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RESEARCH PAPER

Dried blood spot analysis of creatinine with LC-MS/MS in addition to immunosuppressants analysis

**Remco A. Koster · Ben Greijdanus ·
Jan-Willem C. Alffenaar · Daan J. Touw**

Abstract In order to monitor creatinine levels or to adjust the dosage of renally excreted or nephrotoxic drugs, the analysis of creatinine in dried blood spots (DBS) could be a useful addition to DBS analysis. We developed a LC-MS/MS method for the analysis of creatinine in the same DBS extract that was used for the analysis of tacrolimus, sirolimus, everolimus, and cyclosporine A in transplant patients with the use of Whatman FTA DMPK-C cards. The method was validated using three different strategies: a seven-point calibration curve using the intercept of the calibration to correct for the natural presence of creatinine in reference samples, a one-point calibration curve at an extremely high concentration in order to diminish the contribution of the natural presence of creatinine, and the use of creatinine-$[^2H_3]$ with an eight-point calibration curve. The validated range for creatinine was 120 to 480 µmol/L (seven-point calibration curve), 116 to 7000 µmol/L (1-point calibration curve), and 1.00 to 400.0 µmol/L for creatinine-$[^2H_3]$ (eight-point calibration curve). The precision and accuracy results for all three validations showed a maximum CV of 14.0 % and a maximum bias of −5.9 %. Creatinine in DBS was found stable at ambient temperature and 32 °C for 1 week and at −20 °C for 29 weeks. Good correlations were observed between patient DBS samples and routine enzymatic plasma analysis and showed the capability of the DBS method to be used as an alternative for creatinine plasma measurement.

R. A. Koster (✉) · B. Greijdanus · J.-W. C. Alffenaar · D. J. Touw
Department of Clinical Pharmacy and Pharmacology, Laboratory for Clinical and Forensic Toxicology and Drugs Analysis, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands
e-mail: r.koster@umcg.nl

D. J. Touw
Department of Pharmacy, Section Pharmacokinetics, Toxicology and Targeting, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Keywords Dried blood spot · Creatinine · Tacrolimus · Sirolimus · Everolimus · Cyclosporin A

Introduction

Over the last decade, dried blood spot analysis (DBS) has become increasingly popular for outpatient therapeutic drug monitoring (TDM). Analytical methods for DBS analysis of several substances have been successfully developed during the last years. DBS analysis of tacrolimus, sirolimus, everolimus, and cyclosporin A are especially favored because of the need for lifelong TDM in transplant patients. Because DBS is a low invasive and convenient method for the patient, TDM has been gradually changing from whole blood to DBS analysis. The main advantage of DBS analysis is that the patient can perform the sampling at home and can send the DBS card directly to the laboratory [1, 2].

In addition to the concentration of the drug of interest, relevant clinical parameters are mandatory to adjust the dosage of renally excreted or nephrotoxic drugs [3, 4]. DBS would be less efficient if patients still have to travel to the hospital for having a blood sample taken for other laboratory analyses. A more efficient approach of TDM would be the analysis of the drug of interest and relevant clinical parameters in the same DBS. Also, creatinine is the most common analyte measured in relation to drug clearance or toxicity samples from transplant patients. An analytical method for the analysis of creatinine in DBS would be useful for these patients who have a high risk of renal failure. Examples are the immunosuppressants cyclosporine and tacrolimus that are nephrotoxic, in which case creatinine monitoring may help discover nephrotoxicity. Other examples are the early detection of drugs that may accumulate in case of decreasing renal function such as digoxin, metformin, and lithium. Creatinine monitoring is also useful in case of therapy with renally cleared

drugs that cause toxicity when renal function declines such as home intravenous treatment with aminoglycosides. Several DBS procedures have been developed for which a creatinine measurement would be helpful (diabetic patients, patients with heart failure) [5, 6].

To date, only one study described the analysis of creatinine in DBS together with tacrolimus [7]. The authors performed a preliminary validation and showed that it is possible to analyze creatinine along with tacrolimus in the same DBS. There are several disadvantages to their method. The DBS preparation procedure for tacrolimus uses a time-consuming solid phase extraction, while this is not performed for the creatinine extraction. In addition, the two different chromatographic procedures, i.e., normal phase chromatography and reversed phase chromatography, complicate the combined analysis on a single LC-MS/MS system. On could also debate the use of phosphate buffered saline (PBS) as surrogate for creatinine-free whole blood because the change in matrix affects both the formation of the spot as well as the subsequent analysis using LC-MS/MS. As only fixed volume DBS preparation was evaluated without evaluation of the hematocrit (HT) effect, this procedure is not useful for daily practice.

The objective of this study was to develop a fast and reliable method for the analysis of creatinine in the same DBS extract used for tacrolimus, sirolimus, everolimus, and cyclosporin A on a single LC-MS/MS system and to solve the issue of the naturally present creatinine in the reference standards. This would provide a reliable and efficient procedure to perform TDM for outpatient transplant recipients.

Materials and methods

Chemicals and reagents

Creatinine and creatinine-$[^2H_3]$ were purchased from Sigma-Aldrich Inc. (St. Louis, USA). Creatinine-$[^{13}C_3,^2H_3]$ was 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). Citrate whole blood was purchased from Sanquin (Amsterdam, The Netherlands) and was used within 2 weeks after blood donation and was always checked whether the blood was not hemolytic prior to use. The whole blood was stored at 4 °C. Whatman FTA DMPK-C cards were used for method development and validation (Whatman, Kent, UK).

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 (Danbury, CT, USA). The punching machine (punch diameter=8 mm) was purchased from the Technical Support Facilities of the University of Leiden and designed by P.M. Edelbroek PhD (Heemstede, the Netherlands) [1].

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 multiple reaction monitoring (MRM) with unit mass resolution. High-purity nitrogen was used for both the source and collision gas flows. The used mass transitions for creatinine were m/z 114.2 with fragment m/z 72.1, for creatinine-$[^2H_3]$ m/z 117.2 with fragment m/z 75.1, and for creatinine-$[^{13}C_3,^2H_3]$ m/z 120.2 with fragment m/z 91.1. The fragmentor voltage was 110 V for all mass transitions, and the collision energy was 15 V for creatinine and creatinine-$[^2H_3]$ and 8 V for creatinine-$[^{13}C_3,^2H_3]$. 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 40 °C. The column oven was equipped with an integrated Agilent column switch. The mobile phase solutions consisted of methanol and an aqueous 20 mM ammonium formate buffer pH 7.0. Analyses were performed with a 100×2.1 mm 3-μm Waters Atlantis T3 analytical column from Waters Corporation (Milford, MA, USA). Chromatographic separation was performed by means of a gradient with a flow of 0.5 mL/min and a run time of 2.5 min. The gradient starts at 100 % 20 mM ammonium formate buffer pH 7.0 and changes to 95 % methanol between 1.40 and 1.60 min where it remains until 2.00 min. From 2.01 to 2.50 min, the gradient is kept at 100 % 20 mM ammonium formate buffer pH 7.0 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. Analyse-it® software was used for Passing and Bablok regression analysis.

Sample preparation

The preparation of the target HT values was performed by centrifuging tubes with 8 mL of citrate whole blood with a known HT (measured by a Sysmex XN-9000 analyzer) for 5 min at 1972×g. The necessary volumes of plasma were omitted or added to achieve the target HT values [8]. From these blood samples, DBS were created using 50 μL of blood. The DBS were left to dry for 24 h and then stored at −20 °C in a plastic seal bag containing a 2-g silica gel sachet.

The DBS extraction for tacrolimus, sirolimus, everolimus, and cyclosporin A was performed according to a previously described procedure [9]. The extraction solution consisted of methanol/water (80:20 v/v%) and contained the creatinine-[^{2}H$_3$] or the creatinine-[^{13}C$_3$,^{2}H$_3$] internal standard (IS) at a concentration of 20 µmol/L.

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 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, 0.1 µL of the extract was injected to the LC-MS/MS system.

Validation

DBS validation

The analytical method validation was performed at a standardized HT value of 0.38 L/L and included linearity, precision, accuracy, selectivity, specificity, and stability based on international guidelines [10]. For the DBS matrix, the validation was extended with the investigation of the effect of the blood spot volume and HT.

Since creatinine-free blood and thus DBS is not available and cannot be prepared without changing the matrix, it was difficult to fully meet the validation requirements using a standard approach. In order to assess the performance of the analytical method, the method was validated using three different strategies. The first strategy was a seven-point calibration curve, using the intercept of the calibration curve to correct the concentrations for the creatinine present in the used batch of blood. Secondly, a one-point calibration curve at an extremely high concentration of approximately 100 times higher than regular creatinine concentrations was used in order to limit the contribution of the creatinine already present in the used batch of blood. The used IS for these validations was creatinine-[^{2}H$_3$]. The third strategy was the use of a creatinine-[^{2}H$_3$] calibration curve as replacement for creatinine and with creatinine-[^{13}C$_3$,^{2}H$_3$] as IS. Since creatinine-[^{2}H$_3$] is not naturally present in the blood, this provided the possibility to assess the lower limit of quantification (LLOQ) of the analysis method.

Analytical method validation

All reference standards were weighed and dissolved in purified water. 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 and QCs. The prepared blood standards were then tumble mixed gently for 30 min at room temperature directly followed by the preparation of the DBS according to Section 2.3. A seven-point calibration curve was prepared by addition of 60.0, 80.0, 100, 150, 250, 320, and 400 µmol/L creatinine. For the one-point calibration curve, a standard of 7000 µmol/L creatinine was prepared. The following QC concentrations were added for the validation: LLOQ 60.0 µmol/L, Low 100 µmol/L, Medium (Med) 200 µmol/L, and High 320 µmol/L.

The validation of the linearity, precision, and accuracy was also performed with creatinine-[^{2}H$_3$] as target substance and creatinine-[^{13}C$_3$,^{2}H$_3$] as IS.

In order to assess the agreement of the analytical responses of creatinine and creatinine-[^{2}H$_3$], the calibration curves of creatinine and creatinine-[^{2}H$_3$] were compared in extraction solvent. First, two separate calibration curves of creatinine and creatinine-[^{2}H$_3$] were spiked in extraction solvent containing 20 µmol/L creatinine-[^{13}C$_3$,^{2}H$_3$] as IS in order to represent 60.0, 80.0, 100, 150, 250, 320, and 400 µmol/L creatinine or creatinine-[^{2}H$_3$]. The calibration curves were each analyzed in triplicate and the response ratios were compared using Passing and Bablok regression.

The concentrations used for the eight-point calibration curve of creatinine-[^{2}H$_3$] in DBS were 1.00, 3.00, 10.0, 30.0, 100, 200, 320, and 400 µmol/L. The following QC concentrations were used for the validation: LLOQ 1.00 µmol/L, Low 3.00 µmol/L, Med 200 µmol/L, and High 320 µmol/L.

Validation was performed with a maximum tolerated coefficient of variation (CV) and bias of 20 % for the LLOQ and 15 % for all other QC samples including the stability validation. For the determination of the precision and accuracy, all QC concentrations were measured fivefold in three separate runs on separate days. For each precision and accuracy concentration, CV and bias were calculated per run. Within-run, between-run, and overall CVs 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 fivefold without addition of creatinine and at Low and High creatinine concentrations and compared to simultaneously prepared DBS fivefold which were stored at −20 °C. Stability at −20 °C was assessed by preparing fresh DBS after 29 weeks of storage at −20 °C. Stability of creatinine was assessed fivefold as processed sample in the autosampler at 10 °C after 7 days. Stability of creatinine in DBS at 22, 32, and 50 °C was assessed fivefold at multiple time points. All stability results were calculated with the use of the one-point calibration curve.

Table 1 The precision and accuracy results obtained with the use of the seven-point calibration curve with creatinine-[^{2}H$_3$] as internal standard

Concentration (μmol/L)	Within-run CV n=15 (%)	Between-run CV n=15 (%)	Overall CV n=15 (%)	Measured mean (μmol/L)	Overall bias n=15 (%)
60	3.3	2.5	4.1	66	9.4
120	2.1	2.7	3.4	125	4.3
160	2.2	2.0	3.0	168	5.1
260	2.8	2.2	3.6	265	2.0
380	2.5	2.8	3.8	395	4.0

Blood spot volume

To assess the effect of the blood volume used to create a blood spot, blood was prepared with a HT of 0.38 L/L. DBS were prepared at Low and High 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 and CV of 15 %.

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 the extraction recovery, matrix effects, and process efficiency of creatinine and creatinine-[^{2}H$_3$], a defined amount of blood has to be used to create the DBS. Blank paper spots were punched and transferred to an Eppendorf cup, and 15 μL blood with a HT of 0.38 L/L at Low, Med, and High concentration was spiked on the punched spots and analyzed the next day fivefold (solutions A Low, Med, and High). In order to determine the extraction recovery, matrix effect, and process efficiency, DBS without creatinine should be used to obtain blank DBS extracts. These extracts could be spiked with creatinine at the concentrations that represent 100 % recovery, matrix effect, or process efficiency. Since it is impossible to obtain creatinine-free blood, a batch of blood was used from which the creatinine concentration was established by LC-MS/MS measurements. For the extraction recovery, extracts of DBS containing only naturally present creatinine were spiked at Low, Med, and High concentration (solutions

B Low, Med, and High) and were compared to solutions A. Since solutions A and B both contained the naturally present creatinine, there was no need to correct for this phenomenon.

For the matrix effect and process efficiency of creatinine, the established concentration of the naturally occurring creatinine was added up to the Low, Med, and High concentrations of the extraction solvent (solutions C Low, Med, and High).

This was not an issue for the matrix effect and process efficiency testing of creatinine-[^{2}H$_3$], where the extraction solvents were spiked at Low, Med, and High concentration (solutions C Low, Med, and 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 follows: recovery=A/B×100, matrix effect=(B/C×100)−100, and process efficiency=A/C×100. A, B, and C refer to the prepared solutions mentioned above.

Ion suppression chromatograms were obtained by injection of six processed DBS samples obtained from healthy volunteers with the use of a t-piece to combine the flows of the syringe pump with creatinine stock solution and the LC pump.

Influence of the hematocrit

To test the influence of the HT, blood samples at Low and High concentration were prepared at the following HT values: 0.23, 0.28, 0.33, 0.38, 0.43, 0.48, and 0.53 L/L. For the remaining, the procedure described in Section 2.3 was followed. The HT of 0.38 L/L was considered the standard HT values and the biases of the other HT values were calculated with a maximum acceptable bias and CV of 15 %.

Table 2 The precision and accuracy results obtained with the use of the one-point calibration curve with creatinine-[^{2}H$_3$] as internal standard

Concentration (μmol/L)	Within-run CV n=15 (%)	Between-run CV n=15 (%)	Overall CV n=15 (%)	Measured mean (μmol/L)	Overall bias n=15 (%)
56	3.6	1.9	4.0	56	0.0
116	2.2	2.9	3.7	111	−4.2
156	2.3	2.6	3.4	150	−3.2
256	2.9	2.9	4.1	240	−5.9
376	2.5	3.6	4.4	361	−3.9

Table 3 The precision and accuracy results obtained with the use of the eight-point calibration curve with creatinine-[²H₃] as target substance and creatinine-[¹³C₃,²H₃] as internal standard

Concentration (µmol/L)	Within-run CV $n=15$ (%)	Between-run CV $n=15$ (%)	Overall CV $n=15$ (%)	Measured mean (µmol/L)	Overall bias $n=15$ (%)
1.0	2.2	13.8	14.0	1.0	2.9
3.0	2.5	5.8	6.3	2.8	−5.8
200	3.9	0.0	3.9	208	3.4
320	3.6	0.0	3.6	326	1.9

Correlation study: patient analysis in venous blood and DBS from venous blood

The correlation study included EDTA blood samples of solid organ transplant recipients that were obtained for routine TDM. Venous EDTA blood samples received by the laboratory for routine immunosuppressant analysis were analyzed daily according to a previously published method [11]. The venous EDTA blood samples were used to create venous DBS according to Section 2.3. The DBS samples were analyzed with the one-point calibration curve and correlated with the results from the plasma analysis performed for routine patient monitoring in the hospital using Passing and Bablok with Analyse-it® software. The creatinine routine plasma analysis was performed with a Roche enzymatic creatinine assay on a Roche Modular (Roche Diagnostics Limited, West Sussex, UK).

In order to assess the possibility to use creatinine-[²H₃] to calculate creatinine levels, the patient correlation study was also performed with the use of the creatinine-[²H₃] calibration curve, while creatinine Low, Med, and High controls were used for run acceptance. In addition, the results obtained by both calibration curves were correlated with each other using Passing and Bablok with Analyse-it® software.

Results

Validation results

Analytical method validation

All three validation approaches yielded excellent validation results. The regression equations of the seven-point calibration curves which were analyzed on each of the 3 days were used to calculate the mean creatinine concentration of the un-spiked blood. This resulted in a creatinine concentration of 60 µmol/L, which was added to the theoretical validation concentrations.

The seven-point calibration curve with the use of creatinine-[²H₃] as IS was validated with a linear range of 120 to 460 µmol/L and a correlation coefficient of 0.9992 without weighing. In Table 1, the validation results regarding

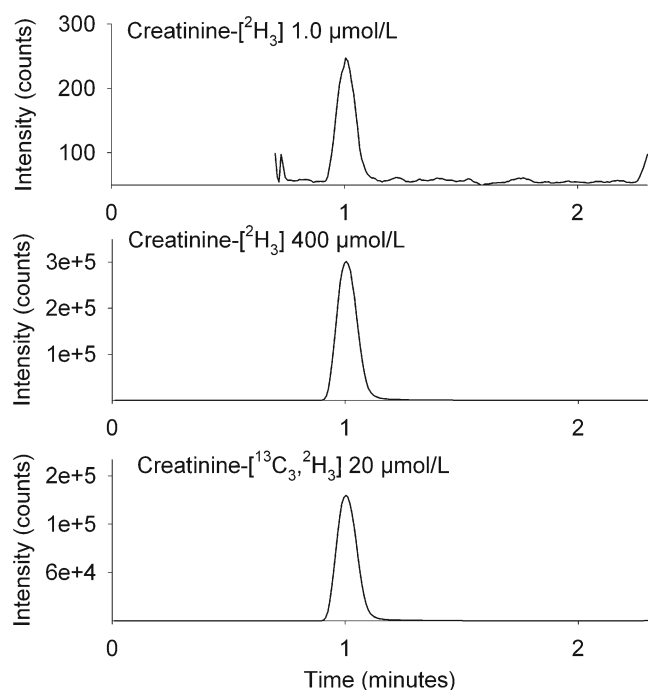precision and accuracy are shown which were obtained with the seven-point calibration curve.

For the one-point calibration curve, which was analyzed on each of the 3 days, the mean un-spiked blood concentration was calculated to be 56 µmol/L, which was added to the theoretical validation concentrations. In Table 2, the validation results regarding precision and accuracy are shown which were obtained with the one-point calibration curve.

The peak area ratios of the seven-point calibration curves prepared with creatinine and creatinine-[²H₃] in extraction solvent showed corresponding peak area ratios. The following Passing and Bablok correlation equation was found: $y=1.02x -0.01$ (95 % confidence intervals; slope=0.98–1.08, intercept=−0.01–0.00). This proved that the area response ratios of creatinine and creatinine-[²H₃] were equal.

The eight-point calibration curve with the use of creatinine-[²H₃] as target substance and creatinine-[¹³C₃,²H₃] as IS was validated in DBS with a range of 1.0 to 400 µmol/L and a correlation coefficient of 0.9971 with $1/X^2$ weighting with 0.5 µL injection volume. In Table 3, the validation results
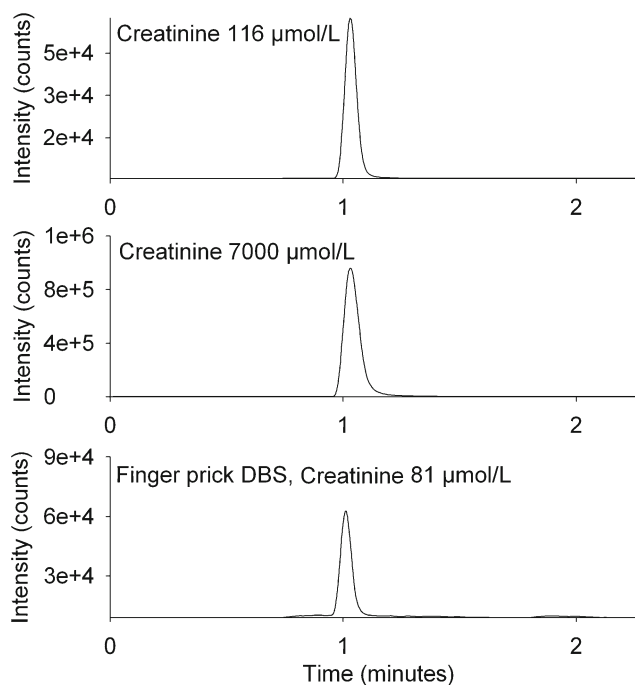
Fig. 1 Representative LLOQ and ULOQ chromatograms for creatinine and a chromatogram obtained from a finger prick DBS from a healthy volunteer

Fig. 2 Representative LLOQ and ULOQ chromatograms for creatinine-[^{2}H$_3$] and a chromatogram of creatinine-[^{13}C$_3$,^{2}H$_3$]

regarding precision and accuracy are shown which were obtained with the eight-point calibration curve with the use of creatinine-[^{2}H$_3$] as target substance.

Representative LLOQ and upper limit of quantification (ULOQ) chromatograms for creatinine, followed by a chromatogram obtained from a finger prick DBS from a healthy volunteer, are shown in Fig. 1. In Fig. 2, representative LLOQ and ULOQ chromatograms are shown for creatinine-[^{2}H$_3$] and a creatinine-[^{13}C$_3$,^{2}H$_3$] chromatogram.

The results of the stability validation with the use of the one-point calibration curve are shown in Table 4. The

creatinine DBS showed stability issues, which depended on the temperature and the concentration of the creatinine. At higher temperature and lower concentration, the creatinine showed increased biases. The measurements of the un-spiked creatinine DBS showed the largest biases. The un-spiked creatinine DBS showed to be stable for 1 week at 22 and 32 °C.

Blood spot volume

The volume of the blood spot showed to be of minor impact on the measured concentrations. The 30-μL spots showed biases of −6 % for the Low and −7 % for the High, while the 70-μL spots showed biases of +1 % for the Low and +2 % for the High concentrations.

Extraction recovery, matrix effect, and process efficiency

With the used extraction and analysis method, high process efficiencies were obtained without any significant matrix effects. The recoveries for creatinine were 87.3, 101.0, and 110.5 % for Low, Med, and High, respectively. The matrix effects for creatinine were 1.6, 4.7, and 0.7 % for Low, Med, and High, respectively. The total process efficiencies for creatinine were 88.7, 105.7, and 109.7 % for Low, Med, and High, respectively.

The recoveries for creatinine-[^{2}H$_3$] were 100.4, 92.2, and 94.6 % for Low, Med, and High, respectively. The matrix effects for creatinine-[^{2}H$_3$] were −4.3 %, −5.1 %, and -5.8 % for Low, Med, and High, respectively. The total process efficiencies for creatinine-[^{2}H$_3$] were 96.0, 87.5, and 89.1 % for Low, Med, and High, respectively.

Table 4 The results of the stability validation with the use of the one-point calibration curve

Stability	Time weeks	Concentration 56 μmol/L		156 μmol/L		376 μmol/L	
		CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
AS 10 °C	1	1.7	−3.9	2.8	−4.6	2.6	−5.3
DBS −20 °C	29	0.9	7.7	2.1	2.9	1.7	1.5
DBS 22 °C	1	2.1	−4.1	2.3	−7.3	2.3	−9.2
DBS 22 °C	2	3.5	39.7	1.7	−2.8	1.8	−6.6
DBS 22 °C	4	n.p.	n.p.	1.3	−0.6	1.6	−7.1
DBS 32 °C	1	0.7	4.7	1.1	−2.0	2.2	−11.1
DBS 32 °C	2	5.9	44.5	1.8	1.5	3.0	−7.5
DBS 32 °C	4	1.4	59.4	2.0	7.8	1.4	−7.9
DBS 50 °C	1	1.8	77.1	1.5	19.2	4.1	−4.2
DBS 50 °C	2	3.3	133.1	3.4	35.0	0.9	−1.8
DBS 50 °C	4	0.9	165.8	1.5	42.4	1.8	4.0

AS autosampler stability in processed sample, *n.p.* not performed

Table 5 Effect of the hematocrit on the bias at Low and High level with the standard hematocrit set at 0.38 L/L

Hematocrit (L/L)	Concentration			
	156 µmol/L		376 µmol/L	
	CV n=5 (%)	Bias n=5 (%)	CV n=5 (%)	Bias n=5 (%)
0.23	4.1	−5.0	2.1	−4.2
0.28	2.5	0.4	1.7	−1.4
0.33	1.5	0.1	1.4	−0.6
0.38	3.0	0.0	1.7	0.0
0.43	4.0	6.3	3.5	3.8
0.48	2.6	8.2	3.1	7.3
0.53	2.2	9.9	1.5	9.0

The ion suppression chromatograms showed very little ion suppression at the dead time, partly because of the extremely low injection volume of 0.1 µL. No ion suppression was observed near the retention time of creatinine.

Influence of the hematocrit

The HT value showed not to be of major influence on the analytical results. With the HT of 0.38 L/L as the standardized HT value, positive biases were observed for increasing HT values and vice versa. The highest biases were found at the Low level where the HT of 0.23 L/L showed a bias of −5.0 %, while the HT of 0.53 showed a bias of 9.9 % (see Table 5).

Correlation study: patient sample analysis in venous blood and DBS prepared from venous blood

For the correlation study, 50 patient samples were analyzed in plasma and DBS with HT values ranging from 0.28 to 0.56 L/L. The following Passing and Bablok fit was found with the

Fig. 3 Patient correlation study of plasma and DBS for creatinine using the one-point calibration curve. The *dotted line* is the identity line; the *continuous line* is the Passing and Bablok regression line. The simple linear regression coefficient and Passing and Bablok fit are the following: R^2 of 0.9904 ($n=50$), $y=0.80x-2.92$ (95 % confidence intervals; slope=0.77–0.84, intercept=−8.64–0.04)
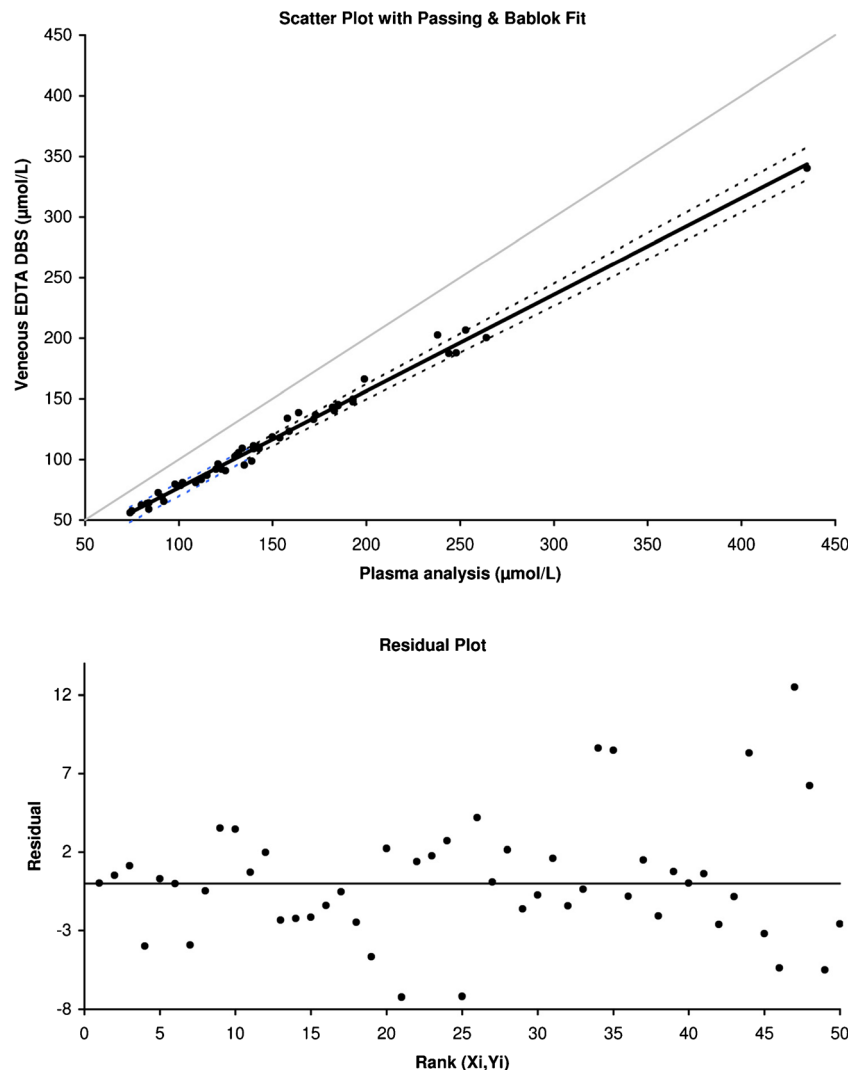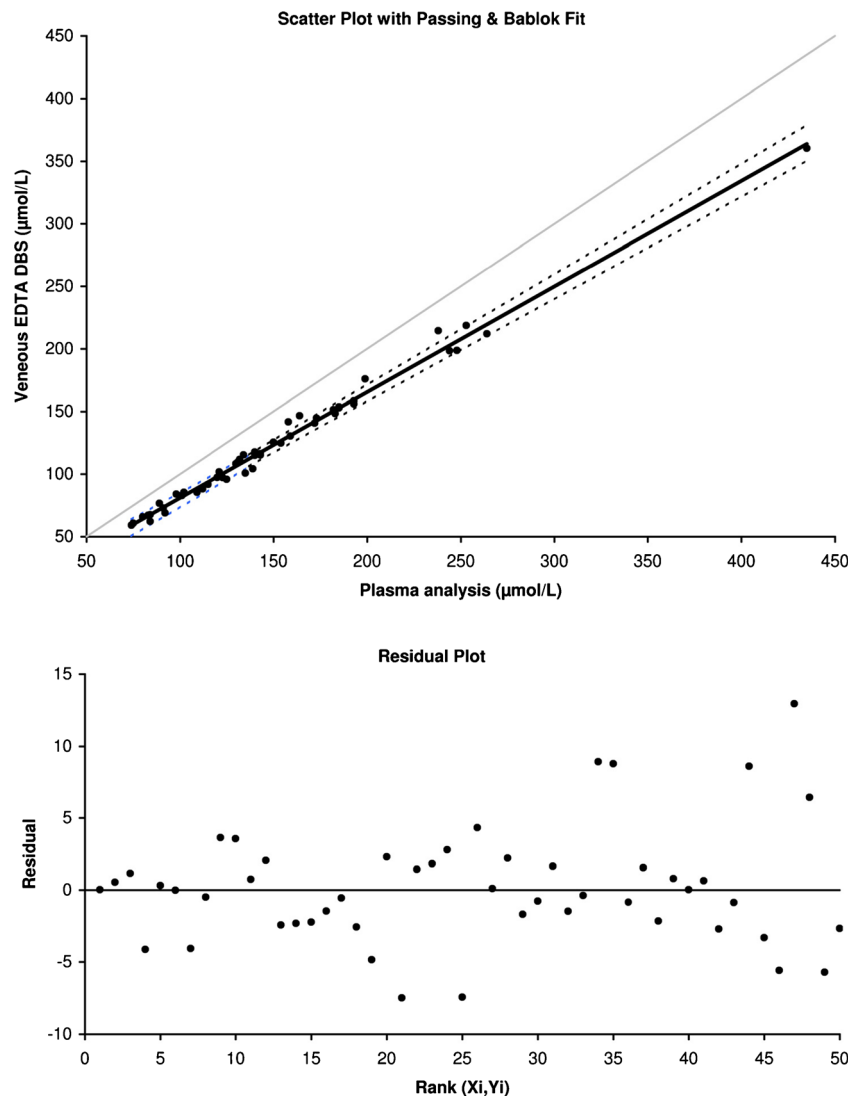
creatinine one-point calibration: $y=0.80x-2.92$ (95 % confidence intervals; slope=0.77–0.84, intercept=−8.64–0.04) with a R^2 of 0.9904 using simple linear regression. The 95 % confidence intervals of the Passing and Bablok results showed a systematic difference of 20 % lower concentrations of DBS from venous blood compared to plasma results. The correlation results for the creatinine one-point calibration curve, obtained with Passing and Bablok regression, are shown in Fig. 3.

The patient correlation study calculated with the creatinine-[^{2}H$_3$] eight-point calibration curve and creatinine controls showed the following Passing and Bablok fit: $y=0.84x-3.34$ (95 % confidence intervals; slope=0.82–0.89, intercept=−9.40 to −0.20) with a R^2 of 0.9904 using simple linear regression. These results showed a systematic difference of 16 % lower concentrations of creatinine in DBS from venous blood compared to plasma results. The correlation results for the creatinine-[^{2}H$_3$] eight-point calibration curve, obtained with Passing and Bablok regression, are shown in Fig. 4.

The correlated patient results obtained by creatinine and creatinine-[^{2}H$_3$] DBS calibration curves showed the following Passing and Bablok fit: $y=1.06x-0.25$ (95 % confidence intervals; slope=1.06–1.06, intercept=−0.25 to −0.25) with a R^2 of 1.0000 using simple linear regression. The patient sample results of the creatinine-[^{2}H$_3$] calibration curve showed overlapping 95 % confidence intervals with the patient results calculated with the creatinine one-point calibration curve with very small confidence intervals. This further underlines the suitability of both the creatinine one-point calibration curve as well as the eight-point creatinine-[^{2}H$_3$] calibration curve for creatinine DBS patient analysis.

Discussion

Our developed analytical procedure showed that the determination of creatinine in addition to tacrolimus, sirolimus,

Fig. 4 Patient correlation study of plasma and DBS for creatinine using the eight-point creatinine-[^{2}H$_3$] calibration curve. The *dotted line* is the identity line; the *continuous line* is the Passing and Bablok regression line. The simple linear regression coefficient and Passing and Bablok fit are the following: R^2 of 0.9904 (n=50), $y=0.84x-3.34$ (95 % confidence intervals; slope= 0.82–0.89, intercept=−9.40 to −0.20)

everolimus, and cyclosporine A in the same extract of a single DBS sample with only a simple reinjection of the sample produced accurate and precise results [9].

All three validation approaches yielded excellent results. The use of a seven-point calibration curve required a correction for the naturally occurring creatinine in the used batch of blood, while the use of a one-point calibration curve did not require this correction. This simplifies the concentration calculation process and will be less prone to errors. The concentration of the one-point calibrator of 7000 μmol/L was not adjusted because concentration enhancement by the natural presence of creatinine in the calibrator would be only 1 %. The purpose of this approach was that the calibration curve would be independent of the naturally occurring creatinine in the used batch of blood. Although the use of a one-point calibration curve may not be a standard approach, the here described methodology proved to be practical, provided valid results and its use can be scientifically supported [12].

The lowest calibrator would normally also represent the LLOQ. Because of the naturally occurring creatinine, the lowest calibrators were 120 and 116 for the seven-point and one-point calibration curves. In order to calculate the un-spiked creatinine DBS, the seven-point calibration curve needed to be extrapolated, while this was technically not the case for the one-point calibration curve. The replacement of the creatinine calibration curve by the creatinine-[^{2}H$_3$] calibration curve proved to provide equal analytical results. Therefore, it would be feasible to use a creatinine-[^{2}H$_3$] calibration curve to calculate creatinine patient and QC concentrations. In addition, this validation showed that the analytical method was capable of measuring accurate and precise creatinine levels below 120 or 116 μmol/L.

It is known that creatinine concentrations significantly increase in serum and plasma within 24 h to 3 days [13–15]. Ring closure of creatine in the matrix occurs while losing a molecule of water to form creatinine under influence of time, temperature, and pH [16–19]. DBS is often promoted as a more stable medium for a blood sample, and this is also true for creatinine [20]. Our experiments showed that stability is now extended to 7 days at a maximum temperature of 32 °C.

For clinical practice, the DBS stability of 1 week should provide enough time to send the sample to the laboratory. There the DBS could be stored at −20 °C up to 29 weeks until analysis. A validation without the assessment of the un-spiked DBS could have provided misleading stability results, especially when creatinine-free blood or another matrix is used for the stability assessment. This would have resulted in seemingly acceptable stability results during the validation, while DBS patient results could be wrongly reported after implementation.

Although HT effects are widely acknowledged, the HT effects observed in this research were of minor influence on the analytical results. This could be explained by the fact that creatinine is not bound to proteins and equally distributes into the red blood cells. This causes a homogeneous distribution of creatinine through the blood sample which is unaffected by the HT value. Only changes in the DBS spot size due to the HT value may be observed.

The correlation study showed good correlations for plasma and venous DBS samples calculated with the use of the one-point DBS creatinine calibration and with the eight-point creatinine-[^{2}H$_3$] calibration. The observed differences could be ascribed to differences in the analysis techniques or to the blood-to-plasma ratio and distribution of creatinine. This should be further investigated before the clinical validation with real DBS finger prick samples is performed. The correlation study showed the capability of the DBS method to be used as an alternative for creatinine plasma measurement. When creatinine DBS analysis is applied for routine analysis, the reference ranges of creatinine DBS should be evaluated and if required adapted according to the results of the clinical validation.

After clinical validation, the developed DBS method would be a useful addition to the monitoring of creatinine levels and therapeutic drug monitoring of renally excreted or nephrotoxic drugs.

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