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Distribution of 25-hydroxyvitamin D_3 in dried blood spots and implications for its quantitation by tandem mass spectrometry

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ABSTRACT

Dried blood spots (DBS) are a convenient collection and archiving method for blood specimens. The interest in screening certain analytes in neonatal DBS continues to increase for a variety of paediatric disorders. 25-Hydroxyvitamin D_3 ($25OHD_3$) is one such analyte. We investigated potential factors that may affect the analysis of $25OHD_3$ in prospective cohorts of DBS, such as blood spot volume, hole punch position, and paper type. All of these factors were shown to affect $25OHD_3$ levels measured. When blood volumes of <50 μL were spotted, $25OHD_3$ concentrations extracted were significantly lower ($P < 0.0001$). We also observed a chromatographic effect across the surface of blood spots, with $25OHD_3$ concentrations significantly higher in outer punched spots compared to those punched from the centre ($P < 0.0001$). This also correlates with a heavier net weight of blood from outer punched spots ($P < 0.0001$). This effect was reproducible on two types of paper cards (Whatman 903® and FTA®), and paper type was shown to be highly relevant. We also show that the distribution of $25OHD_3$ in whole blood is essentially extracellular, with over 98% of $25OHD_3$ residing in the serum component. This may potentially explain why the diffusion properties of blood and type of chromatographic paper may significantly influence the distribution of such analytes in DBS. These factors should be taken into consideration for the prospective collection of DBS and analysis of $25OHD_3$ in DBS.

1. Introduction

Dried blood spots (DBS) have been recognised as a convenient matrix for collecting blood samples in neonatal screening of in-born errors of metabolism [1,2], but also in clinical [3] and pharmacokinetic studies [4,5]. The DBS technique is minimally invasive and only requires a very small amount of blood which greatly facilitates handling, storage and transport [6]. This has resulted in DBS being evaluated as the method of choice for large clinical studies [7,8]. An increasing number of studies are using DBS to investigate neonatal risk factors and biomarkers for diseases such as type 1 diabetes [9,10], cerebral palsy [11], autism [12] and schizophrenia [13,14]. Owing to its high specificity and sensitivity, LC–MS/MS is becoming the new gold standard for the analysis of vitamin D metabolites such as 25-hydroxyvitamin D_3 ($25OHD_3$) in serum [15–17]. An increasing number of paediatric disorders are being linked with low vitamin D and a number of groups have produced assays for $25OHD_3$ using DBS [18–20]. Given that we are aware of a number of studies where prospective collection is underway using DBS to quantify $25OHD_3$, we thought it prudent to examine several issues regarding sample collection. For instance, as many such studies will be collected by a variety of health professionals, we considered whether the amount of blood spotted could affect $25OHD_3$ concentrations. We also examined the punch location in blood spots, as this has been shown to affect the reported concentrations of other analytes such as amino acids [21,22] or highly protein-bound species such as retinol [23]. DBS are being increasingly used for genomic studies in which specific pre-prepared substrates such as Whatman FTA® paper are being employed for the qualitative preservation of DNA [24]. Older studies have by and large employed the use of Whatman 903® for collecting blood, therefore paper type may vary depending on which study variable is most relevant. We currently have no knowledge regarding the effects of paper substrate on $25OHD_3$ concentration or distribution. Finally like retinol, $25OHD_3$ is highly protein-bound [25] and therefore likely to be excluded from erythrocytes. We therefore also explored certain features related to how $25OHD_3$ distributes within whole blood in order to see if this could contribute to sample variability when quantifying $25OHD_3$ in DBS.

Abbreviations: $25OHD_3$, 25-hydroxyvitamin D_3; APCI, atmospheric pressure chemical ionization; DBS, dried blood spots; LC–MS/MS, liquid chromatography tandem mass spectrometry.

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2. Experimental

2.1. Materials and reagents

Acetonitrile (>99.9%, LiChrosolv) was purchased from Merck (Darmstadt, Germany). Formic acid (98%, Cat. No. 56302) and 4-phenyl-1,2,4-triazioline-3,5-dione (PTAD, 97%, Cat. No. 280992) were from Sigma Aldrich (St. Louis, USA). ^{125}I-25OHD$_3$ (Cat. No. 68100E, specific activity 0.004 mCi (148 kBq) diluted in ethanol-phosphate buffer) was purchased from DiaSorin (Stillwater, USA). (6,19,19-*d*3)-25OHD$_3$ (>98%, 97 at.% D, Cat. No. 4163) used as an internal standard, and 25OHD$_3$ (Cat. No. 4163UNL) were supplied from Isosciences LLC. DBS were spotted on either grade 903® filter paper (Item No. 10535097, thickness 490 μm, weight 192 g/m^2), or FTA® MiniCard (Cat. No. WB120055, thickness 545 μm, weight 215 g/m^2) from Whatman (GE Healthcare, Sanford, USA).

2.2. Blood samples

Whole blood was collected by venipuncture from a healthy adult volunteer using a standard butterfly blood collection set (21 gauge × 3/4 in. needle) in VACUETTE® 9-mL tubes containing lithium heparin as an anticoagulant (ref. 455084, Greiner Bio-One GmbH), and kept at room temperature on a roller mixer for no longer than 60 min (no visible clot). Fresh blood was spotted perpendicular to the paper at a constant flow rate (ca. 360 μL/s) using an automatic pipette (Multipette® Stream, Eppendorf AG, Hamburg, Germany) with a 5 mL multi-dispensing tip (Eppendorf Combitip® plus, Cat. No. 0030 069.250). The pipette tip was not allowed to touch the DBS paper, and blood was not re-applied (spotted once only). DBS were allowed to dry at room temperature overnight (ca. 22 °C and 45% air humidity), then stored at −20 °C, in a resealable bag containing silica desiccant packs until they were punched and analysed (within 6 months). A subset of matching sera samples were prepared using an aliquot of the same blood used for DBS, and collected by centrifugation (10,000 × *g* for 5 min) in VACUETTE® 4-mL tubes (Z Serum Clot Activator, ref. 454092, Greiner Bio-One GmbH), and stored at −20 °C until analysed by the same method [18]. As 25OHD$_3$ levels are usually reported in sera and because 25OHD$_3$ is excluded from erythrocytes, data is reported as sera equivalents using a haematocrit value of 0.455, which was determined by centrifuging heparinised blood in a capillary tube at 10,000 × *g* for 5 min (average of 4 replicates). Hence, 25OHD$_3$ concentrations in DBS were corrected according to the following equation:

$$\frac{\text{DBS}\,[25\text{OHD}_3]\,(\text{nM})}{1-\text{Haematocrit fraction}} = \text{Serum}\,[25\text{OHD}_3]\,(\text{nM})$$

DBS calibration standards were prepared as described previously [18]. Standard 25OHD$_3$ stock solutions (ca. 2 mg/mL) were tested by UV (265 nm) using $\varepsilon = 18{,}200\ \text{M}^{-1}\,\text{cm}^{-1}$ [26], and dilutions in ethanol were made up to prepare 5, 12.5, 25, 50 and 125 nM spike solutions. These respective spike solutions were mixed with blood using an ethanol volume of 5 μL per 995 μL blood (0.5%, v/v).

For DBS analyses, 3.2-mm disks were punched out either from the centre or the periphery of the spots, and collected into a 96-well microtiter plate using a BSD700 semi-automated punching instrument from BSD Robotics (Brisbane, Australia). Outer punches are defined as those punched approximately 1 mm from the edge, but not overlapping with the centre of a 50 μL spot. DBS were photographed, weighed and spot area determined using Image-J, software version 1.45 (W. Rasband, National Institute of Health, Bethesda MD, USA).

In order to investigate whether concentrations of 25OHD$_3$ in DBS may be affected by the volume of blood applied onto paper, a control blood sample was individually spotted onto Whatman 903® cards with a volume of 10.0, 25.0, 50.0 or 100.0 μL. A single punch was taken from the centre of the DBS from 16 replicates at each spotted volume.

2.3. LC–MS/MS analysis of DBS

DBS were extracted and assayed as previously described [18]. Analysis of 25OHD$_3$ was performed on a 4000QTRAP tandem mass spectrometer with an APCI TurboIonSpray® source (AB Sciex, Concord, ON, Canada), connected to a Shimadzu Nexera UHPLC (Kyoto, Japan) equipped with a Kinetex column XB-C18, 50 × 2.1 mm, 1.5 μm (Phenomenex, CA, USA), and eluting in isocratic mode (72% acetonitrile/water containing 0.1% formic acid) and at a flow rate of 0.5 mL/min.

2.4. Radioactivity measurements of ^{125}I-25OHD$_3$

We determined the distribution of 25OHD$_3$ in whole blood using a radioactively labelled tracer (^{125}I-25OHD$_3$). An aliquot of blood (2 mL) was spiked with ^{125}I-25OHD$_3$ (50 μL, 2.5%, v/v), allowed to equilibrate on a roller mixer for 30 min, and separated by centrifugation (600 × *g* for 10 min) to collect sera. The erythrocyte fraction was then washed three times sequentially by addition of 1× PBS buffer (1 mL, pH 7.39), gentle mixing, and centrifugation (600 × *g* for 10 min) to remove the sera component. To collect the intracellular content of the erythrocyte, cells were lysed with 1× PBS/1% Triton X-100 (500 μL) and the mixture centrifuged (10,000 × *g* for 10 min). The supernatant and precipitate were counted to assess the intracellular ^{125}I-25OHD$_3$ content and that bound to the cell membrane pellet, respectively. The levels of radioactivity were measured using a 2470 WIZARD^2 automatic gamma counter (Perkin Elmer Inc., Waltham, MA, USA).

2.5. Statistical analysis

Group comparisons were made using one-way ANOVA with post hoc Dunnett or Bonferroni multiple comparisons test, where appropriate. Box plot graphs show values for the lower and upper quartiles and median, including the smallest and largest observations as whiskers. Pairwise comparisons were made using paired *t*-tests with two-tailed *P*-value (95% confidence intervals). A *P*-value of < 0.05 was considered significant.

3. Results

3.1. The effect of DBS volume on 25OHD$_3$ concentrations

25OHD$_3$ concentrations in the 100 μL spot were no different to the 50 μL spot (Fig. 1a). However, 25OHD$_3$ concentrations were significantly lower in both the 25 and 10 μL spots ($F = 17.4$, $P < 0.001$, $n = 16$). This experiment was repeated three times yielding the same result with mean 25OHD$_3$ concentrations of 49.3 ± 3.3 (100 μL), 49.2 ± 3.4 (50 μL), 43.8 ± 2.8 (25 μL), and 44.2 ± 2.0 nM (10 μL). Prior to assay, the weights of the punched disks were measured for the corresponding DBS. Again, there were no differences in the net weight of blood absorbed in punches from the 100 and 50 μL spots, but significantly less blood was absorbed on the 25 and 10 μL spots ($F = 34.4$, $P < 0.001$, $n = 16$) (Fig. 1b).

3.2. Effect of punch position on the determination of 25OHD$_3$

Given that spotted blood volume affects 25OHD$_3$ concentrations, we suspected that the distribution of 25OHD$_3$ across DBS may not be homogeneous. We confirmed this as when the 3.2 mm disks were punched from the periphery of the spots, the mean

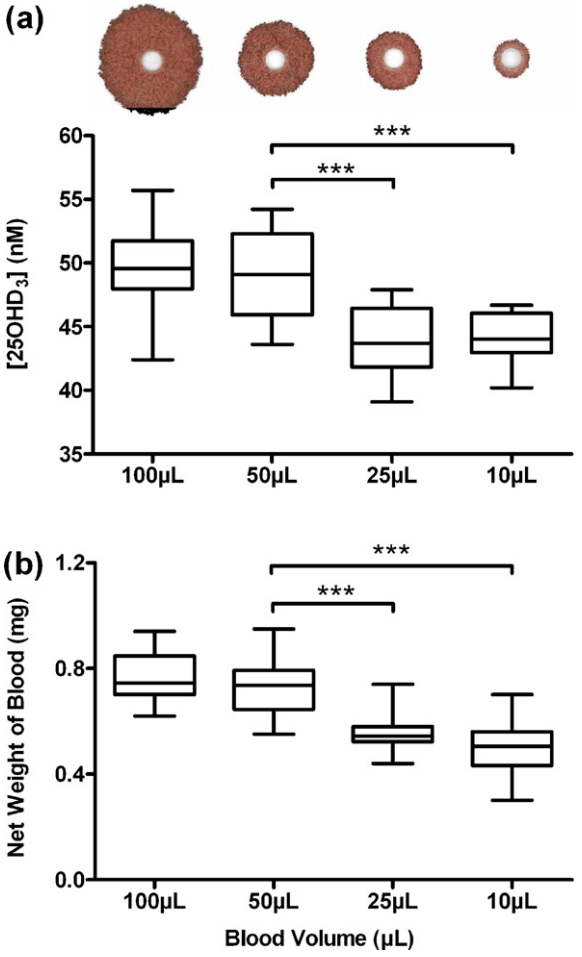

Fig. 1. Influence of total spot volume on (a) recovery of $25OHD_3$ from dried blood spots on Whatman 903®, $P < 0.0001$ vs 50 µL, $F(3, 60) = 17.37$, $n = 16$. (b) Relative weight of blood spot punches. $P < 0.0001$ vs 50 µL, $F(3, 60) = 24.66$, $n = 16$. One-way ANOVA followed by Dunnett's multiple comparisons test. Top row: images of punched DBS corresponding to applied blood volumes. Box and whisker plots show the median, lower and upper quartiles, and the minimum and maximum values.

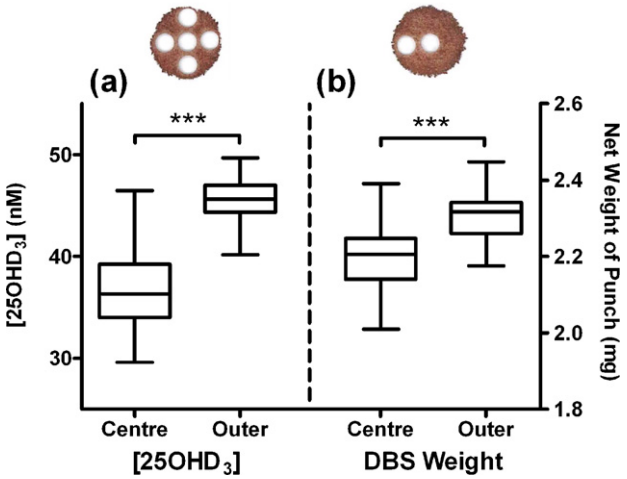

Fig. 2. (a) Effect of punch location on recovery of $25OHD_3$ from dried blood spots on Whatman 903® ($P < 0.0001$, $t(15) = 9.635$, paired t test, $n = 16$ centre vs average of 4 outer spots), and (b) comparison with the relative weights of centre and outer blood spots ($P < 0.0001$, $t(15) = 8.482$, paired t test, $n = 16$).

concentration of $25OHD_3$ in the four outer spots was consistently higher than at the centre. The mean $25OHD_3$ concentrations of DBS on Whatman 903® for centre and outer spots were 36.7 ± 4.4 nM, and 45.3 ± 2.8 nM, respectively ($P < 0.0001$, paired t-test, $t = 9.635$, df = 15, Fig. 2a). The outer spots were also significantly heavier ($P < 0.0001$, paired t-test, $t = 8.482$, df = 15, Fig. 2b).

3.3. Differences observed depending on paper type

We investigated whether the phenomenon observed between centre and outer spots on Whatman 903® was also prevalent in other paper types. The basis weight of FTA® paper (215 g/m^2) is heavier relative to that of 903® (192 g/m^2), and thicker (545 µm compared to 490 µm), so in principle, the former may be able to absorb more blood. Once again the disparity in the weights between centre and outer spots, previously observed on 903® (Fig. 2b) was clearly reproduced using FTA® ($P < 0.0001$, $n = 16$, Fig. 3a). We found no significant difference in the net weight of blood in punches from the centre of the spot from DBS on Whatman 903® or FTA®, with mean weight of absorbed blood of 0.71 ± 0.09 and 0.78 ± 0.07 mg, respectively. However the net weight of blood in punches from outer locations was again significantly heavier in FTA® compared to 903® ($P < 0.05$, $n = 16$, Fig. 3a). We also found the area of DBS on FTA® to be significantly smaller than those on 903® ($P < 0.01$, $n = 19$, Fig. 3b).

The apparent heavier net weight of blood in the periphery of DBS on FTA® also corresponded to a significantly higher concentration of $25OHD_3$ in outer punched spots compared to the centre punched spot ($P < 0.0001$, paired t-test, $n = 15$, Fig. 4a). However, the $25OHD_3$ concentrations on FTA® were significantly overestimated if the DBS calibration was based on standards created on 903® paper (compare values between Fig. 4(a) and (b) ($P < 0.0001$, $n = 15$, Fig. 4)). The use of an appropriate calibration on FTA® paper led to an excellent agreement between the mean $25OHD_3$ levels in reference serum (44.2 ± 1.6 nM) and centre spots (43.0 ± 2.4 nM). However significantly elevated values in outer spots remained (49.5 ± 2.8 nM) ($P < 0.0001$, $n = 15$, Fig. 4b).

3.4. Effect of exogenous ethanol spike on the determination of $25OHD_3$

The concentration of $25OHD_3$ measured in punches from the centre of DBS spotted on Whatman 903® paper was not significantly different to that in the reference serum (Fig. 5a). In contrast, outer spots showed a systematic bias (+23.3%) to higher values ($P < 0.0001$, $n = 16$, Fig. 5a). The variance in outer spots is also generally greater than in centre spots (Table 1).

Somewhat surprisingly, we found that when DBS were spiked with ethanol at levels used to generate standards (0.5%, v/v), the disparity in $25OHD_3$ concentrations between the centre and outer spots disappeared on both paper types (Fig. 5a and Table 1). The weight of absorbed blood was also normalised (Fig. 5b). This effect was replicated in all $25OHD_3$ standards that also contained 0.5% (v/v) ethanol.

3.5. Distribution of $25OHD_3$ in whole blood

We established that virtually all ^{125}I-$25OHD_3$ in whole blood is distributed in the serum compartment (>98%, Table 2), confirming that the metabolite is essentially completely protein-bound, and completely excluded from intra-cellular and membrane component of red blood cells.

4. Discussion

In addition to disorders of calcium and phosphate mobilisation, vitamin D deficiency continues to be linked to a variety of paediatric outcomes such as autism [27], asthma [28], juvenile diabetes [29],

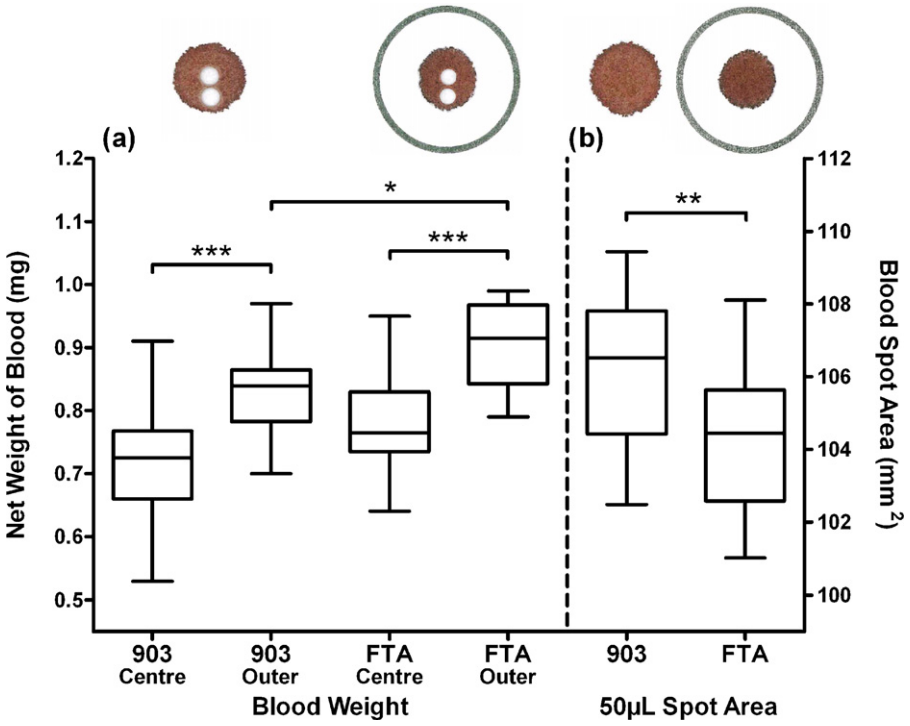

Fig. 3. (a) Weight of absorbed blood on 3.2 mm punches from Whatman 903® and FTA® paper. $P < 0.0001$, $F(3, 60) = 17.41$, $n = 16$, one-way ANOVA, Bonferroni's post hoc test. (b) Relative surface area of the complete dried blood spot (50 μL). $P < 0.01$, $t(37) = 2.867$, unpaired t test.

Table 1
Imprecision and inaccuracy in the relative concentration of $25OHD_3$ on Whatman 903® and FTA® papers depending on punch position in DBS spiked or unspiked with 0.5% (v/v) ethanol.

DBS Sample Type	Mean [$25OHD_3$] (nM)	Standard Deviation	Imprecision (%CV)	Inaccuracy (%d)
Whatman 903®				
Unspiked				
Centre	37.4	4.7	12.6	−15.4
Outer	54.5	9.2	16.9	+23.3
Spiked (0.5%, v/v)				
Centre	42.7	3.4	8.0	−3.4
Outer	44.7	7.9	17.7	+1.1
Whatman FTA®				
Unspiked				
Centre	43.0	2.4	5.5	−2.7
Outer	49.5	2.8	5.7	+12.0
Spiked (0.5%, v/v)				
Centre	41.5	6.1	14.7	−6.1
Outer	41.4	4.5	10.8	−6.3
Serum	44.2	1.6	3.6	(Reference)

autoimmune dysfunction [30]. Most importantly, it is increasingly being linked with non-bone related developmental conditions such as schizophrenia [31], multiple sclerosis [32], type 1 diabetes [33], as well as some cancers [34,35]. Therefore, a number of prospective studies have now been undertaken to more closely examine such associations. The purpose of this study was to ascertain what variables during sample collection could contribute to assay imprecision.

DBS are now also being collected from adults to explore the epidemiological links between vitamin D and adult disorders such as colorectal cancer [36]. Given that some studies will use a number of health professionals to obtain spots we first assessed whether spot volume was a significant variable. The application of 50 μL of whole blood is the recommended volume for blood spot collection [37,38]. Our data confirm that smaller spot volumes lead to spuriously lower results. We presume that blood volumes less than 50 μL

Table 2
Distribution of ^{125}I-25-OHD_3 in whole blood. Radioactivity measured in gamma counts per minute. This experiment was repeated twice on six replicates.

Fraction	[^{125}I-$25OHD_3$] (cps)		[^{125}I-$25OHD_3$] in whole blood (%)		Average Distribution of [^{125}I-$25OHD_3$] (%)
Serum	38,007	28,115	98.5	98.3	98.4
Red blood cells	464	248	1.2	0.9	1.1
Cell lysate	133	61	0.3	0.2	0.3

Background count (blank) = 5 cps.

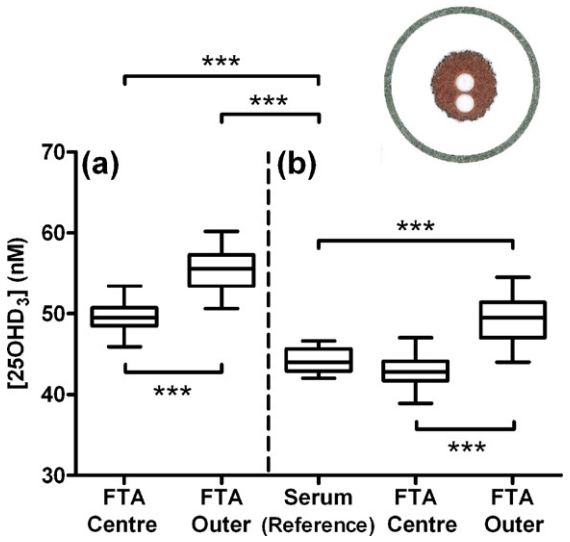

Fig. 4. Relative concentration of $25OHD_3$ in serum (reference) and in matched DBS spotted on Whatman FTA®, with respect to punch location (centre vs outer), derived using (a) standard calibration on 903®, or (b) using the FTA® calibration. $P < 0.0001$, $F(4, 62) = 58.84$, one-way ANOVA, Bonferroni's post hoc test.

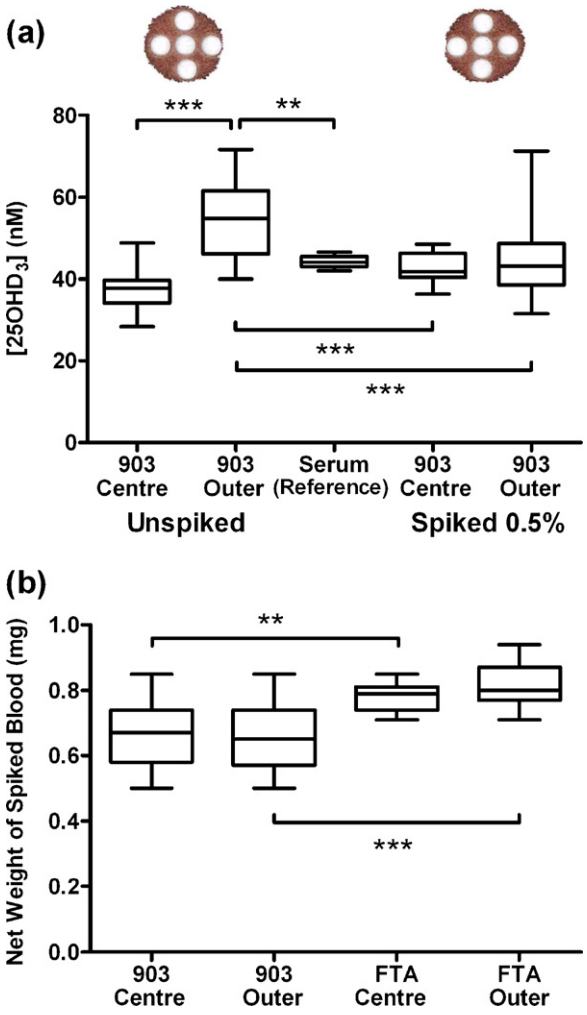

Fig. 5. (a) Relative concentration of $25OHD_3$ in serum (reference) and in matched DBS on Whatman 903® depending on punch location (centre vs outer), for unspiked blood, compared to blood spiked with ethanol (0.5%, v/v). $P < 0.0001$, $F(4, 137) = 21.85$, one-way ANOVA, Bonferroni's post hoc test. (b) Differences in the relative net weight of blood in centre and outer spots punched from Whatman 903® and FTA® cards, prepared from blood spiked with ethanol (0.5%, v/v). $P < 0.0001$, $F(3, 56) = 13.87$, one-way ANOVA, Bonferroni's post hoc test.

do not fully saturate the paper, or induce different flow rates across the paper. Our results show a remarkable agreement between the measured $25OHD_3$ concentrations with respect to spotted volume and the net weight of blood absorbed on Whatman 903® (Fig. 1). Therefore the most likely reason for the lower $25OHD_3$ measures is due to diminished blood absorption in spots that are less than 50 μL. Therefore, as recommended it is important to ensure that DBS samples are sourced from a minimum volume of 50 μL.

The next variable investigated was paper type. Whatman FTA® is commonly used as a blood resource for the preservation of DNA, and the concurrent analysis of $25OHD_3$ in those samples may be desirable. The weight of blank FTA® paper (215 g/m^2) is markedly greater than that for 903® paper (192 g/m^2) [39]. Therefore, we reasoned that the absorption of blood may also be greater in FTA® paper. This was examined and we show that at least in the outer spots that there was a greater amount of blood in FTA® paper compared with 903® (Fig. 3a). Additionally outer FTA® spots were heavier than inner spots implying increased blood deposition on the periphery. Further confirmation that greater blood was absorbed in FTA® compared with 903® paper was the reduction in spot area on FTA® paper for the application of the same volume of blood ($P < 0.0001$, Fig. 3b).

We next went on to confirm that the greater amount of blood absorbed into FTA® paper correlated with a greater $25OHD_3$ concentration. When we measured the $25OHD_3$ concentration in both central and peripheral FTA® spots, reported values were clearly much greater than the reference sera value (Fig. 4a). However when a standard curve was prepared from FTA® based standards, $25OHD_3$ concentrations were normalised such that again the value from the centrally located sample was not significantly different from the sera reference.

We also examined the effect of spot punch position. In both Whatman 903® and FTA® paper, we found a consistent increase in $25OHD_3$ concentrations when punches from the periphery were compared with central locations (Figs. 2a and 4b). This correlated with an increase in DBS punch weight in punches from the periphery compared with centrally located punches (Figs. 2b and 3a). We were next curious to examine how punch location site could affect assay imprecision and inaccuracy. We show that centrally located punched spots from both paper sources were not significantly different from the reference sera value. However, the $25OHD_3$ concentrations in outer punched spots were significantly greater than that of the reference sera value again regardless of paper type (Figs. 4 and 5, and Table 2). Coefficients of variation were also greater for outer punched spots compared to inner punches independent of paper type, indicating sampling from outer spots increased imprecision (Table 2).

There are two plausible explanations for the increased $25OHD_3$ concentrations seen in the periphery compared to the centre. The most obvious is an increased amount of blood, given the greater weight of blood in peripheral punches. Alternatively, chromatographic effects may also be at play. Ren et al. [40] studied the autoradiograms of several ^{14}C-labelled compounds spiked in DBS, and observed a variety of chromatographic effects depending on paper types. These chromatographic effects result from the inhomogeneous nature of blood, and the slower diffusion of red blood cells relative to the sera component. Such a mechanism would lead to increased sera at peripheral sites and more red blood cells at the centre of the spot. These results are consistent with those of Erhardt et al. [23] for the analysis of another highly protein-bound compound, retinol in DBS. This group used sodium concentrations to gauge relative sera content and observed higher levels of sodium, i.e. more sera at the periphery of DBS [23]. We have shown that

virtually all $25OHD_3$ is distributed in the sera component of whole blood, consistent with $25OHD_3$ known to be highly protein-bound [25]. Therefore, a chromatographic mechanism could be affecting the diffusion of blood/serum on paper, similarly to what was observed with retinol.

Finally, we examined the effects of adding ethanol (0.5%, v/v) to blood prior to spotting. This was done for both a practical and a theoretical reason. We made up all $25OHD_3$ DBS standards using $25OHD_3$ diluted in 0.5% (v/v) ethanol. We have confirmed that this percentage of ethanol does not produce any haemolysis. Therefore, we wanted to know whether the addition of this solvent could affect the apparent levels of $25OHD_3$ based on punch location. Surprisingly, it appears that this robust effect was completely abolished in the presence of the ethanol, which was also the case for punch weights. It is possible that this level of solvent altered the surface tension within blood sufficiently to change its flow properties. There are reports where chromatographic effects are not apparent for the analysis of a number of drugs in DBS, where a solvent such as ethanol or acetonitrile has been used to spike drugs in DBS [41,42]. We conclude that low amounts of organic solvents could be sufficient to alter the surface tension of the applied blood and therefore collapse the so-called chromatographic effect.

We have clearly shown that spotted blood volume, paper type and punch location are all confounding variables regarding DBS $25OHD_3$ levels. We therefore make a number of recommendations in order to minimise assay variance. Firstly, the blood volume applied should be 50 μL or greater. Secondly, within a study $25OHD_3$ DBS comparisons should only be made within the same paper source. If paper source is known to have been altered during sample collection, as we have already experienced from a large cohort collected over 15 years, then standards should be prepared from that same paper source. Thirdly, if possible, case/control type comparisons should be made from punches collected from the same position within the spot. This is obviously extremely difficult in archived samples where numerous punches are likely to have been made. Therefore, these findings are probably of greater relevance to prospective cohorts collected for the specific purpose of measuring analytes such as $25OHD_3$. Centrally based punches would appear to be both more accurate and precise. However, if replicates are to be analysed we would make the practical suggestion to assess peripheral spots in order to obtain such replicates.

Competing interests

The authors have declared no financial involvement or any conflicts of interest.

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