ASSESSMENT OF FACTORS INFLUENCING METHOD PRECISION IN A HUMAN DRIED BLOOD SPOT ASSAY FOR THE DETERMINATION OF A NOVEL ANALYTE

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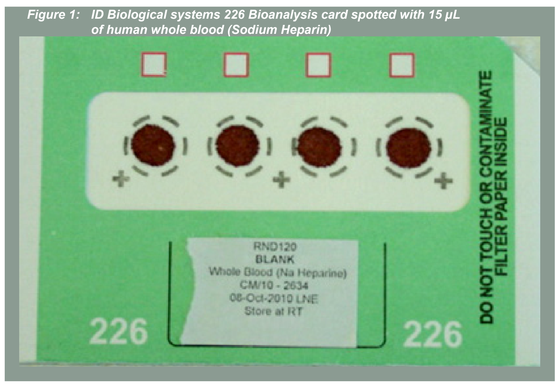
INTRODUCTION

A robust bioanalytical LC-MS/MS method for the determination of a novel analyte in human dried blood spots (DBS) was established in our laboratory. Quantitation used a d6-labeled internal standard targeting an LLOQ of 0.100 ng/mL. In order to fully evaluate the variables for the assay and further optimiseour processes for dried blood spot analysis, this assay was used as a model for testing the factors that may affect assay precision. Assay precision particularly at challenging LLOQs can have a significant impact on data quality for bioanalytical assays. This also has implications for the quality of repeat analysis and incurred sample reproducibility. Several factors are unique to DBS bioanalysis in influencing this precision and are focused around on card sample homogeneity and blood spot thickness. Spotting technique, spotting volume, punching size, punching method, card type, anticoagulant choice and internal standard addition may all contribute to the reproducibility of the assay. This presentation explores which factors may influence the precision and outlines the potential methodology optimisations for overall method reproducibility.

Figure 1: ID Biological systems 226 Bioanalysis card spotted with 15 µL of human whole blood (Sodium Heparin)



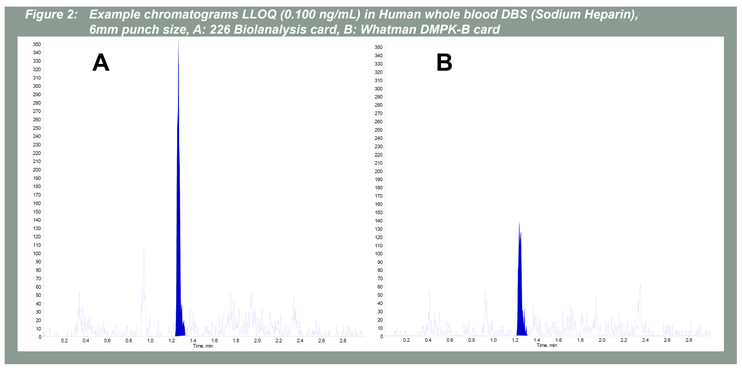
METHOD OVERVIEW

Fresh human whole blood (stored at 5°C for a maximum of two weeks after bleed date) was fortified with analyte (2% by volume) and immediately spotted onto appropriate DBS cards (226 Bioanalysis card, ID Biological Systems and Whatman DMPK-B, GE Healthcare). Cards were dried at ambient temperature for a minimum of two hours and spots of defined size were punched into a 96-well plate using automated or manual punching. Spots were then extracted by the addition of 300 µL of Methanol containing d6-labeled internal standard at appropriate concentration. The plate was sealed and mixed on a horizontal shaker for 10 min at ambient temperature. A 200 µL subaliquot of supernatant was evaporated under nitrogen (40°C, approx. 20 min) prior to reconstitution in 0.1% Formic acid (aq.) and submission for LC-MS/MS analysis using conditions as noted in table 1. Example chromatograms are presented in figure 2.

Table 1: LC-MS/MS conditions

Analytical column	Ascentis Express C18, 50 x 2.1 mm, 2.7 µm (Supelco)
Column temperature	40 °C
Injection volume	20 µL
Mobile Phase A	Water / Formic acid, 100:0.1, v/v
Mobile Phase B	Methanol / Formic acid, 100:0.1, v/v
	Gradient elution at 0.5 mL/min
Instrument	AB Sciex API 4000
Source	ESI, positive
Scan mode	MRM for analyte ant internal standard
Resolution (Q1 / Q3)	unit / unit
Dwell time	75 msec

Figure 2: Example chromatograms LLOQ (0.100 ng/mL) in Human whole blood DBS (Sodium Heparin), 6mm punch size, A: 226 Biolanalysis card, B: Whatman DMPK-B card



RESULTS AND DISCUSSION

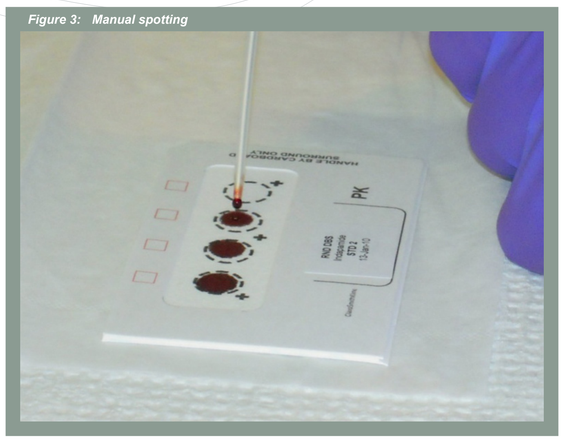
Factors potentially affecting the precision of DBS assays were systematically evaluated. Defined sets of samples were prepared according to the standardized procedure as described in the method overview section. The standard procedure was only modified at one variable per sample set (as described in the corresponding sections) allowing appropriate impact evaluation of the particular factor tested. Sample sets consisted of Human whole blood (Sodium Heparin) fortified with analyte at three concentration levels (eight replicates assessed per concentration level). A sample volume of 20 µL per spot was applied to the DBS card by Multipipette using a 1-mL tip allowing the automated punching of one 6mm punch per spot. Samples from each assessment were then extracted and analysed in the same analytical batch.

Results are presented as analyte/internal standard peak area ratio reflecting the response algorithm as used for data regression.

IMPACT OF SPOTTING TECHNIQUE AND SPOTTING VOLUME

Spotting technique and spotting volume were assessed as these may have crucial impact on the blood distribution and spot thickness across the DBS card material. Since the technique of application of whole blood to the card may slightly differ between the clinical site and the bioanalytical laboratory this impact needs to be carefully considered. This applies not only to precision but also to accuracy as clinical samples will be analyzed against standards and quality controls prepared in the laboratory.

Figure 3: Manual spotting



Manual and automated spotting techniques with different volumes were tested and the results are presented in table 2. No significant differences in precision and absolute response could be observed. Overall precision was found to be in line with measured baseline precision (as evaluated by replicate pooled matrix extract injections at corresponding concentration levels).

Table 2: Impact of spotting technique and spotting volume on precision (CV% on peak area ratio)

Spotting technique / Volume	LLOQ 0.100 ng/mL	LOW 0.300 ng/mL	MED 5.00 ng/mL
Extracted matrix samples, pooled (baseline precision)	8.4	5.4	1.7
Multipipette (1-mL-tip), spotting volume 20 µL	11.3	4.9	2.6
Multipipette (1-mL-tip), spotting volume 15 µL	5.2	5.7	2.9
Multipipette (0.1-mL-tip), spotting volume 20 µL	7.0	5.8	4.9
Capillary (20 µL), spotting volume: full spot	4.8	5.9	3.2
Tecan (1-mL-tip), spotting volume 20 µL	6.3	5.7	2.8
Tecan (0.2-mL-tip), spotting volume 20 µL	3.8	5.7	4.7

IMPACT OF CARD TYPE

DBS card material may also have an effect on the on-card blood spot distribution. Two different types of DBS cards were investigated, the 226 Bioanalysis card (untreated card material) and the DMPK-B card (chemically treated card material for protein denaturation and cell lysis).

Figure 2 shows the chromatograms obtained with cards spotted with 20 µL whole blood fortified with analyte at LLOQ concentration level (0.100 ng/mL). Both types of cards showed good baseline selectivity without interference from matrix or card material. The 226 Bioanalysis card shows better absolute response and improved S/N leading to marginally better precision at the LLOQ (table 3). Nevertheless precision was found to be in line with baseline precision (as evaluated in table 2) on both types of cards. The higher response measured from the 226 Bioanalysis card may be explained by the absence of suppressing species in the untreated card material. This is in line with our previous investigations with other analytes.

Table 3: Impact of DBS card type on precision (CV% on peak area ratio)

DBS card type	LLOQ 0.100 ng/mL	LOW 0.300 ng/mL	MED 5.00 ng/mL
Whatman DMPK-B	10.3	5.7	4.9
ID Bioanalytics card 226	6.9	4.1	3.6

IMPACT OF ANTICOAGULANT

Another factor which can affect blood viscosity and therefore on card homogeneity and spot thickness is the nature of the anticoagulant. Note that during the collection of clinical samples an addition of anticoagulant is not always possible for practical reasons. This may also lead to a different behavior of the blood on card. Human whole blood containing two common anticoagulants (Sodium heparin, K2 EDTA) was tested at a spotting volume of 20 µL. Results are presented in table 4. No significant differences in precision were observed but analyte response in whole blood containing K2 EDTA was found to be about 15-20% higher at all concentration levels compared to whole blood containing Sodium Heparin. It was not further investigated whether this difference in response was due to ionisation, recovery or blood spot thickness. Nevertheless it states the importance of the clinical sample collection procedure.

Table 4: Impact of anticoagulant on precision (CV% on peak area ratio)

Blood type (Anitcoagulant)	LLOQ 0.100 ng/mL	LOW 0.300 ng/mL	MED 5.00 ng/mL
Human whole blood (Sodium Heparin)	5.7	6.8	3.3
Human whole blood (K2 EDTA)	8.4	4.0	3.5

IMPACT OF SPOT PUNCHING TECHNIQUE

In order to address sufficient sample throughput automation of spot punching and its impact on data precision were evaluated. For this purpose semi-automated punching using a BSD 600 duet punching instrument (BSD Robotics, figure 4) was compared to manual punching using a Harris Uni-Core (GE Healthcare, figure 4) at 6mm diameter punch size in order generate inter spot precision data.

Figure 4: Automated punching by BSD 600 punching robot and manual punching by Harris Uni-Core

Manual and automated punching methods gave similar absolute response at all analyte concentration levels. However, the automated punching method showed overall improved precision (Table 5).

Table 5: Impact of punching method on precision (CV% on peak area ratio)

Punching method (punch size)	LLOQ 0.100 ng/mL	LOW 0.300 ng/mL	MED 5.00 ng/mL
Harris Uni-Core, manual (6mm)	10.0	6.9	3.5
BSD 600 punching robot (6mm)	6.8	3.2	3.1

IMPACT OF SPOT PUNCH SIZE

Another aspect to be considered within bioanalytical DBS studies is punch size as this can impact how repeat analysis either for analytical reanalysis or assessment of incurred sample reproducibility will be performed. Validation of appropriate intra and inter spot precision is therefore important. Since the spot punch diameter directly corresponds to sample volume this will consequently directly impact assay sensitivity. Therefore the following evaluations were performed at one concentration level only using automated punching at a spot size of 6mm (representing 11.2 µL blood and allowing 1 punch per spot) and 3.2mm (representing 3.2 µL blood and allowing a maximum of 3 punches per spot).

Results are presented in table 6. Although inter and intra spot precision were found to be in line with measured baseline precision, intra spot precision (total 24 replicates) was found to be marginally worse than inter spot precision from the corresponding punch size. Inter spot precision on 6mm punch size was found to be the best since this punch size almost covered the complete on-card area of a single spot.

Table 6: Impact of spot punch size on intra and inter spot precision (CV% on peak area ratio)

Punch spot size	MED 5.00 ng/mL	
6mm (BSD 600 punching robot)	4.2	(n = 8)
3.2mm, inter spot (BSD 600 punching robot)	6.7	(n = 8)
3.2mm, intra spot (BSD 600 punching robot)	9.9	(n = 24)

IMPACT OF INTERNAL STANDARD ADDITION PROCEDURE

The addition of internal standard prior to sample extraction is common in bioanalytical assays to overcome potential precision issues derived from the sample extraction variability. In the standardized procedure previously described the addition of internal standard directly in extraction solvent will not correct for any extraction recovery differences. Therefore alternative methods of internal standard addition prior to extraction were tested and compared against the standardized procedure.

Two alternative approaches were used. Firstly a defined volume of internal standard solution prepared in methanol was spotted onto the DBS cards (with addition of a visual marker dye) prior to the spotting of whole blood sample. Secondly whole blood was fortified directly with internal standard as well as analyte and this was then spotted in the normal manner. This represents an ideal control for comparison only and was not intended for practical application. These samples were then extracted using methanol only and compared to samples prepared according to the standardized protocol.

The results presented in table 7 show that the overall precision is not significantly worse when internal standard is added in extraction solvent in this case. As expected the different procedures for internal standard addition did not provide comparable absolute values since any recovery and on-card distribution issues are not covered by the standard extraction procedure. Addition of internal standard directly to the card resulted in wider distribution of internal standard compared to the blood spot itself. This lead to a reduced internal standard response for a defined punch area. Additionally this pre-addition of internal standard may also affect the final blood distribution even though the cards were allowed to dry in between spotting steps.

Table 7: Impact of internal standard addition procedure on precision

IS addition technique	IS added by extraction solvent			IS spotted on DBS card prior spotting of Human whole blood			IS fortified into Human whole blood
CONC.	LLOQ 0.100 ng/mL	LOW 0.300 ng/mL	MED 5.00 ng/mL	LLOQ 0.100 ng/mL	LOW 0.300 ng/mL	MED 5.00 ng/mL	MED 5.00 ng/mL
Mean Analyte Area	555	1529	27271	406	1422	23071	23102
% CV	15.8	14.0	6.5	10.4	5.1	6.4	11.0
Mean IS Area	103929	10279	115215	11065	12880	11977	46627
% CV	9.0	9.8	7.5	9.2	9.9	10.6	9.9
Mean Ratio	0.00560	0.0148	0.2369	0.0366	0.111	1.997	0.495
% CV	11.3	4.9	2.6	4.9	6.5	6.7	2.1

CONCLUSION

These evaluations indicate the general robustness of the dried blood spot technique in respect of sample precision. Whilst many of the factors evaluated here did not cause additional impact on method precision it is clear that a defined and consistent procedure should be used both for blood spotting and card punching to maximise method performance. This needs to be balanced with practicalities of applying the DBS technique to facilitate throughput for larger sample numbers associated with clinical studies. Choice of punch size (often driven by required LLOQ) also has implications for method reproducibility. In our laboratory we have chosen to standardise on 3.2mm and 6mm punch diameters. Precision assessment therefore needs to reflect how repeat analysis will be performed in each case.

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