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Prediction of the Hematocrit of Dried Blood Spots via Potassium Measurement on a Routine Clinical Chemistry Analyzer

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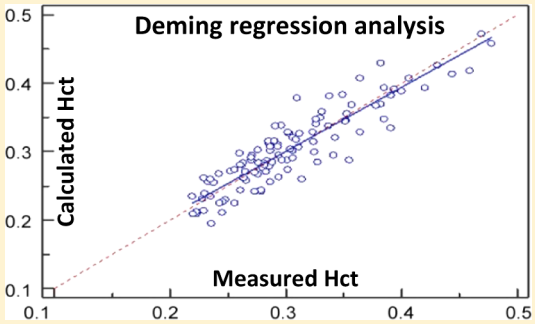
Supporting Information

ABSTRACT: The potential of dried blood spot (DBS) sampling as an alternative for classical venous sampling is increasingly recognized, with multiple applications in, e.g., therapeutic drug monitoring and toxicology. Although DBS sampling has many advantages, it is associated with several issues, the hematocrit (Hct) issue being the most widely discussed challenge, given its possible strong impact on DBS-based quantitation. Hitherto, no approaches allow Hct prediction from nonvolumetrically applied DBS. Following a simple and rapid extraction protocol, K^+ levels from 3 mm DBS punches were measured via indirect potentiometry, using the Roche Cobas 8000 routine chemistry analyzer. The extracts' K^+ concentrations were used to calculate the approximate Hct of the blood used to generate DBS. A linear calibration line was established, with a Hct range of 0.19 to 0.63 (lower limit of quantification, LLOQ, to upper limit of quantification, ULOQ). The procedure was fully validated; the bias and imprecision of quality controls (QCs) at three Hct levels and at the LLOQ and ULOQ was less than 5 and 12%, respectively. In addition, the influence of storage (pre- and postextraction), volume spotted, and punch homogeneity was evaluated. Application on DBS from patient samples (n = 111), followed by Bland and Altman, Passing and Bablok, and Deming regression analysis, demonstrated a good correlation between the "predicted Hct" and the "actual Hct". After correcting for the observed bias, limits of agreement of ±0.049 were established. Incurred sample reanalysis demonstrated assay reproducibility. In conclusion, potassium levels in extracts from 3 mm DBS punches can be used to get a good prediction of the Hct, one of the most important "unknowns" in DBS analysis.



Dried blood spot (DBS) sampling is increasingly used as a minimally invasive tool to acquire a representative blood sample in the context of therapeutic drug monitoring (TDM) and toxicology.[1−4] However, the analysis of DBS is associated with several issues, such as contamination risk, blood volume spotted, blood spot homogeneity, and hematocrit (Hct).[3−5] Of these, the Hct is undoubtedly the most widely discussed challenge, as strongly deviating Hct values may significantly impact DBS-based quantitation.[3,5−15] First of all, the Hct strongly influences the spreading of a blood drop on filter paper, with higher Hct values leading to smaller, more concentrated spots.[3,5−9] Second, the Hct may influence parameters such as recovery and matrix effect.[6,10] Third, when DBS results are to be compared with those obtained from plasma, the distribution of an analyte in red blood cells and plasma needs to be examined on a case-by-case basis.[3,15] These Hct-associated issues, when compared to conventional plasma analysis, make DBS-based quantitation suffer from an additional unknown factor of uncertainty.

Several strategies have been proposed to cope with the so-called "hematocrit effect".[11] The most easy approach is the analysis of complete, volumetrically applied DBS, obtained by pipetting or using precision capillaries or other microsampling devices, delivering a fixed amount of blood to filter paper.[6,10] Indeed, this approach copes with the most evident Hct effect, i.e., the differential spreading of blood with varying Hct. However, volumetric application requires some training and may be difficult to sustain when DBSs are to be obtained by patients at home (e.g., in the context of TDM programs). In these cases, direct application from a cleaned fingertip may be the best feasible approach. As this implies, per definition, nonvolumetric application, DBS punches rather than complete DBS should be evaluated, necessitating the definition of a Hct range and a volume range in which the results for a given analyte still fulfill the acceptance criteria for precision and accuracy.[5,8]

Whereas some have considered the correlation between the DBS diameter and the Hct of volumetrically applied blood,[8,16] hitherto, there is no approach available that allows one to trace back the Hct of DBS obtained by nonvolumetric application of blood. A "marker" allowing one to trace back the Hct should fulfill several criteria: first, it should correlate with the amount of red blood cells; second, it should be universal (i.e., show minimal interindividual variation); third, it should be stable

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(i.e., being applicable to both freshly prepared and old DBS, we found hemoglobin to not fulfill this criterion); fourth, its determination should be possible on a minimal area of the DBS, while still being easy enough to allow universal implementation. Keeping these requirements in mind, potassium (K^+) was chosen as a candidate marker to predict Hct. This electrolyte is primarily located intracellularly, its intracellular concentration (at approximately 140 mM) being about 35 times higher than its extracellular concentration.[17] In addition, K^+ levels are under tight physiological control, with normal serum or plasma levels ranging between 3.5 and 5 mM.[17] As erythrocytes are the predominant cells in the blood, roughly outnumbering white blood cells with a factor of 500−1000, these are the major contributors to the total blood K^+ concentration. Hence, the contribution by the serum or plasma and by other cells accounts for only a low % of the total blood K^+ concentration. As a consequence, physiological changes in serum or plasma concentration and/or in white blood cell count are only expected to have a minimal, if any, impact on total blood K^+ concentration. In this report, we describe that K^+ measurement in extracts from 3 mm DBS punches, using a routine clinical chemistry analyzer, allows one to trace back the Hct of a DBS with acceptable accuracy and precision. Satisfactory results were obtained after evaluation of the developed procedure on DBS from patients.

EXPERIMENTAL SECTION

Preparation of DBS. Venous blood, used for preparation of DBS, was obtained from healthy volunteers and collected in tubes containing Li-heparin as anticoagulant (Venosafe 9 mL VF-109SHL, Terumo, Leuven, Belgium). DBSs were generated the same day of blood collection by applying 25 μL of blood (unless mentioned otherwise) on Whatman 903 filter paper (WHA10334885, GE Healthcare, Dassel, Germany), followed by air drying for a minimum of 2 h. Blood with different Hct was prepared by centrifuging an aliquot of the blood in 2 mL safe-lock tubes in an Eppendorf 5804R centrifuge (Hamburg, Germany) for 5 min at 1000*g* and by removing or adding plasma. The obtained Hct was measured at all instances in duplicate using a Sysmex XE-5000 hematology analyzer (Sysmex Corporation, Kobe, Japan); the mean of these duplicate measurements was considered as the actual Hct. For comparing the Hct of Li-heparin blood and ethylenediaminetetraacetic acid (EDTA)(K_2) blood, coupled samples were obtained from both patients and healthy volunteers. Both groups received information and signed informed consent before entry into this study, which was approved by the Ethics Committee of Ghent University Hospital (project number 2012/314). Harris cutting mats and micropunchers (Uni-Core, 3.00 mm diameter) were obtained from Sigma-Alrich (Bornem, Belgium).

Extraction of Potassium from DBS. Evaluation of the optimal conditions to extract K^+ from DBS was done using 3 mm punches from 2 day old and freshly prepared DBS. Different elution solvents were tested: ultrapure water with or without Triton X-100 (0.45%), PBS, and hypotonic (1:10) PBS with or without Tween 20 (0.05%). We opted to use solvents readily containing 2.5 mM KCl, as the final K^+ concentrations of the resulting solutions were within the validated range of the chemistry analyzer (see below), even at the extreme Hct of 0.19 and 0.63. Two subsequent extractions were performed at room temperature in a 2 mL tube by adding 70 and 30 μL, respectively, to the DBS punch and shaking for 15 min at 1400 rpm on an Eppendorf Comfort Thermomixer. After spinning down the punch, the resulting supernatants were transferred to microcups (Sample Cup Micro 13/16, Roche Diagnostics, Mannheim, Germany). The results obtained for the DBS were corrected for those obtained for the extraction buffer. In line with the findings by Langer et al.,[18] we did not find a measurable contribution from blank paper. Comparison of the different elution solvents (4−6 replicates for each solvent; 3 independent experiments) revealed that none outperformed elution with a 2.5 mM KCl solution in ultrapure water (Supplementary Figure S-1A, Supporting Information). Using 2.5 mM KCl in ultrapure water, the optimal extraction conditions were evaluated further (4−6 replicates for each condition). This revealed that (i) two subsequent elutions with 50 μL equaled elution with 70 and 30 μL respectively, and was better than a single elution with 100 μL; (ii) maximal extraction was already obtained within 1 min of shaking at 1400 rpm; (iii) extraction at 37 °C did not improve the extraction efficiency (Supplementary Figure S-1B, Supporting Information).

Analyses. Routine Hct and K^+ measurements were performed in the ISO 15189 accredited Laboratory of Clinical Biology at Ghent University Hospital. Hct determinations were performed using a Sysmex XE-5000 hematology analyzer, having a measurement range of 0.17 (arbitrarily set, i.e., the lowest quality control (QC) applied in the routine laboratory) to 0.75. K^+ measurements were performed by indirect potentiometry using an ion-selective electrode (ISE) using the ISE module of the Roche Cobas 8000 chemistry analyzer (Roche Diagnostics), disposing of two measurement units and having technical limits of 1.5 and 10 mM. The validation data of both clinical Hct and K^+ determinations on these analyzers can be found in the Supporting Information. For the validation and application, K^+ and Hct measurements were performed in duplicate (unless otherwise mentioned) and the average of these duplicates was used for the calculations.

Validation. Homoscedasticity (homogeneity of variances), linearity, and the choice of the calibration model were evaluated by generating five 7-point calibration curves from DBS, prepared from blood with a Hct of ±0.25, 0.30, 0.39, 0.45, 0.50, 0.55, and 0.61 (prepared from a single donor). Homoscedasticity was examined by plotting the absolute residuals versus the Hct and by performing the F-test at the lowest and highest calibrator levels, at the 99% confidence interval (CI).[19,20] Linearity was assessed by performing Fisher's test.[21] Calibration curves were generated by unweighted, $1/x$, $1/x^2$, $1/y$, and $1/y^2$ weighted linear regression. The choice of the calibration model was based upon the sum % relative error (RE) and the % RE plot versus Hct.[19]

For the evaluation of accuracy (% bias) and precision (% RSD, relative standard deviation), we prepared on each of 4 different days, two 7-point calibration curves from blood with a Hct of ±0.19, 0.26, 0.33, 0.42, 0.48, 0.55, and 0.63. In conjunction with every calibration line, three DBS QCs (with a Hct of ±0.24, 0.41, and 0.58, further referred to as low, medium, and high QC) were prepared. For details we refer to the Supporting Information.

The impact of the punching site and of the applied volume was evaluated at the three Hct QC levels. K^+ concentrations obtained from central punches were compared with those obtained from peripheral punches, excluding the very edge (n = 6). The impact of the volume applied was evaluated in DBS prepared by spotting 15, 17.5, 20, 25, 30, 35, 40, or 50 μL of blood (n = 6).

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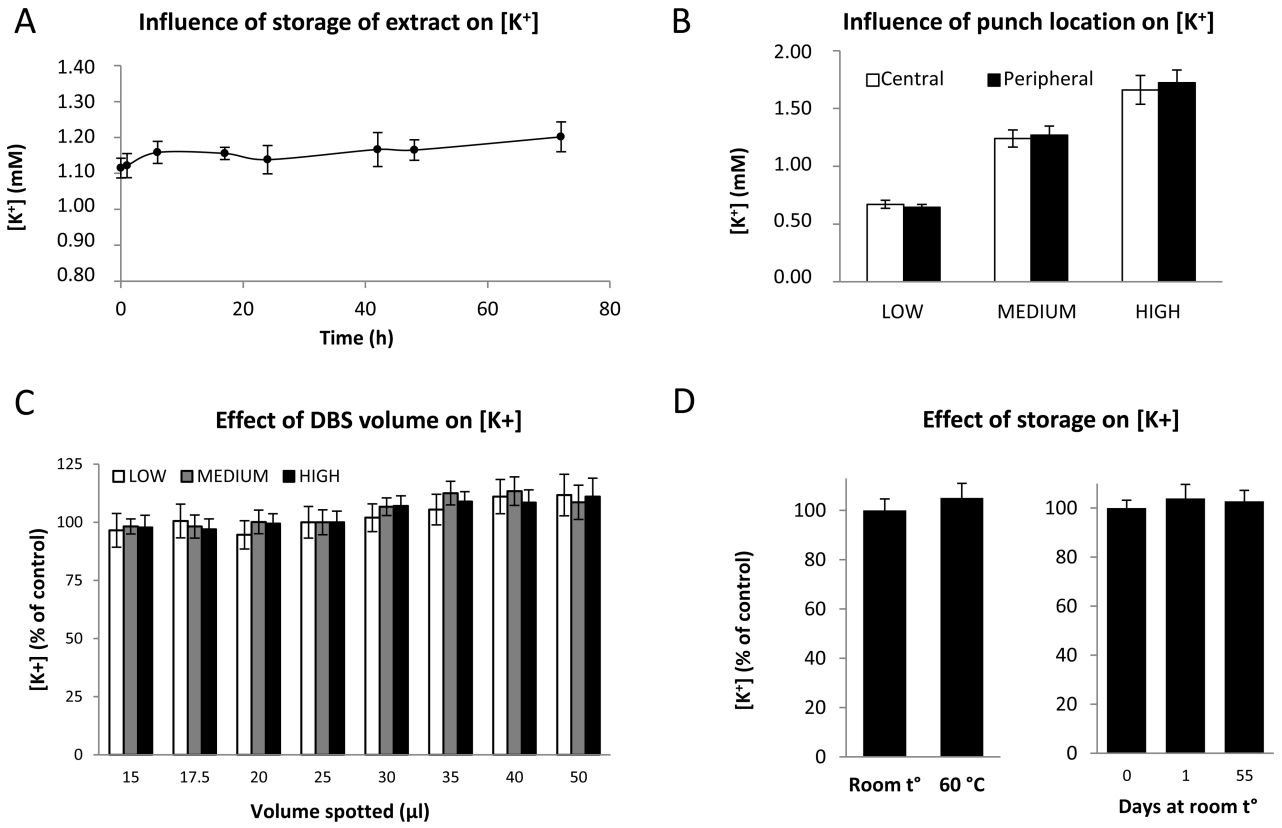

Figure 1. Influence of storage of extracts on the K^+ concentration in the extracts. The mean K^+ concentration at the different time points is shown, together with the standard deviation (n = 6) (A). Influence of the punch localization on K^+ concentration in the extracts, at three different Hct levels. The bars show the mean K^+ concentration, together with the standard deviation (n = 6) (B). Influence of the volume used to generate DBS on the K^+ concentration in DBS extracts, at three Hct levels. The bars show the mean of 3 independent experiments (each with n = 6 for every volume), with indication of the pooled standard deviation. A 25 μL DBS was taken as the reference (C). Influence of long-term storage (up to 55 days) at ambient temperature or short-term storage (20 h) at elevated temperature (60 °C) on K^+ concentrations in DBS. The bars show the mean and standard deviation (n = 6) (D).

The influence of storage was evaluated both before and after extraction of DBS. Postextraction stability of the K^+ concentrations was evaluated by combining the extracts of 3 punches in 1 microcup (n = 6), followed by direct evaluation and after 1, 6, 17, 24, 42, and 72 h. The influence of storage on K^+ in DBS was evaluated by comparing the K^+ concentrations from freshly prepared DBS (dried for 2 h) with those from DBS, stored at room temperature for 1 or 55 days and stored at 60 °C for 20 h.

Finally, we performed incurred sample reanalysis (with 7 days in between) on a subset (n = 49) of patient DBSs (see below). More than 2/3 of the repeated measurements should fulfill the acceptance criterion, i.e., lie within the limits of ±20% of the mean of the original and the corresponding reanalysis result.[22]

Application to Patient Samples. Evaluation of the procedure was performed using blood samples destined for routine clinical chemistry and hematology analysis, collected in Li-heparin tubes (Venosafe 2 mL VF-052SHL, 6 mL VF-106SAHL, or 9 mL VF-109SHL) and in 4 mL EDTA(K_2) tubes (Venosafe VF-054SDK) (all from Terumo, Leuven, Belgium). DBSs were prepared upon arrival at the clinical laboratory by pipetting 25 μL of Li-heparin-anticoagulated blood onto filter paper (n = 118). Relevant routine clinical parameters evaluated in the plasma obtained from these tubes included hemolytic index and plasma K^+ concentration. Hct from corresponding EDTA(K_2) tubes was determined via a single measurement on the Sysmex XE-5000. Following extraction from one 3 mm DBS punch per patient, K^+ levels were measured in duplicate. Samples with evidence of (or no data on) hemolysis (hemolytic index above 88) (n = 2) and samples where no duplicate results of extracted K^+ were obtained (n = 5) were excluded, resulting in a final data set of 111 samples.

Data Analysis. Statistical evaluation of the data was carried out using IBM SPSS Statistics 19 and Microsoft Excel 2010. Comparisons between the different extraction conditions were made using unifactorial ANOVA analysis. An unpaired t test (α = 0.05; 95% CI) was used to evaluate the influence of storage pre- and postextraction and to evaluate the effect of site of punching and applied volume.

Microsoft Excel 2010 was used to generate a Bland and Altman plot, while Medcalc software version 12.3.0.0 (http://www.medcalc.org) was used for Passing and Bablok and Deming linear regression analysis, for generating boxplots, and for generation of a mountain plot, depicting the distribution of the differences between the compared methods. The % RSDs, necessary for performing Deming linear regression analysis, corresponded to 0.81% for the Sysmex-measured Hct (i.e., the % RSD for the highest QC) and were derived from the duplicates (calculated from duplicate K^+ measurements) for the calculated Hct.

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RESULTS AND DISCUSSION

Optimization of the extraction of K^+ from 3 mm DBS punches resulted in the following optimal extraction conditions: two subsequent 5 min extractions at room temperature, under continuous shaking (1400 rpm), using 50 μL of a 2.5 mM KCl solution in ultrapure water. After the first extraction, 40 μL was transferred to a microcup; after the second extraction, another 50 μL extract was added to the microcup. Although a 1 min extraction gave equivalent results (Supplementary Figure S-1B, Supporting Information), we opted in the final protocol for a 5 min extraction for practical reasons. The combined extracts can be stored in the microcups at 4 °C for at least 72 h (Figure 1A) before analysis by the routine clinical chemistry analyzer. The developed procedure is easy and straightforward and is in principle fully automatable, allowing high-throughput analyses.

Several parameters have been shown to potentially influence the distribution of analytes in DBS. Therefore, it is important to control whether the K^+ concentration measured in the 3 mm DBS punches is affected by the punch location (peripheral versus central) or by the blood volume that was applied to prepare the DBS. Irrespective of the Hct, no significant difference (95% CI) was seen between the K^+ concentrations in discs punched out from 25 μL DBS peripherally versus centrally (Figure 1B). Evaluation of the applied volume revealed that differences in K^+ concentrations never exceeded 15%, taking 25 μL DBS as the reference and as extreme volumes 15 and 50 μL, respectively, irrespective of the Hct. However, although limited in extent, we did observe a trend of increasing K^+ concentrations upon increasing DBS volume (Figure 1C), as also others did, albeit for other analytes.[5,9] To minimize this volume effect, we propose that a volume criterion is set using filter paper with two preprinted concentric circles, in which a DBS should fill the inner circle (8 mm diameter) completely, while not exceeding the outer circle (13 mm diameter). Doing so, the volume of DBS will always lie between approximately 20−25 and 40−50 μL, whatever the Hct (Supplementary Figure S-2, Supporting Information). In our experience, this volume range covers the typical volumes obtained from a single drop of blood following a fingerprick.

Statistical evaluation of the calibration data revealed that the data were homoscedastic (i.e., had homogeneous variances) and that the calibration lines were linear, with no need for weighting. The slope and intercept of the calibration curve were 3.15 and −0.09, respectively, with respective 95% CI of 2.96−3.34 and −0.20 to −0.05. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were arbitrarily set at the lowest (0.19) and highest (0.63) points of the mean calibration curve, respectively. Even in a hospital setting, with an obvious overrepresentation of critically ill patients, this Hct range of 0.19−0.63 covers over 99.5% of the patients (1-year data, Ghent University Hospital). As shown in Table 1, precision and accuracy for the QCs (3 levels), LLOQ, and ULOQ fulfilled the predefined acceptance criteria at all instances (<15% RSD and bias). To confirm the validity of our approach, this part of the validation was duplicated, using blood from another donor, giving similar results (Supplementary Table S-1, Supporting Information). Moreover, when the QCs from this second donor were fitted into the final regression equation, obtained using blood from the first donor (and vice versa), precision and accuracy acceptance criteria were still met (Table 1 and Supplementary Table S-1, Supporting Information). Furthermore, evaluation of the influence of storage indicated that no significant change in K^+ concentration occurred upon prolonged storage of DBS at room temperature or after storage for 20 h at 60 °C (Figure 1D).

Table 1. Overview of the Data for Accuracy and Inter- and Intrabatch (n = 8 Duplicate Measurements) Precision for Donor 1[a]

	accuracy (% bias)	intrabatch precision (% RSD)	interbatch precision (% RSD)
A			
QC low	−1.07	8.49	11.69
QC medium	0.09	3.30	5.05
QC high	−2.25	3.42	6.72
B			
LLOQ (0.19)	4.20	9.25	9.25
ULOQ (0.63)	2.75	4.76	9.17
C			
QC low (donor 2)	−2.05	8.75	10.57
QC medium (donor 2)	0.57	3.37	6.26
QC high (donor 2)	−1.31	3.54	6.61

[a]A and B, respectively, give the data obtained for QCs (3 Hct levels) and LOQs (LLOQ and ULOQ), prepared from blood from the same donor as the one in which the calibrators were prepared. C gives the data for QCs prepared from blood from another donor than the one in which the calibrators were prepared.

The developed procedure was evaluated using paired EDTA(K_2) and Li-heparin blood samples, collected from patients (n = 111) for routine clinical purposes. These were used for direct Hct determination and for generating DBS, respectively. For the latter, the developed procedure was applied to calculate the Hct. Figure 2A shows the Bland and Altman comparison of the actually measured Hct and the calculated Hct, with indication of the mean difference and the limits of agreements (LoAs), all with their 95% CI. A first conclusion, which can be inferred from this comparison, is that there is a negative bias of 0.019. As suggested by Bland and Altman, when a consistent bias is observed between two methods (a reference method and a new method), this bias can be adjusted in the new method.[23] We opted to do this for three reasons. First, the slightly negative bias was consistently observed: when splitting the total data set of 111 patients into three subdata sets (each analyzed on a different day), a negative bias was observed for all subdata sets (Supplementary Figure S-3, Supporting Information). Second, a trend line through all data points of the Bland and Altman comparison had a slope close to zero, demonstrating the absence of a proportional difference (i.e., the bias does not depend on the Hct) (data not shown). Third, at least part of this bias can be explained by the fact that Hct values obtained from Li-heparin blood, used for setting up the calibration lines and for generating the DBS, are significantly lower than the Hct values obtained from the "gold standard" for Hct measurement, i.e., EDTA(K_2) blood. We made this observation in paired blood samples of both patients and healthy volunteers and found it to be related to the mean corpuscular volume of the red blood cells, which is significantly lower in Li-heparin blood (data not shown). Figure 2B shows a mountain plot, depicting the distribution of the differences between both methods for all data points, after bias correction. This plot nicely demonstrates that the differences are centered around zero. Passing and Bablok linear regression analysis was performed, also after correcting for the bias (Figure 2C, Table 2). A linear model fits

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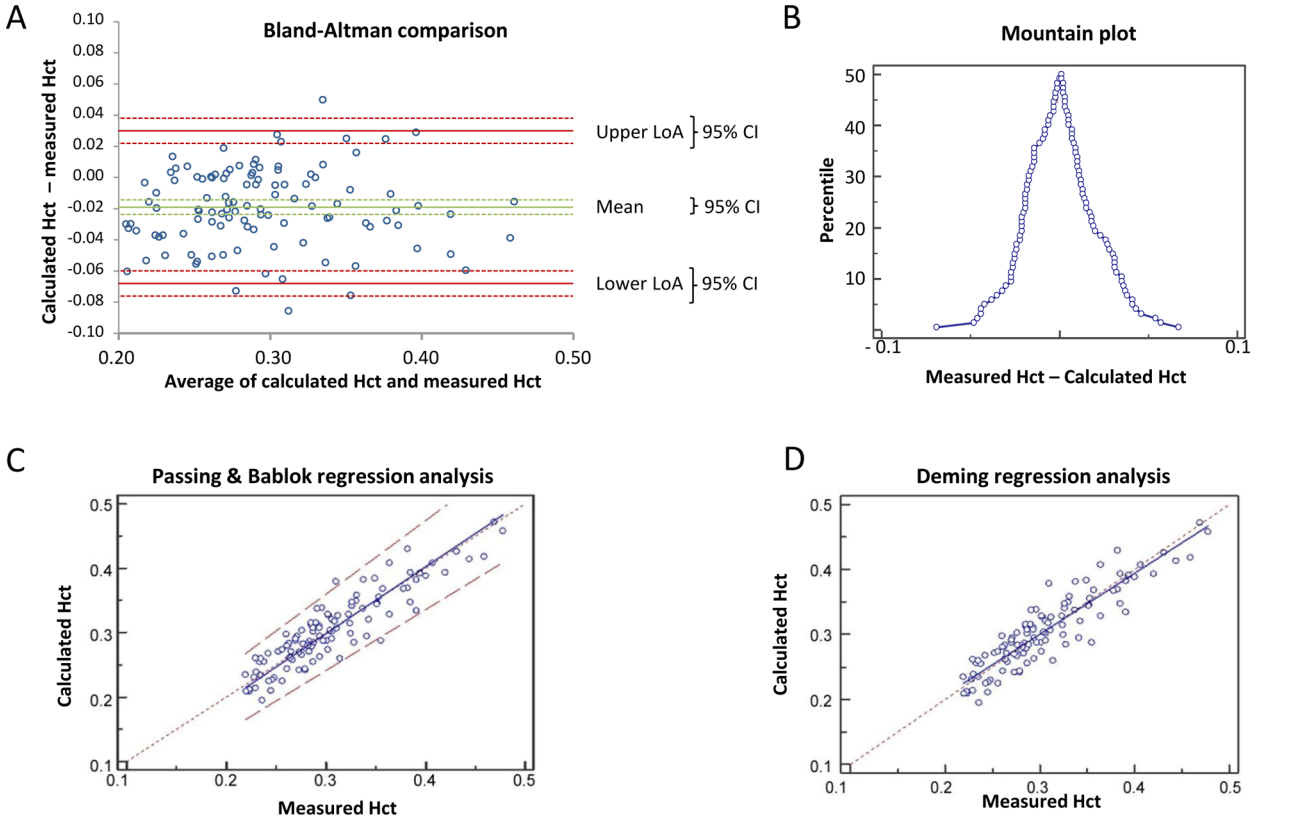

Figure 2. Bland and Altman comparison of calculated Hct and measured Hct. The mean difference, together with the upper and lower LoAs is indicated, with the respective 95% CI (A). Mountain plot depicting the distribution of the differences between the calculated and the measured Hct, after correction for the bias (B). Passing and Bablok (C) and Deming (D) regression analysis of the calculated and measured Hct, after correction for the bias.

Table 2. Results of the Passing and Bablok and Deming Linear Regression Analysis of the Comparison of the Calculated Hct with the Measured Hct[a]

	Passing and Bablok linear regression		Deming linear regression	
regression equation	$1.036x - 0.012$		$0.938x + 0.019$	
95% CI slope	0.943	1.141	0.861	1.015
95% CI intercept	−0.042	0.018	−0.004	0.042

[a]The regression equations are given, together with the 95% CI for the slope and intercept.

the data, and the 95% CI of the intercept contains the zero value. Hence, after correction for the bias, there is no systematic difference between the two methods. In addition, as the 95% CI for the slope contains the value of 1, there is no proportional difference between the two methods. The same conclusions were obtained when performing Deming regression analysis (Figure 2D, Table 2). A second conclusion that can be drawn from the Bland and Altman comparison is that the LoAs, after correcting for the bias, lie at ±0.049. These LoAs are acceptable, given the purpose of the method, i.e., getting an approximate estimation of the original Hct. As such, the developed procedure allows one to make a statement whether results obtained with a certain analytical method are indeed valid (i.e., whether the Hct of any given DBS lies within the predefined range of a validated method) or rather provide an under- or overestimation of the actual analyte concentration. The variation of the K^+-measurements themselves (i.e., technical variation) accounts for less than 0.01 of the observed LoAs. Although this is overall limited, it may be worthwhile to evaluate whether the use of alternative methodologies for K^+ measurement, such as flame photometry, atomic absorption, or inductively coupled plasma-based methods, may further improve the LoAs. However, the possible benefit in increased sensitivity and/or precision of alternative configurations should be weighed against the advantage of high-throughput and simplicity offered by fully automated high-speed clinical analyzers (e.g., the Cobas 8000 configuration used here allows up to 600 samples/hour to be analyzed for Na^+, K^+ and Cl^-). The prime responsible factors for the LoAs are the variation induced by manipulation (manual punching, extraction, and transfer) and the biological variation. With respect to the former, it can be expected that the LoAs may be narrowed further by automation, as in this work all steps of the sample preparation procedure were performed manually. With respect to the biological variation, we evaluated whether there was a possible influence of the plasma K^+ concentration. To this end, we looked if a correlation could be observed between the deviation of the calculated Hct from the expected Hct and the deviation of the plasma K^+ concentration (range of 2.8−5.2 mM) from the median plasma K^+ concentration (4.0 mM). As expected, given the minor contribution of the plasma K^+ concentration, no such correlation was observed (Supplementary Figure S-4, Supporting Information).

Incurred sample reanalysis, performed on a subset of the patient samples (n = 49), demonstrated that, with one single exception, all repeated measurements lie within ±10% of the mean of the repeated and the original measurement. Hence, the

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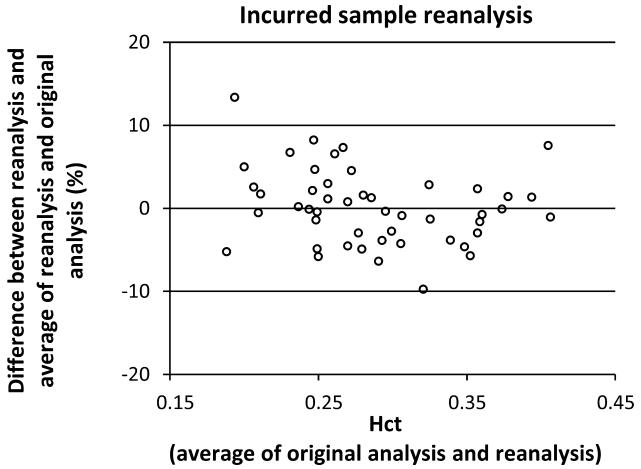

Figure 3. Incurred sample reanalysis, performed on patient DBS samples (n = 49). The difference (in %) between the result of the reanalysis and the average of the reanalysis and the corresponding original analysis is plotted versus the Hct (average of the original analysis and reanalysis).

acceptance criterion (2/3 lying within ±20%) was more than met, demonstrating good assay reproducibility (Figure 3).[22]

CONCLUSION

In conclusion, the successful validation of the developed procedure and its application on real patient samples demonstrate its practical applicability, covering a Hct range of 0.19−0.63. The developed methodology, which because of its simplicity and speed can be easily introduced into any automated clinical laboratory, allows one to make a good prediction of the Hct, one of the most important "unknowns" in DBS sampling. Every analyst working with DBS is aware of the Hct effect and, in our opinion, any validated method using DBS punches should define a Hct range in which precision and accuracy for a given analyte are still acceptable. However, hitherto, there was no methodology available to actually confirm that the Hct of blood used to generate a given DBS actually lies within the acceptable range. Being able to predict the Hct of any given DBS may also render it possible in the future to cope with, and possibly even to adjust for, the "hematocrit effect" in any given DBS-based analytical method. In addition, even though analysis of complete DBS, requiring volumetric application, has been advocated as one of the best solutions to overcome the Hct effect,[11] still, correct interpretation of the obtained results, and correlation with plasma data, requires knowledge of the Hct of the DBS. It will be important to extend this study in the future to true capillary blood samples. Importantly, in these cases, capillary Hct should be determined as a reference, as this may differ from venous Hct;[3,24,25] a finding we also observed in our preliminary experiments.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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