Simple Paper-Based Test for Measuring Blood Hemoglobin Concentration in Resource-Limited Settings

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BACKGROUND: The measurement of hemoglobin concentration ([Hb]) is performed routinely as a part of a complete blood cell count to evaluate the oxygen-carrying capacity of blood. Devices currently available to physicians and clinical laboratories for measuring [Hb] are accurate, operate on small samples, and provide results rapidly, but may be prohibitively expensive for resource-limited settings. The unavailability of accurate but inexpensive diagnostic tools often precludes proper diagnosis of anemia in low-income developing countries. Therefore, we developed a simple paper-based assay for measuring [Hb].

METHODS: A 20-μL droplet of a mixture of blood and Drabkin reagent was deposited onto patterned chromatography paper. The resulting blood stain was digitized with a portable scanner and analyzed. The mean color intensity of the blood stain was used to quantify [Hb]. We compared the performance of the paper-based Hb assay with a hematology analyzer (comparison method) using blood samples from 54 subjects.

RESULTS: The values of [Hb] measured by the paper-based assay and the comparison method were highly correlated ($R^2 = 0.9598$); the standard deviation of the difference between the two measurements was 0.62 g/dL. The assay was accurate within 1 g/dL 90.7% of the time, overestimating [Hb] by ≥1 g/dL in 1.9% and underestimating [Hb] by ≥1 g/dL in 7.4% of the subjects.

CONCLUSIONS: This study demonstrates the feasibility of the paper-based Hb assay. This simple, low-cost test should be useful for diagnosing anemia in resource-limited settings, particularly in the context of care for malaria, HIV, and sickle cell disease patients in sub-Saharan Africa.

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laboratories measure [Hb] with automated hematology analyzers as well as spectrophotometers, blood gas analyzers, and stand-alone CO-oximeters (14). The need for rapid and accurate measurement of [Hb] at the bedside has driven the development and implementation of [Hb] testing on the point-of-care (POC) platform (15). Currently available POC devices are designed to operate in close proximity to the patient, provide accurate results (within 1 g/dL of clinical laboratory analyzers), require small volumes of blood, and be used by persons without expert training, making the nearly instantaneous measurement of [Hb] in operating room or emergency care settings a reality (16–18).

Notwithstanding their obvious advantages, the relatively high per-test and analyzer costs make the use of conventional POC devices for measuring [Hb] prohibitively expensive in resource-limited settings of low-income developing countries in sub-Saharan Africa, on the Indian subcontinent, and throughout Southeast Asia (19, 20). Currently available low-cost alternatives including the “hematocrit/3” method (21) and the WHO Hb color scale (HbCS) test (22) have several drawbacks that limit their use in the field. In addition to being somewhat inaccurate, hematocrit/3 relies on knowledge of hematocrit and, therefore, requires a centrifuge (21, 23). The HbCS test is highly sensitive to the ambient lighting conditions, operator bias, and deviations from the recommended readout time (22, 24). The lack of confidence in the quality of [Hb] measurements may result in clinicians relying exclusively on their clinical judgment to prescribe transfusions in resource-limited settings (25). Therefore, our goal was to develop a simple, low-cost, paper-based Hb assay that addressed these limitations.

Methods

BLOOD SAMPLES

The study protocol was approved by Tulane University Biomedical Institutional Review Board. After written informed consent was obtained, blood samples were collected into 4- or 9-mL Vacutainer tubes (K3EDTA, BD) during routine blood draws (January–April 2013) from patients of the Pediatric Hematology-Oncology Clinic (Tulane University Hospital) and the Sickle Cell Center of Southern Louisiana (New Orleans, LA). We measured standard hematological parameters including [Hb] using a Medonic M-Series hematology analyzer (Medonic M16C/M20C, Boule Medical). The Hb content of blood samples was adjusted artificially to prepare samples with [Hb] ranging from 2.5 to 25 g/dL by adding autologous plasma (for lower [Hb]) or pelletted RBCs (for higher [Hb]) to the original sample.

FABRICATION OF THE MICROFLUIDIC PAPER-BASED ANALYTICAL DEVICES

The microfluidic paper-based analytical devices (µPADs) were fabricated by a previously published method (26, 27). Briefly, the pattern of the µPADs was designed with illustration software (Canvas 11, ACD Systems International). The devices were printed onto sheets of chromatography paper (No. 1, Whatman) with a solid-ink (wax) printer (Phaser 8560N, Xerox), and then heated on a hot plate at 150 °C for 3 min.

QUANTIFICATION OF THE BLOOD STAIN COLOR

Blood samples were mixed at 1:10, 1:15, or 1:20 ratios (vol/vol) with Drabkin reagent (Ricca Chemical Co.) and incubated at room temperature (20–25 °C) for 10 min. The components of Drabkin reagent lysed red blood cells and reacted with all forms of Hb except sulfhemoglobin present in the sample, converting them to cyanmethemoglobin, a stable brownish-colored compound. A 20-µL drop of the mixture was placed onto the center of each µPAD. The resulting blood stain was allowed to dry for 25 min (28).

Quantification of [Hb] was accomplished by scanning the sheets of chromatography paper containing arrays of µPADs on a portable flatted scanner (CanosoScan LiDE110, Canon USA) and then analyzing the images automatically with a custom-coded algorithm (MATLAB, The Math Works). The quantitative analysis of blood stains was based on the red/green/blue (RGB) color model values of the digitized images. The color data in the red, green, and blue channels were extracted from the RGB values of each pixel within a blood stain. We defined the color intensity of each channel as 255 – (R, G, or B); e.g., for red channel, color intensity = 255 – R. The mean color intensity of the blood stain for each color channel was calculated from the color values of all pixels within the blood stain, including the pixels corresponding to the darker ring on the periphery of the stain. The correlation between the mean color intensity and the actual [Hb] was evaluated for all color channels; the green channel showed the best linear fit and was selected to quantify [Hb] in the blood samples.

STATISTICAL ANALYSIS

Pearson correlation coefficient and regression were calculated to directly compare data obtained from the paper-based Hb assay and the Medonic M-Series hematology analyzer (comparison method). Three research associates performed both types of measurements on the same day. A Bland–Altman plot (29) was also constructed, with consideration for limitations, to visualize the comparison.
DESIGN AND OPERATION OF THE PAPER-BASED Hb ASSAY

Figure 1 illustrates the design and operation of the paper-based Hb assay. Each individual μPAD comprised a 2.8-cm-diameter circle (Fig. 1a) designed to create a constant area for quantification with an image analysis algorithm and prevent potential cross-contamination of samples. Each sheet of chromatography paper contained a 5-by-4 array of μPADs and included an alignment mark to simplify automation of the subsequent image analysis. We fabricated the μPAD arrays by printing their pattern with a solid-ink (wax) printer (Fig. 1A). We then used a hot plate to heat the printed chromatography paper above the melting point of wax to allow the wax to reflow and form hydrophobic barriers through the full thickness of the paper (Fig. 1B). When designing the pattern of the μPAD (Fig. 1A), we accounted for the natural widening of the printed lines (to about 1 mm thick) during the melting process (Fig. 1B).

We performed this paper-based Hb assay by first mixing a sample of whole blood with Drabkin reagent and then depositing a 20-μL droplet of the mixture onto the μPAD and letting the blood stain develop (Fig. 1C). The droplet spread radially from the center through the paper substrate toward the periphery of the μPAD due to wicking by capillary action (30, 31), forming the characteristic blood stain pattern (Fig. 1D) reminiscent of the stains produced by drying coffee (32). We selected the volume of the droplet (20 μL) and size of the μPAD (2.8 cm) so that the outermost margin of the stain could not reach the periphery of the μPAD (Fig. 1D).

We used a portable flatbed scanner to digitize the sheets of chromatography paper carrying μPADs with developed blood stains. We analyzed the images to determine the mean color intensity for the blood stain contained within each μPAD using a simple image processing algorithm developed specifically for this purpose (Fig. 1, D and E). The algorithm used the alignment mark on the sheets of paper to determine the location of each μPAD (Fig. 1D). The algorithm applied a circular binary mask to the image to select the area within the hydrophobic border of the μPAD, and then isolated the pixels of the blood stain within the circular region by removing pixels below a threshold value on the basis of the difference in color intensity between the paper substrate and the blood stain (Fig. 1D, dashed circle). Finally, the algorithm used the color information from all pixels within the blood stain to calculate the mean color intensity of the blood stain (Fig. 1E, red solid line). We used the mean color intensity of the stain (Fig. 1E) and a calibration curve (Fig. 2B) to measure [Hb] in the blood sample.

All operations of the paper-based Hb assay, including sample preparation, placement of the sample onto the μPAD, drying of the blood stain, image scanning, and the automated image analysis, could be completed within 35 min.

EFFECT OF DILUTION ON THE MEAN COLOR INTENSITY OF THE BLOOD STAIN

Figure 2 illustrates the effect of dilution on the mean color intensity of the stains produced by blood on paper. To perform these experiments, we prepared a series of calibration samples with their [Hb] adjusted to 2.5, 5, 10, 15, 20, and 25 g/dL (as described in Methods) using blood from 3 consenting volunteers. We then mixed a small volume of each calibration sample with

Results

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Drabkin reagent at 1:10, 1:15, and 1:20 ratios (vol/vol), waited 10 min, and placed a 20-μL droplet of each mixture onto paper. Droplets with markedly different Hb content, due to either a difference in [Hb] or dilution, produced blood stains with different color intensities that were easily distinguishable visually. As expected, the blood stain color intensity increased with increasing [Hb] for each mixing ratio (dilution) and decreased with increasing dilution for each [Hb] value (Fig. 2A). The mean color intensity of each blood stain correlated very strongly with its [Hb] (Fig. 2B–D). The combination of 1:15 mixing ratio and green channel showed the best linear fit (mean color intensity = 2.0986 × [Hb] + 3.7226, $R^2 = 0.996$). Therefore, we used the 1:15 mixing ratio to prepare samples in all further experiments, the mean color intensity in green channel, and the linear equation produced by this graph (Fig. 2C) as the calibration curve for estimating the [Hb] of the sample on the basis of the mean color intensity of its blood stain.

To determine the limit of detection (LOD) and the limit of quantification (LOQ) of the assay, we varied [Hb] of blood samples from 0.5 to 25 g/dL by diluting whole blood with autologous plasma or concentrating whole blood with packed RBCs (as described in Methods). We defined the LOD as the lowest [Hb] at which the blood stain could be identified and measured using our image processing algorithm, and the LOQ as the lowest [Hb] at which the disagreement with the comparison method (Medonic hematology analyzer) was within 1 g/dL. We found that the LOD for our paper-based Hb assay was 1 g/dL and the LOQ was 2.5 g/dL.

**RELATIVE ACCURACY AND PRECISION OF THE PAPER-BASED Hb ASSAY**

We tested the relative accuracy of the paper-based Hb assay by comparing the values of [Hb] measured using...
our assay and the Medonic M-Series hematology analyzer for blood samples from 54 subjects. Subjects included individuals with Hb AA, Hb AS, Hb SC, and Hb SS genotypes; the presence of Hb S in the blood samples did not affect the [Hb] measurements (data not shown). Figure 3 illustrates the results of this side-by-side comparison. The data points representing these measurements were distributed in close proximity to the diagonal line (Fig. 3A, red dashed line), indicating a good agreement between the [Hb] estimated from the mean color intensity of blood stains in our paper-based Hb assay and the actual [Hb] obtained from the standard analysis (Fig. 3A). The linear least-squares regression analysis (Fig. 3A, black solid line) showed a high degree of correlation between the 2 measurements ($y = 1.05x - 0.58, R^2 = 0.9598$).

We also constructed the Bland–Altman plot (29) to evaluate the agreement between the paper-based Hb assay and the Medonic hematology analyzer (Fig. 3b). The standard deviation (SD) of the difference between the comparison method (hematology analyzer) and the paper-based Hb assay was 0.62 g/dL. The limits of agreement between the 2 methods were $-1.30$ and $1.18$ g/dL. The paper-based Hb assay agreed with the hematology analyzer within 1 g/dL 90.7% of the time, overestimating [Hb] by $\geq 1$ g/dL in 1.9% of subjects and underestimating [Hb] by $\geq 1$ g/dL in 7.4% of subjects (Fig. 3B).

In a separate experiment, we tested the precision of the paper-based Hb assay by measuring repeatedly ($n = 5$) the [Hb] for a series of blood samples obtained from the same subject with the actual [Hb] of each sample adjusted (as described in Methods) to 2.5, 5, 10, 15, 20, and 25 g/dL. The SD for the [Hb] measurements performed with different droplets from the same blood sample was consistently $<0.5$ g/dL for all values of the true [Hb] tested: 0.21 g/dL (CV 8.3%) for the sample with true [Hb] of 2.5 g/dL; 0.19 g/dL (CV 3.9%) for 5 g/dL; 0.11 g/dL (CV 1.1%) for 10 g/dL; 0.21 g/dL (CV 1.4%) for 15 g/dL; 0.34 g/dL (CV 1.7%) for 20 g/dL; and 0.78 g/dL (CV 3.1%) for 25 g/dL. For comparison, the [Hb] measurement range for the Medonic hematology analyzer was 0 – 35 g/dL, with CV $<1.0\%$ (values provided by the manufacturer).

**EFFECT OF DROPLET VOLUME VARIATION AND LONG-TERM STABILITY OF THE PAPER-BASED Hb ASSAY**

We measured the effect of variation in the volume of the sample droplet, which may occur during the use of the assay in the field, on the [Hb] measurement for samples with true [Hb] of 5, 10, 15, and 20 g/dL. A 20% variation in the volume of the sample droplet (16 – 24 μL) resulted in a $\leq 0.21$ g/dL SD (CV 4.3%) of the estimated [Hb] for samples with true [Hb] of 5 g/dL; $\leq 0.66$ g/dL (CV 5.6%) for 10 g/dL; $\leq 0.67$ g/dL (CV 4.2%) for 15 g/dL; and $\leq 0.93$ g/dL (CV 4.4%) for 20 g/dL (see Supplemental Fig. 1A, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue10). As expected, the size of the blood stain depended on the droplet volume. For droplets of the same volume, but with different true [Hb] values (5, 10, 15, and 20 g/dL) the normalized size of the blood stains produced by the droplets...
had an SD of $\leq 0.03$ (CV 4.7%) for 16 $\mu$L; $\leq 0.03$ (CV 3.9%) for 18 $\mu$L; $\leq 0.03$ (CV 4.5%) for 20 $\mu$L; $\leq 0.04$ (CV 4.1%) for 22 $\mu$L; and $\leq 0.03$ (CV 3.7%) for 24 $\mu$L. (see online Supplemental Fig. 1B).

Finally, we tested the long-term stability of the paper-based Hb assay measurements by scanning the sheets of paper containing the blood stains 4 times during the first 20 min, while the stain was still drying, and 13 times over the next 24 h, for samples with true [Hb] of 5, 10, 15, and 20 g/dL. The color of the dried blood stain remained stable after the initial drying period. We therefore were able to scan and analyze the blood stains at any point over the next 24 h without any significant change in the [Hb] measurement (see online Supplemental Fig. 2). During this study, the room temperature in our laboratory varied from 18 °C to 30 °C, and humidity varied from 20% to 80%. We did not systematically test the effect of room temperature or humidity variations on the performance of the assay.

Discussion

The measurement of blood [Hb] is one of the most frequently performed laboratory tests within a wide range of medical settings, from acute trauma care to chronic disease management (1). While the primary reason for initial [Hb] screening is to diagnose (or rule out) anemia, frequent monitoring of [Hb] is often required in the context of many conditions to quantify blood loss, track disease progression, assess treatment efficacy, or monitor patients during procedures. When reliable diagnostic devices are available, they are often not used because they are considered to be prohibitively expensive for the resource-limited settings of developing countries (33). For example, in Kenyan district hospitals, nearly 15% of children with clinical histories indicative of malaria or anemia did not have their [Hb] tested (20, 34). In this context, a reliable, low-cost assay for measuring [Hb] would improve the diagnosis of anemia and could help minimize waste of limited resources on unneeded treatments and reduce the risk of complications associated with misdiagnosis (20, 25, 33). The urgent need for such an assay in sub-Saharan Africa (in areas endemic with sickle cell disease and malaria) was the primary motivation for this study.

Our paper-based Hb assay measures [Hb] by simply quantifying the appearance of a stain produced by the blood sample on paper. Perhaps the most widely used alternative low-cost method for estimating [Hb] in resource-limited settings is the WHO HbCS. To perform this test, the user must visually compare the color of a paper strip stained with blood to a standard at a certain time after the blood was applied to the strip. Unlike the HbCS, our paper-based Hb assay is not affected by changes in ambient light because the flatbed scanner itself provides consistent illumination, and the color can be read at any time within a 24-h period after collection of the blood stain. The result is not affected by operator bias because the measurement is performed completely automatically.

Very little training is required to perform our paper-based Hb assay. All a user would need to do is to mix a finger-prick volume of blood with Drabkin reagent at the appropriate ratio and deposit a droplet of this mixture on paper. The complexity of these operations is about the same as that of the sample preparation steps required from consumers for performing at-home rapid diagnostic tests for sperm count (35) or Hb A1c (36). All subsequent operations of the paper-based Hb assay are completely automated and user-independent, including the scanning and analysis of the stains. Notwithstanding its simplicity, our paper-based Hb assay demonstrated excellent comparison (SD = 0.62 g/dL, n = 54) with the Medonic hematology analyzer. The performance of our assay also compares favorably with conventional POC devices (14, 19, 37), particularly in the context of our target applications: (a) providing the measurement of [Hb] when no other methods are available, and (b) enabling public health screening in resource-limited settings where the lowest cost, rather than the highest accuracy, is most important.

We used a USB-powered flatbed scanner (Canon CanoScan LiDE110, $44 on amazon.com as of April 21, 2013) and a laptop computer to digitize and analyze the blood stains. Any computer that has a functional USB port and complies with the system requirements of the scanner could in principle be used, including older salvaged or refurbished models. Importantly, the scanner and laptop could be used for many other purposes, not exclusively for performing our paper-based Hb assay. The primary reason for using these multipurpose, consumer-grade electronic devices as the capital equipment part of a low-cost diagnostic assay is the ability to tap into the already well-established market of consumer electronics. These devices are much more available in resource-limited settings than specialized medical equipment such as a spectrophotometer and can be used for performing the [Hb] measurements without any modification of their original function. This approach represents an important advantage of our work with respect to recent studies that follow the conventional paradigm of POC development and propose to measure [Hb] by use of a new, potentially low-cost electronic device (38, 39). Such a device, designed specifically for measuring [Hb], would have to be separately developed, manufactured, distributed, calibrated, and maintained. The use of these POC devices,
particularly the maintenance and/or replacement of broken devices, requires a level of medical and engineering infrastructure that is largely unavailable in resource-limited settings (40).

Another important advantage of our approach is that the use of existing scanners and laptops, which may already be present in even the most remote facilities because they were purchased by consumers for their personal use, could allow for an effectively “zero-cost” analyzer, compared with analyzer costs for conventional POC devices ranging from $800 to $15 000. In addition to the prohibitively high cost of the analyzers, conventional POC devices often require proprietary disposable supplies with per-test costs ranging from $0.02 to $3.67 (19). In contrast, the components required to perform our paper-based [Hb] assay are available generically at an estimated combined cost of <$0.007 (0.7 US cents) per test.

In summary, we developed and validated a paper-based, point-of-care Hb assay for the low-cost assessment of [Hb]. This assay represents a major step toward effective intraoperative care as well as [Hb] testing at the bedside or in urgent care settings where traditional methods of the clinical laboratory are unavailable or prohibitively expensive. The paper-based Hb assay will be useful for diagnosing anemia in resource-limited settings of low-income developing countries (e.g., sub-Saharan Africa), particularly in the context of malaria, HIV, and sickle cell disease.

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