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Original Clinical Science—General

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

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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.

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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

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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

Patients 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.[9,18,19]

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. T1

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" 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 F1

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)

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

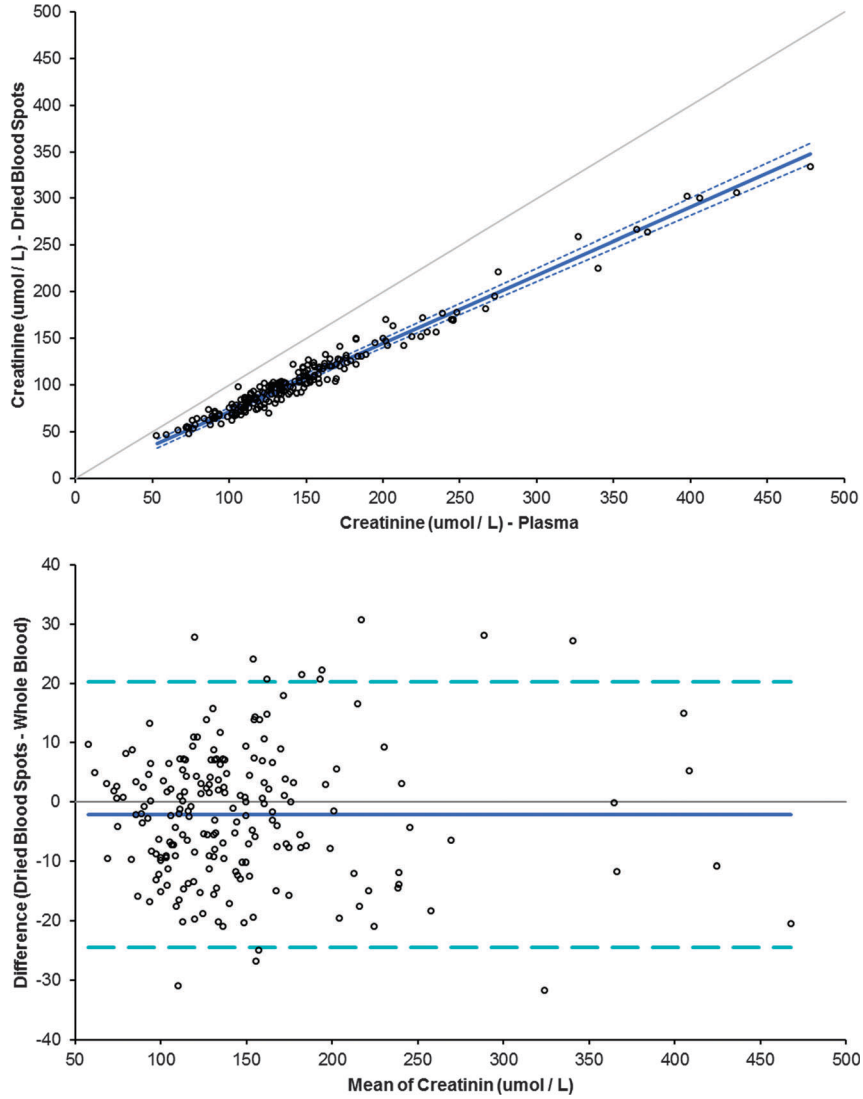

Fig 1 4/C

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.

–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.

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.

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.

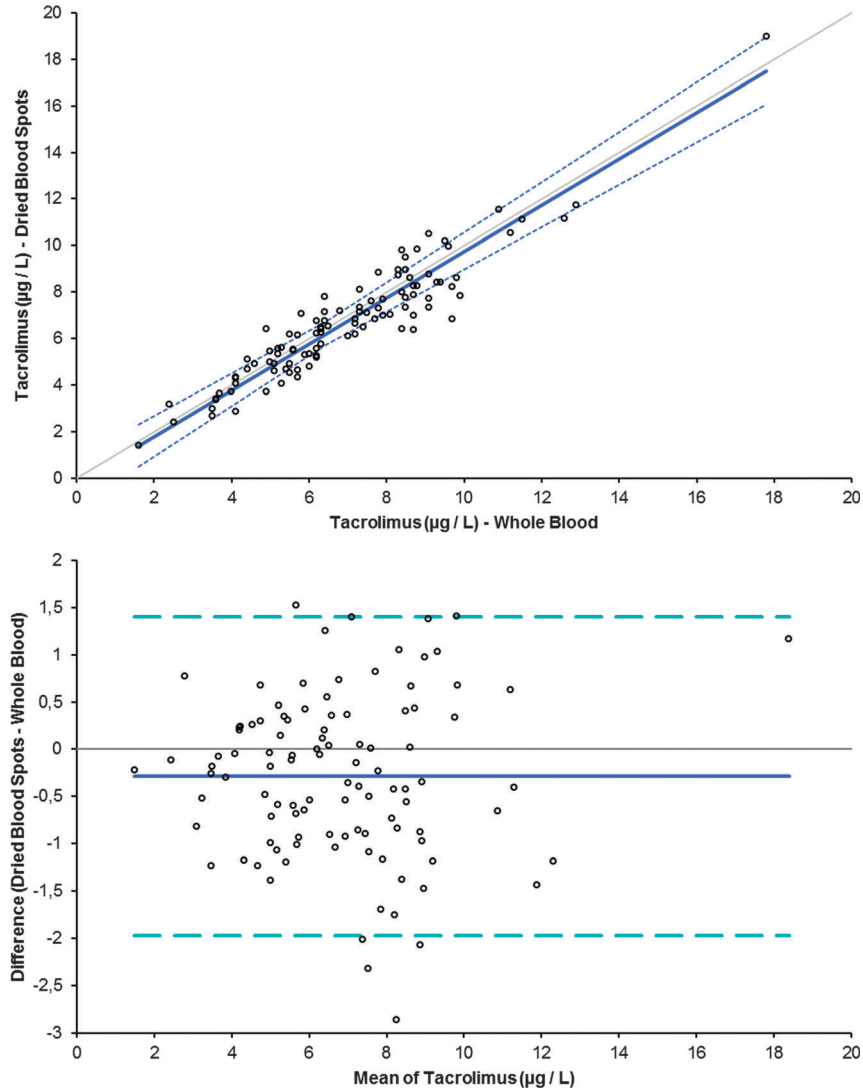

Fig 2 4/C

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.

Fig 3 4/C

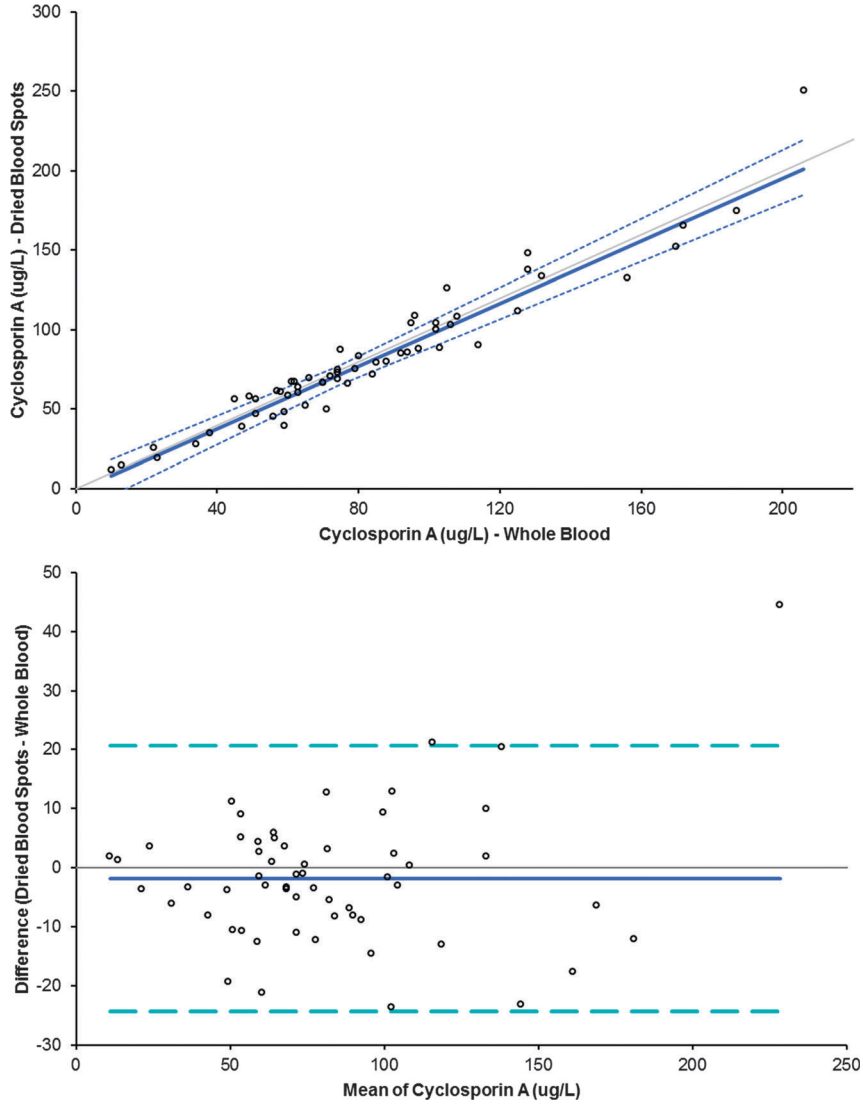

FIGURE 3. Method comparison between venous whole blood cyclosporin A concentrations and DBS concentrations (n = 58). In the upper panel, the dotted line is the line of identity, the continuous line is the Passing & Bablok regression line $y = 0.99x - 1.86$ (95% CI slope, 0.91-1.08; intercept, –8.31 to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

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 analytical 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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