Measurements of 25-Hydroxyvitamin D Concentrations in Archived Dried Blood Spots Are Reliable and Accurately Reflect Those in Plasma

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Context: Recognition that vitamin D might be associated with many chronic diseases has led to large-scale epidemiological and clinical studies. Dried blood spots (DBS) are a useful resource for these studies. Consequently, accurate, efficient, and inexpensive assays to quantify 25-hydroxyvitamin D (25OHD) in DBS are required.

Objective: This study evaluated the validity and reliability of a liquid chromatography-tandem mass spectrometry assay for measuring 25OHD in archived DBS and compared measurements of 25OHD in DBS with those in plasma.

Design and Participants: Sixty-two participants in the Melbourne Collaborative Cohort Study who had plasma and matching DBS stored since study entry in the early 1990s were randomly selected for a study calibrating 25OHD concentrations in DBS with plasma. As part of a study of vitamin D and mortality, cancer, and diabetes, we also assessed the reliability of measurements from DBS using 500 replicates placed randomly within 31 batches run over 15 months.

Outcome Measure: 25OHD concentrations were measured by liquid chromatography-tandem mass spectrometry.

Results: There was good agreement between measurements of 25OHD from DBS and plasma; \( R^2 = 0.73 \) from a regression of plasma concentration on DBS concentration. The within-batch and between-batch intraclass correlations from the 500 replicate measurements were 0.82 (95% confidence interval, 0.80, 0.85) and 0.73 (95% confidence interval, 0.68, 0.78), respectively.

Conclusions: Measuring 25OHD in DBS is a valid and reliable alternative to measuring 25OHD in sera or plasma. A simple calibration model was developed to convert measurements from DBS to equivalent plasma measurements, thus enabling comparisons against clinical reference ranges and with studies using sera or plasma samples. (J Clin Endocrinol Metab 99: 3319–3324, 2014)

Low vitamin D status is associated with musculoskeletal diseases and increased mortality and a range of other diseases including type 2 diabetes mellitus, cardiovascular disease, autoimmune diseases, and some cancers (1–5). Emerging evidence that vitamin D is essential for optimal health has led to a surge in demand for measuring vitamin D.
D, particularly for research purposes (6). There is therefore a need for economical, high-throughput, and reliable assays to quantify circulating vitamin D levels.

The predominant circulating form and best indicator of vitamin D status is 25-hydroxyvitamin D (25OHD), which is the total of the 25-hydroxy forms of vitamin D2 and vitamin D3 (7). Sera or plasma are the standard biological specimens used for measuring the circulating 25OHD concentration, but in some circumstances, dried blood spots (DBS) are the only blood specimen available and are increasingly being exploited for both pediatric and adult studies (8). DBS are readily collected, require no refrigeration, and are easily stored. This makes them ideal for archiving.

Recently, we developed an assay to measure 25OHD from DBS using liquid chromatography-tandem mass spectrometry (LC-MS/MS). As part of the initial assay evaluation, fresh blood samples were spotted onto Guthrie cards, and measurements from the resulting DBS were compared with measurements from plasma. Measurements from the two sources were highly correlated. As we have previously shown, almost all (98.4%) of the 25OHD is found in the sera compartment of whole blood (8). This is because almost all circulating 25OHD is bound to vitamin D binding protein, which is excluded from red blood cells. Therefore, as expected, DBS values were substantially lower than corresponding plasma values.

In most epidemiological studies that use DBS, the samples have been stored for many years. For example, in the Melbourne Collaborative Cohort Study (MCCS), all participants donated a blood sample between 1990 and 1994, from which various blood fractions (including plasma and DBS) were stored. Thus, samples have been archived for at least 18 years. The plasma samples of many participants have been used, leaving only their DBS for measuring 25OHD. The validity and reliability of the assay have not been fully evaluated for such archived samples.

As part of a study of vitamin D and the risk of death, cancer, and diabetes nested within the cohort study, we performed a calibration study to assess how well measurements from stored DBS correspond with measurements from stored plasma. We also assessed the repeatability of the measurements of 25OHD within the calibration study and within the main study under routine study conditions.

Subjects and Methods

Study population

The MCCS is a prospective cohort study of 41,514 people aged 27–75 years at baseline. Details of the design, recruitment, and study procedures are outlined elsewhere (9). Recruitment occurred between 1990 and 1994. Participants are all white, and 24% are southern European migrants. Blood samples were collected at baseline, from which 2–3 mL of plasma was stored in liquid nitrogen. For approximately 75% of participants, whole blood was spotted onto Whatman 903 paper. Spots were air dried and then stored in the dark. The Cancer Council Victoria Human Research Ethics Committee approved the study, and participants gave written informed consent to participate.

Participants—calibration study

Because 250 samples could be measured per 384-well plate for the LC-MS/MS assay, a sample size of 62 was chosen to allow two DBS replicates and two plasma replicates from each participant to be measured on a single plate.

A random sample of 62 MCCS participants who had plasma and DBS available was selected, with stratification by season of blood collection. Half of the selected participants had blood samples collected in February, and the remaining samples were collected in August to reflect the zenith and nadir of an Australian summer and winter, respectively. Plasma samples were thawed overnight in a refrigerator and then at room temperature, and were gently agitated or vortexed before an aliquot was removed. For each participant, four samples derived from a single blood sample collected at baseline were prepared as follows: two DBS from Guthrie cards were placed into separate envelopes, and two 25-μL aliquots of plasma were placed into separate tubes labeled with new identification numbers. The samples were assayed by LC-MS/MS (10). The laboratory analysts were blind to sample identity.

Participants—reproducibility within case-cohort study

To assess the reliability of the assay under routine study conditions, a reproducibility study was undertaken within a nested case-cohort study of 7043 participants from the MCCS, in which 25OHD was measured from DBS using the LC-MS/MS assay. Five hundred participants were randomly selected to have two DBS from the same baseline blood sample measured. Their replicate samples were randomly interspersed between the 7045 samples from the whole case-cohort study.

Measurement of 25OHD—calibration study

The two DBS and the two plasma samples were assessed in the laboratory of D.W.E. at the Queensland Brain Institute. This laboratory participates in the Vitamin D External Quality Assurance Scheme and routinely calibrates relative accuracy using standard reference material (SRM 968e and SRM 1950) serum calibrants supplied by the National Institute of Standards and Technology (NIST) (NIST calibrants for DBS do not yet exist). 25OHD was extracted and assayed using LC-MS/MS as previously described (8, 10). For the plasma analyses, only 3 μL of plasma was required; for the DBS analyses, 3.2 mm disks were punched from archived blood spots using an automated puncher (BSD700; BSD Robotics). Data are reported as total 25OHD, which was calculated by adding 25OHD2 and 25OHD3 concentrations.

Because 25OHD is virtually completely excluded from erythrocytes (8), 25OHD concentrations in DBS were corrected for mean sex-specific hematocrit fractions of 0.41 for females and
0.45 for males (11) using the following formula (10) to estimate equivalent plasma levels:

\[
\frac{\text{Plasma}_{25\text{OHD}} \text{ (nmol/L)}}{1 - \text{Hematocrit fraction}} = \frac{\text{DBS}_{25\text{OHD}} \text{ (nmol/L)}}{
\]

Hematocrit-corrected DBS 25OHD values were used for direct comparisons with plasma measurements. Crude DBS 25OHD values were used for the reliability analyses and development of the calibration model.

Measurement of 25OHD—reproducibility within case-cohort study

DBS were analyzed in the laboratory of D.W.E. as described in Measurement of 25OHD—calibration study. 25OHD measurements were made over a period of 15 months in 31 batches of approximately 230 samples. Laboratory analysts were unaware of the identity and number of replicates.

Statistical analysis—calibration study

Construct validity was assessed by examining whether 25OHD measurements from each of the sample types showed seasonal variation. Independent two-sample t tests were used to compare mean 25OHD concentrations between summer and winter.

Measurements for plasma and DBS were compared by performing a paired t test using one plasma and one DBS measurement randomly selected for each participant. The correlation between plasma and DBS measurements was quantified by the intraclass correlation (ICC) obtained from a mixed effects model fitted on the two plasma and two hematocrit-corrected DBS measurements for each participant, allowing for a constant bias between measurements on the two sample types and with random variability differing for the two sample types.

Multiple linear regression was used to develop a calibration model to predict plasma 25OHD levels from 25OHD concentrations in DBS. For this analysis, one plasma measurement and one DBS measurement (not corrected for hematocrit) were randomly chosen. These pairs were used to develop the calibration equation. Key predictors of vitamin D status, ie, age at sample collection, sex, country of birth, and month of blood sample collection, were evaluated as potential covariates by including them one at a time in the model and assessing their contribution to the model fit using the likelihood-ratio test. Interactions between each of these covariates and the DBS measurement were also assessed using the likelihood-ratio test. Given that men and women tend to have different hematocrit fractions and that this would affect the association between DBS and plasma 25OHD levels, sex was retained in the model.

Sex-specific 95% prediction ranges were estimated from the calibration model. The calibration equation and the other DBS replicate measurements were then used to estimate the plasma concentrations and 95% prediction intervals. The remaining plasma replicate measurements were used to see whether the measured plasma concentrations fell within the estimated prediction interval.

Reliability of replicate measurements, for both plasma and DBS, was quantified by calculating the ICC using one-way ANOVA, fitted separately for each sample type. Agreement between replicate measurements, for both plasma and DBS, was assessed using Bland-Altman plots of the difference of the two replicate measurements against the average of the two measurements and by calculating 95% limits of agreement. Bland-Altman plots facilitate visual assessment of the agreement between the measurements, including evaluation of whether the differences between the two replicate measurements depend on concentration.

Statistical analysis—reproducibility within case-cohort study

The repeatability of the assay for measuring 25OHD in DBS was assessed using a Bland-Altman plot and calculating the 95% limits of agreement. The within-batch and between-batch ICCs were obtained from a mixed effects linear regression model with random effects for batch and participant.

Analyses were performed using Stata 12.1 software (Stata Corp).

Results

Calibration study

Complete measurements were made for all 62 participants (Table 1). The mean age of participants was 54.2 years (SD, 10.1 y), and 45 (72.6%) of the participants were female. The samples had been stored for an average of 19.0 years (SD, 0.6 y). The assay exhibited excellent performance, with the four external NIST sera calibrants showing –1.2, +8.0, –0.3, and –1.5% relative inaccuracies at concentrations of 17.7, 32.2, 49.6, and 61.85 nmol/L, respectively. The overall mean relative inaccuracy over the four external calibrants was 2.75%.

25OHD concentrations were substantially higher at the end of summer than at the end of winter for both plasma and DBS samples (all P < .001). The median (interquartile range [IQR]) 25OHD concentrations from the two plasma replicates were 32.2 nmol/L (24.3–39.3 nmol/L) and 32.2 nmol/L (22.9–41.7 nmol/L) in winter compared with 45.0 nmol/L (37.7–58.9 nmol/L) and 45.5 nmol/L (32.4–61.4 nmol/L) in summer. For the hematocrit-uncorrected DBS replicates, the median (IQR) 25OHD concentrations were 15.0 nmol/L (10.5–18.1 nmol/L) and 15.1 nmol/L (11.0–19.9 nmol/L) in winter, compared with 21.9 nmol/L (18.9–34.8 nmol/L) and 23.9 nmol/L (17.2–35.3 nmol/L) in summer.

Measured 25OHD levels in DBS were on average 19.7 nmol/L lower (95% confidence interval [CI], –22.6, –16.8 nmol/L) than the equivalent plasma levels (P < .0001). When the DBS value was corrected for mean sex-specific hematocrit, on average levels were still 4.5 nmol/L lower (95% CI, –7.0, –2.1 nmol/L; P = .0005). The ICC comparing plasma to hematocrit-corrected DBS values was 0.84 (95% CI, 0.76, 0.92).

Age (P = .53), month of blood collection (P = .87), and ethnicity (P = .18) did not improve the fit of the regression
model relating plasma and DBS measurements. Sex ($P = .02$) was an important covariate, and there was also an interaction between sex and the DBS value ($P = .02$). The regression model (with adjusted $R^2$ of 0.73) for prediction of plasma 25OHD from DBS 25OHD measurements was:

$$\text{Plasma}_{25\text{OHD}} = 1.36 \times \text{DBS}_{25\text{OHD}} + 0.66 \times \text{Male} + 10.33,$$

where male = 1 for males, 0 for females.

Thus, the regression equation predicts that the difference in plasma levels for men and women increases with increasing 25OHD concentration in the DBS. For example, the predicted plasma levels for females and males with a DBS concentration of 13.6 nmol/L (25th percentile) are 28.8 and 30.3 nmol/L, whereas for a DBS concentration of 24.7 nmol/L (75th percentile), the predicted plasma values are 44.0 and 52.8 nmol/L, respectively.

To test the ability of the model to predict plasma 25OHD concentrations from DBS 25OHD, the regression equation was applied to the second set of DBS replicate measurements to predict plasma concentrations and 95% prediction intervals (Figure 1). Almost all (95.2%) of the observations fell within the 95% prediction range.

Reproducibility within case-cohort study

The relative inaccuracies for 31 runs over 15 months using the same individual NIST calibrants as previously mentioned were $-2.3$, $+3.4$, $+2.0$, and $-0.5\%$, yielding an overall inaccuracy of $2.0\%$, with an interassay coefficient of variation of 8.46%.

In the reproducibility study, two DBS measurements were made on 493 of the 500 participants selected to have replicate measurements. The mean age was 54.8 years (SD, 8.8 y), and 246 (49.9%) of the participants were female. The DBS had been stored for an average of 18.1 years (SD, 0.8 y).

The median (IQR) 25OHD concentrations for the two replicates were 23.7 nmol/L (16.8–32.6 nmol/L) and 24.0 nmol/L (16.7–32.0 nmol/L). The average difference between the two replicate DBS measurements was $-0.28$ nmol/L (95% limits of agreement, $-16.9, 16.3$ nmol/L).

Table 1. 25OHD Concentrations in DBS Replicates (Crude Values and Values Adjusted for Mean Sex-Specific Hematocrit Fractions) and Plasma Replicates in the Calibration Study, and DBS Replicates in the Reproducibility Within the Case-Cohort Study

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* 25th and 75th percentiles.

Figure 1. Prediction performance of the calibration model. Measured plasma 25OHD concentrations (dots) are superimposed on predicted plasma concentrations (dashed lines), using the measurements that were not used in developing the calibration equation.

Figure 2. Agreement between duplicate measurements from plasma and DBS samples. The average differences between the two replicate measurements were $-0.77$ nmol/L (95% limits of agreement, $-16.24, 14.70$ nmol/L) for plasma and $-0.46$ nmol/L (95% limits of agreement, $-10.00, 9.08$ nmol/L) for DBS. The variability of the difference between replicate measurements increased slightly as the 25OHD concentration increased (Figure 2). The ICCs for plasma and DBS replicates were 0.89 (95% CI, 0.84, 0.94) and 0.88 (95% CI, 0.82, 0.93), respectively.
Variability was low when the 25OHD concentration was low, but the variability increased slightly with increasing 25OHD concentrations (Figure 2, E and F). The correlation between two measurements made for the same participant in the same batch (the within-batch ICC) was 0.82 (95% CI, 0.80, 0.85), and the correlation between two measurements made for the same participant in two different batches (the between-batch ICC) was 0.73 (95% CI, 0.68, 0.78).

**Discussion**

There was good agreement between measurements of 25OHD from our DBS and plasma that had been stored for 19 years. As expected, measurements from DBS were substantially lower but highly correlated with plasma measurements, and a regression equation was developed that accurately predicted plasma concentrations from the concentration in DBS and participants’ sex. The assay was also very reliable, showing high ICCs between duplicate samples in a 15-month study involving multiple batches.

The calibration study has limitations because all the samples were measured in the same run, the test samples and training samples were from the same people, and the laboratory analysts were aware of its purpose. It is also limited by its small sample size. By chance, the sample comprised 17 males and 45 females, and this limited the ability to account for sex in the calibration model. By contrast, the larger reproducibility analysis was from a standard epidemiological study, and laboratory analysts were unaware of the duplicates.

The use of DBS for measuring vitamin D offers several advantages over serum or plasma for large-scale epidemiological studies. Collection of blood spots is minimally invasive. For our LC-MS/MS assay, only a small volume is required, with a mere 3.3 μL of whole blood in a 3.2-mm DBS punch being sufficient for the extraction and quantification of 25OHD (10). DBS are stable in the dark at room temperature, are suitable for long-term storage, and can be easily transported. This creates many useful applications for this methodology in medical research; for example, DBS routinely collected on Guthrie cards at birth can be used to determine how neonatal vitamin D status affects health outcomes later in life.

DBS 25OHD concentrations were substantially lower than corresponding plasma 25OHD. This was expected given the exclusion of 25OHD from erythrocytes. Other studies have compared DBS 25OHD levels with those in matching sera. These studies used the same method that converts DBS concentrations to sera concentrations based

![Figure 2. Reliability of the LC-MS/MS assay. The reliability of the LC-MS/MS assay for measuring 25OHD in plasma (A and B) and DBS (C and D) in the calibration study, and DBS (E and F) in the reproducibility within the case-cohort study, is shown as scatter plots and Bland-Altman plots. In A, C, and E, the line represents identity.](https://academic.oup.com/jcem/article/99/9/3319/2538473)
on the amount of sera in a DBS of known size (12). In one study, 25OHD$_3$ concentrations were shown to be lower in DBS compared with matching serum, whereas 25OHD$_3$ concentrations were not significantly different (12). Another study found that DBS measurements of 25OHD were slightly lower than both whole blood and serum measurements (13). In our study, correction of DBS 25OHD measurements to equivalent plasma levels using sex-specific mean hematocrit values did not completely account for the differences between DBS and plasma values. Using mean sex-specific hematocrit fractions may not fully adjust for hematocrit because there is also physiological variation in hematocrit that can depend on factors such as age, season, smoking, and exercise (11). It is also possible that the difference is due to under-recovery of 25OHD from the DBS due to diminished sample extraction, ie, due to an inaccessible compartment in cellulose-bonded blood proteins compared with plasma. Alternatively, there may be some slight degradation during storage of DBS relative to plasma (14), or due to inadvertent sunlight exposure during transportation (15).

In conclusion, measurement of 25OHD in DBS using LC-MS/MS is a valid alternative to conventional methods of quantifying vitamin D. This technique is particularly useful for long-term cohort studies. The calibration equation will enable investigators to report DBS measurements in terms of plasma concentrations, thereby facilitating comparison with previous studies and with current threshold levels for vitamin D adequacy or insufficiency.

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**References**