Dried Plasma Spot Measurements of Ferritin and Transferrin Receptor for Assessing Iron Status

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Background: Efforts to reduce the high global prevalence of nutritional anemia require the use of both reliable laboratory assays to distinguish iron deficiency from other causes of anemia and cost-effective methods for collection of blood specimens under field conditions. The suitability of using small plasma samples spotted and dried on filter paper for measurements of plasma ferritin and transferrin receptor was evaluated in the present study.

Methods: Blood specimens obtained from 73 male and 83 female subjects (19–40 years) representing a wide range of iron status were used to perform parallel measurements of plasma ferritin and transferrin receptor on whole plasma and spotted plasma samples.

Results: Ratio plots, evaluating the acceptability and precision of the spot method in ferritin and transferrin receptor assays, showed the expected proportion of data points within the 95% prediction interval. In the composite group of 156 subjects, both the whole plasma and plasma spot methods gave a geometric mean transferrin receptor/ferritin ratio of 18. The regression equation for the ratio was log y = 1.045 log x – 0.05126; r = 0.986; P < 0.0001. The ratio of transferrin receptor/ferritin determined from plasma spots correctly identified all 12 subjects with iron deficiency anemia compared with 11 of the 12 for whole plasma measurements.

Conclusions: Measurements of ferritin and transferrin receptor on plasma spotted and dried on filter paper are comparable to whole plasma values for the identification of iron deficiency anemia. The use of dried plasma spots will facilitate the collection, storage, and transport of samples in epidemiological studies of anemia prevalence.

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It has been estimated that approximately one-quarter of the world’s population, or more than one billion people, are anemic and that the cause in more than one-half of these individuals is iron deficiency (1). A variety of approaches for controlling iron deficiency anemia (IDA)1 have been proposed, including iron supplementation, food fortification, nutrition education, reducing intestinal parasites, and improving the bioavailability of dietary iron (2). Because of the subtle manifestations of iron lack, the choice of a particular intervention strategy and the assessment of its effectiveness are heavily dependent on laboratory methods that will identify IDA specifically and distinguish it from other causes of anemia that may be encountered in prevalence surveys. The majority of traditional measurements of iron status, such as serum iron, iron-binding capacity, and red cell indices, require venous blood because of the relatively large sample volumes required for these assays. In recent years, highly sensitive and specific assays of iron status, such as those for plasma ferritin and plasma transferrin receptor (TfR), have been developed that require only a few microliters of sample and are, therefore, readily performed on blood specimens obtained by finger-stick rather than venous sampling (3, 4). These assays would become even more effective if techniques were used that improved the cost and efficiency of processing, transporting, and storage of specimens obtained under field conditions.

An important evolving technology in the handling of blood specimens obtained in epidemiological studies is the use of small volumes of blood or plasma dried on filter paper. This approach has been used effectively for detecting iodine deficiency (5, 6) and for evaluating vitamin A status by assay of retinol-binding protein (7). A spot method using dried serum for ferritin measurements was reported recently (8). In another recent study, the use of dried whole-blood spots for tandem measurements of

1 Nonstandard abbreviations: IDA, iron deficiency anemia; TfR, transferrin receptor; ID, iron deficiency without anemia; PBS-t, phosphate-buffered saline + 0.5 mL/L Tween 20; CVr, total assay variance for plasma spot method; CVw, total assay variance for whole plasma method; and ACD, anemia of chronic disease.
ferritin and TfR was evaluated (9) and was highly reliable for identifying IDA. However, the contribution of erythrocyte ferritin diminished the sensitivity of whole blood compared with plasma for detecting milder iron deficiency without anemia (ID). The purpose of the present investigation was to assess the suitability of using dried plasma spots rather than whole blood for tandem measurements of plasma ferritin and TfR to assess iron status.

**Materials and Methods**

**STUDY POPULATION**

Venous blood samples were obtained from 73 men and 83 women ranging in age from 19 to 40 years (mean, 25 years). All subjects without anemia were considered in good health. Separation into two degrees of severity of iron deficiency was based on the hematocrit and plasma ferritin concentration as follows: ID was considered present in subjects with a plasma ferritin \( \geq 12 \, \mu g/L \) and a hematocrit \( \geq 37\% \) in women or \( \geq 40\% \) in men (10), whereas IDA was defined by the same plasma ferritin criteria and a hematocrit <37\% in women or <40\% in men. All specimens were obtained according to procedures approved by the Human Subjects Committee of the University of Kansas Medical Center.

**LABORATORY METHODS**

Blood was collected into Vacutainer Tubes containing EDTA as the anticoagulant. After the microhematocrit was measured in duplicate, the blood was centrifuged to remove red cells and 25 \( \mu L \) of plasma was spotted in duplicate onto filter paper (filter paper no. 903; Schleicher & Schuell) and dried at room temperature for at least 3 h. The plasma spot samples were stored at 4 °C for up to 3 weeks in sealed plastic bags containing 10 g of calcium sulfate (Drierite; W.A. Hammond Drierite) as a desiccant, until the day of assay. The Drierite was contained within a porous pouch constructed in our laboratory, as commercially available packets did not have sufficient desiccating capacity. The remaining volume of plasma was stored in microcentrifuge tubes at −30 °C.

Measurements on the eluted plasma spot samples and thawed whole plasma from each subject were performed in the same immunoassay. To prepare the plasma spot samples for assay, the entire spot was cut from the filter paper and transferred to a conical microcentrifuge tube. One milliliter of phosphate-buffered saline, pH 7.2 containing 0.5 mL/L Tween 20 (PBS-t) was then added. The tube was sealed and placed on an end-over-end rotator for 2 h at 4 °C. Prior studies on 10 replicate samples of whole blood demonstrated an elution efficiency for measurements of ferritin and TfR ranging from 93% to 97% with a mean of 95% ± 0.01% (9).

Plasma ferritin and TfR were measured by ELISA using monoclonal antibodies as both capture and indicator antibodies. For the ferritin determination, 200 \( \mu L \) of the plasma spot eluate or whole plasma diluted 1:20 in PBS-t was transferred to a microtiter plate, coated previoulsy with anti-ferritin antibody in 0.2 mol/L carbonate buffer at pH 9.6, and incubated for 2 h at room temperature as described previously (3). After the wells were washed with PBS-t, 200 \( \mu L \) of horseradish peroxidase-conjugated antiferritin antibody in PBS-t containing 10 g/L bovine serum albumin was added to each well and incubated an additional 2 h. The plate was again washed with PBS-t, and after the addition of 200 \( \mu L \) of 3,3',5,5' tetramethylbenzidine substrate, the plate was incubated for 30 min in the dark. The reaction was stopped with 50 \( \mu L \) of 2.5 mol/L sulfuric acid, and the absorbance was measured at 450 nm with a Bio-Tek EL808 microplate reader (Bio-Tek Instruments). Ferritin values were determined using a calibration curve constructed with known concentrations of purified human liver ferritin plotted against the absorbance. The purified calibrators were calibrated against the WHO reference standard obtained from the National Bureau of Standards (11). The TfR was assayed by a similar protocol using two monoclonal antibodies to TfR and using intact transferrin receptor purified from human placenta as the calibrator (4).

To determine the stability of dried spots under different storage temperatures, plasma from 10 subjects was spotted onto filter paper in three sets of four spots. Each set was stored at a different temperature in zipsealed plastic bags containing Drierite in the previously described desiccant pouches. Previous studies have shown that desiccation is vital to the stability of the proteins during storage (9). One set was stored at ambient temperature (22–25 °C), the second at 4 °C, and the third at 37 °C. On days 0, 7, 14, and 28, the spots were cut from the filter paper, eluted as described above, and assayed for ferritin and TfR.

**STATISTICAL ANALYSIS**

The variability and precision of the whole plasma and plasma spot methods for the measurement of ferritin and TfR were evaluated using ratio plots as described by Andersen et al. (12). The mean ratios were calculated by dividing the plasma spot values by the whole plasma values, and the distribution of the ratios was plotted. The total assay variances of the whole plasma and plasma spot measurements for ferritin and TfR were calculated as the sum of the within-assay variance, determined from 70 triplicate measurements performed in the same assay, and the between-assay variance, calculated from 30 triplicate measurements assayed on different days. The within-subject variability was calculated on measurements obtained from three different samples from each of 30 subjects, drawn on separate days at 2-week intervals. Paired results were evaluated using the 95% prediction interval (13). The acceptability of the plasma spot method was evaluated according to analytical quality specifications for imprecision as proposed by Cotlove et al. (14).

Because of skewed distributions, statistical analyses on ferritin, TfR, and TfR/ferritin values were performed on log-transformed data. The difference between various
laboratory values in normal ID, and IDA subjects was assessed by ANOVA followed by comparisons between the groups with the Scheffé test. Least-squares regression analysis of log plasma spot vs log whole plasma values was used to compare ferritin, TfR, and TfR/ferritin. Correlation was evaluated by the Pearson product-moment. Statistical calculations were performed using the Abstat statistical program (Anderson Bell).

Results

The stability of plasma spotted onto filter paper during storage, as assessed by assay of ferritin and TfR, is shown in Table 1. There was no loss with either measurement when samples were stored at 4 °C. At ambient temperature, the ferritin values were constant with 4 weeks of storage, but the TfR values fell at day 14 and 28 by 7% and 11%, respectively. Higher losses occurred with storage at 37 °C. The ferritin and TfR values declined by 22% and 21%, respectively, at 7 days and by 51% and 56% at 14 days. These results indicate that plasma spot samples can be stored with desiccant at an ambient temperature <25 °C for up to 14 days and for at least 4 weeks at 4 °C without substantial loss of activity.

Of the 156 participants in the study, 124 had normal iron status, 20 had ID, and 12 had IDA. The group with normal iron status was composed of 55 women and 69 men who had mean hematocrit values of 42.3% ± 2.4% and 45.7% ± 2.3%, respectively, and geometric mean plasma ferritin values of 64.6 μg/L (± 1 SD, 36.5–115.9 μg/L) and 30.2 μg/L (16.2–56.4 μg/L), respectively. The ID group was composed of 18 women and 2 men. The composite mean hematocrit in these subjects was 42.3% ± 2.0% and the geometric mean plasma ferritin was 7.2 μg/L (± 1 SD, 4.0–12.7 μg/L). Ten of the 12 individuals with IDA were women. The mean hematocrit in the total group was 31.7% ± 4.4%, and the geometric mean plasma ferritin was 4.3 μg/L (± 1 SD, 2.6–6.9 μg/L). The composite geometric mean whole plasma ferritin was 30.4 μg/L compared with of 46.5, 7.2, and 4.3 μg/L in normal, ID, and IDA subjects, respectively (Table 2).

For plasma spot ferritin measurements, the CVs for the within-, between-, and total-assay variability were 7.1%,
11%, and 13% (CV\textsubscript{S}), respectively. For whole plasma measurements the within-, between-, and total-assay CVs were 4.9%, 9.2%, and 10% (CVP), respectively. Based on the sum of the total variances for whole plasma and plasma spot ferritin, the 95% prediction interval was $0.9268 \pm 0.312 (12)$ (Fig. 1). Seven data points out of 156 were outside the prediction interval, which is within the acceptance limits of analytical imprecision. All of these data points were below the cutoff value of 12 µg/L for IDA, where a larger variation would be expected. The within-subject variability of 18% obtained by our laboratory was the same as that reported previously by Cooper and Zlotkin (15). Applying the alternative acceptability criterion for imprecision as described by Cotlove et al. (14), we calculated a value of 17% compared with the total analytical CV of 17%.

Regression analysis gave a high correlation between whole plasma and plasma spot measurements. However,
in the composite group, the plasma spot ferritin values were significantly lower than whole plasma ($P < 0.001$). The paper ferritin measurements averaged 9% less than whole plasma in the composite sample but varied from 7% less in normal subjects to 12% and 21% less in ID and IDA subjects, respectively. This trend to a greater disparity at lower ferritin values is seen in Fig. 2, although the absolute difference in plasma ferritin values below 10 mg/L was <1 mg/L. These lower values are presumably the result of incomplete elution of proteins.

Similar findings were observed with TfR measurements. For plasma spot TfR measurements, the within-, between-, and total-assay CVs were 3.3%, 7.9%, and 8.5% (CV$_S$), respectively. For whole plasma TfR measurements, the within-, between-, and total-assay CVs were 3.8%, 6.8%, and 7.8% (CV$_P$), respectively. Based on the sum of the total variances for whole plasma and plasma spot TfR, the 95% prediction interval was 0.9212 ± 0.211 (Fig. 3). Nine data points were outside the 95% prediction interval a compared with the predicted number of eight. The within-subject variability for TfR was 10%, similar to 13% reported previously (15). Applying the acceptability criterion for imprecision, we calculated a value of 9.2%, compared with the total analytical CV of 11%.

Regression analysis yielded a high correlation between whole plasma and plasma spot values (Fig. 4). As in the case of ferritin determinations, plasma spot TfR measurements were consistently lower than whole plasma values, with a mean disparity in the composite group of 8% ($P < 0.001$). However, in contrast to ferritin measurements, greater differences occurred at high rather than low TfR values. The geometric mean plasma TfR of 5.2 mg/L in normal subjects (Table 2) was similar to the mean of 5.7 mg/L reported previously (4). The mean plasma TfR in ID was identical to the mean in normal subjects and remained within the normal range in all subjects with ID (Fig. 5A). There was minimal overlap between normal subjects and the increased whole plasma values observed in subjects with IDA; the value in one normal subject fell within the range of IDA and the value in one IDA subject fell within the range of normals. The degree of overlap in plasma spot TfR values between normal and IDA subjects was roughly similar to that observed with whole plasma measurements (Fig. 5B).

The major advantage of including TfR measurements in studies of anemia prevalence and cause is the ability to distinguish between IDA and the anemia of chronic disease (ACD), using the ratio of TfR/ferritin (16–18). It was, therefore, of interest to assess the correspondence in this index when based on whole plasma and plasma spot measurements. Because the ferritin falls with iron depletion whereas the TfR increases, the disparities observed between whole plasma and plasma spot values of the ferritin and TfR were offset when calculating the ratio (19). This produced identical values of 11 for the ratio in normal subjects and only minor differences of 73 and 77 in

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**Fig. 5.** Assay values for plasma spot TfR (A) and whole plasma TfR (B). The values are separated into three groups: normal, ID, and IDA.

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**Fig. 6.** Relationship between log TfR/ferritin ratios obtained from plasma spot and whole plasma samples. The solid line represents the regression equation: log$y = 1.043 \log x + 0.05126; r = 0.986; P < 0.0001$. The dashed line is the line of identity.
ID subjects and 333 and 384 in subjects with IDA (Table 2). The similarity between whole plasma and plasma spot results was again reflected in a high correlation by regression analysis (Fig. 6). The ability to identify IDA with the TfR/ferritin ratio was comparable in whole plasma and plasma spot measurements (Fig. 7). Neither measurement showed any overlap between normal and IDA subjects. The ratios in subjects with ID demonstrated a similar degree of overlap between normal subjects and those with IDA when based on whole plasma or plasma spot determinations.

**Discussion**

One type of anemia that is often confused with IDA in prevalence studies is ACD, which results from the impaired stimulation of red cell production associated with a wide spectrum of infectious or inflammatory illnesses. Several laboratory measurements that are used to identify IDA, such as mean corpuscular volume, transferrin saturation, and erythrocyte protoporphyrin concentration, are affected similarly in IDA and ACD. This problem is often circumvented by including plasma ferritin determinations, which are a reliable index of IDA when a low value is detected in the presence of anemia. However, the plasma ferritin concentration is increased in the presence of inflammation or infection and therefore cannot reliably identify concurrent IDA. There is mounting evidence that chronic disease with or without iron deficiency accounts for a large proportion of the anemia detected in certain developing countries. For example, in a recent study of 93 anemic pregnant women living in Malawi, less than one-half were found to have IDA (20).

The usual clinical method for identifying IDA when chronic illness is common is to perform a bone marrow examination to detect the presence or absence of stainable iron (21). This approach clearly is impractical for large epidemiological studies. The measurement of plasma TfR recently has emerged as a suitable alternative, especially when performed in conjunction with plasma ferritin measurements. In a recent clinical study, 129 patients with anemia underwent a diagnostic bone marrow examination to determine the cause (18). There were 48 patients with IDA, 64 with ACD, and 17 with both. Plasma TfR was more reliable than plasma ferritin in distinguishing the cause of anemia, but the optimal index was the TfR/ferritin ratio. The ratio correctly identified all patients with either IDA or ACD and, with the exception of a single patient, correctly identified the presence of IDA in the 17 patients with concurrent infection or inflammation. If confirmed in other studies, tandem measurements of plasma TfR and ferritin will be a valuable addition to studies of anemia prevalence.

The present study was undertaken to determine whether dried plasma spots can be used for measurements of plasma TfR and ferritin in prevalence studies. Because of the small volume of specimen required for these assays, blood can be obtained by finger-stick sampling. The transportation and storage of specimens can be simplified when studies are performed in rural areas without access to proper laboratory facilities. We observed in the present study that spotted paper samples could be stored at 4 °C for at least 4 weeks before assay when sealed in plastic bags containing desiccant. Prior studies using dried whole-blood spots demonstrated that storage up to 1 year at 4 °C with desiccation does not lead to deterioration of the samples or loss of protein activity. Because loss of ferritin and TfR activity can occur at higher temperatures, it seems unwise to transport samples by regular mail during summer months.

In a prior investigation, we evaluated the use of whole blood spotted onto filter paper for measurements of TfR and ferritin (9). The whole blood TfR concentration was nearly identical to plasma TfR when corrections were made for the displacement of plasma by red cells in the whole blood, but the whole blood ferritin concentration was threefold higher than the plasma ferritin concentration because of the contribution of erythrocyte ferritin. Despite the reduced utility of the ferritin determination, the TfR/ferritin ratio in whole-blood spots was able to correctly identify all 10 individuals with IDA. As in the
present investigation, subjects with ID but no anemia had TfR/ferritin ratios that were intermediate between normal subjects and those with IDA.

The choice between using whole blood or plasma for dried paper spots depends on several considerations. The preparation of paper samples in the field is easier with whole blood than with plasma because blood can be spotted directly onto the filter paper, whereas plasma must obtained either by centrifugation or by allowing samples to stand long enough for the separation of red cells. On the other hand, an important advantage of using plasma rather than blood is the highly predictable relationship in ferritin determinations. In populations with a relatively low prevalence of ID or IDA, the assessment of iron reserves based on ferritin measurements is less reliable with whole blood than with plasma. This consideration is less important in regions where the prevalence of anemia is high because the separation between normal individuals and those with IDA is similar when either whole-blood or plasma spots are used. It should be emphasized, however, that subjects with ACD, with or without concurrent IDA, were not included in the prior evaluation of dried whole-blood spots (9) or in the present study of dried plasma spots. It will be important to compare these approaches in a setting where the prevalence of ACD and IDA is high and can be distinguished clearly by a concurrent bone marrow examination.

References