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# Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery

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ABSTRACT

We developed a method for the analysis of four immunosuppressants in dried blood spot (DBS) samples to facilitate therapeutic drug monitoring for transplant patients outside the hospital. An 8 mm disc from the central part of the DBS was punched, extracted and followed by LC-MS/MS analysis. The method was validated with ranges from 1.00–50.0 μg/L for tacrolimus, sirolimus and everolimus, and from 20.0–2000 μg/L for cyclosporin A. The validation showed a maximum overall bias of 13.0% for the sirolimus LLOQ, while the maximum overall CV was 15.7% for the everolimus LLOQ. All four immunosuppressants showed to be stable in DBS for at least 7 days at 22 °C. The volume of the blood spot showed to have minor effect on measured concentrations. A cross-validation test between the 31 ET CHR paper and the Whatman FTA DMPK-C cards showed no significant difference between the two types of paper. During validation the hematocrit (HT) showed to have significant influence on the analytical results. When the measured concentrations were corrected for the effect of the HT, biases improved significantly. Additional recovery tests proved that the combination of especially low HT and high concentration does not only affect the spot size but can also affect the extraction recoveries of sirolimus and especially everolimus. Although the tested parameters like HT and concentrations are extreme and unlikely for routine analysis of outpatients, the fundamental effect of the combination of these parameters on extraction recoveries are proven with this research. The protein binding in the blood and hydrogen binding to the cellulose of the paper is suggested to influence extractions and gives new insights in the extraction methodology of DBS samples.

The observed HT effect during the validation appeared to be negligible during the correlation study as no concentration corrections for the HT values were needed. Nevertheless, results from DBS samples with extremely high concentrations combined with extremely low HT values should be interpreted with caution. The patient correlation study showed good correlations with $R^2$ values higher than 0.87 between venous whole blood and venous DBS samples were observed for all four immunosuppressants. The Passing & Bablok plots showed positive biases of the slopes of 18% for tacrolimus and less than 12% for sirolimus, everolimus and cyclosporin A. The validated method, proved stability of the immunosuppressants in DBS, and the correlation study showed the capability of the DBS method to be used as an alternative for whole blood analysis in therapeutic drug monitoring.



## 1. Introduction

Allograft rejection still remains one of the most important obstacles in allogeneic solid organ transplantation. Tacrolimus (TaC), sirolimus (SiR), Everolimus (EvE) and cyclosporin A (CyA) are successfully applied in solid organ transplantation [1]. But their narrow therapeutic ranges require individualized dosing and continuous therapeutic drug monitoring to balance between subtherapeutic and toxic effects of these drugs.

Outpatients need to travel to the hospital on a regular basis to have their blood samples taken and analyzed. The use of DBS sampling allows the patient to sample at home and send the DBS sample to the laboratory by mail. This sampling is simple, saves patients transportation costs and time, and therefore considered to be patient friendly. In addition, early transfer of the DBS sample from the patient to the laboratory provides the clinician with analysis results before the patient visits the clinic for their routine check-up [2]. Other advantages of DBS sampling are lower risk of

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bio-hazard and a smaller amount of blood required for sampling [2,3]. The hematocrit (HT) represents the relative volume of the red blood cells in the blood and has a direct effect on the viscosity of the blood. The permeability through the paper is influenced by the HT of the blood. A high HT has a low permeability through the paper and thus forms a smaller spot. The fixed diameter punch would then contain a higher blood volume causing a higher bias. Earlier publications have proven the effect of the HT on the measured concentration and it is suggested to correct for this effect with the use of a linear relation between HT and measured concentration [4,5]. Because the effects of the HT and volume of the blood to create DBS samples may be drug dependant, they need to be investigated during the validation. The available DBS analysis methods are focussed on of one or two immunosuppressant drugs only [6–11]. Only one study described the simultaneous analysis for all four immunosuppressants [5]. However, no clinical validation was presented in this study and the sample preparation required a time consuming extraction [5]. With the simultaneous analysis of all four immunosuppressants, one sample preparation procedure and one LC-MS/MS setting can be used for the analysis, creating an efficient workflow in the laboratory.

The objective of this study was to develop a fast and reliable method for the simultaneous analysis of TaC, SiR, EvE and CyA in DBS to provide a more efficient way to monitor outpatient transplant recipients.

## 2. Materials and methods

### 2.1. Chemicals and reagents

TaC was purchased from USP (Rockville, MA, USA). EvE was purchased from Sigma-Aldrich Inc. (St. Louis, USA). SiR was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and CyA was purchased from EDQM (Strasbourg, France). Deuterated internal standards (IS) were used for all drugs. TaC [$^{13}C,^{2}H_2$], SiR [$^{13}C,^{2}H_3$], EvE [$^{13}C_2,^{2}H_4$] and CyA [$^{2}H_{12}$] were purchased from Alsachim (Illkirch Graffenstaden, France). Analytical grade methanol was purchased from Merck (Darmstadt, Germany). Purified water was prepared by a Milli-Q Integral system (Billerica, Massachusetts, USA). Ammonium formate was purchased from Acros (Geel, Belgium).

Human packed red blood cells (RBC) were provided by the department of Hematology, University Medical Center Groningen according to local medical ethical regulations. Pooled human serum was acquired in the laboratory from immunosuppressant free patient samples.

Whatman 31 ET CHR paper was used for method development and validation, while Whatman FTA DMPK-C cards were used for the cross-validation test between the two kinds of paper (Whatman, Kent, UK).

### 2.2. Equipment and conditions

Vortexing was performed with a Labtek multi-tube vortexer (Christchurch, New Zealand). Sonification was performed at 47 kHz using a Branson 5210 ultrasonic bath (Danburry, CT, USA). The punching machine (punch diameter=8 mm) was supplied by the Technical Support Facilities of the University of Leiden (NL) and designed by P.M. Edelbroek Ph.D., (Heemstede, the Netherlands) [2].

All experiments were performed on an Agilent 6460A (Santa Clara, Ca, USA) triple quadrupole LC-MS/MS system, with an Agilent 1200 series combined LC system. The Agilent 6460A mass selective detector operated in heated electrospray positive ionization mode and performed dynamic multiple reaction monitoring (DMRM) with unit mass resolution. High purity nitrogen was used for both the source and collision gas flows. In the first quadrupole ammonium adducts $[M+NH_4]^+$ were selected for all four immunosuppressants. All precursor ions, product ions, optimum fragmentor voltages and collision energy values were tuned and optimized in the authors' laboratory and are shown in Table 1. For all substances the capillary voltage was set at 4500 V, gas temperature at 200 °C, gas flow at 13 L/min, nebulizer gas at 18 psi, sheath gas temperature at 200 °C, sheath gas flow at 12 L/min and the nozzle voltage at 0 V. The Agilent 1290 autosampler was set at 10 °C and the 1260 TCC column oven was set at a temperature of 60 °C. The gradient was based on an earlier published method developed in the authors' laboratory using a quaternary LC pump [12]. The method was adapted and chromatographically optimized for this specific binary LC-pump. The mobile phase consisted of methanol and a 20 mM ammonium formate buffer pH 3.5. Analyses were performed with a 50 × 2.1 mm 3 μm HyPURITY® $C_{18}$ analytical column from ThermoFisher Scientific (Waltham, MA, USA) equipped with a separate 0.5 μm Varian frit filter (Palo Alto, CA, USA). Chromatographic separation was performed by means of a gradient with a flow of 0.5 mL/min and a run time of 3.1 min. The gradient starts at 30% methanol and 70% 20 mM ammonium formate buffer pH 3.5 and changes to 73% methanol at 0.36 min and increases slowly to 77% methanol in 1.52 min. From 1.88 min to 2.08 min the methanol increases to 95% and is maintained until 2.71 min. From 2.71 to 3.10 min the gradient is kept at 30% methanol to stabilize the column for the next injection. Peak area ratios of the substance and its internal standard were used to calculate concentrations. Agilent Masshunter software for quantitative analysis (version B.04.00) was used for quantification of the analysis results.

**Table 1**
Mass spectrometer settings for all substances.

| Substance | Precursor ion (m/z) | Product ion (m/z) | Fragmentor voltage (V) | Collision energy (V) |
|---|---|---|---|---|
| Tacrolimus | 821.5 | 768.4 | 190 | 11 |
| Tacrolimus [$^{13}C,^{2}H_2$] | 824.5 | 771.4 | 140 | 15 |
| Sirolimus | 931.5 | 864.4 | 140 | 6 |
| Everolimus | 975.6 | 908.5 | 121 | 10 |
| Everolimus [$^{13}C_2,^{2}H_4$] | 981.6 | 914.5 | 165 | 13 |
| Cyclosporin A | 1219.8 | 1202.8 | 200 | 30 |
| Cyclosporin A [$^{2}H_{12}$] | 1231.8 | 1214.8 | 170 | 16 |

### 2.3. Sample preparation

For the preparation of the DBS, RBC were washed and a precise volume of serum was added to produce blood with the desired HT according to a previously described procedure [4]. For the preparation of the DBS samples 50 μL blood was pipetted on the paper. The DBS were left to dry overnight and then stored at −80 °C in a plastic seal bag containing a 2 g silica gel sachet.

The extraction solution consisted of methanol:water (80:20 v/v%) and contained the deuterated internal standards TaC [$^{13}C,^{2}H_2$], EvE [$^{13}C_2,^{2}H_4$] and CyA [$^{2}H_{12}$] at concentrations of 2.5 μg/L, 1.0 μg/L and 10 μg/L respectively.

For the preparation of the DBS samples 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 vortexed for 60 s, sonicated for 15 min and then vortexed 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 11,000 rpm for 5 min, 20 μL of the extract was injected to the LC-MS/MS system.

### 2.4. Validation

DBS validation

The analytical method validation was performed at a standardized HT value of 0.35 L/L and included linearity, accuracy, precision, selectivity, specificity and stability according to international guidelines [13]. For the DBS matrix the validation was extended with the investigation of the effect of the blood spot volume, HT and the cross-validation test between the 31 ET CHR paper and Whatman FTA DMPK-C cards. Both types are non impregnated cellulose paper.

#### 2.4.1. Analytical method validation

All reference standards were weighed and dissolved in methanol. One set of stock solutions was used for the preparation of the calibration curve, while another set was used for preparation of all other quality control (QC) concentrations. To prevent cell lysis, the volume of the spiked stock solution never exceeded 5% of the total blood volume used for the preparation of the blood standards. The prepared blood standards were then mixed gently for 30 min at room temperature directly followed by the preparation of the DBS according to section 2.3. For each substance an eight point calibration curve was used. TaC, SiR and EvE were prepared at 1.00, 3.00, 10.0, 20.0, 25.0, 30.0, 40.0 and 50.0 μg/L. The calibration curve for CyA was prepared at 20.0, 50.0, 100, 250, 500, 1000, 1600 and 2000 μg/L. The following QC concentrations were used for the validation. The Lower Limit Of Quantification (LLOQ) was 1.00 μg/L, Low 3.00 μg/L, Medium (Med) 25.0 μg/L and High 40.0 μg/L for TaC, SiR and EvE. For CyA, the LLOQ was 20.0 μg/L, Low 50.0 μg/L, Medium (Med) 1000 μg/L and High 1600 μg/L. Validation was performed with a maximum tolerated bias and CV of 20% for the LLOQ and 15% for all other QC samples including the stability validation. For the determination of the accuracy and precision, all QC concentrations were measured in five fold 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 CV's were calculated with the use of one-way ANOVA. One calibration curve was analyzed each day to determine linearity on three separate days.

For stability testing DBS were prepared at Low and High concentrations in five fold and compared to simultaneously prepared DBS in five fold which were stored at −80 °C. Stabilities of the substances were assessed in five fold as processed sample in the auto-sampler at 10 °C after 5 days. Stability of the substances in DBS at 22 °C and 37 °C was assessed in five fold at multiple time points.

#### 2.4.2. Blood spot volume

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 High concentrations with volumes of 30, 50, 70 and 90 μL. The 50 μL spots were considered the standard spot and the biases of the other volumes were calculated with a maximum acceptable bias and CV of 15%.

#### 2.4.3. Extraction recovery, matrix effect and process efficiency

The creation of DBS with an unknown blood volume and a fixed punch diameter makes it impossible to exactly know the amount of blood that is used for the extraction. The amount of blood used for the extraction is estimated to be approximately 20 μL. To assess extraction recovery, matrix effect and process efficiency a defined amount of blood has to be used to create the DBS. Blank paper spots were punched, transferred to an eppendorf cup and 15 μL blood with an HT of 0.35 L/L at Low and High concentration were spiked on the punched spots and analyzed the next day (solutions A Low and A High). For the extraction recovery, extracts of blank DBS were spiked at Low and High concentration (solutions B Low and B High). For the matrix effect and process efficiency, extraction solvent was spiked at Low and High concentration (solutions C Low and C High). The average 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 followed: recovery $= A/B \times 100$, matrix effect $= (B/C \times 100) - 100$, process efficiency $= A/C \times 100$. Where A, B and C refer to the prepared solutions mentioned above.

#### 2.4.4. Influence of the hematocrit

To test the influence of the HT, blood samples at Low and High concentration were prepared by adding the necessary volume of red blood cells and serum to obtain the following HT values: 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 L/L. From these blood samples DBS were created using 50 μL of blood. For the remaining, the procedure described in section 2.3 was followed.

#### 2.4.5. Influence of the hematocrit and concentration on the recovery

It seems common to state that the effect of the HT on the analytical bias is due to the viscosity of the blood and its permeability through the paper. Thus the DBS spot size is assumed to be of main influence on the observed analytical biases. To assess whether the analytical biases were caused by the spot size of the created DBS or by another mechanism, DBS were made at different HT values and concentrations without the effect caused by the spot size. In this way the parameters which cause the effect at varying HT values could be investigated.

A similar approach was used as in the extraction recovery tests. Concentrations in whole blood were prepared at 3.0, 20.0, 40.0, and 50.0 μg/L for TaC, SiR and EvE, and at 50.0, 800, 1600 and 2000 μg/L for CyA. Every concentration was prepared in whole blood at HT concentrations of 0.25, 0.35 and 0.45 L/L. Blank paper spots were punched, transferred to an eppendorf cup and 10 μL whole blood was spiked on the punched spots. For the remaining, the procedures described in sections 2.3 and 2.4.3 were followed.

#### 2.4.6. Cross-validation test between the 31 ET CHR paper and Whatman FTA DMPK-C cards

To assess significant differences between the paper types of the Whatman 31 ET CHR paper and the Whatman FTA DMPK-C cards, an eight point calibration curve was analyzed from each paper type according to the described sample preparation and analytical procedure. The 95% confidence intervals (CI) of the intercepts and slopes of each calibration curve were compared to determine a significant difference between the two types of paper.

### 2.5. Correlation study: patient analysis in venous blood and DBS from venous blood

The correlation study included patients who had a heart, kidney, lung, liver, pancreas or small intestine transplantation. Trough levels were used to determine the immunosuppressant concentrations. Venous EDTA blood samples received by the laboratory for routine immunosuppressant analysis were analyzed daily according to a previously published method [12]. The venous EDTA blood samples were used to create venous DBS according to section 2.3. The DBS were analyzed and correlated with the results from the venous blood analysis using Passing & Bablok with the help of Analyse-it® software. In addition, this patient population was used to calculate the average HT values of the outpatients and inpatients.

## 3. Results

### 3.1. Validation results

#### 3.1.1. Analytical method validation

During method development it became clear that some deuterated internal standards were contaminated with one of the immunosuppressants. TaC [$^{13}C,^{2}H_2$] was 1.1% contaminated with TaC, SiR [$^{13}C,^{2}H_3$] was 2.9% contaminated with SiR, EvE [$^{13}C_2,^{2}H_4$] was 0.5% contaminated with EvE and was 0.7% contaminated with SiR. For this reason it was decided to validate without SiR [$^{13}C,^{2}H_3$] and to use EvE [$^{13}C_2,^{2}H_4$] as the internal standard for SiR instead. Low concentrations of the internal standards were used to minimize the effect of the contaminants, while still providing high and reliable peak areas.

In Table 2 the validation results regarding accuracy and precision are shown. TaC, SiR and EvE were validated with a linear range of 1.00–50.0 μg/L with correlation coefficients of respectively 0.9975, 0.9962 and 0.9960. CyA was validated with a range of 20.0–2000 μg/L and a correlation coefficient of 0.9992. For CyA a quadratic fit showed to be most suitable for this large calibration range. For the accuracy & precision the highest overall bias found during the validation was 13.0% for the LLOQ of SiR, while the highest overall CV was 15.7% for the LLOQ of EvE. In Fig. 1 representative LLOQ chromatograms are shown for each immunosuppressant. The results of the stability validation of all four immunosuppressants are shown in Table 3. The auto-sampler stability was proven for 48 h at 10 °C for all substances with a maximum overall bias of 12.6%. All substances showed to be stable for at least 7 days at 22 °C at Low and High concentration. SiR and especially EvE showed degradation at 37 °C over a 4 week period (data not shown).

#### 3.1.2. Blood spot volume

The volume of the bloodspot showed minor impact on the measured concentrations. The 30 μL spots showed negative biases with a maximum of −6.6% for the SiR at Low concentration, while the 90 μL spots showed positive biases with a maximum of 13.9% for the CyA at High concentration.

#### 3.1.3. Extraction recovery, matrix effect and process efficiency

With the used extraction and analysis method high process efficiencies are obtained for TaC, SiR and EvE, without any significant matrix effects. For CyA however, high extraction recoveries are found but there showed to be a significant negative matrix effect of about −64% (Table 4). The matrix effect also showed to be present with an equal effect at the M+1 mass as with the ammonium adduct (data not shown). During method development it became clear that chromatographically separating the matrix effect from the peak of CyA was impossible. The use of a deuterated IS corrects for the occurring matrix effect, as can be seen in Table 4. And since CyA showed good validation results the presence of the matrix effect was accepted.

**Table 2**
Accuracy and precision results of all substances.

| Substance | Concentration (μg/L) | Within-run CV (%) | Between-run CV (%) | Overall CV(%) | Overall bias (%) |
|---|---|---|---|---|---|
| Tacrolimus | LLOQ (1.00) | 12.4 | 0.0 | 12.4 | −1.2 |
| | Low (3.00) | 5.9 | 7.0 | 9.2 | 7.5 |
| | Med (25.0) | 4.4 | 0.5 | 4.4 | 1.6 |
| | High (40.0) | 3.8 | 0.0 | 3.8 | −1.4 |
| Sirolimus | LLOQ (1.00) | 13.4 | 0.0 | 13.4 | 13.0 |
| | Low (3.00) | 10.3 | 0.0 | 10.3 | 4.2 |
| | Med (25.0) | 4.8 | 2.1 | 5.3 | −5.1 |
| | High (40.0) | 6.8 | 0.0 | 6.8 | −4.5 |
| Everolimus | LLOQ (1.00) | 15.7 | 0.0 | 15.7 | −2.2 |
| | Low (3.00) | 7.8 | 0.0 | 7.8 | 12.0 |
| | Med (25.0) | 5.4 | 0.0 | 5.4 | 8.6 |
| | High (40.0) | 4.9 | 0.0 | 4.9 | 1.9 |
| Cyclosporin A | LLOQ (20.0) | 4.0 | 8.8 | 9.7 | 4.1 |
| | Low (50.0) | 3.9 | 5.8 | 6.9 | 0.4 |
| | Med (1000) | 2.8 | 6.4 | 6.9 | −3.1 |
| | High (1600) | 5.9 | 12.3 | 13.7 | −0.5 |

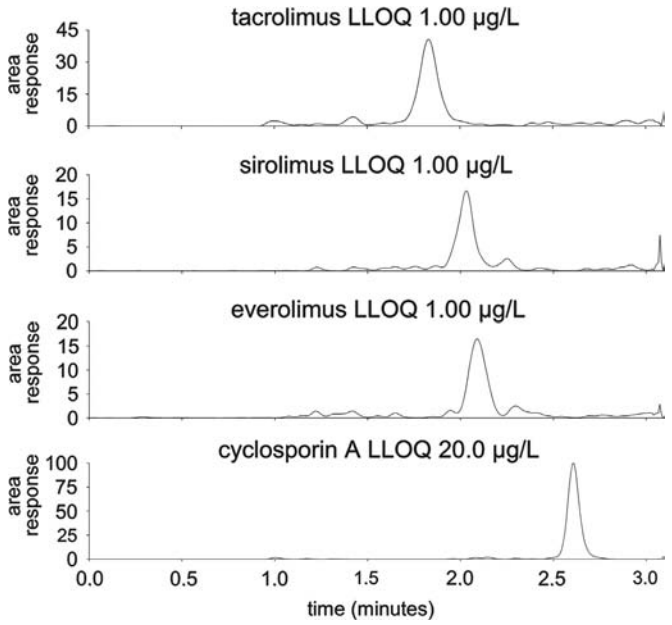


**Fig. 1.** Chromatograms of all LLOQ concentrations for tacrolimus, sirolimus, everolimus and cyclosporin A.

**Table 3**
Results of the stability testing for all four substances. AS is autosampler stability in processed sample. Low is 3.00 μg/L and High is 40.0 μg/L for tacrolimus, sirolimus and everolimus. For cyclosporin A, Low is 50.0 μg/L and High is 1600 μg/L.

| Substance | Stability | Time (days) | LOW | | High | |
|---|---|---|---|---|---|---|
| | | | Within-run CV (%) | Within-run bias (%) | Within-run CV (%) | Within-run bias (%) |
| Tacrolimus | AS 10 °C | 5 | 4.1 | 1.1 | 4.0 | 4.4 |
| | DBS 22 °C | 28 | 5.7 | −5.1 | 4.7 | −3.6 |
| | DBS 37 °C | 28 | 8.2 | −14.2 | 4.5 | −9.8 |
| Sirolimus | AS 10 °C | 5 | 10.1 | 12.6 | 7.0 | 10.9 |
| | DBS 22 °C | 7 | 9.6 | −4.9 | 4.1 | −1.3 |
| | DBS 37 °C | 7 | 13.6 | 1.1 | 6.3 | −12.4 |
| Everolimus | AS 10 °C | 5 | 6.1 | 11.0 | 5.6 | 8.1 |
| | DBS 22 °C | 13 | 5.2 | −7.8 | 5.1 | −12.7 |
| | DBS 37 °C | 2 | 7.5 | −6.2 | 6.8 | −10.8 |
| Cyclosporin A | AS 10 °C | 5 | 3.4 | 0.5 | 3.0 | 10.2 |
| | DBS 22 °C | 28 | 6.5 | −0.2 | 4.4 | 10.5 |
| | DBS 37 °C | 28 | 3.7 | −1.7 | 3.8 | 11.4 |

### 3.1.4. Influence of the hematocrit

As expected the HT showed to be of significant influence on the analytical results. With the HT of 0.35 L/L as the standardized HT value, positive biases were observed for increasing HT values and vice versa. A multivariate regression equation with the use of Microsoft Excel was applied to calculate the expected analytical bias with HT value and nominal concentration as covariates. This expected analytical bias was then used to correct the measured concentration. After correction most biases were within the acceptable range of 15% bias. Only SiR and EvE still showed −20% and −28% bias respectively at the extreme HT of 0.20 L/L at High concentration.

**Table 4**
Results of the extraction recovery, matrix effect and process efficiency testing. IS is internal standard.

| Substance | Concentration µg/L | Extraction recovery % | Matrix effect % | Process efficiency % | Matrix effect corrected with IS % |
|---|---|---|---|---|---|
| Tacrolimus | 3.00 | 95.4 | 0.6 | 95.9 | −4.1 |
| | 40.0 | 95.2 | 14.3 | 108.8 | 0.2 |
| Sirolimus | 3.00 | 92.4 | 3.1 | 95.2 | 5.3 |
| | 40.0 | 88.7 | 3.5 | 91.8 | 4.0 |
| Everolimus | 3.00 | 98.8 | −4.3 | 94.5 | −1.8 |
| | 40.0 | 85.7 | 0.1 | 85.7 | 0.5 |
| Cyclosporin A | 50.0 | 121.3 | −66.5 | 40.7 | −8.9 |
| | 1600 | 89.1 | −60.5 | 35.2 | −2.2 |

### 3.1.5. Influence of the hematocrit and concentration on the extraction recovery

The results of the performed tests show that varying HT and concentrations have no significant influence on matrix effects. For TaC and CyA the extraction recoveries remained constant at 99% (CV 2.3%) and 87% (CV 4.3%) respectively and showed not be influenced by varying concentrations and HT values. For SiR and EvE however, the extraction recovery was influenced by the concentration as well as the HT value, as seen in Fig. 2. The lowest extraction recoveries were observed at the highest concentrations in combination with the lowest HT values. For SiR the highest extraction recovery of 93% was found at 3 µg/L and a HT of 0.45 L/L, while the lowest extraction recovery of 69% was found at 50 µg/L and a HT of 0.25 L/L. For EvE the highest extraction recovery of 87% was found at 3 µg/L and a HT of 0.35 L/L, while the lowest extraction recovery of 49% was found at 50 µg/L and a HT of 0.25 L/L. This proves that the effect of the DBS spot size caused by the HT is not the only parameter that can cause the deviating biases. For SiR and EvE an additional negative effect is caused by the deteriorated extraction recoveries especially at extremely low HT values combined with extremely high concentrations.

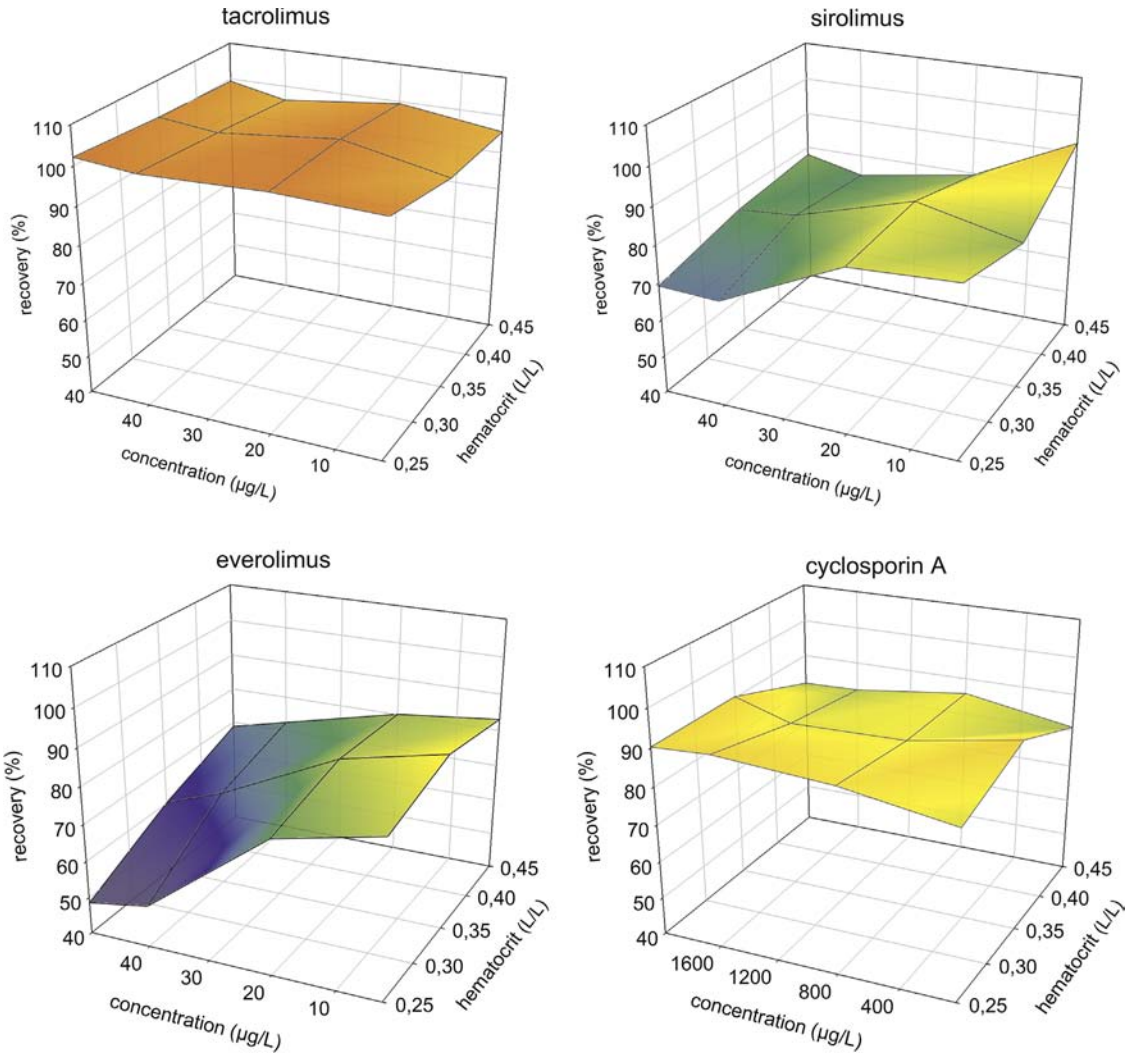


**Fig. 2.** Recoveries of tacrolimus, sirolimus, everolimus and cyclosporin A influenced by concentration and HT, without the chromatographic effect of the paper.

**Table 5**
Results of the correlation between the calibration curves of the Whatman 31 ET CHR paper and Whatman FTA DMPK-C cards for all four substances using a 95% confidence interval (95% C.I.).

| Substance | Whatman paper | Intercept (95% C.I.) | Slope (95% C.I.) | Regression coefficient | Correlation coefficient |
|---|---|---|---|---|---|
| Tacrolimus | 31 ET CHR | 0.0427 (0.0222–0.0632) | 0.1059 (0.0982–0.1136) | 0.9947 | 0.9947 |
| | FTA DMPK-C | 0.0288 (0.0135–0.0441) | 0.1134 (0.1076–0.1191) | 0.9974 | 0.9987 |
| Sirolimus | 31 ET CHR | 0.01518 (0.0006–0.0297) | 0.06159 (0.0561–0.0671) | 0.9922 | 0.9961 |
| | FTA DMPK-C | 0.005827 (−0.0023–0.014) | 0.05608 (0.053–0.0591) | 0.9970 | 0.9985 |
| Everolimus | 31 ET CHR | 0.008848 (−0.0064–0.0241) | 0.07945 (0.0737–0.0852) | 0.9948 | 0.9974 |
| | FTA DMPK-C | 0.003898 (−0.0156–0.0234) | 0.08730 (0.0800–0.0946) | 0.9930 | 0.9965 |
| Cyclosporin A | 31 ET CHR | 0.004576 (−0.0177–0.0268) | 0.003503 (0.0031–0.0039) | 0.9850 | 0.9924 |
| | FTA DMPK-C | 0.01001 (−0.0113–0.0313) | 0.00399 (0.0036–0.0044) | 0.9893 | 0.9947 |

*3.1.6. Cross-validation test between the Whatman 31 ET CHR paper and Whatman FTA DMPK-C cards*

In Table 5 the results of the compared curves are shown for each substance. The overlapping 95% confidence intervals of the intercepts and slopes between the two types of paper concluded no significant differences between the two types of paper for each of the four immunosuppressants. Although it is preferred to use a single kind of paper for the DBS of the calibration curve, quality control samples and patients this test showed that the use of either type of paper will provide similar results.

*3.2. Correlation study: patient analysis in venous blood and DBS from venous blood*

For TaC, SiR, EvE and CyA, 50, 36, 55 and 57 samples were analyzed respectively. The correlation results obtained with Passing & Bablok regression are shown in Fig. 3. The 95% confidence intervals showed that the slopes for TaC, EvE and CyA were significantly higher than 1, concluding a systematic difference between venous blood and DBS from venous blood. The Passing & Bablok results for SiR showed no systematic difference between venous blood and DBS from venous blood. The Passing & Bablok plots showed a positive bias of the slope of 18% for tacrolimus and biases less than 12% for sirolimus, everolimus and cyclosporin A. Since the DBS method was initially developed for the analysis of outpatients the average HT values and their 95% confidence interval were calculated for the inpatients and the outpatients. The outpatients showed to have an average HT of 0.384 L/L ($n=102$, 95% CI: 0.379–0.389), while inpatients showed to have a lower average HT of 0.295 L/L ($n=55$, 95% CI: 0.289–0.302). This significant difference in HT values between inpatients and outpatients shows that the standardized HT used for the calibration curve must depend on the population which is monitored.

## 4. Discussion

We developed a method for the analysis of DBS samples to facilitate TDM of immunosuppressants outside the hospital. The developed method is fast and the extract can be injected into the LC-MS/MS without a time consuming concentration step like evaporation to dryness and subsequent re-dissolving of the sample. The method was extensively validated, including the effect of the blood spot volume and by testing a wide range of HT concentrations. The HT and recovery tests in section 3.1.5 showed that the viscosity of the blood due to the HT is not the only parameter that affects the measured concentration. At extremely high concentrations combined with extremely low HT values, recoveries deteriorated for EvE and SiR.

Van der Heijden et al. also found that extraction recoveries of EvE (60.8% recovery at 8 μg/L) deteriorated at higher concentrations and suggested that EvE adsorbed to the paper [7]. Impregnation of the paper with a mixture of ammonium acetate, formic acid and pasteurized plasma-protein solution would prevent the adsorption and a filtration extraction procedure improved the recovery of EvE. Although our results showed the same phenomenon, the extraction recoveries with the here described extraction method at Low and High concentrations were 98.8% and 85.7% respectively, without the inconvenient impregnation of the paper. Van der Heijden et al. explained the concentration dependant recovery by the ability to easily extract the superficial EvE from the DBS, while the EvE that permeated deeper into the DBS was alleged to be extracted less well [7].

TaC and SiR have protein bindings of approximately 99% and 92% respectively, while EvE has a lower protein binding of approximately 74% [14]. To improve oral availability, EvE was synthesized by chemists at Novartis, who made a 2-hydroxyethyl chain substitution at position 40 of the SiR structure [15,16]. A high number of hydrogen bond acceptors may explain the level of affinity to form hydrogen bonds with the cellulose of the paper. The molecules of TaC, SiR and EvE have 12, 13 and 14 hydrogen bond acceptors respectively [17]. For EvE this resulted in a lower protein binding affinity and higher affinity to form hydrogen bonds with the cellulose of the paper compared to its structural analogs TaC and SiR. An alternative hypothesis that we propose is that at high concentrations and low HT the free fraction of EvE is increased which forms hydrogen bonds with the cellulose of the paper. The protein bound EvE is easily extracted, while the EvE, which is bound to the cellulose of the paper is more difficult to extract. The same hypothesis could be applied for SiR, but to a lesser extent.

The HT value also showed to be of significant influence on the measured DBS concentration of CyA. Additional testing, which excluded the effect of the spot size during the creation of the DBS, showed no decrease in the extraction recovery of CyA at varying concentrations and HT values. This proved that the chromatographic effect during the formation of the DBS is of significant influence on the distribution of CyA in the DBS and is HT and concentration dependant. In blood, the distribution of CyA is concentration dependent with approximately 33%–47% in plasma and 41%–58% in erythrocytes. At high concentrations, the binding capacity of leukocytes and erythrocytes becomes saturated [14]. The high affinity of CyA to distribute to plasma may explain the chromatographic effect seen at extremely low HT combined with a high concentration. This causes a high degree of plasma bound CyA which has a lower viscosity than the erythrocytes, creating a chromatographic effect in the distribution of the DBS.

The observed HT effects during the validation showed to be negligible at the patient correlation study, where a good correlation was observed without concentration corrections of the HT values. The less extreme HT values and concentrations at the patient correlation study minimize the observed effect during the validation and allow DBS analysis without HT correction.

The patient correlation study showed good correlations for all four immunosuppressants with $R^2$ values higher than 0.87 between venous

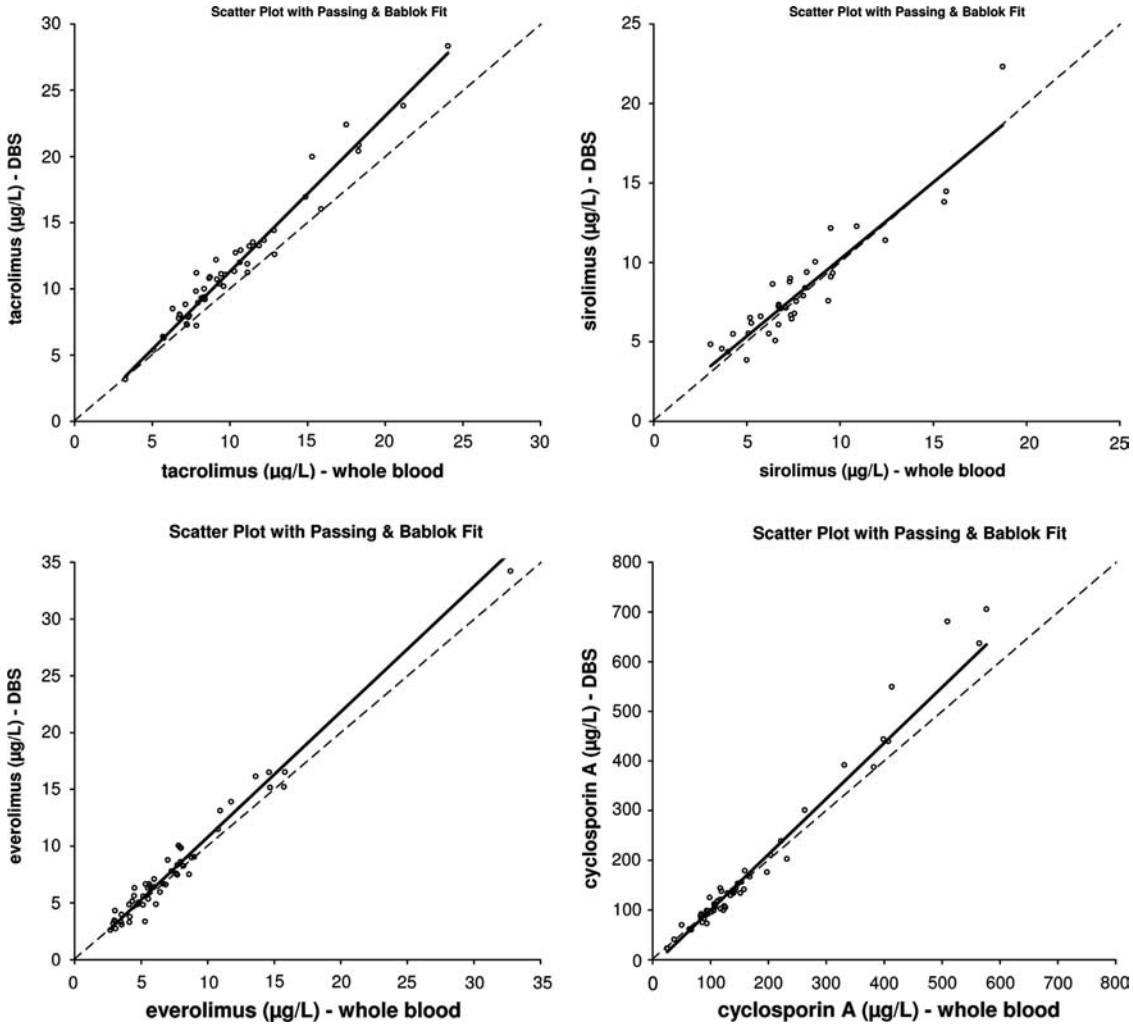


**Fig. 3.** Patient correlation study of venous blood and DBS for tacrolimus, sirolimus, everolimus and cyclosporin A. The dotted line is the identity line, the continuous line is the Passing & Bablok regression line. Simple linear regression coefficients and Passing & Bablok regression equations are the following. tacrolimus: $R^2=0.9607$ ($n=50$), $y=1.18x-0.45$ (95% CI slope: 1.11 to 1.25, intercept: −1.02 to 0.19); sirolimus: $R^2=0.8708$ ($n=36$), $y=0.97x+0.52$ (95% CI slope: 0.80 to 1.21, intercept: −1.04 to 1.66); everolimus: $R^2=0.9698$ ($n=55$), $y=1.10x-0.26$ (95% CI slope: 1.04 to 1.21, intercept: −0.96 to 0.19) and cyclosporin A: $R^2=0.9775$ ($n=57$), $y=1.12x-12.26$ (95% CI slope: 1.05 to 1.18, intercept: −20.55 to −4.06).

whole blood and venous DBS samples. In addition, the $R^2$ of CyA was 0.9775, showing that the ion-suppression found for CyA in this study was well corrected with its deuterated internal standard during venous DBS patient analysis. The correlation study showed the capability of the DBS method to be used as an alternative for whole blood analysis in therapeutic drug monitoring.

## 5. Conclusion

A fast analysis method for TaC, SiR, EvE and CyA in DBS was developed and fully validated. The validation showed significant effects by the combination of extreme HT values and concentrations on the analytical results. Additional recovery tests proved that the combination of especially low HT and high concentration does not only affect the spot size but can also affect the extraction recoveries of SiR and especially EvE. Although the tested parameters like HT and concentrations are extreme and unlikely for routine analysis of through levels, the fundamental effect of the combination of these parameters on recoveries are proven with this research. The suggested influence of protein binding in the blood and hydrogen binding to the cellulose of the paper give new insights in the extraction methodology of DBS samples. Future research is needed to further determine the mechanism of binding and extraction of these immunosuppressants regarding the paper matrix. For the patient correlation study, which showed no extreme HT values or concentrations, these effects showed to be of minimal influence and patients analysis acquired from venous whole blood and DBS showed good correlation results. Nevertheless, results from DBS samples with extremely high concentrations combined with extremely low HT values should be interpreted with caution. The validated method and proved stability of the immunosuppressants of at least 7 days at 22 °C in DBS makes the method suitable to replace the whole blood analysis for outpatients.

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