Comparison of HbA1c detection in whole blood and dried blood spots using an automated ion-exchange HPLC system

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Aim: Hemoglobin A1c (HbA1c) is a widely recognized analyte for diagnosing and monitoring diabetes. Dried blood spot (DBS) constitutes a useful alternative to blood collection by venipuncture. Analytical and clinical validation of DBS use is, however, necessary before implementation. **Results/methodology:** HbA1c levels from whole blood or DBS from a cohort patients with diabetes were compared. DBS specimens were stable at ambient temperature. HbA1c detection on DBS was accurate, robust, and the correlation and agreement with whole blood values was excellent. **Conclusion:** This study provides for the first time a complete method comparison and validation under the ISO15189 guideline using an automated HPLC system. This approach constitutes, therefore, a useful tool for diagnosing diabetes.

Keywords: clinical biology • DBS • dried blood spot • glycated hemoglobin • HbA1c • HPLC • ion exchange • ISO15189 • method validation

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The prevalence of Type 2 diabetes has been constantly increasing during the last 15 years, in parallel with the prevalence of obesity and the aging of the world's populations [1]. The main chronic complications of diabetes are retinopathy, nephropathy and cardiovascular diseases [2,3]. Since the risks of complications increase in diabetic patients, glycemia monitoring and control play an essential role in the management of this condition [4]. Glycated hemoglobin A1c (HbA1c), which results from the irreversible glycation of the hemoglobin A (HbA) beta chain, is the main analyte used as a marker to detect and control diabetes [5]. The ratio between HbA1c and whole HbA red blood cells is correlated with the mean blood glucose level present during red blood cells' lifespan (about 120 days) [6]. HbA1c is, therefore, a reliable biomarker which can be used both to screen diabetes in subjects at risk and to monitor diabetic individuals' glycemic levels. Patients with HbA1C values above 6.5% are taken to be diabetic [5]. In these patients, the HbA1C levels are significantly correlated with the risk of chronic complications such as microangiopathic problems [7,8]. HbA1c is usually measured in blood specimens obtained by venipuncture, which has to be performed by properly trained medical staff. Among the analytical methods generally used to determine patients' HbA1c levels in blood specimens, HPLC is among the validated ones [9].

In the early 1960s, dried blood spot (DBS) sampling on filter paper was developed for screening inherited metabolic disorders in newborn infants [10]. Many methods to quantify clinical analytes on DBS were subsequently validated ([11]). The main advantages of DBS include the fact that it involves less invasive capillary sampling and requires very small volumes of the patient's blood. In addition, the stability of the samples and the simplicity of the methods of shipment and storage involved make DBS method an attractive

suitable alternative to venipuncture for collecting blood. The measure of HbA1c in DBS using turbidimetric immunoassays [12–14] and HPLC analysis [15–19] was previously studied in individuals with and without diabetes. The results obtained have shown that using DBS to measure HbA1c is a useful potential alternative to classical venipuncture [12,14]. Before this procedure can be implanted at clinical level, however, it has to be completely validated in order to comply with the requirements of ISO Standard norm 1589 for medical laboratories [20].

In this study, an ion-exchange HPLC system (ADAMS HA-8180V® from Arkray/AxonLab) was used to assess the use of DBSs for detecting HbA1c. The imprecision, sensitivity, linearity and accuracy of the results obtained on DBS were found to be satisfactory and comparable to those of classical sampling methods. The HbA1c analytes present in DBS remained stable for a period of at least seven days when stored at ambient temperature. The analyses were performed on a cohort of 217 diabetic and control patients. Taken together, our study adds significantly to the previously published article on the subject. It is in fact the first time that a complete method comparison on an automated HPLC system that includes a full validation under the ISO15189 guideline was realized. This notably included the use of international standard on DBS. Hence, this study demonstrates the possibility to use DBS in an automated way in a routine clinical laboratory for diabetes detection and follow-up.

Experimental procedure

Population & samples

Blood specimens from 217 diabetic and control patients were analyzed in this study. Patients gave their informed consent to donating blood specimens to the officially registered and ethically approved biological collection (#DC-2008-417) housed at the Montpellier CHU's certified (NFS 960-900) biobank [21]. Most of these patients were being treated at the unit headed by Professor Ariane Sultan (Département Nutrition-Diabète, CHU Lapeyronie, Montpellier) for the diagnosis and follow-up of Type 1 and 2 diabetes (see Table 1).

Whole blood (WB) was collected by venipuncture in 5-ml EDTA tubes. HbA1c quantification was then performed within 4 h. DBS samples were prepared from a 75 µl drop of WB spotted onto preprinted 12-mm diameter circular filter paper discs (DBS cards from Spot-ToL-ab®, France). Cards were dried for 2 h at room temperature before being placed in individual zipped plastic bags and stored at -20°C or room temperature for subsequent HbA1c analysis.

Extraction of HbA1c from DBS

One disk of 6-mm diameter was punched out of the cards using an automatized DBS Puncher® Instrument (PerkinElmer, CT, USA). The punch was placed in either a 96-well deep-well plate (Axgen, P-DW-20-C) or a 1.5-ml Eppendorf tube (Lobind, Eppendorf, Hamburg, Germany), immersed in 400 or 450 µl of DW buffer (Spot-To-Lab®, Montpellier, France). After gentle shaking of the samples (at 500 rpm/min) at room temperature for 1 h, the supernatant was recovered and tested using ADAMS HA-8180V sample cups.

HbA1c measurements

HbA1c was measured using the ADAMS HA-8180V ion-exchange HPLC system from Arkray/AxonLab in line with the manufacturer's instructions. This analyzer is based on an ion exchange column containing resin composed of hydrophilic methacrylate ester copolymer. The column was maintained at 40°C and the elution was performed using a five-step phosphate-buffered gradient with increasing ionic strengths. The colorimetric method of detection involved the use of dual wavelengths of 420 and 500 nm. The linear HbA1c detection range extended from 3 to 20% (9–195 mmol/mol) with a runtime of 48 s per sample.

Analytical performances

DBS stability study

The stability of HbA1c in DBS was tested in 11 independent samples. Blood was spotted onto five circles on a single DBS card and the specimens were stored with desiccant at room temperature (20–25°C), for a period of up to 2 weeks. HbA1c measurements were performed on DBS and WB on the day of collection as well as on DBS on the subsequent days 1, 3, 5, 7, 10

Table 1. Characteristics of the study population (gender, age, %HbA1c).

Studied population	All	No diabetes	Type 1 diabetes	Type 2 diabetes
Number of samples	217	61	45	107
Gender (male/female)	126/91	31/30	23/22	67/42
Age (years:mean + SD)	56.0 ± 16.5	60.1 ± 13.2	43.6 ± 16.2	60.8 ± 12.7
% HbA1c (mean + SD)	6.7 ± 1.7	5.4 ± 0.4	7.1 ± 1.5	7.3 ± 2.0

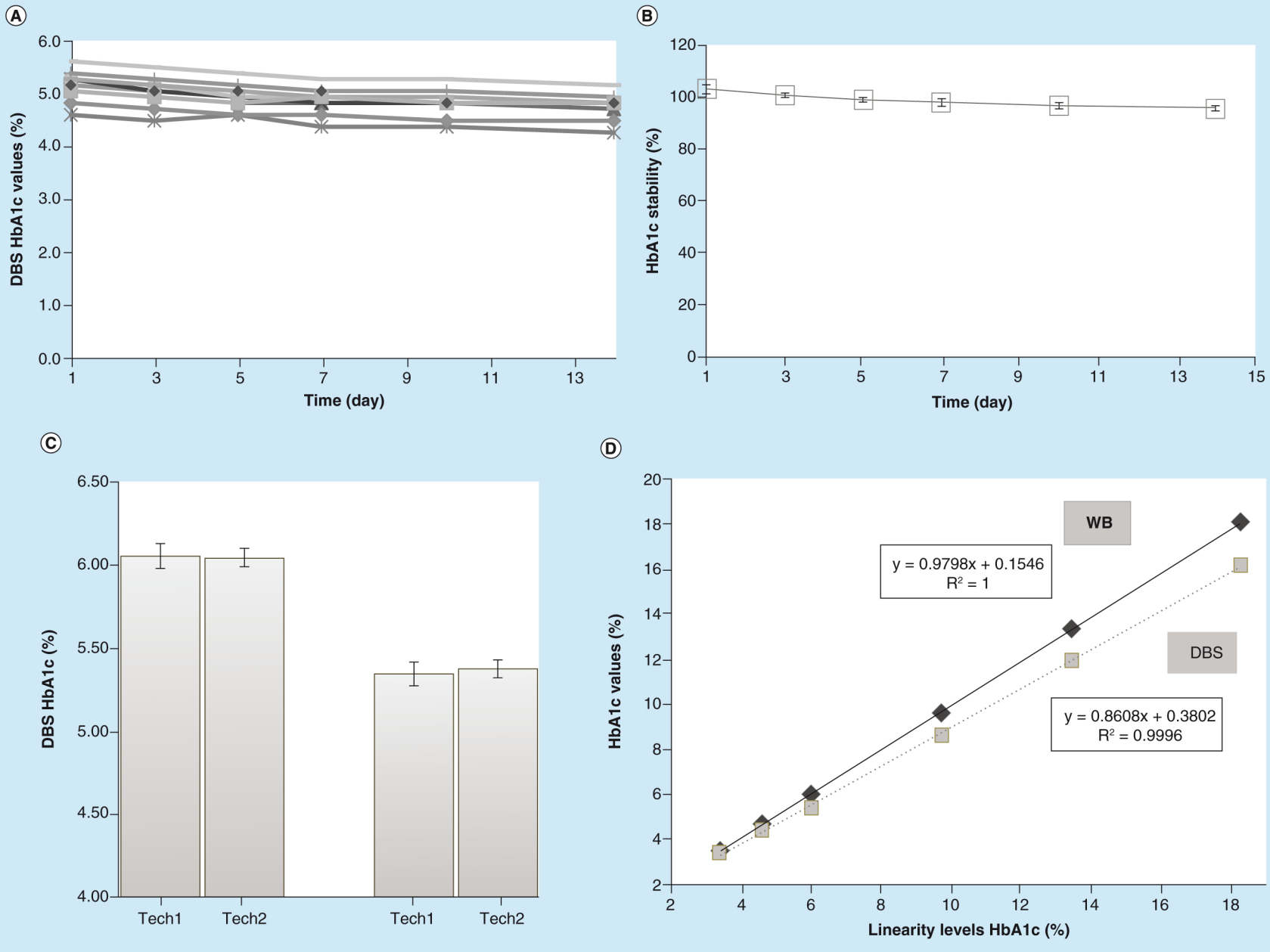
A
DBS HbA1c values (%)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1
3
5
7
9
11
13
Time (day)
B
HbA1c stability (%)
120
100
80
60
40
20
0
1
3
5
7
9
11
13
15
Time (day)
C
DBS HbA1c (%)
6.50
6.00
5.50
5.00
4.50
4.00
Tech1
Tech2
Tech1
Tech2
D
HbA1c values (%)
20
18
16
14
12
10
8
6
4
2
2
4
6
8
10
12
14
16
18
Linearity levels HbA1c (%)
WB
y = 0.9798x + 0.1546
R² = 1
DBS
y = 0.8608x + 0.3802
R² = 0.9996

Figure 1. Analytical characteristics of HbA1c measurement in dried blood spots (see previous page). Stability during 2 weeks: **(A)** individual samples; **(B)** mean. Robustness during a period of 10 days **(C)**. Linearity of whole blood and dried blood spot sample **(D)**.
DBS: Dried blood spot; WB: Whole blood.

and 14. Samples were stored at -20°C before being analyzed. This timeframe was chosen in order to test and establish the future transport conditions.

Robustness & carry-over

Robustness was assessed by calculating the coefficient of variation of the HbA1c detected in two DBS samples collected by two technicians and measured independently for 10 days. Carryover was assessed on five consecutive measurements on DBS samples having low HbA1c (L) (5.1%) and high HbA1c (H) (10.4%) values, using the sequence H1-H2- L1-L2-L3 and the formula (L1-L3)/(H mean – L1 mean) × 100.

Imprecision study

Repeatability (intra-assay variability) was assessed by performing 12 replicate measurements on three DBS samples with low, medium and high HbA1c levels. Reproducibility (interassay variability) was tested on three DBS samples at a rate of one analysis per day for 10 days. The same DBS samples with low, medium and high HbA1c concentrations as those used previously in the repeatability study were analyzed.

Linearity & accuracy

To check the linearity and accuracy of the DBS HbA1c measurements, we used a human WB-based commercial set (Lyphochek Hemoglobin A1C Linearity Set, BioRad, France) consisting of six samples with expected values ranging from 2.7 to 3.8%, 4.1 to 5.1%, 5.5 to 6.5%, 8.4 to 10%, 12 to 15% and 16 to 22%, as recommended by the College of American Pathologists and the 'National Glycohemoglobin Standardization Program' [19]. After being reconstituted, these samples were spotted onto DBS cards, dried and processed in the same way as regular blood samples. HbA1c levels measured in WB and DBS were then compared with these linear target values for HPLC method. Accuracy was measured in the same experiment, and the percentage DBS accuracy was calculated with respect to the results obtained on WB samples.

Comparison between DBS & WB measurements

Results of HPLC analysis of HbA1c performed on DBS were compared with those obtained on WB. Statistical analyses were performed with the MedCalc (12.1.4.0) software program. Comparisons were made between the two procedures by performing least squares linear regression analyses on the slopes, intercepts and coefficients of correlation. In addition, the scattering of the differences was displayed on Bland–Altman plot [22] and Passing & Bablok regression. Concordance between methods was assessed using Cohen's Kappa test [23]. Excellent agreement was taken to exist at Kappa values ≥0.81; good agreement, at Kappa values ranging from 0.61 to 0.80; and moderately good agreement, at Kappa values ranging between 0.41 and 0.60. Clinical concordance between the two methods was assessed by analyzing individual patients' classification in the three categories (normal/increased risk/diabetes) defined by the American Diabetes Association [5].

Results & discussion

A total number of 217 participants (126 male and 91 female) were included in the study. Mean age was 56.0 ± 16.5 years; 45 participants (20.7%) had been previously diagnosed with Type 1 diabetes, 111 (51.2%) with Type 2 diabetes and 61 (28.1%) had no diabetes. Overall mean WB HbA1c levels were 6.7 ± 1.7% (5.4 ± 0.4% in participants without diabetes, 7.1 ± 1.5% in those with Type 1 diabetes, and 7.3 ± 2.0% in those with Type 2 diabetes). Characteristics of the participants are summarized in Table 1. This cohort was fairly representative of a screening population with WB HbA1c levels ranging from 3.9 to 18.0%.

The stability study (Figure 1A & B) showed that the HbA1c levels detected in DBS decreased slightly during storage at room temperature. This decrease was not significant, however, up to 7 days of storage. Similar small variations with time have been previously observed [12,14,19,24,25].

To ensure that DBS can be used in clinical applications, it was necessary to assess whether the perfor-

Table 2. Imprecision study of HbA1c measurements in dried blood spots.

Within-run imprecision (n = 12)				Between-run imprecision (n = 10)			
HbA1c (%)	Mean	SD	CV %	HbA1c (%)	Mean	SD	CV %
High	9.79	0.06	0.61	High	9.80	0.08	0.83
Medium	6.65	0.07	1.03	Medium	6.65	0.07	1.03
Low	5.00	0.00	0.00	Low	5.03	0.00	0.93

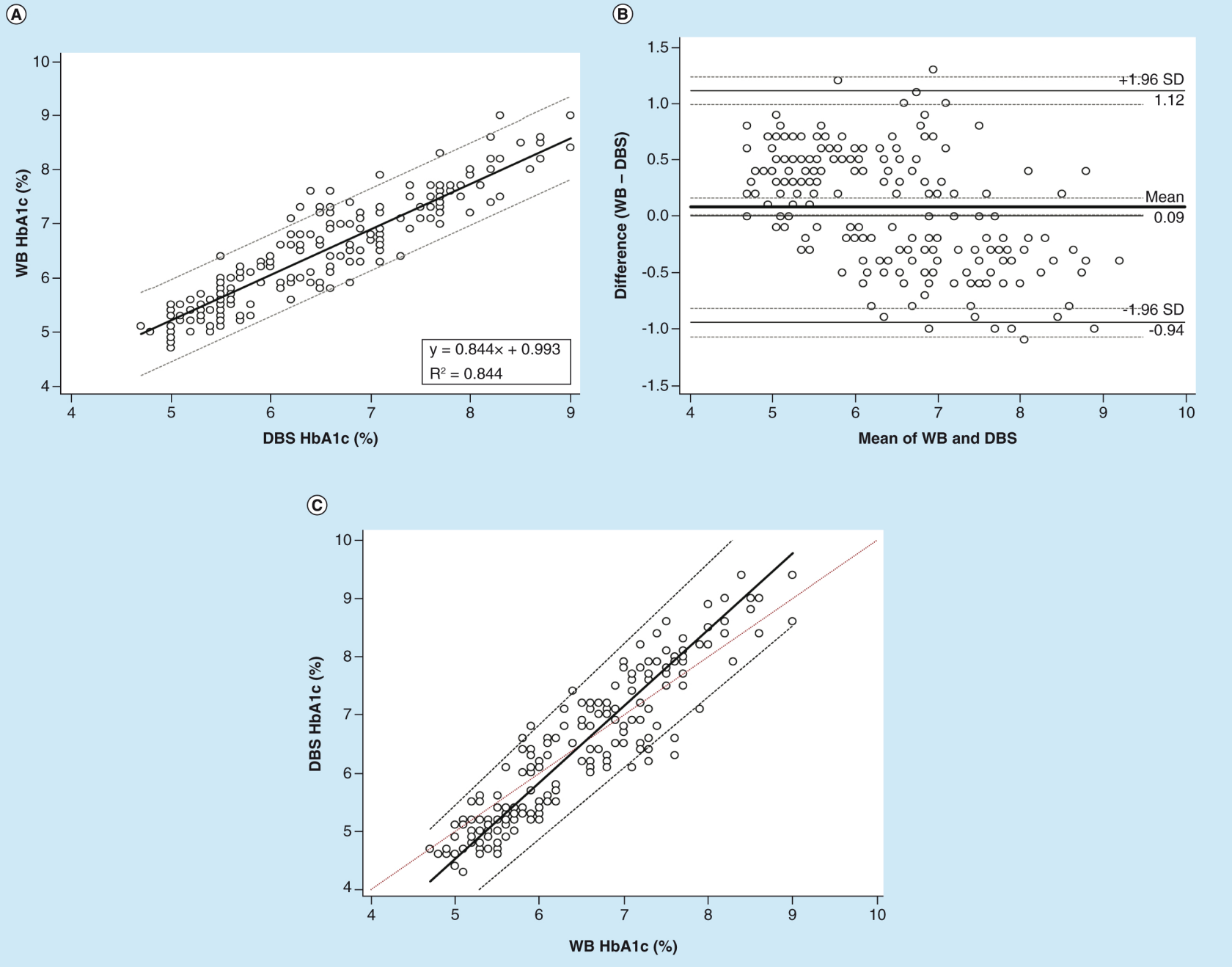
A
WB HbA1c (%)
DBS HbA1c (%)
y = 0.844x + 0.993
R² = 0.844
B
Difference (WB – DBS)
Mean of WB and DBS
+1.96 SD
1.12
Mean
0.09
-1.96 SD
-0.94
C
DBS HbA1c (%)
WB HbA1c (%)

Figure 2. Comparison of whole blood and dried blood spot measurements (see facing page). Plot of whole blood versus dried blood spot values **(A)**. Bland–Altman difference plot **(B)** and Passing & Bablok plot **(C)**.
DBS: Dried blood spot; WB: Whole blood.

mances of the assay were in keeping with the requirements of ISO Standard 15189. We first tested the robustness of the assay by comparing independent measurements obtained by two technicians (Figure 1C). We also computed the carryover effect between samples with high and low HbA1c values, which worked out at <0.01%. The imprecision, in terms of the within-DBS and inter-run DBS measurement variability of high, medium and low HbA1c concentrations was found to be highly satisfactory, since a CV < 1.03% was obtained (Table 2).

Linearity and accuracy were determined using a commercial linearity set including six levels. The correlations found to exist between the values measured in reconstituted WB and DBS (Figure 1D), reflected high levels of accuracy and linearity in the case of both WB and DBS methods (CV < 1% at defined concentrations, and r^2 = 1 and 0.996, respectively). However, the two regression lanes had different slopes: the DBS method showed a bias of -0.08 on average. This relative bias was confirmed with clinical samples measured using the two methods, as illustrated in Figure 2A at HbA1c values ranging from 4 to 10%. The overall comparison between the two methods showed the existence of a strong correlation (r = 0.9191) and a mean difference (WB/DBS) of only 0.09 (Figure 2B). The Bland and Altman plot [26] was used to investigate whether the two methods can be used interchangeably. Most of the values recorded were in fact located around the mean difference, and at the limits of agreement, defined as the mean difference plus and minus 1.96-times the standard deviation of the differences. In addition the Passing & Bablok regression (Figure 2C) confirmed that there was no significant deviation from linearity (p = 0.21) between the two measurements, most of the values being included into the confidence interval (dash lines). In view of this bias in the DBS measurements, we decided, as in previous comparisons between DBS and classical methods [19,24,27], to adjust the HbA1c values obtained, using the regression equation to obtain agreement between WB and DBS.

To confirm the clinical interest of the DBS detection method, a concordance study was performed in terms of the patients' classification in the three clinical categories defined by the American Diabetes Association [5]: normal (HbA1c <5.7%); at an increased risk of developing diabetes (HbA1c 5.7–6.4%); and diabetes (HbA1c ≥6.5%). Excellent agreement (Kappa value ≥0.81) was observed here between the DBS and WB methods (Table 3). Exact matches were obtained in 83% of the patients between WB and DBS, and most of the discrepancies involved the middle HbA1c range and the borderline values. Importantly, no samples with pathological or normal values were mistaken for each other with either method. Reference intervals using a total of 72 values from control nondiabetic subject were also computed. Using a normal distribution, the values were 5.4 (4.6–6.3) for DBS and 5.5 (4.7–6.4) for WB. This confirmed that the two methods were very close with reference values within the expected range as given by the American Diabetes Association (ADA) (4.0–6.0).

The results obtained in this study confirm that HbA1c analysis of Spot-To-Lab DBS constitutes a clinically valid alternative method. One of the main advantages of this method is that samples can be sent by post to a central test laboratory. This method therefore provides an excellent opportunity for simplifying the screening of diabetes in the general population. In addition, this method of glycemic monitoring, which requires no venipuncture and can easily be performed at home, could help to improve diabetic patients' quality of life.

Future perspective

The results obtained in this study confirmed that HbA1c analysis on DBS constitutes a clinically valid alternative method to venipuncture. Importantly, in this approach samples can be sent by post to a central test laboratory. This method, therefore, provides

Table 3. Concordance between whole blood and dried blood spot measurements of HbA1c using HPLC method.

Concordance	DBS <5.7%	DBS 5.7–6.4%	DBS ≥6.5%
WB <5.7%	53	8	0
WB 5.7–6.4%	5	25	21
WB ≥6.5%	0	3	102

Patients were classified into the three ADA categories based on both WB and DBS values. This allowed to compute the concordance between the two methods (see text).
DBS: Dried blood spot; WB: Whole blood.

an excellent opportunity to simplify the screening of diabetes in the general population and its follow-up of patient. The perspective is that in few years from now this DBS method will be used for mass screening for preventive action in most countries. HbA1c DBS measurement when combined to other DBS measurement for creatinine and protein in urine for example could also represent the method of choice for the follow-up of diabetic patients.

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Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Background

- Hemoglobin A1c (HbA1c) is a widely recognized biomarker for diagnosing and monitoring of diabetes.
- Dried blood spot (DBS) constitutes a useful alternative to blood collection by venipuncture and necessitates a full method validation before implementation.

Methods & results

- HbA1c levels in whole blood or DBS using HPLC (Arkray ADAMS HA-8180V) from a cohort patient with diabetes were measured. A full validation under the ISO15189 guideline was realized.
- HbA1c detection on DBS and whole blood were comparable and with appropriate clinical performance.

Conclusion

- DBS represents a valuable and promising tool for detection and follow-up of diabetes.

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