Research Article

Validation of a Ligand Binding Assay Using Dried Blood Spot Sampling

Daniel Burns,¹ Laura Brunner,² Surendran Rajendran,³ Beth Johnson,¹ Mark Ma,¹ and Jin Wang^{1,4}

Received 15 August 2012; accepted 20 October 2012; published online 8 November 2012

Abstract. Dried blood spots (DBS) technology has been introduced as a microsampling alternative to traditional plasma or serum sampling for pharmacokinetics or toxicokinetics evaluation. The application of DBS has been established for many small molecule drugs at discovery, nonclinical, and clinical stages. However, the application of DBS for large molecule therapeutics development is not yet well-established. This article describes the method validation of a ligand binding assay (LBA) for DBS sampling of a therapeutic monoclonal antibody—AMG 162 (Denosumab). The original serum LBA was modified for the DBS method. A fit-for-purpose method validation was performed to evaluate accuracy and precision, selectivity, dilutional linearity, and stability. In addition, the parameters relevant to DBS, such as spot volume, extraction recovery, whole blood stability, and hematocrit effects, were evaluated. The validation results demonstrated assay robustness with inter-assay precision of $\leq 19\%$, inter-assay accuracy of $\leq 9\%$, and total error of $\leq 24\%$. Selectivity, extraction recovery, dilutional linearity, and stability were demonstrated. The validation results revealed some limitations of the possible effect of blood hematocrit on therapeutic concentration measurements and the caution required using whole blood for standards and quality controls preparation. This is the first article to describe a thorough method validation of an LBA using DBS for a therapeutic monoclonal antibody. The lessons learned can serve as a model process for future method validation of other LBAs for large molecule therapeutics or biomarkers using the DBS sampling method.

KEY WORDS: dried blood spot (DBS); large molecule therapeutics; ligand binding assay (LBA); method validation; therapeutic monoclonal antibody.

INTRODUCTION

Dried blood spot (DBS) sampling is a microsampling technique providing an alternative to measure the systemic exposure of pharmaceuticals in plasma or serum (1). The method involves spotting of 10 to 20 μL of whole blood onto an absorbent filter paper. The blood spot is punched out and extracted with buffer/solvent and the extract is subjected to bioanalysis for the analytes of interest, such as drug compounds (small and large) and their metabolites or biomarkers (2–4).

The use of DBS has been spreading from its modest beginning in the diagnostic arena for neonatal screening (5) to drug development stages, including discovery research (6,7), nonclinical and clinical toxicokinetic and pharmacokinetic evaluation, and therapeutic drug monitoring (1,8,9). This increase in the use of DBS is due to its multifold advantages relating to scientific, practical, ethical, and cost savings in both nonclinical and clinical arena (4,10–13).

DBS sampling has been in practice for the small molecule at drug discovery, nonclinical, and clinical stages (14–16). The increase in drug development of large molecule therapeutics posts the need of considering DBS for large molecule therapeutics development. The use of DBS on large molecule therapeutics has the potential of reducing animal and therapeutic use, enabling serial sampling in rodent studies, removing the need to collect plasma or serum, enabling capillary blood sampling (especially in pediatric studies), and the ability to maintain sample integrity without refrigeration (makes in-house and remote area studies possible). The assessment of DBS technology for the detection of therapeutic antibodies had been previously reported (3). Our initial evaluation results comparing traditional serum or plasma sampling to DBS for large molecule therapeutics of antibody, peptibody, protein, and fusion protein modalities and biomarker indicated that the ligand binding assays (LBAs) with DBS sampling were feasible and comparable to those of plasma or serum methods (17,18).

The main drawback of DBS has been the lack of acceptance by regulatory agencies. The main concern of the regulators is on the ability of DBS to provide robust data to allow reliable risk assessment (19). It is germane that the DBS method must provide the same level of confidence as the traditional serum-based or plasma-based methods. One way to address this issue convincingly is the increased applications of DBS in the regulated bioanalysis environment.

¹ Pharmacokinetics and Drug Metabolism Department, Amgen Inc., Mail Stop 30E-3-C, One Amgen Center Drive, Thousand Oaks, California 91320, USA.

² Key Account Management, Sartorius Stedim Biotech North America, Bohemia, New York, USA.

³ Bioanalytical Science, Bristol-Myers Squibb, Princeton, New Jersey, USA.

⁴ To whom correspondence should be addressed. (e-mail: jinw@amgen.com)

Such application first requires thorough method validation of the DBS method providing assay performance data with the similar stringency in acceptance criteria as that of the plasma/serum method. The potential differences of sample collection, processing, and analysis in DBS compared to plasma/serum methods must be considered and included in the validation characterization (19). To ensure data quality, the effect of whole blood as a matrix, blood hematocrit, blood spotting practice, sample preparation steps, spot volume, and storage conditions have to be evaluated. This study presents a thorough method validation of an LBA to measure a therapeutic monoclonal antibody—AMG 162 (Denosumab, a fully human IgG₂ monoclonal antibody that blocks receptor activator of nuclear factor kappa-B ligand (RANKL), also known as osteoprotegerin ligand (OPGL), from binding to its receptor RANK), while sample collection was performed using DBS technology. The thorough processes and reliable results illustrated a rigorous framework for good blood spotting practice, sample preparation and processing, storage conditions, and assay acceptance criteria that allow the application of DBS with confidence.

MATERIALS AND METHODS

Materials

AMG 162 used as reference standard (STD), recombinant human OPGL used as capture reagent, and biotinylated rat monoclonal antibody against AMG 162 (clone 3.14.1) used as detection reagent were from Amgen Inc. (Thousand Oaks, CA). Fresh human whole blood with K₂EDTA was from Bioreclamation (Hicksville, NY). Streptavidin-HRP was from R&D Systems Inc. (Minneapolis, MN). I-Block reagent was from Applied Biosystems Inc. (Carlsbad, CA). Phosphate-buffered saline (PBS) was from Gibco Life Technologies (Grand Island, NY). Tween 20 was from Thermo Scientific (Ashville, NC). Bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Sodium carbonate and sodium bicarbonate were from Mallinckrodt Inc. (Hazelwood, MO). Sodium chloride (NaCl) and sulfuric acid were from JT Baker (Center Valley, PA). KPL Wash Solution was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Tetramethylbenzidine (TMB) peroxide substrate solution was from BioFX Laboratories Inc. (Owings Mills, MD).

DBS cards (Whatman FTA® DMPK-C), Harris 6-mm punches with cutting mats, zip-locked storage bags, and desiccant packs were from GE Healthcare (Piscataway, NJ). Tecan Freedom EVO® automated liquid handler was from Tecan US (Research Triangle Park, NC). Centrifuge, tube rocker, plate shaker, multitube vortexer, plate washer, and plate reader were used in this study.

Methods

DBS Sample Preparation

STDs, validation samples (VSs)/quality controls (QCs), and test samples were prepared by spiking AMG 162 stock in PBS buffer with 1% BSA at 50 times of the final concentrations using Tecan Freedom EVO® automated liquid handler. The intermediate stocks for STD, VS, QC, and test sample were then

manually diluted 1:50 into 100% human whole blood. The final STD concentrations were 5, 10, 20, 50, 125, 250, 400, 750, 1,000, and 1,500 ng/mL, in which 5 and 1,500 ng/mL were anchor points. The final VS concentrations were 10 ng/mL (lower limit of quantitation [LLOQ]), 30 ng/mL (low quality control [LQC]), 200 ng/mL (middle quality control [MQC]), 600 ng/mL (high quality control [HQC]), and 1,000 (upper limit of quantitation [ULOQ]). To mimic *in vivo* sample conditions, the spiked whole blood STDs, VSs/QCs, and test samples were incubated at 37°C with gentle shaking for 1 h. Twenty microliters of each spiked whole blood sample was spotted on Whatman FTA® DMPK-C cards and allowed to air dry at ambient room temperature (ART) for a minimum of 2 h to form DBS.

DBS cards were then extracted immediately for analysis or placed in zip-locked bags with desiccant and stored at desired temperatures for a prespecified time period for later extraction and analysis. The extraction was carried out by punching out a 6-mm disk from the center of DBS spots and placing the punched disk in a well of a 96-well polypropylene microtiter plate. Two hundred microliters of extraction buffer (1× PBS with 1 M NaCl, 0.5% Tween 20, and 1% BSA) was added to each well containing DBS disk. The plate was shaken on a multitube vortexer at ART for approximately 1 h, followed by incubation at 4°C for a minimum of 12 h. The plate was centrifuged and the supernatant from each well was removed for analysis.

Ligand Binding Assay Procedures

The extracted STDs, QCs, test samples, and blank were analyzed following a modified LBA. The modification made was changing the assay minimum required dilution (MRD) from 1:40 for the serum samples to 1:6 for the DBS samples. Each bioanalytical assay consisted of one set of standards, blank, two replicates of three levels QCs, and test samples. The extracted STDs, QCs, test samples, and blank were diluted 1:6 into assay buffer (1× PBS with 1 M NaCl, 0.5% Tween 20, and 1% BSA). One hundred microliters of diluted samples were added to a 96-well microtiter plate that was passively coated with OPGL. AMG 162 in the sample was captured by the OPGL and the unbound materials were removed by a wash step. Biotin-conjugated rat monoclonal antibody against AMG 162 was added. After another wash step, a streptavidin-HRP was added to bind to the complex. After a final wash step, TMB peroxide substrate solution was added to produce a colorimetric signal, which was proportional to the amount of AMG 162 bound by the capture reagent. Color development was stopped by the addition of H₂SO₄. The plate was read on a SpectraMax 340 PC plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm, with reference at 650 nm.

Analytical Data Regression and Acceptance Limits

The conversion of the optical density units for the test samples, VSs, and QCs to concentrations was performed using the Watson LIMS software (version 7.0.0.01 and 7.4; Thermo, Pittsburgh, PA). The absorbance *versus* concentration relationship was regressed according to a four-parameter logistic regression model with a weighting factor of 1/Y. The back-calculated values of each standard point must meet the target acceptance criteria of 15% of the nominal value, for at

Table I. Accuracy and Precision Results

Sample type	Nominal concentration (ng/mL)		Inter-assay accuracy (%RE)		Inter-assay precision (%CV)		Total error of method (%TE)	
	DBS	Serum	DBS	Serum	DBS	Serum	DBS	Serum
Standards	10–1,000	20–2,000	–3.0 to 7.0	–3.4 to 8.0	3.8 to 7.0	0.8 to 3.2	N/A	N/A
VS1 (LLQC)	10	20	–5.2	2.6	19.1	7.1	24.3	9.8
VS2 (LQC)	30	60	–2.7	1.9	11.5	6.9	14.2	8.8
VS3 (MQC)	200	400	–9.1	1.2	7.8	4.9	16.9	6.0
VS4 (HQC)	600	1,200	–5.1	3.4	7.9	11.9	12.9	15.3
VS5 (ULQC)	1,000	2,000	–2.3	–3.9	9.1	8.5	11.3	12.3

$n=60$ for the DBS method; $n=24$ for the serum method

least 75% of the acceptable standard points. Two thirds of the QC should be acceptable within 25% of the nominal values.

Extraction Recovery Evaluation

The AMG 162 extraction recovery from DBS was evaluated by comparing the DBS STDs to the plasma STDs that were processed from the same set of whole blood samples. The STDs, used as the test samples, were prepared by spiking AMG 162 into K₂EDTA human whole blood and then divided into two sets—one set processed to plasma and the other processed to DBS. Each DBS STD (a 6-mm DBS disk) was extracted with 200 μ L of extraction buffer. To mimic the DBS sample condition and to account for the sample volume difference between the whole blood and the plasma due to hematocrit, a 5- μ L volume of plasma STD was also added to 200 μ L of extraction buffer. The AMG 162 concentration in a 6-mm DBS disk (equals to approximately 10 μ L of whole blood) should be close to the concentration in a 5- μ L volume of plasma because the average normal adult blood hematocrit is about 50% (20). The processed plasma STDs and extracted DBS STDs were analyzed in the same assay for the comparison.

Dilutional Linearity Sample Preparation

The dilutional linearity experiment was performed by preparing a sample in 100% human whole blood at a nominal concentration of 15.0 μ g/mL of AMG 162 and processed to DBS. The DBS samples were then extracted and further diluted 1:15, 1:25, 1:60, 1:500, and 1:1,500 into assay range using extracted blank or sample diluent. The extracted blank was prepared by pooling the extracted solution from DBS made with normal human blood. The sample diluent was prepared by adding the approximate whole blood volume in a 6-mm DBS disk to 200 μ L of extraction buffer, which is about 5% of whole blood in the extraction buffer.

Hematocrit Sample Preparation

To prepare the blood samples at various hematocrit levels, the human whole blood was spun down and then diluted into human plasma at dilution factors of 1:5, 1:3.33, 1:2.5, 1:2, and 1:1.67 to make the blood samples with hematocrit levels of 20%, 30%, 40%, 50%, and 60%. The prepared blood samples were further spiked with AMG 162 at HQC (600 ng/mL) and LQC (30 ng/mL) levels.

RESULTS

Accuracy and Precision

A total of ten accuracy and precision assays were performed by three analysts varying incubation times and using two different lots of FTA® DMPK-C cards over a 3-day period. Two independent sets of DBS STDs and one bulk set of DBS VSs were prepared and stored at nominal 25°C in zip-locked bags with desiccant for a minimum of 12 h and up to 3 days prior to assay.

Table I displays the accuracy and precision summary results. For comparison purposes, the table includes the

Fig. 1. **a** Comparison of plasma standards to DBS standards. The STDs were prepared by spiking AMG 162 into K₂EDTA human whole blood and then divided into two sets—one set processed to plasma and the other processed to DBS. Both plasma STDs and DBS STDs ($n=1$) were tested in the same assay. The value of 50% hematocrit was used in the evaluation. **b** AMG 162 concentration ratio (DBS/plasma) evaluation. The graph displays the concentration ratio of DBS over plasma at each standard level ($n=1$) covering the assay range of 10 to 1,000 ng/mL (STD 2 to STD 9)

Fig. 2. Selectivity evaluation. Human whole blood obtained from 20 healthy individuals ($n=40$ at LQC and LLOQ levels) and 6 diseased individuals ($n=12$ at LQC and LLOQ levels). The dotted lines indicate the assay acceptance limits

accuracy and precision results of a validated method for measuring serum samples using the same pair of capture and detection reagents. The inter-assay precision for the DBS method ranged from 7.8% to 19.1% versus from 4.9% to 11.9% for the serum method; the total error for the DBS method ranged from 11.3% to 24.3% versus from 6.0% to 15.3% for the serum method.

Extraction Recovery

The DBS sample extraction recovery was evaluated to assess whether the extraction recovery was consistent throughout the standard curve range with DBS sampling in comparison to the conventional plasma method that did not require extraction. Figure 1a shows a good correlation between the DBS STDs and the plasma STDs ($R^2=0.9984$). When regressed to the plasma STDs, the relative concentration ratios of DBS STDs to plasma STDs were from 0.95 to 1.13 covering the assay range of 10 to 1,000 ng/mL (STD 2 to STD 9), indicating the consistency extraction recovery throughout the assay range (Fig. 1b).

Selectivity (Matrix Effect)

To confirm that the assay measures AMG 162 accurately without significant matrix interference, human whole blood from 20 healthy individuals (12 males and 8 females) and from 6 targeted diseased population (3 males and 3 females) were tested unspiked and spiked with AMG 162 at the LQC (30 ng/mL) and LLOQ (10 ng/mL) levels. Spike recoveries were within

Fig. 3. Dilutional linearity evaluation. A spiked sample at the concentration of predicted C_{max} was diluted at 15-, 25-, 60-, 500-, and 1,500-fold with sample diluent (filled circles) or extracted blank (open squares) ($n=3$). The dotted lines indicate the assay acceptance limits

acceptance limits for 100% of the healthy individual lots and 83.3% of diseased population lots (Fig. 2). The %CV were $\leq 15\%$ and 20% for the healthy and diseased groups, respectively, demonstrating selectivity of the method.

Dilutional Linearity

The dilutional linearity experiment was performed by preparing a sample in 100% human whole blood at a nominal concentration of 15.0 $\mu\text{g/mL}$ of AMG 162 and processed to DBS. The DBS samples were then extracted and diluted 1:15, 1:25, 1:60, 1:500, and 1:1,500 into assay range using extracted blank or sample diluent. The extracted blank was prepared by pooling the solution processed from extracted DBS blanks. The sample diluent was 5.0% whole blood in extraction buffer. The results in Fig. 3 demonstrated acceptable dilutional linearity and agreement between dilutions made with extracted blank and sample diluent. Therefore, for ease of use, the sample diluent was used for subsequently study sample dilution. Assay hook effect was examined and no hook effect was observed.

DBS Spot Volume

The impact of DBS spot volume on the method accuracy and precision was evaluated. The whole blood test samples prepared at 600 ng/mL (HQC) and 30 ng/mL (LQC) of AMG 162 were spotted at volumes of 10, 20, and 30 μL . The spotted DBS samples were analyzed with DBS STDs and QCs that were spotted at 20 μL whole blood. No significant differences were observed within each volume or among different spot

Table II. Comparison of DBS Spot Volume

Nominal concentration		600 ng/mL				30 ng/mL			
DBS spot size	n	Mean concentration (ng/mL)	Percent different from nominal concentration	Intragroup %CV	Intergroup %CV	Mean concentration (ng/mL)	Percent different from nominal concentration	Intragroup %CV	Intergroup %CV
30 μL	3	605	0.8	3.1	2.5	27.7	-7.7	5.9	5.1
20 μL	3	627	4.6	1.2		27.5	-8.3	6.2	
10 μL	3	614	2.3	1.8		28.1	-6.2	5.1	

volumes for both QC levels as shown by the low %CV values in Table II.

Hematocrit Effect

To assess the hematocrit effect, various whole blood pools were prepared at hematocrit levels of 20%, 30%, 40%, 50%, and 60% and spiked with AMG 162 at HQC (600 ng/mL) and LQC (30 ng/mL) levels. The test DBS samples were analyzed with STDs and QCs that were prepared in whole blood with 47% hematocrit. As shown in Fig. 4, recoveries of all tested hematocrit samples were within the acceptance range. However, there was a positive trend on the measured concentrations with the increasing levels of hematocrit—the test samples with 30% to 50% hematocrit levels recovered from -10% to 7% of baseline concentrations, while those with 20% or 60% hematocrit levels recovered at -25% and 17%, respectively. The discrepancy between the highest and lowest tested samples can be as much as 42%.

Whole Blood Matrix Stability

To ensure the integrity of STDs and QCs prepared for a regulated study, the whole blood matrix stability was performed. Limited data from prevalidation experiments (data not shown) indicated the instability of AMG 162 after 2 weeks storage at 4°C for samples prepared from whole blood of multiple-used vials. Therefore, during method validation, only the first-time-use whole blood vials were used in the whole blood matrix stability evaluation. The stability samples were assayed with STDs and QCs prepared in a fresh lot of whole blood. DBS samples were tested at levels of ULOQ, HQC, MQC, LQC, and LLOQs, which were prepared in various lots of whole blood that were stored at 4°C for 7, 16, and 23 days. No downward trend was observed up to 23 days, as shown in Fig. 5.

Process Stability for DBS Preparation

Various process stability conditions, listed in Table III, were tested to confirm the process stability for DBS

Fig. 4. Hematocrit effect on analyte recovery. The whole blood pools at hematocrit levels of 20%, 30%, 40%, 50%, and 60% were used to prepare the test samples at HQC and LQC levels ($n=3$). The test samples were analyzed with STDs and QCs in whole blood of 47% hematocrit. The graph displays the %Bias from baseline (Y axis) of the HQC (lined bar) and LQC (dotted bar) at different hematocrit levels (X axis). The dotted lines indicate the assay acceptance limits

Fig. 5. Whole blood matrix stability. Different lots of whole blood stored at nominal 4°C for 7, 16, and 23 days were used to prepare DBS samples at ULOQ, HQC, MQC, LQC, and LLOQ levels ($n=3$). Prepared DBS samples were tested against the STDs and QCs prepared in fresh whole blood received within 2 days. The dotted lines indicate the assay acceptance limits

preparations which includes the blood draw, the storage of whole blood samples, the spiking of AMG 162 into whole blood, the 37°C incubation of whole blood samples, and the DBS spotting, drying, and extraction. The demonstrated process stability is shown in Table III.

Long-Term DBS Storage Stability

Sample storage stability was assessed to ensure the sample integrity during long-term storage and in shipping. DBS samples were tested at conditions of 37°C, ART, 4°C, -20°C, and 25°C with 60% humidity. Results showed that DBS samples can be stored at 37°C for at least 10 days, at ART for up to 94 days, at 25°C with 60% humidity for up to 12 days, at 4°C for up to 180 days, and at -20°C for at least 384 days. As shown in Fig. 6, samples stored at ART and 25°C with 60% humidity showed downward trends in recovery, whereas samples stored at -20°C showed acceptable recoveries for the longest period, suggesting that the ideal long-term storage condition for DBS samples is nominal -20°C.

DISCUSSION

It is a novel approach to use DBS in an LBA in support of pharmacokinetics evaluation of a therapeutic large molecule drug. Special considerations must be included in a DBS method validation especially when there is no regulatory guidance or industrial consensus to follow. We adopted the regulatory method validation guidance on plasma and serum methods as a basic framework (21,22) to evaluate accuracy and precision, selectivity, dilutional linearity, and relevant stabilities. In addition, we performed evaluations on DBS specific method characteristics, such as extraction recovery, hematocrit effect,

Table III. Process Stability for DBS Preparation

Test condition	<i>n</i>	Demonstrated stability boundary
Spiked WB samples at ART	3	Up to 24 h
Spiked WB samples at 4°C	3	Up to 24 h
Spiked WB samples at 37°C	3	30 to 90 min
DBS dry time at ART	3	2 to 22 h
DBS extraction at ART	3	30 to 90 min
DBS extraction at 4°C	3	12 to 48.5 h

spotting volume, and DBS relevant stability. Table IV lists the validation parameters that are routinely tested in a traditional LBA *versus* the DBS method that we described. Compared to a traditional plasma/serum method validation, the DBS method requires additional evaluation on the DBS specific parameters. The only validation parameters for a traditional LBA method that would not be required for the DBS method are the freeze and thaw stability and -70°C long-term storage stability.

DBS sample preparation requires the extraction of dried blood samples into solution. This is a critical step unique to DBS for large molecule sample analysis and this extraction step is crucial to the method success. For a given analyte, a poor extraction recovery or a poor correlation between the serum/

plasma and DBS samples may indicate that further method development is necessary or that the analyte may not be a good candidate for the DBS method (18). Therefore, the sample extraction condition and extraction recovery should be evaluated during method development and confirmed in method validation. Ideally, extraction recovery should be performed by comparing the DBS samples to the spiked whole blood samples. However, when the whole blood assay is not available for the therapeutics, comparing DBS samples to plasma/serum samples may be used for assessing the DBS sample extraction recovery if the therapeutics' pharmacokinetics in the plasma/serum and the blood/plasma ratio of the therapeutics were well understood. The blood/serum ratios for AMG 162 were between 0.45 and

Fig. 6. Long-term stability evaluation. Prepared stability samples at HQC (filled diamonds) and LQC (open diamonds) levels ($n=3$) were stored at different test conditions (a 37°C , b ART, c 4°C , d -20°C , and e 25°C with 60% humidity) and were tested at planned time points against freshly prepared STDs and QCs. %Bias were calculated from the nominal concentrations. The graphs represent the %Bias change over time. Stability was demonstrated when the %Bias from both HQC and LQC samples were within the acceptance limits (dotted lines)

Table IV. Validation Parameters—Traditional LBA Method *Versus* DBS Method

Validation parameters		Plasma/serum method	DBS method
Accuracy and precision		✓	✓
Extraction recovery		NA	✓
Selectivity		✓	✓
Specificity		✓	✓ ^a
Dilution linearity		✓	✓
Hematocrit effect		NA	✓
DBS spot volume		NA	✓
Stability	Whole blood matrix	NA	✓
	Spiked whole blood	NA	✓
	Process	✓	✓
	Freeze thaw	✓	NA
	Long-term stability		
	37°C	NA	✓
	25°C at 60% humidity	NA	✓
	ART	NA	✓
	4°C	NA	✓
	–20°C	✓	✓
	–70°C	✓	NA

NA not applicable

^a Not tested in this validation

0.61 (data not shown). We used a value of 0.5 in the extraction recovery evaluation. The use of K₂EDTA whole blood allowed a head-to-head comparison of the plasma and DBS samples from the same spiked sample. As shown in Fig. 1a, the extraction recovery results demonstrated excellent correlation between plasma and DBS samples. Our experience with various types of large molecule therapeutics also revealed good correlation between DBS and serum/plasma samples (3,17,18). The extraction of DBS may serve as an MRD for an LBA. However, for this DBS method, an MRD of 1:6 was also required in addition to DBS extraction.

A robust procedure of STD and QC preparation has to be established due to the challenge of the pipetting of whole blood required for DBS methods. Previously, we had good experience using Tecan Freedom EVO® automated liquid handler to dilute analyte solutions to improve accuracy and reproducibility (23), so we used Tecan automation instruments for the STDs and QCs preparation for this method. To minimize the manual pipetting of whole blood, the Tecan instrument was used to dilute the drug solution in buffer at levels of 50 times the final STD or QC concentrations followed by a 1:50 manual dilution of spiked buffer STDs and QCs in whole blood prior to DBS spotting. Another challenge that we encountered in preparing DBS STDs and QCs was the use of whole blood matrix. Ideally, fresh whole blood should be used to prepare STDs and QCs; however, its criticality has not been tested. In the field of transfusion, units of whole blood are kept refrigerated at 1°C to 6°C with maximum permitted storage periods (shelf life) of 35 days. A set of biochemical and biomechanical changes (storage lesion) will occur during storage (24,25). To assess whether whole blood storage will impact bioanalysis, we evaluated the effect of whole blood matrix stability on the performance of STD and QC. Our preliminary results indicated no more than 2 weeks of whole blood stability when the multiple-used whole blood vials were used. The validation results demonstrated extended whole blood stability when the first-time-use whole blood vials were used in the evaluation. Thus, we purchased the whole blood specimens in one-time-use

aliquot size for each subsequent STD and QC preparation. A recent invention extending the shelf life of red blood cells reported that the shelf life can be extended 100% by storing the red blood cells under oxygen-depleted conditions to reduce oxidative damage (26). Therefore, the proper use of whole blood matrix, with caution to avoid oxygen contact, for bioanalysis should be applied and evaluated for large molecules stability in whole blood.

It may be conceivable that DBS methods can be less sensitive than that of plasma/serum methods because of the additional sample extraction step and/or the matrix effect from the whole blood. However, our DBS method validation results demonstrated comparable assay sensitivity when compared to a serum enzyme-linked immunosorbent assay (ELISA) that utilized the same pair of capture and detection reagents as the DBS method. Our validation results did reveal that the DBS method for AMG 162 had higher inter-assay variability and higher total error than that of the serum assay. However, the acceptance criteria of 20% bias and 15% CV on QCs for this DBS method met the industrial standards and FDA and EMA's requirements of an LBA (21,22). For the evaluation of new technology, the DBS in this case, we purposely set a wider acceptance limit of 25% bias, instead of 20%, for the potential inclusion of more data for the evaluation.

Due to classical ELISAs' relatively narrow assay range, a majority of large molecule study samples require dilutions prior to analysis. For a traditional serum or plasma assay, study samples are diluted with pooled serum or plasma. For the DBS assays, study samples should be diluted with the blank blood spots extracted solution (extracted blank). However, due to extensive work required in obtaining sufficient amount of extracted blank for study sample dilution, we evaluated an alternative diluent—sample diluent for study sample dilution. The demonstrated comparability between these two sample dilution solutions enabled the use of sample diluent for future study sample dilution.

One of the advantages of DBS sampling is the ability to store DBS samples at ART. The question is for how long.

Edelbroek (1) stated that “a slow but steady decrease of the amino acids has been revealed over long term storage in DBS cards.” McDade (4) commented that DBS samples from multiple large molecule biomarkers varied in their degradation rate, indicating the importance of establishing sample handling and long-term stability parameters prior to initiating studies. To demonstrate stability for anticipated DBS card storage during handling and shipping, we tested DBS card storage at 37°C, at 25°C with 60% humidity, and at ART. To evaluate the optimal long-term storage condition, we also tested storage conditions at 4°C and –20°C. Our results showed that the high temperature and humidity had a negative impact on the DBS sample stability and the optimal long-term storage condition for DBS samples was not ART but –20°C. A recommended strategy for DBS sample storage is to keep DBS cards at ART during processing and shipping and then to place it at –20°C for long-term storage.

DBS sample integrity and homogeneity is critical for the DBS method to be robust, accurate, and reproducible. The White Paper from the European Bioanalysis Forum recommended setting good blood spotting practice boundary limits (27). One of the applications of using the DBS method is to support pediatric studies. In a clinical setting, it may be difficult to ensure that the strict guidelines or procedures for sample processing and storage are followed. Therefore, it is useful to test the stability for DBS preparation and storage in method validation to provide a buffering zone for DBS sample handling. We evaluated the DBS spot volumes from 10 to 30 µL and the stability of spiked whole blood samples and DBS samples at ART and at 4°C. The comparable results of various spot volumes of 10 to 30 µL provide latitude of whole blood spotting for accurate bioanalytical measurement. Sample stability of spiked whole blood and DBS at ART and at 4°C resulted in flexible options for handling and managing study samples.

Hematocrit is known to affect the dispersion of blood on DBS cards that impacts spot formation, spot size, drying time, homogeneity, and ultimately, the assays' performance (27). Since low hematocrit samples spread over a larger area on the card than samples with high hematocrit levels, STDs and QCs prepared in whole blood of a different hematocrit level from those of the study samples could cause inaccurate analysis results (28). Our data confirmed that hematocrit levels can impact the bioanalytical results. The higher hematocrit samples tend to yield higher observed drug concentrations. Therefore, study samples' blood hematocrit levels should be monitored. For samples with extremely low or high hematocrit levels, the measured concentration levels may be either normalized or reanalyzed against a standard curve made in blood with the hematocrit level close to that of the samples.

From operational perspectives, several limitations of DBS assay are worth noting. (1) Because of the lack of supporting automation technology, the processing of DBS preparation and sample extraction requires extra time and manual effort when compared to the traditional LBAs. This will result in slower assay throughput. (2) Compared to the plasma or serum matrix, whole blood matrix is stable for a relatively short period of time which requires changing assay matrix frequently during the study support. This could affect assay consistency. (3) The hematocrit effect on the bioanalytical measurement may require monitoring hematocrit levels especially in patients with

suspected anemia to ensure bioanalytical accuracy (28). Due to these limitations, the expected advantages and potential operational efficiency and cost saving with DBS sampling technology might not be fully achieved.

CONCLUSION

We have successfully validated an LBA for the quantification of AMG 162 using the DBS sampling method. The validated method was demonstrated to be accurate, precise, robust, and is comparable to the serum assay for the quantification of the same therapeutic monoclonal antibody, indicating the potential of utilizing the DBS sampling method for large molecule bioanalysis. The validation results also revealed the limitations of the possible effect of blood hematocrit on drug concentration measurements and the caution required using whole blood for STD and QC samples preparation. The experiences and the lessons learned that are shared in this article can serve as a model process for other methods of validation of LBAs for large molecule therapeutics and biomarkers using the DBS sampling method.

ACKNOWLEDGMENTS

We would like to thank Dr. Jean Lee and Dr. Theingi Thway for their contributions to the critical review of this manuscript.

REFERENCES

1. Edelbroek PM, Heijden J, Stolk LML. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit*. 2009;31(3):327–36.
2. Tanna S, Lawson G. Analytical methods used in conjunction with dried blood spots. *Anal Methods*. 2011;3:1709–18.
3. Prince PJ, Matsuda KC, Retter MW, Scott G. Assessment of DBS technology for the detection of therapeutic antibodies. *Bioanalysis*. 2010;2(8):1449–60.
4. McDade TW, Williams S, Snodgrass JJ. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population based research. *Demography*. 2007;44(4):899–925.
5. Guthrie R, Susi A. A simple phenylalanine method for detecting PKU in large populations of newborn infants. *Pediatrics*. 1963;32:338–43.
6. Beaudette P, Bateman KP. Discovery stage pharmacokinetics using dried blood spots. *J Chromatogr B*. 2004;809:153–8.
7. Clark GT, Haynes JJ, Bayliss MAJ, Burrows L. Utilization of DBS within drug discovery: development of a serial micro sampling pharmacokinetic study in mice. *Bioanalysis*. 2010;2:1477–88.
8. Evans CA. Dried blood spot analysis: a paradigm shift. *AAPS Newsmagazine*. April 2010.
9. Spooner N, Lad R, Barfield M. Dried blood spot as sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of qualitative bioanalytical method. *Anal Chem*. 2009;81:1557–63.
10. Arnaud C. Technology renews a basic approach: dried blood spots offer advantages, but also challenges for pharmaceutical analysis. *Chem Eng News*. 2011;89(3):13–7.
11. Patel P, Mulla H, Tanna S, Pandya H. Facilitating pharmacokinetic studies in children: a new use of dried blood spots. *Arch Dis Child*. 2010;95:484–7.

12. Corran P, Cook J, Lynch C, *et al.* Dried blood spots as a source of anti-malarial antibodies for epidemiological studies. *Malar J.* 2008;7:195.
13. Solomon SS, Solomon S, Rodriguez I, *et al.* Dried blood spots: a valuable tool for HIV surveillance in developing/tropical countries. *Int J STD AIDS.* 2002;13:25–8.
14. Li W, Tse FLS. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr.* 2010;24(1):49–65.
15. Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis.* 2010;2(11):1791–6.
16. Heinig K, Wirz T, Bucheli F, Gajate-Perez A. Determination of oseltamivir (Tamiflu®) and oseltamivir carboxylate in dried blood spots using offline or online extraction. *Bioanalysis.* 2011;3(4):421–37.
17. Rajendran S. Dried blood spot (DBS) assays for determination of biologics in whole blood. Oral presentation at the Mini Symposium Session—Dried Blood Spot Analysis for Biotherapeutics and Biomarkers (#190), 2011 AAPS—National Biotechnology Conference, San Francisco, CA, 17 May 2011.
18. Burns D, Rajendran S, Wang J, DeSilva B, Ma M. Validation feasibility of large molecules of differing modalities using dried blood spot samples. Poster presented at the 2011 AAPS—National Biotechnology Conference, San Francisco, CA, 17 May 2011.
19. Beharry M. DBS: a UK (MHRA) regulatory perspective. *Bioanalysis.* 2010;2(8):1363–4.
20. Davis CP. Hematocrit blood test. http://www.emedicinehealth.com/hematocrit_blood_test/article_em.htm. Accessed 13 July 2012.
21. FDA. Guidance for industry—bioanalytical methods validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), May 2001.
22. EMA Guideline on Validation of Bioanalytical Methods. Committee for Medicinal Products for Human Use, 1 Feb 2012.
23. Wang J, Lee JW, Burns D, Doherty D, Brunner L, Peterson M, DeSilva B. “Fit-for-purpose” method validation and application of a biomarker (C-terminal telopeptides of type 1 collagen) in denosumab clinical studies. *AAPS J.* 2009;11:385–94.
24. Blood Bank. Wikipedia, the free encyclopedia. http://en.wikipedia.org/wiki/Blood_bank. Accessed 13 Jul 2012.
25. Wang SS. What's the shelf life of blood? *Wall Street Journal.* 1 December 2009. <http://online.wsj.com/article/SB10001424052748703939404574567771148801570.html>. Accessed 13 Jul 2012.
26. Mascheroni M. Extending the shelf life of donated blood. *Innovation.* 2008; 6(3). <http://www.innovation-america.org/extending-shelf-life-donated-blood>. Accessed 13 Jul 2012.
27. Timmerman P, White S, Globig S, Ludtke S, Brunet L, Smeraglia J. White paper/EBF recommendation on the validation of bioanalytical methods for dried blood spots. *Bioanalysis.* 2011;3(14):1567–75.
28. Denniff P, Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis.* 2010;2(8):1385–95.