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Implementing Dried Blood Spot sampling in transplant patient care

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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.

<u>Agilent LC-MS/MS settings</u>

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.

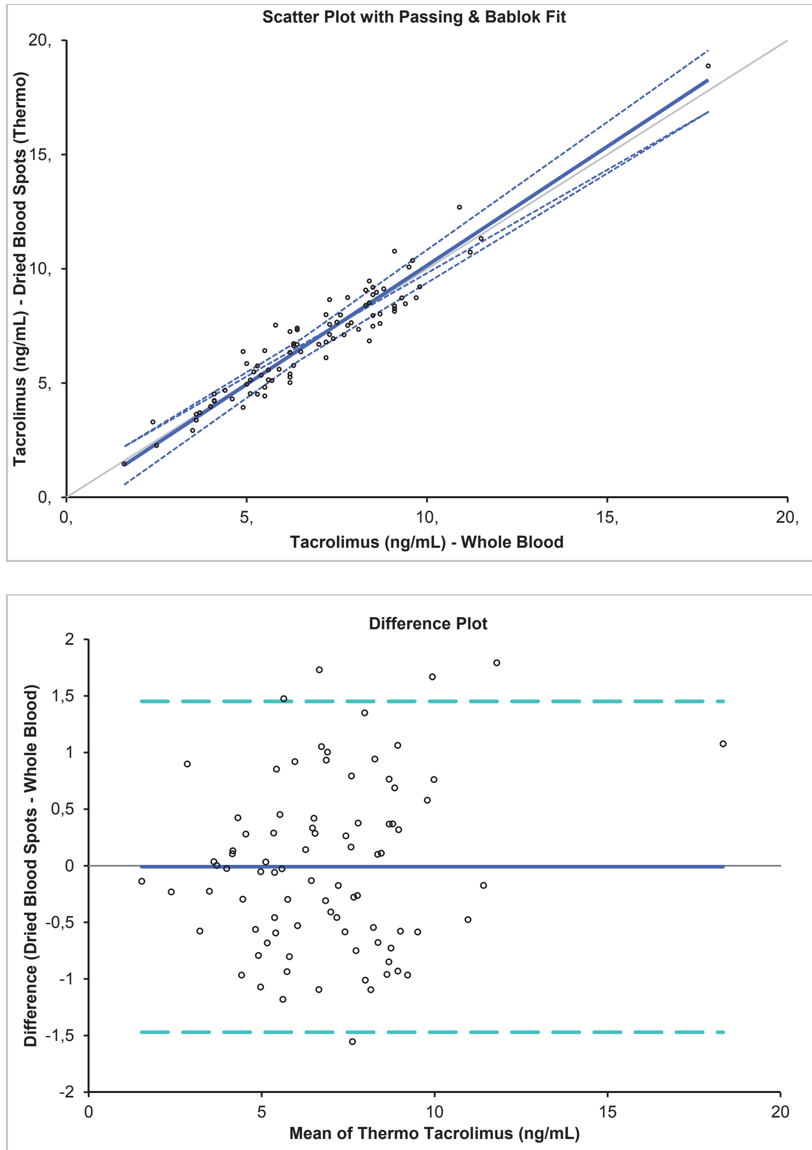

Figure 1: *Comparison of paired whole blood tacrolimus concentrations and Dried Blood Spots (DBS) tacrolimus concentrations measured on a Thermo LC–MS/MS (n = 85). In the upper panel the dotted line is the line of identity, the bold line is the Passing & Bablok regression line y = 1.04 × -0.25 (95% CI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

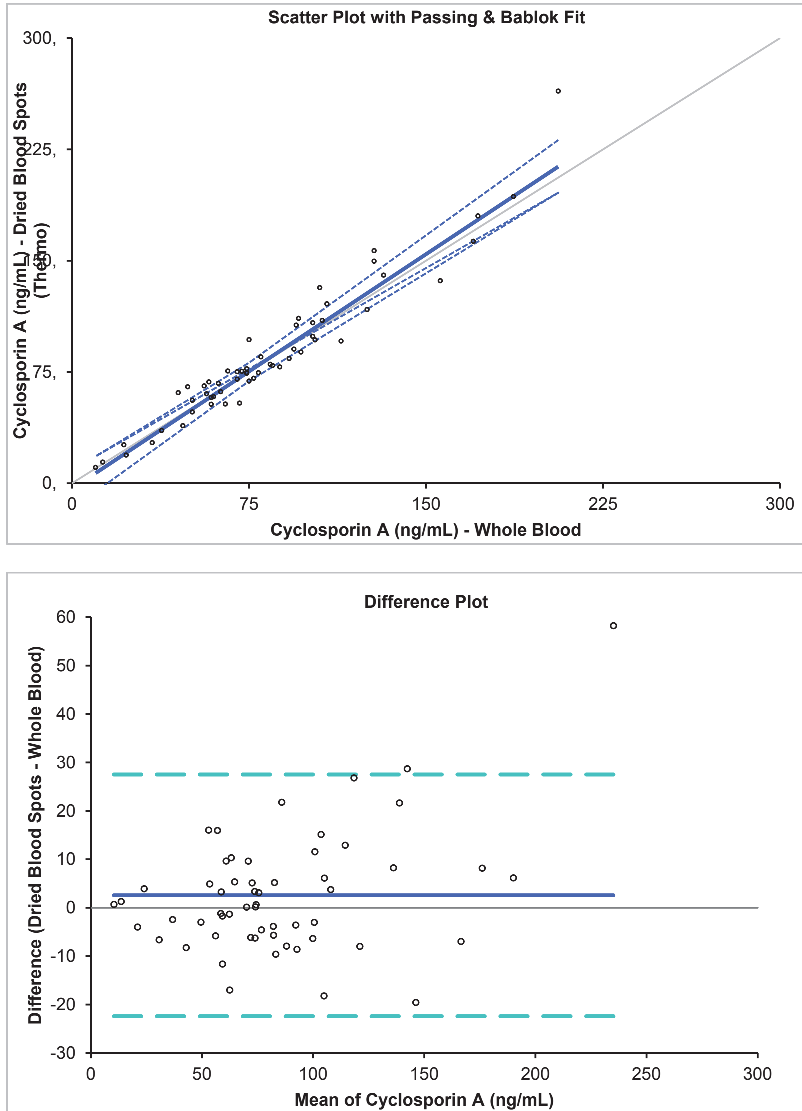

__Figure 2:__ Comparison of paired whole blood cyclosporin A concentrations and Dried Blood Spots (DBS) cyclosporin A concentrations measured on a Thermo LC–MS/MS (n = 57). In the upper panel the dotted line is the line of identity, the bold line is the Passing & Bablok regression line y = 1.05× – 3.64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