caused by hemolysis of whole blood during the drying process. Erythrocytes contain very low concentrations of homocysteine, \( \sim 10\% \) of plasma values (11). The DBS homocysteine assay showed considerably poorer precision than the analysis of plasma by the same method, but the CV of the DBS assay compared well with other DBS homocysteine assays (5, 6). Increased variability may be attributable to the process of blood-spot preparation, the small sample volume, elution of homocysteine from the blood spot, and the greater sample dilution. The assay may not be able to quantify DBS homocysteine in all individuals because the lower limit of detection is above the concentrations found in some healthy neonates (7, 12).

The results of this study show that homocysteine is stable in DBS at room temperature for 24 h, followed by a small but consistent decrease by day 3 and a more modest reduction after 28 days of storage. This is acceptable for the purposes of a screening program in which the samples are sent by mail to the laboratory. A possible explanation for the stability is that during the process of drying the blood on the filter paper, the enzymes in the sample become denatured as a result of dehydration. The continuous enzymatic production of homocysteine that occurs in vitro may therefore be inhibited in dried whole blood. The slight decrease in homocysteine concentration between days 1 and 28 may be attributable to bacterial breakdown of amino acids in the sample.

Further investigation into the analysis of homocysteine in the diagnosis of neonatal homocystinuria is required; for example, the concentration of homocysteine in blood of neonates with CBS deficiency needs to be determined followed by establishment of appropriate diagnostic cutoff values. However, for neonatal screening of homocystinuria by measurement of blood spot homocysteine to be considered, it is important to establish the suitability of these samples for homocysteine analysis. We have described a method that is robust, reproducible, and suitable for the detection of increased homocysteine concentrations and have demonstrated that homocysteine is sufficiently stable in DBS to meet the requirements of a screening program.

References


Serum 25-Hydroxyvitamin D Is Unaffected by Multiple Freeze-Thaw Cycles, Diana M. Antoniucci,* Dennis M. Black,2 and Deborah E. Sellmeyer1 (1 Division of Endocrinology, Department of Medicine, and 2 Department of Epidemiology and Biostatistics, University of California, San Francisco, 74 New Montgomery St., Suite 600, San Francisco, CA 94105; * author for correspondence: fax 415-597-9213, e-mail dantoniucci@psg.ucsf.edu)

Vitamin D deficiency is a common disorder believed to affect as many as 57% of hospitalized patients (1) and 9–50% of outpatients, depending on the characteristics of the study population (2–6). Vitamin D deficiency can cause secondary hyperparathyroidism and, if severe, osteomalacia. Both of these conditions are associated with loss of bone density, and both are reversible by repletion of vitamin D (7–10). Recently, the association of vitamin D deficiency with an increased risk for a broader range of diseases, such as prostate cancer (11, 12) and colon cancer (13), has come to attention. Dietary vitamin D supplemen-
tation has even been implicated in decreasing the risk of developing type 1 diabetes mellitus (14), and ultraviolet irradiation has been shown to decrease systolic blood pressure in patients with mild untreated hypertension (15). Determining whether vitamin D deficiency is present usually involves assessing the stores of vitamin D by measuring serum 25-hydroxyvitamin D (25OHD) concentrations, in combination with an assessment of parathyroid hormone concentrations. Currently, the preferred clinical 25OHD assay is a two-step procedure that involves a rapid extraction step of 25OHD from serum or plasma, followed by an equilibrium RIA procedure. The RIA is based on an antibody with specificity toward 25OHD. Specimens are obtained and immediately frozen at –20 °C or lower, then thawed just before the assay is performed. Vitamin D analytes, however, appear to be quite stable even at other temperatures, as was demonstrated by the International Quality Assessment Scheme for Vitamin D metabolites (DEQAS), a multinational study run out of the UK. Studies in the laboratory of the DEQAS organizer have shown no significant change in results for vitamin D metabolites during storage for up to 2 weeks at 30 °C (16). Consequently, DEQAS samples of vitamin D metabolites are sent to participants at ambient temperature, by first class mail, and typically arrive within 2 weeks of dispatch without any evidence of deterioration of the vitamin D metabolites.

Assaying serum for 25OHD is relatively expensive and is therefore often forfeited in large clinical trials of osteoporosis drugs. Undetected vitamin D deficiency may increase a patient’s risk of hypocalcemia when treated with bisphosphonate therapy (17). Many prospective studies have stored serum from participants and therefore

<table>
<thead>
<tr>
<th>Freeze-Thaw cycle</th>
<th>Median (interquartile range), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57.4 (39.9–73.6)</td>
</tr>
<tr>
<td>2</td>
<td>53.7 (41.2–77.4)</td>
</tr>
<tr>
<td>3</td>
<td>53.7 (38.7–81.1)</td>
</tr>
<tr>
<td>4</td>
<td>57.4 (38.7–82.4)</td>
</tr>
</tbody>
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Table 1. Serum vitamin D concentrations at each freeze-thaw cycle.

Fig. 1. Correlations between serum 25OHD measurements at each freeze-thaw cycle.
offer the opportunity to further study the relationship between vitamin D deficiency and diseases such as colon, breast, and prostate cancer and diabetes. However, the serum samples that are stored as part of these large epidemiologic studies are often limited and desired by multiple investigators, and not uncommonly have been thawed and refrozen at least once. There are no published data about the reproducibility of vitamin D assays in serum samples that have undergone multiple freeze-thaw cycles. Experts often informally state that the assay should be reliable when performed in samples that have undergone up to three freeze-thaw cycles [Drs. M.F. Holick (Boston University Medical Center, Boston, MA) and R. R. Recker (Creighton University, Omaha, NE), personal communications]. The instruction manual that accompanies the DiaSorin 25OHD assay reports that no significant changes in values were observed for samples subjected to three freeze-thaw cycles (18). This information is based on internal data from DiaSorin, which studied serum 25OHD concentrations in five individuals. The concentrations were measured at baseline, then after three freeze-thaw cycles, and the correlation between these two measurements was 0.61. We feel that this correlation coefficient is too low to comfortably use samples that have previously been thawed. Furthermore, the data do not provide information on the correlation between the baseline value and each subsequent value. For these reasons, we sought to determine the correlation between baseline serum 25OHD concentrations and those obtained after the same samples underwent each of three freeze-thaw cycles.

We recruited 20 healthy adults. All participants gave informed consent before participation in the study, which was approved by the University of California, San Francisco (UCSF), Institutional Review Board. Individuals were excluded if they were on medications or had medical conditions known to alter vitamin D metabolism, such as malabsorption conditions, renal or hepatic disease, therapy with vitamin D exceeding the dietary recommended intake (19), or therapy with antiepileptic medications. A single serum sample was collected from each participant and immediately aliquoted into four vials. All four vials were frozen at −70 °C. We define each freeze-thaw cycle (FT) as the vial being frozen and thawed once. The four collected vials were exposed to one, two, three, or four freeze-thaw cycles and were maintained fully frozen for 3 days between thaws. This meant that one vial was never thawed until the day of the 25OHD assay performance (FT1), one vial was thawed once (FT2), one vial was thawed twice (FT3), and one was thawed three times and refrozen (FT4) before the day of the 25OHD analysis. Specimens were analyzed by the UCSF clinical laboratory by use of the commercially available DiaSorin RIA with antibodies specific to 25OHD.

Correlation coefficients for the 25OHD result were calculated between the FT1 and each subsequent freeze-thaw cycle, with a P < 0.05 considered as significant. All analyses were performed by use of Stata 8.0 (Stata Corporation).

Among the participants, 13 were women and 7 were men; the mean age was 39.8 years (range, 23–60 years). The median (interquartile range) 25OHD concentration in the baseline specimen was 57.4 (39.9–73.6) nmol/L (Table 1). The correlation between the baseline 25OHD measurement and the measurement obtained after each successive freeze-thaw cycle was 0.99 (Fig. 1).

Our data show that 25OHD results obtained for specimens that have been thawed and refrozen up to four times are reliable. The data also suggest that investigators can use stored specimens from epidemiologic studies to measure 25OHD even if the samples have been previously thawed for other purposes.

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References


Rheumatoid factor (RF) is currently the most accepted laboratory test for rheumatoid arthritis (RA) and is part of the revised American College of Rheumatology (ACR) classification criteria for RA (1). The specificity of RF is, however, often low (2–5). A newer diagnostic marker for RA is anti-citrullinated peptide antibodies (ACPs), which can be identified by tests such as a line immunoassay (LIA™) for the detection of anti-pepA and anti-pepB antibodies (6), the anti-cyclic citrullinated peptide ELISA (7), an ELISA using citrullinated recombinant rat filaggrin (8), and an ELISA using deiminated fibrinogen (9). ACPAs have excellent specificity (89–100%) for RA, with good sensitivity (41–80%) (3–7, 10–15). Furthermore, the HLA shared epitope (SE) has been described, which is found more often in RA patients than in controls (16–18). Most studies of these newer tests have used control populations consisting of selected groups of patients with defined diseases and healthy controls. This does not represent real-life clinical practice because the composition of the control group does not reflect the natural prevalences of diseases in cases for which serologic markers for RA are requested. Data about specificity, positive predictive value (PPV), and negative predictive value (NPV) are thus hard to interpret. We designed the present study to reflect everyday rheumatology practice.

In this prospective study, we included 1003 consecutive patients in three academic and nonacademic centers: the Department of Rheumatology, Ghent University Hospital (Ghent, Belgium); the Locomotor Center, Elisabeth Hospital (Sijsele, Belgium); and the Department of Rheumatology, St-Augustinus Hospital (Wilrijk, Belgium). Patients were entered in the study when they presented with a new diagnostic problem for which RA was included in the differential diagnosis. This means that the rheumatologist would typically request RF determination, although the patients did not necessarily have early arthritis. Diagnoses were established after 1 year of follow-up. The clinicians were unaware of the test results obtained through the study. No ACPA results were available to the clinicians during the follow-up period. The study was conducted after receipt of approval by the local ethics committees. Oral informed consent was obtained from all patients.

RF was determined with the Waaler Rose (RF WR) and with the latex fixation method (RF LF). Anti-pepA and anti-pepB antibodies were detected by a research LIA (INNO-LIA™RA; Innogenetics) (6). We used scan values to obtain continuous data. This test can also be reported as positive or negative by use of a reference strip. In that case, cutoffs cannot be varied, and the results may differ from the ones reported here. The HLA SE was determined by INNO-LiPA (line probe assay) technology (Innogenetics). Details of the methods are available in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue12/.

We computed sensitivities, specificities, PPVs, and NPVs together with their 95% confidence intervals (CIs) (19). We compared sensitivities and specificities by use of the McNemar test. ROC curve analysis was performed. Statistical analysis was performed with SPSS 10.0.

Clinical diagnoses were established by the treating rheumatologist after 1 year of follow-up. The distribution was as follows: definite RA (n = 153), probable RA (n = 72), potential RA (n = 75), non-RA (n = 629), and lost to follow-up (n = 74). Of the patients with clinically “definite” RA, 144 fulfilled the revised ACR criteria for RA (2) and were further considered as RA patients. We used ACR criteria to improve the comparability of our results, although RF is part of these criteria and the diagnostic value of RF might thus be overestimated. However, by starting with a clinical diagnosis of RA, we minimized this problem. The non-RA patients were taken as the control group.

The mean age of the RA patients was 58.0 years (range, 21–84 years), which was significantly higher than the mean age of the non-RA patients (51.4 years; range, 12–88 years; P < 0.001). The male-to-female ratio was 50:94 in the RA group and 213:414 in the non-RA group (not significantly different). There were no significant differences between the two groups for duration of symptoms (mean of 19.3 months in the RA group and 15.9 months in the non-RA group).

We performed ROC curve analyses to compare the diagnostic accuracies of RF LF, RF WR, and anti-pepA and anti-pepB antibodies (Fig. 1). RF LF and RF WR had higher areas under the curves than anti-pepA and anti-pepB antibodies. The areas under the ROC curves were 0.84 (95% CI, 0.80–0.88) for RF LF, 0.82 (95% CI, 0.77–0.87) for RF WR, 0.78 (95% CI, 0.73–0.84) for anti-pepA anti-