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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.

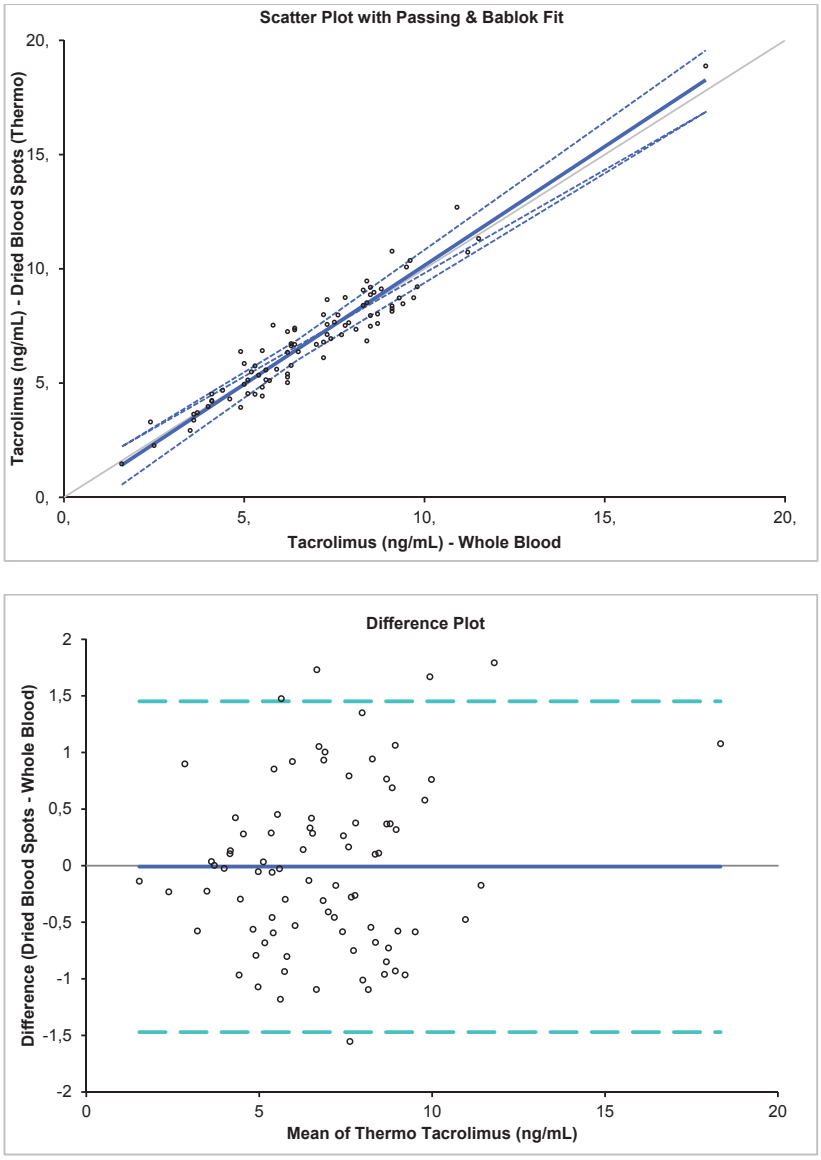

Figure 1: *Comparison of paired whole blood tacrolimus concentrations and Dried Blood Spots (DBS) tacrolimus concentrations measured on a Thermo LC–MS/MS (n = 85). In the upper panel the dotted line is the line of identity, the bold line is the Passing & Bablok regression line y = 1.04 × -0.25 (95% CI 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.*

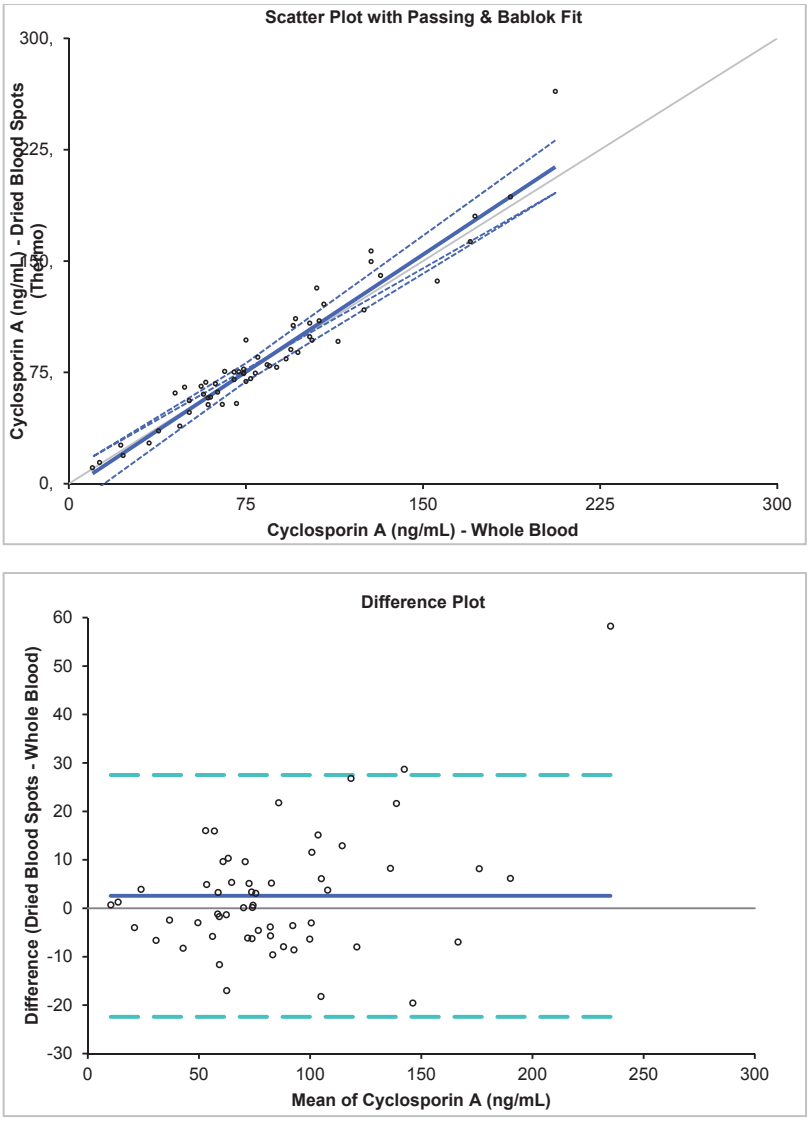

Figure 2: *Comparison of paired whole blood cyclosporin A concentrations and Dried Blood Spots (DBS) cyclosporin A concentrations measured on a Thermo LC–MS/MS (n = 57). In the upper panel the dotted line is the line of identity, the bold line is the Passing & Bablok regression line y = 1.05× – 3.64 (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"

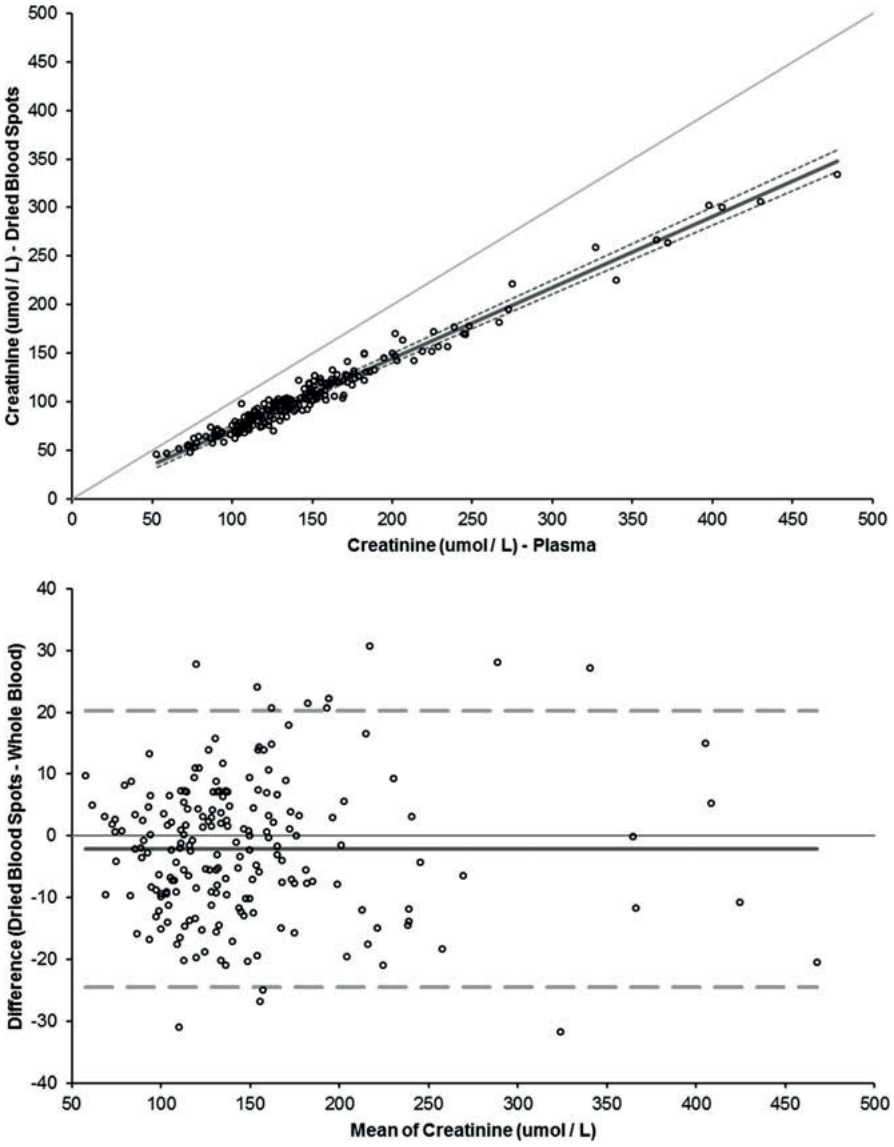

Figure 1. *Method comparison between plasma creatinine levels and DBS creatinine levels (n = 199). In the upper panel the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line y = 0.73x - 1.55 (95% CI slope, 0.71-0.76; intercept, −4.58 to 1.65). The lower panel 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.

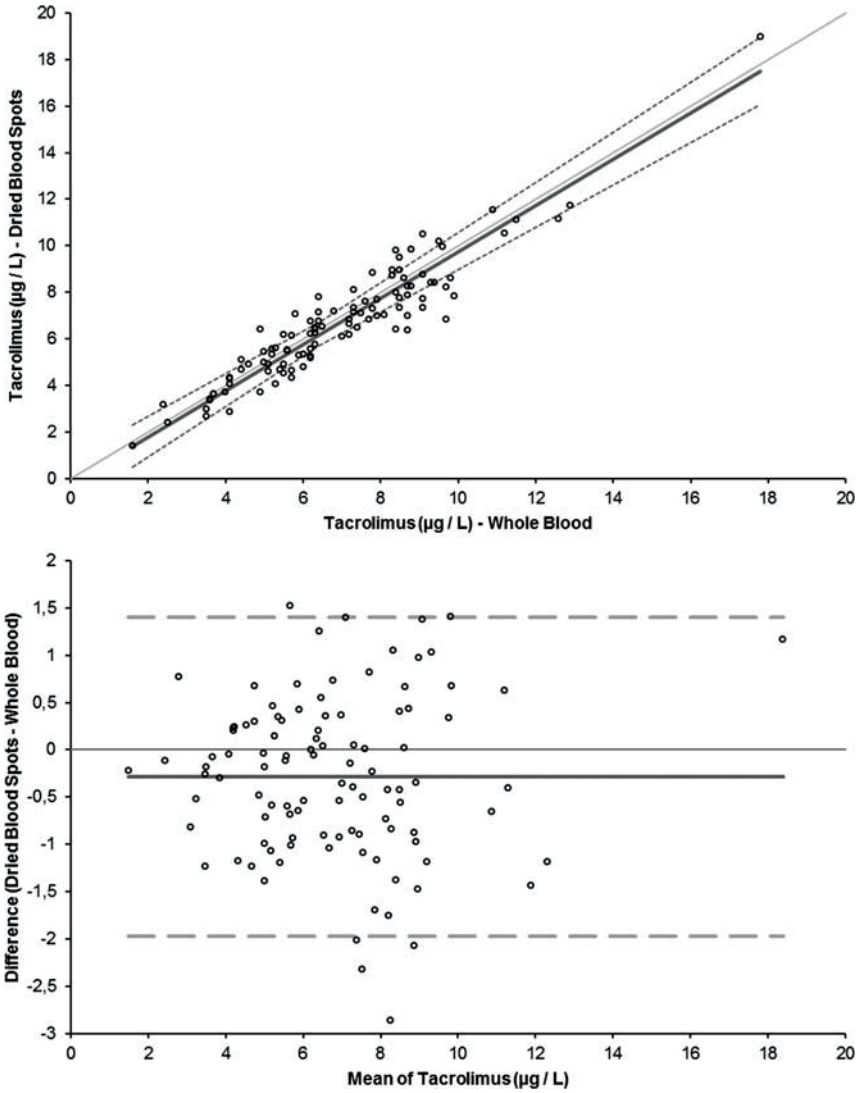

***Figure 2**. Method comparison between venous whole blood TaC concentrations and DBS concentrations (n = 104). In the upper panel, the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line y = 1.00x - 0.23 (95% CI slope, 0.91-1.08; intercept, −0.69 to 0.30). 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.

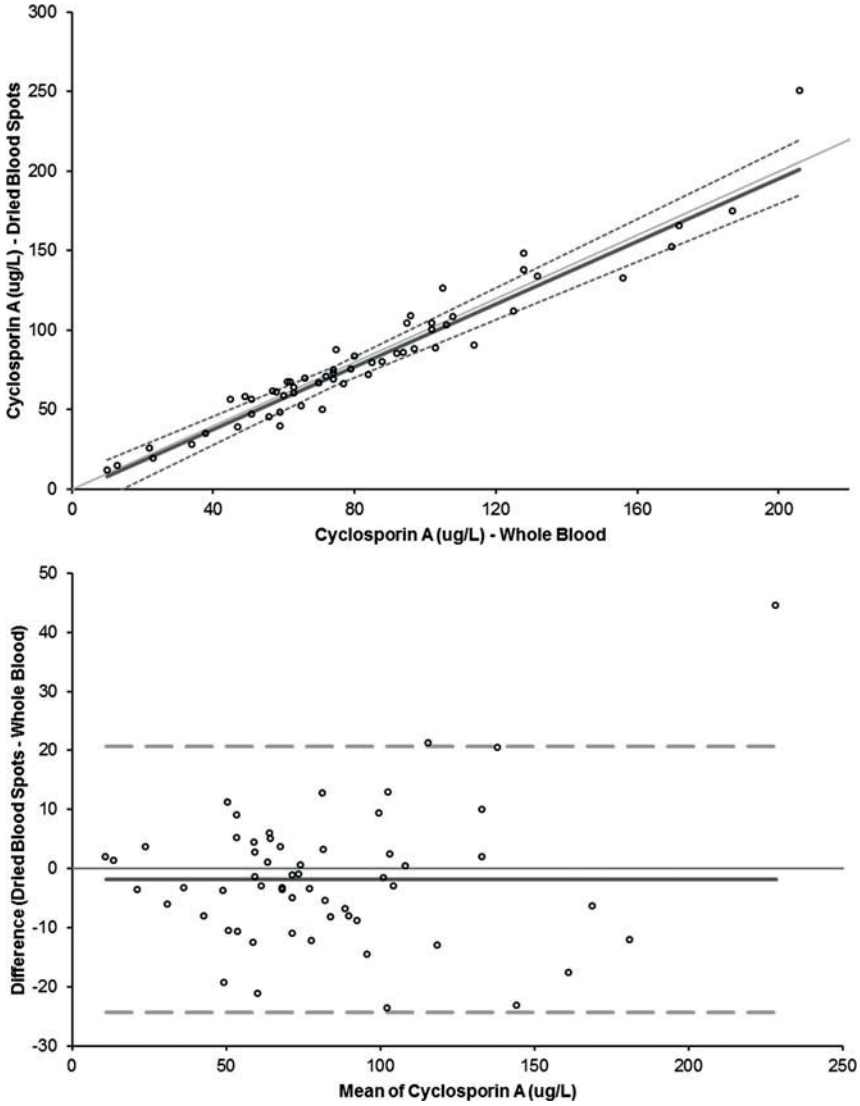

Figure 3. *Method comparison between venous whole blood cyclosporin A concentrations and DBS concentrations (n = 58). In the upper panel, the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line y = 0.99x - 1.86 (95% CI slope, 0.91-1.08; intercept, −8.31 to 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).

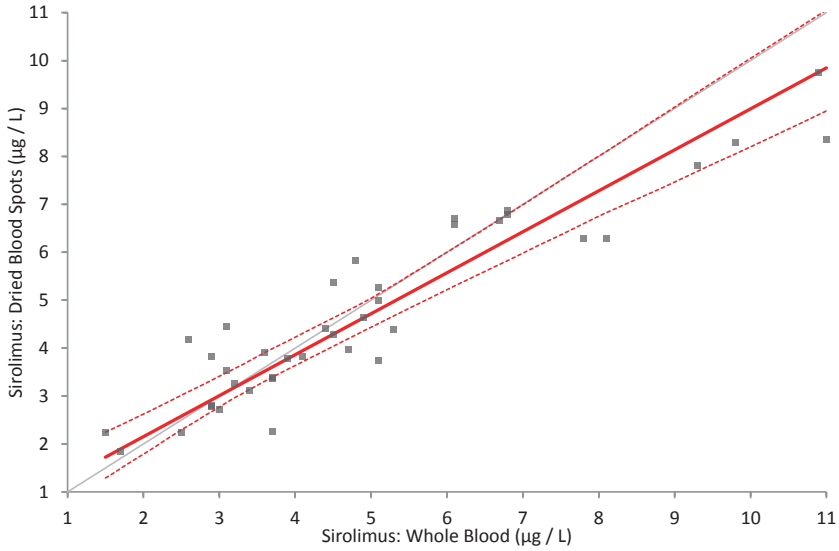

Figure 1. *Method comparison for sirolimus concentrations in WB and DBS (n = 39). The continuous line is the Passing-Bablok regression line y = 0.86x + 0.44 (95% CI slope, 0.72–1.02; 95% CI intercept −0.23 to 1.11). The dashed line is the 95% CI.*

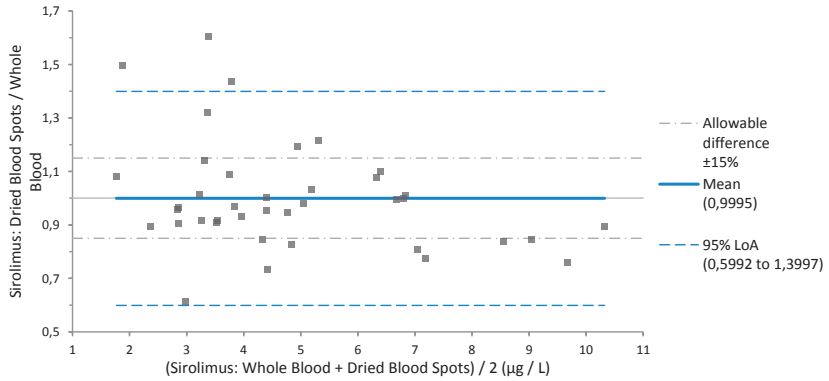

***Figure 2**. Method comparison for sirolimus concentrations in WB and DBS (n = 39).The continuous line is the Bland-Altman bias estimation of 1.00 (95% CI 0.93–1.07). The dashed line is the 95% LoA and the dotted/ dashed line is the limit of clinical relevance set at 15%.*

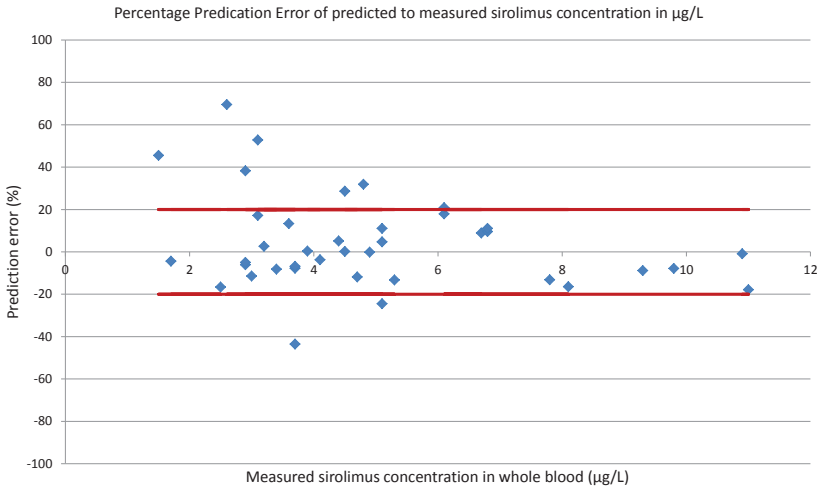

***Figure 3**. Percentage prediction error or predicted to measured sirolimus concentrations with acceptable prediction error set at –20% and 20%.*

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%.

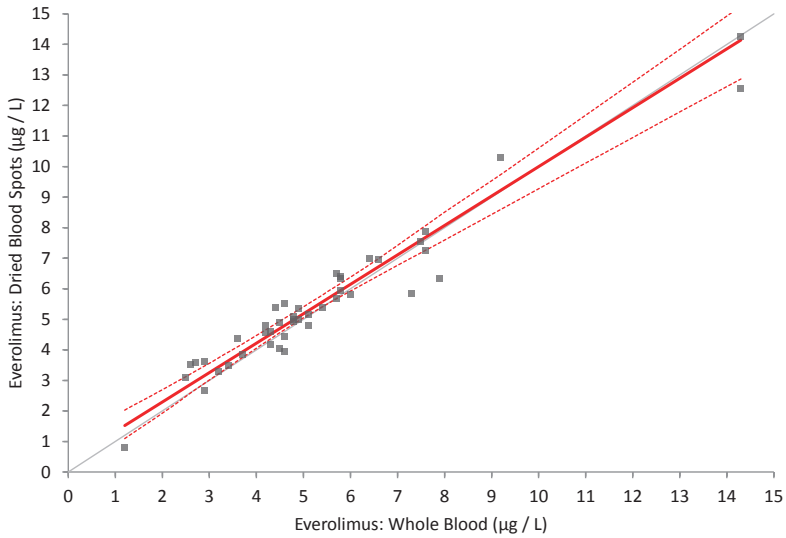

Figure 4. *Method comparison for everolimus concentrations in WB and DBS (n = 44).The continuous line is the Passing-Bablok regression line y = 0.96x + 0.37 (95% CI slope, 0.84–1.06; 95% CI intercept −0.11 to 0.99). The dashed line is the 95% CI*

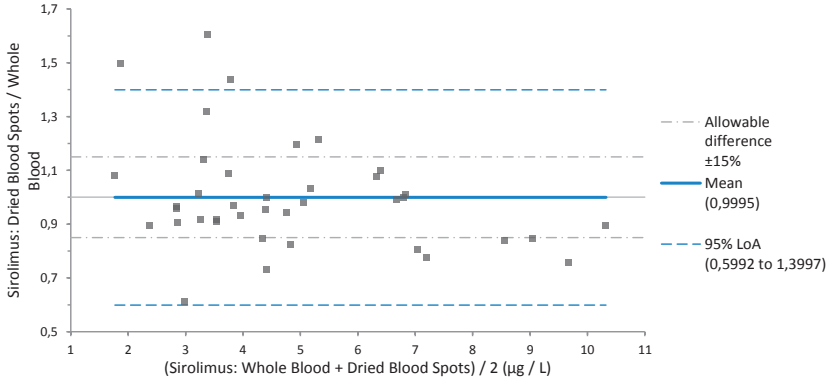

Figure 5. *Method comparison for everolimus concentrations in WB and DBS (n = 44).The continuous line is the Bland-Altman bias estimation of 1.05 (95% CI 1.00–1.08). The dashed line is the 95% LoA and the dotted/dashed line is the limit of clinical relevance set at 15%.*

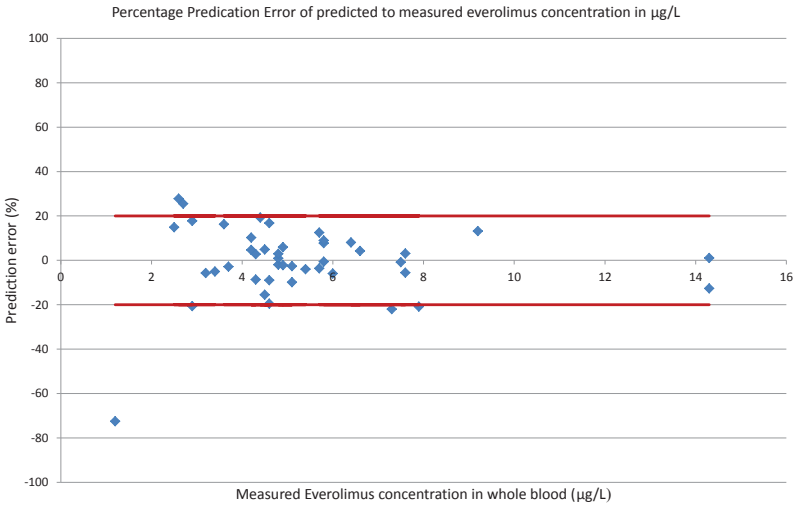

Figure 6. *Percentage prediction error or predicted to measured everolimus concentrations with acceptable prediction error set at –20% and 20%.*

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 5

Quality Assessment of Dried Blood Spots from Patients With Tuberculosis from 4 Countries

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Abstract

Background: Dried blood spot (DBS) sampling is a blood collection tool that uses a finger prick to obtain a blood drop on a DBS card. It can be used for therapeutic drug monitoring, a method that uses blood drug concentrations to optimize individual treatment. DBS sampling is believed to be a simpler way of blood collection compared with venous sampling. The aim of this study was to evaluate the quality of DBSs from patients with tuberculosis all around the world based on quality indicators in a structured assessment procedure.

Methods: Total 464 DBS cards were obtained from 4 countries: Bangladesh, Belarus, Indonesia, and Paraguay. The quality of the DBS cards was assessed using a checklist consisting of 19 questions divided into 4 categories: the integrity of the DBS materials, appropriate drying time, blood volume, and blood spot collection.

Results: After examination, 859 of 1856 (46%) blood spots did not comply with present quality criteria. In 625 cases (34%), this was due to incorrect blood spot collection. The DBS cards from Bangladesh, Indonesia, and Paraguay seemed to be affected by air humidity, causing the blood spots not to dry appropriately.

Conclusions: New tools to help obtain blood spots of sufficient quality are necessary and environmental specific recommendations to determine plasma concentration correctly. In addition, 3% of the DBS cards were rejected because the integrity of the materials suggesting that the quality of plastic ziplock bags currently used to protect the DBS cards against contamination and humidity may not be sufficient.

Introduction

Tuberculosis (TB) is a disease that continues to affect people all around the world. In 2017, 6.4 million new TB cases were reported.[1] Bangladesh and Indonesia combined made up 19% of all incident TB cases in 2017.[1] Drug-susceptible TB is treated with the first-line anti-TB drugs isoniazid, rifampicin, pyrazinamide, and ethambutol for 2 months, followed by isoniazid and rifampicin for another 4 months, leading to successful outcomes in 82% of the cases.[1,2] To optimize TB treatment in patients responding poorly to standard treatment or with risk factors for low drug exposure, therapeutic drug monitoring (TDM) can be performed.[3,4] TDM was recently recommended in the TB treatment guidelines of the ATS/CDC/IDSA.[5] One of the barriers to introduce TDM at a large scale is the stability of plasma or serum samples. This can be overcome by implementing dried blood spot (DBS) sampling.[6] This procedure uses a single drop of blood on special absorbent paper to determine the blood drug concentration. DBS has several other advantages, such as easy sample collection and shipment through regular mail, which saves costs for shipment on dry ice.[7] Also, DBS minimizes the biohazard risk due to the use of dried samples, which is a major advantage in countries with a high prevalence of HIV coinfection.[6,7] The small sample volume of 20–100 mL blood is a major advantage compared with the 4–10 mL of venous blood collected for conventional blood sampling, making this method suitable for children,[7] but also for those who oppose to large volume venous blood sampling. There are 2 DBS sampling methods: The first method involves letting the blood drop fall directly within the premarked circle, followed by partial spot analysis. The second method requires the application of the required blood volume by capillary or pipette onto the DBS card, followed by full-spot analysis.[7] The first method is often preferred because it can be performed by untrained personnel but inaccurate sampling may influence analytical results.[8] Although preliminary results are promising, some hurdles still need to be overcome before DBS sampling can be implemented in TB programs.[9] Laboratories need to clinically validate DBS methods comparing venous samples with fingerprick samples to be able to offer a range of assays. Hematocrit has shown to be of influence on analytical results and should therefore be included during clinical validation.[10–12] Health care workers have to get familiar with the sampling procedure, and pitfalls need to be examined. The aim of this study was to assess, using a structured checklist, whether DBSs of sufficient quality can be obtained with limited training and to give recommendations on how to improve the quality of DBS samples.

Materials and methods

Data sets

Feasibility of DBS was evaluated in 5 different settings: International Centre for Diarrhoeal Disease Research in Bangladesh, Grodno Oblast Clinical Centre "Ftiziatria" in Belarus, Lung Hospital Respira, Yogyakarta in Indonesia, Instituto Nacional de Enfermedades Respiratorias y del Am- biente Juan Max Bohener in Paraguay and Indigenous Hospital of Limpio in Paraguay. Health care workers received DBS packages containing an instruction form, a patient letter, a request form, Whatman FTA DMPK-C DBS cards, plastic ziplock bags to store and ship the cards, desiccant sachets to be included in the ziplock bag for storage and shipment, and lancets to puncture the finger. As DBS sampling was considered to be a simple procedure, health care workers only received written instructions and an instruction video in English.[13,14] DBS samples were collected from patients with TB receiving standard first-line anti-TB treatment. Ethical clearance was obtained at all sites, and patients gave written informed consent.

Validity of the DBS Cards

DBS cards, containing 4 blood spots, were scored on 4 categories: integrity of the DBS materials, appropriate drying time, blood volume, and blood spot collection. The aspects that a DBS card has to comply with for an accurate analysis were based on the "Blood Collection and Handling—Dried Blood Spots" manual of the World Health Organization (WHO).[15,16] For the category integrity of the DBS materials, the plastic ziplock bags were checked if the bag was sealed and had maintained its integrity (holes and rips). Also, the DBS card itself was checked for folds or rips.

For the next category, drying of the DBS card was examined. The card should not be put in the ziplock bag before it was laid to dry for at least half an hour at room temperature in a non-humid environment avoiding direct sunlight. The inside of the ziplock bag and the outer surface of the desiccant sachet were inspected for blood stains. DBS cards that have not been dried long enough show blood spots of a lighter color.[15]

We also assessed whether a 3-, 5-, or 8-mm punch could be made without punching the ink from the predefined circle. In addition, we examined if the blood spot was consisting of a single drop of blood or multiple overlaying drops.

For the last category, blood spot collection, we examined if the blood spot was collected appropriately, without touching the paper and without using a capillary. This was assessed by judging the shape and consistency of the blood spot. We also looked for the presence of a light ring around the blood spot, which can result from squeezing the blood from the finger.

For each category, a series of questions have been included in a checklist (see Supplemental Digital Content 1, http://links.lww.com/TDM/A345). The DBS cards were accepted for analysis if at least 2 blood spots complied with all quality requirements. Two spots will enable a second analysis if something went wrong during sample processing, to confirm the results from the first DBS and to allow different sample preparations to be performed for different assays.[17]

The checklist was filled in for all DBS cards by 2 independent DBS experts: M.A.Z. and H.V. Disagreements were solved by consensus. If the integrity of the DBS materials was compromised or if the drying time was disputable, all 4 DBSs were rejected for analysis. For the other 2 categories, the DBSs were scored individually.

Results

In total 464 DBS cards with 4 blood spots from 4 countries were assessed; 117 from Bangladesh, 90 from Belarus, 129 from Indonesia, and 128 from Paraguay. The amount of blood spots that could be analyzed based on punch size per country is shown in Figure 1. Rejection rate of the DBSs ranged from 13% for Indonesia, 20% for Paraguay, 37% for Belarus to 52% for Bangladesh. Overall, 46% of all DBSs could not be used according to the predefined criteria for analysis.

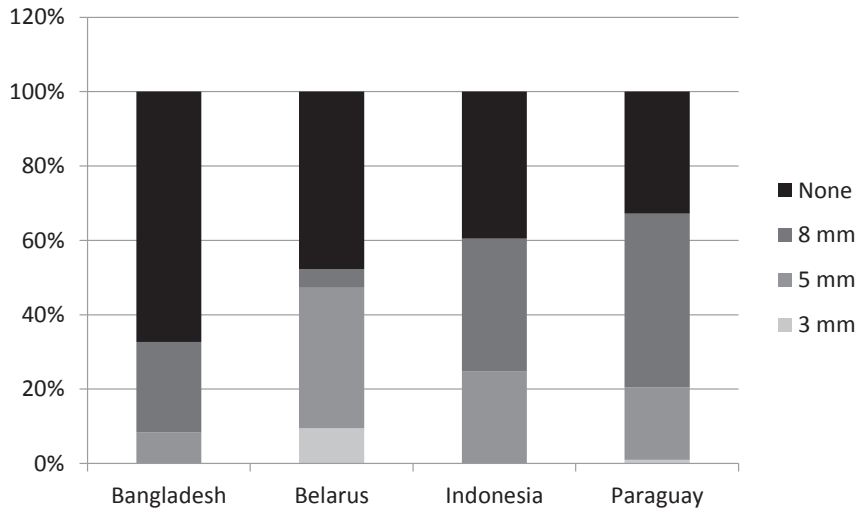

Figure 1. *The DBSs per country and per punch size. This figure shows how many DBSs could be punched and with which minimum size. If a DBS could be punched with an 8- mm puncher, then it is part of the percentage that could be punched with an 8-mm puncher and not with a 5- or 3-mm puncher.*

Most DBSs could be punched with an 8-mm puncher. This was not the case for the blood spots from Belarus. Only 5% of the blood spots were of sufficient size to be punched with an 8-mm puncher, 43% with a 5-mm puncher, and 52% with a 3-mm puncher.

In Figure 2, the amount of DBSs that were rejected based on 4 categories is shown. DBS cards could not be analyzed in 3% of the 464 cases because of the integrity of the materials. Only for the DBS cards from Indonesia, this did not pose a problem. In Indonesia, different bags, foil barrier ziplock bags (Whatman, 10534321; GE Healthcare, Little Chalfont, United Kingdom), were used instead of the provided plastic ziplock bags. As can be seen in Table 1, the biggest issues were due to the plastic bag being open or a missing sachet with desiccant. On 6 DBS cards from Indonesia, a fungus, typed as Aspergillus species, was present. Also, the bags from Indonesia contained 3 DBS cards, only separated by filter papers, whereas there should only be 1 card included per bag.

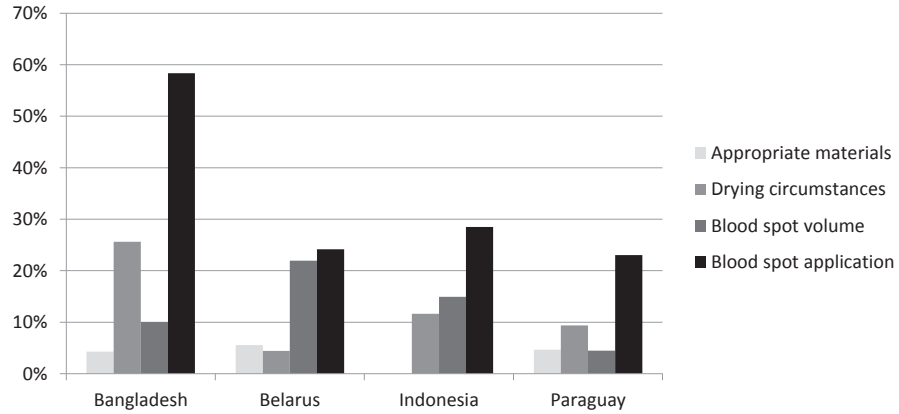

Figure 2. *Percentage of DBSs that were rejected, divided in 4 categories. The results are shown for the 4 categories separately and not as overall results. One DBS can be part of multiple categories.*

The second most important reason for the rejection of DBSs was insufficient drying time before placing the DBS cards in the ziplock bag. As can be seen from Table 1 and Figure 2, this problem was most apparent for the DBSs from Bangladesh and Indonesia.

The biggest cause of rejection was inaccurate blood spot collection. The DBS cards were mostly rejected because of contamination of the DBSs, caused by touching the card with either a finger or a capillary. The latter could be recognized by small round circles on the filter paper. A total of 15% of the DBSs from Bangladesh were rejected

Table 1. *Percentage of DBS Cards That Could be Used per Question From the Checklist*

Category	Question	Bangladesh, % N = 117 DBS Cards With 4 BloodSpots	Belarus, % N = 90 DBS Cards With 4 Blood Spots	Indonesia, % N = 129 DBS Cards With 4 Blood Spots	Paraguay, % N = 128 DBS Cards With 4 Blood Spots	Overall, %
Integrity of the DBS materials	In bag with desiccant	95	87	100	98	96
	Bag intact	97	96	100	95	97
	DBS card intact	98	100	100	98	99
Appropriate drying time	Only blood on the DBS card	76	96	88	92	88
	No lighter color blood spots	99	99	100	100	100
Blood volume	One drop of blood	93	98	87	97	94
	Blood soaked through filter paper	96	80	95	98	93
Blood spot collection	Separate blood spots	99	100	100	100	95
	DBS card not touched during application	85	76	77	83	83
	No light rings around the blood spot	55	100	92	92	73

for touching of the card; however, an additional problem here was the formation of light rings. This eventually caused 58% of the blood spots from Bangladesh to be rejected. For the blood spots from Indonesia, the main issue was that 13% of the spots consisted of multiple overlaying drops of blood. Besides contamination, blood spot volume was an important reason for rejection in Belarus and Indonesia (Fig. 2). This low blood volume caused the blood to not soak through the filter paper of the DBS card, as can be seen in the low per- centage stated in Table 1.

Keeping all criteria in mind, an average of 65% of the DBS cards could be used. For Bangladesh, this was only 40%, for Belarus 59%, for Indonesia 80%, and for Paraguay 77% (Fig. 3).

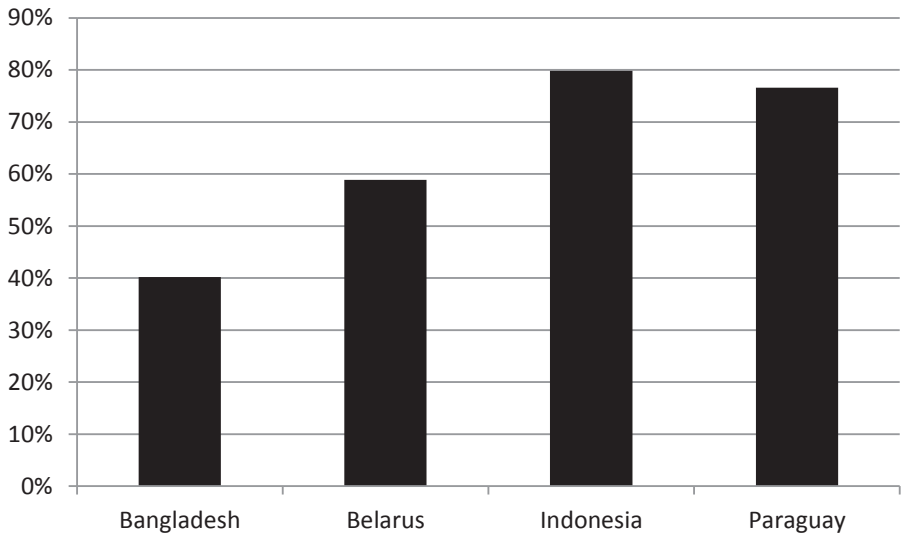

Figure 3. *Percentage of DBS cards that could be used per country. The percentage of DBS cards that could be used for analysis, based on the need for 2 DBSs per DBS cards for analysis.*

Discussion

Ultimately, the goal is to implement DBS sampling for TDM of anti-TB drugs, to provide a more patient-friendly way of sampling in remote settings. DBS sampling can also be used in other infectious diseases such as HIV, for which not only plasma concentrations of antiretroviral drugs can be determined with DBS, but also viral load.[18] Unfortunately, 46% (859/1856) of the spots in our study were rejected based on strict criteria, leading to an average of 65% of DBS cards that could be used. The high rejection rate of samples in our study showed that DBS sampling would require more training to produce bloods spots of sufficient quality to be analyzed. This confirms the study of Hoogtanders et al. which showed that DBSs of sufficient quality for analysis could not be obtained with the first method, which is letting the blood drop fall directly onto the DBS card, without appropriate training.[8,19] This article can help guide health care workers and researchers to collect DBSs of sufficient quality.

We found that the currently used plastic ziplock bags were not of sufficient quality to protect the DBS card from damage, with around 3% of the bags being torn and 4% being open or missing a desiccant sachet. The reason for an open ziplock back, next to opening during transport, could also be because they are difficult to close. In Indonesia, different bags were used, which were of thicker material. These light protected seal bags did not have any problems as far as opening or ripping and could therefore be a better option. A fungus was found on 6 DBS cards, likely caused by contamination of the DBS cards or filter papers dividing the DBS cards, since the fungus is commonly found on skin. This warrants further study to exclude that the bag itself aided the growth of the fungus. If this is not the case, light protected biohazard seal bags are preferred over the currently used plastic ziplock bags. A study by Winter et al. showed that the analysis results were affected by many contaminants, that is, feces, disinfectants, and urine,[20] showing the need for hygiene when performing DBS sampling.

We also assessed whether punches could be obtained without ink from the premarked circle. As it is not known what influence the ink has on the outcome of the analysis, we have decided to reject blood spots that could only be obtained with ink. This was only the case for blood spots that could be obtained with an 8-mm puncher; therefore, it had no influence on the amount of blood spots that could ultimately be analyzed.

Drying conditions was shown to be one of the most important reasons for the rejection of samples from Bangladesh, Paraguay, and Indonesia. Light rings were also more common in these countries, for example, for Bangladesh, 45% of the blood spots needed to be rejected because of this (Table 1). Because the light rings were more common in these countries, it is more likely that a high humidity is the cause of the

light rings instead of squeezing of the finger. A high humidity requires a longer drying time.[21] It could also be that the DBS cards were humid before use because the DBS cards were not stored in a plastic ziplock bag with a desiccant sachet before use and were therefore not protected against humid conditions.[22] To prevent this from happening, DBS sampling should be performed in a temperature- and humidity-controlled environment. DBS sampling is not a viable option in remote settings with high humidity or home sampling. An alternative could be to store the DBS cards in a box or package with desiccant sachets. In such an environment, storage of the collected sample can also be done in the box. It needs to be tested whether this would improve drying of the samples.[23]

DBS sampling can be difficult in case of callous skin. Callous skin is difficult to penetrate with the lancet causing issues with obtaining enough blood for the DBS sampling. This was seen for blood spots from Belarus, as the blood spots were too small. Use of another type of lancet or an alternative sampling position compared with the finger, that is, the ear lobe, could solve this problem.

A study by Martial et al.[24] showed that DBS sampling would not only be more patient-friendly, but also cost-effective, if it could be performed at home. Without practical training, it is shown to be difficult to obtain a blood spot from which the drug concentration can be accurately determined. As TDM for the optimization of TB treatment will likely be performed using 2– 4 rounds of TDM,[25,26] it will be easier and more cost-effective to train health care workers instead of patients. Because patients with TB visit the public health service regularly, the DBS sampling could be performed during a scheduled visit. Based on the results from this study, the instructions need to be amended. Instructions could also be made available in native languages to overcome interpretation issues.

Conclusions

More practical training is needed to adequately perform DBS sampling. Compliance with storage and shipment conditions is important to have the DBS sample arrive in the laboratory and pass quality checks.

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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]

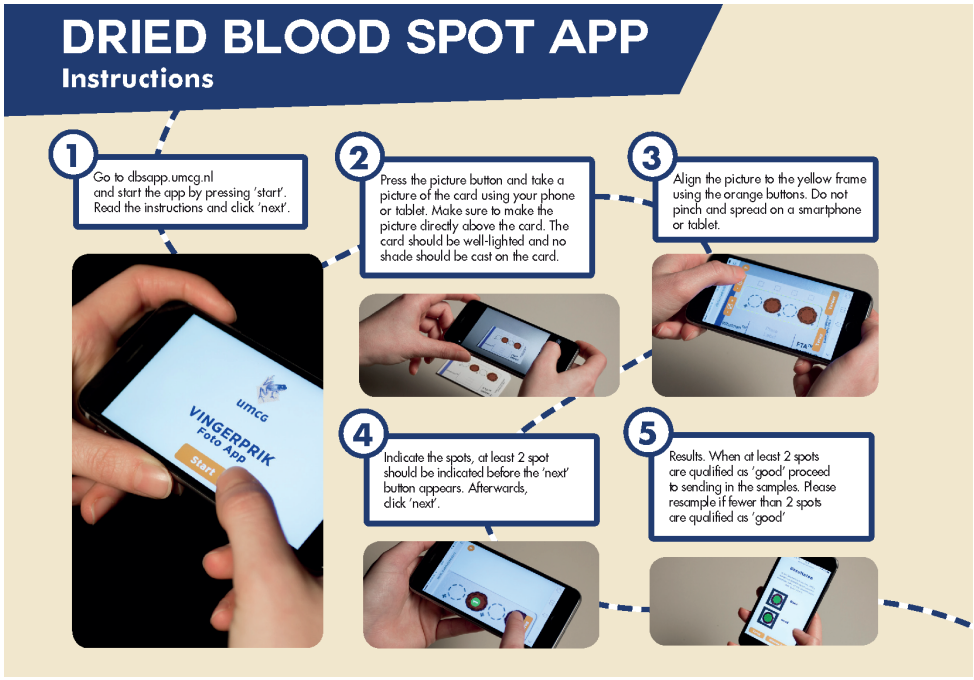

Figure 1. *Instructions for using the DBS app*

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.

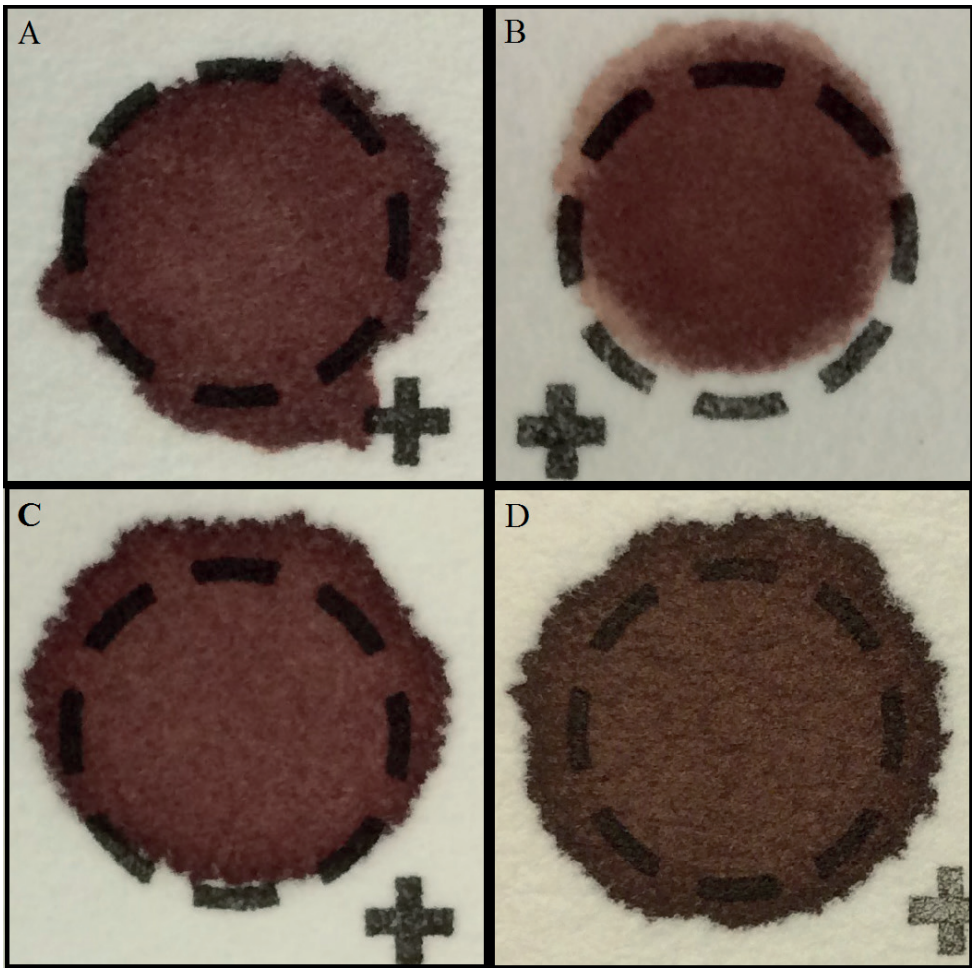

Figure 2. *Different types of spot quality in DBS sampling. (A) Layered spot consisting of multiple droplets of blood. (B) Discoloration because of hemolysis or humidity. (C) Spot that is not perfectly circle-shaped. (D) A good quality blood spot meeting all requirements: round, filling at least the 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 7

Effects, costs and implementation of monitoring kidney transplant patients' tacrolimus levels with Dried Blood Spot sampling: a randomized controlled hybrid implementation trial

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Abstract

Aims: Dried Blood Spot (DBS) home sampling allows monitoring of creatinine levels and tacrolimus trough levels as an alternative for blood sampling in the hospital, which is important in kidney transplant patient follow-up. This study aims to assess whether DBS home sampling results in decreased patient travel burden and lower societal costs.

Methods: In this single-center randomized controlled hybrid implementation trial, adult kidney transplant patients were enrolled. The intervention group (n=25) used DBS home sampling on top of usual care in the first 6 months after transplantation. The control group (n=23) received usual care only. The primary endpoint was the number of outpatient visits. Other endpoints were: (1) costs per patient (2) patient satisfaction and (3) implementation.

Results: There was no statistical significant difference in the average number of outpatient visits between the DBS group (11.2, SD: 1.7) and the control group (10.9, SD: 1.4) (p = 0.48). Average costs per visit in the DBS group were not significantly different (€542, 95%CI €316 - €990) compared to the control group (€533, 95%CI €278 - €1093) (p = 0.66). Most patients (n=19/23, 82.6%) were willing to perform DBS home-sampling if this would reduce the number of hospital visits. Only 55.9% (n=143/256) of the expected DBS samples were received and one-fifth analyzed on time (n=52/256).

Conclusions: Adult kidney transplant patients are willing to perform DBS home sampling. However, to decrease patient travel burden and costs in post-transplant care, optimization of the logistical process concerning mailing and analysis of DBS samples is crucial.

Introduction

Tacrolimus is currently the most used immunosuppressant in allograft rejection prevention in kidney transplant patients.[1] While effective at the correct dose, high tacrolimus trough levels are associated with severe adverse effects, while low tacrolimus levels increase the risk of acute rejection.[2] To find a balance between sub therapeutic- and toxic effects of this drug in transplant patients, lifelong monitoring of blood drug levels using Therapeutic Drug Monitoring (TDM) is therefore mandatory.[2,3] Current clinical practice requires transplant patients to frequently travel to the hospital for venous blood sampling. In general, TDM is performed weekly in the first month post-discharge after renal transplantation. Over a period of approximately one year, the frequency is tapered to 3-monthly visits. Given the time delay between blood sampling and availability of analytical results, tacrolimus blood trough levels are usually not yet available when the nephrologist sees the patient and only become available in the evening of the day of sampling or the following day. This requires the patient to sample a few days earlier, or requires the nephrologist to schedule another appointment (usually by telephone) to discuss the TDM results. For both patient and nephrologist, this workflow is suboptimal.

Recently, Dried Blood Spot (DBS) sampling was introduced as a novel tool that allows patients to sample at home. Using a fingerprick, blood can be applied to a sampling card which can subsequently be mailed to the hospital laboratory a few days before a consultation with the nephrologist. DBS provides reliable results for both tacrolimus and creatinine levels.[4-7] This results in up-to-date blood-drug levels at the time the patient consults the nephrologist. In theory, DBS sampling may result in a decreased patient travel burden, a more efficient workflow for the nephrologist, fewer outpatient visits and lower societal costs with improved quality of care.[4] While promising, apart from a scenario analysis evaluating DBS home sampling for TDM of immunosuppressants, no clinical studies have assessed the cost-effectiveness of DBS home sampling.[8] Only one study addressed the feasibility and implementation of home-based micro sampling for tacrolimus TDM in children, but this study lacked a control group.[9] Other studies focused on the feasibility of DBS home sampling in the context of patient sampling performance and sample quality.[10-14]

Given that using DBS home sampling could result in up-to-date blood levels of tacrolimus readily available at every consultation, we hypothesized that implementing DBS would increase the rate of tapering of outpatient visits and therefore would lower total health care costs. In this randomized controlled hybrid implementation trial, we aimed to assess whether the use of DBS home sampling in the first six months after kidney transplantation would result in fewer clinical consultations and lower costs from a societal perspective compared to usual care. In addition, the implementation of DBS home sampling was evaluated with regards to sampling logistics.

Materials and Methods

Study design

In this single-center randomized controlled trial, the intervention group used DBS sampling on top of usual care in the first six months after kidney transplantation, while the control group received usual care only. This study was designed as a hybrid implementation trial where a clinical intervention is tested while observing and gathering information on implementation.[15] Therefore, an implementation strategy was not part of this study. This study is reported in accordance with the Standards for Reporting Implementation Studies (StaRI), see supplement S1, available online.[16,17] This study was conducted in accordance with the declaration of Helsinki, the EMA guideline for good clinical practice E6(R2) and the CONSORT 2010 guideline, see Supplement S2, available online.[18,19] The study protocol was approved by the medical ethical committee of the UMCG (NL56927.042.16.). The study protocol stated that, because of the nature of the study, no (serious) adverse events have to be reported. The trial protocol was registered in the Dutch Trial Register (Trial NL7721).

Study population

All adult patients who were hospitalized at the University Medical Center Groningen (UMCG) after receiving a renal transplantation were screened. The inclusion criteria were: age ≥ 18 years, still hospitalized after renal transplantation, use of tacrolimus, proficiency of the Dutch language and ability to use the DBS sampling method.[20] The study follow-up period was six months. Patients who withdrew from the study during the enrollment period for any reason were replaced. Patients who switched, during the study, to another immunosuppressant that could also be monitored with DBS such as cyclosporin A, sirolimus or everolimus were not excluded.[4,21] Written informed consent was obtained from all participants included in the study as described by the ICMJE.[22]

DBS training & administration schedule

The intervention group received training in DBS sampling using a previously described method while still hospitalized after transplantation.[20] This 15-minute training included studying and practicing the complete sampling procedure under supervision of an experienced study-coordinator until deemed satisfactory.[9,23] At each patients consultation, the nephrologist placed orders for DBS home sampling for the following consultation. During the first four weeks after kidney transplantation, patients were scheduled to visit the outpatient clinic every week and sampled 5 days prior to the visit. Subsequently, patients received instructions to sample 7 days prior to a scheduled visit. Upon receiving the DBS samples, the hospital laboratory routinely analyzed the blood extracted from the DBS samples twice weekly, using previously validated method..[4-6]

Study context and usual care

Patients in both the DBS- and control group received usual care as described by transplantation protocols in het UMCG. During the first year post-transplantation, all patients are treated in the academic hospital (the UMCG) where the transplantation was performed. This treatment consists of transplantation nephrologist consultation in the out-patient clinic. Patients arrive in the out-patient clinic before 10 AM for venous blood sampling because of the need to obtain a tacrolimus trough concentration, in addition to other clinical chemical parameters and serum creatinine. Nephrologist consultation is usually between 9 and 12 AM and takes on average 10 minutes. Tacrolimus trough concentrations are usually available in the late afternoon. Therefore, a telephone consult of, on average, 7.5 minutes takes place in the evening or the next day if, based on tacrolimus trough concentration, adjustment of the tacrolimus dose is needed. The frequency of outpatient visits are pre-determined (weekly visits) in the first month post-discharge after adult renal transplantation. After that, follow-up visit frequency is planned based on clinical observation by the nephrologist. Given that using DBS home sampling would result in up-to-date blood levels of tacrolimus and creatinine readily available at every outpatient visit, we hypothesized that implementing DBS would increase the rate of tapering of outpatient visits and therefore would lower the number of outpatient visits during the first 6 months after transplantation. On a yearly basis, approximately 170 adult kidney transplantations are performed in the UMCG. Patient traveling distance to the outpatient clinic is usually between 1 and 150 km.

Effectiveness outcomes

The primary endpoint of this study was the number of outpatient visits per patient. Secondary endpoints were costs and patient satisfaction. Cost differences were measured between the DBS group and the control group using standard health resources use questionnaires and the formal Dutch reference prices of care, further detailed in the following 'Data collection' section and 'Cost evaluation' section.[24]

Implementation outcomes

Implementation outcomes were the number of tacrolimus dose adjustments communicated by phone. If DBS results were available during the patient' visit, no phone calls discussing tacrolimus dosing would be needed. Other implementation and logistics measures included: (1) the number of DBS results that were on time, defined as the analytical results that were available in the patient's electronic health record (EHR) prior to the outpatient visit to the nephrologist, (2) the time between sampling by the patient and receiving the sample at the laboratory as well as the analysis time, obtained from the EHR, and (3) the number of printed prescriptions for tacrolimus, obtained from the EHR.

Cost evaluation

To perform the cost evaluation from a societal point of view, three cost categories were identified: patient costs (parking costs and traveling expenses), costs related to loss of productivity (patients' travel time and time in the hospital, caregivers time) and healthcare costs (outpatient clinic visits, laboratory costs, nephrologist phone call, DBS sampling kit and analysis costs).[8] Patient parking costs and traveling distance (in km) were obtained from the iMTA questionnaire (Supplement S3, available online).[25-27] To calculate traveling time (by car) an average speed of 80 km/hour was assumed. The time in the hospital was calculated by the time difference between the moment of venous blood sampling and the start of the scheduled appointment plus 30 minutes to account for the duration of the scheduled appointment with the nephrologist (15 minutes) and the queue for venous sampling by a phlebotomist (15 minutes). Information about the presence of a caregiver and the patients occupation was obtained from the iMTA questionnaire. Depending on the occupation of a patient (employed, not-employed), a rate for loss of productivity was chosen for the cost calculation as stated by the formal Dutch national tariff, cost year 2017 (Table 1).[24]

Table 1. *Reference prices as defined by the Dutch national tariff of 2017 and the University Medical Centre Groningen tarrif list of 2017.*

Type	Costs	Per
Patient costs		
Travel expenses (car)	€0.19	kilometer
Parking costs	€3.00	visit
Loss of productivity		
Productivity cost, paid, working women	€32.00	hour
Productivity cost, paid, working men	€38.00	hour
Unpaid work, replacement costs	€14.00	hour
Caregiver, replacement costs	€14.00	hour
Healthcare costs		
Patient visit to an academic outpatient clinic	€163.00	visit
Nephrologist time	€113.00	hour
Nephrologist phone call	€14.13	call
Study coordinator costs	€24.70	hour
DBS training (time and materials)	€13,68	training
DBS sampling kit	€7.50	kit
DBS analysis (tacrolimus + creatinine)	€50.00	analysis
'small' lab	€41.52	analysis
'normal' lab	€110.49	analysis
'extensive' lab	€348.34	analysis
Tacrolimus whole blood	€44.03	analysis
Mycophenolic acid whole blood	€44.03	analysis
BK IgG	€93.87	analysis
CMV IgG	€227.17	analysis
EBV IgG	€227.17	analysis

BK IgG, BK virus antibodies. CMV IgG, Cytomegalovirus antibodies. EBV IgG, Epstein-Barr virus antibodies.

All laboratory results from the patients were obtained and prices were calculated using the tariff listing of the UMCG of 2017. Lab ordering was done using fixed sets of clinical chemical and hematological parameters. There are 3 types of order sets used which are defined as 'small', 'normal' and 'extensive' (table 1). Additional parameters such as blood drug levels and viral antibody titers are ordered separately (table 1). The costs for an outpatient visit, the nephrologist telephone call and DBS training was based on the Dutch national tariff.[24] Since DBS-training was performed while patients were still hospitalized, no loss of productivity occurred. The costs for the DBS sampling kit and analysis was fixed (see table 1). The one-time costs of the implementation were calculated. Since, prior to the start of the implementation, the DBS analytical method and DBS instruction method were already present, the development of these methods was not added to the implementation costs.[28] Based on the labor tariffs of the UMCG in the year 2017, the hourly costs of the study coordinator who performed the implementation was calculated (Table 1).

Data collection

Demographic and clinical data

Demographic, clinical and biochemical data were retrospectively collected from the EHR at baseline and during study follow-up. This included total bodyweight, height, time and date of transplantation, donor (living/deceased), donor age, donor sex, diabetes at baseline, immunosuppressive medication, delayed graft function and hospitalization time after transplantation.

The number of visits to the transplantation outpatient clinic was recorded, including date, tacrolimus dose (adjustments) and accompanying physician notes. The number of tacrolimus dose changes communicated by nephrologist' phone calls was obtained from the EHR. The number of written prescriptions for tacrolimus was obtained from the Electronic Prescribing System (EPS). For the DBS samples, data about date and time of sampling were recorded by patients at time of sampling. Data about reception of the sample at the lab, analysis time and time the results were available were collected from the EHR.

Cost and patient satisfaction data

Four weeks after inclusion, all patients received a questionnaire about loss of (work) time due to the routine outpatient visits based on the Medical Consumption Questionnaire (MCQ), the iMTA Valuation of Informal Care Questionnaire (iVICQ) and the Productivity Cost Questionnaire (PCQ), developed by the Institute for Medical Technology Assessment (iMTA) of the Erasmus University Rotterdam.[25-27] These questionnaires are validated to perform cost-effectiveness research from a societal point of view in the Netherlands. Patients in the DBS group, also completed a survey

on patient satisfaction and feasibility of DBS sampling. The (Dutch) questionnaires can be found in supplement S3, available online. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Sample size

Based on the kidney transplant protocols used in our hospital and retrospective data, we expected an average of 10 visits to the outpatient clinic per patient during the study period. Based on expert opinion by several experienced nephrologists, the number of visits per patient could be reduced to 9 when using the DBS sampling method. With an expected reduction of 10%, assuming a standard deviation (SD) of 1,5% error and a power of 90% resulted in a sample size of 22 patients per group. To account for 10% expected loss to follow-up during the study, a minimum of 25 patients per group were to be included.

Randomization

Randomization was done using computer-generated random numbers with equal allocation in two groups by an independent researcher who was not part of this study. The allocation was concealed in sequentially numbered, sealed envelopes. Patients were screened, enrolled and trained by one study coordinator. Due to the nature of the intervention, participant blinding was not possible.

Statistical analysis

All categorical data were expressed as percentages, numeric data were expressed as average ± standard deviation (SD). All categorical data were expressed as percentages, normally distributed numeric data were expressed as average ± standard deviation (SD). Normality was tested using a Shapiro-Wilk test. When not normally distributed, the data were expressed as median with an interquartile range (IQR). Normally distributed cost data was expressed as a average with 95% confidence interval (95%CI) based on SD. When cost data was not normally distributed, the 95%CI was obtained by non-parametric bootstrapping (n=1000 resampled data sets, bootstrap estimates 2.5th to 97.5th percentiles).The differences in patient visits were analyzed both per-protocol and as intention-to-treat. Differences in continuous variables were assessed by a two-tailed, unpaired T-test. All statistical analyses were performed using SPSS, version 23 (SPSS, Chicago, IL, USA) or Anlayse it® for Excel 4.81.6 (Leeds, UK). A value of $p < 0.05$ was considered statistically significant.

Results

Study Population

In total, 83 patients were screened between May 2016 and May 2017 of which 54 patients were randomized (for flow diagram, see Figure 1). In the DBS group, three patients were excluded, resulting in 25 patients included in the analysis. In the control group, three patients were excluded resulting in 23 patients included in the analysis. Reasons for exclusion can be found in figure 1. All patients were Caucasian and received standard triple immunosuppressive therapy after transplantation consisting of tacrolimus, mycophenolic acid and prednisolon. Baseline characteristics were comparable in both groups (Table 2).

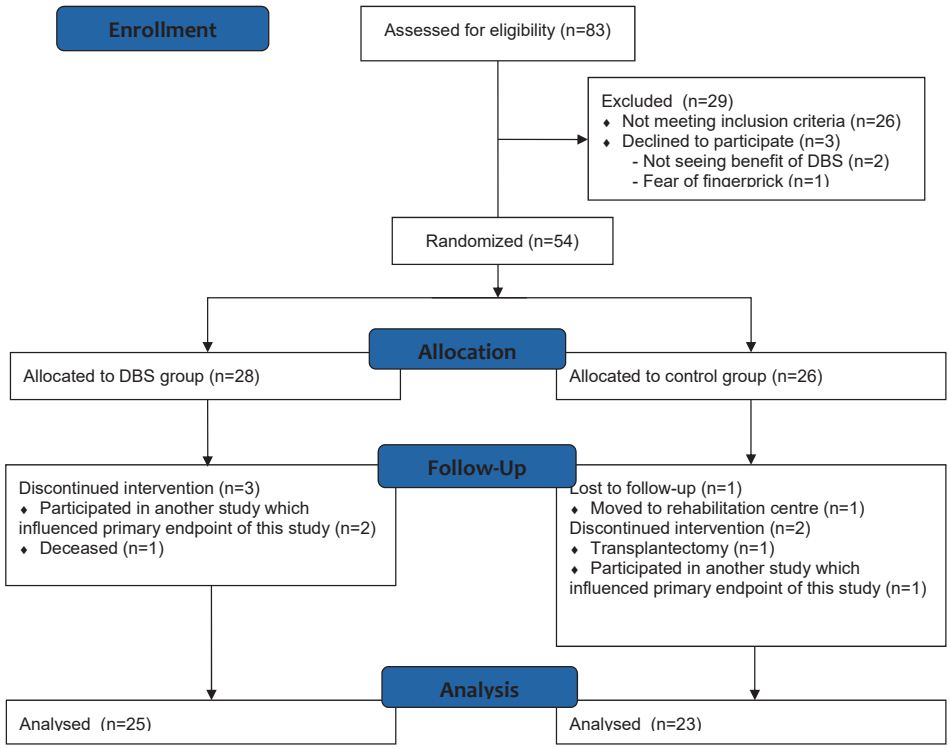

Figure 1. *Consort diagram*

Table 2. *Demographics and baseline charecteristics of the patients*

Participants	DBS group (n=25)	Control group (n=23)
Male sex, n (%)	17 (68.0)	12 (52.2)
Age, years ± SD	52.8 ± 14.1	50.9 ± 14.3
Body weight, kg ± SD	83.7 ± 15.6	79.2 ± 15.1
BMI, kg/m^2 ± SD	27.7 ± 4.6	26.3 ± 4.7
Hospitalization time after transplantation, days ± SD	8.3 ± 2.3	9.3 ± 4.1
Delayed graft function, n (%)	5 (25.0)	4 (17.4)
Diabetes at baseline, n (%)	5 (25.0)	4 (17.4)
Induction, n (%)		
rATG	1 (4.0)	2 (8.7)
Basiliximab	5 (20.0)	5 (21.7)
Rituxima	2 (8.0)	0 (0.0)
Alemtuzumab	0 (0.0)	1 (4.3)
Donor		
Average age, years ± SD	55.3 ± 11.3	50.0 ± 15.9
Male, n (%)	18 (72.0)	17 (73.9)
Donor category, n (%)		
Living	17 (68.0)	12 (52.1)
Deceased heart-beating	0 (0.0)	5 (21.7)
Deceased non-heart-beating	8 (32.0)	6 (26.1)

BMI, Body Mass Index. DBS, dried blood spots. rATG, Rabbit Anti-Thymocyte Globulin. SD, standard deviation.

Effectiveness outcomes

Number of visits

There was no statistically significant difference in the average number of visits between the DBS group and the control group (p = 0.48) as shown in Table 3.

Table 3. *Average number of visits to the outpatient clinic per patient per group.*

	DBS group		Control group (n=23)	p value	
	Intention to treat (n=25)	Per protocol* (n=6)		Intention to treat	Per protocol*
Average number of visits per patient ± SD	11.2 (1.7)	11.2 (0.9)	10.9 (1.4)	0.48	0.63
Average number of tacrolimus dose adjustments communicated by phone ± SD	3.9 (1.6)	3.8 (2.0)	3.3 (2.0)	0.23	0.57
Average number of printed prescriptions for tacrolimus per patient ± SD	3.4 (1.2)	4.0 (1.2)	3.1 (1.5)	0.45	0.18

*The per protocol group consists of 6 patients who had ≥4 visits where results of DBS analysis were available in the Electronic Health Records (EHR) at the time of nephrologist consultation.

Patient satisfaction

In the DBS group, 23 out of 25 patients (92%) completed the questionnaire on DBS sampling satisfaction. Qualitative results can be found in Table 4. DBS sampling was manageable for most patients (82.6%, n=19/23) and most patients (82.6%, n=19/23) were willing to perform DBS home-sampling if this would reduce the number of hospital visits.

Table 4. *Qualitative results of patient questionnaire on DBS sampling feasibility (n=23).*

Already experienced with finger prick sampling	No (11)	Yes (8)	Slightly (4)
Able to produce a sufficient quality DBS sample with given instructions	Too hard (3)	Hard, but manageable (3)	Reasonably/Easily (17)
Preferred way of blood sampling	Finger prick (5)	Venous sampling (6)	No preference (12)
Willing to provide multiple DBS a year if this can reduce the number of hospital visits	No (3)	Yes (19)	I don't know (1)

Costs

A total of 23 patients in the DBS group and 23 patients in the control group completed the cost questionnaire and were included in the cost analysis. Because only 56% (see Results section 'implementation outcomes') of the expected number of DBS were analyzed, the costs related to DBS analysis were corrected (Table 5). A Shapiro-Wilk test showed that costs were not normally distributed in both the DBS ($p<0.0001$) and control ($p<0,0001$) group. Therefore, costs are shown as a median in Table 5. However, because average costs are often used in decision making, this is also shown in Table 5. In the DBS group almost 80% (n=18/23) of the patients were accompanied by a caregiver during the visit, in the control group this was about 50% (n=12/23). Average costs in the DBS group were slightly higher, but not significantly different from the control group ($p = 0.66$). If the cost for DBS were subtracted, the average total cost for a patient' visit in the DBS and control group were similar (€508; 95%CI €294 - €949 and €533; 95%CI €278 - €1093, respectively). The costs of patient training were €13.68 per patient, since these costs are one-time only, they were not included in Table 5.

Table 5. *Average costs per visit of the patient to the outpatient clinic from a societal point of view.*

Time and costs per visit to the policlinic	DBS group (n=23)				Control group (n=23)			
	Average Volume/ Unit n ± STD	Average costs € (95%CI)	Median Volume/ Unit n (IQR)	Median costs € (IQR range)	Average Volume/ Unit n ± STD	Average costs € (95%CI)	Median Volume/ Unit n (IQR)	Median costs € (IQR range)
Patient costs								
Parking costs		€2.61 (€0.00 - €3.00)		€3.00 (€3.00 - €3.00)		€1.62 (€0.00- €3.60)		€3.00 (€0.00 - €3.00)
Travel expenses	73 ± 46 km	€29.52 (€2.13 - €70.47)	76 km (42 - 92)	€29.15 (€15.81 - €39.14)	90 ± 60 km	€34.02 (€0.18 - €88.16)	94 km (32 - 140)	€35.61 (€12.29 - €53.20)
Loss of productivity								
Travel time	109 ± 69 min	€40.88 (€2.13 - €149.32)	115 min (62 - 137)	€32.03 (€20.62 - €60.55)	132 ± 85 min	€43.21 (€5.53 - €117.41)	120 min (49 - 210)	€32.80 (€17.27 - €61.09)
Time in hospital	90 ± 34 min	€36.66 (€7.00 - €87.89)	87 min (70 -111)	€30.10 (€18.67 - €51.20)	91 ± 40 min	€32.93 (€7.00 - €93.84)	91 min (67 - 109)	€30.10 (€17.03 - €45.33)
Companion		€37.30 (€17.06 - €90.67)		€45.96 (€36.43 - €58.22)		€33.06 (€21.67 - €109.50)		€45.96 (€36.43 - €58.22)
Healthcare costs								
Visit to the policlinic		€163.00*		€163.00*		€163.00*		€163.00*
Nephrologist telephone call		€4.97 (€0.00- €14.13)		€0.00 (€0.00 - €14.13)		€4.25 (€0.00 - €14.13)		€0.00 (€0.00 - €14.13)
Laboratory costs		€194.94 (€57.29 - €653.25)		€154.52 (€85.55 - €179.42)		€218.77 (€84.01 - €731.14)		€154.52 (€85.55 - €248.39)
DBS set	0.56	€4.33 (€0.00 - €7.50)		€7.50 (€0.00 - €7.50)				
DBS analysis	0.56	€28.85 (€0.00 - €50.00)		€50.00 (€0.00 - €50.00)				
Total		€541.55 (€315.78 - €990.19)		€492.68 ± (€415.63 - €601.20)		€532.73 (€278.09 - €1092.57)		€479.55 (€399.83 - €581.86)

*costs are fixed, so no IQR or 95%CI is given. SD = Standard Deviation. IQR = Interquartile range. DBS = Dried Blood Spots. 95%CI = 95% confidence interval obtained by non-parametric bootstrapping.

Implementation outcomes

There were no statistically significant differences in the average number of tacrolimus dose adjustments communicated by phone and the number of printed tacrolimus prescriptions (Table 3).The implementation took a total of 6 months' time for the study coordinator. The total (one-time) implementation costs were €19,244.

In the DBS group, according to the study protocol, a total of 256 DBS samples were expected to be sent to the hospital laboratory. During the study, a total of 143 (55.9%) of the expected number of DBS samples were received at the hospital. Of those, slightly over one third (n=52) of the results were on time. Seven DBS samples (4.9%) were rejected because of insufficient quality. For the intention-to-treat group, the average number of DBS samples per patient that was on time was 2.1 ± 2.0 (range 0-7). A per protocol analysis was performed on a subset of 6 patients who had ≥4 visits, where results of the DBS analysis were available in the EHR at the time of nephrologist consultation. There was no statistically significant difference in the per protocol group compared to the control group for the number of visits, tacrolimus dose adjustments communicated by phone and number of printed prescriptions as shown in Table 3.

If dose adjustment occurred, this was communicated to the patient by the nephrologist per phone in 92.6% of the cases in the control group. For the intention-to-treat and per-protocol group this was 89.9% and 79.3%, respectively. This difference was not statistically significant for both groups compared to the control group (p = 0.66 and 0.34 respectively).

The average time between patient home sampling and receiving the sample in the hospital was 3.9 ± 6.6 days. The average time between receiving the sample and results available in the EHR (analysis time) was 2.6 ± 2.3 days. The total time between patient home sampling and results available in the EHR was 6.5 ± 6.6 days.

Discussion

Although DBS sampling is a promising tool to improve kidney transplant patient healthcare from both costs and patient satisfaction perspectives, this study showed no decrease in the number of outpatient visits. In addition, there were no reductions in costs, written tacrolimus prescriptions and dose adjustments communicated by phone when comparing the DBS group to the control group. Of note, implementation of DBS sampling, analysis and logistics were far from optimal with 56% of DBS samples received and only one out of five DBS results being available on time.

The low availability of timely DBS results (20%) seems a plausible explanation for the absence of differences in primary or secondary endpoints in this study. Although these values are low, this is in line with the low DBS availability in a study by Al-Uzri et al. where 28 pediatric transplant patients were expected to provide a total of 279 DBS samples in a 12-month period.[9] In this latter study, a total of 77% of the expected DBS were received by the lab, and 38% were considered on time (within 7 days after sampling). Yet, in contrast to our study, in the study by Al-Uzri et al., patients received a reminder phone call when no DBS sample was received.[9] Upfront, we expected that patients would be highly motivated to perform DBS sampling at home, because the results of DBS would be available to them at the time of nephrologist consultation. A possible explanation for the low adherence to DBS sampling could be that patients performed DBS on top of conventional venous sampling instead of a complete substitute for venous sampling. Another possibility is the logistical problems concerning sending of DBS samples. The Dutch public posting service assures that if a medical sample is sent during working hours, it should be delivered the next morning. However, we calculated an average of 3.9 ± 6.6 days between patient sampling and receiving the sample at the laboratory. It is not possible to assess the reason for this, but possible explanations could be: (1) the patient forgot to send the sample, (2) delays by the Dutch posting service, and (3) delays in the hospitals' internal distribution system. Logistical delays might have led to a number of DBS results not being available on time, which could have resulted in decreased motivation and adherence to DBS sampling, as was mentioned by three patients.

Only three out of 23 patients found DBS home sampling too difficult to perform, mainly due to tremor in the hands, a well-known side-effect of tacrolimus. This is in accordance with other studies in which 91% (n=55) and 93% (n=36) of the patients were able to perform DBS sampling at home.[11,13] Only 4.9% of the received samples was of insufficient quality, which is comparable to the performance of DBS sampling by trained phlebotomists[4,23] and is better than reported in other DBS feasibility studies, where 20% of the obtained samples were unfit for analysis.[9,10] This shows that

the used instruction method is adequate for patients to perform DBS home sampling. Preference of DBS sampling over venous sampling in this study was relatively low (21.1%) compared to other studies that report values between 37-61%.[10-13] This can likely be explained by the previously mentioned reasons on patient motivation. However, 82.6% of the patients were willing to perform DBS sampling, if this leads to a reduced number of outpatient visits.

This study showed that kidney transplant patients required an average of 11 outpatient clinic visits in the first 6 months after transplantation at an average cost of €520 per visit (excl. costs for DBS analysis). If DBS home sampling is used as intended and a reduction of 1 visit per patient in the first six months after transplantation is realized, DBS sampling will save around €3 per patient if it is used on top of usual care. If DBS sampling would replace venous sampling for tacrolimus and creatinine, is used as intended and therefore reduces 1 visit per patient in the first six months after transplantation this will lead to a cost reduction of €399 per patient. In our center, 192 adult kidney transplantations were performed in 2017. Thus, this could potentially lead to an annual societal cost-reduction of €76,608. Patients who are >12 months post-transplantation usually visit the outpatient hospital on a 3-monthly basis. It is possible that introduction of DBS home sampling for these patients on a 2-3 monthly basis might reduce the need for an outpatient visit every 3 months resulting in even further cost reduction.

Although not significant, this study shows a trend towards fewer telephone calls needed to discuss tacrolimus dose adjustments if DBS results are available on time. This indicates that the workflow for the nephrologist is potentially less time consuming and might increase the cost-reduction.

A strength of this study is that this is the first study that calculates costs of DBS sampling from a societal perspective in an outpatient setting. The design was chosen to be highly reflective of real-world practice of kidney transplant outpatient follow-up. To reflect daily practice, we deliberately chose to not interfere with usual care (e.g. send reminders to nephrologists and patients to sample DBS) and make the patient and nephrologist responsible for timely DBS sampling. This study shows that implementation of a novel home sampling method requires more time and a proper reminder system before it can be a part of routine patient care, replacing conventional venous sampling. Another implementation study including an implementation strategy and evaluation, prior to performing a cost evaluation study, might help in achieving this goal.[29] In addition, logistical challenges need to be overcome. Possible solutions could be: (1) automated reminders by (smart)phone or e-mail to tell the patient when to perform DBS home sampling, (2) increasing the time between sampling and the

visit to the outpatient clinic (>7 days) to account for logistic delays and (3) sending the samples with track-and-trace to be able to gain insight in logistical processes.

A limitation is that this study was performed in a Dutch setting, which can be different from other countries. However, since the Netherlands is more densely populated than most countries, patient costs saved due to DBS sampling might be higher in other countries due to longer travel distances to the hospital. Another limitation is the sample size which was calculated on the primary endpoint. The sample size might not be fit for measuring secondary endpoints, meaning that caution is warranted when interpreting these results.

Although DBS home sampling seems promising, improving logistical methods for DBS samples is required to reduce the average of four days between patient sampling and receiving the sample at the lab to make DBS sampling feasible. A standard day for sampling, sending the sample and analysis in the laboratory might reduce both the time between sampling by the patient and analysis, as well as the time between analysis and results becoming available. However, this might make DBS sampling less feasible in the first four weeks after transplantation when visits are scheduled weekly. In future trials, a feasibility study should be performed prior to a cost-evaluation study on DBS to account for logistical hurdles. This study should be designed as an implementation study using available implementation strategies, such as the Consolidated Framework for Implementation Research (CFIR).[29] The results from a feasibility study can also help provide a more accurate sample size calculation. If the number of visits and the SD observed in this study were used for the power calculation mentioned in the Methods' section the number of patients per group would be 48.

In conclusion, this study did not show a reduction in the number of outpatient visits when DBS home sampling for tacrolimus monitoring was offered to kidney transplant patients. Although DBS seems promising, the logistical process concerning timely sending and analysis of DBS samples should be optimized first, before effectiveness assessment. Potentially, successful implementation of DBS offers a more efficient workflow for nephrologists, requiring less telephone calls to communicate dose adjustments. Transplant patients are willing to perform DBS home sampling if logistical hurdles are overcome and DBS home sampling is properly implemented in routine transplant care. If DBS is optimally implemented, eventually, this might lead to increased patient satisfaction, lower patient travel burden and lower societal costs.

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Chapter 8

Official International Association for Therapeutic Drug Monitoring and Clinical Toxicology Guideline: Development and Validation of Dried Blood Spot–Based Methods for Therapeutic Drug Monitoring

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Abstract

Dried blood spot (DBS) analysis has been introduced more and more into clinical practice to facilitate Therapeutic Drug Monitoring (TDM). To assure the quality of bioanalytical methods, the design, development and validation needs to fit the intended use. Current validation requirements, described in guidelines for traditional matrices (blood, plasma, serum), do not cover all necessary aspects of method development, analytical- and clinical validation of DBS assays for TDM. Therefore, this guideline 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 are described covering sample collection procedure, type of filter paper and punch size, sample volume, drying and storage, internal standard incorporation, type of blood used, sample preparation and prevalidation. Second, common parameters regarding analytical validation are described in context of DBS analysis with the addition of DBS-specific parameters, such as volume-, volcano- and hematocrit effects. Third, clinical validation studies are 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 is discussed, covering changes made to existing sampling- and analysis methods. This guideline of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology on the development, validation and evaluation of DBS-based methods for the purpose of TDM aims to contribute to high-quality micro sampling methods used in clinical practice.

Introduction

Dried blood spot (DBS) analysis has been introduced more and more into daily practice.[1] To assure the quality of bioanalytical methods and to assure that the results obtained with those methods are valid, it is of utmost importance that newly developed methods are fit for purpose. Those methods must have undergone adequate method validation and are monitored through a suitable quality control (QC) program. Absence of DBS-specific method validation guidelines results in DBS-based methods lacking essential validation aspects resulting into reduced credibility.[1–4] Validation requirements described in guidelines for the quantitative analysis of traditional matrices (ie, liquid blood, plasma, or serum) are not always easily translated to analysis of DBS.[5,6] Moreover, several additional parameters, such as volume and hematocrit (HT) effects, which are not part of traditional guidelines, are often overlooked or not adequately assessed.[7]

Therefore, this guideline aims at defining the parameters necessary for the validation of quantitative DBS-based 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. The recommendations in this guideline are based on existing guidelines for traditional matrix analysis, in particular, the bioanalytical method validation guidelines issued by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA),[5,6] the guideline for measurement procedure comparison provided by the Clinical and Laboratory Standards Institute (CLSI),[8] several white papers on dried matrix analysis,[9–11] as well as other published work and the personal experience of the authors.

The focus of this guideline is the analysis of DBS for the quantitative determination of small molecule drugs and drug metabolites using chromatographic techniques for therapeutic drug monitoring (TDM) purposes. However, many elements of this guideline are also relevant for the analysis of samples obtained through volumetric absorptive micro sampling (VAMS) and for dried plasma spot (DPS) analysis, as well as for the analysis of DBS for purposes other than TDM.

As the successful validation of a DBS-based analytical method starts with method development, this guideline commences by outlining the potential pitfalls encountered during that stage (see Considerations Regarding Sample Collection, Considerations Regarding Sample Preparation, and Other Important Considerations). Furthermore, the importance of prevalidation stress testing is highlighted (Prevalidation—Stress Testing). In a next section, the actual method validation is extensively discussed (see ANALYTICAL VALIDATION and CLINICAL VALIDATION). This validation section encompasses both the analytical validation (comprising both the classical and the DBS-specific validation parameters) and the clinical validation (ie, demonstration of equivalence between DBS-based results and results obtained in the classical matrix). Finally, QC is briefly discussed (see CROSS-VALIDATION). A summary of this guideline can be found in Supplemental Digital Content 1 (see Supplement S1, http://links.lww.com/TDM/A342).

Method development: considerations for successful validation

Before embarking on the set-up of a DBS-based procedure, it is essential to carefully think about the purpose of the method. Certain considerations need to be made to ensure the suitability of the method for a given application (ie, to ensure the method is fit for purpose) already in this early stage. These considerations are discussed below, and the different options are schematically summarized in Figure 1. Furthermore, stress testing of the method during method development will allow potential issues to be detected at an early stage, which will eventually increase the chances of a successful method validation and application.

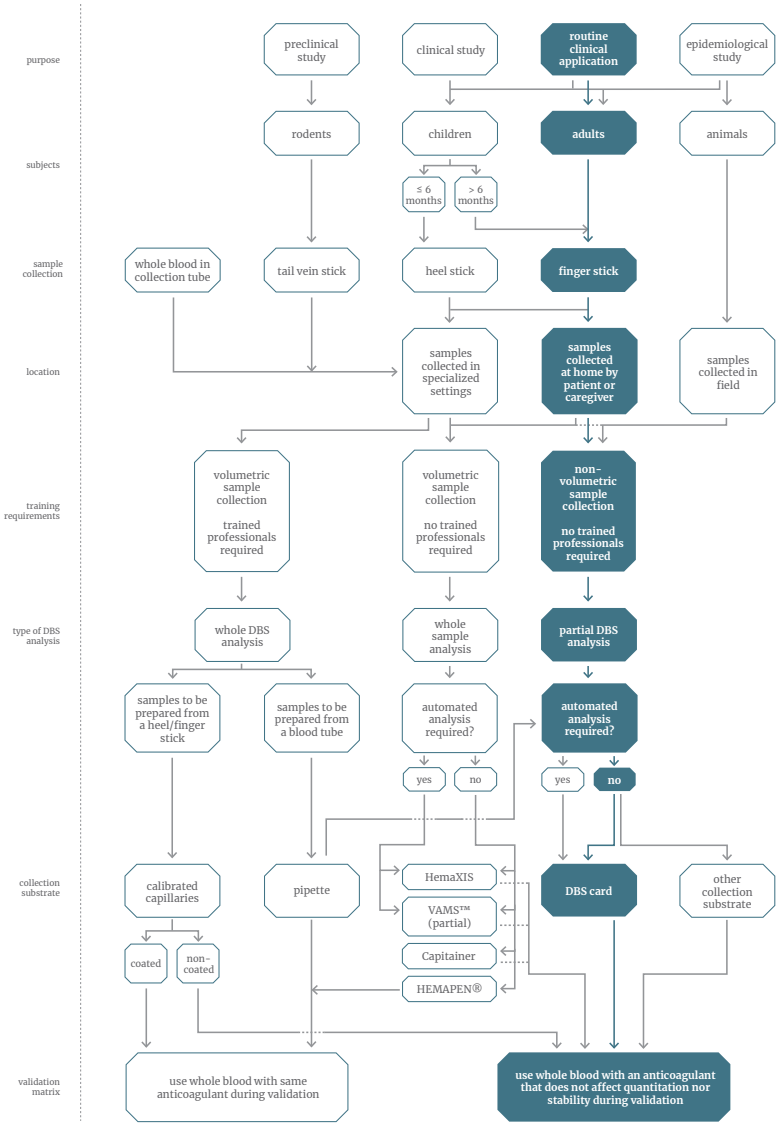

Figure 1. *Flowchart depicting different options for the set-up of a dried blood spot–based method which can be used before setting up a dried blood spot–based procedure. The highlighted "flow path" shows the procedure for therapeutic drug monitoring of immunosuppressants following home sampling by adult patients and partial spot analysis of DBS cards sent to the laboratory. Reprinted with permission from Anoek Houben. Copyright 2018. Adaptations are themselves works protected by copyright. So to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.*

Considerations Regarding Sample Collection

Nowadays, the most frequently used dried blood sample collection method is the collection of a nonvolumetric drop of blood (DBS), free falling or by touching onto a filter paper (ie, directly from a finger prick or heel stick). Alternatively, the blood sample may be deposited volumetrically using a capillary or a pipette. Furthermore, several blood collection strategies exist in which a volumetric dried blood sample can be directly generated from a nonvolumetric drop of blood, without the use of pipettes or handheld capillaries. These strategies include, HemaXis,[12] hemaPEN,[13] Capitainer-B,[14–16] and VAMS.[17–19] In addition, DPS may be collected rather than DBS. These DPS may be generated either by centrifugation of a liquid blood sample and subsequent application of an amount of plasma onto a filter paper or by using a device that allows in situ DPS generation.[20–23] Although some of the above-mentioned collection strategies may allow patient self-sampling (eg, nonvolumetric DBS collection,[24] VAMS,[25] and in situ generated DPS), other collection methods (eg, volumetric DBS collection using exact volume capillaries and DPS generation after centrifugation) require trained professionals and/or laboratory equipment. Although the latter strategies are not suitable for home sampling, they may still be valuable in another context. DPS generation through whole blood centrifugation and pipetting may, for example, be a suitable approach if DPS are prepared in a laboratory in a remote or resource-limited setting to allow more convenient transport to a centralized or reference laboratory.[26] In addition, other parameters such as required sample volume, automation capabilities, commercial availability, the cost of a given microsampling device, as well as overall costs may also play an important role in the selection of the sample collection method.

Selection of the Type of Filter Paper

If samples are to be collected on filter paper, the type of filter paper (card) that will be used needs to be carefully chosen. The type of filter paper may affect the occurrence of interferences, the blood's spreading behavior, sample homogeneity, as well as analyte stability and recovery.[27–29] Commercially available filter paper can either be untreated (eg, Whatman 903, Ahlström 226, DMPK-C), or pretreated with for example, denaturing agents or enzyme inhibitors (eg, DMPK-A or DMPK-B).[30] Furthermore, in certain DBS- based methods, in-house pretreated filter paper has been used to increase analyte stability or recovery.[31–34] Moreover, some types of collection devices have been reported to be less affected by the HT effect and may help to overcome this issue.[35,36] In addition, chitosan and alginate foams have

been proposed as collection substrates to help increase analyte recovery, as they dissolve during sample extraction.[37] Although most DBS-based bioanalytical methods use regular, cellulose-based, untreated filter paper (cards), for certain applications, it may be valuable to evaluate the use of pretreated or noncellulose-based alternatives. However, it needs to be kept in mind that the use of noncommercially available substrates may hinder a generalized application of the method and requires in-house assessment of batch to batch quality.[38]

Interferences Originating From the Collection Substrate

It is advised to analyze some blank collection cards during early method development to assess whether the collection material itself is blank and whether there are any interferences present that need to be separated chromatographically from the target compound(s).[28] If one of these issues occurs, it might also be valuable to evaluate different collection substrates.

Sample Volume

The amount of sample that is required for a certain analysis will mainly depend on the envisaged lower limit of quantification (LLOQ) and is inherently linked to the available instrumentation. However, the minimally required volume should always relate to how the samples are collected. For the set-up and validation of the method, a sample volume representative of the sample volume of the patient samples needs to be used. Most people will typically generate DBS of 20–70 µL if free falling drops of blood are collected, whereas somewhat smaller DBS typically 15–50 µL will be obtained if a hanging blood drop is collected by bringing it into contact with the filter paper. With the latter approach, it is essential that only the blood drop and not the fingertip touches the filter paper. If a DBS is smaller than what is typically expected, this may be an indication that the fingertip came into contact with the filter paper. On the other hand, if a DBS is larger than expected, multiple drops were likely collected. Obviously, whenever samples are collected volumetrically, the sample volume will be determined by the used device. If a larger volume of blood is required to reach the LLOQ, sometimes punch stacking is used.[39] Nonetheless, the number of punches required for a single analysis should remain as small as possible, to limit the amount of good quality samples that needs to be collected and to allow incurred sample reanalysis (ISR).

Drying and Storage Process

A parameter that is often neglected in DBS-based methods is the impact of drying time. If the sample is not completely dry before putting it in a zip-locked bag for storage, microbiological growth may occur and compromise sample quality.[40] Furthermore, improper drying might also affect analyte stability and recovery.[41,42] Therefore, it is advised to dry samples at least 3 hours under ambient conditions (preferably without

direct sunlight) and to store them with a desiccant, which will remove an additional 5% of water from the dried samples.[40,43] In certain settings, however, the required drying time may be longer because this depends on the ambient temperature and humidity, the sample volume, and the type of filter paper.[42] In other settings, shorter drying times may suffice. Therefore, it is relevant to evaluate during early method development whether the drying time is adequate under the conditions likely to be encountered during the collection of the patient samples. This evaluation is preferably performed using DBS with an HT in the upper range of the HT of the target population and, if applicable, a large sample volume, as these will dry the slowest.[27] Furthermore, the ambient temperature and humidity during drying have been suggested to affect DBS homogeneity (although this effect also depends on the type of filter paper that is used).[44] Similarly, also the storage conditions should mimic the ambient conditions encountered during patient sample transport/storage.[45]

Considerations Regarding Sample Preparation

Punch size

For volumetric DBS applications, the punch size needs to be large enough to punch out the entire DBS, independent of the HT of the sample. Hence, it is advised to select the required punch size based on samples with an HT of approximately 0.15, since this HT level will be lower than the lowest HT level of the patient population and will therefore yield DBS that are (slightly) larger than the largest expected patient DBS. The punches can either be made after application of the blood spot to the substrate or in advance.[46–48] For nonvolumetric DBS applications, partial DBS punches are made that exclude the outer edge of the sample. If relatively small punches are made (#4 mm or approximately 5.7 μL), most patients should be able to generate multiple DBS that are large enough to analyze. However, larger punch sizes may be required to obtain the desired LLOQ to increase method accuracy and imprecision or to exclude DBS homogeneity issues. Although generating larger DBS will be somewhat more difficult for a patient, when properly educated and trained, most patients will be able to provide at least 1 or 2 samples that are large enough to make punches up to 8 mm (± 20 μL). The latter will also be easier if falling-drop-collection is used rather than hanging-drop-collection.

Internal Standard Incorporation

Ideally, an internal standard (IS) is mixed homogenously with the biological sample before sample preparation to compensate for any variability throughout the entire analytical process. Unfortunately, this is difficult to achieve with a DBS. For DBS analysis, the closest alternative is to spray the IS evenly onto the sample before extraction.[49] However, this requires the availability of a validated dedicated spraying system, which

is not available in most laboratories. Another option is to precoat the filter paper with the IS.[50] However, in that case, the IS needs to be applied to a larger surface, as it is not known where exactly the sample will be deposited. Furthermore, the IS should be stable for a sufficiently long period (ie, during sample collection, transport, storage, and analysis). In addition, the same batch of IS solution should be used for calibrators, QCs, and patient sample collection cards, which is not feasible on a large scale. Another potential side-effect of precoating filter paper with IS (in the absence of matrix) is that the IS may show different recovery than the target analyte. To the best of the authors' knowledge, such strategies have not yet been evaluated for other dried blood samples nor has a successful application of IS-precoated microcapillaries been described. Again, such an approach would require the availability of tailor-made devices, which will be at the expense of additional costs. In most DBS-based methods, the IS is added to the extraction solution or directly to the DBS punch before extraction and will hence not compensate for variability in analyte recovery.[9,51] Therefore, analyte recovery must be investigated extensively under different conditions (see Evaluation of the Robustness of the Extraction Procedure and Short-Term Stability) during method development and validation.

Other Important Considerations

Type of Blood Used

For the set-up of calibration curves and internal QCs, it is from a practical point of view impossible to use capillary blood samples derived from a finger prick. Instead, spiked samples generated from venous whole blood containing an anticoagulant are used. Which type of blood is best suited for this purpose largely depends on how patient samples will be collected. If the DBS collection device that is used to generate the patient DBS contains a certain anticoagulant, the venous whole blood also needs to contain that same anticoagulant. On the other hand, if no anticoagulant is used during the collection of the patient samples, theoretically, the blood used to set up the calibration curves and QCs also has to be non-anticoagulated. Unfortunately, it is very impractical to prepare spiked samples from non-anticoagulated blood because blood will start coagulating almost immediately after collection. Therefore, in most cases, a suitable anticoagulant will have to be selected. It is essential that the use of this anticoagulant does not impact the obtained results, and that the stability of calibrators and QCs reflects that of real samples. Hence, we strongly advise to compare in an early stage results obtained from a non-anticoagulated sample with results from patient samples anticoagulated with different anticoagulants.[52] These blood samples should all be obtained venously from the same volunteer or patient at (approximately) the same time and should be analyzed in quintuplicate. Based on the knowledge about the (lack of) impact of certain anticoagulants in liquid blood, some anticoagulants may

readily be excluded. For example, if analytes are, for example, stabilized by oxalate/NaF, this type of blood should preferentially not be used to assess the analyte's stability in DBS (which in practice would not contain that stabilizing anticoagulant). On the other hand, if the anticoagulant stabilizes the analyte, and anticoagulant-containing DBS are commutable in any other way with DBS without anticoagulant, the former could be used for the set-up of calibrators and QCs as the prolonged analyte stability could help ensure consistent calibration.

Preparation of Spiked Samples

A first step in the preparation of spiked samples is to adjust the HT or erythrocyte volume fraction of the whole blood to the desired HT value. For most experiments, the latter will correspond to the mean or median HT value of the target population.[53] Although there are several ways of preparing samples with a certain HT, the preferred procedure is to measure the HT of the original blood sample with a hematology analyzer and to calculate how much plasma needs to be added or removed to obtain the desired HT value.[54] After the addition or removal of the plasma, it is important to measure the HT again, to ensure the sample was prepared correctly.

In a next step, the analyte needs to be spiked into the blood. It is important to only spike a limited volume of analyte solution to the blood (ie, 5% of the sample and preferably even less) to not change the nature of the sample.[5] Moreover, the addition of a larger volume of solvent would also change the sample's viscosity and/or cause cell lysis, thereby affecting its spreading behavior through the DBS filter paper. Furthermore, organic solvents may denature proteins. To further minimize the effect of the spiking volume on the sample's spreading behavior, stock solutions can be diluted with plasma, rather than with water or another solvent, if solubility allows for it. After spiking the blood with the target analyte, the samples should equilibrate for a sufficient amount of time at a suitable temperature to mimic the analytes' in vivo RBC/plasma distribution.[55]

Prevalidation—Stress Testing

Exploratory Tests

As with a traditional bioanalytical method, several exploratory tests need to be performed to assess whether a developed method is good enough to proceed toward validation. As with any chromatographic method, several technical aspects should be checked early on during method development, for example, the absence of carryover and the influence of the sample matrix on the chromatographic method. Furthermore, the stability of the stock solutions used for the spiking of the calibrators and QCs should be guaranteed. Particular points of attention during prevalidation for DBS-based methods are short-term stability and extraction efficiency.

Although DBS generally tend to improve analyte stability, this is not always the

case. Enzymatic analyte degradation may readily occur during the drying process.[56] Furthermore, oxidation sensitive analytes are likely to suffer from stability issues, since DBS are exposed to air during drying and/or storage.[30] If low signals are obtained from fresh samples (eg, compared with a standard solution with the same concentration), this might be due to stability issues during the drying process. In addition, these low signals may also be caused by matrix effects (MEs), poor extraction efficiency, or a combination of the above.

When using liquid chromatography tandem mass spectrometry (LC-MS/MS), the presence of MEs can be evaluated using postcolumn infusion. If present, these MEs may be eliminated by further optimization of the sample preparation and/or the chromatography. Poor extraction efficiency may be due to the analyte's interaction with the carrier or with endogenous matrix compounds.[29,57,58] However, the differentiation between extraction efficiency issues and actual analyte instability may not be so straightforward.[34] To get an idea about potential stability issues, existing literature about the stability of the analyte in whole blood or about the chemical and physical properties of the analyte may be a good starting point. If degradation during sample drying is anticipated (eg, for compounds with a very short in vitro half-life), flash heating may improve the analyte's stability (at least if the analyte is thermostable) because this inactivates the enzymes.[56] Unfortunately, this strategy is not suitable for home sampling. Nonetheless, it may help to figure out the cause of the poor method outcome. Other strategies to help improve the analyte stability may include preimpregnating the collection substrate with antioxidants or buffers.[34,59] However, these strategies may hamper generalized application of the method. For some analytes, instability issues remain unsolved, even when taking into account a restrictive time frame for transportation of DBS. In those cases, it should be decided that dried blood sampling for that analyte is not feasible. In specific situations, a volumetrically obtained sample could be brought into a stabilizing sampling buffer shortly after.[60] When poor extraction efficiency is suspected, further optimization of the extraction procedure may be required (ie, the evaluation of different extraction solvents, additives and extraction temperatures, as well as more rigorous extraction techniques (such as sonication). Furthermore, the use of different (pretreated) collection cards/devices may also help to improve the extraction efficiency.

At this stage, it should also be evaluated whether the obtained results are affected by the time between sample collection and analysis. More particularly, the results from samples analyzed at T0 (typically between 30 minutes and 3 hours after sample generation, depending on the required drying time) should be compared with results obtained at later time points, preferably up to 48 or 72 hours. This experiment is important since time-dependent extraction issues have been described.[61] More specifically, if the recovery decreases for the first (couple of)

time points, but remains stable afterward, it may still be possible to obtain good analytical results. In such a case only samples older than a specified time point should be analyzed. Obviously, this strategy should not only be implemented for the patient samples, but also for the calibrators and QCs.

Evaluation of the Robustness of the Extraction Procedure and Short-Term Stability

In a next step, the robustness of the extraction procedure should be thoroughly investigated. This is a crucial experiment because in most DBS applications, the IS is not capable of correcting for variability in extraction efficiency. The extraction efficiency may be concentration, HT, and time-dependent, and importantly, these parameters may also affect each other.[41,62–64] HT-dependent extraction efficiency may be present or more pronounced at one concentration level compared with another.[64] Similarly, time-dependent extraction efficiency issues may occur earlier at a more extreme HT level.

For nonthermolabile compounds, the occurrence of HT- and time-dependent extraction issues can be evaluated by comparing the results from fresh DBS at low, medium, and high HT levels (with these HT levels encompassing the HT range of the target population; eg, 0.20, 0.40, and 0.60) with a second set of samples stored at 50–60°C for at least 2 days. This second set mimics thoroughly dried (aged) samples. This experiment should be performed at both the low and high QC levels (Fig. 2). Furthermore, to simultaneously determine the actual extraction efficiency at both QC levels, and to evaluate the presence of MEs, also samples spiked after extraction and standard solutions should be included in this experiment. Moreover, each of these samples should be analyzed in quintuplicate. In addition, along with these samples, a calibration curve and QCs have to be analyzed. Importantly, in case of partial DBS analysis, these samples should be pre- pared by the accurate pipetting of a fixed amount of blood onto prepunched filter paper disks to rule out any influence of the HT spreading effect on the amount of sample being analyzed.

When no relevant differences (ie, <15%) can be observed between the results obtained from fresh DBS and those stored at 50–60°C, it is unlikely storage will have an impact on extraction efficiency. A good outcome in this set- up may also readily indicate good stability under ambient conditions, although this needs to be formally evaluated during method validation. However, it needs to be mentioned that the latter can also be affected by other parameters such as humidity and exposure to sunlight. Furthermore, by comparing the results of the samples at the 3 different HT levels (both for the fresh and the stored samples), the occurrence of HT-dependent extraction efficiency issues can be evaluated. Moreover, using the Matuszewski approach, recovery and ME can be evaluated at both concentration levels and at 3 HT levels.[65] While performing this experiment may seem fairly elaborate at first, it may prevent serious problems at a later stage, which may require a complete

revalidation (eg, if the extraction needs to be adapted). Moreover, if successful, the evaluation of ME and recovery may not have to be repeated at different HT levels during the actual method validation, as long as the method remains unchanged. Also, the evaluation of short-term stability at fairly extreme storage conditions (ie, 50–60°C) is already incorporated in this experiment (see Classical Validation Parameters to Be Evaluated).

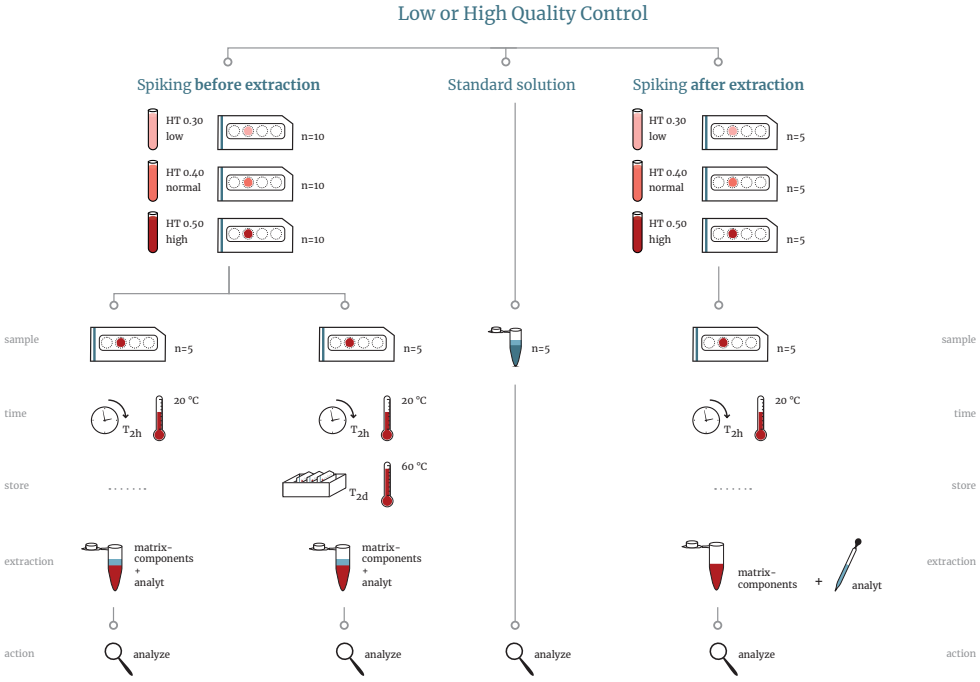

Figure 2. *Schematic set-up of the experiments needed to assess the robustness of the extraction procedure and short-term stability. The total amount of samples to be analyzed for this experiment is 100 (plus calibrators and QC samples). Reprinted with permission from Anoek Houben. Copyright 2018. Adaptations are themselves works protected by copyright. So to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.*

For more thermolabile compounds, a similar experiment can be performed with samples stored at room temperature for 2 weeks instead of at 60°C for 2 days. Although this is a less harsh experiment than the previously described one, it does cover a time span in which most clinical samples in a laboratory will have been analyzed. Alternatively, even lower storage temperatures may be used. However, if the analyte is not stable at room temperature for at least a couple of days, the method will not be suitable for routine use. Obviously, if satisfactory, these data can also be used as part of the stability data required for method validation.

To minimize the number of samples that has to be analyzed at this stage, a simplified

experimental set-up is suggested in Figure 3. In particular, this set-up does not include "spiked after extraction" samples or standard solutions, and all samples are only analyzed in triplicate. This simplified set-up offers the advantage that if the extraction procedure has to be adjusted (and consequently, this evaluation has to be repeated), the number of samples that needs to be analyzed will not increase drastically. However, with this experiment, recovery and ME will still need to be evaluated at different HT levels in a separate experiment during method validation.

If the results of the above-mentioned experiments are nonsatisfactory, this may be due to instability of the target analyte or to extraction efficiency issues. If the results for the different HT levels differ significantly and/or substantially (ie, >15%), this is due to an HT-dependent extraction efficiency issue, and the extraction procedure needs further optimization. In this context, heated extraction and the use of a mixture of organic solvents rather than a single organic solvent may be helpful.[62,63,66,67] Furthermore, the use of a different collection card may also help to resolve this problem. Possibly, depending on the target population, the procedure can be repeated with less extreme low and high HT values, to evaluate whether acceptable results are obtained for a more limited HT span.

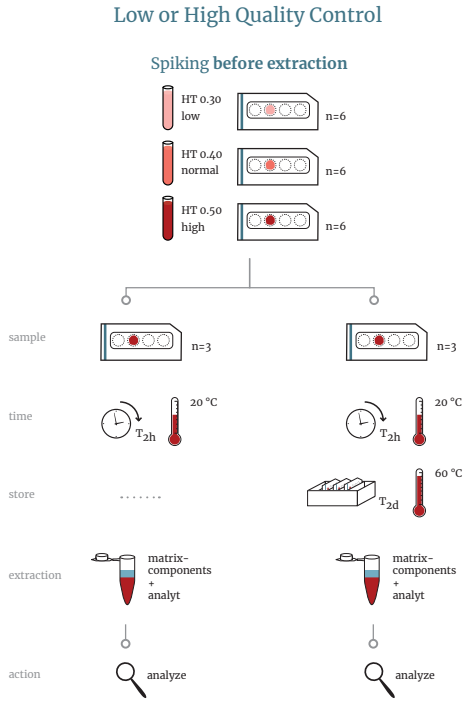

Figure 3. *A simplified schematic set-up of an experiment to assess the robustness of the extraction procedure and short-term stability, requiring a minimum number of samples. The total number of samples to be analyzed for this experiment is 36 (plus calibrators and QC samples). Reprinted with permission from Anoek Houben. Copyright 2018. Adaptations are themselves works protected by copyright. So to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.*

A difference between the fresh and the stored samples, on the other hand, might be due both to a time-dependent extraction efficiency issue and to actual instability of the target analyte.[68] However, if this difference is not observed at all HT levels, it is unlikely that analyte instability is the culprit. If the difference is observed at all HT levels, it may be worthwhile to repeat the experiment at a lower storage temperature, as this may indicate analyte instability.

DBS Homogeneity

In case of partial DBS analysis, it is essential to evaluate DBS homogeneity, that is, to assess whether results from central punches are equivalent to peripheral (or decentral) ones.[69] By already evaluating this parameter during prevalidation, one knows whether, during the next experiments, it is required to make a central punch or whether a peripheral punch or multiple punches can be made from a single DBS. This evaluation must be performed at 2 concentration levels (low QC and high QC), at different HT levels (low, medium, and high) and at sample volumes representative of the anticipated patient sample volumes. Each of the evaluated conditions should be analyzed in quintuplicate. All samples should be compared with a calibration curve prepared with samples of medium HT level and average volume, of which a central punch was extracted. When both central and peripheral punches yield results within the standard bioanalytical acceptance criteria (typically, within 15% of their target value), the use of both types of DBS punches is considered acceptable.[69]

Obviously, this experiment only needs to be conducted if a central and a more peripheral punch can be made from a sample, which in turn will depend on the used punch size. When making peripheral punches, the very outer edge of the DBS should be excluded because this has a different composition than the rest of the DBS (eg, a higher amount of red blood cells, when using conventional Whatman 903 filter paper). In addition, the back of the filter paper should always be checked to ensure that the peripheral punch is made in a part of the DBS in which the filter paper is saturated. Importantly, the samples should be prepared under similar conditions as the patient samples because the drying process is known to influence DBS homogeneity.[27,70] Other parameters that may influence the equivalence between central and peripheral punches include the filter paper type, the position of the DBS card during drying, and the punch size (with larger punches being less affected by inhomogeneities within the DBS sample). The presence of an anticoagulant, on the other hand, does not seem to influence DBS homogeneity.[27]

Analytical validation

None of the currently existing bioanalytical validation guidelines have been set up for dried blood sample–based methods. Certain experiments described in these guidelines may not be applicable (eg, freeze-thaw stability, depending on the storage and transport conditions), whereas others may require some refinement (see Classical Validation Parameters to Be Evaluated). Moreover, some additional parameters will have to be evaluated (see DBS-Specific Validation Parameters).[9,71] An overview of the required additional investigations can be found in Table 1. These will result in a slightly larger number of samples that will have to be analyzed during method validation (Table 2). Before starting any analytical validation, it is essential to contemplate what the desired quality of the method should be. Although the analytical performance requirements described in, for example, the FDA or EMA guidelines are widely applied and accepted, they may not always be suitable for DBS methodology. Depending on the analyte and the purpose of the method, these requirements can be set either more or less strict based on scientific evidence. In this context, some have suggested to use acceptance criteria based on biological variation, as is common practice in other areas of clinical chemistry.[72]

Table 1. Overview of the analytical validation parameters that require additional evaluation in dried blood spot-based methods, and how to assess them.

Validation parameter	Evaluation	Statistical test/Acceptance criterion
Recovery, matrix effect, process efficiency	Evaluate at both high and low QC levels using 6 different donors, (with one donor evaluated at minimally 3 HT levels), with each condition determined in quintuplicate*.	Should be reproducible, both between matrices and HT values (%RSD ≤ 15%).
Volume effect	Evaluate at both high and low QC levels and at least at 3 HT levels and 3 volumes*.	One-way ANOVA with bonferroni post-hoc analysis ($p \leq 0.05$). Back calculated values deviate ≤15 % of medium volume.
Hematocrit effect	Evaluate at both high and low QC levels and at least at 3 HT levels*.	One-way ANOVA with bonferroni post-hoc analysis ($p \leq 0.05$). Back calculated values deviate ≤15 % of medium HT values.
Volcano effect	Compare central and peripheral measurements. Evaluate at both high and low QC levels and at least at 3 HT levels and one volume (typically, the highest)*.	Paired t-test ($p \leq 0.05$) Back calculated 'peripheral' values deviate ≤15% of 'central' values

*HT levels should cover the entire HT range of the target population and the volumes should be representative of the sample volumes that will be generated by the patient.

Table 2. An overview of the minimally required amount of analyses for the analytical validation of dried blood spots vs. whole blood.

Validation parameter	Amount of samples (dried blood spot-based)	Amount of samples (liquid whole blood)
Selectivity	n = (6 + 6) x 1 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo	n = (6 + 6) x 1 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo
Calibration model	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo
Accuracy & precision	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate
Dilution integrity	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate
Carry-over	n = (1 + 1) x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo	n = (1 + 1) x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo
Recovery, matrix effect, process efficiency	n = 2x (2 x 5 x 1 x 1 x 5) + 2x (2 x 1 x 3 x 1 x 5) + (2 x 1 x 5) = 170 2 QC levels, 6 donors, of which 1 donor at 3 HT levels, 1 day, in quintuplicate (spiked before/after) 2 QC levels, 1 day, quintuplicate (standard solutions)	n = 2x (2 x 6 x 1 x 1 x 5) + (2 x 1 x 5) = 130 2 QC levels, 6 donors, 1 HT level, 1 day, in quintuplicate (spiked before/after) 2 QC levels, 1 day, quintuplicate (standard solutions)
Stability	n = 2 x 1 x 4 x 5 = 40 2 QC levels, 1 HT level, 4 points: T_0, T_{1w}, T_2w @ RT, T_{2d} @ 60°C, in quintuplicate	n = 2 x 1 x 7 x 5 = 70 2 QC levels, 1 HT level, 7 points, in quintuplicate: Bench-top stability: T_0 & T_{24h} @ RT Storage stability: T_{1w}, T_{2w} @ 4°C/-20°C Freeze thaw stability: min. 3 cycles
Volume effect, hematocrit effect, volcano effect	n = 2 x 3 x 4 x 5 = 120 2 QC levels, 3 HT levels, low, medium and high volume central punch + high volume peripheral punch, all in quintuplicate	N.A.
TOTAL	**412**	**282**

RT = room temperature, T = time point, T_0 = starting point = at the minimum drying time (e.g. 2 hours) = at the minimum drying time (e.g. 2 hours), d = day, w = week.
*samples are prepared in blood of median HT, unless mentioned otherwise.

Classical Validation Parameters to Be Evaluated

Most of the validation parameters described in traditional bioanalytical method validation guidelines will have to be assessed for DBS-based methods as well.[5,6] Therefore, those documents will need to be consulted too when performing a DBS method validation. However, the particular points of attention when evaluating those classical validation parameters in the context of a DBS method are given below. Furthermore, to assist the reader, a brief overview of these classical validation parameters is given in Table 3.

Table 3. An overview of the classical validation parameters and how to assess them.

Validation parameter	Evaluation	Statistical test/ Acceptance criterion
Selectivity	6 individual blank matrices	≤ 20% of LLOQ (analyte) ≤ 5% (IS)
Calibration model	Use min. 6 calibrators + zero + blank. Zero and blank samples should not be included in the calibration curve.	Backcalculated concentrations ≤ 15% of nominal value (≤ 20% at LLOQ). ≥ 75% of all calibrators and ≥ 50% per calibration level should comply.
Accuracy & precision	Evaluate at 4 QC levels: LLOQ Low = ≤ 3 x LLOQ Medium = 30 - 50% of range High = ≥ 75% of highest calibrator	≤ 20% for LLOQ ≤ 15% for other QC levels
Dilution integrity	Evaluate a dilution factor (e.g. 1:9) applicable to the patient samples.	Accuracy and precision ≤ 15%
Carry-over	The analysis of (zero and) blank samples after the highest calibrator	≤ 20% of LLOQ (analyte) ≤ 5% (IS)
Recovery, matrix effect, process efficiency	Evaluate at both low and high QC, using 6 different blank matrices. Recovery: spiked before/spiked after. Matrix effect: spiked after/ standard solutions Process efficiency: spiked before/ standard solutions	CV ≤ 15%
Stability	Evaluate at both low and high QC levels. Store stability QCs under representative conditions for a representative time frame and measure against fresh calibrators.	≤ 15% of nominal value (or ≤ 15% of value at T_0)

T_0 = starting point = when samples were fresh.

Selectivity

analyzed without IS, as well as 2 zero samples (blank DBS extracted with extraction solvent containing IS). These blank samples should be obtained using the same sampling approach as the one that will be used to collect the patient samples. In addition, DBS prepared from blank blood spiked with common comedications, metabolites, and other potential interferences could be tested. At this stage, it may also be worthwhile to run a few authentic patient samples to ascertain there is no nonanticipated coelution of a metabolite that may not be available as a standard.

Calibration Model, Accuracy and Precision, Measurement Range

For the evaluation of the calibration model, the LLOQ and upper limit of quantitation (ULOQ), accuracy, and precision, all experiments should be performed in accordance with existing guidelines.[5,6] The only difference is that all calibrators, blank, zero, and QC samples should be prepared in blood with the median HT of the target population and should have a volume representative of the patient samples.[53] As with any bioanalytical

method, the measurement range should be representative of the concentration range in patient samples. For the purpose of TDM, a calibration range minimally spanning from half of the lower end of the therapeutic interval to twice the upper end of the therapeutic interval should suffice. Furthermore, intracard and intercard variability do not need to be evaluated separately, as these variables will be inherently included throughout the method validation.[9] For a method to be applied in a routine context, interbatch variability should be assessed. The latter can be performed by including cards from multiple batches in the validation experiments. However, if noncertified filter paper is used, a more elaborate evaluation of the filter paper may be warranted.

Dilution Integrity

Contrary to traditional liquid blood samples, DBS cannot be diluted directly. Hence, to analyze samples with a concentration above the measurement range, DBS extracts are typically diluted with blank DBS extracts or extraction solvent. Furthermore, IS-tracked dilution can be performed.[6,73] With this approach, a higher concentration of IS is added to the extraction solvent, with the exact amount of IS depending on the envisaged dilution factor. This approach renders the dilution a volume-noncritical step. In addition, for DBS, the donut punch approach can be used.[74] With this approach, a small central punch (ie, smaller than the regular punch size for a given DBS method) is made from a DBS sample and is extracted simultaneously with a donut punch prepared from a blank DBS sample. This donut punch is a regular sized DBS punch from which a small central punch (with the same punch size as used for the actual DBS sample) has been removed. However, to use the latter approach successfully, DBS homogeneity should be adequate for the small punch size, and the extraction efficiency should not depend on the punch size.

Carryover

Aside from classical carryover, in a DBS workflow, the punching step could be considered a potential source of contamination. Hence, we propose to include in the method validation, the processing of one or more blanks after the processing of the highest calibrator.[9] To the authors' knowledge, however, no punch-mediated carryover has been described for (therapeutic) drugs, although it has been observed for PCR-based methods.[75] In addition, physical carryover between cards should be avoided by storing the cards separately. However, if multiple cards will be stored together, potential carryover between cards requires evaluation.[9] The same acceptance criteria as for classical carryover should be applied.[5,6]

Matrix Effect, Recovery, and Process Efficiency

ME, recovery, and process efficiency should be evaluated in line with the set-up proposed by Matuszewski et al.[65] (also see METHOD DEVELOPMENT: CONSIDERATIONS FOR

SUCCESSFUL VALIDATION). For this experiment, blood from at least 6 different donors should be used, and 2 concentration levels should be evaluated (ie, low and high QC levels). In addition, since it is known that the HT may strongly impact the recovery—and possibly also the ME—it is essential to evaluate recovery and ME at different HT levels, prepared from the blood of at least one donor. These HT levels should encompass the anticipated HT range of the target population. Alternatively, this experiment could also be performed using 5 HT levels (0.20, 0.30, 0.40, 0.50, and 0.60). The latter set-up has the advantage that whenever the most extreme HT values do not yield acceptable results, a narrower, acceptable HT range (regarding recovery and ME) may still be determined, without having to repeat the experiment. This set-up is schematically depicted in Figure 4. As mentioned before, to accurately perform this experiment, a fixed volume of blank or spiked blood needs to be applied on prepunched filter paper discs.

Although MEs are preferably as small as possible, recovery and process efficiency as high as possible, the exact values are not that relevant. It is essential, however, that they are reproducible (ie, relative SD or %RSD within 15% after IS normalization). It is relevant to note that observations by Abu-Rabie et al.[49] suggest that extraction procedures with lower recoveries may be more subject to an impact of HT (see DBS-Specific Validation Parameters).

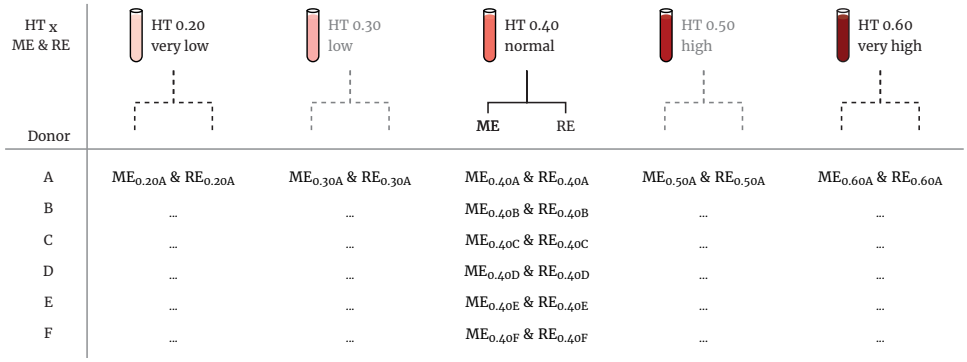

Figure 4. *A schematic set-up for the evaluation of ME and recovery (RE). The experiment can either be performed at 5 HT levels or at 3 (ie, without the gray samples). This experiment allows to evaluate whether ME and RE are constant for different matrices and for different HT levels. Each condition is analyzed in quintuplicate. Reprinted with permission from Anoek Houben. Copyright 2018. Adaptations are themselves works protected by copyright. So to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.*

Stability

The stability assessments performed during method validation should be representative of the ambient conditions encountered during sample transport, storage, and processing. Therefore, stability should be evaluated at room temperature

(the exact temperature depending on where the method will be applied) and the investigated time frame should cover the maximum expected time frame between sample collection, analysis, and potential reanalysis. Furthermore, because temperatures may be significantly higher during transport (eg, in a mail box in the sun during summer time), short-term stability at elevated temperatures (ie, 2 or 3 days at 50–60°C, or higher temperatures depending on the country) should also be tested.[45,76] If stability under ambient conditions is only sufficient for a couple of days (but long enough to allow transport to the laboratory), it may be evaluated if storage at lower temperatures in the laboratory may help stabilize the DBS until (re)analysis.

Importantly, stability may also be affected by other parameters such as humidity and exposure to (sun)light, conditions which are harder to replicate in the laboratory. To evaluate the effect of actual sample transport, samples which are generated in the laboratory can be analyzed immediately after drying, after storage for a certain time under controlled conditions, and after sending them to the laboratory through mail service. Preferably, the samples are deposited in a mail box that is relatively far from the laboratory. Furthermore, it may be relevant to repeat this experiment under different weather conditions, to rule out any seasonal effects on the stability of the samples. Although stability is typically evaluated using spiked samples, it may be worthwhile to also evaluate the stability of incurred samples, as spiked samples may not always display the same stability profile as actual samples.[77] In addition, postpreparative stability should be assessed.

DBS-Specific Validation Parameters

The analytical validation of DBS methods requires the evaluation of several additional parameters (Table 2): that is, the volume effect, the volcano effect (ie, DBS homogeneity), and the HT effect.[1,9,71] It is essential that these parameters are assessed simultaneously because they may affect one another. These parameters can be evaluated in a single day experiment in which the obtained results are compared with those obtained from the reference condition (ie, central DBS punches generated from DBS of average or median volume and HT). Alternatively, this evaluation can be combined with the accuracy and precision experiments (ie, by measuring 2 series of DBS samples with different volumes, different HT levels, etc., on each of 3 days). The latter approach has the advantage that accuracy profiles can be established.[78,79] Importantly, if a certain effect is observed (ie, a relevant volume, HT, or volcano effect), appropriate measures need to be taken to ensure patient samples are within the validated limits and patient results are reliable. Obviously, it should also be demonstrated that these measures are indeed adequate.

Volume Effect

The volume range in which DBS-based results are still acceptable should be defined during method validation. Typical volume ranges to be evaluated are 10–50 µL for hanging-

drop-collection and 20–70 µL for falling-drop-collection. The volume effect should also be evaluated at low (0.30), medium (0.40), and high (0.50) HT and at both the low and high QC level as shown in Figure 5. Whether a sufficient volume is collected from a patient should always be evaluated in the laboratory before DBS analysis. This evaluation should be performed based on the diameter of the DBS. More particularly, the diameter of the patient DBS should be between the diameter of the DBS prepared from the smallest validated volume at low HT and the diameter of the DBS prepared from the largest validated volume at high HT. To help patients to collect DBS of adequate volume, filter paper with 2 concentric circles may be used (Fig. 5).[80] These circles should correspond to the minimally required volume and the maximally allowed volume (also taking into account different HT levels, as described above).[80] It should be noted, however, that this type of filter paper is not commercially available. Furthermore, although these circles may be printed onto commercially available filter paper, it should be considered that the printing itself may affect the analysis (interferences from ink or toner, potential effect on blood flow, eg, caused by paper compression or wax-like materials present in toner). Therefore, the printed filter paper should be used during the entire method validation. Alternatively, equivalence between the in-house printed filter paper and the filter paper used during validation should be demonstrated at both low and high QC levels, and at low, medium, and high volume and HT. In addition, the volcano effect might have to be re-evaluated, depending on the DBS punch size. Another option is to use a phone app to assess whether the generated DBS are within the validated volume ranges.[81] Again, correct performance of the app should be verified during method validation using samples of known volume, covering the entire validated volume and HT range.

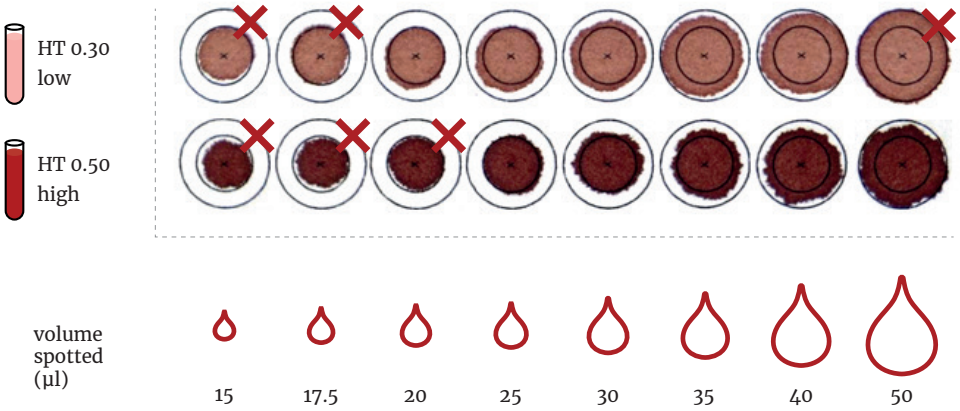

Figure 5. *Example of filter paper with 2 concentric samples corresponding to the minimally required volume (eg, 20 mL) and the maximally allowed volume (eg, 50 mL), also taking into account different HT levels. Figure adapted from Capiau et al.[80] Reprinted with permission from Anoek Houben. Copyright 2018. Adaptations are themselves works protected by copyright. So to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.*

Volcano Effect

Spot homogeneity should be evaluated when embarking upon partial spot analysis (also see part 2, prevalidation). If a relevant volcano effect is observed (eg, punches from the central part of the spot yield different analytical results then punches from edges of the spot), only central punches should be analyzed.

HT Effect

As mentioned before, it is important to actually determine the HT of the calibrators and the samples used during method validation. This will ensure the exact HT value and, consequently, the validated HT range. At least 3 HT levels should be evaluated, more particularly, a QC generated with blood that has the same HT as the blood that was used to generate the calibrators, bracketed by HT values that encompass the expected patient HT range. At each HT level, 2 concentrations should be tested. The HT range that needs to be evaluated depends on the target population (Fig. 6). For a quasiuniversal method, the range should span from 0.20 to 0.65, although a narrower range will suffice for most applications.[80] The exact range will depend on the target population and should encompass at least 95% of the target population.[53]

Unless no relevant HT effect is observed over the entire HT range (both during analytical and clinical validation, see CLINICAL VALIDATION) or unless it is reasonable to assume that all patient HT values will be within the validated HT range, a method should be used to assess the HT of the patient samples. Besides confirming that the

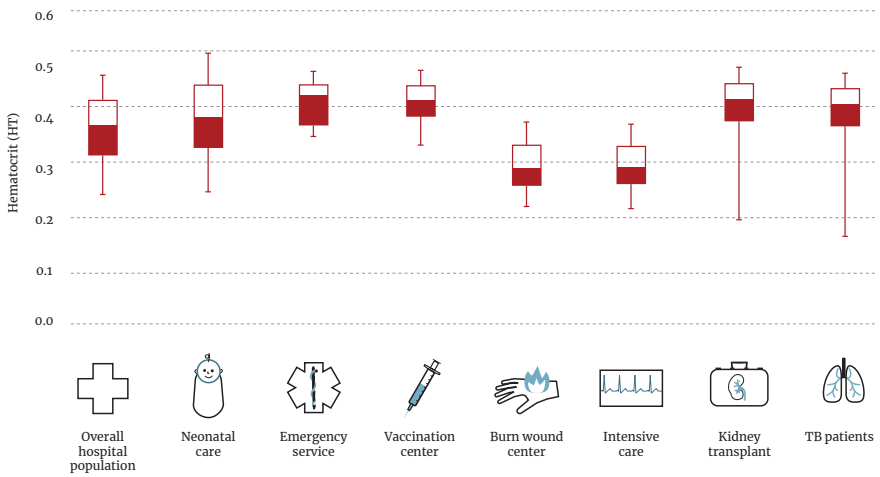

Figure 6. *Overview of the expected hematocrit (HT) range in different patient populations. The boxplots depict the distribution of HT values per patient population. The boxes show the HT values between the 25th and 75th percentile, as well as the median HT value. The flags show the 2.5% and 97.5% percentiles. Adapted from De Kesel et al.[53] Reprinted with permission from Anoek Houben. Copyright 2018. Adaptations are themselves works protected by copyright. So to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.*

HT of the patient sample effectively lies within the validated HT range, this may also allow to perform an HT correction, to alleviate the HT bias.[82,83] Other options are to use volumetric dried blood samples (if there is no HT effect on recovery or ME) or DPS (if there is no HT effect on DPS generation).[36]

Validation of Online DBS Analysis

Whether the sample preparation and analysis are performed online or not does not affect the validation parameters that need to be evaluated. The way in which certain parameters (more particularly, recovery, ME, and process efficiency) are evaluated, however, will need to be adapted.[84–87]

Recovery is typically evaluated by comparing the peak areas from blank matrix samples spiked before extraction with the peak areas from blank matrix samples spiked after extraction. However, with an online sample preparation procedure, there is no option to spike the samples after extraction. Instead, the analytes are introduced to the system during the extraction step. Depending on the type of system used, this can be performed through the IS loop or by spiking the extraction solvent. The results of the samples spiked during extraction are then compared with those of DBS samples containing the same absolute amount of analyte. This requires the entire DBS to be analyzed. When adding the analyte during extraction, the analyte passes through the filter paper and dried blank blood matrix, during which, theoretically, some analyte adsorption may occur. If such adsorption occurs, this will yield a falsely lowered "100% extracted" reference value, which in turn will result in an overestimation of the analyte's recovery. Alternatively, recovery may be evaluated by comparing the peak area resulting from a single extraction with the sum of peak areas resulting from, for example, 10 consecutive extractions. It needs to be considered that even after 10 extractions, not all the analyte may be extracted, again leading to an overestimation of the recovery. Moreover, these multiple extractions may technically not be possible because of filter paper deterioration (depending on the type of filter paper used).

For the evaluation of the ME, the peak areas resulting from the analysis of blank DBS samples and blank DBS cards can be compared. In both cases, the analyte will be introduced during extraction.

Clinical validation

It is generally accepted that a DBS sampling method can only be implemented in the routine care for the purpose of TDM—and thereby (partly) replacing the standard venous whole blood sampling with blood, serum, or plasma analysis—after it has been successfully validated in a clinical validation study.[1,88–91] In a clinical validation study, paired DBS and venous blood, plasma, and/or serum samples are obtained and analyzed. The analytical results are compared and statistically evaluated. The purpose of a clinical validation is to demonstrate that results from DBS are interchangeable with those obtained with the standard method used for TDM, that is, a blood, serum, or plasma analysis. The aim of this part of the guideline is to provide recommendations on how to clinically validate a DBS assay for TDM in daily practice. Current recommendations regarding clinical validation are largely based on published clinical validation studies that used genuine finger prick blood-derived DBS, paired DBS and traditional matrix samples from at least 20 patients, and appropriate statistical analysis to compare both methods.[90–102]

Concentration Range, Number of Clinical Samples, and Patients

The concentration range that needs to be covered during clinical validation depends on the sampling time points of interest (ie, trough and peak) and the shape of the pharmacokinetic time curve of a particular drug and the intra- individual and/or interindividual variability.[2] The CLSI guideline states that at least 40 patient samples should be analyzed for a clinical validation, ideally covering the entire measuring interval of the measurement procedures.[8] This sample size is based on linear regression described by Linnet et al.[103] The sample size that is necessary mostly depends on the coefficient of variation (CV%) of the method and the range ratio (maximum value divided by minimum value). Because most DBS methods have a CV% >5% and a range ratio >25, the number of samples needed after Linnet's calculation will always be 36 or 45. Therefore, using fewer than 40 samples is only possible if the CV% of the method is <5% and/or the range ratio <25. Depending on the situation, these 40 samples could either be paired capillary DBS venous blood samples from at least 40 different patients collected at a single time point (ie, trough or peak), or paired samples taken at 2–3 time points and from a smaller cohort, covering the whole concentration range of interest.[8,103] Ideally, a total of 80 samples obtained from at least 40 different patients should be acquired for validation. This allows using one set of 40 randomly selected samples for fitting a line between DBS and blood (or serum or plasma) concentrations using appropriate statistical tests (see next paragraphs). If required, this will derive a conversion formula or factor to convert, for example, capillary DBS concentrations into venous plasma concentrations. The other set of 40 samples can be used to validate this

conversion.[104] Despite the limitation of collecting multiple samples from the same patient, this approach does not require a new cohort of 40 subjects. If the amount of patients is limited and multiple samples from the same patient (eg, trough and peak) are acquired, it is our recommendation to have a minimum of 40 samples from at least 25 different patients to account for variation in MEs. In those cases where there is only a limited number of paired samples available, the conversion of a concentration in one matrix to that of another can also be checked for by a jackknife method. In this approach, the original set of n samples is resampled n times by systematically creating all possible subsets of n-1 samples. Each of these subsets is then used to set up a conversion equation, which is subsequently applied to the nth sample (ie, that sample which was not included in the subset that was used to set up the conversion equation).[105] To assess the predictive performance of the conversion equation, the median percentage predictive error (MPPE) = median (corrected $[analyte]_{test\ matrix}$ - $[analyte]_{reference\ matrix}$/$[analyte]_{reference\ matrix}$) x 100% and median absolute percentage predictive error (MAPE) = median (|corrected $[analyte]_{test\ matrix}$ – $[analyte]_{reference\ matrix}$/-$[analyte]_{reference\ matrix}$|) x 100% can be calculated. These provide a measure of bias and imprecision, respectively.[106,107]

Comparing DBS Concentrations With Plasma or Whole Blood Concentrations and Effects of HT

Peripherally, collected blood consists of a mixture of venous and arterial blood and interstitial fluids. Therefore, the drug concentration in peripherally collected blood may differ from venously collected blood. This effect is mostly present during the distribution phase of the drug. Although drugs are usually rapidly distributed throughout the body, this process sometimes can take up to several hours, leading to unreliable results when samples are collected during the distribution phase.[2,108–110] To detect a potential capillary-venous difference (Fig. 7), the results obtained from a DBS collected from a finger prick (sample A) can be compared with those from a DBS prepared from venously collected blood (sample B). This venous blood (sample C) can be used to generate plasma (sample D). Both sample C and D can be compared with blood collected by finger prick (sample A). Alternatively, another blood sample needs to be collected at the same time point if serum (sample E) is to be prepared. Serum or plasma is typically used for routine TDM. It is essential that samples B and C should give the same result. If they do not, this points to an effect of the DBS approach in se.

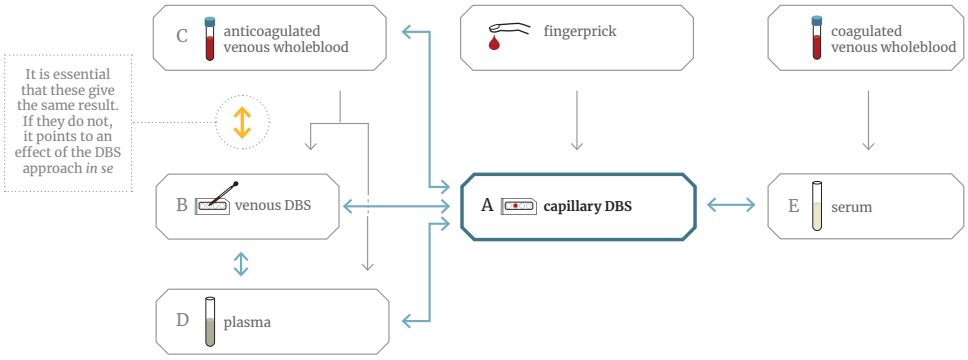

Figure 7. *A schematic overview of the samples that could be collected during a clinical validation study. The bold blue lines depict which samples could be compared with one another. The gray lines show which samples can be generated from which sampling method. Reprinted with permission from Anoek Houben. Copyright 2018.*

In vivo, drugs can bind to components of plasma or accumulate in red blood cells, leading to differences between observed concentrations in whole blood (and hence DBS) and in plasma (or serum, depending on the matrix that is routinely used for an analyte).[98,108] The difference in drug concentration between blood (DBS) and plasma can be explained by the fraction of drug in plasma relative to whole blood, the HT, and the drug's affinity for red blood cells. The study design may allow for the generation of this blood–plasma relationship. If a blood concentration has to be expressed as a plasma or serum concentration for easy interpretation by the clinician, HT values should ideally be measured, known, or calculated for each blood (DBS) sample. Furthermore, when acceptance limits for the HT have been set based on the analytical validation, one should actually know whether the HT of a given sample effectively lies within these limits. When comparing capillary DBS values with reference whole blood values, correction factors (sometimes based on HT) can be necessary and should be derived from clinical validation studies comparing whole blood values to finger prick (capillary) DBS values.[89,91,92,95,97,111–115]

If, for a specified HT range, the analytical validation has demonstrated that a DBS analytical method is independent of HT (or dependency is within acceptable analytical limits, see above), confirmation is required in a clinical validation study by plotting the differences between DBS results and reference method results versus the HT. The slope of the resulting curve should not be significantly different from zero.[80] When this has been confirmed, plasma or serum concentrations can be calculated based on the equation derived from the Passing–Bablok or weighted Deming regression line.[91,101,116–120] If an analytical method has proven to be dependent on HT values during analytical and clinical validation using appropriate statistical tests, a conversion formula should include a correction for HT.[121,122] An example is the estimation of plasma values from

DBS concentrations using the formula 1 – (HT/100).[122] This will only be possible if there is a systematic effect from HT on estimated venous blood concentrations, which is fixed within the relevant clinical range.[123] If this is not the case, the method might not be suitable for clinical application. If an HT-dependent method is to be used in routine care, the HT of the DBS should ideally be known. Procedures to derive HT from a DBS card include potassium measurements,[80] noncontact diffuse reflectance spectroscopy,[52,83] near-infrared spectroscopy,[124] or the use of sulfolyser reagent.[125] If, for an HT-dependent method, it is—because of technical or other reasons—not possible to know the HT of a DBS, clinical validation can be performed for a specific patient population, provided the HT range in that specific population is narrow and lies within the method's acceptance limits (Fig. 6).[94,98] In many instances, the mean or median HT and range for a given patient population can be calculated from historical patient data.[53] For a different patient population, it should be determined whether a new clinical validation should be performed.[10,98,122] Another approach to cope with the HT effect is whole blood spot analysis using a fixed spot volume. A volumetric capillary or pipet can be used to apply a fixed volume of finger prick blood to the filter paper.[14,126,127] In this situation, no conversion formula to correct for HT is needed. However, it should be clear from the analytical validation that the HT has no impact on recovery or MEs.[89,91,95,97,115] Moreover, this can be at the expense of the simplicity of sampling and/or bring along additional costs.

Statistical Methods and Interpretation

Technically, a DBS clinical validation is a cross-validation study because a candidate method (DBS-based) is compared with a reference method (blood-, serum- or plasma-based). Although guidelines from the EMA, FDA, and CLSI include cross-validation and subsequent statistical analysis of results, this paragraph provides additional recommendations and guidance for the interpretation of results.[1,5,6,8]

As part of a clinical validation, the results obtained from DBS and the reference method should be compared using appropriate statistical tests. To compare 2 methods, regression analysis should be performed to measure the correlation, followed by an agreement and bias estimation test.[8] As both the reference and the DBS method have some inherent variability, so that either Passing–Bablok or weighted Deming regression should be used instead of standard linear regression.[8,128–130] Both approaches have been used in various clinical validation studies.[91–102,131] Deming regression takes variability of both x and y into account; Passing–Bablok regression makes no assumptions about the distribution of data points and is more resistant toward outliers.[8,129,132] Various clinical validation studies have shown that the absolute difference between results from a reference and a DBS method is proportional to the concentration, at least at higher concentrations. However, in these studies, sometimes only a few high concentration samples were available.[91,96,120] Theoretically, an outlier in this region would impose an inflated or deflated estimate of

proportional difference. In this case, a Passing–Bablok regression analysis is the preferred statistical method.[8,133] After regression analysis, a Bland–Altman difference plot should be made to assess the agreement between both methods and estimate the bias.[8] When using a (HT-dependent) conversion formula obtained from Passing–Bablok or weighted Deming regression, the Bland–Altman difference plot should be made using the (blood, plasma, or serum) concentrations that were calculated from the DBS concentrations.[1,91] Most clinical validation studies show some level of bias when performing a Bland–Altman test. Although it may seem obvious that Bland–Altman graphs should be generated and interpreted in a correct manner, this is not always the case.[133] Several things can be deduced from a Bland–Altman difference plot. First, it can be observed whether there is an average bias between both methods and whether the 95% CI of this bias contains zero. Importantly, if the latter is not the case, it should have been formally decided beforehand what a clinically relevant or acceptable bias and corresponding limits of agreement (LoA) should maximally be. For instance, for tacrolimus, where trough concentrations in blood are usually between 5 and 20 mcg/L, a bias of 0.28 mcg/L (LoA 20.45 to 20.12 mcg/L), which is at most a bias of 5.6% (LoA 9.0%– 2.4%) would not impact clinical decision making, whereas a higher bias or LoA might.[134] Second, the LoAs can be derived from the Bland–Altman plot. Here, the same holds true: preset criteria are needed to define what concentration or %difference span between the LoAs is still considered acceptable. This is a critical point that, in many instances, is lacking: for example, although, on average, there may be no bias between a DBS- and blood-based procedure, the span of the LoA's may be too wide (implying there is too much variation) to be acceptable. What is considered acceptable in terms of bias or LoA will largely depend on the clinical setting, the laboratory's internal policy, the availability of guidelines (eg, RCPA criteria)[135] and the drug of interest. Acceptance criteria should be decided by a multidisciplinary team of experts based on both clinical and analytical acceptance criteria. In addition, during a clinical validation, it can be investigated for each measured pair of samples whether the clinical decision by the health care provider would differ, based on the DBS concentration versus the concentration in the reference sample.[92,93,99,136] Again, acceptance criteria should be stated beforehand in the study protocol. The EMA guideline states for cross-validation study samples, "the difference between the 2 values obtained should be within 20% of the mean for at least 67% of the repeats."[5] It has been suggested that this guideline could also be applied to assess agreement between DBS-based analytical results and reference results.[1] For example, a study, in which for 30% of the samples, a difference of more than 20% of the mean is observed, would theoretically fulfill the criteria put forward by the EMA guideline. However, this would likely be clinically unacceptable, and in this case, stricter LoA would be preferred. It is also possible that, at lower concentrations, a maximum absolute deviation may be tolerated, while at higher concentrations, a maximum allowable percentage deviation may be set.

Type of Card/Paper Used

In a clinical validation study, it should be stated which type of paper or DBS card is used. This type of paper should be the same as the one that was used during analytical validation.[29]

Sampling Method and Spot Quality

A major problem during clinical validation is that the provided DBS may be of insufficient quality for analysis due to incorrect sampling.[42,137] Therefore, during clinical validation, the method of sampling and spot quality assessment by either an analyst or an automated quality assessment method should be mentioned in the study protocol.[138,139] As drug concentrations are dynamic, it is important to collect all paired samples within 5–10 minutes of each other.[91,116] Time-dependent changes in drug concentration are determined by pharmacokinetics and should be taken into account for the preparation of a sampling scheme. This is particularly relevant for drugs with a very short half-life or during the absorption and distribution phase of the drug.

The sampling method that is used during clinical validation should be the same as the sampling method that will be used in daily practice. For example, if the method is intended for home sampling by patient finger prick, the DBS samples obtained for clinical validation should also be obtained by finger prick. Spotting of venous blood on a DBS card is only appropriate if in clinical practice venous blood will be spotted on DBS cards. For instance, this may be the case when transport of tubes of whole blood is not possible due to instability of the compound or because of logistic difficulties (eg, in remote areas or in resource-limited settings).[58] This is highly relevant as for some analytes venous capillary differences may, or are known to, be present. If a method is designed for home sampling, patients should ideally perform a finger prick to collect a DBS sample themselves during clinical validation. However, in most clinical validation studies, a trained phlebotomist collects or helps to collect samples, to rule out variability due to inexperienced sampling by the patient.[91,95,97,99,116,123] Alternatively, both approaches can be used successively during clinical validation.

Proper finger prick DBS sampling technique has been described earlier by the WHO, CLSI, and in several studies[11,42,131,138,140,141] and is also shown in Supplemental Digital Content 1 (see Figure S-2, http://links.lww.com/ TDM/A342). In short, sampling should be performed after disinfecting the finger without excessive "milking" or squeezing of the puncture site to avoid hemolysis or dilution by tissue fluid. When possible, finger prick blood should fall on the sampling paper instead of applying the droplet of blood to the sampling paper with the finger (without touching the sampling paper with the finger). Both patient and phlebotomist should be trained before samples can be obtained. This training should include practicing the whole sampling procedure under supervision of someone experienced in DBS sampling using either a test kit or a real finger prick aided by educational material such as a movie or a written instruction.[25,131,137,138,140]

All spots provided in a clinical validation study should be checked for quality by an experienced analyst or through a validated automated quality assessment method. Some requirements for a good quality spot depend on the analytical method and should be stated on beforehand, such as minimum spot size imposed by punching size. Other requirements are independent of the analytical method. Criteria are stated in Supplemental Digital Content 1 (see Figure S-3, http://links.lww.com/TDM/A342). In short, all spots should be round, dried, consisting of one droplet of blood, and not touching other droplets.

ISR, Duplicates, and Outliers

In their guideline, the FDA mentions ISR as a validation parameter for DBS methods.[6] In a clinical validation, ideally at least 2 replicate spots are available for analysis, to allow ISR and/or duplo analysis. However, reanalysis of the same spot (through a second punch) will not be possible when the protocol involves the use of larger punching sizes (eg, 6 or 8 mm).[64] During clinical validation, it is recommended to analyze 2 different spots per sample, when possible, to evaluate within-card precision, which can be calculated as the percentage difference: %difference = ((repeat value – initial value)/mean value)x100.[5,24] The %difference between duplicates should not be greater than 20% of their mean for at least 67% of the samples.[5,6] In addition, ISR of the same spot is recommended when decentral punches may be used, provided spot homogeneity is supported by the analytical validation, and small punch sizes (eg, 3 mm) are used.[27] The presence of an outlier may be explained by several reasons such as contamination of the sample, errors in sampling, extreme drying, or storage conditions during transport or analytical errors.[42] In a clinical validation study, most of the possible errors can be accounted for by, for instance, checking of spot quality of the sample upon arrival in the laboratory or checking and logging the drying time. When an outlier cannot be explained by such errors, the extreme studentized deviate technique[8] or a standardized score test can be used to exclude outliers.[121] However, outliers should be discussed in the context of clinical application of the DBS method. Therefore, outliers require an argumented discussion considering clinical setting and the aforementioned statistics tests.[8]

Clinical Validation of Automated Analysis Methods

Automation of a DBS assay could improve DBS sample and workflow efficiency and reproducibility. Several examples exist of automated (online or offline) DBS assays using techniques such as online extraction and solid phase extraction.[87,142,143] If an automated method is designed without a previous manual DBS method, the same recommendations for clinical validation apply. If a manual DBS assay used in clinical practice is replaced by an automated DBS method which is fully analytically validated, it is recommended to perform a cross-validation including sample size of 40 samples

from at least 25 different patients.[5,6,8] Because of the nature of DBS, it will most likely be challenging in real practice to measure the same spot using both an online and offline method. Therefore, if during the clinical validation the within-card precision is found to be acceptable and 2 spots per finger prick DBS sample are provided, it is recommended to analyze one spot using the automated method and one spot using the manual method. Evaluation of agreement can again be performed by Passing–Bablok or Deming analysis and through a Bland– Altman plot, as described earlier.

QC

Laboratories should participate in external QC programs if a DBS assay is implemented in routine care or provide objective evidence for determining the reliability of their results.[2,38] Apart from a proficiency test pilot for the immunosuppressant tacrolimus, no external QC programs are currently available for DBS assays for drugs.[144] There is an urgent need for DBS proficiency testing programs to facilitate the uptake of DBS in routine care. Although external QC materials developed for the evaluation of liquid blood-based methods may be used to evaluate the quality of a DBS-based method, it should be taken into account that these materials typically have a different viscosity than true blood samples and will therefore yield DBS of deviating sizes. Therefore, when using these materials, they should always be analyzed using a full-spot approach.[145] Furthermore, the extraction efficiency of an artificial matrix may always differ from the extraction efficiency of an actual sample. Since most external QC materials are only available for plasma analysis and not for whole blood analysis, another option might be to remove part of the plasma of a blank whole blood sample and to replace it with the external QC material. The resulting blood can then be used to generate DBS, as was successfully applied for, for example, conventional antiepileptics.[67]

Cross-validation

Once a DBS assay has been successfully applied in clinical practice, it is possible that changes have to be made to the sampling method, filter paper, or analytical method. For some of these changes, the standard guidelines for crossvalidation are applicable.[5,6] This part will focus on additional recommendations when DBS assays or sampling methods are altered.

Different Punch Size

As stated before (see METHOD DEVELOPMENT: CONSIDERATIONS FOR SUCCESSFUL VALIDATION), a punch size is preferably less than 4 mm because punching the sample in the laboratory will be easier, and patients do not need to produce large blood spots. When the desired LLOQ, accuracy, and precision can be met with a different punch (eg, smaller or "donut" punch)[74] than currently used in practice, a cross-validation

study should be performed. If during the clinical validation the within-card precision is within analytical limits and 2 spots per sample are provided, it is recommended to analyze 1 spot with the new punch size and 1 with the old punch size. In total, 40 samples of at least 25 different patients should be analyzed. In addition, extraction efficiency and DBS homogeneity should be re-evaluated. The extraction volume used with smaller punches can be downscaled accordingly. Although theoretically possible, we do not recommend to use a surface-based formula to convert a result from a small (eg, 3 mm) DBS punch to a theoretical bigger (eg, 6 mm) DBS equivalent.

Different Type of Filter Paper

In routine practice, several types of DBS filter paper are used such as the Whatman 903, Whatman FTA DMPK cards (type, A, B and C) (GE Healthcare, Chicago, IL), and Perkin Elmer 226 cards (Ahlstrom, Helsinki, Finland).[29] Although performance of the FDA-approved Whatman 903 (GE Healthcare) and Perkin Elmer 226 paper is consistent and comparable in newborn screening,[146] the influence of drug concentration and HT can lead to a difference in recovery of up to 20% between cards.[29,147] This may be caused by the drugs' ability to form hydrogen bonds with the cellulose paper, leading to decreased recoveries,[57] differences in spot homogeneity, or differences in background signal.[27] Not only the recovery of the analyte may be altered, also matrix, volcano, volume, and HT effects may have changed, as well as the analyte's stability. These parameters should all be re- evaluated as discussed before. Furthermore, QC samples for the new filter paper should be made using the same method as was performed for the old filter paper.[54] Both old and new QC samples should be analyzed, and the obtained mean accuracy should be within 15%.[5] The equivalence between both filter papers should be confirmed using a minimum of 40 samples obtained from at least 25 different patients. If not all parameters prove to be similar for both types of filter paper, full analytical validation and clinical validation are required.

Different Sampling Method

Switching the sampling method will, most likely, be accompanied by some change in the method. For instance, it is likely that whole spot analysis rather than partial-punch analysis will be performed when a fixed volume of finger prick blood is deposited on a card instead of direct application of blood from the fingertip to the card. Moreover, it is possible that DBS-based assays are replaced by newer alternatives such as the earlier discussed VAMS technique because of the convenience of sampling and/or automation possibilities.[25] Importantly, as stated earlier, volumetric sampling does not necessarily eliminate the effect of HT or aging on recovery, so this remains an important parameter to be studied.[7,29,57,62,148] In addition, a new sampling technique might influence spot homogeneity, thereby introducing a possible unknown error in analytical results.[27] Therefore, when changing sampling technique, sample vehicle, or

changing to whole spot analysis, it is recommended to perform a full clinical validation study, comparing the new method to the reference method, provided this change has been appropriately analytically validated.[25]

Conclusion

To successfully incorporate DBS-based methods in routine practice, good quality methods are a prerequisite. Since the quality of a method starts with its design, a sound method set-up not only ensures the method is suitable for a given application, it also increases the chances of a successful method validation. The quality of a method needs to be assessed both during analytical and clinical validation and should be compared with preset acceptance criteria. This is the first guidance document discussing how to evaluate the quality of a DBS-based method. This guideline outlines which traditional and nontraditional validation parameters should be assessed for this type of method and provides suggestions on how to do this. Most importantly, each parameter should be evaluated in a way that reflects the real-life situation in which the method will eventually be applied. Furthermore, to ensure the method's quality on a day-to-day basis, the first QC programs for quantitative DBS-based methods have been established recently. It is important to keep in mind that DBS for TDM applications only has a future if the quality of the result can be guaranteed. A proper analytical validation and clinical validation are essential to achieve this.

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Chapter 9

A volumetric absorptive microsampling LC–MS/MS method for five immunosuppressants and their hematocrit effects

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Abstract

Aim: The aim of this study was to develop and validate a LC–MS/MS assay for tacrolimus, sirolimus, everolimus, cyclosporin A and mycophenolic acid using volumetric absorptive microsampling tips as a sampling device and to investigate the effect on the recoveries of the analyte concentration in combination with the hematocrit (HT), which included temsirolimus (a structural analog).

Results: The maximum observed overall bias was 9.6% for the sirolimus LLOQ, while the maximum overall coefficient of variation was 8.3% for the everolimus LLOQ. All five immunosuppressants demonstrated to be stable in the volumetic absorbtive microsampling tips for at least 14 days at 25°C. Biases caused by HT effects were within 15% for all immunosuppressants between HT levels of 0.20 and 0.60 l/l, except for cyclosporin A, which was valid between 0.27 and 0.60 l/l. Reduced recoveries were observed at high analyte concentrations in combination with low HT values for sirolimus, everolimus and temsirolimus. Conclusion: A robust extrac- tion and analysis method in volumetric absorptive microsampling tips was developed and fully validated. HT- and concentration-related recovery effects were observed but were within requirements of the pur- pose of the analytical method.

Introduction

Therapeutic drug monitoring of immunosuppressant drugs is of major importance in the treatment of transplant patients. Based on the concentration in the blood, the immunosuppressant dose is adjusted to balance between toxicity and allograft rejection in an individual patient.[1] Therefore, lifelong monitoring is necessary, which requires transplant patients to travel to the hospital for venous sampling. Introduction of easy-to-use microsampling techniques can enable home sampling and reduce patient burden.[2] Recently introduced volumetric absorptive microsampling (VAMS) tips could be used for this purpose. They are designed to wick-up an exact volume of, that is, 10, 20 or 30 µl blood. A high blood wicking volume would allow lower LLQs, but could also be more vulnerable to decreased recoveries due to insufficient penetration of the extraction solvent into the VAMS tip during the extraction. The volume of 20 µl was thought to provide the best of both. The absorbed volume of blood is claimed to be independent of the blood hematocrit (HT) value which is to be considered a significant improvement compared with dried blood spot (DBS) sampling followed by partial spot analysis.[3] DBS samples created by a drop of blood of unknown volume (approximately 50 µl) and followed by partial spot analysis suffer from HT-related variation of the formation of the spot size. A drop of blood with a low HT value creates a larger spot than a drop of blood with a high HT value.[4] This is due to the respectively low and high viscosity of the blood. This affects the amount of blood that is captured with the partial spot punch. Low HT values will cause negative biases and high HT values positive biases compared with a standardized HT value and this is generally known as the HT effect.[4–10] In addition, low HT values in combination with high analyte concentrations can influence the recoveries of specific analytes due to binding of the analytes to the sampling matrix.[4,5] The combination of these two HT effects adds up to unacceptable biases at low HT levels and high analyte concentrations. Because a fixed volume is absorbed, the VAMS tips should not suffer from the effect of the HT on the blood volume, but the effect of low HT values and high analyte concentrations on its recoveries is unknown. Recently, various methods to analyze a single immunosuppressant such as tacrolimus or everolimus in VAMS tips were published but, to date, no multi-analyte VAMS analysis method exists covering all relevant immunosuppressants. Several publications described possible recovery issues encountered during the optimization of the VAMS extraction method. The conclusions drawn from these research projects underline the importance of the development of an extraction method that provides stable recoveries at various HT values, analyte concentrations, VAMS drying times and after storage of the sampled VAMS tips.[8–14] For this study, the following analytes were included: tacrolimus (TAC), sirolimus (SIR), everolimus (EVE) temsirolimus (TEM), cyclosporin A (CYA) and

mycophenolic acid (MPA). All analytes except TEM are immunosuppressants, while TEM is an anticancer agent and ester analog of SIR. TAC, SIR, EVE and TEM are all structural analogs and the addition of TEM is used to assess the extraction behavior and the possible adsorption to the VAMS sampling material.[5,15] The objective of this study was to develop and validate an extraction and analysis method for the VAMS tips and to investigate whether the recovery is influenced by the combination of the HT, analyte concentration and drying time.

Materials and methods

Table 1. Mass spectrometer settings for all analytes.

Analyte	Precursor ion (m/z)	Product ion (m/z)	RF Lens (V)	Collision energy (V)
Tacrolimus	821.5	768.4	82	20
Tacrolimus [$^{13}C,^{2}H_2$]	824.5	771.4	82	20
Sirolimus	931.5	864.4	83	15
Everolimus	975.6	908.5	88	16
Temsirolimus	1047.6	980.5	90	16
Everolimus [$^{13}C_2,^{2}H_4$]	981.6	914.5	88	16
Cyclosporin A	1219.8	1202.8	93	15
Cyclosporin A [$^{2}H_{12}$]	1231.8	1214.8	93	15
Mycophenolic acid	321.1	207.0	58	22
Mycophenolic acid [$^{13}C,^{2}H_3$]	325.1	211.0	58	22

Chemicals & reagents

TAC, SIR and EVE were purchased from Cerilliant (TX, USA). TEM and MPA were purchased from Sigma-Aldrich GmbH (Buchs, Switzerland) and CYA was purchased from EDQM (Strasbourg, France). Stable isotope labeled internal standards (SIL IS) were used when possible. TAC [$^{13}C,^{2}H_2$], EVE [$^{13}C_2,^{2}H_4$], CYA [$^{2}H_{12}$] and MPA [$^{13}C,^{2}H_3$] were purchased from Alsachim (Illkirch Graffenstaden, France). EVE [$^{13}C_2,^{2}H_4$] was used as IS for EVE, SIR and TEM because no suitable isotopically labeled IS with high enough purity was available for SIR and TEM. 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 anticoagulated whole blood was purchased from Sanquin (Amsterdam, The Netherlands). The whole blood was stored at 4°C and was used within 2 weeks after donation. The blood was checked for hemolysis prior to use. A total of 20 µl Mitra VAMS tips were acquired from Neoteryx (CA, USA).

Equipments & conditions

Experiments were performed on a triple quadrupole LC–MS/MS and consisted of a Vanquish UPLC system in combination with a TSQ Quantiva triple quadrupole mass spectrometer, from Thermo Fisher (MA, USA). The mass selective detector operated in electrospray positive ionization mode and performed multiple reaction monitoring 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, TEM and CYA $[NH4]^+$ adducts are selected in the first quadrupole. The autosampler temperature was set at 10°C and the column oven temperature was set at 60°C. The binary pump LC method was optimized for UHPLC analysis (including separation of the MPA glucuronide) using a Thermo Accucore C18 2.6 µm 50 × 2.1 mm analytical column equipped with a 5 µm Thermo inline frit filter. The mobile phase consisted of 20 mM ammonium formate buffer pH 3.5 and methanol. Chromatographic separation was achieved with the use of a gradient using a flow of 1.0 ml/min and a run time of 1.5 min, see Table 2 for the gradient settings.

Table 2. Chromatographic gradient.

Time (min)	20 mM ammonium formate buffer pH 3.5 (%)	Methanol (%)
0.000	70	30
0.300	70	30
0.310	27	73
0.950	22	78
0.960	5	95
1.250	5	95
1.251	70	30
1.500	70	30

Sample preparation

The preparation of the different target HT values was performed as described previously by removing or adding plasma to achieve the different target HT values.[16] The following HT values were prepared during the research: 0.10, 0.20, 0.27, 0.30, 0.40, 0.50 and 0.60 l/l. The prepared HT values were confirmed by analysis on a XN-9000 hematology analyzer from Sysmex (Hyogo, Japan).[16] For the preparation of the different blood concentrations, the volume of the spiked stock solution never exceeded 3% of the total blood volume in order to prevent cell lysis. The prepared blood standards were then gently mixed on a roller mixer for 30 min at room temperature directly followed by sampling of the VAMS tips. During method development, the optimal extraction method proved to consist of a two-step extraction. The first extraction solution

(extraction solution 1) consisted of methanol:water (40:60 v/v%) and contained the SIL IS. In the first step, extraction solution 1 was added in order to redissolve the red blood cells with the use of a high percentage of water, while the presence of methanol still has a positive influence on the solubility of the analytes and SIL IS. In the second step, methanol (extraction solution 2) was added to optimally extract the analytes and to optimize the solubility of the analytes and SIL IS. The complete extraction is as follows: The VAMS tips are removed from the holder and placed in a 2-ml Eppendorf cup and 100 µl extraction solution 1 is added. The Eppendorf cups are sonicated at 47 kHz for 30 min, then 200 µl extraction solution 2 is added, followed by 15 min vortexing at medium speed and 1 min at maximum speed, 15 min of sonification at 47 kHz, 15 min vortexing at medium speed and 1 min at maximum speed and 5 min centrifuging at 10,000 × g. The extraction solvent is transferred into a vial with an insert, and the vials are placed at -20°C for 10 min, centrifuged for 5 min at 10,000 × g and 20 µl of the upper layer is injected into the LC–MS/MS. The autosampler needle height was set high enough in order to avoid injection of precipitated blood, which will otherwise cause blockage of the autosampler needle and injection loop.

General recovery experiments

For every combination of HT and analyte concentration, blood was spiked with all analytes, gently mixed for 20 min, sampled with the VAMS tips in fivefold, dried for the designated time and extracted. The defined amount of blood absorbed by the VAMS tip that was used for the preparation of calibrators and quality control (QC) was 21.6 µl (according to the VAMS tips package insert for that batch). For calculating the recoveries, enough blank blood samples of every HT value were sampled with the VAMS tips, dried for the designated time, extracted and spiked in fivefold with recovery solution which represents 100% recovery of a 21.6 µl VAMS sample. Analyte/SIL IS area response ratios were used to calculate recoveries. For each analyte, every concentration and HT value was extracted and measured in fivefold and the analyte/SIL IS area response ratio results were averaged. For each averaged value to be accepted, the coefficient of variation (CV) was calculated and should be within 15%. The percentage recoveries were calculated as follows: mean ratio for the analyte divided by the mean ratio of the spiked blank extracts multiplied with 100.

Investigation of the HT- & concentration-related recovery effects

Previous DBS research showed that at decreasing HT in combination with increasing concentration the recoveries for SIR, EVE and TEM declined, while this phenomenon was not observed for TAC and CYA.[5,6] To evaluate this for the VAMS sampling, the following concentrations were tested for TAC, SIR, EVE and TEM: 3.00, 20.0, 40.0, 50.0, 80.0 and 100 ng/ml. The following concentrations were tested for CYA: 30.0, 200, 400, 500, 800 and 1000 ng/ml. The following concentrations were tested for MPA: 300,

2000, 2500, 4000 and 5000 ng/ml. All concentrations were tested at the following HT values: 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 l/l. All sampled VAMS tips were dried for 24 h and subsequently extracted and analyzed. Each mean recovery was evaluated using a 3D graph with the analyte concentration on the x-axis, the recovery on the y-axis and the HT on the z-axis.

Analytical method validation

The validation was performed for all analytes except for TEM, which was only used for investigating the relation between HT, concentration and analyte recovery. The analytical method validation was executed using a standardized HT value of 0.38 l/l based on an earlier study in transplant patients and included linearity, accuracy, precision, selectivity, specificity and stability based on the US FDA and EMA international guidelines.[17–19] Additional validation experiments were performed considering the use of the VAMS matrix. This included the investigation of the effect of the HT and drying time. All sampled VAMS tips were dried for at least 48 h before extracting and analysis. Different preparations of stock solutions were used for the preparation of the calibration curve, and all other QC concentrations.

Calibration

For each analyte, an eight-point calibration curve was used, except for CYA were a seven-point calibration curve was used. The following concentrations were prepared for TAC, SIR and EVE at 1.00, 3.00, 10.0, 20.0, 25.0, 30.0, 40.0 and 50.0 ng/ml. The calibration curve for CYA was prepared at 10.0, 30.0, 200, 250, 300, 400 and 500 ng/ml. The calibration curve for MPA was prepared at 100, 500, 2000, 5000, 7500, 10,000, 12,500 and 15,000 ng/ml. One calibration curve was analyzed each day for three separate days to determine linearity.

Accuracy & precision

The QC concentrations used for the validation were as follows. The LLOQ was 1.00 ng/ml, low 3.00 ng/ml, medium 25.0 ng/ml and high 40.0 ng/ml for TAC, SIR and EVE. For CYA, the LLOQ was 10.0 ng/ml, low 30.0 ng/ml, medium 250 ng/ml and high 400 ng/ml. For MPA, the LLOQ was 100 ng/ml, low 300 ng/ml, medium 7500 ng/ml and high 12,500 ng/ml. For the validation to be accepted, the maximum bias and CV for the LLOQ was 20%. While for all other QC samples including the stability validation 15% was acceptable. The accuracy and precision were performed on separate days and in three separate runs by measuring all QC concentrations in fivefold. The bias and CV were calculated for each run at each accuracy and precision concentration. One-way ANOVA was used to calculate within-run, between-run and overall CVs.

Dilution

Dilution was validated by dilution of an over-curve (OC) blood sample extract in fivefold for 3 days. Each OC extract was diluted ten-times with extract of blank blood with SIL IS. For TAC, the OC was spiked at 200 ng/ml, for SIR and EVE, the OC was spiked at 140 ng/ml and for CYA, the OC was spiked at 2000 ng/ml. Due to the large linear range of MPA, dilution was not validated for MPA.

Stability

For stability testing, VAMS tips were prepared at low and high concentrations in fivefold and compared with freshly prepared VAMS tips in fivefold. Analyte stability in the autosampler was assessed in fivefold with the use of the extracts stored at 10°C for 7 days. Stability of the analytes in VAMS was evaluated at 25, 37 and 50°C in fivefold at multiple time points.

Extraction recovery, matrix effect & process efficiency

As stated before, the amount of blood that was wicked up by the VAMS tip was 21.6 µl. This volume was used for the assessment of the extraction recovery, matrix effect and process efficiency. VAMS tips were sampled with blank blood or blood spiked at low, medium and high analyte concentration levels (at a HT of 0.38 l/l) and dried for 48 h. The VAMS tips were handled and processed as described above in the recovery experiments section. These spiked low, medium and high levels correspond with solutions A low, A medium and A high. In order to represent 100% extraction recovery, the calculated theoretical amounts of analytes were added to the extracts of the VAMS tips sampled with blank blood (solutions B low, B medium and B high). In order to represent 0% matrix effects and 100% process efficiency, the final extraction solvent composition, which contained the final concentrations of ISs, was spiked with all analytes in order to obtain the final concentrations at low, medium and high levels (solutions C low, C medium and C high). The average ratios of the analyte peak area responses and SIL IS peak area responses were used to calculate recovery, matrix effect and process efficiency. The calculations of the recovery, matrix effect and process efficiency were as follows: recovery = A/B × 100, matrix effect = (B/C × 100)-100, process efficiency = A/C × 100.

Influence of the HT

The effect of the HT on the bias was tested at low and high analyte concentrations at the following HT values: 0.20, 0.30, 0.38, 0.40, 0.50 and 0.60 l/l with the standard HT set at 0.38 l/l and 24 h drying time.

Influence of the drying time & HT on the recovery

The effect of the drying time was investigated at the low and high levels at the following

HT values: 0.20, 0.30, 0.40, 0.50 and 0.60 l/l. Except for MPA, where the high level was tested at 2000 ng/ml instead of 12,500 ng/ml. The drying times of the VAMS tips were assessed at 3, 24 and 48 h and recoveries were calculated as described above. Differences in recoveries were calculated as the subtracted difference between the percentage recovery found at 48 h drying at HT 0.40 l/l and the percentage recovery found at a certain HT and drying time. This was evaluated for each concentration.

Statistical analysis, software & calculations

Peak area ratios of the analyte and its internal standard were used to calculate concentrations. Thermo Xcaliber software (version 3.0) was used for quantification of the analytes and the calibration curves. For each analyte, the most simple linear calibration curve fit was chosen that best described the relation between analyte response and concentration. All calculations performed for the validation were made with Excel (version 2010, Microsoft, WA, USA) spreadsheets that were developed in-house. An in-house developed and validated Excel spreadsheet was used to calculate within-run, between-run and overall CVs with the use of one-way ANOVA.

Results & discussion

Trained setting

All analyte recovery data were plotted in 3D graphs and are shown in Figure 1. TAC and MPA show a fairly flat recovery pattern, which is not affected by the combination of HT and concentration. SIR, EVE and TEM show declining recoveries as concentration increases and HT decreases, and all these three 3D plots show the same pattern. The lowest recoveries were observed at the HT of 0.1 l/l in combination with the concentration of 100 ng/ml for SIR, EVE and TEM. For TEM, the recovery of 56% was the lowest observed, while for EVE and SIR higher recoveries of 60 and 63% were observed respectively. CYA showed lowered recoveries of 71–86% at the lowest concentration of 30 ng/ml for all HT values, while higher concentrations showed recoveries increasing up to 100%. For SIR, EVE and TEM, this corresponded to previous observations with DBS analysis methods.[5,6,15] The increasing number of hydrogen bond acceptors of SIR, EVE and TEM, respectively, was inversely related to the recoveries. Therefore, it was previously theorized that more hydrogen bond acceptors in the analyte molecule would lead to increased binding to the sampling matrix at lowered HT and increased analyte concentration. The increased amount of analyte and the decreased amount of red blood cells to bind to induces binding of the analyte to the sampling matrix.[5,6,15]

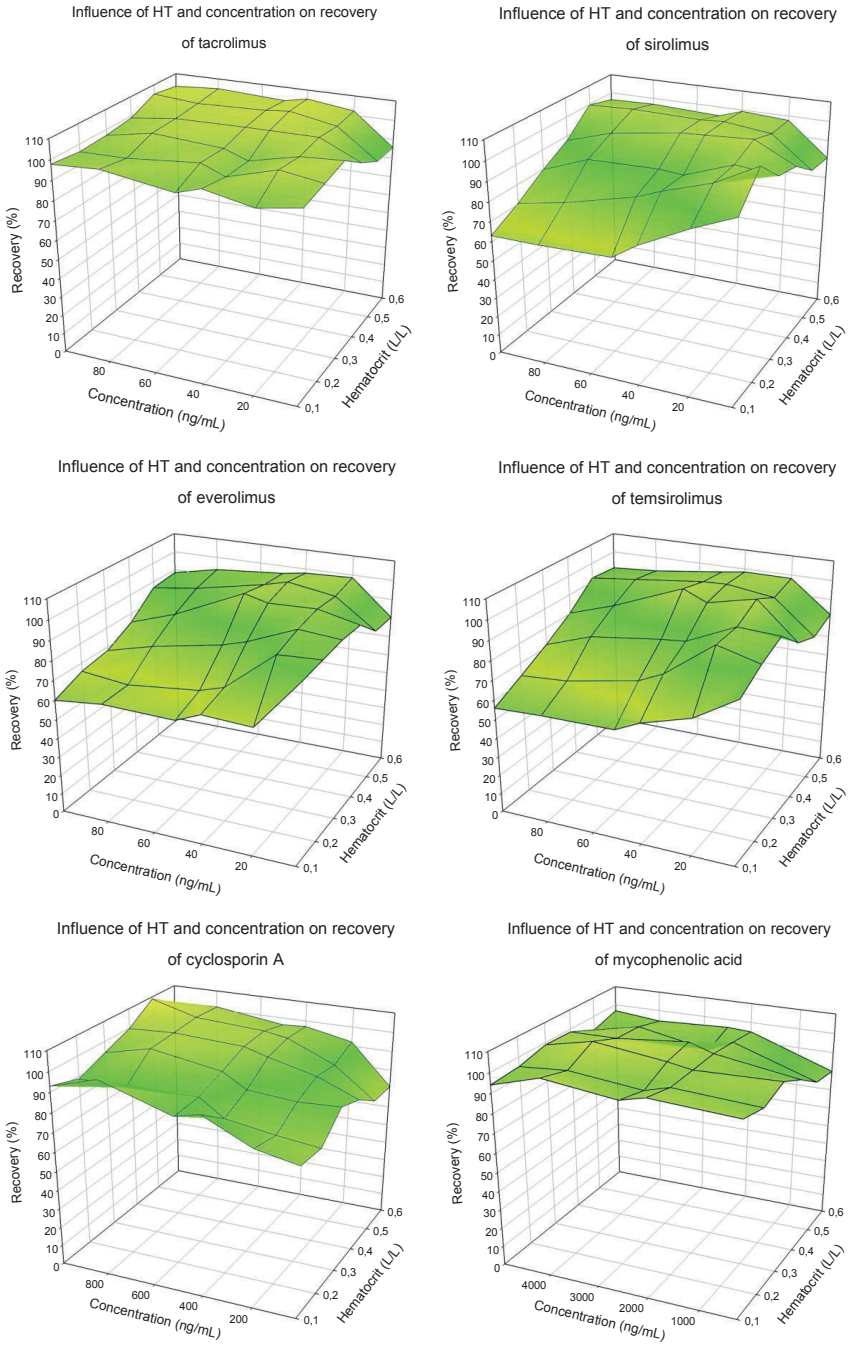

Figure 1. *3D plots of the recoveries of tacrolimus, sirolimus, everolimus, temsirolimus, cyclosporin A and mycophenolic acid. The following concentrations were tested. For tacrolimus, sirolimus, everolimus and temsirolimus: 3.00, 20.0, 40.0, 50.0, 80.0 and 100 ng/ml. For cyclosporin A: 30.0, 200, 400, 500, 800 and 1000 ng/ml. For mycophenolic acid: 300, 2000, 2500, 4000 and 5000 ng/ml. All concentrations were tested at the following hematocrit values: 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 l/l. HT: Hematocrit*

Analytical method validation

Calibration

TAC, SIR and EVE were validated with a linear range of 1.00–50.0 ng/ml with mean correlation coefficients of, respectively, 0.9987, 0.9984 and 0.9970. CYA was validated with a range of 10.0–500 ng/ml and a mean correlation coefficient of 0.9989. MPA was validated with a range of 100–15,000 ng/ml and a mean correlation coefficient of 0.9988. In Figure 2, chromatograms of all LLOQs are shown. For MPA, a linear curve fit with 1/X weighting was applied, while for all other analytes a linear curve fit with 1/X2 weighting was applied. All validated linear ranges are suitable for the measurement of trough concentrations and in most cases also for peak concentrations. For CYA, the linear range might not be sufficient for all peak concentration samples and a dilution would then be necessary.

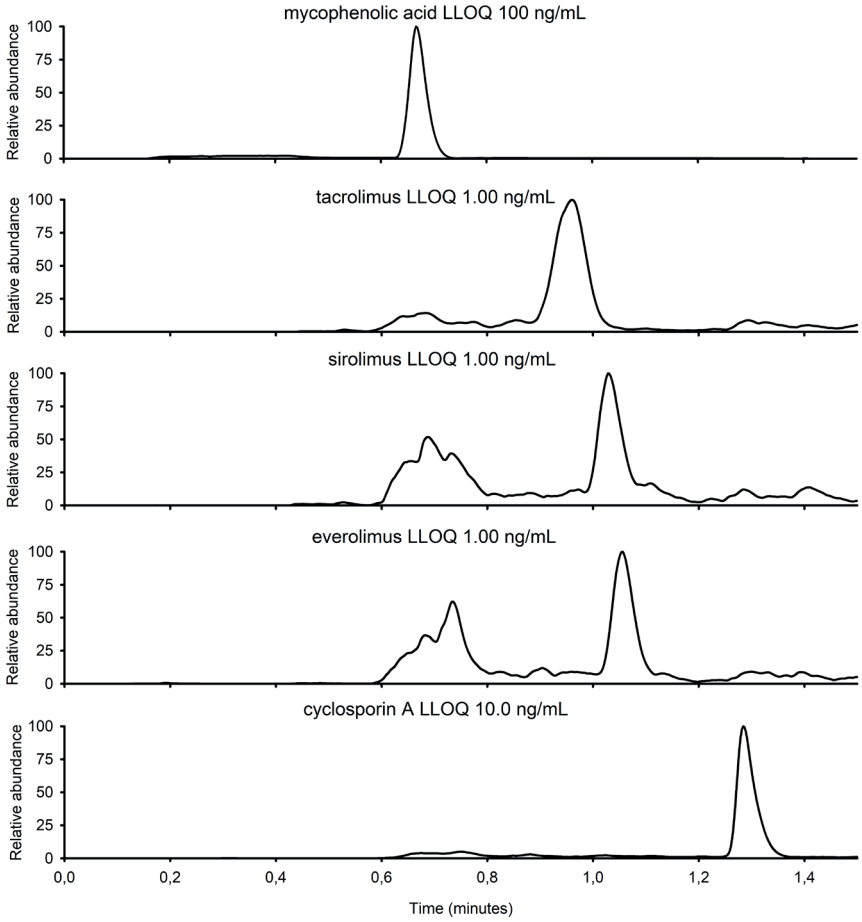

Figure 2. *Chromatograms of all validated analytes at LLOQ level.*

Accuracy & precision

The accuracy and precision results of the validation were well within requirements for all analytes and are summarized in Table 3. The maximum overall bias was observed at the LLOQ of SIR and was 9.6%. Although at the LLOQ of EVE the maximum overall CV was observed with 8.3%.

Table 3. *Mitra VAMS validation results of the accuracy (bias) and precision (coefficient of variation).*

Analyte	Concentration (ng/ml)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall bias (%)
Tacrolimus	LLOQ (1.0)	4.5	1.2	4.7	8.8
	Low (3.0)	6.1	2.9	6.8	-0.8
	Med (25)	4.6	2.7	5.3	4.1
	High (40)	5.5	2.4	6.0	2.8
	OC (200)	3.9	0.0	3.9	-9.0
Sirolimus	LLOQ (1.0)	4.6	6.0	7.5	9.6
	Low (3.0)	7.0	4.3	8.2	-1.4
	Med (25)	6.0	4.7	7.6	6.6
	High (40)	6.4	3.9	7.6	6.8
	OC (140)	7.5	5.8	9.5	-12.0
Everolimus	LLOQ (1.0)	8.3	0.0	8.3	7.6
	Low (3.0)	6.8	3.5	7.7	-0.4
	Med (25)	5.9	5.1	7.8	4.9
	High (40)	5.9	3.5	6.9	6.6
	OC (140)	7.1	3.9	8.1	-26.5
Cyclosporin A	LLOQ (10.0)	5.2	3.1	6.0	5.2
	Low (30.0)	5.8	0.0	5.8	-1.4
	Med (250)	4.5	3.6	5.8	3.8
	High (400)	5.1	0.0	5.1	3.6
	OC (2000)	3.6	0.4	3.6	-7.6
Mycophenolic acid	LLOQ (100)	3.6	4.5	5.8	9.3
	Low (300)	5.0	2.2	5.5	8.7
	Med (7500)	5.1	1.5	5.3	9.3
	High (12,500)	5.4	0.0	5.4	7.9

CV and Bias should be within 15% (20% for the LLOQ). OC stands for overcurve concentration where the extract was diluted ten-times with blank extract (N = 15). CV: Coefficient of variation; OC: Over-curve.

Dilution

Dilution of OC samples showed acceptable results for TAC, SIR and CYA, but unacceptable results for EVE (-26.5% bias). This is in-line with the observed deteriorated recoveries for EVE at high concentrations and low HT values. We hypothesize that the high

concentration of EVE of 140 ng/ml causes increased binding with the sampling material, which negatively influences the recovery. This is also in-line with the theory described in the section 'Investigation of the HT- and concentration-related recovery effects'. Although the standardized HT of 0.38 l/l was used to evaluate the OC dilution, the tested concentration was so high that the negative effect on the recovery was already present without the combination with a low HT. This effect was also noticed, but within acceptable limits, for SIR, with a bias of -12.0%. Dilution of an EVE sample extract is therefore not permitted. However, the large linear range of EVE of 1.00–50.0 ng/ml is sufficient for the measurement of trough levels and PK curves. Due to the large linear range of MPA, dilution was deemed not necessary and thus not validated for MPA. For VAMS sampling tips, dilution due to a too low amount of sample will not occur, since a VAMS tip will either be completely filled or is not suitable for analysis.

Stability

The validation of the stability results of TAC, SIR, EVE, CYA and MPA are shown in Table 4. Autosampler stability was proven for 7 days at 10°C for all analytes with a maximum overall bias of 7.4% for EVE. All analytes showed to be stable for at least 14 days at 25°C, 30 days at 37°C, 2 days at 50°C and 50 days at -20°C.

Table 4. *Results of the stability testing for all five analytes.*

Analyte	Stability	Time (days)	Low		High	
			CV (%)	Bias (%)	CV (%)	Bias (%)
Tacrolimus	AS 10°C	7	4.8	2.5	5.3	4.2
	Mitra 25°C	60	5.5	-6.5	4.2	-8.1
	Mitra 37°C	60	4.4	-10.8	6.0	-14.2
	Mitra 50°C	2	4.4	0.7	5.0	-1.2
	Mitra -20°C	50	2.9	-6.3	3.4	-4.7
	F/T 3	n.a.	9.9	-2.9	7.3	4.5
Sirolimus	AS 10°C	7	3.4	1.8	6.0	5.3
	Mitra 25°C	30	7.4	-12.9	5.2	-3.3
	Mitra 37°C	30	12.5	1.9	5.2	-2.2
	Mitra 50°C	2	9.3	-2.8	8.6	2.3
	Mitra -20°C	50	6.3	5.2	5.3	-9.8
	F/T 3	n.a.	12.0	7.0	7.1	2.1
Everolimus	AS 10°C	7	6.5	-0.3	6.2	7.4
	Mitra 25°C	60	6.0	-7.0	3.6	-12.3
	Mitra 37°C	30	9.9	6.0	2.6	-5.9
	Mitra 50°C	2	10.2	6.3	9.0	-1.6
	Mitra -20°C	50	6.9	-4.6	4.8	-6.5
	F/T 3	n.a.	7.5	-1.3	12.7	0.5

Table 4. *Continued*

Analyte	Stability	Time (days)	Low		High	
			CV (%)	Bias (%)	CV (%)	Bias (%)
	Mitra 25°C	14	5.8	-4.5	3.1	-9.5
	Mitra 37°C	30	5.5	-2.8	2.9	-3.1
	Mitra 50°C	2	5.0	3.4	5.7	5.5
	Mitra -20°C	50	2.4	10.0	4.0	7.7
	F/T 3	n.a.	4.6	2.1	7.1	6.9
Mycophenolic acid	AS 10°C	7	4.8	-0.8	5.5	3.7
	Mitra 25°C	60	2.1	-9.8	3.4	-13.5
	Mitra 37°C	30	2.5	-0.7	2.7	-7.0
	Mitra 50°C	2	2.5	2.5	5.5	-2.1
	Mitra -20°C	50	3.9	-4.4	3.2	-5.1
	F/T 3	n.a.	5.7	-0.6	5.4	-0.8

Low concentrations are 3.00 ng/ml for tacrolimus, sirolimus and everolimus, 30.0 ng/ml for cyclosporin A and 300 ng/ml for mycophenolic acid. High concentrations are 40.0 ng/ml for tacrolimus, sirolimus and everolimus, 400 ng/ml for cyclosporin A and 12,500 ng/ml for mycophenolic acid. AS is autosampler stability in processed sample. F/T 3 stands for three freeze/thaw cycles, n.a. stands for not applicable.
CV: Coefficient of variation

Extraction recovery, matrix effect & process efficiency

Extraction recoveries higher than 85% and process efficiencies higher than 87% were observed for all analytes, with the use of SIL IS correction at the standardized HT of 0.38 l/l and 48 h drying time. With the use of SIL IS correction, no matrix effect was observed for all analytes (Table 5). SIR was the only analyte that was validated without its own SIL IS, but with the SIL IS of EVE. The IS corrected matrix effects showed a maximum of -7.3% matrix effect for SIR with the EVE [$^{13}C_2$,$^{2}H_4$] as internal standard. This concludes that there is either no ion suppression or that it is corrected for with the used SIL IS of SIR.

Table 5. *Results of the extraction recovery, matrix effect and process efficiency calculated with analyte/internal standard response ratios.*

Analyte	Concentration (ng/ml)	Extraction recovery (%)	Matrix effect (%)	Process efficiency (%)
Tacrolimus	Low (3.0)	102.8	-0.5	102.3
	High (40)	100.0	0.3	100.3
Sirolimus	Low (3.0)	105.3	-7.3	97.6
	High (40)	91.1	-3.6	87.7
Everolimus	Low (3.0)	98.1	0.7	98.8
	High (40)	87.6	2.0	89.4
Cyclosporin A	Low (30.0)	85.3	3.1	88.0
	High (400)	85.7	2.3	87.6
Mycophenolic acid	Low (300)	97.5	1.6	99.1
	High (12,500)	92.6	2.2	94.7

Influence of the HT

The possible HT effect on the bias at low and high concentrations with the standard HT set at 0.38 l/l and 24 h drying time is shown in Table 6. TAC showed not to be affected by the various HTs, with a maximal bias of -8.3% for HT 0.20 l/l at 40 ng/ml. This is in-line with the findings of Kita et al.[12] The biases of CYA exceeded 15% for the HT of 0.20 l/l (low: -24.8% and high: -20.9%). The bias was within 15% for all other analytes, with the maximum bias being -11.7% for both SIR and EVE at the high level and HT 0.20 l/l. Extra HT levels were evaluated for CYA and at the HT of 0.27 l/l and the biases were acceptable with -10.7 and -13.0% for low and high, respectively.

With the previously described DBS analysis method, there was no need for HT correction for TAC, SIR, EVE, CYA and MPA.[6] However, this was only valid between HT values of 0.23–0.53 l/l and concentrations of 3.0–10.0 ng/ml for TAC, SIR and EVE, 60.0–200 ng/ml for CYA and 300–12,000 ng/ml for MPA.[6] With the use of VAMS, this is significantly improved for TAC, SIR and EVE and there is no need for HT correction in the HT range of 0.20–0.60 l/l at tested concentrations of 3.0–40.0 ng/ml. For CYA, compared with DBS, a larger HT and concentration range with the use of the VAMS tips was established with a HT range of 0.27–0.60 l/l at tested concentrations of 30.0–400 ng/ml .[6] Although the HT range for CYA was increased with the use of the VAMS tips, the lowest acceptable HT value was 0.27 l/l, while the DBS method showed no significant biases at the lowest HT of 0.23 l/l.[6] For the intended setting with transplant outpatients, the lowest applicable HT of 0.27 l/l is very likely to be sufficient to cover the HT range of the patient population.[19]

Table 6. *Effect of the hematocrit on the bias at low and high concentrations with the standard hematocrit set at 0.38 l/l and 24 h drying time.*

Hematocrit	Tacrolimus				Sirolimus				Everolimus				Cyclosporin A				MPA			
(l/l)	3.0 ng/ml		40 ng/ml		3.0 ng/ml		40 ng/ml		3.0 ng/ml		40 ng/ml		30 ng/ml		400 ng/ml		300 ng/ml		12,500 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 (%)
0.20	3.1	-5.0	2.7	-8.3	4.4	-2.4	1.3	-11.7	7.5	-2.8	1.3	-11.7	3.6	-24.8	2.0	-20.9	3.4	2.7	2.0	-2.1
0.30	3.1	2.8	5.1	-3.4	4.2	6.1	5.6	-3.8	4.6	3.4	3.8	-2.7	4.7	-7.2	5.2	-8.0	1.7	1.4	5.4	-2.7
0.38	2.8	0.0	3.8	0.0	2.6	0.0	7.1	0.0	3.8	0.0	4.4	0.0	4.5	0.0	3.8	0.0	2.7	0.0	4.2	0.0
0.40	3.4	-1.3	4.3	-4.1	6.1	-1.2	4.3	-5.0	6.1	0.1	3.7	-3.2	3.2	-0.2	6.5	-2.1	2.1	-1.2	3.1	-3.8
0.50	1.7	3.5	3.8	-2.0	7.1	3.9	5.3	-4.0	5.4	2.4	4.9	-0.3	4.0	7.3	3.9	0.8	2.0	-2.3	3.6	-1.0
0.60	4.1	0.1	2.0	-5.9	6.9	-0.1	1.1	-10.5	6.2	-1.8	2.3	-5.9	9.7	12.7	2.2	-2.1	4.2	-0.3	1.8	-6.5

CV: Coefficient of variation.

Influence of the drying time & HT on the recovery

To evaluate the differences between the drying times and HT values on the recovery at each analyte concentration level, the percentage recovery found at 48 h drying at HT 0.40 l/l was set as the standard and the difference in percentage recovery found at a certain HT and drying time was calculated. The evaluation of the drying time showed several biases exceeding 15% (Table 7). SIR showed a bias of -18.3% at the high analyte concentration level, HT 0.20 l/l and 48 h drying. EVE showed several biases exceeding 15%, where the biases at the high analyte concentration level, HT 0.20 l/l and 24 and 48 h drying were -18.3 and -32.4%, respectively. These biases of SIR and EVE can be explained by the binding of SIR and EVE with the sampling matrix.[5,6,15] The other biases of EVE exceeding 15% were for 3 h drying at the low analyte concentration level (bias 15.1%, HT 0.60 l/l) and at the high analyte concentration level (bias 16.0%, HT 0.40 l/l). For CYA, a bias of 15.3% is observed at the high analyte concentration level, HT 0.40 l/l and 3 h drying. These biases of 16.0, 15.1 and 15.3% for EVE and CYA at 3 h drying are somewhat random and cannot be explained by interaction of the analyte with the sampling matrix, nor is a trend relating to the HT or concentration observed. Insufficient drying of the sampled blood may cause higher but unstable recoveries. This could explain the observed positive biases. It can be concluded that a drying time of 3 h is not recommended. This is in-line with the findings of Fang et al.[10] For CYA, where two additional biases of -16.8 and -20.3% were observed at the low and high level, HT 0.20 l/l and 48 h drying, respectively. These results for CYA are in-line with the evaluation of the results of the HT effects presented in the previous paragraph, which were performed in a separate previous experiment. For TAC and MPA, all biases were within 15% with the maximum biases being -14.4% (high, HT:0.20 l/l, 48 h drying) and 11.3% (high, HT:0.20 l/l, 24 h drying), respectively.

It can be concluded that the drying time of the VAMS tips is of no structural influence for all analytes within a HT range of 0.30–0.60 l/l. At the extremely low HT of 0.20 l/l SIR, EVE and CYA have deteriorating recoveries, which leads to unacceptable biases. The tested HT of 0.20 l/l is very unlikely to be encountered within the home sampling patient population, for which this sampling technique and analysis method is intended.[19]

Before application of this analysis method in a clinical setting, a clinical validation study should be performed to assess if results from VAMS tips are comparable to results from venous whole blood.[19–21] The observed HT-related recovery effects are the only HT effects that influence the final results of the VAMS tips. Although for DBS, an additional HT effect is caused when using partial spot analysis.[4] The lower viscosity of the blood at low HT decreases the amount of blood that is present in the punched DBS and vice versa at high HT, causing negative and positive biases, respectively. For both DBS and VAMS tips, decreasing recoveries at high HTs could be caused by a suboptimal extraction procedure, which is unable to diffuse through the increased amount of dried blood cells at high HT values. For TAC, no analyte binding of the sampling matrix and no influence of

the drying time and HT on the recovery was observed (Table 7). In the study of Kita et al., minimal impacts of the HT on the accuracy of TAC were found, which are in-line with our findings for TAC.[12] However, Kita et al. found that ambient stability tests showed lowered accuracies after 3 days of storage, which were attributed to reduced recovery rather than instability.[12] In our stability experiments, TAC proved stable at ambient temperature for 60 days (Table 4). The reduced recovery could be caused by a suboptimal extraction procedure.

In the study of Verheijen et al., the EVE VAMS assay showed positive biases at the HT of 0.31 l/l and negative biases at the HT of 0.49 l/l. This is not in line with our findings, but this phenomenon seems to be frequently encountered with VAMS extraction methods. Reduced recoveries at high HT values and after prolonged drying time indicate a suboptimal extraction procedure.[8–13] Since TAC and MPA showed no HT and concentration dependent adsorption to the VAMS sampling material, these analytes give good insight in the efficiency of the developed extraction procedure. The possibility of reduced HT and concentration dependent recoveries after extensive drying of the VAMS tip should be taken into account when an extraction method is developed. With this in mind, the developed 2-step extraction procedure provides good recoveries for the whole HT range of TAC and MPA and proves that the developed extraction procedure performs well in the whole HT range.

Conclusion

A robust extraction and analysis method for TAC, SIR, EVE, CYA and MPA in VAMS tips has been developed and extensively validated. HT- and concentration-related recovery effects were observed but less pronounced when compared with DBS analysis and the HT-related effects were within requirements of the purpose of the analytical method.

Future perspective

This study showed that the analyte adsorption to the sampling matrix does not only occur with the DBS card matrix but also with the VAMS matrix. For future dried microsampling methods, the effect of the combination of the HT and analyte concentration should always be evaluated. In the near future, newly developed microsampling materials are hopefully able to fixate the analyte on a dried sampling material without irreversible analyte adsorption. Before this method can be used in clinical practice, the method should be clinically validated, where patient whole blood samples are compared with fingerprick VAMS samples.[21] Various studies proving clinical validity are currently being conducted.[22] In addition, because currently no external control programs exist for immunsuppresant fingerprick methods, an external quality control scheme will have to be setup in order to independently monitor the performance of the VAMS analysis method.[23]

Table 7. *Influence of the drying time and hematocrit on the recovery at low and high concentrations compared with the standard drying time of 48 h and HT of 0.40 l/l*

Analyte	Hemato-crit (l/l)	3 h drying				24 h drying				48 h drying			
		Low		High		Low		High		Low		High	
		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
Tacrolimus	0.20	4.2	6.4	3.3	3.6	5.7	3.8	3.3	-1.7	4.6	1.2	5.4	-14.4
	0.30	3.5	6.6	9.0	-3.4	3.2	3.6	3.3	-1.6	3.5	1.6	5.8	-1.0
	0.40	3.8	5.4	4.2	7.3	5.0	1.8	4.4	4.4	2.4	0.0	4.8	0.0
	0.50	3.2	12.2	3.7	5.1	3.9	3.7	5.1	0.8	2.9	0.8	4.3	1.0
	0.60	5.9	11.5	4.8	-3.6	4.5	2.5	8.1	-9.3	3.7	2.6	5.2	-5.2
Sirolimus	0.20	7.1	8.4	5.8	4.9	10.8	5.0	3.2	-5.0	5.5	-2.0	4.2	-18.3
	0.30	6.8	10.2	14.7	-6.0	9.4	-0.7	2.5	-0.8	6.6	0.3	6.5	-3.7
	0.40	3.8	7.3	8.3	14.6	11.1	-0.3	5.1	8.4	3.9	0.0	5.1	0.0
	0.50	4.1	9.2	4.4	8.8	9.4	-4.5	5.2	3.4	3.6	4.0	4.1	2.2
	0.60	6.9	14.2	7.0	-2.2	9.4	-4.3	8.5	-9.4	8.8	2.5	7.6	-3.5
Everolimus	0.20	6.2	12.7	4.2	1.0	10.7	6.0	2.8	-18.3	5.2	0.8	5.0	-32.4
	0.30	5.0	10.5	13.9	-6.3	5.9	7.0	2.8	-6.5	6.1	0.4	7.3	-10.3
	0.40	5.1	8.4	9.6	16.0	8.5	2.5	6.0	8.2	5.0	0.0	6.5	0.0
	0.50	5.2	11.6	3.8	11.2	5.6	3.5	6.0	3.3	3.3	0.6	5.2	2.0
	0.60	6.4	15.1	6.0	-0.4	5.5	1.0	8.9	-7.2	4.6	1.3	7.6	-2.9
Cyclosporin A	0.20	3.1	0.8	4.7	1.3	7.4	-10.7	4.4	-9.9	5.3	-16.8	5.5	-20.3
	0.30	2.7	6.8	4.1	1.8	3.9	0.3	3.7	-3.9	5.3	-4.6	5.4	-4.3
	0.40	3.9	6.9	6.7	15.3	6.4	2.2	4.5	7.7	2.8	0.0	4.4	0.0
	0.50	2.0	13.9	4.6	10.9	5.3	8.7	4.2	4.6	2.8	6.3	3.9	2.4
	0.60	5.5	14.5	5.0	-1.2	5.2	9.0	8.4	-4.9	3.2	6.2	4.9	-2.0
Mycophenolic acid	0.20	2.7	8.0	3.4	9.8	7.2	6.4	3.6	11.3	4.2	1.7	5.0	4.6
	0.30	2.7	7.4	4.5	5.8	5.0	5.1	3.3	4.0	3.8	2.2	5.9	6.2
	0.40	3.4	6.5	3.8	6.6	5.4	0.5	2.8	0.7	2.1	0.0	5.0	0.0
	0.50	1.3	10.5	5.0	6.8	5.0	3.4	3.8	0.8	1.5	3.2	4.2	0.4
	0.60	4.4	10.4	4.8	8.3	4.5	2.0	8.2	1.3	4.0	1.8	4.9	4.0

Low concentrations are 3.00 ng/ml for tacrolimus, sirolimus and everolimus, 30.0 ng/ml for cyclosporin A and 300 ng/ml for mycophenolic acid. High concentrations are 40.0 ng/ml for tacrolimus, sirolimus and everolimus, 400 ng/ml for cyclosporin A and 2000 ng/ml for mycophenolic acid. Calculated as the substracted difference between the percentage recovery found at 48 h drying at HT 0.40 l/l and the percentage recovery found at a certain HT and drying time. This was evaluated for each concentration.

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Chapter 10

Volumetric Absorptive Micro Sampling and Dried Blood Spot Micro Sampling versus Conventional Venous Sampling for Tacrolimus Trough Concentration Monitoring

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Submitted

Abstract

Background: Monitoring tacrolimus blood concentrations is important for preventing allograft rejection in transplant patients. Our hospital offers Dried Blood Spot (DBS) sampling, giving patients the opportunity to sample a drop of blood from a fingerprick at home, which can be sent to the laboratory by mail. In this study, both a Volumetric Absorptive Microsampling (VAMS) device and DBS sampling are compared to venous whole blood sampling.

Methods: 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 Passing-Bablok regression. Bias was assessed using Bland-Altman. A multidisciplinary team pre-defined an acceptance limit requiring >80% of all paired samples within 15% of the mean of both samples. Sampling quality was evaluated for both VAMS and DBS samples.

Results: 32.3% of the VAMS samples and 6.2% of the DBS samples were of insufficient quality, leading to 88 paired samples fit for analysis. 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). Both VAMS (after correction for the slope) and DBS showed no significant bias in Bland-Altman analysis. For VAMS and DBS, the acceptance limit was met for resp. 83.0% and 96.6% of the samples.

Conclusion: VAMS sampling can replace whole blood sampling for tacrolimus trough concentration monitoring, but VAMS sampling is currently inferior to DBS sampling, both regarding sample quality and agreement with whole blood tacrolimus concentrations.

Introduction

Therapeutic Drug Monitoring (TDM) of immunosuppressant drugs has been part of routine transplant patient care for decades. Sub therapeutic dosing of immunosuppressants such as tacrolimus can lead to rejection of the allograft, while overdosing can lead to toxicity and side-effects, including diabetes and nephrotoxicity.[1] Because of great inter- and intra-individual variation in pharmacokinetics (PK), dosing of these drugs is tailored for each patient based on the blood drug concentration. This makes frequent patient visits to the hospital for venous blood sampling mandatory. In the past years, several Dried Blood Spot (DBS) microsampling methods for tacrolimus have been introduced, enabling patient home sampling.[2-11] Through a fingerprick, capillary blood is directly applied to special filter paper. After drying, the sample can be send to the laboratory by mail. This decreases patient burden and allows more flexible immunosuppressant monitoring.[8,12] Several of these DBS methods have shown to yield interchangeable results with venous whole blood (WB) and are routinely applied in transplant patient care since a few years, including in our hospital.[2,3,11,13] A drawback of DBS application is that sampling by the patient does not always lead to sufficient quality DBS samples, rates of up to 20% invalid samples for patient home sampled DBS have been reported.[11,14-16] Volumetric Absorptive Micro Sampling (VAMS) was introduced as a potential successor of DBS sampling. VAMS tips are designed to have several advantages compared to DBS. They wick-up an exact amount of sample volume, independent of hematocrit, and potentially improve the ease of sampling for the patient.[17-19] Although the effects of the hematocrit on the sample volume can be overcome by VAMS, this does not necessarily apply for the effect of hematocrit on extraction recovery from VAMS tips.[20-23]

A recent study shows that tacrolimus can be reliably measured in VAMS throughout the complete dose interval of tacrolimus in renal transplant patients when comparing fingerprick VAMS (Mitra®) results to paired venous whole blood samples.[24] However, in the latter study, sample quality of VAMS was not discussed. In addition, there are no studies that directly compare the performance of fingerprick VAMS to fingerprick DBS for immunosuppressants. Only one study exists where fingerprick VAMS (Mitra®) samples and fingerprick glass capillary tube samples (Drummond Aqua-Cap®) were compared to venous WB samples for the drug radiprodil showing an underestimation of radiprodil exposure in VAMS (but not for capillary tube sampling) compared to venous WB.[25]

In the current study, we compared both a novel VAMS sampling device (Mitra®) and conventional DBS sampling to venous WB sampling with regards to interchangeability of analytical results and sample quality.

Methods

Training of phlebotomists

For the DBS sampling, all phlebotomists were trained at the time DBS sampling was introduced (2016). At that time, the training consisted of a 15-minute lecture explaining the sampling procedure, including common pitfalls and how to avoid them.

Because VAMS sampling was new in our hospital, the same phlebotomists were trained specifically for the VAMS sampling procedure. Although individual training of phlebotomists, including performing the sampling method, is preferred, this was not feasible for one study coordinator for approximately 75 phlebotomists.[26,27] Therefore, similar to the previous DBS validation studies performed in our hospital, all phlebotomists were trained in a 15-minute lecture explaining the sampling procedure, including common pitfalls and how to avoid them based on information provided by literature and the manufacturer of the VAMS tips (Neoteryx, Torrance, CA, USA).[2,13,19,28]

Patients, sample collection and sample quality

Patient samples were collected from tacrolimus-using adult kidney transplant patients during routine visits to the University Medical Center Groningen (UMCG, the Netherlands) for nephrologist consultation and TDM. 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 UMCG (Metc 2011.394). All samples were obtained within 10 minutes of each other by the same phlebotomist following written instructions available at time of sampling. First, the WB sample was obtained. Afterwards, a fingerpick was performed, and a DBS sample was obtained by letting 2 drops of blood fall freely on a Whatmann DMPK-C cards (GE Healthcare, Chicago, IL, USA) following a previously described method.[27] From the same fingerpick, two 20 µL VAMS tips (Mitra®, Neoteryx) were filled according to the manufacturer's instructions. Because the WB samples were part of routine care, they were analyzed within a day. After receiving the DBS and VAMS samples, they were inspected independently by two experienced lab technicians for quality, based on predefined criteria described earlier.[15,27,29,30] If the judgment of the technicians differed, consensus was obtained by discussing each other's judgment. The DBS and VAMS samples were dried for at least 24 hours at room temperature and packed in sealed plastic bags with a desiccant. The samples were stored at -20°C until analysis was performed. Stability of tacrolimus in DBS samples was validated for 29 weeks and in VAMS samples for 50 days at -20°C, so analysis occurred within these timeframes, respectively.[23,31,32]

Equipment and procedures

Hematocrit of the WB samples was measured using a XN10/XN20 hematology analyzer (Sysmex, Kobe, Japan).

Tacrolimus concentrations were analyzed in the WB samples using a validated analysis

method on a Thermo Fisher Scientific triple quadrupole Quantiva MS/MS system with a Thermo Fisher Scientific Vanquish UPLC system (Waltham, MA, USA).[33] Tacrolimus DBS samples were analyzed using a validated method on the aforementioned Thermo Fisher Scientific LC-MS/MS system.[31,32,34] The VAMS samples were analyzed for tacrolimus using a validated method on the aforementioned Thermo Fisher Scientific LC-MS/MS system.[23]

Statistical analysis

Clinical validation was performed based on relevant guidelines by the CLSI, FDA, EMA and the recently published Guideline on Development and Validation of Dried Blood Spot–Based Methods for Therapeutic Drug Monitoring.[27,35-37] In short, method comparison was performed using Passing-Bablok regression analysis.[38] Bland-Altman analysis was used to calculate bias.[39] The limit of clinical acceptance was set a priori at 85%-115% around the ratio of paired WB-DBS and paired WB-VAMS samples for at least 80% of the samples in accordance with earlier studies.[13,27] These limits were chosen in a multidisciplinary team consisting of transplantation nephrologists, pharmacists and analysts and were based on current trough concentration targets and the relevant concentration window for tacrolimus in kidney transplantation in combination with the aspects of the analytical method used for VAMS, DBS and WB.[1,13,31-34] It is unlikely that a difference of <15% between WB and either DBS or VAMS would lead to a different choice by the clinician in dosing tacrolimus. The predictive performance of both the DBS and VAMS method was established using the method described by Sheiner and Beal.[40] In short, WB concentrations were predicted from both DBS and VAMS concentrations according to a previously described method.[3,13,27] The bias of the prediction 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). The following equations were used:

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

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

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

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

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%.[3,6,13,41] Statistical analysis was performed using Analyse-it® Method Validation Edition for Microsoft Excel version 4.18.6 (Analyse-it, Leeds, UK) and Microsoft Excel 2010 (Microsoft Inc., Redmond, WA, USA). All categorical data were expressed as percentages, numeric data were expressed as mean ± standard deviation (SD) and range or as median and range.

Results

Sample Quality

In total, 130 paired samples were obtained from 107 adult kidney transplant patients between June 2018 and October 2018. For the VAMS samples, 42 (32.3%) of the samples were rejected because of insufficient quality, 26 samples (20.0%) contained one sufficient quality tip and 62 samples (47.7%) contained two sufficient quality tips. Consensus between technicians was needed for eight (6.2%) of the VAMS samples. Three reasons for VAMS sample rejection were identified: (1) For 31 individual tips, the tip touched the cap of the sampling container caused by improper closing of the cap (Figure 1B), (2) For 30 individual tips, the tip was oversaturated, caused by letting blood fall on the tip instead of dipping the tip in the blood (Figure 1C), (3) For 39 individual tips, the tip was undersaturated, caused by not enough blood obtained from the fingerprick or not dipping the tip into the blood long enough (Figure 1D). An analysis was performed to evaluate if a learning effect over time could be observed on VAMS sampling. The percentage of sufficient quality tips for the first half of the samples was similar to the percentage of sufficient quality tips for the last half of the samples (63.8% and 66.9% respectively), showing no learning effect. For the DBS samples, eight samples (6.2%) were rejected because of insufficient quality, 23 samples (17.7%) contained one sufficient quality spot and 99 (76.2%) of the samples contained two sufficient quality spots.

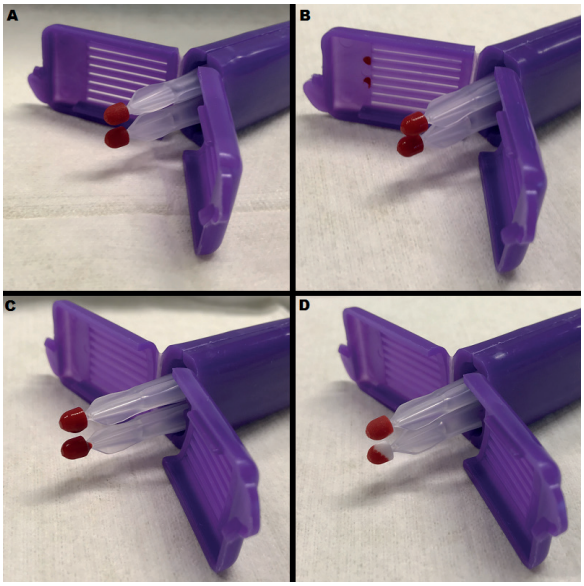

Figure 1. *Different types of quality in 20 µL Volumetric Absorptive Micro Sampling (VAMS) samples. A: Sufficient quality VAMS sample meeting all requirements. B: Insufficient quality VAMS sample because the containers' cap touched the tip, blood is visible on the inside of the cap. C: Insufficient quality VAMS sample because of oversaturation, blood is visible on the tip holder. D: Insufficient quality VAMS sample due to undersaturation, the tip is not completely filled with blood.*

Patients

In total, 88 paired samples from 72 unique patients were included in the method comparison analysis. Patient demographics are summarized in Table 1. The average concentrations of tacrolimus in WB, DBS and VAMS, and the average hematocrit values can be found in Table 2. All tacrolimus concentrations were within the analytically validated range. All hematocrit values were within the analytically validated range.

Table 1. *Patient demographics*

Patient demographics	n	Median (range)
Age, years	72	58 (21 – 78)
Sex	72	42 male (58.3%) 30 female (41.7%)
Time since transplantation	72	1 year, 7 months, 25 days (22 days – 16 years, 4 months)

Table 2. *Average tacrolimus and hematocrit concentrations including SD and range*

Concentration	n	Average ± SD (range)
Tacrolimus in WB, µg/L	88	6.5 ± 3.1 (3.0 – 24.3)
Tacrolimus in DBS, µg/L	88	6.4 ± 3.1 (2.8 – 23.5)
Tacrolimus in VAMS, µg/L	88	5.8 ± 2.8 (2.8 – 15.8)
Hematocrit (v/v)	88	0.39 ± 0.05 (0.25 – 0.50)

WB, whole blood; DBS, dried blood spot; VAMS, volumetric absorptive micro sampling; SD, standard deviation

Clinical validation of VAMS

The Passing-Bablok fit was y = 0.88x + 0.01 (95% CI slope, 0.81 – 0.97; 95% CI intercept, -0.47 – 0.39) showing no significant constant difference. A significant systematic difference of 12% lower tacrolimus concentration in VAMS compared to WB was observed (Figure 2). This systematic difference was used to derive the following conversion formula: [Tacrolimus WB concentration] = [Tacrolimus VAMS concentration] / 0.88. This conversion formula was used to recalculate all VAMS values, these recalculated values were used in Bland-Altman analysis.[27] No significant bias was found in Bland-Altman analysis, with a mean ratio WB/VAMS of 1.00 (95% CI 0.98-1.02) as shown in Figure 2. In total, 83.0% of the paired samples are within the limits of clinical acceptance meeting the requirement of at least 80%. Because of the correction factor used, the bias estimation in the predictive performance was small with an MPE of 0.00 µg/L and a MPPE of 0.00%. The predictive performance of imprecision as shown by the RMSE was small with a value of 0.54 µg/L. The MAPE was within acceptable limits (<15%) with a value of 8.74%. The acceptance limit for MAPE

(>67% of samples with a value <20%) was met with 82 out of 88 samples (93.2%) (Figure 3).

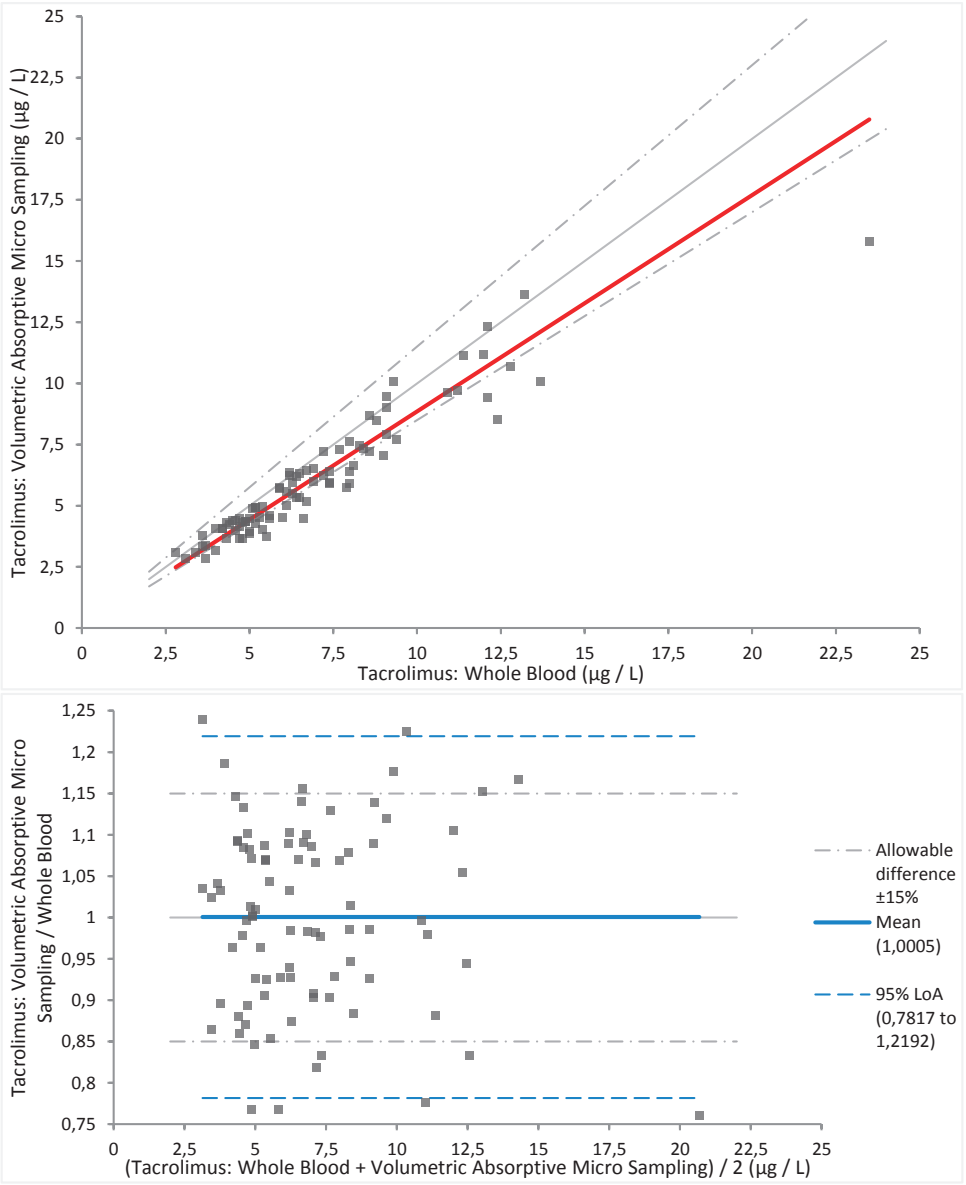

Figure 2. *Method comparison between whole blood tacrolimus levels and Volumetric Absroptive Micro Sampling (VAMS) tacrolimus levels for 88 paired samples. In the upper panel, the continuous line is the Passing-Bablok regression line y = 0.88x + 0.01 (95% CI slope, 0.81 – 0.97; 95% CI intercept, -0.47 – 0.39). The dotted/dashed line is the 15% limit of clinical acceptance. The lower panel shows the Bland-Altman analysis bias estimation based on recalculated values for VAMS using the formula [Tacrolimus WB concentration] = [Tacrolimus VAMS concentration] / 0.88. Calculated bias is 1.00 (95% CI 0.98-1.02). The dotted/dashed line is the 15% limit of clinical acceptance. The dashed line is the 95% Limits of Agreement (LoA).*

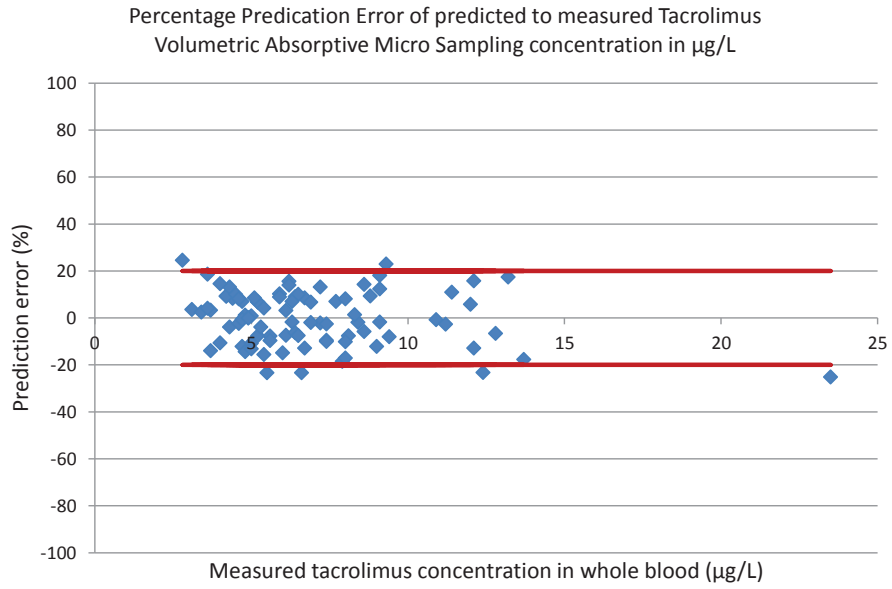

Figure 3. *Percentage prediction error of predicted to measured Tacrolimus Volumetric Absorptive Micro Sampling concentrations with acceptable prediction error set at -20% and 20% after applying the formula [Tacrolimus WB concentration] = [Tacrolimus VAMS concentration] / 0.88.*

Clinical validation of DBS

The Passing-Bablok fit was y = 0.99x + 0.02 (95% CI slope, 0.95 – 1.04; 95% CI intercept, -0.26 – 0.28) showing no significant systematic or constant difference between WB and DBS as shown in Figure 4. Bland-Altman analysis shows no significant bias, with a mean ratio WB/DBS of 1.01 (95% CI 0.99-1.02) as shown in Figure 4. The 95% Limits of Agreement (LoA) are within the limits of clinical acceptance set at ±15%. In total, 96.6% of the paired samples are within the limits of clinical acceptance meeting the requirement of at least 80%. The bias estimation in the predictive performance was small with an MPE of 0.00 µg/L and a MPPE of -0.04%. The predictive performance of imprecision as shown by the RMSE was small with a value of 0.32 µg/L. The MAPE was within acceptable limits (<15%) with a value of 5.18%. The acceptance limit for MAPE (>67% of samples with a value <20%) was met with 87 out of 88 samples (98.9%) (Figure 5).

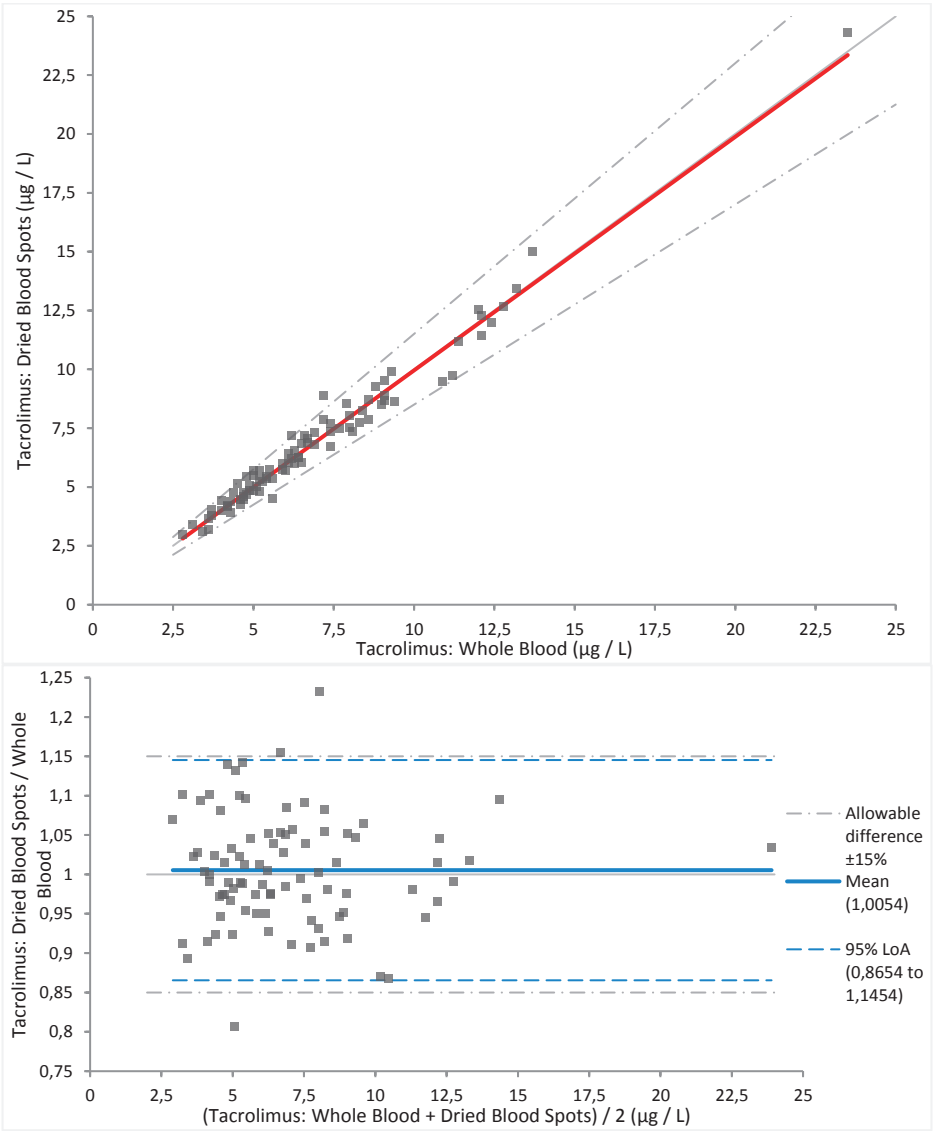

Figure 4. *Method comparison between whole blood tacrolimus levels and Dried Blood Spot tacrolimus levels for 88 paired samples. In the upper panel, the continuous line is the Passing-Bablok regression line y = 0.99x + 0.02 (95% CI slope, 0.95 – 1.04; 95% CI intercept, -0.26 – 0.28). The dotted/dashed line is the 15% limit of clinical acceptance. The lower panel shows the Bland-Altman analysis bias estimation of 1.01 (95% CI 0.99-1.02). The dotted/dashed line is the 15% limit of clinical acceptance. The dashed line is the 95% Limits of Agreement (LoA).*

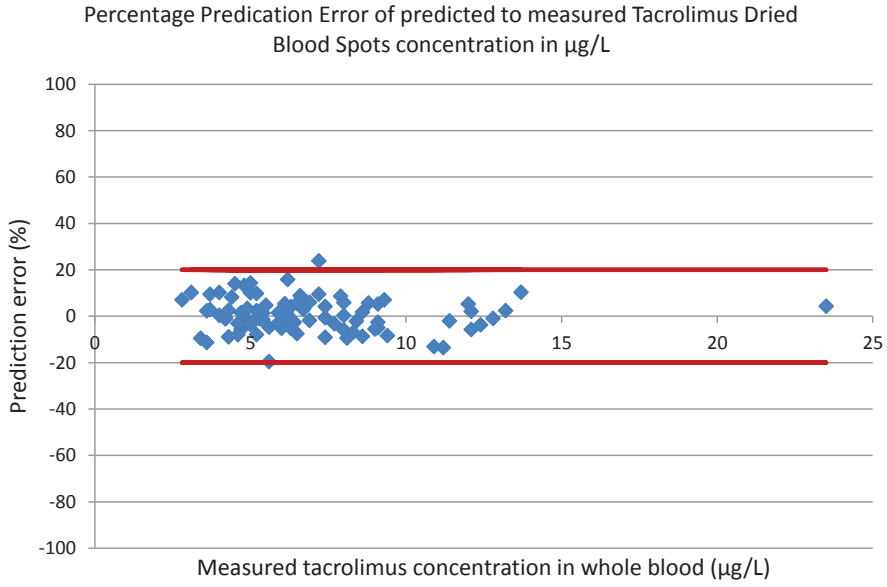

Figure 5. *Percentage prediction error of predicted to measured Tacrolimus dried blood spots concentrations with acceptable prediction error set at -20% and 20%.*

4. Discussion

This study showed good agreement between tacrolimus VAMS and tacrolimus WB concentrations, and very good agreement between tacrolimus DBS and tacrolimus WB concentrations in kidney transplant patients over a relevant range of trough concentrations. The predictive performance of both the VAMS and DBS meet the predefined criterion of >67% of the samples to have a prediction error of <20%. Both VAMS and DBS meet the predefined limits of clinical acceptance and can be used in transplant patient care. For our VAMS method, the analytical results should be corrected with the conversion formula [Tacrolimus WB concentration] = [Tacrolimus VAMS concentration] / 0.88.

The conclusion that DBS performs better than VAMS was unexpected. We considered that this might be caused by the fact that DBS sampling has been used for over 3 years in our hospital, allowing DBS sampling and DBS analysis to improve. In our previous validation studies, performed prior to DBS implementation in routine care, no limits of clinical acceptance were set.[2,32] In order to get more insight in the performance during the early adoptive phase of DBS, we applied the limits of clinical acceptance used in this study to the data of the previous studies. These limits would not be met with only 78.9% (n = 82/104)[2] and 80.0% (n = 70/85)[32] of paired samples having a WB/DBS ratio between 85%-115%. It will be difficult to conclude what the exact improvements in the complete DBS chain were. The fact that the performance has improved over time can be attributed

to improvements in DBS sampling and/or DBS analysis method or even the whole blood analysis which is used as the golden standard.

During VAMS analytical validation, recovery of tacrolimus was stable across a wide hematocrit range (0.20 – 0.60 v/v) and concentration range (3.0 µg/L – 40 µg/L), with a maximal bias of -8.3% at extreme values for hematocrit and tacrolimus concentration (respectively 0.20 v/v and 40 µg/L).[23]Therefore, it was unexpected that the VAMS method showed a significant systematic difference of 12% lower tacrolimus concentration in VAMS compared to WB samples.

Because of insufficient sample quality, only 62 duplicate VAMS samples were available for analysis.[27] Method comparison using the mean value of the duplicate samples yielded a similar conversion formula for VAMS in Passing-Bablok analysis and similar bias in Bland-Altman analysis (data not shown). The duplicate analysis also showed that 17 of the 62 analysis results of the two duplicate VAMS tips differed >10% compared to the mean of both samples. When these results were excluded, the Passing-Bablok analysis and bias in the Bland-Altman analysis results were still similar (data not shown). Since the analytical method was validated for the use of VAMS analysis in singlicate and proved to be accurate and precise, the duplicate VAMS analysis showed that two correctly sampled VAMS tips will generate the same results. It can thus be concluded that duplicate VAMS sample analysis has no positive effect on the quality of the analysis results and has no added benefit.

Other studies report both lower and higher concentrations in VAMS compared to WB for various drugs.[20,25] The study by Kita et al. reported an average of 14% higher AUC for tacrolimus in rat tail blood collected in VAMS compared to wet rat tail blood samples.[42] In the study by Vethe et al., who performed a clinical validation study for tacrolimus with paired WB and VAMS samples from 2 full 12-hour PK curves of 27 adult renal transplant patients totaling 679 paired samples of which 105 were trough concentrations, no significant systematic differences are observed between WB and VAMS samples for tacrolimus across the entire concentration- and hematocrit range.[24] We consider three possible explanations for the lower concentrations of tacrolimus in VAMS compared to WB in our study. The first is the possible influence of the anticoagulant on the analytical results.[27] During method validation and sample analysis for this study, citrate anticoagulated blood was used for the calibration and quality control (QC) samples for both the DBS and VAMS samples.[23,32] The obtained patient samples consisted of capillary blood which does not contain an anticoagulant. Although this proves to be of no influence on DBS analytical results, the absence of the citrate anticoagulant in patient samples might lower the VAMS extraction recovery. It is interesting to see that Vethe et al. describe the use of water as the first extraction solvent while other studies used organic extraction solvents (e.g. methanol or methanol/water).[20,21,23,24,42] The application of pure water as the first added extraction solvent might overcome the potential effects of anti-coagulants from the VAMS tips. However, Vethe et al. did not specify the anticoagulant of the blood used during

method validation and patient sample analysis. The second reason might be the batch-to-batch differences in blood wicking volume of the Mitra® tips. However, we observed only a slight difference of 3% lower blood wicking volume in the batch of VAMS tips used for patient sampling compared to the batch of VAMS tips used during method development and validation, according to the certificates of conformance. The third reason might be the influence of 'invisible undersampling' of VAMS samples. Oversaturated VAMS tips will all be identified and excluded from analysis. Although obviously undersaturated VAMS tips (see Fig. 1D) will be identified and excluded, this might not be the case for slightly undersaturared VAMS tips. According to the sampling instruction, the VAMS tip should remain in the drop of fingerprick blood for 2 seconds after the tip turns completely red to allow the complete filling of the inside of the tip.[28] When removed earlier, the tip might not be completely filled with blood, without the possibility of identifying this during sample inspection. To investigate this, we assumed that, for samples that passed quality control where the values of the two duplicate VAMS tips differed >10% compared to the mean of both samples, this was caused by invisible undersaturation. We assumed that only the highest of these two values would represent a properly saturated tip. This was the case for 17/62 samples. When using only the highest values in the Passing-Bablok analysis, we still found a 7% lower concentration of tacrolimus in VAMS compared to WB. Combined with the 3% lower blood wicking volume a difference of 4% lower tacrolimus concentration in VAMS compared to WB remains, which might be attributed to the earlier mentioned effect of the anticoagulant combined with the extraction method.
When using the aforementioned conversion formula to calculate VAMS tacrolimus concentrations, the results from this study are comparable to the results of the study by Vethe et al. In their study, a limit of clinical acceptance of 20% was defined.[24] In total 97.1% of the trough concentration samples (n=105) were within these limits. If a limit for clinical acceptance of 20% was applied to our study, 94.3% of the VAMS samples would be within these limits.

The rejection rate of 32.3% for the VAMS samples was unexpected because VAMS sampling was perceived as similar, if not easier, than DBS sampling. In addition, phlebotomists were trained using a similar training method (15 minute lecture) that was used for the previous DBS clinical validation studies performed in our hospital. In these previous studies, rejection rates of DBS samples were 0.0% – 4.8%.[2,13,32] In the study by Vethe et al. no data is provided on sample quality of VAMS tips.[24] The study stated that only 7 sample pairs were excluded because of technical or logistical reasons, suggesting that a maximum of 7 samples were excluded because of insufficient quality. This difference is likely due to a different study setting by Vethe et al. compared to our study. Although their study does not state how many phlebotomists obtained the samples or how they were trained, it is likely that only a limited number of study coordinators obtained the samples because it was a full-curve PK study involving 27 patients. Involving only a few study coordinators

who's training included practicing all steps of the sampling method can lead to up to 100% sufficient quality samples.[26] In our hospital, a total of 75 different phlebotomist could have performed the VAMS sampling. Considering the variation in VAMS sampling quality between different studies, it can be concluded that training is of essence in order to ensure acceptable sample quality. Even experience of phlebotomists with other micro sampling techniques like DBS seems to be no guarantee for good quality VAMS samples.

DBS sampling requires a drop of blood to fall freely on a sampling card, while VAMS sampling requires the droplet of blood to be on top of the finger so the VAMS tip can be placed into the droplet of blood. Although this difference was clearly stated in the instruction method, performing DBS sampling prior to VAMS sampling might have led to erroneously letting a drop of blood fall onto the VAMS tip, explaining the high number of oversaturated VAMS tips (Figure 1C). In addition, performing DBS sampling prior to VAMS sampling might result in not enough blood available from the finger prick to fill this VAMS sample. Combined with the possible hesitation by the phlebotomists to perform another finger prick and the often long queues for patient blood sampling at our hospital, this might have resulted in the high number of undersaturated VAMS tips (Figure 1D). If the lids of the purple Mitra® cartridge are closed incorrectly, they are able to touch the blood sample, making the sample unusable (Figure 1B). Improvement of the cartridge, or using another type of sample container can overcome this type of sampling error.

In future clinical validation studies, sample acquisition by only a limited number of well-trained personnel is key in obtaining high quality samples. However, the intended use of both the VAMS and DBS sampling method is patient home sampling. Therefore, the sampling method should be as easy as possible. Based on the results in this study, we hypothesize that at this time the introduction of VAMS sampling instead of DBS sampling does not improve the amount of sufficient quality samples produced by patients at home. However, studies where patients perform both DBS and VAMS sampling, preferably at home, are needed to assess true differences in sample quality and patients' sampling method preference. Although meeting the predefined limits of clinical acceptance, at this moment VAMS results are inferior to DBS results, regarding agreement with WB results. Therefore, conventional DBS home sampling by transplant patients is currently the preferred micro sampling method in our hospital for TDM of tacrolimus.

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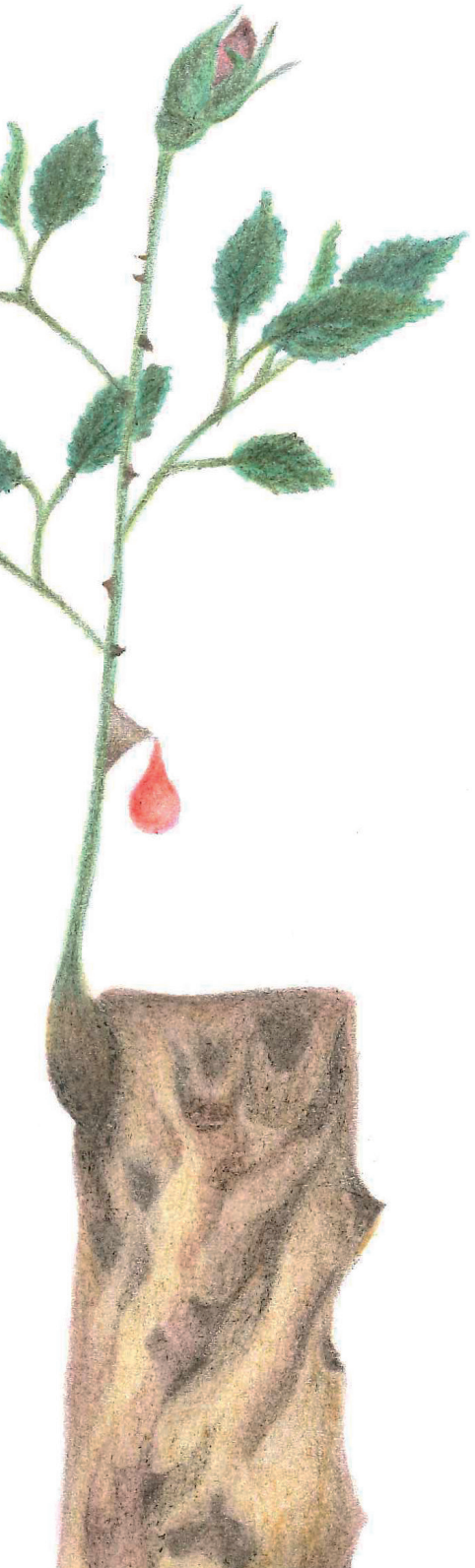

Chapter 11

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.

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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.

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