FEASIBILITY ASSESSMENT FOR AN ELISA METHOD FOR THE QUANTITATION OF A THERAPEUTIC HUMAN ANTIBODY IN DRIED BLOOD SPOTS

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INTRODUCTION

Dried blood spot (DBS) bioanalysis has risen to prominence in the last few years particularly in application to small molecules. The aim of this assessment was to evaluate the applicability in our laboratory of DBS to large molecules. The assessment evaluated the quantitative determination of a therapeutic human antibody in blood samples using an established ELISA method and compared this to previously generated human serum data. The potential advantages of DBS are well documented and include small sample volume requirements, easier sample handling at the clinical site, easier storage and shipping logistics and improved sample stability.

METHOD OVERVIEW

The method was based on a previously validated ELISA assay in human serum. The target molecule specific for the therapeutic human antibody is immobilized on an ELISA plate. The samples are incubated in the plate and the captured therapeutic human antibody is detected using a mouse anti-human antibody conjugate with colorimetric readout at 450 nm. The validated analytical range in human serum is 233-10'000 ng/mL of therapeutic human antibody with a minimal required sample dilution of 1:400. For application of DBS, blood samples are first spotted onto an untreated filter paper card (ID Biological Systems 226 Bioanalysis Cards) and air dried at ambient temperature for a minimum of two hours. Untreated card material was chosen to avoid protein denaturation which would severely impact the ELISA procedure. The analyte is then eluted with an ELISA compatible buffer and resulting extracts measured using the standard ELISA procedure. Standard DBS parameters used were: i) blood spot volume of 25 µL, ii) punch size of 6 mm (using semi automated punching) and iii) elution volume of 300 µL, the eluted samples are further diluted in elution buffer to a final dilution of 1:400 prior to application of the ELISA procedure.

PRECISION, ACCURACY AND SENSITIVITY

Duplicate standard curves and replicate QCs were prepared in human whole blood (EDTA and Heparin were tested with no apparent impact of anticoagulant noted) and spotted onto filter paper cards, dried and analysed in a number of analytical runs. Standard curves were shown to be comparable in DBS and serum. A representative DBS curve is shown in Figure 1. The inter-assay precision data from these experiments and equivalent data from the human serum validation are presented in Table 1. The accuracy (% Bias) of both methods is very comparable, however, in DBS samples higher %CV values are apparent at all concentration levels. This poorer precision is probably from the additional handling steps associated with the quantitative extraction. Additionally there was a higher background signal evident from DBS. As a consequence, it is to be expected that the DBS method sensitivity may be a factor of 1.5-2 lower as compared to the serum method to be considered truly robust.

Figure 1: Representative DBS standard curve

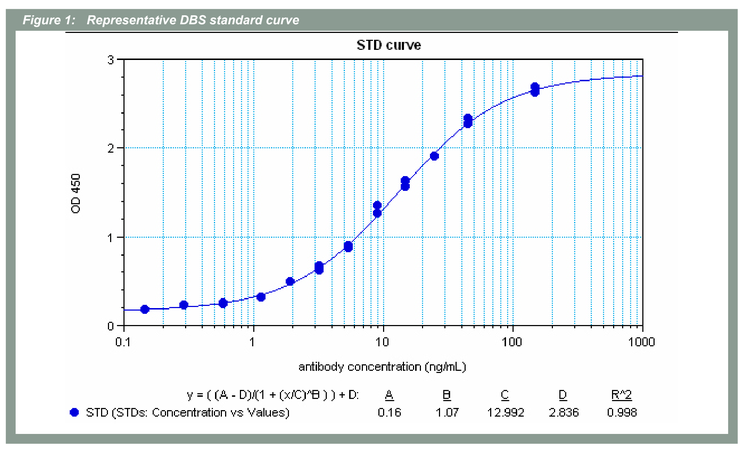


Table 1: Inter-run Precision and Accuracy

Inter-run P&A of STDs

DBS

Theor. Conc.	STD10	STD09	STD08	STD07	STD06	STD05	STD04	STD03
ng/mL	233.2	476	776	1296	2160	3600	6000	10000
Mean	237.8	461.8	805.9	1251	2152	3848	5848	9689
%CV	21.9	10.1	9.0	7.7	8.0	8.7	9.3	9.5
% Bias	2.0	-3.0	3.9	-3.5	-0.3	6.9	-2.5	-3.1
n	12	12	12	12	12	12	12	12

Serum

Mean	247.2	456.6	818.9	1263	2132	3607	6174	10010
%CV	10.5	6.2	6.0	4.6	3.3	4.3	3.8	4.6
% Bias	6.0	-2.4	5.5	-2.5	-1.3	0.2	2.9	0.1
n	12	12	12	12	12	12	12	12

Inter-run P&A of QC's

DBS

ng/mL	233.2	466	600	2400	4800	10000
Mean	260.2	459.5	573.6	2387.6	4602.7	9704.3
%CV	25.4	5.4	13.5	21.7	15.3	9.0
% Bias	11.7	-1.4	-4.4	-0.5	-4.1	-3.0
n	14	6	20	18	20	16

Serum

Mean	231.5		547.5	2321	4551	9257
%CV	15.8		9.3	7.2	8.3	9.4
% Bias	-0.6		-8.8	-3.3	-5.2	-7.4
n	18		18	18	18	18

STABILITY

The data from a number of published studies suggest that antibodies are particularly stable in DBS. This potential advantage of DBS was evaluated by measuring QCs samples as dried blood spots spotted on untreated cards and stored desiccated at room temperature for 11 days. These were measured against a freshly prepared standard curve in the fresh blood lot. The data in Table 2 shows that both the precision and bias from the theoretical concentration are both < 20% at all three concentration levels tested indicating a high degree of anticipated stability.

Table 2: Stability of DBS QCs at room temperature after 11 days

	LQC	MQC	HQC
Theor. Conc.	600	2400	4800
	ng/mL	ng/mL	ng/mL
#1	505.3	2530	4455
#2	462.4	2166	4251
#3	491.1	2373	4776
#4	530.5	2598	4710
#5	534.1	2168	4299
#6	534.6	2272	4559
Mean	509.7	2351	4508
%CV	5.7	7.8	4.7
% Bias	15.1	2.0	6.1
n	6	6	6

SELECTIVITY

Selectivity was assessed by measuring the therapeutic antibody spiked into 10 individual human whole blood lots at low and high QC levels. Results are presented in Table 3. All 10 blank blood lots were below the LLOQ level. In 4 out of 10 lots and 1 out of 10 lots at low and high QC level respectively, the measured concentration deviated from the expected theoretical value by > 20%. This relatively pronounced matrix effect is in contrast to the measured selectivity human serum, where all 10 individual serum lots fulfilled the acceptance criteria. Additional experiments are required to investigate this issue in more detail. At this time it could not be established if this was due to extraction differences between blood lots or if matrix effect could be eliminated by further post extraction dilution.

Table 3: Selectivity in individual whole blood lots

		Low (600 ng/mL)		High (4800 ng/mL)	
Blood ID	Blank (ng/mL)	% CV	% Bias	% CV	% Bias
Wb1	153.8	3.0	50.3	7.4	1.9
Wb2	4.2	14.1	4.9	0.5	14.5
Wb3	BLQ	12.3	-1.6	12.1	-5.8
Wb4	27.8	5.0	4.1	0.5	2.8
Wb5	BLQ	2.2	23.3	1.6	5.9
Wb6	97.5	4.3	-10.0	6.7	8.5
Wb7	BLQ	15.1	24.2	6.2	25.9
Wb8	BLQ	19.6	24.7	7.5	6.1
Wb9	BLQ	3.9	-7.6	1.9	3.4
Wb10	BLQ	5.7	-2.7	1	-4.8

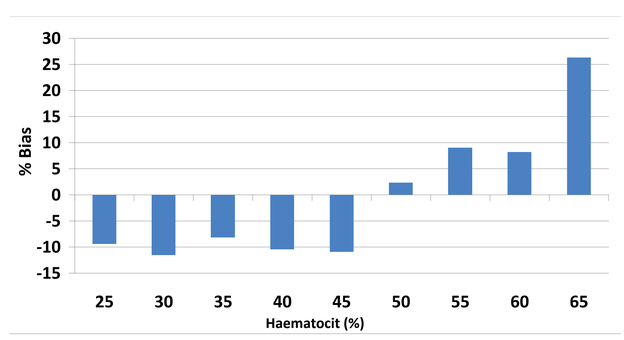
DBS SPECIFIC PARAMETERS

In addition to the normal method parameters applied to the ELISA procedure, those specifically applicable to the DBS sampling and subsequent sample preparation were evaluated. These parameters may significantly influence the outcome of the analytical measurement. Haematocrit effect and blood spot volume are sampling parameters which cannot be influenced by the bioanalytical laboratory. The first being related to the properties of the blood sample itself and the second related to the non quantitative spotting of blood onto the blood spot cards at the clinical site. Automated sample punching was used with sequential punching of samples from the card without a cleaning step in between. Card punching carry-over was therefore evaluated.

HAEMATOCRIT EVALUATION

In the first instance, the hematocrit level (volume % of red blood cells) influences the blood viscosity and thus the distribution of sample on the filter paper card. This has obvious consequence for blood spot diameter from a defined sample volume. Samples were prepared with varying haematocrit levels by recombining red cell and plasma fractions. These were then spiked with the analyte to the same concentration, spotted and measured following extraction. Figure 2 shows a positive correlation between the hematocrit level and the measured analyte concentration, with a positive and a negative relative bias above and below the 50% haematocrit level (a normal average haematocrit in human population). At haematocrit levels typical in the human population (40-60%), the differences to the theoretical value are within 11%, thus not significantly biasing the accuracy. The observed trend is obviously only applicable to the blood lot tested and may vary slightly between blood lots and with blood age.

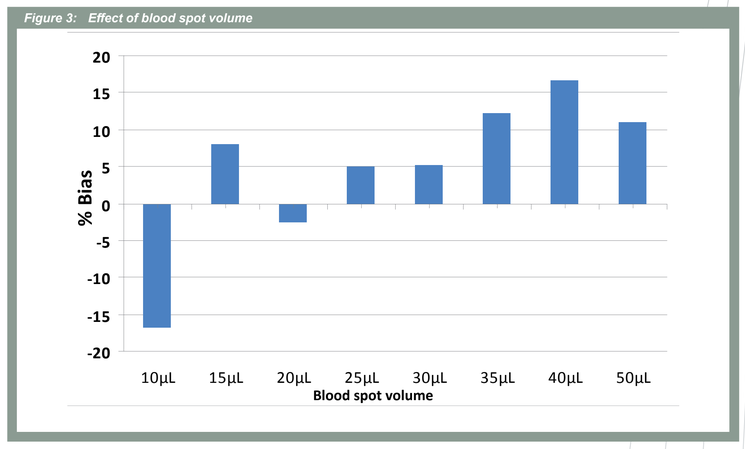
Figure 2: Effect of haematocit



BLOOD SPOT VOLUME

The effect of amount of blood spotted on the filter cards was also assessed and was shown to have little effect on the concentration measured in this case over a relatively wide range of spotting volumes (15-50 µL). Only at volumes at or below 10 µL was the negative bias shown to be significant. This representing a spot diameter equivalent or less than the 6mm sampled. Results are presented in Figure 3.

Figure 3: Effect of blood spot volume



EXTRACTION RECOVERY

Blood samples are spotted on filter paper cards and dried in the DBS method. Consequently, the analyte needs to be quantitatively extracted from the card in an ELISA compatible buffer system after punching. A 6 mm diameter punch size was used and various elution buffers tested during method development. The aim was to minimize the background signal and to achieve the largest possible signal to background ratio. Table 4 shows the results of the extraction buffer optimisation. Best results of ULOQ/blank ratio response were used to assess choice of buffer system. The extraction of the analyte from the blood spot card is quite a rapid process, as no significant differences in measured analyte concentrations were observed by varying the elution time from 15 min to 4 h at room temperature or overnight at 5°C (data not shown). An estimation of analyte recovery was made by measuring DBS samples against a standard curve in serum. Recovery was shown to be approximately 70% and consistent across the calibration range. Further experiments to evaluate the recovery more precisely without any potential matrix effect bias could be conducted.

Table 4: DBS extraction buffer optimisation

	Analyte concentration (ng/mL)	OD450
Elution Buffer 1	0	0.133
	ULOQ	2.963
Elution Buffer 2	0	0.110
	ULOQ	2.794
Elution Buffer 3	0	0.091
	ULOQ	2.777
Elution Buffer 4	0	0.122
	ULOQ	3.097
Elution Buffer 5	0	0.410
	ULOQ	2.756
Elution Buffer 6	0	0.105
	ULOQ	2.646
Elution Buffer 7	0	0.110
	ULOQ	2.878
Elution Buffer 8	0	0.062
	ULOQ	2.987

CARRY-OVER ASSESSSMENT

To estimate the level of potential cross-sample carry-over QCs spiked at 400 µg/mL (representing anticipated highest level in clinical samples) were punched followed by a blank blood spot. Blank samples were then extracted and measured. No significant carry-over contamination was observed in any of the blank samples (maximum measured values were less than 20% of the LLOQ).

CONCLUSION

This assessment indicates the potential applicability of the dried blood spot format to ligand binding assays for large molecule quantitation. Whilst the data obtained for DBS may not be as precise as that obtained for serum, it does indicate that the quantitative extraction of target analyte from DBS in an ELISA compatable system is possible. Additional matrix effects may be evident either as a result of co-extracted blood constituents or due to non reproducibility of extraction in certain instances.

When assessing DBS for ligand binding methodologies additional DBS specific parameters need to be considered as potential variables. The wider applicability of DBS is not yet fully understood but may be expected to be less applicable to large molecules than small molecules.

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