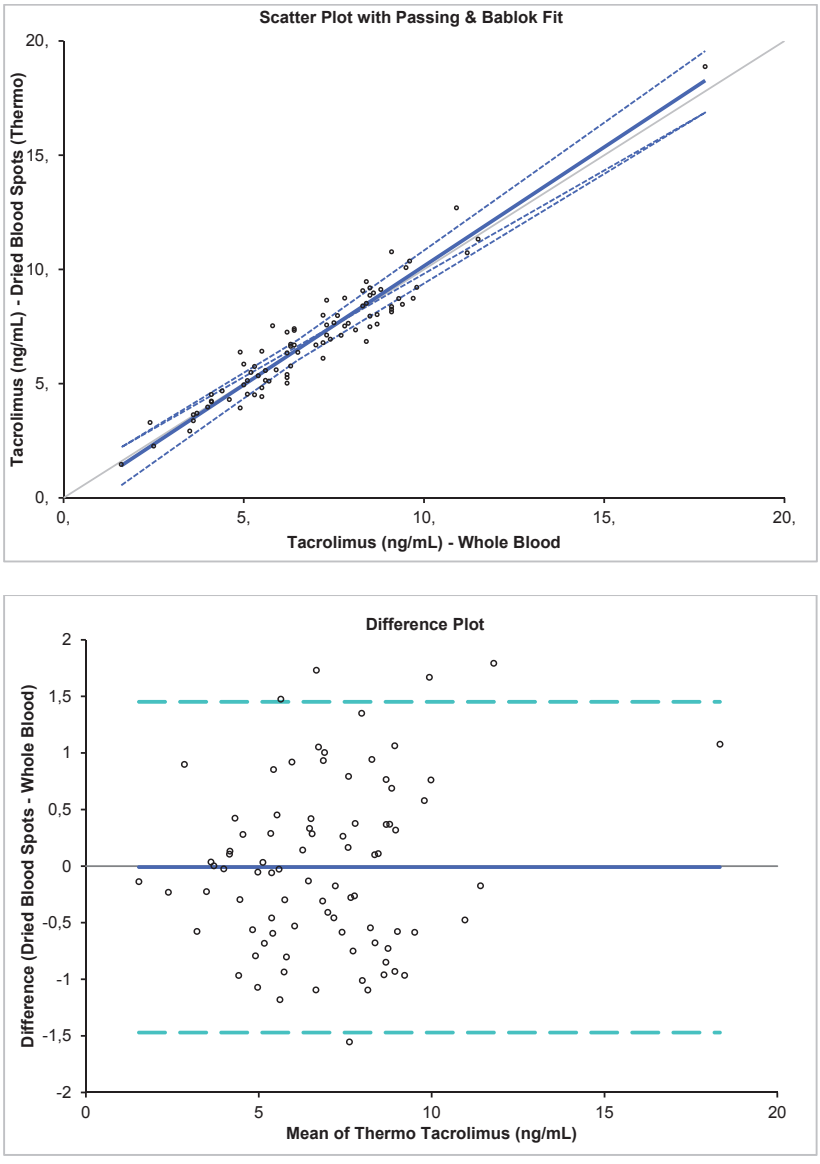

Figure 1: *Comparison of paired whole blood tacrolimus concentrations and Dried Blood Spots (DBS) tacrolimus concentrations measured on a Thermo LC–MS/MS (n = 85). In the upper panel the dotted line is the line of identity, the bold line is the Passing & Bablok regression line y = 1.04 × -0.25 (95% CI slope 0.96–1.12; intercept -0.73,0.16). The lower panel shows Bland–Altman analysis with a non-significant bias of -0.01 (95%CI -0.17 – 0.15) shown by the bold line, the dashed line indicates 95% limits of agreement.*

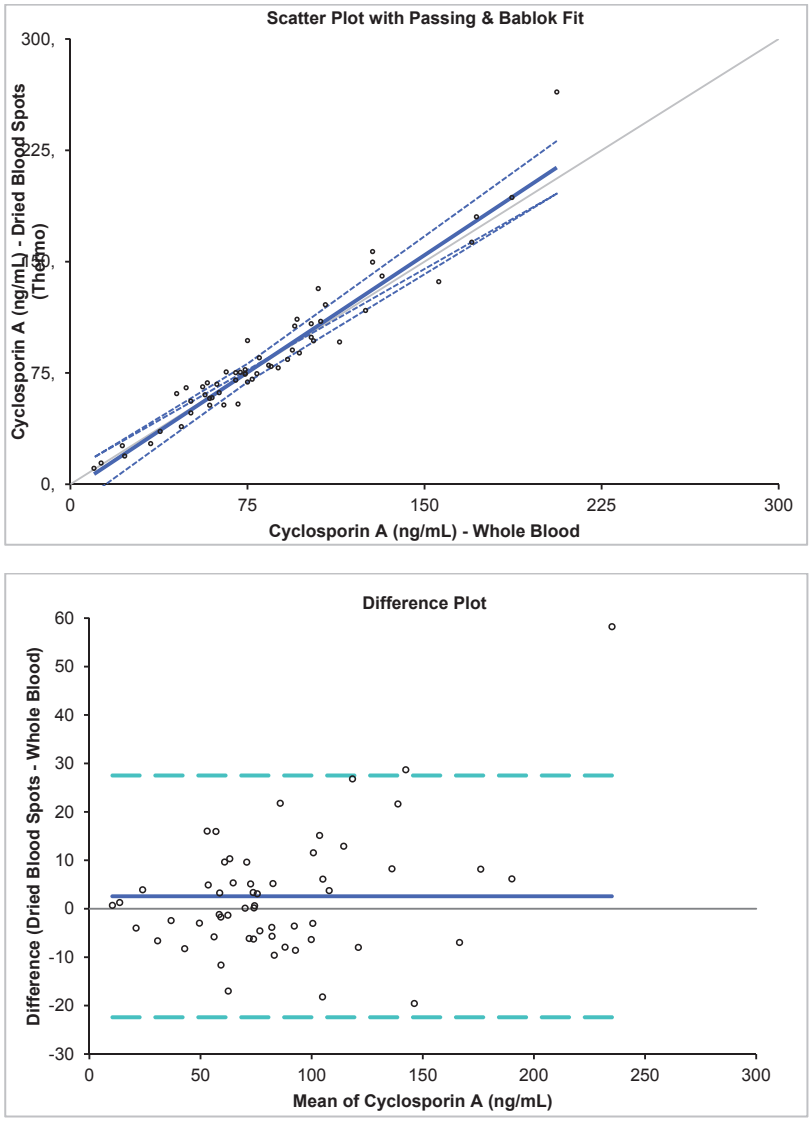

Figure 2: *Comparison of paired whole blood cyclosporin A concentrations and Dried Blood Spots (DBS) cyclosporin A concentrations measured on a Thermo LC–MS/MS (n = 57). In the upper panel the dotted line is the line of identity, the bold line is the Passing & Bablok regression line y = 1.05× – 3.64 (95% CI slope 0.97,1.15; intercept -10.17,2.23). The lower panel shows Bland-Altman analysis with a non-significant bias of 2.6 ng/mL (95% CI: -0.8 – 5.9) shown by the bold line, the dashed line indicates 95% limits of agreement.*

Conclusion

The DBS analysis methods showed to have good performance for the accuracy and precision, and the Ht effects were within the set criteria (with two exceptions) in the therapeutic trough concentration window. In addition, the validation was now performed on two LC–MS/MS systems, which showed comparable performance. Instead of correcting for the Ht of the DBS, the method was validated within an adequate concentration and Ht window, which was still suitable for the intended patient population. It can be concluded that the presented method is patient friendly because the sample collection is non-invasive and since no extra blood samples are needed to determine the Ht value of the patient. Furthermore the DBS method is cost-efficient because samples can be collected at home and shipped at room temperature: no visits to the out-patient clinic are needed. It was shown that the two LC–MS/MS systems are both suitable for the routine analysis of TaC and CsA in DBS in transplant patients. A clinical validation will be performed for SiR, EvE and MPA as soon as sufficient samples are collected.

Future perspectives

More and more transplant patients will be transferred from whole blood analysis to DBS analysis. As a consequence, healthcare costs will decrease and patient burden will be reduced due to less hospital visits. Once transferred to DBS, patients can also be easily introduced and transferred to improved home sampling techniques.

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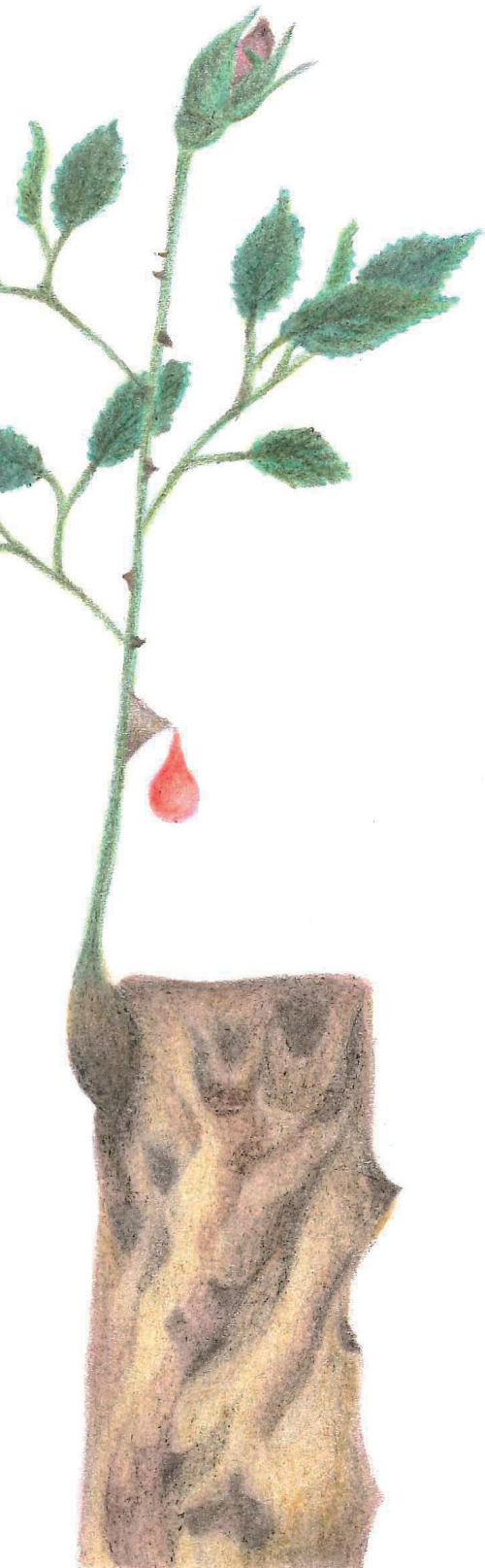

Chapter 3

Clinical Validation of Simultaneous Analysis of Tacrolimus, Cyclosporine A, and Creatinine in Dried Blood Spots in Kidney Transplant Patients

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Abstract

Background: monitoring of creatinine and immunosuppressive drug concentrations, such as tacrolimus (TaC) and cyclosporin A (CsA), is important in the outpatient follow-up of kidney transplant recipients. Monitoring by dried blood spot (DBS) provides patients the opportunity to sample a drop of blood from a fingerprick at home, which can be sent to the laboratory by mail.

Methods: we performed a clinical validation in which we compared measurements from whole-blood samples obtained by venapuncture with measurements from DBS samples simultaneously obtained by fingerprick. After exclusion of 10 DBS for poor quality, and 2 for other reasons, 199, 104, and 58 samples from a total of 172 patients were available for validation of creatinine, TaC and CsA, respectively. Validation was performed by means of Passing & Bablok regression, and bias was assessed by Bland-Altman analysis.

Results: for creatinine, we found y = 0.73x - 1.55 (95% confidence interval [95% CI] slope, 0.71-0.76), giving the conversion formula: (creatinine plasma concentration in μmol/L) = (creatinine concentration in DBS in μmol/L)/0.73, with a nonclinically relevant bias of −2.1 μmol/L (95% CI, −3.7 to −0.5 μmol/L). For TaC, we found y = 1.00x - 0.23 (95% CI slope, 0.91-1.08), with a nonclinically relevant bias of −0.28 μg/L (95% CI, −0.45 to −0.12 μg/L). For CsA, we found y = 0.99x - 1.86 (95% CI slope, 0.91-1.08) and no significant bias. Therefore, for neither TaC nor CsA, a conversion formula is required.

Conclusions: DBS sampling for the simultaneous analysis of immunosuppressants and creatinine can replace conventional venous sampling in daily routine.

Introduction

Calcineurin inhibiting immunosuppressants such as tacrolimus (TaC) and cyclosporine A (CsA) are successfully applied in solid organ transplantation to prevent allograft rejection for many years. Because of their narrow therapeutic range and significant interindividual and intraindividual variabilities in absorption and metabolism, therapeutic drug monitoring is an important tool to help physicians to balance between subtherapeutic and potentially toxic concentrations of these drugs.[1] In combination with the blood drug concentration, the creatinine concentration is used to monitor the renal graft function and toxicity of immunosuppressants.[2,3] As lifelong monitoring is required, patients need to travel to the hospital on a regular basis to have their blood samples drawn and analyzed. This logistical burden can be overcome by the use of dried blood spots (DBS) sampling. This method, using a drop of blood from a fingerprick, is patient friendly and allows patients to sample at home and send the DBS card to the laboratory by mail. When appropriately timed, the results will be available for the clinician upon routine check-up of the patient.[4] In time, monitoring patients using DBS might decrease the frequency of routine check-ups saving time for the patient and clinician. In literature, various methods for analyzing immunosuppressants and creatinine in DBS have been described.[2,5-10] Current challenges in DBS sampling include matrix effects, the effect of the hematocrit (Ht) on the formation of the blood spot, and the combined effect of Ht and immunosuppressant concentration on the analytical results.[4,6,9,11,12] Although DBS assays are analytically sound, clinical validations comparing whole blood samples to capillary blood obtained by fingerprick and applied on a DBS card are of utmost importance before the assay can be implemented in daily practice.[10,13,14] There is consensus that spotting of defined amounts of whole blood on a DBS card using a pipette by a laboratory technician as alternative for capillary sampling is not acceptable as clinical validation.[15] There is less consensus about the number of subjects and amount of samples to be included for clinical validations. For TaC and CsA, Hinchliffe et al.[8] report good agreement between DBS samples and venous sampling for, respectively, 42 and 45 samples from heart lung transplant patients. Wilhelm et al.[16] reported no significant difference between venous and DBS samples in 40 samples of 36 stem cell transplant patients for CsA. Dickerson et al. reported a significant mean lower concentration of 0.6 ng/mL in DBS compared to whole blood for TaC in pediatric transplant patients.[7] Only 1 study reported a preliminary validation of creatinine using a time consuming solid phase extraction showing a correlation coefficient of 0.890 for 19 samples.[2] In the absence of robust clinical data to support DBS in clinical practice for creatinine, TaC and CsA monitoring, we aimed to clinically validate our method for analyzing creatinine, TaC and CsA in a single bloodspot to implement DBS in routine outpatient care.

Materials and methods

Patient and sample collection

Patient samples were collected during routine clinical follow-up in the hospital from adult kidney transplant patients. Because of the nature of this study, being implementation of DBS in routine care, the need to provide informed consent by the subjects was waived by the ethics committee of the University Medical Center Groningen (Metc 2011.394). A trained phlebotomist obtained both the venous and DBS samples.[17] Finger prick blood samples were collected within 10 minutes of the venous sample. The fingertip was disinfected using chloorhexidinegluconate 0.5% m/v in alcohol 70% v/v and dried. Finger prick blood samples were collected using a Microtainer Contact-activated Lancet (Blue, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The first drop was discarded and the next 2 drops were collected by letting the blood freely drop onto two 10-mm premarked circles on the Whatman FTA DMPK-C sampling card (Whatman Schleicher & Schuell, Dassel, Germany). The blood spots were allowed to dry for 1 to 7 days at room temperature and packed in resealable plastic mini bags. These bags were stored in a −20 °C freezer ensuring stability until they were analyzed.[9,18]

Equipment, Conditions and Procedures

The routine plasma creatinine analyses were performed with a Roche enzymatic creatinine assay on a Roche Modular (Roche Diagnostics Limited, West Sussex, UK). Our reference procedure was measurement of TaC and CsA in whole blood obtained by venapuncture, with analyses performed on a Thermo Fisher Scientific (Waltham, MA) triple quadrupole Quantum Access LC-MS/MS system with a Surveyor HPLC system.[19] For the DBS analyses of creatinine, TaC, CsA, an Agilent 6460A (Santa Clara, CA) triple quadrupole LC-MS/MS system, with an Agilent 1200 series combined HPLC system was used.[9] The Ht of the venous sample was measured using an XN10/XN20 hematology analyzer (Sysmex, Kobe, Japan). The blood spots were visually inspected for completeness, homogeneity and symmetric filling of the 10-mm circle and dark red color on both sides of the paper according to prespecified criteria.[17,20] The whole blood and DBS extraction and analysis procedures were performed as described previously with minor alterations.[9,18,1]

Statistical analysis

Statistical analysis was performed using Analyse-it® Method Validation Edition for Microsoft Excel version 2.30 (Leeds, United Kingdom). Standard linear regression analysis was used to calculate the correlations between methods. Only values within analytically validated ranges were analyzed. Method comparison was done using Passing and Bablok regression analysis and Bland-Altman was used for bias calculation.

Passing and Blablok regression, Bland-Altman method and Deming regression were used to calculate systematic difference between the DBS and plasma creatinine measurements. Using these differences an optimal conversion formula for creatinine was determined.[21-23] Statistical significance was set at 0.05, results are presented with 95% confidence intervals (CI).

Results

Patients

In total 210 paired DBS and whole blood samples were collected from 172 adult kidney transplant patients between August 2015 and May 2016. All patients received multiple immunosuppressive therapy consisting of a calcineurin inhibitor (TaC or CsA) in conjunction with mycophenolate mofetil and prednisolone. After visual inspection 10 DBS were discarded because of insufficient sample quality making 95.2% of all collected samples suitable for analysis. One sample, which was intended to be used for validation of creatinine and TaC, was excluded because of an outlier value of Ht of 0.537. In total 199 paired creatinine, 106 paired TaC and 61 paired CsA samples were analyzed. Some patients used other immunosuppressive drugs (sirolimus or everolimus). Table 1 summarizes demographic patient characteristics. All evaluated drug and creatinine concentrations were within the validated analytical ranges.

Table 1. Patient demographic and clinical laboratory data

	N	Mean ± SD (range)
Age, y	172	55 ± 14 (20-84)
Sex	172	105 male, 67 female
Plasma creatinine, µmol/L	199	149 ± 65 (53-478)
Venous whole blood TaC trough concentrations, µg/L	106	7.1 ± 3.3 (1.6-17.8)
Venous whole blood CsA trough concentrations, µg/L	61	109 ± 112(10-206)
Ht (v/v)	199	0.387 ± 0.054 (0.252-0.514)
Time from transplantation	172	6 y, 10 mo ± 7 y, 10 mo (10 d to 36 y, 10 mo)

Clinical validation

Creatinine

Linear regression analysis showed a significant relationship between creatinine concentrations in plasma derived from whole blood obtained by venapuncture and creatinine concentrations in DBS capillary whole blood obtained by fingerprick (R^2 = 0.97, P < 0.0001). Passing & Bablok regression found y = 0.73x - 1.55 (95% CI slope,

0.71-0.76; 95% CI intercept, −4.58 to 1.65), consistent with a significant systematic difference of a 27% lower concentration of creatinine in DBS from capillary whole blood, with no significant intercept difference compared to plasma results as shown in Figure 1. This was expected because creatinine concentrations in DBS are "diluted"

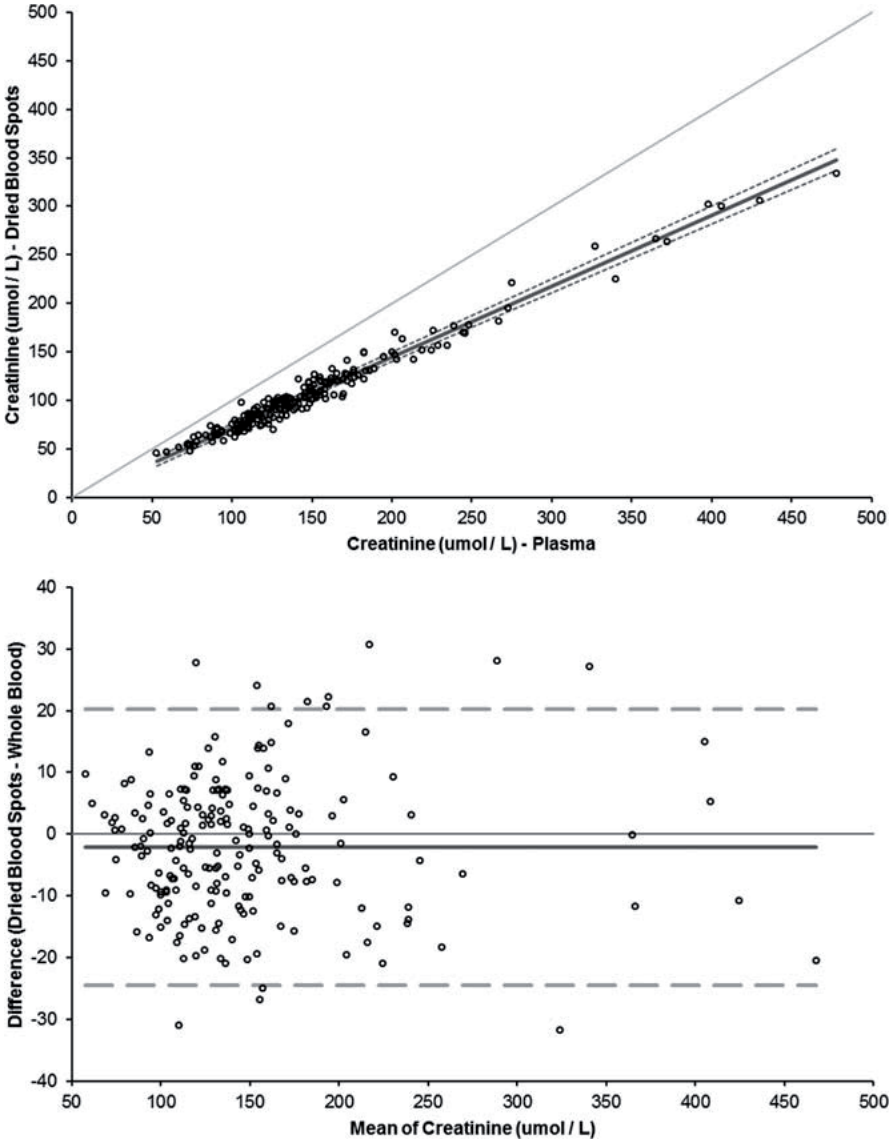

Figure 1. *Method comparison between plasma creatinine levels and DBS creatinine levels (n = 199). In the upper panel the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line y = 0.73x - 1.55 (95% CI slope, 0.71-0.76; intercept, −4.58 to 1.65). The lower panel shows Bland-Altman analysis based on recalculated values for DBS using the formula [creatinine plasma concentration in µmol/L] = [DBS concentration in µmol/L]/0.73. Calculated bias is significant at −2.1 µmol/L (95% CI, −3.7 to −0.5) shown by the continuous line, the dashed line indicates 95% limits of agreement.*

by the red blood cells obligatory present in these samples. Results from Bland-Altman analysis and Deming regression showed similar results but systematic differences between DBS and plasma of 33% and 28%, respectively. All data were reanalyzed using recalculated DBS concentrations based on the 3 systematic difference percentages. Unlike the 33% and 28% differences, correction for the 27% systematic difference gave no significant constant or proportional differences in Passing & Bablok analysis. A fixed bias of −2.1 μmol/L (95% CI, −3.7 to −0.5) was observed in Bland-Altman analysis for the recalculated values using the 27% difference as seen in Figure 1. We deem a fixed bias of −2.1 μmol/L as not clinically relevant and therefore propose the following conversion factor*: [creatinine plasma concentration in μmol/L] = [DBS concentration in μmol/L]/0.73*. Subanalysis of samples with a creatinine level of less than 177 μmol/L (n = 163) showed a comparable bias of −2.0 μmol/L (95%CI, −3.5 to −0.4). Using this conversion factor for creatinine, the DBS analytical results can be interchanged with plasma analytical results.

TaC

In total, 106 samples were analyzed. One sample was excluded because of high Ht. One sample was excluded because it was a peak concentration instead of a trough concentration and therefore not clinically relevant. Linear regression analysis showed a significant relationship between DBS TaC levels and venous whole-blood TaC levels (R^2 = 0.93, P < 0.0001). Passing & Bablok fit was y = 1.00x − 0.23 (95%CI slope, 0.91-1.08; intercept, −0.69 to 0.30) showing no systematic difference as seen in Figure 2. The Bland-Altman analysis showed a significant bias of a 0.28 μg/L (95% CI, −0.45 to −0.12 μg/L) lower concentration in DBS compared with venous blood which we consider not clinically significant. These results prove that for TaC DBS analytical results are interchangeable with venous whole-blood analytical results.

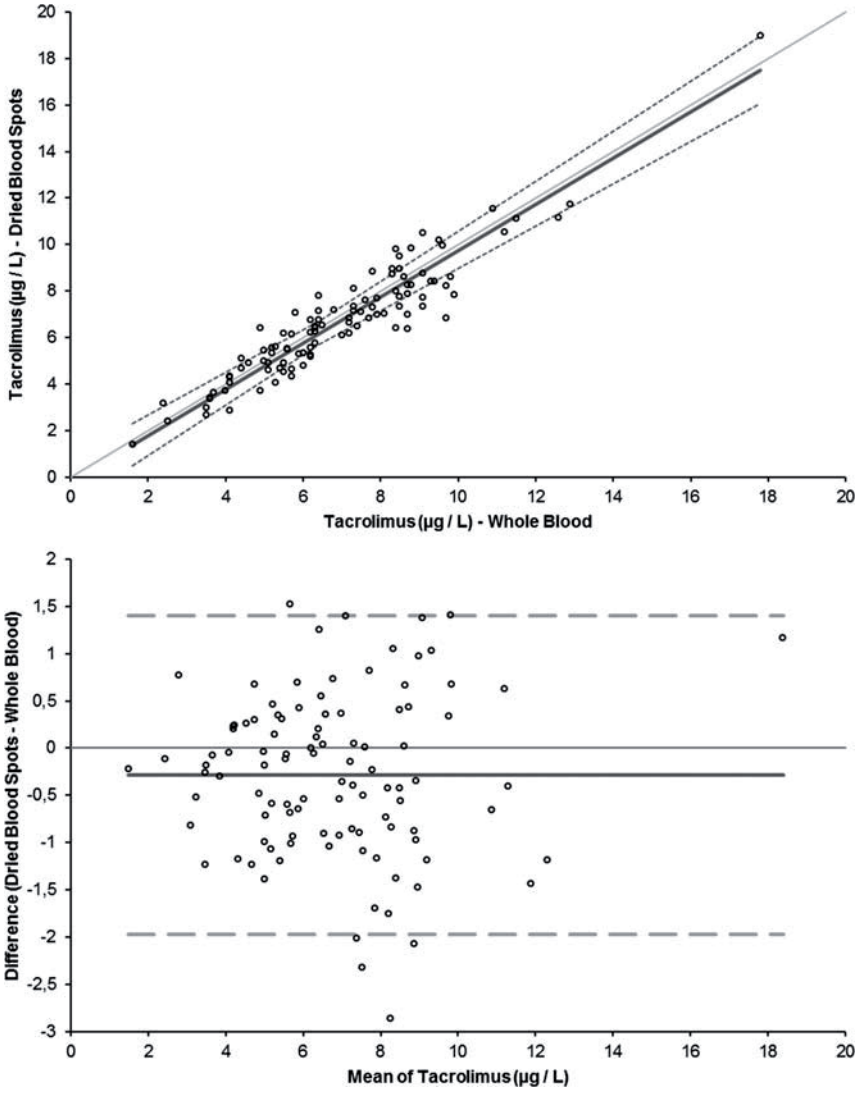

***Figure 2**. Method comparison between venous whole blood TaC concentrations and DBS concentrations (n = 104). In the upper panel, the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line y = 1.00x - 0.23 (95% CI slope, 0.91-1.08; intercept, −0.69 to 0.30). The lower panel shows Bland-Altman analysis with a significant bias of −0.28 µmol/L (95% CI, −45 to −0.12) shown by the continuous line, the dashed line indicates 95% limits of agreement.*

CsA

In total, 61 DBS CsA samples were analyzed, 3 samples were excluded because they were peak concentrations. Linear regression analysis showed a significant relationship between DBS CsA levels and venous whole-blood CsA levels (R^2 = 0.93, P < 0.0001). Passing & Bablok fit was y = 0.99x - 1.86 (95% CI slope, 0.91-1.08; intercept, −8.31 to 3.64), showing no systematic difference as seen in Figure 3. The Bland-Altman analysis

showed a nonsignificant bias. These results show that for CsA, DBS analytical results are interchangeable with venous whole-blood analytical results.

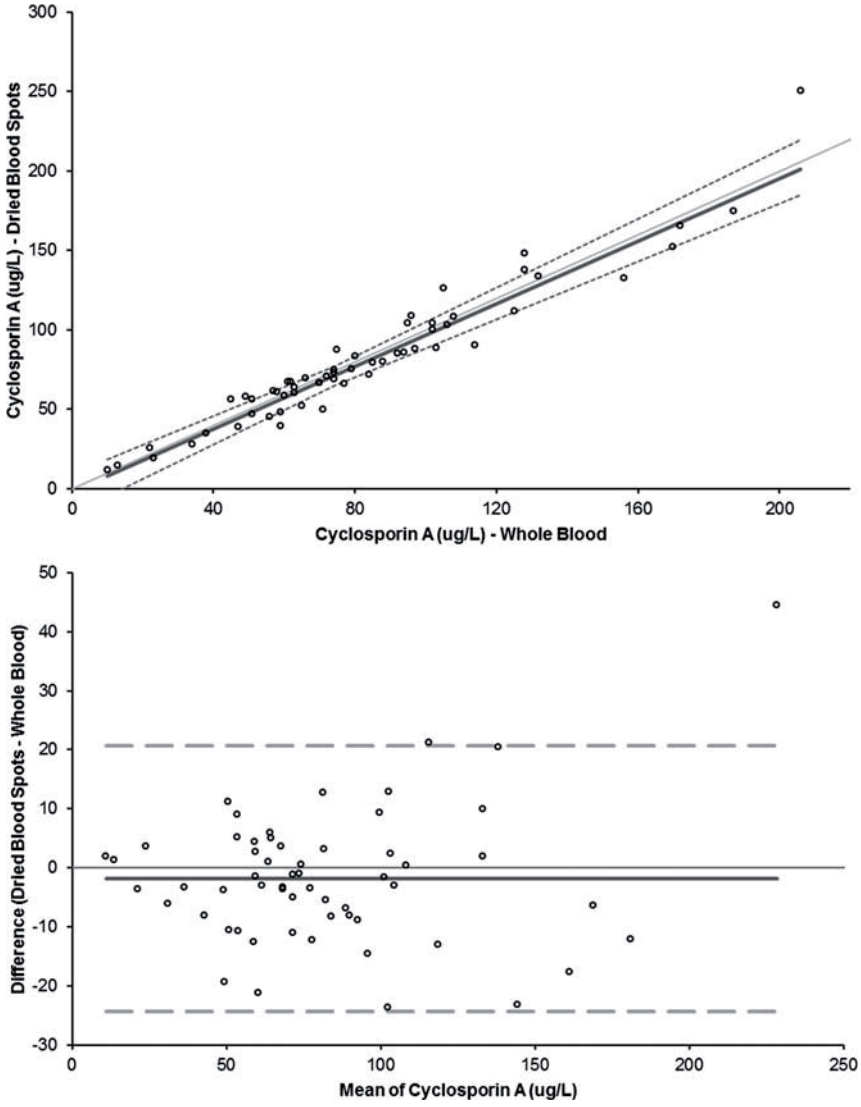

Figure 3. *Method comparison between venous whole blood cyclosporin A concentrations and DBS concentrations (n = 58). In the upper panel, the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line y = 0.99x - 1.86 (95% CI slope, 0.91-1.08; intercept, −8.31 to 3.64). The lower panel shows Bland-Altman analysis with a nonsignificant bias of −1.8 µmol/L (95% CI, −4.8 to 1.3) shown by the continuous line, the dashed line indicates 95% limits of agreement.*

Discussion

This study showed that DBS sampling for the simultaneous analysis of creatinine and immunosuppressants TaC and CsA can replace conventional venous sampling methods in daily routine.

Before monitoring creatinine and immunosuppressive therapy using DBS in transplant patients can be clinically applied, several steps must be taken. The analytical method for DBS samples must be simple, robust, and validated. This study shows excellent linearity of CsA, TaC, and creatinine in DBS compared with venous samples. Ht has been shown to have effect on CsA recovery; however, its influence is within analytical limits, except for CsA concentrations greater than 200 µg/L at Ht of 0.53 or greater.[9,24] This has been deemed not clinically relevant because in outpatient practice trough concentrations are usually targeted at less than 200 µg/L. Because the DBS method for creatinine, TaC, and CsA has been shown to be independent of Ht,[9,18] there is no need for Ht corrections by means of potassium measuring or near-infrared spectroscopy as described in the literature.[6,25] Our results are in agreement with Wilhelm et al.[16] who reported no bias or systematic error for a comparison of CsA in whole blood and DBS in 40 samples in 36 patients. Hinchliffe et al.[8] reported a significant bias for CsA of 2.6 µg/L and a significant bias of −0.7 µg/L for TaC resulting in a correction formula based on the Passing & Bablok analysis. Dickerson et al.[7] reported a mean lower concentration of 0.6 µg/L in DBS compared with venous whole blood for TaC. We report no correction factor and only a small bias of 0.28 µg/L for TaC which is within analytical limits for concentrations greater than 2.0 µg/L.[7,8,24,26] Although the used LC-MS/ MS methods are comparable both Hinchliffe and Wilhelm used Whatman 903 sampling paper, Dickerson did not report the used paper. We previously demonstrated the performance of Whatman DMPK-C cards used in our study is superior to the Whatman 903 paper when using the analysis method developed by our institution.[27] This may have contributed to the observed differences.

Koop et al.[2] were the first to compare clinical DBS and venous samples for simultaneous determination of immunosuppressants and creatinine. Although the correlation coefficient for creatinine was 0.890, the bias found with Bland-Altman was 17.7 µmol/L. In their study, only 19 samples were analyzed, which means that no reliable correction factor could be derived from the results. Our study is the first to propose a correction factor for creatinine concentrations in DBS based on a clinical validation with a larger sample size than any clinical validation of immunosuppressants or creatinine measured in DBS reported in literature. We found a slightly lower concentration of creatinine (−2.1 µmol/L) in DBS compared with plasma samples. In clinical practice, the range of creatinine concentrations in kidney transplant patients is often between 100 and 300 µmol/L, so the lower concentration of creatinine would imply a negative bias of approximately 2.1% and 0.7% at the respective

clinical creatinine concentrations. We doubt that in any clinical situation, this small negative bias would lead to different decision making by clinicians or patients, and therefore we deemed this difference not clinically relevant. As described, the creatinine measurement only requires a reinjection of the extract on a different HPLC column making the simultaneous analysis of immunosuppressants and creatinine relatively simple requiring no complicated techniques like solid phase extraction.[2,18] For immunosuppressants, this study only describes validation in the range of clinically relevant trough concentrations. This limits the use to monitoring trough concentrations in the home setting. Validation at higher concentrations needs to be done before DBS can be applied in studies measuring peak concentrations. In his study, patients did not perform the DBS sampling method themselves. Application of DBS in the home setting will require patients to perform DBS based on training received in the hospital and (video or written) instruction.[17] Incorrect sampling by the patient may lead to insufficient blood spot quality due to overlapping spots, insufficient spot size, blood smearing, and excessive squeezing of the finger leading to hemolytic samples. However, this limitation reduces bias and gives a true comparison of DBS versus venapuncture analytical results. The phlebotomist in our hospital used the same instruction method and DBS sampling method as the patients use at home.[17] Our instruction material contains examples of the most frequently observed incorrect sampling methods. In addition, patients receive training by an experienced phlebotomist before their first application of DBS in the home setting. Another factor influencing successful application are logistical challenges. Because dose adjustments should be done based on a recent trough concentration, time between DBS sampling and arrival of the samples at the laboratory by mail needs to be as short as possible. Although theoretically possible, this could prove to be a challenge in the early posttransplant period when patients frequently visit the hospital. This results in relatively short time intervals between visits, whereas the time between visits must be long enough to allow for completion of the logistic process necessary for routine outpatient application of the DBS method, which includes sampling, sample transport by mail, analysis in the laboratory, and reporting of the analytical results. Although DBS samples are proven to be stable at various temperatures (−80°C to 37°C), extreme conditions during shipment may influence nalytical results.[9,18] We expect that kidney transplant patients are able to perform DBS sampling because kidney transplant patients are experienced with self-monitoring of glucose and/or international normalized ratio due to new-onset diabetes after transplantation.[24] In addition, we expect that the patient's own interest in the performance of their allograft as described by immunosuppressant concentrations and creatinine and the possibility that DBS sampling may lead to distant monitoring by the clinician, reducing the need for clinical check-ups and saving the patients' time and money will contribute to high-quality DBS samples. In the future, studies should be done to evaluate costs and efficacy of DBS in clinical practice to investigate the

possible impact of logistical errors and incorrect sampling by patients using the DBS method.

In summary, we have demonstrated the feasibility of the clinical application of simultaneous detection of immunosuppressants TaC, CsA, and creatinine in DBS. The results from the clinical validation show that the DBS sampling method can produce reliable results and therefore can replace conventional venous blood sampling for these key parameters in the routine care of transplant patients. Implementation of DBS monitoring is feasible and may help with achieving target trough levels, flexible monitoring of graft function and at the same time may reduce patient burden.

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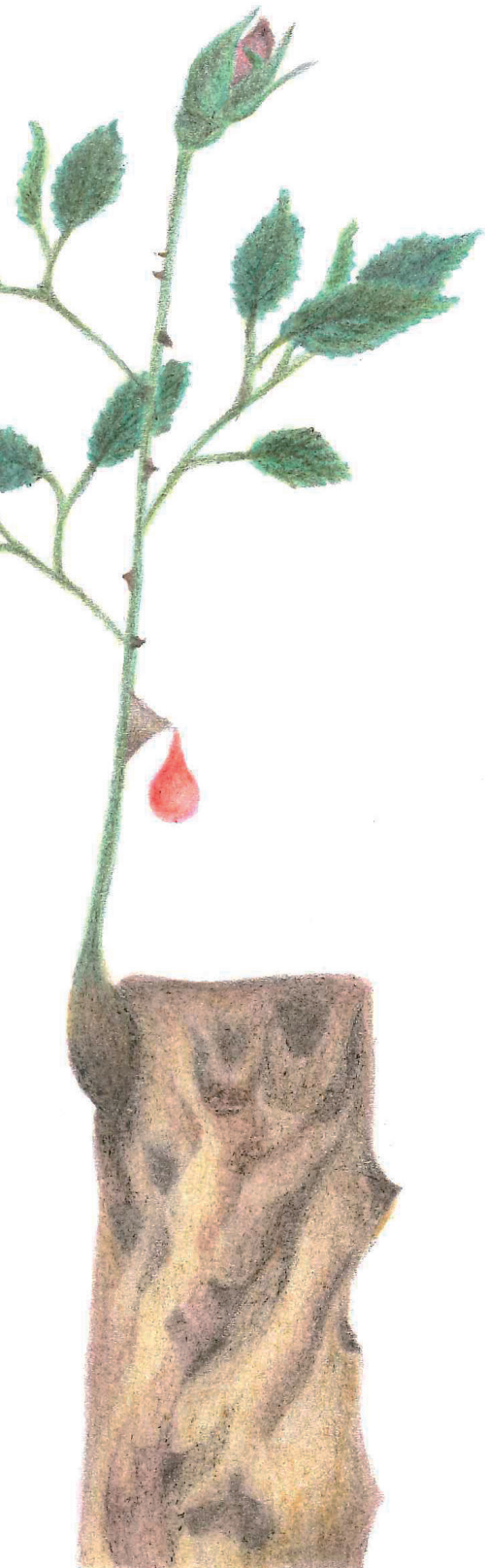

Chapter 4

Clinical application of a dried blood spot assay for sirolimus and everolimus in transplant patients

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Abstract

Background: Monitoring of immunosuppressive drugs such as everolimus and sirolimus is important in allograft rejection prevention in transplant patients. Dried blood spots (DBS) sampling gives patients the opportunity to sample a drop of blood from a fingerprick at home, which can be sent to the laboratory by mail.

Methods: A total of 39 sirolimus and 44 everolimus paired fingerprick DBS and whole blood (WB) samples were obtained from 60 adult transplant patients for method comparison using Passing-Bablok regression. Bias was assessed using Bland-Altman. Two validation limits were pre-defined: limits of analytical acceptance were set at >67% of all paired samples within 20% of the mean of both samples and limits of clinical relevance were set in a multidisciplinary team at >80% of all paired samples within 15% of the mean of both samples.

Results: For both sirolimus and everolimus, Passing- Bablok regression showed no differences between WB and DBS with slopes of 0.86 (95% CI slope, 0.72–1.02) and 0.96 (95% CI 0.84–1.06), respectively. Only everolimus showed a significant constant bias of 4%. For both sirolimus and everolimus, limits of analytical acceptance were met (76.9% and 81.8%, respectively), but limits or clinical relevance were not met (77.3% and 61.5%, respectively).

Conclusions: Because pre-defined limits of clinical relevance were not met, this DBS sampling method for sirolimus and everolimus cannot replace WB sampling in our center at this time. However, if the clinical setting is compatible with less strict limits for clinical relevance, this DBS method is suitable for clinical application.

Introduction

Lifelong therapy with immunosuppressive drugs is a cornerstone in the prevention of rejection of allografts in transplant patient care.[1] Because of their narrow therapeutic range, many immunosuppressive drugs, including the mammalian target of rapamycin inhibitors everolimus and sirolimus are subject to therapeutic drug monitoring (TDM) to allow for balancing between toxic- and sub- therapeutic drug concentrations. Tacrolimus is currently the most widely used calcineurin inhibitor in kidney transplant patient care.[2] However, the recent TRANSFORM trial suggests efficacy of maintenance therapy with everolimus in combination with low dose tacrolimus is comparable to a standard regimen of tacrolimus and mycophenolate mofetil.[3,4] An additional advantage is the reduced viral infection risk. This might lead to an increase in everolimus use in transplant patients. Traditionally, venous blood samples are used for monitoring of immunosuppressive drug concentrations and patients have to travel to the hospital on a regular basis to have their blood drawn. To decrease the burden for patients, dried blood spot (DBS) home sampling has been developed among various micro sampling methods for several drugs, including immunosuppres sants, to enable home sampling.[5-16] For this, a drop of blood from a fingerprick is applied to a sampling card and dried. This card is sent to the laboratory by mail a few days prior to routine check-up of the patient in the hospital. At the time of the check-up, blood-drug concentrations and creatinine levels will be available for the clinician and the patient. Current challenges of DBS implementation include the influence of the hematocrit and logistical hurdles.[9,13,17,18] Although DBS analytical methods can meet the required analytical standards, analysis of clinically collected samples does not always result in sufficient agreement between the standard (venous) method and the novel fingerprick DBS method.[17] Therefore, a clinical validation study showing inter- changeability between DBS and venous sampling is required before clinical application.[18] This is shown for tacrolimus, cyclosporin A and creatinine.[5,7–15,19] For sirolimus, Dickerson et al. report agreement between fingerprick DBS and venous samples in 25 pediatric transplant patients, where mean DBS concentrations were on average 0.8 µg/L lower than venous samples.[15] This difference between the two methods increased with increasing concentrations of sirolimus. Willemsen et al. reported agreement between everolimus fingerprick DBS and venous samples in 20 patients with cancer with a mean ratio of whole blood (WB) to DBS concentrations of 0.90.[20] The current Clinical and Laboratory Standards Institute (CLSI) guideline suggests at least 40 paired samples for comparison, therefore, the number of samples collected in both studies for cross-validation was low.[21] In addition, no clinical validation study for everolimus using fingerprick DBS has been published for transplant patients. Therefore, the aim of this study was to clinically validate our method for analyzing sirolimus and everolimus in DBS to enable implementation in routine care.

Materials and methods

Patient and sample collection

Patient samples were collected from adult transplant patients during routine clinical check-ups in the hospital. Because of the nature of this study, the need to provide written informed consent by the patients was waived by the Ethics Committee of the University Medical Center Groningen (Metc 2011.394). A trained phlebotomist obtained both the venous and DBS samples within 10 min of each other using a collection method described elsewhere.[9,22,23] In short, after a fingerprick, two drops of blood were allowed to fall freely on a Whatman FTA DMPK-C sampling paper (GE Healthcare, Chicago, IL, USA). The WB samples were analyzed within a day as they were part of routine care. DBS are stable for at least 7 days at room temperature, therefore the DBS samples were allowed to dry for 24–74 h at room temperature and packed in zip lock plastic mini bags with a desiccant.[24–26] Upon receiving the DBS samples in the laboratory, the samples were inspected for spot quality based on pre-defined criteria.[22,23,27] DBS samples fit for analysis were stored at -20°C until analysis. DBS samples are stable for at least 29 weeks at -20°C so analysis occurred within this timeframe.[25]

Equipment, Conditions and Procedures

Our reference procedure was a measurement of sirolimus and everolimus in WB obtained by venipuncture, with a previously validated analysis method performed on a Thermo Fisher Scientific (Waltham, MA, USA) triple quadrupole Quantiva LC-MS/MS system with a Vanquish HPLC system.[28] For the DBS analysis of sirolimus and everolimus, a previously validated method was used using the aforementioned Thermo Quantiva LC-MS/MS.[24,25] The analytical range for both the WB and DBS assay for sirolimus and everolimus was 1.0–50.0 µg/L. Hematocrit of the venous samples was measured using an XN10/XN20 hematology analyzer (Sysmex, Kobe, Japan).

Statistical analysis

Statistical analysis was performed using Analyse-it® Method Validation Edition for Microsoft Excel version 4.18.6 (Analyse-it, Leeds, UK) and Microsoft Excel (Microsoft Inc., Redmond, WA, USA). Method comparison was done using Passing-Bablok regression analysis and a Bland-Altman analysis was used for bias calculation.[29,30] Because no official guideline exists for clinical validation of DBS assays, we set two limits of acceptance a priori. The first is the limit of analytical acceptance which is based on the EMA guidelines for cross-validation and the 2018 version of the FDA guideline for studies required to bridge two analytical methods.[31,32] As acceptance criteria, both FDA and EMA guidelines state that at least two-thirds (67%) of the paired samples should be <20% of the mean of both methods. The second is the

limit of clinical relevance which was set at a range of 85%–115% around the ratio of the paired DBS and WB samples for at least 80% of the samples. This range was chosen by a multidisciplinary team consisting of clinicians, pharmacists and analysts and was chosen based on the therapeutic window given in the summary of product characteristics of 3–8 μg/L for everolimus and 4–12 μg/L for sirolimus trough concentrations for stable transplant patients >3 months after transplantation.[33,34] A difference of 15% in the acceptable range ratio for a high everolimus trough concentration (8 μg/L) in WB would lead to a DBS concentration range of 6.8–9.2 μg/L. For a low everolimus trough concentration (3 μg/L) in WB this would lead to an acceptable DBS concentration range of 2.6–3.5 μg/L. These values are comparable to the acceptable variability of 15% for accuracy and precision that are mentioned in the FDA and EMA guidelines for bioanalytical methods.[31,32] If 80% of all patients are within this range this was deemed feasible by the clinicians. The predictive performance of the DBS analytical method was established using the method described by Sheiner and Beal.[35] In short, DBS concentrations were used to predict WB concentrations. For each paired WB and DBS sirolimus and everolimus sample, the slope and intercept of the Passing-Bablok regression was calculated using the whole population of sirolimus and everolimus samples, respectively, excluding the data of that specific paired sample. The error of this prediction is determined by bias and imprecision. The bias is the median difference between the predicted and true concentration and is shown by the median prediction error (MPE) and the median percentage prediction error (MPPE). The imprecision is the variance of the predicted values which is measured by the root median squared prediction error (RMSE) and the median absolute percentage prediction error (MAPE). For analyzing the predictive performance the following equations were used:

$$Median\ Prediction\ Error\ (MPE) = median\ (Predicted\ WB - WB) \quad (1)$$

$$Median\ Percentage\ Prediction\ Error\ (MPPE) = median\ \left(100\% * \frac{Predicted\ WB - WB}{WB}\right) \quad (2)$$

$$Root\ Median\ Squared\ Prediction\ Error\ (RMSE) = \sqrt{Median}(Predicted\ WB - WB)^2 \quad (3)$$

$$Median\ Absolute\ Percentage\ Prediction\ Error\ (MAPE) = median\ \left(100\% * \frac{|Predicted\ WB - WB|}{WB}\right) \quad (4)$$

In accordance with other studies, acceptable values for MPPE and MAPE were set at <15% and at least 67% of all samples should have an absolute prediction error of <20%.[5,20]

Results

Patients and samples

A total of 90 paired DBS and WB samples were taken from 60 adult transplant patients between January 2017 and December 2017. All DBS cards had at least one spot of sufficient quality for analysis. Three samples were excluded because no paired WB sample was taken. Another three samples were excluded because the WB and DBS sample were not taken within 10 min of each other. One sample was excluded because it was not a trough concentration. A total of 39 paired sirolimus and 44 paired everolimus samples were available for method comparison from 29 and 27 unique transplant patients, respectively. The hematocrit ranged from 0.23 to 0.51 (v/v) with a mean hematocrit of 0.40. All hematocrit values were within the analytically validated range, which means that the hematocrit value had no influence on the DBS analytical results.[24] Mean concentrations of sirolimus and everolimus in WB and DBS can be found in Table 1. All evaluated concentrations were within the analytically validated range.[24] Patient demographics and transplantation type can be found in Tables 2 and 3.

Table 1. Mean drug concentrations, range and SD of sirolimus and everolimus in whole WB and DBS

Drug concentrations	N	Mean ± SD (range)
Sirolimus in WB (µg/L)	39	5.0 ± 2.4 (1.9 - 10.9)
Sirolimus in DBS (µg/L)	39	4.7 ± 1.9 (1.8 - 9.7)
Everolimus in WB (µg/L)	44	5.4 ± 2.6 (1.2 - 14.3)
Everolimus in DBS (µg/L)	44	5.0 ± 2.4 (1.9 - 10.9)

Table 2. Patient demographics and transplantation type

Patient demographics and clinical laboratory data	N	Median (range)
Age (years)	56	61 (23-77)
Sex	56	38 male (67.9%)
		18 female (32.1%)
Time from transplantation	56	2 years,3 monts, 5 days
		(10 days - 22 years, 7 monts)

Table 3. Patient transplantation type per sample type

Transplantation type	Sirolimus samples	Everolimus samples	Total samples	Unique patients
Liver	30	0	**30**	22
Lung	2	7	**9**	7
Stem Cell	7	0	**7**	6
Kidney	0	37	**37**	21
Total	**39**	**44**	**83**	**56**

Clinical validation

Sirolimus

For sirolimus, the Passing-Bablok analysis fit was y = 0.86x + 0.44 (95% CI slope, 0.72–1.02; 95% CI intercept −0.23 to 1.11) showing no significant constant or systematic difference as can be seen in Figure 1. The correlation coefficient was 0.93. The Bland-Altman plot (Figure 2) shows that the mean ratio of WB and DBS sirolimus concentrations is 1.00 (95% CI 0.93–1.07), without significant bias. The 95% limits of agreement (LoA) are 0.60 and 1.40, which is wider than the limits of (23.1%) fell outside the limits of analytical acceptance. For the limits of clinical relevance this was 15/39 (38.5%). For the predictive performance, bias was small with an MPE of −0.008 µg/L and an MPPE of −0.16%. The predictive performance of imprecision as measured by the RMSE was small with a value of 0.56 µg/L. The MAPE was within acceptable limits (<15%) with a value of 11.07%. The acceptance limit for MAPE (at least 67% of the samples with a value <20%) was met with 30 out of 39 values (76.9%) (Figure 3).

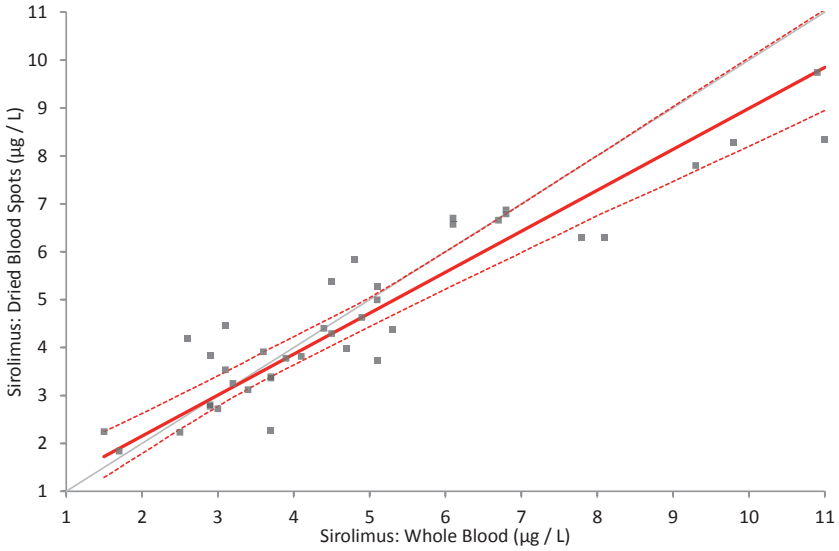

Figure 1. *Method comparison for sirolimus concentrations in WB and DBS (n = 39). The continuous line is the Passing-Bablok regression line y = 0.86x + 0.44 (95% CI slope, 0.72–1.02; 95% CI intercept −0.23 to 1.11). The dashed line is the 95% CI.*

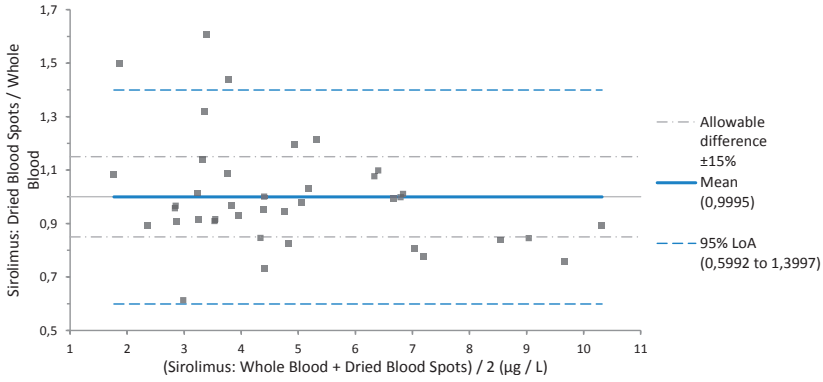

***Figure 2**. Method comparison for sirolimus concentrations in WB and DBS (n = 39).The continuous line is the Bland-Altman bias estimation of 1.00 (95% CI 0.93–1.07). The dashed line is the 95% LoA and the dotted/ dashed line is the limit of clinical relevance set at 15%.*

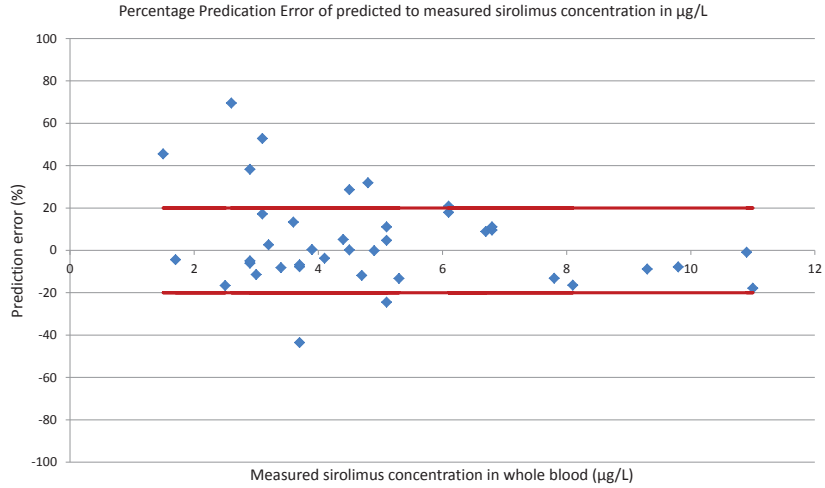

***Figure 3**. Percentage prediction error or predicted to measured sirolimus concentrations with acceptable prediction error set at –20% and 20%.*

Everolimus

For everolimus, the Passing-Bablok analysis fit was y = 0.96x + 0.37 (95% CI slope, 0.84–1.06; 95% CI intercept –0.11 to 0.99), also showing no significant constant or systematic difference as can be seen in Figure 4. The correlation coefficient was 0.97. The Bland-Altman plot (Figure 5) shows that the mean ratio of WB and DBS everolimus concentrations is 1.04 (95% CI 1.00–1.08), which is a small but significant bias of 4%. The 95% LoA are 0.78 and 1.30, which is wider than the limits of analytical acceptance which were set at 0.80 and 1.20. Only eight out of 44 values (18.2%) fell outside the limits of analytical acceptance. For the limits of clinical relevance this was 10 out of 44 (22.7%). For the predictive performance, bias was small with an MPE of 0.003 µg/L and an MPPE

of 0.13%. The imprecision as measured by the RMSE was small with a value of 0.39 µg/L. The MAPE was within acceptable limits (<15%) with a value of 7.9%. The acceptance limit for MAPE (at least 67% of the samples with a value <20%) was met with 39 out of 44 values (88.6%) (Figure 6). One outlier of −72.5% was observed. The outlier prediction error shown in Figure 6 can likely be explained by the low concentration of everolimus (1.2 µg/L in WB), which is just above the lower limit of quantification of the method. In this setting, the influence of the intercept (−0.49) becomes paramount, resulting in a predicted value of 0.33 µg/L, giving a prediction error of −72.5%.

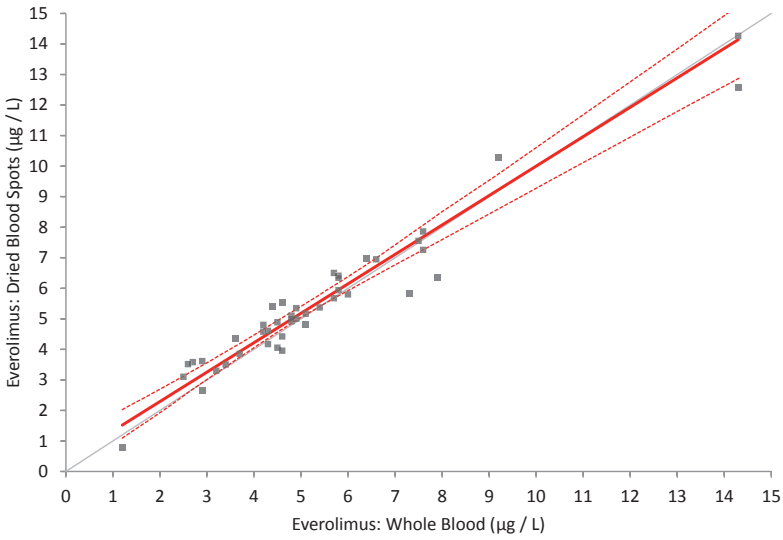

Figure 4. *Method comparison for everolimus concentrations in WB and DBS (n = 44).The continuous line is the Passing-Bablok regression line y = 0.96x + 0.37 (95% CI slope, 0.84–1.06; 95% CI intercept −0.11 to 0.99). The dashed line is the 95% CI*

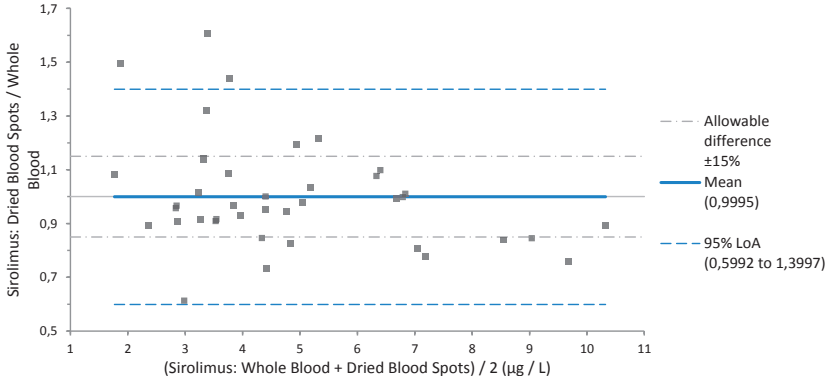

Figure 5. *Method comparison for everolimus concentrations in WB and DBS (n = 44).The continuous line is the Bland-Altman bias estimation of 1.05 (95% CI 1.00–1.08). The dashed line is the 95% LoA and the dotted/ dashed line is the limit of clinical relevance set at 15%.*

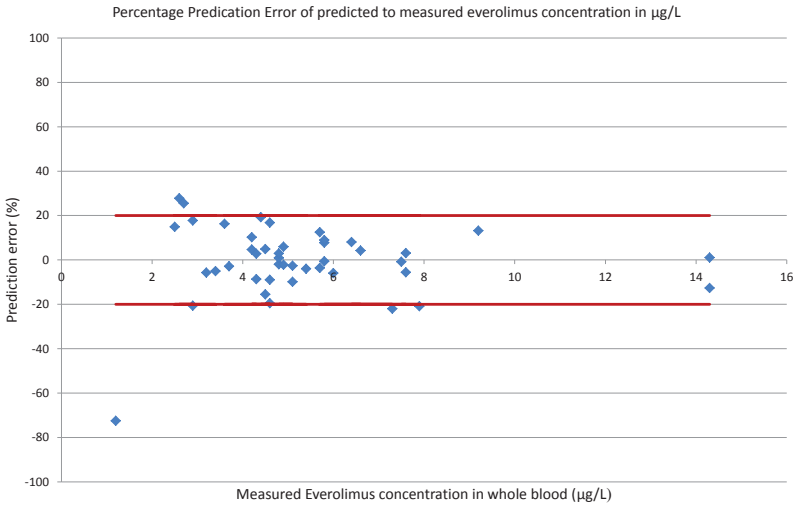

Figure 6. *Percentage prediction error or predicted to measured everolimus concentrations with acceptable prediction error set at –20% and 20%.*

Discussion

This study showed good agreement between DBS sirolimus and everolimus concentrations and venous WB concentrations in transplant patients over a concentration range relevant for TDM of trough concentrations. No correction factor is needed to calculate WB values from DBS values. For sirolimus and everolimus 76.9% and 81.8%, respectively, of all DBS concentrations fell within limits of analytical acceptance. Therefore, this method met the requirements set in the EMA guideline for cross-validation and FDA guidelines for bridging studies.[31,32] The predictive performance of the sirolimus and everolimus DBS method complied with the predefined criteria of >67% of all samples to have a prediction error of <20%. However, the limits set for clinical relevance (>80% of the samples with <15% of the mean) were not met with a value of 77.3% and 61.5% for sirolimus and everolimus, respectively.

Because tacrolimus is the most widely used immunosuppressant in our center to prevent renal allograft rejection, the amount of patients in our institution receiving either sirolimus or everolimus is limited. Therefore, patients from all transplantation types (Table 3) were asked to provide samples. The heterogeneous patient population is a strength of this study, hematocrit values of all patients were within the analytically validated limits and mean hematocrit values were comparable between the different groups of transplant patients (data not shown).

Because a clinical validation of a DBS fingerprick method shows strong resemblance to a cross validation, the CLSI guideline recommends to include at least 40 patient samples.[21] Although the study by Willemsen et al. showed good agreement between WB and capillary blood, the performed power calculation resulting in 20 samples necessary was done prior to this result. The power calculation was based on the assumption that venous blood and DBS are the same matrix and no effect of the hematocrit is expected.[20] It is, however, well-known that hematocrit can affect DBS assays and sometimes results in unacceptable biases.[24,25,36] Capillary collected blood consists of a mixture of venous blood, arterial blood and interstitial fluid which is not the same matrix as a venous WB sample. Therefore, we think making an assumption that the matrix of capillary blood is the same as venous WB is not recommended. Following the CLSI guideline for finding a sample size would, in our opinion, be more appropriate. The recommendation of 40 samples in the CLSI guideline is based on regression analysis described by Linnet, where the amount of samples necessary for a cross-validation can be calculated based on the analytical characteristics of the assay.[37] If Linnets' calculation would be followed for the everolimus DBS assay used by Willemsen et al., the recommended number of samples is 40, and if Linnets'

calculation would be followed for the sirolimus DBS assay used by Dickerson et al. the recommended number of samples is 37.[19,36] Because of the exclusion of several sirolimus samples the required amount of 40 samples was not met. However, with the amount of 39 paired samples available, we do not think that the absence of one paired sample has a great influence on the clinical validation.

For everolimus, our results are in part in agreement with Willemsen et al.[20] Our method did not show a constant or proportional bias as shown by Willemsen et al. where a small but significant proportional bias was found in the Passing-Bablok regression. In addition, they demonstrated a ratio of 0.90 in the Bland-Altman comparison, where our method shows a small but statistically significant ratio of 1.04. It should be noted that the Bland-Altman comparison by Willemsen et al. is shown as a ratio of WB/DBS which is in contrast with this study where the ratio is shown as DBS/WB. However, the spread of the relative difference in our method (Figure 5) and corresponding LoAs are wider than in the method used by Willemsen et al. This is especially true for the low trough concentrations (1–5 μg/L). Although not statistically significant, the analytical validation showed a trend towards more bias at lower concentrations (3 μg/L) compared to higher concentrations (10 μg/L) for everolimus.[24] This might be an explanation for the observed spread of relative difference. Other clinical validation studies usually have few samples and very few samples in the low concentrations range. However, in a study on tacrolimus, 22.2% (n = 63) of the lower (trough) concentrations exceeded <20% limits of the DBS to WB concentration ratio.[5] In this study, the area under the curve (AUC) was calculated for both DBS and WB based on trough concentrations and three sampling points at t = 1, t = 2 and t = 3 h after medication intake. For the AUCs, 90.3% (n = 63) of the paired AUC values were within 20% limits of DBS to WB ratio suggesting higher tacrolimus concentrations show less spread compared to trough concentrations. It is unlikely that the hematocrit has caused these differences, because previous research shows that hematocrit effects are most prominent at high concentrations of everolimus and sirolimus (50 μg/L) and low hematocrits (<0.23 v/v).[24,25] Re-evaluation of the data stratified for either transplantation type or time from transplantations showed that these two factors are not of influence on the results (data not shown). In future studies, introduction of duplicate analysis of both WB and DBS samples or analysis of two individual blood spots might reduce the observed spread in the lower (1–5 μg/L) concentration range. In addition, incurred sample reanalysis (ISR) is recommended for both WB and DBS samples to assess the spread of individual patient samples. Two major differences present in the study by Willemsen et al. are the much broader concentration range of trough concentration samples (3.6–28.5 μg/L in WB) and the broader limits of clinical relevance that were used in comparison to this study.[20] Because dosing of everolimus in patients with cancer is performed in steps of 2.5 mg and the target trough concentration range is much wider

(up to 19.2 µg/L), a larger clinical limit is accepted.[38,39] In transplant patients, dosing can be done in steps of 0.25 mg and the target trough concentration range is 3–8 µg/L, therefore, a much narrower limit of clinical relevance is adjudicated. To the best of our knowledge, no guideline is available to determine limits of clinical relevance for DBS. The available literature suggests that setting a limit of clinical relevance should be done in a multidisciplinary team taking into account the clinical application of the method, the patient characteristics and the properties of the analytical methods.[5,20] In our study, the everolimus DBS method does not meet the limits of clinical relevance set by our team and, at this time, cannot replace conventional WB sampling in the TDM of transplant patients where low trough concentrations are targeted. For sirolimus, Dickerson et al. showed a statistically significant difference of −0.8 µg/L in the Bland-Altman analysis where our method showed no bias.[15] The range of sirolimus concentrations in Dickerson et al. is 4–18 µg/L which is higher than the range of 1.7–10.9 µg/L in our study. The observed increased bias for higher trough concentrations (>10 µg/L) shown in Dickerson et al. might also be present using our method. Although results are shown as a ratio, samples with a WB concentration of >7.5 µg/L (n = 6) also showed lower concentrations in DBS (Figure 1) in this study. Excluding these samples yields a slope of 1.04 in Passing-Bablok regression, this explains the observed slope of 0.86 in the Passing-Bablok regression analysis for all sirolimus samples. However, excluding these samples does still result in not meeting the limits of clinical relevance. Another possibility is that this is a random phenomenon because the amount of samples with sirolimus WB concentration >7.5 µg/L is limited. Additional samples in the range of 5–15 µg/L are needed to assess this. For sirolimus, the limits of clinical relevance are not met in this study and the same trend as for everolimus is present where samples with a concentration of 1–5 µg/L showed the greatest bias. This might be caused by the same factors mentioned before for everolimus. Therefore, at this time, the sirolimus DBS method cannot replace conventional WB sampling in the TDM of transplant patients with low trough concentrations.

In our study the DBS samples were obtained by trained phlebotomists at the hospital and not by the patients themselves at home. Considering DBS methods are intended for home-sampling this might be a limitation of our study. However, the instructions and sampling methods are the same for both phlebotomist and patient. Patients receive instructions before home sampling is initiated including practicing a fingerprick under the supervision of a trained phlebotomist. This should be sufficient for appropriate sampling at home if a patient or caregiver is willing and able to perform home sampling, in addition, paper and video instruction are available.[40]

In the area of transplantation, where narrow therapeutic windows are followed for TDM of immunosuppressants, there are strict requirements for the analytical

performance of assays measuring immunosuppressants in blood. With the current data, this clinical DBS validation study showed that not all predefined requirements set were met. Although Passing-Bablok analysis showed no systematic or constant differences between WB and DBS samples, the spread of samples did not meet the predefined limits of clinical relevance. However, as these limits were set by a local multidisciplinary team these may vary between settings and centers.[18] In addition, in a limited resources setting, where no WB bioanalytical method exists for sirolimus and everolimus, the DBS assay presented here could be used to allow TDM. If future studies show optimization of DBS assays using ISR, and if logistical challenges surrounding DBS home sampling can be overcome, the DBS method could be implemented in routine transplant patient care.[9,13,18] This would help in reducing patient burden, quickly achieving target trough levels the first months after transplantation and flexible monitoring of graft function.

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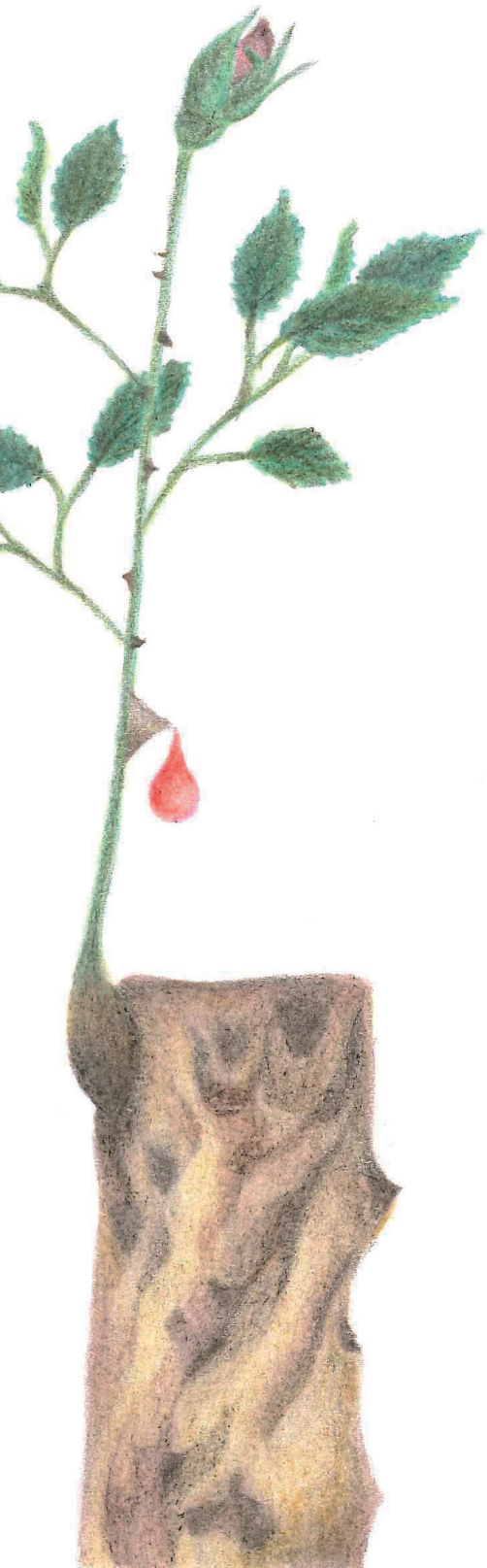

Chapter 6

Performance of a web-based application measuring spot quality in dried blood spot sampling

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Abstract

Background: The dried blood spot (DBS) method allows patients and researchers to collect blood on a sampling card using a skin-prick. An important issue in the application of DBSs is that samples for therapeutic drug monitoring are frequently rejected because of poor spot quality, leading to delayed monitoring or missing data. We describe the development and performance of a web-based application (app), accessible on smartphones, tablets or desktops, capable of assessing DBS quality at the time of sampling by means of analyzing a picture of the DBS.

Methods: The performance of the app was compared to the judgment of experienced laboratory technicians for samples obtained in a trained and untrained setting. A robustness- and user test were performed.

Results: In a trained setting the app yielded an adequate decision in 90.0% of the cases with 4.1% false negatives (insufficient quality DBSs incorrectly not rejected) and 5.9% false positives (sufficient quality DBSs incorrectly rejected). In an untrained setting this was 87.4% with 5.5% false negatives and 7.1% false positives. A patient user test resulted in a system usability score of 74 out of 100 with a median time of 1 min and 45 s to use the app. Robustness testing showed a repeatability of 84%. Using the app in a trained and untrained setting improves the amount of sufficient quality samples from 80% to 95.9% and 42.2% to 87.9%, respectively.

Conclusions: The app can be used in trained and untrained setting to decrease the amount of insufficient quality DBS samples.

Introduction

Dried blood spot (DBS) sampling is a technique that finds its application in clinical research and routine patient care as part of therapeutic drug monitoring (TDM).[1-3] Using a skin-prick, capillary blood is applied to a sampling card that is allowed to dry. From these DBSs, blood drug concentrations, clinical chemical parameters such as creatinine or titers of antiviral antibodies can be measured.[2,4,5] The advantages of DBSs include increased sample stability and ease of sample storage, more convenient and simple sampling procedure with reduced risk of infection, no phlebotomist required for sampling and the possibility of sending samples by regular mail without special precautions.[5,6] Therefore, DBSs are used to facilitate sampling for TDM in remote areas and patient home sampling.[7] One of the major issues in DBS sampling is the quality of the produced blood spots. In short, a good quality blood spot is round, consists of one droplet, does not touch other droplets and is large enough for punching a 3, 5 or 8 mm disc.[8-10] However, even in controlled environments, where trained phlebotomists obtain the DBS samples, 4–5% of the samples are rejected because of insufficient quality.[2] When patients sample at home as part of routine care, 80% of obtained blood spots are of sufficient quality.[10] In clinical research in developing countries, where DBS sampling is performed by untrained researchers, rejection rates can even be as high as 52%.[11] Rejection of DBS samples can lead to delayed monitoring of patients or missing data in clinical research. Other factors impacting DBS sample quality are the choice of filter paper, analyte stability, storage and transport conditions, exposure to direct sunlight, drying time and humidity.[12]

Currently, quality inspection of the DBSs is performed at the laboratory by experienced laboratory personnel (ELP) based on available World Health Organization (WHO) and Clinical and Laboratory Standards Institute (CLSI) guidelines and quality standards that are set by the individual laboratory.[8,11,13] The issue with this workflow is that quality inspection is performed upon arrival at the laboratory and not immediately after the moment of sampling. If samples are of insufficient quality, timely resampling is often not possible.[14]

Although training of sampling can decrease the rejection rate of samples,[9] it would be more convenient if a phlebotomist, researcher or patient is able to determine the quality of a sample at the time of sampling, which would give the possibility of immediate resampling if the sample is of insufficient quality.

In newborn bloodspot screening an optical scanning instrument is available for measuring spot quality, but this method still requires that samples are sent to the laboratory before quality inspection.[15] Currently, no standardized, automated method exists for determining spot quality in fingerskin-prick DBS sampling at the time of sampling. We aimed to develop a tool that can be easily used by patients,

healthcare workers and researchers at the time of sampling and gives reliable results for DBS spotting quality. We describe the development and performance of a web-based application (app) capable of measuring DBS quality by means of capturing images of the blood spot. The app was tested in both a trained and an untrained setting.

Materials and methods

Using the app

The app is a responsive web-based application accessible in the browser of a smartphone, tablet, laptop or desktop PC. The app requires a working Internet connection to load but no installation on a device is required. After the app has been loaded and saved in the browsers cache, the app can be used off-line. A detailed instruction on how to use the app can be found in Figure 1. The app is available in Dutch and English and can be found at www.dbsapp.umcg.nl. The app has been developed by MAD multimedia (Groningen, The Netherlands) in consultation with specialists from the Department of Clinical Pharmacy and Pharmacology from the University Medical Centre Groningen (Groningen, The Netherlands). A detailed description of the app specifications can be found in Supplementary file S1 (available online, Open Access).

Performance qualification

DBS samples were visually inspected for layering, contaminations, hemolysis, dilution, clotting, smearing of blood, saturation of the paper, coloration and intactness of the filter paper based on available guidelines because all of these factors can influence analytical results.[8,11,13] Two experienced technicians (ELP) independently evaluated the test samples and were considered as gold standard (GS) for the app. When the judgment of the ELP differs, the sample was re-evaluated by the ELP until consensus was obtained. The performance of the app was defined as the percentage of samples where the judgment of the app is in agreement with the GS. If the judgment of the app and ELP differ, there can be either a false positive or false negative result. False positives (app judges sample as insufficient, ELP judges as acceptable) will lead to unnecessary resampling but not to delayed monitoring. False negatives (app judges sample as acceptable, ELP judges insufficient) will lead to sending samples of insufficient quality, which would result in delayed monitoring or incomplete data. In clinical validation studies, usually 95% of samples obtained by trained phlebostomists are judged as acceptable.[2] Therefore, we set the performance qualification of the app at 95% prior to testing the app.

Sample size

A sample size calculation was performed based on a non-inferiority hypothesis, a power of 80% and an alpha of 5%. The judgment of the ELP (P1) is 0.99 and the judgment of the app (P2) is expected to be 0.96. A non-inferiority margin is set at 0.01 and sampling ratio at 1:1. This resulted in a sample size of 187. For the trained setting, 221 DBS samples were available. For the untrained setting, 1610 DBS samples were available. To avoid selection bias, we decided to use all samples to test the app.

Ethics statement

For the performance testing, patient samples were used from earlier studies.[2,11] Additionally, patients were asked to participate in the user test. Due to the availability of previously collected samples, the need to obtain written informed consent from the subjects was waived by the Ethics Committee of the University Medical Center Groningen (Metc 2011.394).

Trained setting

In total 221 blood spots were collected from 181 adult kidney transplant patients.[2] Samples were collected during routine visits of transplant patients to the clinic using a standardized method.[16] Trained phlebotomists obtained the samples by fingerprick using a Blue Microtainer Contact-activated Lancet (BD and Co, Franklin Lakes, NJ, USA) and letting a drop of blood fall freely on a Whatman FTA DMPK-C sampling card (GE Healthcare, Chicago, IL, USA).

Untrained setting

A total of 1610 individual spots were collected in a previous study.[11] The samples were collected as part of a TDM study of anti-tuberculosis drugs in Bangladesh (n = 244), Belarus (n = 358), Indonesia (n = 516) and Paraguay (n = 492).[11] DBS samples were obtained by local healthcare workers who did not receive on the job training and only had the written instructions in English before sampling.[16] Although 1856 individual spots were obtained in the aforementioned study, some spots were already analyzed before a photo could be captured resulting into 1610 usable spots for this study.

Testing app performance

The app was tested using an Apple iPhone 5S (Cupertino, CA, USA), equipped with a standard 8 megapixel camera. The DBS card was placed on a clean and flat surface. No extra lighting apart from the standard ceiling chemiluminescent lights (3350 lumen) available in the laboratory was used. To avoid variation, the iPhone 5S was not handheld but fixed in landscape position at 8 cm above the DBS card.

Pictures were taken after auto-focusing of the camera without using the flash light. All pictures of the samples were processed in duplicate in the app on a desktop PC.

Robustness

The International Conference on Harmonization (ICH) states "The robustness/ ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage".[17] To test robustness, factors that could possibly interfere with the performance of the app were identified: person taking the picture, camera type, lighting, casting a shade, use of the camera's flashlight, distance between sample and camera, angle for taking the picture, device on which the app is used. To test the influence of these factors, a library of 16 samples was made from the "trained setting" samples set that were difficult for the app to process during performance testing as experienced by the technicians testing the app and based on the function of the app. The test samples consisted of five false negatives, five false positives, three good spots and three bad spots as was determined by the app during the initial performance testing. Three different investigators using three different phones tested the app for all 16 samples using ideal circumstances as described under "testing app performance" as baseline with alteration of one of the following conditions for each test run: (1) Dimly lit room (no ceiling lights and only limited daylight through a small window), (2) Casting a shade on the sampling card, (3) Using the camera's flashlight, (4) Using a distance of 50 cm between camera and sampling card, (5) Taking the picture from a 45° angle. The success rate was defined as the percentage of samples that yielded the same results in the app as was found in the initial performance testing of the samples. The three phone cameras that were used were the standard equipped cameras using autofocus on the iPhone 5, Nokia C5 2010 version (Espoo, Finland) and Samsung Galaxy S7 Edge (Seoul, South Korea). All pictures were tested in the app both on the device the picture was taken on and on a PC, with the exception of the photos taken with the Nokia C5 which were only tested on a PC.

User test

Usability is defined as "the extent to which a product can be used by specified users to achieve specified goals with effectiveness, efficiency and satisfaction in a specified context of use".[18] A user test was designed based on available literature, details can be found in Supplementary file S2 (available online, Open Access).[19-23] [19–23]. Results were scored using the system usability score (SUS), a score of above 70 was considered acceptable.[22]

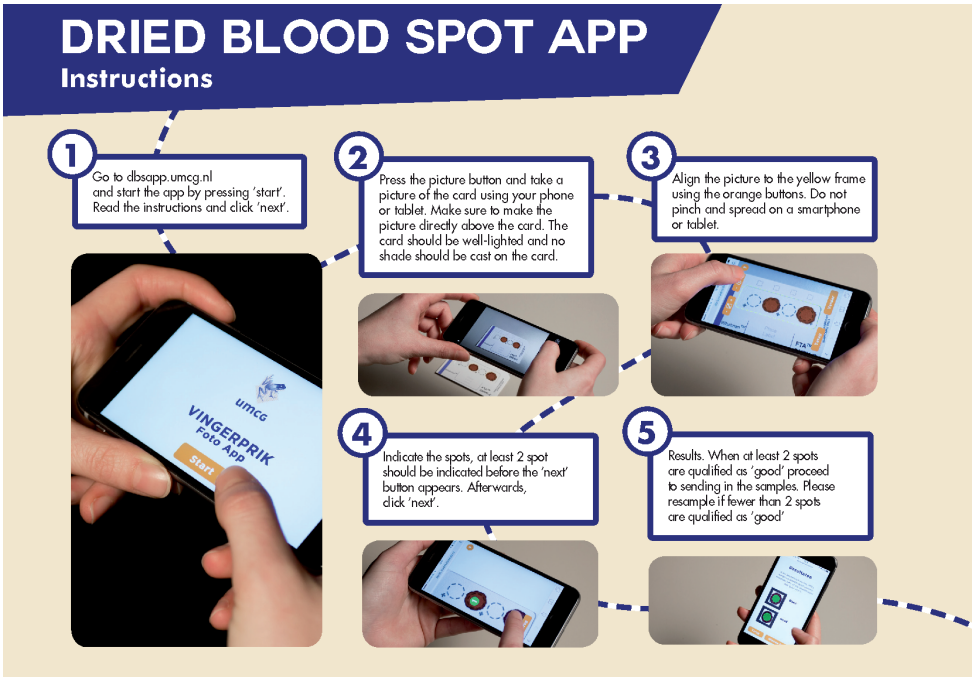

Figure 1. *Instructions for using the DBS app*

Results

Trained setting

In total, 149 (67.4%) samples were judged as acceptable and 72 (32.6%) as insufficient by the GS. The first version of the app showed a performance with accurate judgment of 76.8% of the samples with 10.5% false negatives and 12.7% false positives. For the false negatives, two types of errors were identified. The app could not identify layering of blood spots (Figure 2A) and spots that were hemolytic or discolored due to humidity (Figure 2B). The false positives consisted of spots that were not circle-shaped (Figure 2C). Because this result did not meet the performance qualification of 95%, the app was improved, resulting in a second version. In this version, the nine electronic iterations with a 10° rotation were introduced and width–height ratio was set at 12% based on retesting of false positive and false negatives samples (see Supplementary file S1). The second version of the app resulted in a performance of 90.0%, with 5.9% false positives and 4.1% false negatives. In the second version of the app, the number of layered spots that were identified as false negatives were reduced from 21 to 7 due to the introduction of the nine iterations wherein the picture is rotated. As a result, the number of false positives dropped from 28 to 13 and the number of false negatives dropped from 23 to 9. The second version of the app was used for all remaining tests.

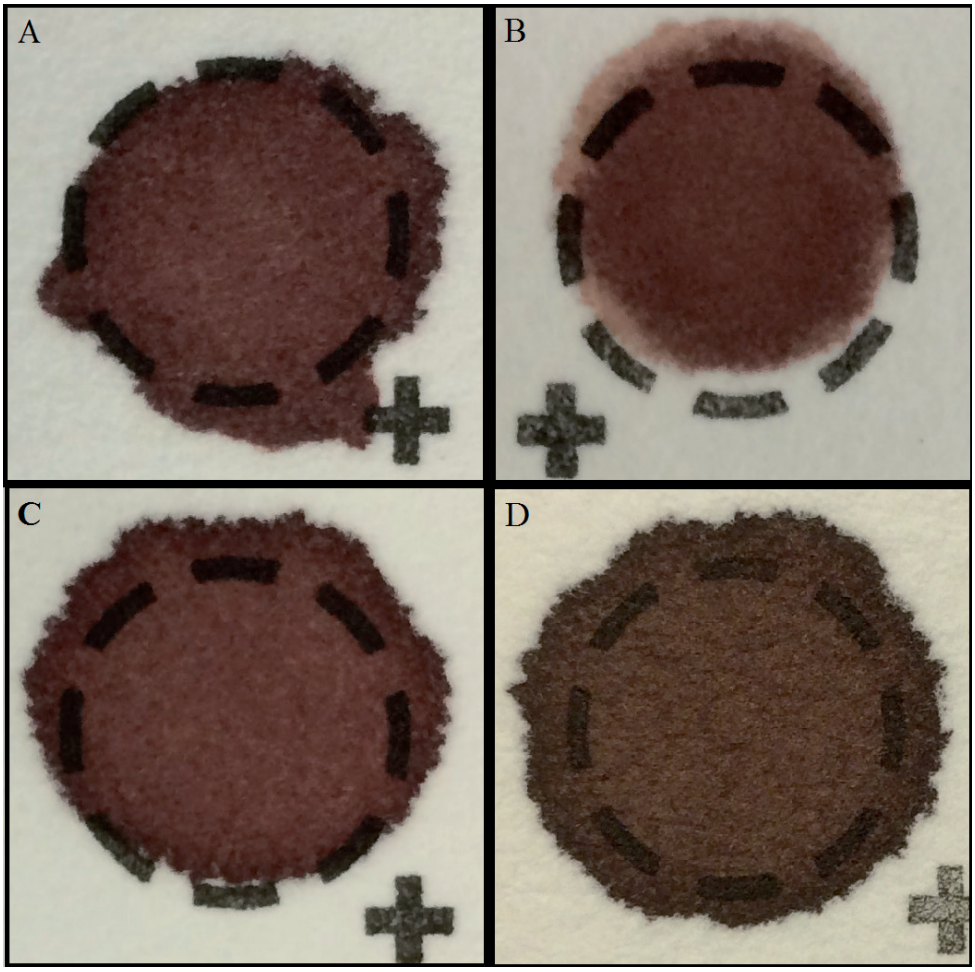

Figure 2. *Different types of spot quality in DBS sampling. (A) Layered spot consisting of multiple droplets of blood. (B) Discoloration because of hemolysis or humidity. (C) Spot that is not perfectly circle-shaped. (D) A good quality blood spot meeting all requirements: round, filling at least the pre-marked circle, consisting of one drop of blood and not touching other drops.*

Untrained setting

The app was used to test the 1610 samples obtained in an untrained setting. The performance was 87.4% with 5.5% false negatives and 7.1% false positives, comparable to the clinical samples. Results per country can be found in Table 1. Only 42.2% of the samples were of sufficient quality for analysis using 8 mm punches as determined by the GS.[11] Hypothetically, if the app was present and used correctly at the time of sampling and if the suggested resampling by the app was performed without error the amount of samples sufficient for analysis would have been 87.9% (Table 2). It should be noted that reasons for insufficient quality of DBS samples differed per country in the untrained setting. For instance, Belarus had a relatively large number of very small

spot sizes (<8 mm), while in Bangladesh, Indonesia and Paraguay humidity-related problems were more abundant.[11]

Table 1. Performance of the app processing samples obtained in an untrained setting.

App performance	Paraguay	Belarus	Bangladesh	Indonesia	Total
Correct	416 (84.6%)	348 (97.2%)	194 (79.5%)	449 (87.0%)	1407 (87.4%)
False negative	23 (4.7%)	3 (0.8%)	29 (11.9%)	34 (6.6%)	89 (5.5%)
False positive	53 (10.8%)	7 (2.0%)	21 (8.6%)	33 (6.4%)	114 (7.1%)
Total	**492 (100%)**	**358 (100%)**	**244 (100%)**	**516 (100 %)**	**1610 (100%)**

Table 2. Amount of research samples that were fit for analysis without using the app and the hypothetical amount of samples that would have been fit for analysis if the app was present, used correctly and the suggested resampling yielded sufficient quality spots.

Samples of sufficient quality	Without app, %	With app, %
Paraguay	57.0	93.0
Belarus	5.6	100.0
Bangladesh	36.8	72.6
Indonesia	58.1	88.4
Total	**42.2**	**87.9**

Robustness

During performance testing the deliberately induced unfavorable circumstances sometimes resulted in the app not being able to identify red pixels in a picture. As a result, the spots could not be indicated in the app (Figure 1, step 4) and the steps in the app could not be completed. This was indicated as an error. Because the error rate of the Nokia C5 was 36% and errors also occurred under perfect circumstances the Nokia C5 was considered not suitable to use with the app and the results were omitted from the performance testing. For each factor, a total of 64 samples were analyzed (16 pictures per phone, measured on both the phone and a PC). The overall performance of the robustness test is shown in Table 3. The success rate of the app was 84% under perfect conditions. The angle, lighting, casting a shade and the distance were all of influence on the performance of the app. Therefore, these specific issues are addressed in the instructions (Figure 1). The use of the flashlight is not of major influence on the app's results. The error rate was 0% for the two newest phones (Samsung Galaxy S7 Edge and iPhone 5S).

Table 3. Results of the robustness test.

Factors in the robustness test	Success rate, %	Error rate, %
Perfect conditions	84	0
Dimly lighted room	67	19
Casting a shade on the sampling card	77	3
Flashlight on	86	0
Distance 50 cm	39	50
Angle of 45°	29	54

User test

After verbal consent, a total of seven patients and one caregiver participated in the user test. Details are provided in Supplementary file 2. None of the patients successfully used the app without prior instructions. Although the app was built to be intuitive, especially the use of the buttons to align the picture to the frame and indicating the spots were steps that could not be completed in the first try. After an instruction explaining the steps and pitfalls in using the app, all patients could complete all steps in the app with a median time of 1 min and 45 s. The average SUS score was 74, which can be classified as an acceptable satisfaction. All patients and the caregiver gave a score >50, showing good overall usability of the app. The most common mistakes made by the patients were trying to pinch and swipe in step 3 (Figure 1) and forgetting to indicate the spots in step 4 (Figure 1).

Discussion

We developed an app to measure spot quality in DBS sampling that can easily be accessed and used by patients and professionals to determine spot quality, collected for TDM, in an objective way. Because the developed app is accessible on different devices, it is flexible and can be used in many different situations including home sampling and research in remote areas. Use of the app will only take a few minutes per sample.

In the first version of the app the acceptable width-to-height ratio was set lower than 12% which resulted in 12.7% false positives in the trained setting. The false positive results in the first version of the app mainly consisted of spots that were rejected by the app because of an unacceptable width-height ratio. In the second version, the acceptable width-to-height ratio was set at 12% lowering the amount of false positives from 12.7% to 5.9%. In clinical practice, the fall of a droplet on a card does not always provide a perfect circle-shaped spot. The ELP can determine whether a spot consists of one droplet without smearing. Even if the spot is not perfectly round, it would be acceptable (Figure 2C). Allowance of higher values for the width-to-height ratio would potentially decrease the amount of false positives, but would introduce an increase in false negatives because more layered spots would wrongfully be judged as acceptable. Allowance of lower values for the width-height ratio would increase the number of false positives, because acceptable spots that are not entirely circle-shaped would be rejected by the app. Therefore, despite limitations of the app, it was concluded that the second version of the app was of sufficient quality.

The app is unable to identify hemolytic or humid spots because hemolytic discoloration of the spots is still red as defined by specified RGB range and therefore is identified as a blood-pixel by the app. In clinical practice, discoloration due to hemolysis or humidity will not be visible until approximately 24 h after application of the blood to the DBS card.[24] Even if the app could identify hemolytic spots this will probably not be in time to allow resampling in a reasonable time frame. For instance, the patient will already have taken the medication, so measuring a trough concentration is not possible within the intended sapling time.

Only eight patients participated in the user test and thus only the major problems in usability of the app could be identified. After introduction of the app, post introduction surveillance should be performed to enable further optimization of the usability and app user instructions. The robustness testing showed a result of 84% repeatability in perfect conditions. This was unexpected because the device on which the app is used should not be of any influence on the app results. In addition, the pictures were taken under the same conditions across three devices. However, the samples that were chosen for the robustness test were deliberately selected based on their difficulty, in order to test repeatability in the most extreme circumstances. For instance, one of

the samples had a spot diameter of an 8.6 mm. The influence of the aligning of the picture (Figure 1, step 3) becomes paramount in this setting because 8.5 mm is judged as acceptable and 8.4 mm as insufficient. Other spots included false negatives with multiple layered spots where the width-height ratio was slightly lower than 12% and false positive spots that are not perfectly circle shaped as shown in Figure 2C. This could explain the observed difference between the used devices. When considering all samples obtained in the untrained setting, the robustness should be higher. In addition, during initial performance testing, the phone was fixed in landscape position above the DBS excluding variation of distance between phone and DBS. During robustness testing, the phone was handheld. Variation in distance between phone and sample might also contribute to reduced repeatability in perfect conditions, especially considering that a distance of 50 cm is of great influence. Because of the difference in results between smartphones, it is recommended, in future studies or applications, to first test the device intended to use with the app for repeatability. Especially, with regards to the setting in which the app will be used and different users.

The performance of 90.0% and 87.4% for samples obtained in respect to a trained and untrained setting did not meet the performance criterion of 95% set beforehand. However, the current version of the app would lead to resp. 5.9% and 7.1% unnecessary resampling. Although this is not optimal, the resampling, when using the app correctly, should lead to (another) good quality spot that will be sent in. No delay in patient monitoring or missing data in research will be introduced. Thus, the current version of the app should lead to sending in good quality samples in resp. 95.9% and 94.5% of the cases.

In a setting where training of healthcare workers is not possible, the app might lead to a major increase in sufficient quality samples (from 42.2% to 87.9%, Table 2). In a setting where training of patients or healthcare worker is possible, the potential benefit of the app is less pronounced. The training of healthcare workers in DBS sampling can lead to 100% sufficient spot quality in a research setting.[9] However, patients trained in DBS sampling who perform sampling at home as part of routine care only produce 80% sufficient quality spots.[10] Therefore, application of the app in a patient home sampling setting might still lead to an increase in the number of sufficient quality spots (from 80% to 95.9%). However, this increase will only be possible if patients are trained in using the app as shown by the user test and robustness is improved after implementation.

One of the limitations of the app is that the current version of the app will only work with DBS sampling paper that has the same size and dimensions as Whatman FTA DMPK-C cards because the frame of the paper is used to measure the size of the spots. However, other commonly used DBS sampling cards such as the Ahlstrom AutoCollect and Whatman FTA DMPK variant A and B have the same dimensions. In addition, the app is calibrated for 8 mm punches. If smaller punches are used, the app needs to

be calibrated for the appropriate punch size. However, other sampling instructions advise to let the blood drop fall freely on the DBS card.[16] A DBS that is generated from a freely fallen blood drop is at least 8 mm in diameter due to the viscosity of the blood and the subsequent formation and falling of a blood drop. Even when smaller punches are being used for analysis, the current app settings would still be correct for the evaluation of a DBS. As mentioned before, insufficient quality spots due to humidity or hemolysis cannot be identified by the app. This can be challenging if sampling and drying is performed in extremely humid conditions such as tropical areas. Additional precautions on sample handling are needed.[13] The app is developed to determine spot quality, after the spot has been made by the subject, based on spot size, color and shape. Other important factors affecting DBS sample quality such as differences between sampling card materials, hematocrit and volcano effects on sport formation and influence of drying time, sample transport and direct exposure to sunlight need to be addressed otherwise.[12,25] Finally, the technician is responsible for the final judgment of the quality of received samples and should always determine if a received DBS sample is fit for analysis.[26] Therefore, the app is only an aid for patients and researchers and is not defined as a medical device.[27]

DBS sampling is a patient friendly and easy-to-use sampling method. However, insufficient spot quality is a major issue in DBS sampling. The DBS app is a quick and easy tool to objectively measure the quality of DBS. Based on our test, the app can increase the amount of sufficient quality spots in an untrained setting from 42.2% to 87.9% and in a trained setting from 80% to 95.9%. The app is accessible in a browser by any patient, caregiver or researcher with a smartphone, tablet or PC. The app can be a valuable asset for increasing the amount of spots of sufficient quality in patient care and to increase the amount of usable data in DBS research studies. The app can contribute to a more widespread use of the DBS technology in bioanalysis and TDM.

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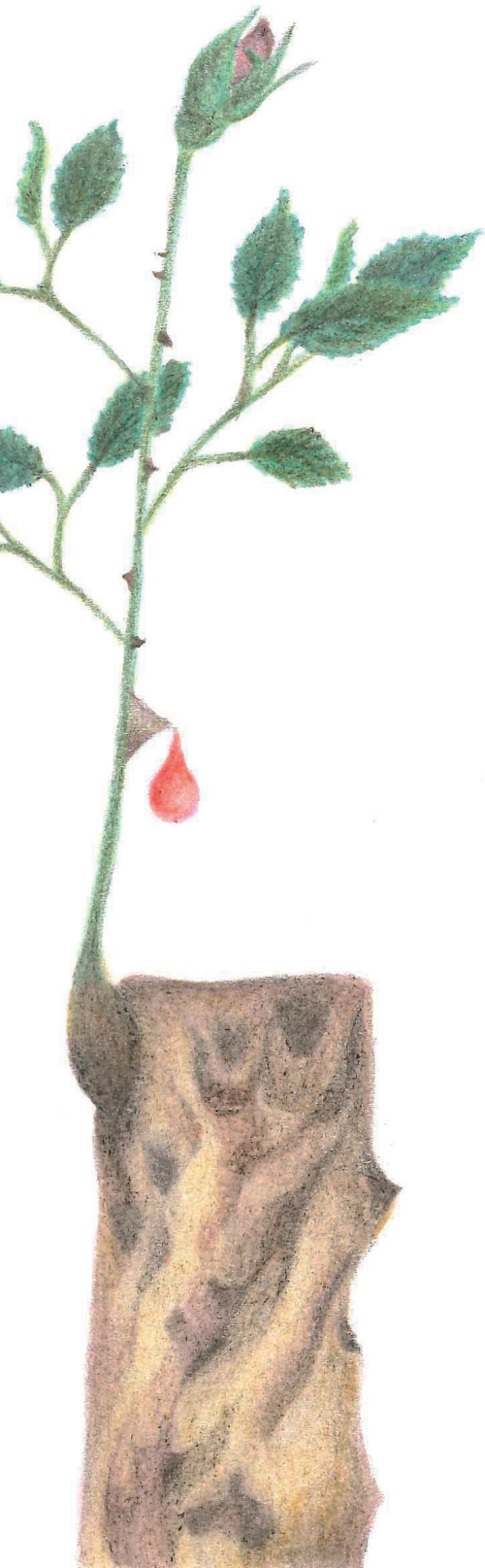

Chapter 11

General discussion and future perspectives

Kidney transplantation is currently the best treatment option for patients suffering from end stage kidney disease.[1] To prevent rejection of the transplanted organ, chronic use of immunosuppressive drugs is required. When these immunosuppressants are inadequately used or when they are dosed to low, there is an increased chance of acute rejection. When these drugs are overdosed, major side-effects and toxicity can occur.[2] Therefore, dosing is based on frequent assessment of blood drug levels. This requires the transplant patients to frequently travel to the hospital for venous blood sampling. With the introduction of Dried Blood Spot (DBS) sampling, transplant patients are enabled to sample at home, potentially reducing patient burden and costs.[3] In this thesis, the implementation of this method was evaluated regarding analytical and clinical performance of the DBS assay, in addition to costs, logistics, patient sampling performance and patient satisfaction.

Analytical performance

Implementation of a DBS method in clinical practice for the purpose of Therapeutic Drug Monitoring (TDM) is only feasible if the method used for analyzing the DBS samples is fast, robust and meets all bio-analytical requirements.[4-6] The analytical method used in our hospital is able to simultaneously assess levels of tacrolimus, sirolimus, everolimus and cyclosporin A.[6] In **chapter 2** we describe an improvement of our multi-analyte assay, including the addition of mycophenolic acid.

Currently, analysis of immunosuppressants in whole blood (tacrolimus, cyclosporin A, sirolimus, everolimus) or plasma (mycophenolic acid) is the standard.[7] These analyses are performed on highly sensitive LC-MS/MS systems. These methods are robust, fast, have been used for over a decade, have external quality control programs and do not suffer from DBS-related problems such as the effect of the hematocrit.[8] A novel DBS method should be in line with these standards. This means that sample preparation should be straight-forward, fast and without difficult and time-consuming steps like solid phase extraction.[9] The assay described in **chapter 2** is slightly more labor-intensive for lab technicians compared to the venous whole blood assay.[7] This is mainly due to the fact that DBS analysis requires manual punching of the blood spots and some additional steps like vortexing, sonication and a freeze step to improve protein precipitation. However, the additional time needed for DBS analysis is limited and analysis of DBS samples can be performed within a day, which is similar to whole blood analysis.

Although DBS assays can meet the quality criteria put forward in relevant guidelines of the EMA and FDA, additional aspects specific to DBS assays need to be addressed.[4,5] One of the most challenging aspects is the influence of hematocrit on analytical results.[8]

The influence of the hematocrit can be interpreted as the influence of hematocrit on spot formation only.[10] This can wrongfully lead to the conclusion that, if whole spots with a known volume are analyzed, hematocrit is not of influence. However, an effect of hematocrit on extraction recovery is always present, irrespective of the sampling device or sampling paper used for the micro sampling method.[11] Therefore, a potential influence of hematocrit should always be taken into consideration during analytical validation. In **chapter 8,** specific steps to investigate and interpret the effect of hematocrit are described. In **chapter 2,** we describe how both the hematocrit and the concentration of the drug of interest are of influence on analytical results. However, only cyclosporine concentrations outside of the target trough concentration range (>200 µg/L cyclosporin A) in combination with extreme values of hematocrit (e.g. 0.20 v/v), resulted in a bias which was higher than the predefined criterion of 15%. Therefore, it was concluded that for application in clinical practice, the assay is independent of hematocrit effects.

In circumstances where hematocrit would be of influence on recovery, several strategies have been suggested to overcome this problem. These are all based on the incorporation of the patients' individual hematocrit values in calculating DBS values.[12] To make this possible, the hematocrit should be known for individual samples. This lead to the development of several strategies of measuring hematocrit in DBS samples, including measurement of potassium, use of near-infrared spectroscopy, use of sulfolyser reagent and use of noncontact diffuse reflectance spectroscopy.[13-17] However, if the hematocrit has such a major impact on analytical result that this becomes necessary, one might argue that the used extraction method is not optimal. For everolimus, a major impact of hematocrit on analytical performance was observed in Volumetric Absorptive Micro Sampling (VAMS) tips.[11] In our VAMS analytical validation, which was described in **chapter 9**, this was not the case. This is best explained by a difference in extraction methods between our analytical method and earlier methods. It should be noted that in literature there is a great variety in extraction methods for immunosuppressants in micro sampling devices.[6,9,11,18-32] Future research should focus on the most optimal extraction procedure which should be independent of hematocrit and the sampling device.

Another advantage of DBS is the possibility of automated analysis. Several strategies to automate punching, extraction and analysis of DBS samples have been described.[33-35] The further development and clinical validation of these methods might greatly contribute to the implementation of DBS in routine care. In future, the most ideal laboratory procedure for DBS analysis is the insertion of a freshly arrived DBS sample into a fully automated LC-MS/MS setup, which can produce an analytical result within a few hours without the need of sample preparation by the lab technician.

Clinical performance

In 2016, a review was published showing a list of 90 drugs that could be determined from DBS.[36] This number has undoubtedly increased in the past years. However, the number of clinical validation studies published is probably just a fraction of this number. In a clinical validation study, a candidate analytical method (DBS or other micro-sampling device) is tested against the standard (usually analysis in whole blood, serum or plasma). The purpose of these studies is to investigate whether there is sufficient agreement between the DBS method and reference plasma, serum or whole blood method. To perform these studies, paired fingerprick DBS samples and venous liquid blood samples are obtained, analyzed and compared using appropriate statistical tests. We describe such studies in **chapters 3,4 and 10**. In **chapter 8**, a guideline on how to perform such studies is presented. In the previous paragraph it was stated that the DBS assay should meet the analytical standards as set by the whole blood method. This is also true regarding the clinical standard.

There can be several reasons why clinical validation studies are not published in literature. A potential reason is that these studies can be labor- and cost intensive and require ethical clearance before they can be conducted. In addition, patients who use the drug of interest need to be included in the study. To realize this, a multidisciplinary approach is needed and the treating physicians, pharmacists, analysts and (sometimes) patients, should be part of the research team. For labs, outside of (academic) hospitals, this can be a challenge, which might be too hard to overcome. Another reason for the lack of published clinical validation studies might be publication bias. There is a possibility that clinical validation studies are performed, but that they show insufficient agreement between the novel DBS method and the reference method, and are therefore not published. Although one of the first clinical validation studies was published in 2005, it took until 2018 for the first 'negative' study to be published by Kloosterboer et al.[24,37] In their study, Kloosterboer et al. describe a clinical validation study for antipsychotics where all drugs investigated did not meet the predefined criteria set for the Bland-Altman analyses. This was interesting, because the DBS analysis method had already been analytically validated in an earlier publication.[38] This underlines the need for clinical validation studies – and independent replication thereof – as a standard part of the development, validation and implementation of DBS assays. In **chapters 3 and 10,** we have shown that tacrolimus, cyclosporin A and creatinine can be reliably measured from DBS. In addition, tacrolimus can also be measured in VAMS, as is described in **chapter 10**. Unfortunately, for everolimus and sirolimus the clinical validation was unsuccessful according to our predefined criteria as is described and discussed in **chapter 4.**

The predefined criteria for acceptance of the method as is described in **chapter 8** and applied in **chapters 4 and 10,** are very important in clinical validation studies. Analytical

results from DBS assays can have direct clinical consequences, such as tacrolimus dose adjustment based on a trough concentration measured in a DBS sample. This clinical decision making should be taken into account in a clinical validation study. Therefore, before starting a study, limits for clinical acceptance should be defined. Ideally, these limits should be defined in such a way that results assessed with DBS sampling will translate in making the same clinical decision as would have been made if results came from a whole blood sample. However, analytical factors such as bias and precision, clinical factors such as target trough concentration range and patient factors such as patient-specific pharmacokinetic and pharmacodynamic parameters are all of influence. Therefore, the limits of acceptance should be set by a multi-disciplinary team which include pharmacists, physicians and lab technicians. Some clinical validation studies are designed in such a way that the clinical interpretation of a DBS sample is done separately from the whole blood sample.[29] This provides the opportunity to assess whether results from a DBS sample and a whole blood sample will result in the same clinical decision. In future clinical validation studies, this approach is highly recommended and should include setting of pre-defined limits for acceptance.

In this thesis, a multi-analyte assay is presented, which is able to determine blood concentrations from 5 immunosuppressants. Unfortunately, only 4 out of 5 of these immunosuppressants are tested in a clinical study. Mycopohenolic acid remains to be tested in a clinical validation study. Although monitoring of mycophenolic acid trough concentrations is done less frequently than tacrolimus, it could prove to be useful. This could be particularly true because it is part of the DBS analysis method, but not of the whole blood analysis method. This means that analyzing mycophenolic acid in DBS requires no additional work from lab technicians.

To date, only a few hospitals use DBS sampling as part of routine transplant patient care for tacrolimus TDM. This might be a reason why no external quality control program, such as proficiency testing exists. The International Organization for Standardization (ISO) states that all medical laboratories are required to participate in inter-laboratory comparison or proficiency testing to ensure quality, comparability and acceptability of analytical results.[39] Therefore, there is an urgent need for proficiency testing programs for DBS. Ideally, this program should contain patient samples as well as spiked samples. In addition, the spiked blood that is used to prepare DBS samples can be used as a sample itself. These samples can be analyzed by participating labs on the routine whole blood analysis method and can serve as a quality control.

If DBS assays prove to be valid in a well-designed and executed clinical validation study and are monitored by external quality control programs in clinical practice, transplant patient treatment can be based on results from DBS samples.

Implementation in clinical care

In this thesis, we present a tacrolimus DBS assay that meets analytical and clinical standards. However, having a high quality analysis method is only the beginning of a trajectory of implementing DBS sampling in standard transplant patient care. As is demonstrated in **chapters 5,6 and 7**, logistical challenges and sample quality are of major concern in implementing DBS in routine care.

Costs, effects and patient satisfaction

In **chapter 7** we have described a study in which the results do not show a cost reduction when transplant patients use DBS home sampling for tacrolimus TDM and creatinine monitoring. Main reasons for this negative finding are logistical issues concerning the sending and analysis of the samples. When it comes to logistics, the standard is set (again) by the whole blood method used for TDM. If a doctor asks a patient to donate a venous blood sample in the hospital, this will result in availability of a tacrolimus trough concentration in the patients' Electronic Health Records (EHR) by the end of the same day in >99% of the cases. Even if a DBS home sampling method results in 80% of the DBS results available in the patient's EHR prior to the outpatient visit to the physician, this still can be perceived as insufficient by both patient and physician. Because of this, the logistical challenges of DBS sampling are as important as the analytical and clinical performance of DBS assays. In **chapter 7**, we have shown a number of important leads for the further improvement of the implementation of DBS home sampling. First of all, adult kidney transplant patients are enthusiastic about the prospect of the possibility of reduction of frequency of outpatient visits. Therefore, if DBS leads to reduced outpatient visits, patients will be highly motivated to correctly perform DBS sampling. In **chapter 7**, we also have described the societal costs involved in one outpatient visit. From this, cost-reduction can easily be calculated for DBS after improved implementation. Although the logistical challenges concerning DBS home sampling are serious, they can be regarded as teething problems. In the future, the logistics can be improved by automatically sending the patient the sampling kit a few days prior to scheduled sampling accompanied with an automated reminder system by e-mail or phone. This will greatly reduce the chance of the patient forgetting to sample. After sampling, a pick-up service could collect the samples at home (or work) and send them with track-and-trace to the laboratory. If there are standardized days of sampling and analysis, the chance that no results will be available during the outpatient visit will be minimized. Disadvantages are the increased costs of such a service, but they will most likely be very small compared to the costs of one saved outpatient visit. Another disadvantage of this system would be that the DBS method will not be feasible for patients who visit the outpatient clinic every week, in the first month after transplantation.

This proposed way of improvement of implementation of DBS should be studied. Inclusion of implementation specialists from the emerging field of implementation science in such a study is recommended.[40] One of the main aspects will be management of expectations from patients, pharmacists and physicians, since it will be likely that a >99% success rate cannot be achieved.

Sampling quality

Even if logistics can be organized perfectly, incorrect sampling by the patient will still result in no tacrolimus trough concentration available during the next outpatient visit. Sample quality and sampling procedures are therefore an important factor in DBS implementation.

Various studies have been performed on sampling performance by patients and researchers.[25,41,42] For patients using DBS for home sampling, rejection rates of samples because of insufficient sample quality of up to 20% are described. However, in **chapter 7**, the rejection rate of patient home-sampled DBS is only 4.9% which is comparable to the rejection rate of DBS samples obtained by trained phlebotomists. The patients that we included were all instructed by one experienced study coordinator, and the instruction protocol included practicing the DBS method by the patient while they were supervised by the study coordinator. In a research setting, a similar training method yielded a 0% rejection rate when trained phlebotomists were asked to perform the DBS sampling.[42] In **chapter 6**, we have shown that total absence of training results into rejection rates of up to 58%. This shows that training is the key factor in achieving a high rate of sample quality.

Various novel sampling devices have been introduced in the past years, which claim improved analytical performance and easier sampling by the patient. Examples include the Mitra© tip, The HemaXis DB device, Capitainer-B and HemaPEN.[8] However, they have rarely been tested in direct comparison to conventional DBS. In **chapter 10**, we have described such a comparison and we demonstrate that the Mitra© tip is inferior to conventional DBS sampling regarding both analytical performance and sampling quality.

Regardless of the sampling device, the person handling the device needs training as described earlier. If this is the case, the kind of sampling device becomes of lesser importance. Even for conventional DBS sampling, it is possible to achieve very low sample rejection rates, even when patients perform sampling at home. We developed an app to aid in judging the quality of a DBS. This app is described in **chapter 6**. The app can indeed contribute to improved sample quality. The benefits of the app are most prominent in a setting where training of people who obtain the samples is not

possible or not feasible. In situations where (repeated) training is possible, the app can serve as a way to identify patients who repeatedly fail to adequately perform DBS sampling. These patients can receive additional training, which will help improve their sampling performance.

Conclusive remarks

In this thesis, we described the steps necessary to implement Dried Blood Spot sampling of immunosuppressant TDM for transplant patients. This thesis shows that this is possible if:

1. The analysis method used for analyzing the DBS samples is fast, robust and meets all general and DBS-specific bio-analytical requirements.

2. DBS assays prove to be valid in a well-designed and executed clinical validation study and are monitored by external quality control programs in clinical practice.

3. It is likely that logistics can be optimized including Track-and-Trace sending of samples, reminder systems for patients and standardized days of sampling and analysis.

4. Patients are trained and re-trained in DBS sampling using a training method that includes practicing the complete sampling procedure under supervision of someone experienced in DBS sampling.

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Summary

Renal transplantation is currently the best treatment option for patients suffering from end stage kidney disease. Once transplanted, patients receive immunosuppressive drugs to prevent rejection of the graft by the recipient. When immunosuppressants are dosed too low, there is an increased chance of acute rejection. When these drugs are overdosed, major side-effects and toxicity can occur. Because of great intra- and interpatient variation in drug exposure, dosing is based on blood drug concentrations which requires the transplant patients to frequently travel to the hospital for venous blood sampling. This process is called Therapeutic Drug Monitoring (TDM). With the introduction of Dried Blood Spot (DBS) sampling, transplant patients are able to sample at home using a finger prick and applying a few drops of blood on a sampling card that can be send to the laboratory by mail. From these blood spots immunosuppressant drug concentrations and serum creatinine levels can be measured. This potentially reduces patient burden and costs. In this thesis, the implementation of this DBS home sampling method for transplant patients was evaluated regarding analytical and clinical performance of the DBS assay, in addition to costs, logistics, patient sampling performance and patient satisfaction.

In **chapter 2** we have improved the available liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis method for immunosuppressant DBS samples. The method is able to analyze 4 immunosuppressants (tacrolimus, everolimus, sirolimus, cyclosporin A). Mycophenolic acid was added to this method. The aim was to analytically validate this DBS assay on two different LC-MS/MS systems (Thermo® and Agilent®) across a clinically relevant hematocrit range without the need to correct for hematocrit. In addition, this validation was performed on Whatman DMPK-C cards instead of 31-ET-CHR cards. On both LC-MS/MS systems the analytical requirements were met for all immunosuppressants. Bias caused by the hematocrit was within 15% for all immunosuppressants for hematocrit levels between 0.23 (v/v) and 0.48 (v/v) across a relevant range of trough level concentrations, meaning no hematocrit correction is needed. The bias caused by the hematocrit for everolimus and sirolimus was higher compared to the other 3 drugs, particularly at lower concentrations (3 µg/mL). The method employed on the Thermo LC-MS/MS was used in a clinical validation study where analytical results from the finger prick DBS samples were compared to the analytical results from the paired venous whole blood samples. For ciclosporin A and for tacrolimus, the results from DBS were interchangeable with the venous whole blood results showing that this DBS analysis method can be used in patient home sampling.

In **chapter 3** the aim was to show interchangeability between analytical results from fingerprick DBS samples and venous samples for both tacrolimus, cyclosporin A and creatinine. The DBS results from the Agilent method mentioned in **chapter 2** were used. All finger prick DBS and venous whole blood samples were obtained by trained phlebotomists within 10 minutes of each other during routine adult kidney transplant patient visits to the hospital for TDM and nephrologist consultation. After exclusion of several samples because of insufficient quality, a total of 172, 104 and 58 samples were available from 172 different patients for method comparison of creatinine, tacrolimus and cyclosporin A, respectively. In Passing & Bablok regression analysis and Bland-Altman analysis no clinical significant differences between DBS and whole blood were found for tacrolimus and cyclosporin A. For creatinine, a difference between DBS and plasma results was found, as was expected because of the different matrices (venous plasma and finger prick capillary blood). A systemic difference was observed, allowing the conversion of DBS results to plasma creatinine results using the formula (creatinine plasma concentration in µmol/L) = (creatinine concentration in DBS in µmol/L)/0.73. In conclusion, this chapter showed that DBS sampling can replace venous sampling for the monitoring of tacrolimus, cyclosporin A and creatinine.

In **chapter 4** a similar clinical validation study was performed as described in **chapter 3**, but for the immunosuppressants sirolimus and everolimus. Because these drugs are not used as frequently as tacrolimus, the sample size was limited (39 and 44 paired DBS and venous samples respectively for sirolimus and everolimus). In addition to the validation steps described **chapter 3,** two additional validation parameters were investigated; the limits of clinical acceptance and the predictive performance as described by Sheiner and Beal. The limits of clinical acceptance were set in a multidisciplinary team consisting of pharmacists, analysts and transplant physicians at >80% of all paired samples to be within 15% of the mean of both samples. The Passing & Bablok regression analysis and Bland-Altman analysis showed no clinically relevant differences between DBS and whole blood. The predictive performance met the predefined criteria, showing that whole blood values can be predicted from DBS values. However, the limits of clinical acceptance were not met showing values of 77.3% for sirolimus and 61.5% for everolimus. In this chapter we concluded that DBS sampling cannot replace venous sampling at this time for sirolimus and everolimus trough concentration monitoring because the pre-defined limits of clinical acceptance were not met. However, if less strict limits are acceptable for clinical practice, this DBS method will be suitable for clinical use.

In **chapter 5** the quality of 464 individual blood spot cards from 4 different countries (Paraguay, Belarus, Bangladesh, Indonesia) were assessed. These samples were obtained as part of a TDM study for drugs used in the treatment of tuberculosis, by

untrained healthcare workers who only had a written instruction available on how to perform DBS sampling. A checklist was developed consisting of multiple criteria to assess the quality of the obtained DBS samples. Two DBS experts used the checklist to score the samples independently of each other and found that only 54% of the samples complied with present quality standards. In most of the cases, this was due to incorrect sampling. In addition, samples from relatively humid countries (Paraguay, Bangladesh and Indonesia) seemed to be affected by the high air humidity causing light-red rings around the blood spots during drying of the samples. This chapter showed that training of health care workers in DBS sampling is very important for yielding a high amount of sufficient quality DBS samples in clinical research.

In **chapter 6,** the development of a web-based application (app) capable of assessing DBS quality at the time of sampling by means of analyzing a picture of the DBS was described. Regarding DBS sample quality, the judgment of an experienced laboratory technician is, based on the criteria mentioned in **chapter 5,** the golden standard. After development, the app was tested by comparing the results of the app to this golden standard. The performance qualification was set a priori at 95%, meaning that the app should make the same decision as the golden standard in 95% of the cases. The datasets of **chapter 3** and **chapter 5** were used to test the app and were defined as the trained and untrained setting, respectively. In a trained setting the app yields an adequate decision in 90.0% of the cases with 4.1% false negatives (insufficient quality DBS incorrectly not rejected) and 5.9% false positives (sufficient quality DBS incorrectly rejected). In an untrained setting this is 87.4%, with 5.5% false negatives and 7.1% false positives. If the app had been present in the trained and untrained setting, was used properly and resampling would have yielded a sufficient quality DBS sample, the amount of sufficient quality samples would have increased from 80.0% to 95.9% and 42.2% to 87.9%, respectively. In conclusion, the app can be used in both a patient care and research setting to increase the amount of sufficient quality DBS samples.

In **chapter 7**, we have described the first randomized-controlled clinical study assessing the costs and effects of the implementation of DBS home sampling in transplant patient care. In this single-center randomized-controlled clinical trial, 25 patients used DBS home sampling on top of usual care 6 months after renal transplantation while 23 patients received usual care only. The aim was to assess whether DBS home sampling would lead to a reduced amount of outpatient visits, reduced costs from a societal point of view and improved patient satisfaction. Unfortunately, the number of outpatient visits was not significantly lower in the DBS group (11.2, SD: 1.7) compared to the control group (10.9, SD: 1.4) ($p = 0.48$). In addition, costs per visit in the DBS group were not significantly different (€537, SD €179) compared to the control group

(€510, SD €229) (p = 0.66). This is probably due to the fact that only 56% of the expected DBS was sent in and that 20% of the expected DBS was analyzed on time, meaning that the result from the DBS analysis was present in the Electronic Health Records of the patient at time of nephrologist consultation. However, 82.6% of the patient are willing to perform DBS home-sampling if this would reduce the number of outpatient visits. Optimization of logistical processes concerning the sending and analysis of DBS samples is crucial in implementation of DBS home sampling.

In **chapter 8**, a guideline was presented on the development, analytical and clinical validation of Dried Blood Spot based methods used for TDM. Current validation requirements, described in guidelines for traditional matrices (blood, plasma, serum), do not cover all necessary aspects for this. Therefore, this chapter provides parameters required for the validation of quantitative determination of small molecule drugs in DBS using chromatographic methods, and to provide advice on how these can be assessed. In addition, guidance is given on the application of validated methods in a routine context. First, considerations for the method development stage were described. Second, common parameters regarding analytical validation were described in context of DBS analysis with the addition of DBS specific parameters. Third, clinical validation studies were described, including number of clinical samples and patients, comparison of DBS with venous blood, statistical methods and interpretation, spot quality, sampling procedure, duplicates, outliers, automated analysis methods and quality control programs. Lastly, cross-validation was discussed, covering changes made to existing sampling- and analysis methods.

In **chapter 9**, we have described the development and analytical validation of an LC-MS/MS assay for tacrolimus, everolimus, sirolimus, cyclosporin A and mycophenolic acid using Volumetric Absorptive Micro Sampling (VAMS) tips (Mitra®). These tips wick up an exact amount of blood which potentially mitigate volume-related hematocrit effects and potentially make patient sampling easier. Biases caused by hematocrit effects were within 15% for all immunosuppressants between hematocrit levels of 0.20 and 0.60, except for cyclosporin A, which is valid between 0.27 and 0.60 v/v. There was a trend visible where higher analyte concentrations combined with low hemacrit values result in reduced recovery. However, for the relevant clinical ranges this bias was within requirements and the values are lower than reported for DBS **(chapter 2).** This analysis method was tested for tacrolimus in a clinical validation study described in **chapter 10**. A total of 130 paired fingerprick VAMS, fingerprick DBS and venous whole blood samples were obtained from 107 different kidney transplant patients by trained phlebotomists for method comparison using the same validation criteria as was described in **chapter 4**. A multidisciplinary team pre-defined an acceptance limit requiring >80% of all paired samples within 15% of the mean of both samples as was

described in **chapters 4 and 8**. Sampling quality was evaluated for both VAMS and DBS samples: 32.3% of the VAMS samples and 6.2% of the DBS samples were of insufficient quality. Passing & Bablok regression showed a significant difference between VAMS and whole blood, with a slope of 0.88 (95%CI 0.81-0.97) but not for DBS (slope 1.00; 95%CI 0.95-1.04). For VAMS and DBS, the acceptance limit was met for respectively 83.0% and 96.6% of the samples. VAMS sampling can replace whole blood sampling for tacrolimus trough concentration monitoring, but VAMS sampling was inferior to conventional DBS sampling, both regarding sample quality and agreement with whole blood tacrolimus concentrations.

In **chapter 11** the thesis was discussed and future perspectives were given. In this thesis, we have described the steps necessary to implement Dried Blood Spot sampling for immunosuppressant TDM for transplant patients. This is possible if the following criteria are met. (1) The analysis method used for analyzing the DBS samples is fast, robust and meets all general and DBS-specific analytical requirements. (2) DBS assays prove to be valid in a well-designed and executed clinical validation study and are monitored by external quality control programs. (3) Logistics are optimal, and might include Track-and-Trace sending of samples, reminder systems for patients and standardized days of sampling and analysis (4) Patients are trained and re-trained in DBS sampling using a training method that includes practicing the complete sampling procedure under supervision of someone experienced in DBS sampling.

Nederlandse samenvatting

Niertransplantatie is momenteel de beste behandeling voor patiënten met een ernstige nierziekte. Eenmaal getransplanteerd worden patiënten behandeld met immuunsysteem onderdrukkende geneesmiddelen (de immunosuppressiva) om te voorkomen dat het lichaam het getransplanteerde orgaan afstoot. Als de immunosuppressiva te laag worden gedoseerd is er een verhoogde kans op acute afstoting. Als deze geneesmiddelen te hoog worden gedoseerd kunnen er ernstige bijwerkingen optreden. Omdat er grote verschillen in blootstelling aan de immunosuppressiva zijn, zowel tussen patiënten als binnen één patiënt, wordt de dosering van deze geneesmiddelen ingesteld op basis van de bloedspiegel. Hierdoor is het nodig dat de transplantatiepatiënt regelmatig naar het ziekenhuis gaan om een veneus bloedmonster af te staan. Dit proces wordt ook wel therapeutisch geneesmiddel monitoring genoemd, in het Engels Therapeutic Drug Monitoring (TDM).

Met de introductie van de Dried Blood Spot (gedroogde bloedspot, DBS) methode hebben patiënten de mogelijkheid om thuis bloed af te nemen. Door middel van een vingerprik kunnen twee druppels bloed op een kaartje worden aangebracht. Na drogen kan dit kaartje met de post verstuurd worden naar het laboratorium. Vanuit deze bloedspotjes kunnen de bloedspiegels van de immunosuppressiva gemeten worden en serum creatinine waarden. Het serum creatinine geeft de functie van de nieuwe nier weer. De DBS methode kan potentieel de last voor transplantatiepatiënten verlichten en kostenbesparend zijn. In dit proefschrift wordt de implementatie van DBS thuismonitoring voor transplantatie patiënten geëvalueerd. Hierbij wordt er gekeken naar de analytische en klinische performance van de DBS methode, kosten, logistiek, de afname prestaties van de patiënt en patiënttevredenheid.

In **hoofdstuk 2** beschreven we een verbetering van de al bestaande analyse methode om immunosuppressiva spiegels te meten in DBS monsters. De analyse wordt gedaan door middel van vloeistof chromatografie gecombineerd met massa spectrometrie, kortweg LC-MS/MS. De bestaande analysemethode kan 4 immunosuppressiva meten (tacrolimus, everolimus, sirolimus en cyclosporine). Een vijfde immunosuppressivum (mycofenolzuur) werd toegevoegd aan deze methode. Het doel was om de DBS methode analytisch te valideren op 2 verschillende LC-MS/MS systemen (van de merken Agilent® en Thermo®) over een bereik van klinische relevante hematocrieten zonder dat het nodig is om te corrigeren voor het hematocriet. Daarnaast werd de validatie uitgevoerd met Whatman DMPK-C DBS kaarten in plaats van de 31-ET-CHR kaarten. Op beide LC-MS/MS systemen voldeed de analyse methode aan de analytische eisen voor alle immunosuppressiva. De systemische afwijking (bias) veroorzaakt door het

hematocriet was binnen de gestelde eis van 15% voor alle immunosuppressiva, voor hematocriet waarden tussen de 0.23 (v/v) en 0.48 (v/v). Dit geldt voor een bereik van klinisch relevante dalspiegels, waardoor er geen hematocriet correctie nodig is. De bias veroorzaakt door het hematocriet bij everolimus en sirolimus was hoger dan bij de andere 3 immunosuppressiva, in het bijzonder voor lage concentraties (3 µg/mL). De resultaten gegenereerd met behulp van het Thermo systeem zijn getest in een klinische validatie studie waarbij de analytische resultaten van vingerprik DBS monsters zijn vergeleken met gepaarde veneus afgenomen volbloed monsters. Voor cyclosporine en voor tacrolimus werd er geconcludeerd dat de resultaten van de DBS analyse inwisselbaar zijn met de resultaten van de volbloed analyse, wat betekent dat de DBS analyse gebruikt kan worden voor thuismonitoring van patiënten.

In **hoofdstuk 3** was het doel om inwisselbaarheid tussen analytische resultaten van vingerprik DBS monsters en veneuze monsters aan te tonen voor tacrolimus, cyclosporine en creatinine. De resultaten van de Agilent analyse methode beschreven in **hoofdstuk 2** werden hiervoor gebruikt. Alle vingerprik DBS monsters en veneuze monsters werden afgenomen door een getrainde doktersassistente in maximaal 10 minuten tijd, tijdens een routine bezoek van een volwassen niertransplantatie patiënten aan het ziekenhuis. Nadat er een aantal DBS monsters werden geëxcludeerd vanwege onvoldoende kwaliteit bleven er respectievelijk 172, 104 en 58 gepaarde monsters over van in totaal 172 verschillende patiënten voor creatinine, tacrolimus en cyclosporine. In de methode vergelijking waarbij er gebruik gemaakt werd van Passing & Bablok regressie analyse en Bland-Altman analyse werden er geen klinisch significante verschillen tussen DBS en volbloed waarden gevonden voor tacrolimus en cyclosporine. Voor creatine werd een verschil gevonden tussen de DBS en plasma resultaten. Dit was volgens verwachting vanwege het verschil in matrix (veneus afgenomen plasma en capillair volbloed uit een vingerprik). Het verschil was systematisch waardoor het mogelijk is om een conversie formule maken om DBS creatinine waarden om te zetten in plasma creatinine waarden: (creatinine plasma concentratie in µmol/L) = (creatinine concentratie in DBS in µmol/L)/0.73. Dit hoofdstuk laat zien dat DBS monsters veneuze monsters kunnen vervangen voor de monitoring van bloedspiegels van tacrolimus, cyclosporine en creatinine.

In **hoofdstuk 4** werd er een soortgelijke klinische validatie studie uitgevoerd als beschreven in **hoofdstuk 3**, maar dan voor de immunosuppressiva everolimus en sirolimus. Omdat deze twee geneesmiddelen minder frequent gebruikt worden dan tacrolimus was er slechts een beperkte hoeveelheid monsters beschikbaar (respectievelijk 39 en 44 gepaarde DBS en veneuze monsters voor sirolimus en everolimus). Naast de genoemde validatiestappen in **hoofdstuk 3** werden er twee additionele validatie parameters onderzocht: de klinisch acceptatie grens en de

voorspellende performance zoals beschreven door Sheiner en Beal. De klinische acceptatie grens werd in een multidisciplinair team bestaande uit apothekers, analisten en nefrologen bepaald. De grens werd als volgt gedefinieerd: de resultaten van minimaal 80% van de gepaarde monsters moet binnen 15% van het gemiddelde van beide monsters zitten. In Passing & Bablok regressie analyse en Bland-Altman analyse werden er geen klinisch relevante verschillen gevonden tussen DBS en volbloed resultaten. De voorspellende performance voldeed aan de vooraf gedefinieerde eis. Hieruit blijkt dat veneuze bloedwaarden voorspeld kunnen worden uit DBS waarden. Echter, de klinische acceptatie grens werd niet gehaald met 77.3% voor sirolimus en 61.5% voor everolimus. In dit hoofdstuk concluderen we dat DBS monsters veneuze monsters niet kunnen vervangen voor het monitoren van sirolimus en everolimus bloedspiegels omdat er niet voldaan werd aan de vooraf gedefinieerde klinische acceptatie grens. Als er een klinische setting is waarin de klinische acceptatie grens minder streng kan worden gedefinieerd is de DBS methode wellicht wel geschikt.

In **hoofdstuk 5** werd de kwaliteit van 464 bloed spot kaarten uit 4 verschillende landen (Paraguay, Wit-Rusland, Bangladesh en Indonesië) onderzocht. Deze DBS monsters werden verkregen als onderdeel van een TDM studie naar geneesmiddelen die gebruikt worden in de behandeling van tuberculose. De DBS monsters werden afgenomen door ongetrainde gezondheidszorgmedewerkers die slechts een geschreven handleiding beschikbaar hadden waarin staat hoe de DBS afname procedure werkt. Er werd een checklist ontwikkeld waarmee de kwaliteit van een DBS monsters kan worden vastgesteld. Twee DBS experts gebruikten de checklist, onafhankelijk van elkaar, om alle DBS monsters te scoren. Slechts 54% van alle DBS monsters voldeed aan de kwaliteitseisen. In de meeste gevallen kwam dit door verkeerde monstername. Daarnaast lijken monsters uit landen met een relatief hoge luchtvochtigheid (Paraguay, Bangladesh en Indonesië) beïnvloed te zijn door de hoge luchtvochtigheid wat zichtbaar was door licht rode ringen rondom de gedroogde bloeddruppels. Dit hoofdstuk laat zien dat het trainen van gezondheidsmedewerkers in het correct uitvoeren van de DBS monstername belangrijk is voor het verkrijgen van een hoog percentage DBS monsters van voldoende kwaliteit in klinisch onderzoek.

In **hoofdstuk 6** werd de ontwikkeling van een web-applicatie (app) beschreven die het mogelijk maakt een DBS te beoordelen op kwaliteit op het moment van monstername, door middel van het analyseren van een foto van het DBS monster. Aangaande DBS monster kwaliteit is het oordeel van een ervaren laboratorium medewerker, gebaseerd op de checklist uit **hoofdstuk 5**, de gouden standaard. Nadat de app is ontwikkeld werd die getest door het oordeel van de app te vergelijken met deze gouden standaard. De performance kwalificatie werd vooraf gesteld op 95%, wat betekent dat de app hetzelfde oordeel moet maken als de gouden standaard in minimaal 95% van de

gevallen. De data uit **hoofdstuk 3** en **hoofdstuk 5** werd gebruikt om de app te testen en zijn gedefinieerd als respectievelijk 'getrainde setting' en 'ongetrainde setting'. In de getrainde setting haalde de app een performance kwalificatie van 90.0% met 4.1% vals negatieven (DBS van onvoldoende kwaliteit wordt incorrect beoordeeld als voldoende door de app) en 5.9% vals positieven (DBS van voldoende kwaliteit wordt incorrect beoordeeld als onvoldoende door de app). In de ongetrainde setting was de performance kwalificatie 87.4% met 5.5% vals negatieven en 7.1% vals positieven. Indien de app aanwezig was geweest in de getrainde en ongetrainde setting, correct gebruikt was en het opnieuw afnemen van een DBS monster resulteerde in een goede kwaliteit DBS, dan was het aantal DBS monsters van voldoende kwaliteit van respectievelijk 80.0% naar 95.9% gegaan en van 42.2% naar 87.9%. De app kan worden gebruikt in zowel een patiëntzorg als en een research setting om het aantal DBS monsters van goede kwaliteit te verhogen.

In **hoofdstuk 7** beschreven we de eerste randomisatie-gecontroleerde klinische studie waarin de kosten en effecten van het implementeren van DBS thuismonitoring in de transplantatie patiëntenzorg werden onderzocht. In deze single-center, gerandomiseerde klinische studie gebruikten 25 transplantatie patiënten DBS thuismonitoring bovenop de gebruikelijke zorg de eerste 6 maanden na transplantatie, terwijl 23 patiënten alleen de gebruikelijke zorg ontvingen. Het doel was om te onderzoeken of het gebruik van DBS thuismonitoring leidt tot een verminderd aantal bezoeken aan de polikliniek, verminderde kosten en verbeterde patiënttevredenheid. Helaas was het aantal bezoeken in de DBS groep niet lager (11.2, standaarddeviatie (SD) 1.7) dan in de controle groep (10.9, SD 1.4) (p=0.48). Daarnaast waren de kosten per polikliniekbezoek in de DBS groep (€537, SD €179) niet verschillend ten op zichtte van de controle groep (€510, SD €229) (p = 0.66). Dit ligt waarschijnlijk aan het feit dat slechts 56% van het verwachte aantal DBS monsters opgestuurd waren en dat 20% van het verwachte aantal DBS monsters op tijd waren geanalyseerd, wat inhoudt dat het resultaat van de analyse beschikbaar is in het medisch dossier van de patiënt op het moment dat de patiënt bij de nefroloog op de polikliniek is. Echter, 82.6% van de patiënten is bereid om thuis DBS monsters af te nemen indien dit er toe leidt dat er minder polikliniek bezoeken nodig zijn. Optimalisatie van het logistieke proces aangaande het versturen en analyseren van DBS monsters is cruciaal in de implementatie van DBS in de patiëntenzorg.

In **hoofdstuk 8** werd er een richtlijn gepresenteerd aangaande de ontwikkeling, analytische en klinische validatie van DBS analyse methoden die gebruikt worden voor TDM. De huidige validatie eisen, beschreven in richtlijnen voor traditionele matrices (bloed, plasma, serum), bevatten niet alle aspecten die nodig zijn hiervoor. Daarom werden er in dit hoofdstuk aanvullende parameters beschreven die nodig zijn voor

het valideren en kwantificeren van klein-molecuul geneesmiddelen in DBS monsters waarbij gebruik gemaakt wordt van chromatografische methoden. Daarnaast werd er advies gegeven over hoe deze parameters onderzocht kunnen worden en werd er advies gegeven over hoe de analyse methoden toegepast kunnen worden in praktijk. Eerst werden er overwegingen beschreven voor de methode ontwikkelings fase. Daarna werden de gebruikelijke parameters aangaande analytische validatie beschreven in de context van DBS analyse met de toevoeging van DBS-specifieke parameters. Als derde werden klinische validatie studies beschreven, inclusief het benodigde aantal klinische monsters en patiënten, vergelijking van DBS waarden met veneus bloed waarden, statistische methodes en interpretatie, spot kwaliteit, afname procedure, duplicaten, uitschieters, geautomatiseerde analyse en kwaliteitsprogramma's. Als laatste werd cross-validatie bediscussieerd aangaande veranderingen aan een bestaande afname procedure of bestaande analyse methode.

In **hoofdstuk 9** beschreven we de ontwikkeling en analytische validatie van een LC-MS/MS methode voor tacrolimus, everolimus, sirolimus, cyclosporine en mycofenolzuur gebruik makend van Volumatric Absoprtive Micro Sampling (VAMS) tipjes (Mitra®). Deze tipjes zuigen een exact volume bloed op wat potentieel de volume-gerelateerde hematocriet effecten elimineert. Daarnaast is de afname procedure voor de patiënt potentieel eenvoudiger. De bias veroorzaakt door het hematocriet effect was kleiner dan 15% voor alle immunosuppressiva tussen een hematocriet bereik van 0.20 to 0.60, behalve voor cyclosporine waarbij het bereik 0.27 tot 0.60 was. Er was een trend zichtbaar waarbij hogere concentraties van het geneesmiddel gecombineerd met lage hematocriet waarden resulteerden in gereduceerde extractie opbrengst (recovery). Echter, voor de relevante klinische concentratie range voldeed de bias aan de eis en was deze kleiner dan gevonden werd bij DBS **(hoofdstuk 2)**. De analysemethode werd getest voor tacrolimus in een klinische validatie studie beschreven in **hoofdstuk 10**. In totaal werden er 130 gepaarde vingerprik VAMS monsters, vingerprik DBS monsters en veneuze bloedmonsters verkregen van 107 verschillende volwassen niertransplantatie patiënten. Methode vergelijking werd op dezelfde manier uitgevoerd als beschreven in **hoofdstuk 4**. Een multidisciplinair team definieerde vooraf de klinische acceptatie grens: de resultaten van minimaal 80% van de gepaarde monsters moet binnen 15% van het gemiddelde van beide monsters zitten zoals beschreven in de **hoofdstukken 4 en 8.** De kwaliteit van de VAMS en DBS monsters werden beoordeeld: 32.3% van de VAMS monsters en 6.2% van de DBS monsters waren van onvoldoende kwaliteit. Passing & Bablok regressie liet een significant verschil zien tussen VAMS en veneus bloed, met een helling van 0.88 (95% betrouwbaarheidsinterval 0.81-0.97), maar niet tussen DBS en veneus bloed (helling 1.00: 95% betrouwbaarheidsinterval 0.95-1.04). Voor VAMS en DBS werd de klinische acceptatie grens gehaald met respectievelijk 83.0% en 96.6%. VAMS monsters kunnen veneuze monsters vervangen voor tacrolimus

bloedspiegel monitoring, maar de VAMS methode is inferieur aan de DBS methode met betrekking tot monster kwaliteit en inwisselbaarheid met volbloed tacrolimus concentraties.

In **hoofdstuk 11** werd dit proefschrift bediscussieerd en werden toekomst perspectieven beschreven. In dit proefschrift werd beschreven welke stappen er nodig zijn om DBS thuismonitoring van immunosuppressiva bloed spiegels te implementeren voor transplantatiepatiënten. Dit is mogelijk als er aan de volgende criteria wordt voldaan. (1) De DBS analyse methode moet snel en robuust zijn en moet voldoen aan alle algemene en DBS-specifieke analytische voorwaarden. (2) DBS analyse methoden moeten valide worden bevonden in een goed ontworpen en uitgevoerde klinische validatie studie. Daarnaast moeten er een extern kwaliteitsprogramma zijn. (3) De logistiek moet optimaal zijn. Deze kan eventueel verbeterd worden door het Track-and-Trace versturen van monsters, herinneringssystemen voor patiënten om thuis een bloedspot af te nemen en gestandaardiseerde dagen waarop de analyse plaats vindt in het laboratorium. (4) Patiënten getraind worden in de DBS afname procedure waarbij onderdeel van de training is dat patiënten de complete afname procedure uitvoeren onder supervisie van iemand met ervaring.

Acknowledgements (dankwoord)

Dear reader,

Most of these two pages are blank. This might spark the thought that I am not grateful to anyone. Please know that the opposite is true. Some people will find that these pages will be filled with words of gratitude. However, these words are for their eyes only. Therefore, they are only readable in their personal, printed copy of this thesis.

Herman Veenhof

About the Author

Herman Veenhof was born on the 19th of February 1989 in the city of Apeldoorn, The Netherlands. He lived in Istanbul, Turkey for the first 2 years of his life. At the age of 2, he moved to Zuidlaren, The Netherlands. He spend another 3 years abroad in Miri, Malaysia, at the age of 7-10. Afterwards, he moved back to The Netherlands, to the village of Ede, to finish primary school. He graduated from high school (VWO, Guido de Brès, Amersfoort) in 2007. In that same year he moved to Groningen to start with the Bachelor of Pharmacy at the University of Groningen (RUG), which he completed in 2012. In 2015, he completed his Master's degree in Pharmacy (PharmD) at the RUG. In 2015 he started working at the University Medical Center Groningen (UMCG) at the Department of Clinical Pharmacy and Pharmacology as a project pharmacist, under supervision of Daan Touw and Stephan Bakker. The goal of the project was to implement Dried Blood Spot sampling for adult kidney transplant patients. After a few months, this project was combined with a job as a clinical pharmacist in the same department of the UMCG under supervision of Prashant Nannan-Panday. During the implementation process, various research projects were initiated, leading to the start of a PhD project with this thesis as a result. Herman continued to work both as a PhD student and a clinical pharmacist until the end of the PhD project in December 2019. In January 2020, Herman started his training to become a hospital pharmacist at the UMCG.

Herman lives together with Annemarieke Veenhof-Bronswijk and their daughters Hanna and Ellen in the village of Haren, close to the city of Groningen. He enjoys craft beer, theology, seeing family and friends, and giving other people computer/laptop purchase advice.

List of publications

Veenhof, H., Koster, R. A., Alffenaar, J. W. C., Berger, S. P., Bakker, S. J., & Touw, D. J. (2017). Clinical validation of simultaneous analysis of tacrolimus, cyclosporine A, and creatinine in dried blood spots in kidney transplant patients. Transplantation, 101(7), 1727-1733.

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