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Effect of blood volume on analytical bias in dried blood spots prepared for newborn screening external quality assurance.

Stuart J Moat[1&2]*, Catherine Dibden[3], Lesley Tetlow[4], Caroline Griffith[5], Jim Chilcott[6], Roanna George[1], Laura Hamilton[4], Teresa Hoi Yee Wu[7], Finlay MacKenzie[8] , S Kate Hall[9]

[1]Department of Medical Biochemistry, Immunology & Toxicology, [2]School of Medicine, Cardiff University, University Hospital Wales, Cardiff, CF14 4XW, UK.
[3]Department of Clinical Chemistry, Sheffield Children's (NHS) FT, Sheffield, UK.
[4]Department of Clinical Biochemistry, Manchester University NHS Foundation Trust, Manchester, UK.
[5]Specialist Laboratory Medicine, St James's University Hospital, Leeds, UK.
[6] School of Health and Related Research (ScHARR), University of Sheffield, Sheffield, UK.

[7]Willink Biochemical Genetics Laboratory, Manchester University NHS Foundation Trust, Manchester, UK.

[8]Birmingham Quality/UK NEQAS, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK.
[9]UK Newborn Screening Laboratories Network.

*Corresponding author: Prof Stuart J Moat, Department of Medical Biochemistry, Immunology & Toxicology, University Hospital Wales, Cardiff, CF14 4XW. Stuart.Moat@Wales.nhs.uk, Tel +44 29 20 743 562, Fax +44 29 20 744 065.

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Key Words
Dried blood spots, calibrators, quality control, external quality assurance, newborn screening, analytical bias, sub-punch location, drop volume.

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Background: Dried Bloodspots (DBS) are used for the analysis of >2000 biomarkers. We assessed a range of analyte concentrations and diameters of DBS created by the application of increasing volumes of whole blood prepared by the UK NEQAS Quality Assurance Laboratory. Samples were analysed in four separate laboratories. **Results:** Volumes <25µL (8mm) and >75µL (14mm) created unsatisfactory analytical biases. Results obtained from peripheral sub-punches tended to be higher than those from a central sub-punch. **Conclusion:** DBS diameters formed from non-volumetric application of blood to filter paper can be used to assess whether measurement bias will be within acceptable limits according to the analyte being quantified. DBS received for newborn screening in the UK with diameters <8mm and those >14mm should be rejected.

Introduction

Guthrie and Susi showed the applicability of dried blood spot (DBS) sampling to newborn screening (NBS) in 1963, Guthrie having developed a method for blood spot phenylalanine a few years earlier [1]. However, recent advances in analytical technologies have led to more than 2000 biomarkers being analysed in DBS samples [2]. DBS samples are an increasingly attractive sample type for use in healthcare surveillance due to their relative stability, small blood volumes required and ease of transport from remote sites for analysis. Analysis of DBS samples is employed routinely for therapeutic drug and treatment monitoring, toxicological analyses and therapy adjustment in patients with inherited disorders [2-5].

The process of DBS specimen collection typically involves the application of a non-volumetric amount of blood (single hanging drop of blood) from a heel or finger prick that disperses by both spreading radially across the filter paper, whilst penetrating the porous fibres to fully soak through the filter paper. Ideally, the distribution of analytes across the filter paper collection device should be constant. However, the plasma component of the blood applied occupies a greater fractional volume of the interior of the filter paper fibres than the erythrocytes and as a result the erythrocytes concentrate at the edge of the bloodspot (which is often visible). This loss of homogeneity across the DBS, results in increased concentrations of analytes associated with erythrocytes in the peripheral sub-punches relative to central sub-punches taken from the DBS [6, 7].

The perceived benefit of DBS sampling is the assumption that a sub-punch (small cylinder of a fixed diameter) of a defined volume can be obtained from a DBS formed from a non-volumetrically applied blood sample. However, the volume and haematocrit (Hct) of the whole blood sample applied to the filter paper collection device are both known to affect the diameter of DBS samples formed and therefore the concentration of the analytes within the DBS [8-12]. However, the effect of Hct cannot be controlled or corrected for as there is no direct method to detect Hct in DBS samples. Whilst non-destructive methods for Hct prediction and thus concentration correction have been developed, these have not yet been conveniently incorporated into available punching equipment [13]. The results obtained by analysis of DBS samples are therefore inherently less precise than those obtained using a

fixed volume of liquid whole blood. It is therefore important to understand and control where possible any pre-analytical variables that may affect the final test result.

Filter paper collection devices usually have printed broken line circles of a predefined diameter to serve as a guide for specimen collectors to obtain appropriate sized samples. The quality of DBS specimens received into the NBS laboratory are assessed subjectively by visual inspection; ensuring that the printed circle is suitably filled with blood, the blood is spread symmetrically and evenly with blood viewed from both sides of the filter paper. Repeat samples are requested on those samples deemed unsuitable for analysis. In the UK, the filter paper collection devices used for NBS have four printed guide circles with an inner diameter of 10mm, which when appropriately filled contain approximately 35-50µL of whole blood. Currently, in the UK a minimum spot diameter of 7mm is recommended for NBS, as this provides two 3.2mm sub-punches [14]. However, it should be highlighted that the diameter of the calibrator, quality control and external quality assessment (EQA) DBS samples used in different jurisdictions internationally vary significantly. Furthermore, the minimum spot size from patients accepted by testing laboratories may also vary and be significantly different to the diameter of the calibrator sample, quality control (QC) sample and EQA sample materials used in the assay. Previous studies assessing the impact of DBS size on analyte concentrations have all used heparinised blood from healthy volunteers and analysis performed in one centre.

The aim of this study was to assess the impact of DBS diameter and sub-punch location on a range of analyte concentrations in external quality assurance (EQA) specimens measured in four separate NBS laboratories and to assess the utility of using the diameter of the DBS sample received by the testing laboratory as a guide for the rejection of inappropriately sized DBS samples. The blood absorption matrix utilised, PerkinElmer 226, is used for newborn screening in all four UK countries and many other jurisdictions globally. Due to minimal funding examination of other substrates such as Whatman 903 was not feasible. Both Perkin Elmer 226 and Whatman 903 comply with CLSI NBS01-A6 specifications [15].

Experimental

Investigation of varying blood volumes on blood spot diameters

DBS samples were prepared as follows; a fresh donor unit of packed red cells, in citrate phosphate dextrose (CPD) anticoagulant (containing anhydrous glucose 129.0 mmol/L; sodium citrate 89.4 mmol/L; citric acid monohydrate 15.6 mmol/L; sodium dihydrogen phosphate dihydrate 16.1 mmol/L) was obtained from the National Health Service Blood and Transplant Service (NHSBT, Colindale, UK). The collection and storage of packed red cells and fresh frozen plasma is a standardised procedure [16]. The packed cells were washed with a solution of saline, adenine, glucose, and mannitol. Additions were made into fresh frozen plasma (obtained from NHSBT) [16] and mixed with the washed red blood cells to achieve a notional 55% haematocrit, typical of a baby in the first few days of life [17]. The haematocrit (Packed Cell Volume) was calculated following centrifugation of the sample by measuring the plasma and erythrocyte lengths in the tube using a ruler. The UK NEQAS Quality Laboratory (Birmingham, UK) routinely use blood products sourced from NHSBT to prepare DBS EQA material that is supplied to all UK newborn screening laboratories to assess performance.

The whole blood was enriched with thyroid stimulating hormone [TSH, 10 mU/L], immunoreactive trypsin [IRT, 20µg/L], phenylalanine [Phe 240µmol/L], tyrosine [Tyr 240µmol/L], octanoyl carnitine [C8, 0.5µmol/L], decanoyl carnitine [C10, 0.5µmol/L], Isovalerylcarnitine [C5, 1.1µmol/L], glutarylcarnitine [C5DC, 0.7µmol/L], leucine [Leu, 600µmol/L], and methionine [Met, 50 µmol/L]. Phenylalanine, Tyrosine, Leucine and Methionine were obtained from Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK. C5, C5DC, C8 and C10 were obtained from Cambridge Isotope Laboratories, Andover, MA 01810, USA. TSH was obtained from SciPak, Sittingbourne, UK and trypsin was obtained from Athens Research & Technology, Athens, GA, USA. Analytes were dissolved in 0.9% saline, with acidification where necessary. The volume of the non matrix material used to enrich the sample was <1% of the total blood volume. Following enrichment, the blood sample was gently roller-mixed for 30 minutes before sample application onto the filter paper. The blood sample was continually mixed throughout the blood application process.

This process was performed by the UK NEQAS Quality Laboratory, which provides DBS EQA samples to all UK newborn screening laboratories.

Thirty replicates per sample volume, per punch location (central and peripheral) per analyte group (MS/MS, IRT, and TSH) were created by applying the whole blood sample onto PerkinElmer Grade 226 filter paper using a pipette (Fisherbrand Finnpipette II, adjustable 10 to 100µL from Fisher Scientific, Loughborough, UK) at the following volumes: 10, 25, 35, 50, 75 and 100µL (Figure 1). Three hundred and sixty bloodspots were created for each analyte group. Therefore a total of 1080 bloodspots were prepared for each laboratory. The samples were allowed to air dry at ambient temperature before shipment at ambient temperature via a courier service to the four newborn screening laboratories for analysis. Samples were stored at ambient temperature until analysis. Samples were analysed within 14 days of receipt into the laboratories. It is known that metabolites can degrade over time. For this reason we analysed / expressed the data relative to the 50µL central sample for each laboratory as this will take into consideration any degradation and bias issues between laboratories.

Blood spot analysis

Thirty replicates each of central (C) and peripheral (P) punches (3.2mm) (Figure 1) were analysed for every bloodspot volume for TSH, IRT, amino acids and the acylcarnitines. Samples were analysed in four UK NBS Laboratories (Cardiff, Leeds, Manchester and Sheffield) to assess consistency of analysis. For the analysis of TSH and IRT commercially available (PerkinElmer, Finland) dissociation enhanced fluorimetric immunoassays were used (these are routinely used in all the UK newborn screening laboratories). Samples for TSH and IRT analysis were prepared for analysis as per the manufacturer's instructions. For the analysis of the amino acids and acylcarnitine species, samples were punched (3.2mm) into 96 well plates and methanol containing the internal standards (Cambridge Isotope Laboratories) was added to each well as the extraction solvent. The plates were covered and shaken for 20 minutes on a plate shaker, the eluent was then analysed using tandem mass spectrometry (MS/MS) using a mobile phase containing acetonitrile, deionised water and

formic acid. This method of analysis is routinely employed in newborn screening laboratories [11,12].

Bloodspot diameters measurements

The diameters of the bloodspot samples from two distributions were measured independently by SKH and LH, using engineering grade callipers. Each scientist made thirty duplicate measurements at each DBS volume either at right angles or along the longest and shortest axes. The two diameter measurements were averaged and then the mean of the 30 averages calculated for each volume. Finally the measurements from each observer were averaged at each volume.

The relationship between the DBS diameters and blood drop volume applied to the filter paper collection devices was examined in three ways:

1. We investigated the hypothesis that the DBS may be considered to be a thin cylinder composed of blood and paper calculated from the area of the bloodspot and thickness of the paper: therefore the drop volume = t x πr^2, where t is the thickness and r is the radius of the spot or diameter/2. Hence diameter = 2 x $\sqrt{A}$, where A = volume / t x π [18].

PerkinElmer 226 collection paper has a nominal thickness of 0.52 mm [19]. This parameter is not required to be formally assessed according to CLSI NBS01-A6 after the manufacture of the filter paper collection device [20]. No attempt was made in this study to measure the thickness across each dried spot or the thickness of cards before the application of blood as such technology is not available in the UK Newborn Screening Laboratories. Using the cylinder model the thickness was back calculated for each drop volume using Python™ software. Additionally the least squares method was used in Python for 50 possible thicknesses from 0.40 to 0.60 mm in 0.004 increments in order to select the best fit theoretical thickness for this lot number of collection devices.

2. The relationship between blood volume applied and diameter of the DBS were examined with best fit calculations in Excel™ using data from this study and data from a

previously published study [12]. In addition, we also plotted the calculated results based upon the cylinder model as described above.

3. A broken stick model (segmented linear regression) comparing the volume of blood applied versus theoretical volume created assuming a paper thickness of 0.52 mm i.e. assessment of blood dispersion was undertaken. The assumption is made that the blood drop disperses uniformly onto the filter paper collection device to form the DBS which comprises a cylinder of blood impregnated filter paper. The bloodspot volume (cylinder volume) was calculated using the following equation $Vs = 2\pi t.d_1d_2/4$, where t = assumed thickness of filter paper (0.52mm), d_1d_2 = observed diameter (minimum and maximum measurements (mm)). The observed dispersion is calculated by dividing the Vs by the volume applied to the filter paper (V) in µL.

Statistical Analysis

Statistical analysis was carried out using one-way ANOVA with post-hoc Tukey test, calculated using SPSS (v16). All statistical analysis was carried out on the analyte concentrations and not on percentage differences. Bloodspot diameter/dispersion statistical analysis utilised a non-parametric Kruskal-Wallis rank sum test with post hoc analysis. P values <0.05 were considered as indicating statistical significance. To overcome any inter-laboratory biases and any potential sample degradation effects as a result of the differences between the timing of sample receipt and analysis, we calculated and analysed the percentage bias differences for each of the volumes (central and peripheral sub-punches) compared to the concentration in a 50µL central spot analysed in each of the laboratories. The results from a central punch from a 50µL bloodspot was used as the standard control value for comparison as this volume fills the 10 mm printed circle on the UK NBS card. A total of 30 replicates were analysed for each analyte for both central and peripheral punches for each volume of blood.

Results and Discussion

Effect of applied blood volume and punch location on measured analytes

With the exception of IRT the smaller bloodspot volume of 10µL produced significantly lower results for all analytes, when compared to bloodspots with volumes ≥50 µL ($P<0.05$) (Figure 2 and Table 1). The mean negative biases observed for the analytes (excluding IRT) in the 10µL central and 10µL peripheral punches vs the 50µL central punch were -11.3% and -7.4% respectively. DBS samples with volumes ≥75µL produced significantly higher results than the 50µL central sub-punch results for most analytes. The mean positive biases observed for the analytes (excluding IRT) in the 50µL peripheral, 75µL central, 75µL peripheral, 100µL central and 100µL peripheral punches vs the 50µL central punch were 7.4%, 6.0%, 12.0%, 11.6% and 19% respectively. These findings confirm previous studies using heparinised volunteer blood [10-12] and are consistent with the phenomenon that the smaller the blood volume applied to the filter paper, the further the blood spreads relative to a sample of higher volume. Therefore, the higher the volume applied to the filter paper the more the concentrated the blood will be in the sub-punch.

The analyte concentrations obtained using peripheral punches were generally higher than those obtained from a central punch (Table 1). This study is consistent with previously published studies which have also shown that results obtained using peripheral punch locations are often higher than those from a central punch location [10-12]. This was statistically significant at all volumes for TSH, for all except the 10µL volume for C8 and C10, for four of the six volumes for IRT and C5 and three of the six volumes for leucine and phenylalanine. Only for tyrosine, methionine and C5DC did the difference not reach statistical significance. Interestingly, other papers that were investigating other factors influencing variability in dried blood spot samples did not investigate sub-punch location in their studies [8, 21].

Effect of bloodspot volume applied to filter paper on DBS diameters

The mean (SD) diameter of the 10µl, 25µL, 35µL, 50µL, 75µL and 100µL volume bloodspots were; 6 (0.5) mm, 8.3 (0.3) mm, 9.7 (0.4) 12.0 (0.3) mm, 13.9 (0.4) mm and 16.6 (0.6) mm respectively (Figure 1). Bloodspot diameters increased non-linearly with increasing volume (Figure 3). The simple diameter equation in Excel permitted a reasonable curve fit to the diameter versus volume to all blood volume diameters except when the data from the 10µL DBS sample was included. Data from this study yielded a curve fit that was comparable to data from the study of George and Moat [12]. Both of these were also comparable to the curve fit reported by Hall and colleagues [8].

The thin cylinder hypothesis permitted the formulation of curves which varied slightly in best curve fit according to the apparent thickness of the filter paper. The calculated thickness of the filter paper at the volumes of 10µL, 25µL, 35µL, 50µL, 75µL and 100µL volume bloodspots were; 0.354, 0.451, 0.474, 0.450, 0.501 and 0.468 mm respectively. The least best fit was for the 10µL sample, with a 0.354 mm calculated thickness. The filter paper thickness with the best overall fit to the measured diameter versus volume from the mathematical testing of 50 different filter paper thicknesses was 0.465 mm. The mean thickness excluding the 10µl sample was 0.469mm.

The thin cylinder equation describing the relationship between DBS diameter and blood volume applied derived from first principles here differs from that of Hall and colleagues [8], as their equation does not take into consideration the thickness of the filter paper. This equation will fail at a volume below 10µL as a drop volume of zero will yield a diameter of greater than zero i.e. 1.69 mm. This is in contrast to the thin cylinder model described here where a drop volume of zero will yield a diameter of zero as anticipated. The best fit curves in this study were similar to that of Hall and colleagues [8]. However, the drying and differences in Hct of the blood into the paper after its spreading is complete may affect the apparent thickness and its deviation from 0.52mm. The diameters of DBS samples appear to be predictable using a cylinder model assuming a free flow of blood through the paper. A calculated paper thickness of 0.465mm gave the best curve fit for volume versus diameter. However, this calculated thickness is 11% lower than the nominal paper thickness of

0.52mm for the PerkinElmer 226 filter paper specification. The drying and clotting of blood could potentially influence this.

The broken stick model demonstrated greatest variation in dispersion at a blood volume of 10µL (Figure 4). The variability of dispersion of blood onto the filter paper at 10µL was significantly greater than the dispersion at all other volumes ($P<0.001$ for 25, 35, 50, 75 and 100µL; $P<0.01$ for 50 µL). Dispersion at volumes ≥25 µL appears to be relatively constant (Figure 4). Although it has been shown that the diameter of a DBS sample is related to the total volume and Hct of blood applied [8], the Hct was kept constant during this study. However, the speed of sample dispensing has been shown to affect DBS diameters [22], and this may explain some of the variability observed in the dispersion of the blood when applied to the filter paper as the samples from this study were pipetted manually onto filter paper.

Limitations of study

The DBS samples produced for this study were prepared by UK NEQAS from washed, anticoagulated, donor red blood cells and fresh frozen plasma, applied manually to filter paper using pipettes. This is significantly different to the processes used to collect newborn heel prick samples and to collect samples for other healthcare and surveillance purposes. However, some of the findings in this study compare to routine NBS test results e.g. higher results observed in peripheral vs central punches. There may have been variation in peripheral punch location between laboratories i.e. how far from the edge was the sub-punch taken as this may have affected the differences in analyte concentrations between the sub-punches. Imaging software may have provided more accurate mean diameters than using callipers. The thickness of the filter paper was not measured before or after the application of blood and this may account for the difference in the calculated thickness using the thin cylinder model. In addition the effect of varying Hct was not examined in this multicentre study.

Conclusion

Our results confirm previous findings that compared to a 50µL sample volume smaller volume DBS samples produce lower analyte concentrations and that results obtained from peripheral punch locations are higher than those from a central punch location. In addition, those samples with larger volumes applied to filter paper produce significantly higher results. A similar type of work has been carried out previously, using the range of analytes that are included in the UK NBS Programme [11, 12]. However, no study to date has assessed these various factors utilising EQA material and analysed in multiple testing laboratories. The results from this study validate previous findings and provide reassurance that blood used in DBS EQA material for NBS behaves in a manner that is analogous to heparinised volunteer blood. Furthermore, we demonstrate that the diameters of DBS samples appear to be predictable using a thin cylinder model assuming a free flow of blood through the filter paper.

Filter paper collection devices for capillary blood collection from heel or finger pricks are Class II Medical Devices and should ideally meet agreed international criteria for performance such as those by the Clinical & Laboratory Standards Institute. Filter paper is produced from cotton linters, and defines how the matrix influences blood collection and therefore affects the precision and reproducibility from lot-to-lot. The NBS Quality Assurance Program at the Centers for Disease Control (CDC), USA, monitors the consistency of the filter paper to ensure uniformity of specimen collection, calibrators, QC and reference materials for NBS assays [15, 21]. Using DBS specimens for disease diagnosis and treatment monitoring adds additional requirements for the precision and accuracy of analyte recovery. The type of matrix used for calibrators and QC materials will influence the analyte recovery. Therefore, any methods testing patient DBS specimens should also use DBS calibration and QC to correct for the filter paper matrix rather than using liquid calibrators and applying a factor to DBS results.

The analysis of DBS whose volume of applied blood differs to that of calibrators may introduce significant biases that may affect the interpretation of the results. It is also likely that analyte concentrations vary across the diameter of any calibrator or IQC DBS material.

To maximise the accuracy of the results, DBS samples should be the same diameter and punched in the same location as the calibrator material / EQA material. UK NEQAS produce DBS samples by the application of 35µL volumes of whole blood at an Hct of 55%. This volume almost fills the 10 mm printed circle used in the UK. UK NEQAS recommends that sub-punches are taken from the centre of DBS EQA material. Conversely, DBS diameters of calibration materials provided by the CDC are much larger (15 - 18mm) derived from the application of sample volumes ≥75µL of enriched blood (now at a 50% Hct, formerly 55%). DBS material provided by PerkinElmer for the calibration of the TSH and IRT assays are usually larger than 10 mm (12mm for IRT and 15mm for TSH) in diameter, which could affect the accuracy of results in newborn DBS samples that differ significantly in volume of blood applied compared to the calibration DBS samples. Furthermore, assay performance on a patient's results cannot be assessed objectively if the patient's bloodspots differ in size and Hct from those in EQA specimens. In addition, some of the materials are prepared by using lysed blood and not whole blood which may have different effects in terms of sample homogeneity and diameter.

Smaller blood volumes applied to filter paper produce analytical results with significant negative biases for many analytes especially at volumes <25µL, which corresponds to a diameter <8mm [11, 12]. A negative bias due to the use of small samples for analysis may risk failing to detect a disorder from the current panel of disorders, which infants are currently screened for in the UK. This is especially so for those disorders such as Medium Chain Acyl Co-A Dehydrogenase Deficiency (MCADD) and Maple Syrup urine Disease (MSUD) where the results in affected infants may be near to the analytical or action screening cut-offs. The cylinder volume approach outlined in this study, demonstrates that the dispersion of blood is less consistent with sample volumes <25µL. In practical terms this means that a DBS sample with a diameter ≥8mm will therefore give more reliable analytical test results. Our results strengthen the recommendation that samples of <8mm diameter are rejected, secondary to the observed negative bias [12]. Current UK guidelines recommend acceptance of all samples >7mm diameter [14]. Our study indicates that consideration should also be given to rejecting those samples >14mm in diameter (i.e. >75µL volume) due to the observed significant positive biases for analytes which can lead to an increased number of false positives cases, especially where absolute values of analytes are used in algorithms and

where the results in affected infants may be near to the screening cut-offs (e.g. TSH for congenital hypothyroidism, leucine for MSUD and C5DC for glutaric aciduria in the UK screening protocols). It should be noted that false positive NBS results are associated with increased parental anxiety and stress, with increased hospital visits for the infant even after follow-up diagnostic tests have excluded a disorder [23, 24]. Larger DBS samples could also pose issues in the future if the aim is to identify analyte results below an action value instead, for example, low T-receptor excision circles (TRECs) for severe combined immunodeficiency (SCID) screening and enzyme activities for the screening of galactosaemia, biotinidase deficiency and lysosomal storage disorders. Improvement in DBS specimen size and quality could be potentially achieved by the use of blood collection devices that collect defined volumes of liquid blood for sampling or to use approaches to estimate the volume or Hct of a DBS [25-28]. However, the cost of such strategies to correct for sample volume is at present prohibitive to newborn screening programmes.

Laboratories need to be aware of the fact that DBS samples of various sizes, and that sub-punches taken from different locations within the DBS produce significantly different analytical results. These differences are not just theoretical but can impact significantly on patient pathways when absolute analyte values are used in screening or diagnostic testing algorithms. Furthermore, understanding and controlling for the pre-analytical factors that affect the final test results can help progress the expansion of DBS samples for use in the analysis of numerous biomarkers.

The results from our study demonstrate that EQA DBS samples behave in a similar manner to patient DBS samples, despite the fact that the red cells are washed and that the samples are enriched with the various analytes. Previous studies have never been undertaken in several laboratories and the results correlated with each other. In addition, this study highlights that the diameter of the DBS sample can be used to assess whether measurement bias will be within acceptable limits according to the analyte being quantified. Furthermore, this study re-enforces the requirement to standardise the blood volumes used to create DBS calibrator and quality control materials.

Future perspective

DBS specimen size and quality can significantly affect an analytical result, which has implications for the use of DBS not only for population screening, but also diagnosis and monitoring. Findings from our study can be utilised to ensure that the appropriate size of samples are accepted for DBS assays, thereby ensuring the correct outcome for the baby being screened and to standardise practice by ensuring that all laboratories are accepting and rejecting samples of the same size and quality. Future work is required to assess the accuracy and homogeneity of the use of calibrator and QC materials prepared using lysates. The development of high throughput scanning technology to routinely assess DBS sample diameter and geometry plus haematocrit correction facilities are required to standardise the rejection of newborn DBS specimens for analysis to minimise inaccurate results.

Executive summary

Background

- To investigate the impact of DBS diameter and sub-punch location on a range of analyte concentrations in external quality assurance (EQA) specimens.

Experimental

- PerkinElmer 226 filter paper collection devices were used for sample collection.
- Whole blood samples were prepared by the UK NEQAS Quality Assurance Laboratory.
- Samples were analysed in four separate laboratories.

Results and Discussion

- Results from this study using DBS EQA material validate previous studies using heparinised volunteer blood.
- Smaller bloodspots (<8mm) produce significantly lower results for most analytes and larger bloodspots (>14mm) produce significantly higher analyte results using the EQA DBS material employed in this study.
- Analyte results obtained from peripheral sub-punches tended to be higher than those from a central sub-punch using EQA DBS material.
- Dispersion of blood on filter paper is less variable at sample volumes ≥25µL.

Conclusion

- DBS diameters may be used as a tool to identify samples that will give negative and positive biases for analyte concentrations.
- DBS samples received for NBS analysis in the UK with diameters <8mm and those >14mm should be rejected as they produce unsatisfactory biases.
- For greatest accuracy DBS samples should be the same diameter and sub-punches should be taken in the same location as the calibrator samples.
- Standardisation of blood volumes to create DBS calibrators and quality control materials is recommended.

Table 1 – Effect of blood volume and punch location on analyte concentrations. Results are shown as mean (SD), n=30 replicates. Amino acids and acylcarnitines (µmol/L), TSH (mU/L), IRT (ng/mL), C = central punch, P = peripheral punch. ‡P<0.05, *P<0.001 (Central vs peripheral).

Analyte		10µL	25µL	35µL	50µL	75µL	100µL
Phenylalanine	**C**	259 (30)	287 (34)	284 (31)	294 (32)	313 (38)	326 (37)
	P	267 (32)	300 (39)	299 (40)	311 (45)‡	323 (48)‡	348 (56)*
Tyrosine	**C**	196 (28)	230 (33)	220 (31)	226 (35)	236 (34)	254 (33)
	P	209 (27)	239 (35)	228 (35)	238 (38)	248 (41)	261 (47)
Methionine	**C**	30.0 (2.3)	33.0 (2.6)	32.3 (2.7)	34.4 (2.8)	34.5 (2.9)	35.9 (3.7)
	P	30.2 (2.7)	33.6 (2.9)	32.6 (2.9)	35.1 (2.8)	34.9 (3.0)	36.4 (3.6)
Leucine	**C**	575 (62)	652 (72)	634 (59)	652 (64)	693 (78)	731 (74)
	P	595 (71)	677 (80)	662 (81)	692 (97)*	721 (104)‡	771 (123)‡
C5	**C**	0.80 (0.11)	0.93 (0.10)	0.90 (0.11)	0.92 (0.12)	1.01 (0.11)	1.06 (0.11)
	P	0.84 (0.10)	0.97 (0.10)	0.95 (0.11)‡	0.98 (0.11)*	1.06 (0.11)‡	1.13 (0.12)*
C5DC	**C**	0.51 (0.15)	0.60 (0.16)	0.60 (0.15)	0.62 (0.16)	0.68 (0.16)	0.72 (0.17)
	P	0.56 (0.13)	0.64 (0.17)	0.64 (0.14)	0.66 (0.16)	0.69 (0.17)	0.74 (0.19)
C8	**C**	0.50 (0.04)	0.56 (0.04)	0.55 (0.04)	0.57 (0.05)	0.60 (0.04)	0.64 (0.05)
	P	0.52 (0.05)	0.59 (0.05)*	0.59 (0.05)*	0.61 (0.05)*	0.65 (0.05)*	0.69 (0.06)*
C10	**C**	0.61 (0.06)	0.68 (0.06)	0.65 (0.05)	0.65 (0.05)	0.68 (0.05)	0.71 (0.05)
	P	0.63 (0.04)	0.70 (0.04)‡	0.69 (0.06)*	0.71 (0.07)*	0.75 (0.07)*	0.79 (0.09)*
TSH	**C**	8.2 (0.51)	9.3 (0.62)	9.03 (0.68)	8.7 (0.73)	9.2 (0.80)	9.5 (0.62)
	P	8.9 (0.64)*	10.1(0.85)*	10.1 (0.89)*	9.9 (1.07)*	10.3 (0.86)*	10.9 (1.4)*
IRT	**C**	21.7 (2.3)	24.3 (2.9)	22.9 (2.5)	20.8 (2.6)	21.9 (2.8)	23.3 (2.8)
	P	22.0 (2.5)	24.9 (2.7)	24.0 (2.7)‡	23.9 (2.6)*	24.6(2.8)*	25.6 (2.7)*

Figure Legend

Figure 1

Effect of sample volume on DBS diameter. The printed circle (dashed line) diameter is approximately 10mm. Sub-punches (3.2mm) were taken from central (second row down) and peripheral locations (third row down).

Figure 2

Effect of sample volume and punch location on analyte concentrations

Figure 3

Relationship between volumes of blood applied to the filter paper collection devices and the measured DBS diameter. Results shown are the mean of the diameter measurements.

Figure 4

Effect of blood volume applied to filter paper on dispersion of blood across the filter paper (n=60 samples per volume). $Vs = 2\prod t.d_1d_2/4$, where t = assumed thickness of filter paper (0.52mm), d_1d_2 = observed diameter (minimum and maximum measurements (mm)). The observed dispersion is calculated by dividing the Vs by the volume applied to the filter paper (V) in µL. * P<0.001 vs other volumes.

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