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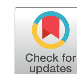

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A B S T R A C T

Blood for determining 25-hydroxyvitamin D_3 [25(OH)D_3] is usually obtained through venipuncture although, as an alternative for serum, dried blood spot (DBS) can be considered. The aim of this proof-of-concept study was to investigate levels of agreement between measurements of 25(OH)D_3 obtained with DBS compared with serum. 301 Chinese participants were included who completed 25(OH)D_3 measurement from DBS and from simultaneously collected blood samples obtained by venipuncture. Measurements of both DBS and serum 25(OH)D_3 were performed using liquid chromatography followed by tandem mass spectrometry. Agreement between the two methods was assessed with Passing and Bablok regression analysis and Bland-Altman plot.

Measurements showed a good correlation (Pearson's correlation coefficient $r = 0.929$, $P < 0.001$) between the two methods. After recalculating for a 13% difference, a regression equation of DBS 25(OH)D_3 = -1.91 + 1.00 serum 25(OH)D_3 was found in Passing and Bablok regression analysis. Bland-Altman analysis showed a fixed bias of 1.7 nmol/L; upper and lower limit of agreement was 24.1 nmol/L and -20.7 nmol/L, respectively.

Sensitivity of recalculated DBS for 25(OH)D_3 concentrations < 30 and < 50 nmol/L was 87.8% and 91.1%, respectively, and specificity was 89.2% and 83.1%, respectively.

In conclusion, a good agreement was found between the measurement of 25(OH)D_3 obtained with DBS compared with serum. DBS may possibly be used in a future screening program, but it is less suitable for individualized vitamin D status assessment.

1. Introduction

Vitamin D_3 is produced in the skin when exposed to ultraviolet light irradiation from its precursor 7-dehydrocholesterol. In the liver, vitamin D_3 is rapidly hydroxylated into 25-hydroxyvitamin D_3 [25(OH) D_3], which is the major circulating metabolite and considered the best indicator of vitamin D status.

In the Netherlands, margarine and baking oils are fortified with vitamin D_3 (7.5 μg/100 g). Vitamin D supplements prescribed by physicians in the Netherlands all contain vitamin D_3. Also, almost all vitamin D_2, originated from irradiation of the plant sterol ergosterol, in multivitamin tablets in the Netherlands has been replaced by vitamin D_3.

The Health Council of the Netherlands uses cut-off points for 25(OH)D_3 of 30 nmol/L for vitamin D deficiency and risk of rickets and of 50 nmol/L above which no further improvement of bone health is expected [1]. Based on their data, the Institute of Medicine suggests that persons are at risk of deficiency relative to bone health at serum 25(OH)D levels of below 30 nmol/L, and that practically all persons are sufficient at serum 25(OH)D levels of at least 50 nmol/L [2].

Many large-scale epidemiological and clinical studies on vitamin D have been conducted. Blood sampling for disease diagnosis and therapeutic drug monitoring is usually obtained by venipuncture. However, large-scale studies may be hampered by this kind of blood collection

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due to emotional, psychological, physical, and/or organizational barriers. For example, i) in older persons it may be difficult to draw blood [3], ii) for infants and children, painless blood collection and a low sample volume are important conditions [4], iii) in developing countries, access to a phlebotomy service may be limited (especially in remote areas) decreasing study participation.

Recently, dried blood spot (DBS) techniques for measurements of 25(OH)D_3 have been developed as an alternative for venipuncture [5–8]. This method for 25(OH)D_3 measurement is minimally invasive, requires a smaller volume of blood than venipuncture and (with adequate training) is suitable for patients to self-sample at home. Dried blood spots of 25(OH)D_3 are stable at room temperature and can easily be transported and long-term stored [5]. The method involves the collection of blood (by a finger prick) that is dropped onto filter paper and allowed to dry.

Observational studies have explored the use of DBS for assessment of vitamin D status [9–11]. Others compared measurement of 25(OH)D_3 using DBS as compared with serum or whole blood and reported relatively good agreement between these two methods [4,12]. However, despite these positive results, DBS is not commonly used in the Netherlands, raising the question as to whether DBS could replace conventional venous sampling for measurement of 25(OH)D_3 concentrations.

Therefore, in a sample of Chinese individuals residing in the Netherlands, this proof-of-concept study examines whether DBS measurements of 25(OH)D_3, using a method developed in the Netherlands, correspond with concentrations measured in serum.

2. Subjects and methods

As part of an observational study with a total of 418 persons to determine vitamin D status in the Chinese population in the Netherlands (performed in March 2014) [13], in the present study a subgroup of 301 participants completed 25(OH)D_3 measurement from blood spot samples and from simultaneously collected blood samples obtained by venipuncture. Briefly, participants aged ≥18 years with a Chinese background and residing in the Netherlands were eligible to participate when they, or at least one of their parents, were born in mainland China, Hong Kong or Taiwan.

The study protocol was approved by the Medical Ethical Committee of the Leiden University Medical Center and was conducted in accordance with the principles of the Declaration of Helsinki. All participants gave written informed consent.

2.1. Methods

Blood spot cards used for the measurement of DBS 25(OH)D_3 were purchased from and analyzed by the Dried Blood Spot Laboratory (DBSL, Geleen, the Netherlands). Certified nurses collected both venous and DBS blood from the participants. Using single-use lancets, a drop of blood was formed and allowed to drop freely onto the filter paper (Whatman 903®) of the sample cards. For each participant, two non-overlapping drops of blood were collected in pre-marked circles printed on the filter cards. After drying for ≥15 min at room temperature and further drying in a special shipping box (containing a silica gel sachet as a drying agent) the cards were sent by postal service to the laboratory of DBSL and analyzed by liquid chromatography followed by tandem mass spectrometry (LC–MS/MS).

2.2. LC–MS/MS settings and detection by DBSL

The LC–MS/MS method used by the laboratory (DBSL) is a further development of Eyles' method [5]. Chromatography (Agilent 1290 binary pump, autosampler, thermostat, and column compartment) was performed using an Acquity C18 column (Waters), 150 mm x 2.1 mm, 1.7 µm particle size, and detection was performed using triple-quadrupole mass spectrometry (Agilent 6460C LC–MS/MS) with an ESI source. The detection of ions of a specific fragment was performed using multiple reaction monitoring (MRM mode). Mobile phase A consisted of water containing 0.38 g/L ammonium acetate and 6 ml/L 2-methyl-2-butanol; mobile phase B consisted of methanol containing 0.38 g/L ammonium acetate and 6 ml/L 2-methyl-2-butanol. The column compartment temperature was 75 °C and the injection volume was 5 µL.

Mass parameter settings were: 25(OH)D_3-PTAD, *m/z* 558.4 Da - > 298.1 Da, collision energy 15 eV; internal standard (25(OH)D3-d3), *m/z* 561.4 Da - > 301.1 Da, collision energy 15 eV.

2.3. Calibration by DBSL

First, calibration standards for 25(OH)D_3 were prepared. As 25(OH)D_3 is an endogenous compound, all human blood samples contain 25(OH)D_3 and, therefore, no blank samples are available. DBSL has chosen to spike artificial vitamin D free serum with standard reference material (SRM) 2972a (in ethanol) (NIST, Gaithersburg, MD, USA) and mixed this with washed red blood cells (RBC) to produce blood with a hematocrit of 0.45. The prepared standards were mixed gently for 30 min at room temperature directly followed by the preparation of the DBS samples by applying 30 µL of the blood standard to the paper. Calibration was performed with these DBS reference samples of known content.

Quality control samples were made using serum reference material (LabQuality, Helsinki, Finland) by mixing these with washed RBC to produce a hematocrit of 0.45.

2.4. Sample preparation by DBSL

Prior to sample preparation, a solution of 75 µL internal standard (IS) (100 nmol/L) [Tri deutero-25-hydroxycholecalciferol (25(OH)D3-d3) Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands] in a solvent containing 1 N NaOH, 0.25% Tween-20 and 2 g/L tetra butyl ammonium bromide (used as an ion-pairing reagent to improve extraction recovery as it is believed to release adsorbed 25(OH)D_3 from the active places on the Whatman 903® filter paper) was transferred into a Sarsted tube followed by the addition of DBS by punching a circle of 8 mm out of the blood spot. Next, the tube was sonicated for 5 min to release 25(OH)D_3 from vitamin D binding protein. Subsequently, for deproteination a 50% acetonitrile/zinc sulphate (0.1 M) solution was added, followed by 30 min sonication. For extraction of 25(OH)D_3 hexane was added. After mixing, the hexane layer was removed and evaporated to dryness. For derivatization, to enhance detectability, a solution of 0.02% 4-Phenyl-1,2,4-Triazoline-3,5-Dione (PTAD) was added. The resulting product was evaporated to dryness and reconstituted in mobile phase B for analysis.

The lower limit of quantitation was 9.75 nmol/L with an overall precision [intra-assay coefficient of variation (CV)] of 13.9%, and an overall accuracy (bias) of 5.7% (N = 6). For concentrations between 48–140 nmol/L [SRM 1 (76.4 nmol/L), SRM 2 (48.3 nmol/L), SRM 3 (49.04 nmol/L), SRM 4 (139.2 nmol/L)] the CV values were 8.7%, 9.8%, 9.9%, and 7.6% (N = 6), respectively; bias was 2.0%, -8.7%, -3.6%, and 7.9% (N = 6), respectively. Recovery, which was about 76%, was established by comparing blood standards with spiked blank samples of the same concentration.

2.5. Measurement of the serum 25(OH)D concentration

Simultaneously with blood spot collection, blood samples were obtained by venipuncture from all participants. After centrifugation, serum samples for 25(OH)D measurement by isotope dilution/online solid-phase extraction liquid chromatography tandem mass spectrometry (ID-XLC-MS/MS) was performed at the Endocrine Laboratory of the Amsterdam UMC, Vrije Universiteit Amsterdam [14], with only minor adjustments [15]. In short, deuterated internal standard [25(OH)

D3-d6] was added to the samples and 25(OH)D was released from its binding proteins with acetonitrile. Samples were extracted and analyzed by XLC-MS/MS [a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands)] coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The limit of quantitation was 4.0 nmol/L; intra-assay CV was < 6%, and interassay CV was < 8% for concentrations of 25–180 nmol/L.

The Endocrine Laboratory participates with this 25(OH)D method in the Vitamin D External Quality Assessment Scheme (DEQAS). The long term CV% (over years) of the internal serum controls is < 10% for all concentrations. Moreover, this method was well standardized by comparing this method with the reference method procedures (RMP) of the University of Ghent in 40 serum samples collected by the US Center for Disease Control and Prevention (CDC, Atlanta). This comparison showed a very good agreement with the reference method [16].

3. Statistical analyses

Initially, both methods were compared with Pearson's correlation coefficient. Further comparison between DBS and serum samples was performed by Passing and Bablok regression analysis, and Bland-Altman analysis was used to calculate bias. A systematic difference (%) found in Passing and Bablok analysis was to recalculate bias in Bland-Altman analysis.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of recalculated DBS for 25(OH)D_3 concentrations < 30 and < 50 nmol/L were also calculated.

Statistical significance was considered at a P-value < 0.05. Analyses were performed using MedCalc® for Windows, version 17.6 (MedCalc Software, Ostend, Belgium).

4. Results

Of the subgroup of 301 participants (83 males, 218 females) who completed 25(OH)D_3 measurements from blood spot samples and blood samples obtained by venipuncture, 12 (4%) DBS samples (analyzed singularly because the blood spots were too small to punch out a spot of 8 mm) were excluded, leaving 289 samples suitable for analysis. The mean age of these men and women was 55.8 ± 14.6 years and 57.2 ± 11.0 years, respectively. Self-reported use of vitamin D supplements was 18.4% in men and 35.5% in women; the estimated average quantity vitamin D was 12.5 μg and 16.25 μg per day, respectively (data not shown).

Since the distribution of serum 25(OH)D_3 concentration was skewed, median 25(OH)D_3 concentrations were calculated. Median 25(OH)D_3 concentration for DBS was 39 [IQR, 27–56] nmol/L and for serum was 47 [IQR, 35–69] nmol/L (P < 0.001). Serum 25(OH)D_3 was < 30 nmol/L in 18.5% of the population, between 30–50 nmol/L in 38.2%, and ≥50 nmol/L in 43.3%.

Results from the two methods of 25(OH)D_3 measurements were acceptably close, as expressed in a Passing and Bablok regression equation DBS 25(OH)D_3 = -1.53 (95% CI, -3.60, 0.64) + 0.87 (95% CI, 0.82, 0.92) serum 25(OH)D_3 (Fig. 1); this implies a systematic 13% lower DBS 25(OH)D_3 concentration compared to serum 25(OH)D_3 concentration, with no significant intercept difference. The Cusum test for linearity did not show a significant deviation from linearity (P = 0.33). Pearson's correlation coefficient was 0.929 (P < 0.001).

Using the systematic difference of 13% as found in the Passing and Bablok equation to reanalyze Bland-Altman analysis, a significant fixed bias of 1.7 nmol/L (95% CI, 0.4, 3.0) was found. Upper (mean + 1.96 SD) and lower (mean – 1.96 SD) limit of agreement was 24.1 nmol/L and -20.7 nmol/L, respectively (Fig. 2).

As we regard a fixed bias of 1.7 nmol/L as not clinically relevant, we used the systematic difference of 13% to recalculate DBS 25(OH)D_3 concentration by multiplying DBS 25(OH)D_3 concentrations with factor 100/87. After recalculation the Passing and Bablok equation was DBS 25(OH)D_3 = -1.91 (95% CI, -4.45, 0.59) + 1.00 (95% CI, 0.95, 1.06) serum 25(OH)D_3, with no significant intercept or slope difference. The Cusum test was not significant (P = 0.26), i.e. no significant deviation from linearity.

Sensitivity of recalculated DBS for 25(OH)D_3 concentrations < 30 and < 50 nmol/L was 87.8% and 91.1%, respectively, and specificity was 89.2% and 83.1%, respectively. The PPVs for recalculated DBS were 62.3% and 86.7%, respectively, and the NPVs were 97.3 and 88.5%, respectively (Table 1).

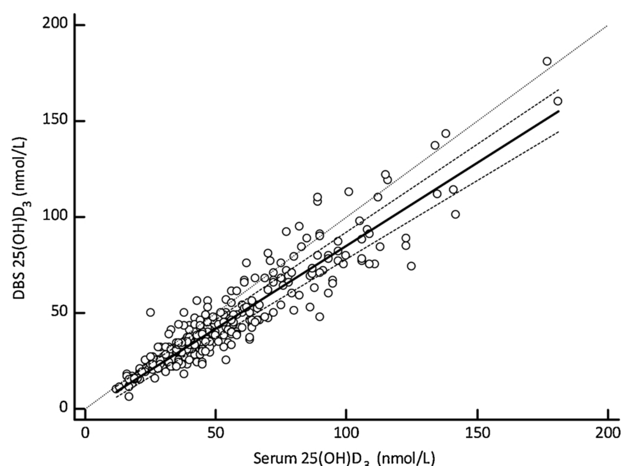

Fig. 1. Passing and Bablok plot of 25(OH)D_3 measured in dried blood spot (DBS) compared to serum in 289 simultaneously collected samples, with regression line and confidence bands for regression line. Identity line is dashed.

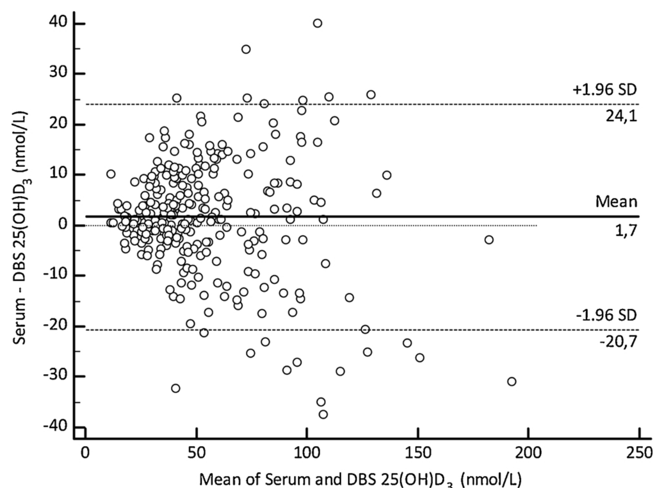

Fig. 2. Bland-Altman plot (difference plot) of 25(OH)D_3 measurement comparing dried blood spot (DBS) samples and venous blood samples using LC–MS/MS.

Table 1
Sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) of recalculated dried blood spot for 25(OH)D_3 concentrations of < 30 nmol/L and < 50 nmol/L.

	< 30 nmol/L	< 50 nmol/L
SE (%)	87.8	91.1
SP (%)	89.2	83.1
PPV (%)	62.3	86.7
NPV (%)	97.3	88.5

5. Discussion

In this study, a good agreement was found between measurements of $25(OH)D_3$ obtained with DBS compared with serum. After correction for the 13% systematic difference a fixed bias of 1.7 nmol/L was found and no significant constant or proportional differences were observed in Passing and Bablok analysis.

Our results support studies that found good agreement between measurements of 25(OH)D from DBS and plasma or serum samples. In one study, no significant difference was found between $25(OH)D_3$ concentrations in DBS compared to serum [7]. Another study reported (on average) lower DBS levels compared to plasma, even after correction of DBS value for mean sex-specific hematocrit levels [12]. This was explained by physiological variation in hematocrit levels, diminished sample extraction of DBS, and degradation of DBS during approximately 20 years of storage. In another study comparing DBS to serum or whole blood samples, a good (but imperfect) agreement was shown, in which DBS was (on average) slightly lower than serum measurement [4].

In the present study, we also found lower $25(OH)D_3$ concentrations in DBS compared to matching sera. However, although we cannot account for the systematic difference found in our analysis, differences in the standardization of the methods may partially explain this. For example, the use of different calibration or preparation standards, and differences in operation procedures between the two methods may have an effect on bias.

As almost all circulating $25(OH)D_3$ is bound to vitamin D binding protein [17] and, since this component is excluded from RBC, almost all of the $25(OH)D_3$ is found in the serum component of whole blood [18]. Therefore, to compare DBS and serum, the influence of the hematocrit fraction should be taken into account. For example, the viscosity of blood increases with increased hematocrit and, thus, the spread of blood on the DBS cards could vary depending on the hematocrit. In general, there is an inverse relationship between blood hematocrit and spot area [19], i.e., a punch of a fixed blood spot will contain more blood in case of a high-hematocrit sample compared to a low-hematocrit sample due to the higher viscosity of blood. However, DBSL uses a comparatively large punch (8 mm), which may partly overcome the variety caused by the hematocrit fraction. We applied no correction factor for hematocrit as we aimed to perform a clinical validation of a standard DBS method versus simultaneously collected blood samples obtained by venipuncture. It was not our intention to develop a DBS method and to investigate the influence of hematocrit. In addition, when one decides to use DBS for measurement of vitamin D, hematocrit is hard to establish so a correction cannot be applied. However, although we did not measure individual hematocrit values, another group examined samples containing a range of RBC and known $25(OH)D_3$ concentrations; in that study, within a hematocrit range of 0.4-0.6 (considered normal for an adult population), very little change in levels was seen [7].

Using a $25(OH)D_3$ concentration of < 30 nmol/L for vitamin D deficiency, the sensitivity of recalculated DBS was 87.8% and specificity was 89.2%. The PPV for recalculated DBS was 62.3%, implying that the advice to start vitamin D supplementation might be given too early in almost 38% of the cases. However, 96% of these false positive-cases had $25(OH)D_3$ concentrations < 50 nmol/L, which still can be regarded as insufficient. Furthermore, recommending vitamin D supplements in a false-positive case will very likely do no harm. The NPV for recalculated DBS $25(OH)D_3$ concentrations < 30 nmol/L was 97.3%, implying that in less than 3% of the samples the DBS method misclassified the vitamin D status as not deficient.

Because of the wide range of $25(OH)D_3$ concentrations, as shown by the limits of agreement in the Bland-Altman plot, DBSL is less suitable for assessing vitamin D status and monitoring vitamin D therapy in individuals. Thus, DBS $25(OH)D_3$ values may be almost 21 nmol/L below or 24 nmol/L above serum values, which is not an acceptable range for clinical decision making.

At this time, it is too early to decide whether DBS could be used in a vitamin D deficiency screening program. First, PPV and NPV are among others dependent on the prevalence of vitamin D deficiency in the target population. Therefore, PPV and NPV that we found may not be applicable for important target groups like young children (because of the risk of rickets) and institutionalized older persons (because of their often low production of vitamin D in the skin and their higher risk of fractures). Second, the cut-off point of DBS as a first step in screening should be chosen with a view to an optimum of PPV and NPV. E.g., one may choose an NPV as high as possible, even if this means a lower PPV, as false-positive cases are not very problematic: repeat measurement by venipuncture is relatively easy and cost of eventual vitamin D therapy is low. For example, if vitamin D deficiency is defined as serum 25(OH)D < 30 nmol/L (venipuncture), in our respondents a cut-off of 40 nmol/L (DBS) would have resulted in a PPV of 38.5% and an NPV of 99.4% (data not shown).

To our knowledge this is the first study performed among a Chinese population to compare DBS and serum for measurement of $25(OH)D_3$. A strength of this study is the relatively large number of participants who completed $25(OH)D_3$ measurement from DBS and from simultaneously collected blood samples obtained by venipuncture, with an adequate number of vitamin D deficient people. However, our study also has several limitations. First, as we did not measure hematocrit levels of individual participants it is uncertain whether participants had a blood hematocrit level beyond the reference values, thereby influencing the assay results. Second, the punch of 8 mm as used by DBSL, although relatively large, carries the risk that small spots may be completely included in the punch, whereas large spots are only partially included, resulting in a negative bias in large spots. However, only 12 DBS (4%) samples were excluded due to an inadequate punch. Third, DBSL did not differentiate between $25(OH)D_3$ and its C-3-epimer, which may result in an over-estimation of the total 25(OH)D concentration. Because 3-epi-$25(OH)D_3$ concentrations are believed to account for a significant percentage of the total 25(OH)D only in infants under one year [20], the assay used by DBSL is less suitable for this age category. Fourth, as vitamin D_2 is generally not used in the Netherlands, we did not measure $25(OH)D_2$ concentrations, which may be regarded as a limitation of our study. Finally, the results may not be generalizable to other populations or age categories, especially not to infants under one year, institutionalized older persons and populations where supplements containing $25(OH)D_2$ are commonly used.

In conclusion, DBS is an easy and less invasive method for collecting blood samples than venipuncture and requires smaller blood volumes for measurement of $25(OH)D_3$. The assay used showed a good agreement between the measurement of $25(OH)D_3$ in DBS and serum, and may adequately exclude vitamin D deficiency in community-dwelling adults and noninstitutionalized elderly persons. However, DBS is less suitable for individualized assessment of vitamin D status because of the wide interval of the limits of agreement in Bland-Altman analysis. Based on our results, it is too early to recommend the DBS method for screening for vitamin D deficiency. Further research is needed, especially for use in young children and institutionalized older persons.

Declaration of Competing Interest

Paul Lips has received a lecture fee from Abiogen. The other authors declare that they have no conflict of interest.

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