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The Application of Dried Blood Spots in Toxicokinetic and Pharmacokinetic Studies

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The Application of Dried Blood Spots in Toxicokinetic and Pharmacokinetic Studies

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Abstract

Dried Blood Spot (DBS) sampling is a microsampling technique used throughout the World for neonatal screening. The work set out in this thesis shows the development and implementation of DBS in the area of preclinical and clinical pharmaceutical drug development, specifically in support of Toxicokinetics and Pharmacokinetics. The advantages of the technique are explored along with the issues faced. The papers discussed in this commentary, include in papers 1 and 2, the concept of supporting both Toxicokinetics and Pharmacokinetics studies and the validation of bioanalytical assays utilising DBS. Commentary paper 3 further explores the practicalities of DBS in the Clinical environment and commentary paper 4 the transferability of DBS technology between laboratories. Commentary paper 5 uses Incurred Sample Reanalysis data to answer questions around specific DBS issues and commentary paper 6 looks at indicating papers for Dried Plasma Spots. Commentary papers 7 and 8 explore the use of consortia to investigate hematocrit and homogeneity when using DBS and finally commentary paper 9 explores the training required to ensure quality DBS samples. The impact and contributions to this field of research are demonstrated through discussion and critical examination of selected examples of the author's peer-reviewed publications in this area. Developments of scientific practices, where the author has contributed intellectual, leadership and practical insight to achieve significant improvements in the generation of knowledge, are highlighted throughout the commentary.

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Glossary, Abbreviations and Definitions

[Bold in the text**]**

Term	**Definition**
3Rs	Replacement, Reduction and Refinement of animals Developed over 50 years ago as a framework for humane animal research
ADME	The investigation of the Adsorption, Distribution, Metabolism and Elimination of a drug
AUC	Area Under Curve
Bioanalytical/bioanalysis	Bioanalysis is the quantitative measurement of a drug and/or their metabolites in a biological matrix typically blood, plasma, serum, urine or tissue
Blanks	A sample of a biological matrix to which no analyte(s) have been added. For internally standardized methods this would contain the internal standard(s).
Blood cell association (BCA)	The partition of drug between the blood cells and plasma content of blood. Measured by spiking into whole blood, centrifuging and quantifying analyte concentration in blood cells and plasma
Calibration curve	Comparison of a set of standard samples of known concentration against samples with unknown concentrations to determine their concentration
Cmax	Maximum concentration reached within a profile

Contract Research Organizations (CRO)	Organisations that provide research services on a contract basis
Cross validation	Validation of an assay across multiple laboratories using QCs or pooled samples
Direct analysis	Analytical techniques that eliminate manual extraction steps required for sample preparation prior to sample analysis
Dried Blood Spots (DBS)	The use of solid support to collect blood samples
Dried Plasma Spots (DPS)	The use of solid support to collect plasma samples
GSK	GlaxoSmithKline Research and Development Ltd.
Haemodynamic effects	Dynamics of blood flow
Hematocrit (hct)	The proportion, by volume, of the blood that consists of red blood cells and is expressed as a percentage. For example, a hematocrit of 50% means that there are 50 mL of red blood cells in 100 ml of blood
Hemolysis	Red blood cells in plasma
HPLC	High Performance Liquid Chromatography
Homogeneity	A mixture that has the same proportions of its components throughout a given sample
Incurred Sample Reanalysis (ISR)	A repeated measurement of analyte concentration from study samples to demonstrate reproducibility
In vitro	In body
In vivo	Out of body (in glass)

LC-MS/MS	Liquid Chromatography interfaced to a Mass Spectrometer (often shortened to this even when MS fragmentation is carried out i.e. LC-MS/MS or LC-MS^n)
Lipemic	Lipids (fats) in the matrix
LLQ (s)	Lower Limit of Quantification; lowest concentration at which the analyte can be detected with predefined bias and precision
Metabolite	Product of metabolism (biotransformation of parent drug, or of another metabolite)
Microsampling	Defined as <50 µL of sample
MS	Mass Spectrometry
No Adverse Effect Level (NOAEL)	The highest experimental point that is without adverse effect
Pharmacokinetics	Study of the time course of drug absorption, distribution, metabolism, and excretion – described through mathematical models
Pharmacodynamics	Study of the biochemical and physiologic effects of drugs in humans
Post Column Infusion	Checks for suppression effects. Compound of interest is infused into the MS and extracted total blanks are injected. A change in the baseline shows suppression or enhancement
Quality controls	A sample with a known quantity of analyte that is used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples

	analyzed in an individual run
Total blank	A blank which does not contain the drug(s) or internal standard(s)
Toxicokinetics	The study of PK at high doses using toxicology studies where absorption and clearance mechanisms may be saturated
Toxicodynamics	Study of the biochemical and physiologic effects of drugs in animals

Figures

Tables

Declaration

I hereby declare that the basis for this work is as described in Appendices to this Commentary and as otherwise acknowledged.

This work has not been previously submitted for a degree to the University of Lincoln or at any other University.

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• My Mum for providing me with the opportunity to step on the path to this in the beginning – I know that she is very proud of me and I thank her from the bottom of my heart

• Last but by no means least, my wife Karen and two daughters, Sophie and Amy who have put up with me whilst I have struggled through this process and have provided love, support and encouragement throughout. I now promise to get to the list of jobs that have been building

Scope of the Commentary

This commentary focuses on the use of Dried Blood Spots **(DBS)** in support of **Toxicokinetic (TK)** and **Pharmacokinetic (PK)**, studies exploring the learnings' and expertise gained by the author and subsequently shared with the industry. The publications give strong evidence of the significant role in which the author took in implementing DBS and **Microsampling** and provide a critical commentary on both the successes and issues associated with the technique.

The papers selected herein span the conceptual idea of using DBS in support of both preclinical/clinical analysis and gives a framework for the experiments required to scientifically demonstrate the validity of the **Bioanalytical** data. In depth investigations into clinical practicalities, both from a patient and nurse's perspective, are explored and further validation of the technique including **cross validation**, **Incurred Sample Reproducibility (ISR),** sampling sites and comparisons with wet samples are considered. Taking the DBS principle and applying to plasma resulted in **Dried Plasma Spots (DPS)** and the novel use of indicating papers for simple sample identification. Experiences of unifying the industry through consortia to give an industry perspective on key issues (**hematocrit** (hct) and homogeneity) are considered. Finally, practicalities of multi-site clinical studies and how to successfully implement DBS in later stage development are discussed.

The impact of the author's contributions to the development of DBS and microsampling approaches, describes the positive impact on aligning the community from pharma, academia and manufacturers together in order to offer solutions and new ideas to make microsampling a reality and standard platform.

1: Introduction

The pharmaceutical industry is obliged to use animals to ensure medicines are fit for human use (Regulation, 1986 (Revised 2013)), but the industry has always been interested and encouraged to look for opportunities to minimize the use of animals in the drug discovery and development process (Cox, 1990, Michael F.W. Festing et al., 1998). The author's interest in DBS began in 2005 on finding a poster exploring DBS as a sample collection technique in support of **metabolite** identification (Bateman, 2005). Further research into the subject revealed its potential benefits from a Reduction and Refinement (2 of the **3Rs)**, clinical and financial perspective compared against conventional sampling and work began to investigate how to introduce the technique and its utility in supporting TK and PK studies.

The thesis will focus on the background information and principles associated with DBS in support of bioanalytical methods for TK and PK studies. In particular validation of quantitative methods utilizing DBS will be addressed along with advantages and disadvantages of the technique in support of the development of medicines. The basis of the commentary will be the published works of this author, demonstrating the novel application of DBS in the drug development process and providing evidence for the significant and sustained contribution that this author has made to microsampling over many years. Finally, the impact of the work to the wider community will be discussed.

1.1: Toxicokinetics, Pharmacokinetics and Bioanalysis

By quantifying the concentration of drug in the systemic circulation of humans, PK models can be built which describe the effect the body has on the drug (e.g. Adsorption, Distribution, Metabolism and Elimination (**ADME**)). The mathematical model of the relationship between PK and **pharmacodynamics** (PK/PD) can be built to help understand the efficacy, or lack of, observed in clinical trials and preclinical animal models. Thus a PK assessment is vital to drug development. Blood, plasma or serum are commonly selected to measure drug concentrations, although the systemic circulation is usually not the target of drug action. However, the systemic circulation is

easily accessible for sampling purposes and there is a fundamental assumption that unbound drug levels in the circulation reflect those in the target tissue (Emmons and Rowland, 2010).

Prior to administration to humans, toxicology studies are undertaken to identify potential hazards to humans. A TK assessment is vital to this risk assessment. Knowledge of the relationship between TK and **toxicodynamics** (TK/TD) can be extremely useful, especially in, for example, cardiovascular studies where TK/TD models can be built to understand **haemodynamic effects**. However, the biggest use of a TK assessment is to determine a safety margin with respect to concentration/exposure rather than dose between animal systemic exposure at the **No Adverse Effect Level (NOAEL)** and human systemic exposure.

To quantify circulating drug concentrations accurately and precisely in humans and animals requires validated bioanalytical methodology which assesses a range of parameters (chapter 1.8). At the relatively high doses used in toxicity studies, assay sensitivity is generally not an issue, but at the relatively low therapeutic doses employed in the clinic, the assay must be sufficiently sensitive to permit an accurate estimation of systemic exposure. Bioanalysis is a heavily regulated area as patient safety is at risk if circulating concentrations are not accurately measured.

Blood is the obvious choice to measure circulating concentrations, but it has always been perceived as a difficult matrix to analyse from a storing and shipping perspective. Typically 50:50 blood:water (to lyse the cells) has historically been used when freezing the blood (more recently it has been proven that whole blood can be used, refer to chapter 2.4.4), which requires an accurate volumetric measurement to obtain the correct ratio. In practice this is not possible in Drug Development with far larger studies spanning across many continents and many people that would need training to carry out the procedure. Generally, in Development, plasma has historically been used to support TK and PK, as it is fairly simple to harvest (centrifuge whole blood at approximately 3000 g) ship, freeze and store. Also, blood is colored and interferes with assays with a colorimetric endpoint, whereas plasma is compatible with such techniques. The main issue with plasma is that approximately 50% (depends on affinity for plasma versus cellular compartments) of the blood sample is wasted when

deriving plasma (Emmons and Rowland, 2010), which means larger sample volumes are required to support developing medicines. A review of methods in 2005/2006 (Fang et al., 2006, Kubo et al., 2005, Trivedi et al., 2005) typically shows aliquot volumes of 100 µL – 500 µL, which would equate to up to 2mL of sample required per time point.

This wastage and the following examples summarised below and discussed further in this chapter are why the author and industry have explored techniques such as DBS in order to reduce and drive down the sample volumes required to develop new medicines (Spooner, 2010):

- increasing pressure from society to promote the ethical use of animals in research embodied as the 3Rs of animals. DBS sampling can lead to notable reductions and refinement in animal use, particularly rodents;
- the recent requirements of pharmaceutical companies by regulators to file their first applications to market new drugs for children, as well as adults where appropriate, has led to the search for techniques to handle the small blood samples to support paediatric drug studies and the juvenile rodent toxicology studies used to support their safety;
- the need to provide appropriate medicines for, and conduct clinical studies in developing nations and emerging markets, where clinics do not always have the equipment and infrastructure for deriving, storing and shipping of plasma samples (e.g., the availability of centrifuges and freezers and/or dry ice for shipping).

1.2: Dried Blood Spots

DBS sampling is a microsampling technique, which is a relatively simple technique for collecting small volumes of blood, and offers a way of removing the need to freeze blood samples and move away from wasteful plasma harvesting. Blood is collected *via* a skin prick (from a finger or heel) or venous cannula. The blood is collected often by a glass capillary or into a tube and then pipetted onto a substrate, typically a cellulose or

polymer based paper (figure 1). The samples are allowed to dry for a minimum of 2 hours and, where suitable analyte stability has been demonstrated, can be stored and shipped at ambient temperature and analysed by a host of different analytical techniques.

For the application of TK and PK Bioanalysis, typically 3 x 15 µL samples are taken at each time point; this allows for an original analysis, ISR (Timmerman et al., 2009) and any repeats.

The first report of DBS dates back to the analysis of glucose in 1913 (Schmidt, 1986) and utilized filter papers. Other early adopters (1925-1950) reported interesting benefits when using DBS for syphilis testing. Notable advantages included reduced sample volumes, inexpensive as using filter papers, enhanced stability and reduced chance of spoiling the sample (Zimmermann, 1939, Anderson et al., 1961, Harris and Olansky, 1951, Chapman, 1924). The most known, and often mistakenly considered as the first publication, using DBS was published by Guthrie in 1961 (Guthrie, 1961) for the detection of phenylketonuria in newborns. Guthrie coupled DBS with an agar diffusion microbial assay and also collected urine on paper for analysis. This was the start of mass screening of infants across the world and today the modern test is still referred to as the Guthrie Test and checks for up to 54 conditions utilizing multiple analytical procedures. Prior to the author's first publication, there were few publications focusing on the technique for TK (Tawa et al., 1998, Posyniak et al., 2002) and PK (Beaudette and Bateman, 2004). As these papers didn't explore the potential benefits that DBS offered (3Rs, paediatrics and home sampling) they did not excite the community and have the impact they deserved.

Figure 1. Example of DBS card's

1.3: Advantages of DBS for TK and PK analysis

DBS offers advantages over conventional 'wet' plasma, which historically has been the main matrix used in TK and PK bioanalysis. Advantages for TK are summarized below and The NC3Rs website (NC3Rs) and ICH SA3 guidelines (ICH, 2014) cover in detail expectations and advantages:

Reduction: The largest influence on the number of animals in toxicity studies is TK profiling (Sparrow et al., 2011). Conventional plasma samples require >250 µL of blood (yielding approximately 125 µL of plasma) compared with <50 µL for DBS per TK time point. Due to the large volume required for plasma samples, TK satellite samples (rodents only) are used so as not to impact the main study animals (e.g. Hematology data can change due to the blood loss). Also regulations govern the amount of blood that can be drawn from an animal per day (Diehl et al., 2001). Taking conventional TK samples along with multiple blood draws to support such studies as hematology from the main study animal would exceed the maximum limit and therefore is not a viable option. The ability to microsample has the potential to drastically reduce rodent use. Even the simplest of studies (7-day dose range finder study) uses 12 TK satellite animals, which would not be required if microsampling were employed.

Higher quality data: The removal of TK satellite animals means that the data for both toxicity and exposure is derived from the same animal. This makes interpretation much simpler as a direct comparison in the same animal can be made. Reduced sample volumes also allows for serial sampling rather than composite. The generation of concentration profiles from an individual animal rather than across multiple animals generally leads to less variability as inter-animal differences are removed (Sparrow et al., 2011, Wickremsinhe and Perkins, 2015).

Refinement: Ability for simplified sampling sites (Stokes et al., 2011) and removal of animal warming. Taking large blood volumes from animals is only possible if the animals are first warmed which makes the blood less viscous. Options for animal warming include the use of hot box's or water baths with the animals warmed for approximately 10 minutes. These benefits result in causing less stress to the animal which is an obvious advantage but also reduces the likelihood of adverse events.

Economic: Removal of satellites reduces the amount of compound dosed and also the cost of animals and housing. A typical 28-day rodent study would save approximately 335g of drug substance (24 fewer animals, weighing 250g for 28 days at 2000mg/kg/day) and result in a saving of approximately £1000 in animal and husbandry costs.

1.4: Advantages for PK are

Access to paediatric populations: Many of today's medicines could have major positive impacts on the welfare of paediatrics. Studies have shown that over 50% of the medicines used for children might not have been tested for use in this specific age group (EU). Regulators have recognized this need and now encourage companies to test medicines in infants as a first intent. Regulators allow extended patents to medicines that comply (EU) . Paediatric studies do pose many challenges, including limited blood volumes and acquiring parent's consent to allow sampling. DBS sampling offers the opportunity to overcome these issues due to the small blood volumes withdrawn and the ability to utilize different sampling techniques. Both

finger and heel pricks can be used to obtain DBS samples (limited number of sticks allowed) and these are seen as less intrusive and more acceptable to parents, which aids recruitment.

Access to remote areas and home sampling: DBS requires less processing than plasma samples; less invasive sampling, no requirement to centrifuge, ambient storage (removal of -20°C and -80°C storage) and ambient temperature shipments (removal of dry ice requirements) where analyte stability has been demonstrated. All these factors combined, allow studies to be conducted in previously unconsidered applications. Examples are sampling in remote locations, home sampling and the cost savings associated (Martial et al., 2016).

1.5: Advantages associated with both TK and PK

Simplified workflow: Figure 2 demonstrates preclinical workflow advantages but also applies to clinical support. DBS removes the centrifugation, decanting, capping and freezing/defrosting steps.

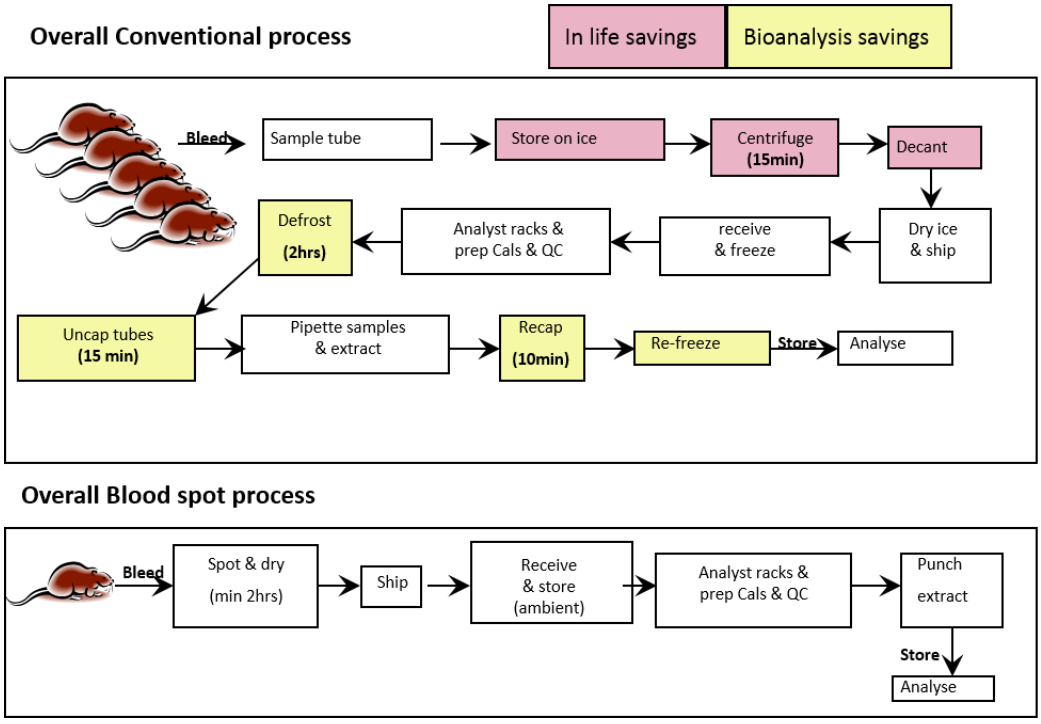


Figure 2. Process flow showing difference between conventional and DBS sampling

Stability: It has been proven that a DBS sample is generally more stable than a liquid sample (Bowen et al., 2010, D'Arienzo et al., 2010). Enzymes are rendered inactive in a DBS sample and are unable to degrade the compound. Generally, if the compound of interest is stable over the drying period, it will be stable on the card for many months and possibly even years. However, the sample must be protected from high humidity (Denniff et al., 2013, Denniff and Spooner, 2010b) as the sample must remain in a dry state.

Storage and shipping: Storage and shipping is at ambient temperature for DBS compared with liquid samples, which are generally stored at -20^{0}C or -80^{0}C and shipped on dry ice. This has major financial advantages, with a dry ice shipment costing in excess of £500 and banks of freezers being costly to run. There is also an environmental advantage through power saving (generating dry ice and freezers). In the US DBS cards are transported using the conventional postal service as DBS cards are considered as nonhazardous if double contained (Service, 2013).

1.6: Issues associated with DBS for TK and PK analysis

Many advantages of DBS have been explored previously but there are issues associated with DBS which the author has explored and reported. Also not all advantages can be fully realised. Shipping companies require minimum box sizes and to make full advantage of reduced shipping costs, other options would need to be developed e.g. the use of Fedex. Also using conventional postal services would never be an option, as supply needs to be guaranteed due to ethical and financial reasons. Also within GSK in **Good Laboratory Practice (GLP)** Toxicology studies, TK satellite animals have not been removed in part due to skepticism and reluctance to change. There is a growing body of evidence in supporting microsampling (Mitchard et al., 2017, Poitout-Belissent et al., 2016, Caron et al., 2015, Chapman et al., 2014, Niu et al., 2016, Prior et al., 2015) but there is still more need for companies to join together to share data covering a diverse chemical space and build a convincing factorial scientific justification that using microsamples to support TK from the main study animals will not impact any other measurement. With satellite animals still being used in GLP

studies at GSK the option of reducing the size of animal facilities is also currently not a potential advantage.

Hematocrit: Hct values vary between healthy humans (table 1) with the greatest variance observed with infants below 1 month of age. Also drugs can change hct based on mechanism of action (e.g., increased production of RBC's), or secondary effects (e.g. dehydration) or disease state/illness (e.g. bone marrow toxicity). In preclinical species hct is generally better controlled but can be affected by drugs and disease states.

Age/Sex	Hematocrit (%)
0-2 years	28-67
2-12 years	34-45
12-18 years female	36-46
12-18 years male	37-49
Adult female	36-44
Adult male	41-50

Table 1. Human hematocrit values across sex and age (Denniff and Spooner, 2010a)

Varying hct impacts the size of the DBS sample. As the hct increases the viscosity of the blood rises, causing the blood to spread less across the substrate (figure 3). This results in a linear, inverse relationship between spot area and hct (De Kesel et al., 2013). Higher hct values give rise to smaller, more concentrated spots. This is an issue when taking a small central sample punch and can drastically impact PK and cause misleading outcomes. A small central punch takes away the necessity for animal technicians and clinical staff to accurately spot a fixed blood volume, and places the burden on the analytical lab who are better placed to deal with accurate measurements. The simplest way to overcome varying hct between subjects is to accurately dispense a fixed volume of blood and punch out the entire spot. This relies on an accurate volume of blood to be spotted, which in reality is not possible with current technology, as calibrated pipettes or capillaries are required which require trained personnel to operate. Novel solutions are being investigated and developed which will be discussed later in the thesis (chapter 2.7.4).

Recovery - To further complicate the hematocrit issue it has been reported (De Vries et al., 2013) that, in some cases, as hematocrit increases the recovery decreases, which can mask the increase in concentration due to a smaller more concentrated spot with higher hct. Also reduced recoveries in aged spots can be observed (De Vries et al., 2013) upon extended storage, which in turn can seriously limit the viability of long term storage for some analytes.

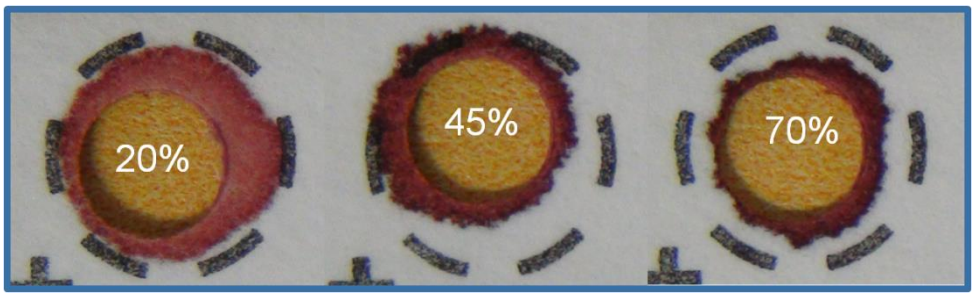


Figure 3. Change in spot size with changing hematocrit

Homogeneity: The distribution of analytes across the card should ideally be constant. Chromatography on the substrate can impact the homogenous nature of the sample, with some analytes having greater affinity for the substrate than the blood and vice versa (O'Mara et al., 2011). This can be likened to affinity chromatography with the paper substrate acting as the solid phase and the blood as the liquid phase (Fanali et al., 2013). Analyte, paper substrate and hct all impact the homogeneity of the sample. Some papers have less impact and blood with a larger hct (70%) tends to produce more homogeneous samples when spotted. There are two ways to overcome this, either spot an accurate volume of blood (which poses its own issue) and punch the entire sample or always punch from exactly the same place for each assay. Both are dependent on the accuracy of the user and cannot be guaranteed. Some paper manufacturers are attempting to create new substrates that guarantee a homogenous sample that would overcome the issue, but with so many variables, this may not be possible.

Regulatory: Microsampling is encouraged by all regulatory authorities (Beharry, 2010, ICH, 2014), including the U.S. Food and Drug Administration (FDA) and accept that blood is a recognized matrix to support PK and TK analysis (guideline, 1994). In such a highly regulated area, agencies must be confident that the data reported is a true reflection of the circulating concentration and public health is critical. As DBS is relatively new, in this setting, the agencies are cautious and require a large body of evidence to allow DBS as the first intent. Agencies for clinical studies have been requesting both conventional and DBS data to be presented (Evans et al., 2015, Xu et al., 2013). However, this has a very large economic burden on the industry and ultimately doubles the cost of the Bioanalytical phase. This is not acceptable to project teams and is causing major concern with companies not wishing to spend the resources and regulators not allowing DBS to be the only method to obtain circulating concentrations. A stalemate is in place with no group making any progress.

Sensitivity: Taking a 3mm center punch from a DBS sample yields a 2.1 µL sample, which can pose issues when attempting to achieve pg/mL assay sensitivities. Mass spectrometers are, however, becoming progressively more sensitive. If data issued by the manufactures can be believed (it is not published), on average each new generation machine should give between 5 - 10 fold increase in sensitivity. From the introduction of **Mass Spectrometry (MS)** into the bioanalytical work stream, currently we have access to the 7th generation MS. With the 1st generation machines pg/mL assays required large samples volumes, typically 1 mL. Using microsamples today should allow equivalent **Lower Limits of Quantitation** (**LLQ)** and in some cases this is the case. However, there is a tradeoff between analyte sensitivity and background (as analyte signal increases so does the background) so often any gain is swamped by a gain in the background noise.

Bioanalytical resource: When DBS was first introduced into the bioanalytical laboratory it was harder to develop assays and no automation was available. Multiple paper types and solvents needed to be evaluated up front before any *in vivo* work could take place. As the technique became more embedded the impact on resource reduced. The implementation of dedicated automation, including **direct analysis** tools and generic workflows, increased efficiencies and this became less of an issue.

There are advantages and disadvantages associated with DBS and some of the disadvantages were only discovered as the technique was further investigated. The advantages to animals, paediatric studies and home sampling are obviously key to the continued interest in DBS and microsampling.

1.7: Early DBS work prior to publication (Evans, 2010)

When the author presented the concept of DBS to the bioanalysis group, the first comment was "this technique has been around for years, why would we use it?" After discussing the potential benefits and gaining interest amongst the group, the author started to investigate the DBS application to support TK. To convince any bioanalyst of any new technique, an analytical validation is required. A validation was carried out on a compound in early development, concentrating on precision and accuracy, on card stability and robustness to spotting volume (described in section 1.8). The technique proved to be simple and resulted in a precise and accurate method (e.g. precision and accuracies less than 15%). This data then allowed extra resource to be given within bioanalysis and 10 compounds were validated across multiple scientists to ensure the technique was robust across multiple people. Simple chemical properties are shown in table 2 to demonstrate that a diverse range of molecules were chosen. Analysts on questioning found the technique easy to use and the assays were fit for purpose.

Compound	cLogP	Polar Surface Area Å	Assay LLQ (ng/mL Rat Plasma)	Blood Spot Recovery (%)	Blood Spot Assay Range (ng/mL)
1	3.02	76.07	20	98.4	2.5-1000
2	5.29	102.49	0.1	91.7	0.1-100
3	5.06	71.53	1000	103.0	0.1-100
4	2.82	46.53	2500 (human)	93.0	2.5-250
5	3.86	73.56	2	93.4	2-1000
6	2.79	84.56	-	88.3	2-1000
7	3.98	71.09	5	92.4	1-1000
8	4.29	79.29	2	90.0	10-1000
9	5.32	75.13	2	47.3	1-1000
10	3.75	97.12	-	94.7	10-10000

Table 2. Chemical properties of 10 compounds for development work

These validations confirmed that, from a bioanalytical standpoint, samples from DBS supported validations that were fit for purpose. The next question was the ***in vivo*** comparison of conventional wet blood (50:50 blood:water) versus DBS. Does DBS give the same concentration from *in vivo* samples when compared against blood:water? To answer this question, 12 ongoing DMPK rat studies were chosen and excess blood was used to generate DBS samples alongside conventional wet blood samples. The bioanalytical approach was to use fit for purpose assays, employing a generic **HPLC** gradient, comparing peak area ratios and the same assays used to support both sample types.

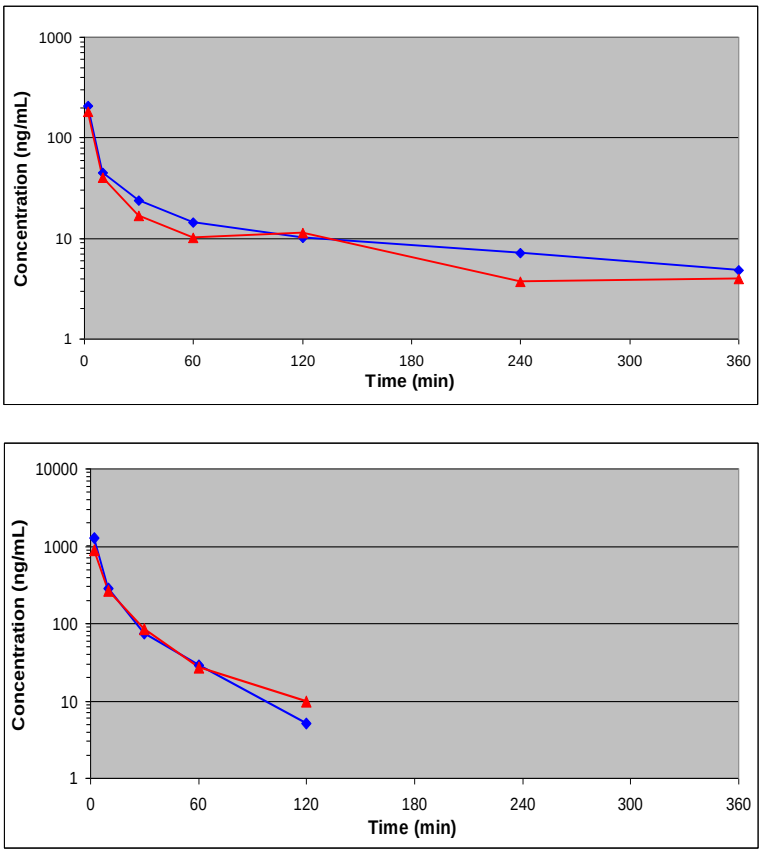


Figure 4. Example from the 12 compounds - Blood:water & DBS comparison

All 12 compounds showed no significant difference (<5% in all AUC) between the two sample types (figure 4). This data allowed the creation of a partnership between Bioanalysis, Toxicology and the Animal Scientists, as it was vital to gain acceptance of the new approach across these disciplines. The team was made up of individuals open to critical assessment of DBS and a strategy was developed to validate DBS across the TK platform worldwide. Unlike Discovery studies, which utilize blood, historically plasma was used to support TK, and therefore this had to be taken into account. 10 non GLP, 7 day, rat TK studies formed the pilot, with 3 samples being generated (plasma, DBS and 50:50 blood:water). Assays were generated for each compound, independent of matrix type and also ***in vitro*** **blood cell association (BCA**) data was generated, to assess if DBS and blood data could be directly compared with plasma using the BCA correction value. Alongside this investigation, the animal scientists were looking at new blood sampling techniques to be able to minimize any blood wastage. This included different bleeding sites and also collection methods.

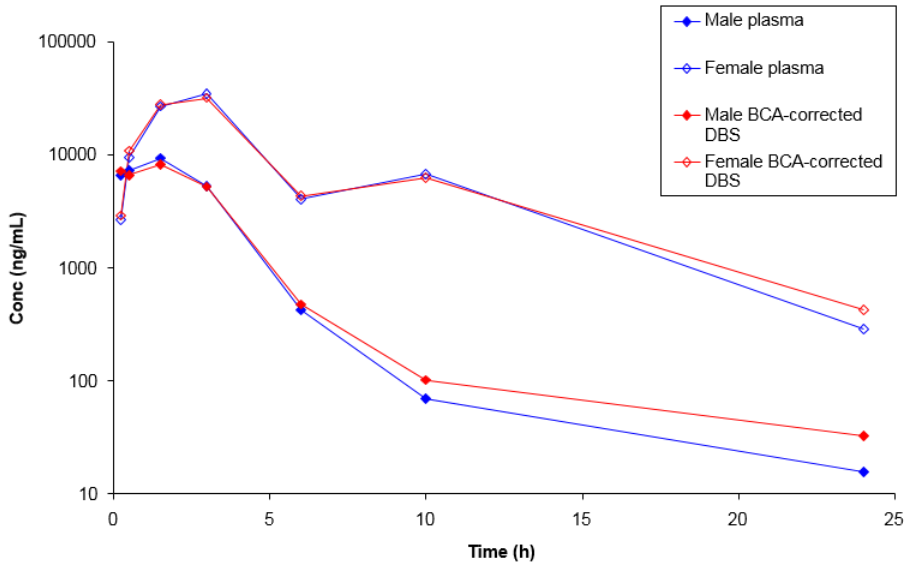


Figure 5. Comparison of plasma against blood

The results showed no notable differences were observed that would impact the interpretation of TK between DBS and blood. The *in vivo* plasma concentrations (figure 5) always had the same profile compared with the blood but were offset, which was expected (different compounds often partition more readily to blood cells or plasma). What wasn't expected is that the *in vitro* BCA value did not always correct and overlay the data, however no pattern to these discrepancies could be identified. This could be due to the magnitude, how the blood was collected, age or even temperature of the blood being used for the test (Abu-Rabie and Spooner, 2010). The result was that DBS was considered to be a viable sampling technique but plasma and blood could not simply be interchanged. Once a compound entered Development the matrix was chosen and that matrix would be used throughout the development of the drug in the pre-clinical and clinical stage (Emmons and Rowland, 2010).

DBS sampling was endorsed as the preferred technique for all non GLP TK studies for the development of small molecule oral drugs.

During these developments, the author forged strong links with the paper manufacturers, initially Whatman, who were subsequently purchased by GE Healthcare. Time was spent focusing on the format of the card for this DBS application and a 4 spot card (figure 6) was designed and implemented, allowing for 3 samples to be taken, (sufficient for original analysis, pharmacokinetic repeats and ISR analysis)

and a spare spot if an error occurred. Room was incorporated for a label and spot rings were included to direct the sampler to where to place the blood. Also small boxes above the spots were added to allow the sampler to indicate if there was anything wrong when conducting the process (a cross could be placed in the box indicating not to use that sample). A rigid card design was chosen for easy handling and this card became the industrial standard format for all manufacturers. The size of the card was equivalent to a credit card, which was also compatible with a commercially available automated puncher (BSD Biosampler punch). This punch was an off-the-shelf product but working with the manufacturer, a camera was included which could detect the center of the spot so as to take a central punch sample.

This method development and validation stage took approximately 3 years and this work led to the first pivotal paper (chapter 2.1) and subsequent publication strategy.

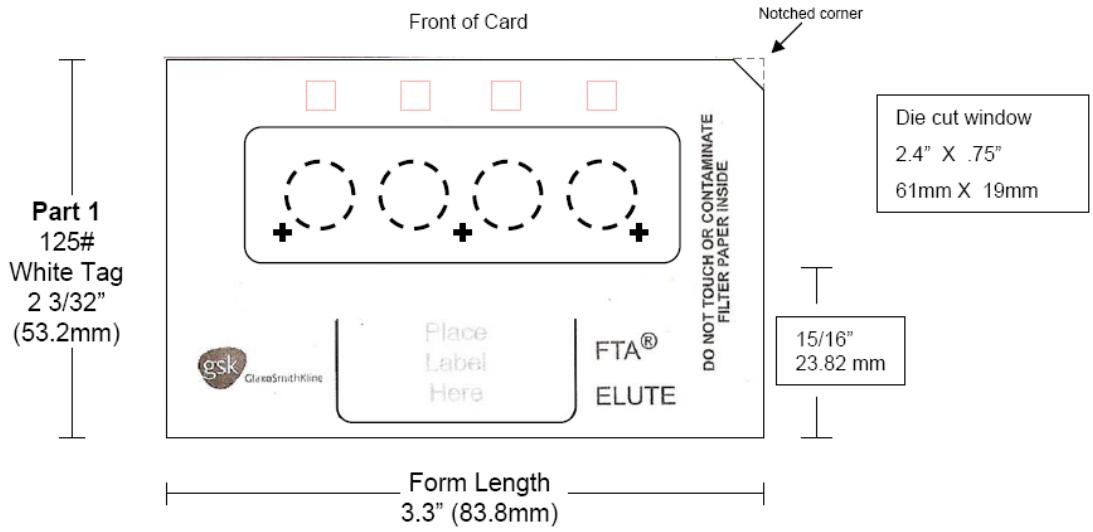


Figure 6. Schematic of a DBS card for TK and PK support at GSK

1.8: Bioanalysis – Validation of LC/MS/MS assays

The aim for any bioanalytical assay is to demonstrate that the measured concentration accurately and precisely reflects the concentration of the circulating drug/metabolite at the time of sample collection (Summerfield et al., 2016). This measurement could be in a different country from where the sample was taken and sometimes years after the sampling occasion. Methods are validated in accordance to regulations (Shah et al., 2000) to ensure data quality. Quantitation is performed by spiking relevant control matrix, with known concentrations of the analyte(s) in question. This is used to construct a **calibration curve** from which the concentration of analyte(s) in control and unknown samples can be determined. **Quality controls (QC)**, **blanks** and **total blank** samples are typically included to monitor the performance of an analytical method during an analytical run.

A Bioanalytical method consists of sample preparation, detection and validation.

Sample preparation and detection

Biological samples are complex in nature, containing not only the analyte(s) to be quantified but also endogenous materials. Sample preparation techniques aim to remove the unwanted materials and ultimately produce a cleaner sample to aid robustness, limit variability and on occasions concentrate the sample (Anjana Vaghela, 2016). The three most common techniques are protein precipitation, solid phase extraction (**SPE**) and liquid-liquid extraction (**LLE**). All methods can be used as a sample clean up for DBS (Oliveira et al., 2014, Liu et al., 2010) but for most methods at GSK, the simple addition of methanol (containing I.S.) to the DBS sample has allowed suitable assays to be developed, validated and supported both TK and PK studies. This makes sample preparation for DBS extremely fast and simple.

The introduction of **LC-MS/MS** into Bioanalysis (Jemal, 2000) in the early nineties revolutionised bioanalysis, allowing greater sensitivity and selectivity, shorter run times and allowing simpler extraction techniques. Mass spectrometers have become increasingly sensitive, and readily available, which allows bioanalytical assays to be

developed that are compatible with the small blood volumes typically used with DBS sampling.

Validation

Often the phase of the molecule in product development dictates the extent of validation that is required. An early discovery assay will have no validation, whereas an assay supporting late phase development asset requires a full validation. That said an assay is never fully tested and only real samples often actually unearth issues that cannot be mitigated through validation e.g. drug-drug interactions. Table 3 details the requirements for a full DBS validation at GSK, and was built around the experimental work and learnings' made by the author and colleagues. This is not an inclusive list and does not include basic validation procedures e.g. linearity, precision and accuracy.

Test	Description	Acceptance
Selectivity	Demonstrate selectivity by analyzing a minimum of six independent sources of the drug-free biological matrix, e.g., individual lots as total blanks.	Any observed interference (as defined by peak height) should be no greater than 20% of the analyte response at the LLQ and no greater than 5% of the internal standard response
Carry Over	Assess carry-over by injecting **blank** sample(s) after a high concentration standard or validation sample.	≤ 20% of LLQ response
Recovery	Assessed at three concentrations (low, medium & high) at three different levels of hematocrit (e.g., 25%, 45% (typical control matrix) and 65%) for a total of nine samples. Compare the response (peak area or peak area ratio) of QCs spiked with analyte prior to extraction (6 replicates per concentration) with the response of blank matrix spiked at the same concentrations post extraction, which represent 100% recovery (6 replicates per concentration).	The recovery of an analyte should be precise (CV ≤ 15%) at each of the concentrations. If the spread of mean recovery values across the three concentrations exceeds 25%, then an investigation should be conducted and documented

Matrix effects	Compare the response of matrix blank samples spiked after extraction with analyte at three concentrations (low, mid and high) with those of matrix free samples at the same concentrations. At least six sources of matrix	The extent of the matrix effects between the separate sources of matrix should be precise (CV ≤15%) at each concentration.
Assay robustness to pipetting error	Performed when taking a fixed diameter sub-punch at three different volumes corresponding to the volume specified in the method and to 5 µL increments above and below the target (i.e., the method states to spot 15 µL; then evaluate the robustness of the method with 10 µL, 15 µL and 20 µL spots). Spot the DMS cards with the appropriate volumes in replicates of six at two concentrations (low & high). The diameter of the disc removed from the spot must be the same as listed in the method.	The within-run precision (CV) and bias (%) at all concentrations should be ≤15%
Impact of hematocrit	To assess any potential impact of hematocrit on the resulting measured quantitative concentrations, test validation samples (in replicates of six) at two concentrations (low & high) in whole blood, at hematocrit levels bracketing the anticipated hematocrit range, are analyzed. For normal subjects, this corresponds to 25% and 65%. These samples should be analyzed using a calibration curve and QC samples with a hematocrit level of 45%. If the anticipated hematocrit range of subjects is expected to be shifted outside of normal, based on population or disease state, the investigated hematocrit levels for validation and calibrants can be adjusted as appropriate	The results of QC samples at each concentration with hematocrit levels (e.g., 25% and 65%) should be within 15% of that for the QC samples with the standard hematocrit level, e.g., 45%.
Analytical stock stability	Determine the storage stability of the analyte(s) in analytical stock solutions for 6 hours at ambient temperature, plus appropriate long term storage conditions (e.g., freezer or refrigerator storage). If working solutions are stored, then test their stability at two concentrations (typically low and high). At the appropriate time interval, compare the stored solution to be tested with a fresh solution which has been accurately prepared at an equal concentration. Dilute the solutions (each in replicates of six) to a concentration within the linear response range of the instrument, using an appropriate diluent containing internal standard.	The difference between the analyte: internal standard mean peak area ratios of the stored and fresh solutions should be ≤5% for stability to be demonstrated

Short term stability	The conditions evaluated should include room (ambient), -20˚C, and 40˚C temperatures and should be evaluated at the maximum anticipated storage time under those conditions. Experiments should include validation samples or other suitably prepared samples (in replicate of six), at two concentrations (low & high), maintained at typical ambient conditions, -20˚C, and 40˚C for a minimum of 48 hours, to evaluate a "worst" case for sample stability (Bowen et al., 2011).	The average result of the short term storage samples at each concentration should be within 15% of the nominal concentration (or of the Time 0 response in the case of whole blood stability).
Whole blood stability	Test fresh whole blood (≤48 hours from collection) up to 37°C for 2 hours. Use for this stability experiment where possible, with the same anti-coagulant. At each concentration to be tested, directly extract six replicate aliquots (using internal standard) immediately after spiking into blood at 37°C, and again after typically 2 hours' incubation at 37°C and if required at ambient and on wet-ice.	At each concentration and temperature tested, the difference in mean (analyte: internal standard) peak area ratio between fresh and stored whole blood must be ≤15%.
Long term stability	Determine long term storage stability at ambient temperature of analyte(s) in the appropriate biological matrix, to cover the longest time interval between collection of individual samples and the date of their last analysis, including analytical and PK repeats, and incurred sample reanalysis. This determination may be made through ongoing assessment of results for study QCs, validation samples, or other appropriate samples that are prepared at or before the time of sample receipt, stored with the study samples under identical storage conditions, and analyzed against freshly prepared calibration standards. Assess stability using six replicates per concentration, measured against a freshly prepared calibration line. Include freshly prepared QCs in the same run, in order to verify the integrity of the calibration standards.	Within 15% of the nominal concentration
Re-injectability	This may be evaluated by re-injecting an entire validation run at 24 hours, 48 hours, or other appropriate time interval after completion of its initial run.	Acceptance criteria as any validation run.
ISR	Take a minimum of 10% of study samples and re-extract in a unique run. Not required for all studies.	At least 66.7% (2/3) of the repeat results should be within ±20% of the mean (of original and repeat result).

Table 3. Validation requirements for DBS assays

2: Principle Papers Published as basis for this Commentary

1. **Matt Barfield**, Neil Spooner, Rakesh Lad, Simon Parry, Susan Fowles. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies: Journal of Chromatography B, 2008.

2. Neil Spooner, Rakesh Lad, and **Matt Barfield**. DBS as a sample collection technique for the determination of pharmacokinetics in clinical studies: Anal Chem, 2009.

3. Neil Spooner, Y Ramakrishnan, **M Barfield**, O Dewit & S Miller. Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations: Bioanalysis, 2010.

4. Phillip E Turpin, Josephine EC Burnett, Lee Goodwin, Amanda Foster & **Matthew Barfield**. Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories: Bioanalysis, 2010.

5. **Matthew Barfield**, Sheelan Ahmad & Maria Busz. GlaxoSmithKline's experience of incurred sample reanalysis for DBS samples: Bioanalysis, 2011.

6. **Matthew Barfield** and Robert Wheller. Use of dried plasma spots in the determination of pharmacokinetics in clinical studies: validation of a quantitative bioanalytical method: Anal Chem, 2011.

7. Ronald de Vries, **Matthew Barfield**, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Zoe Cobb, Steve White & Philip Timmerman. The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium: Bioanalysis, 2013.

8. Zoe Cobb, Ronald de Vries, Neil Spooner, Stephen Williams, Ludovicus Staelens, Mira Doig6, Rebecca Broadhurst, **Matthew Barfield**, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Steve White & Philip Timmerman. In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium: Bioanalysis, 2013.

9. Tina Panchal, Neil Spooner, **Matthew Barfield**. Ensuring the collection of high quality dried blood spot samples across multisite clinical studies: Bioanalysis 2017.

These specific papers were selected as they provide an in depth understanding and demonstrate the author's contribution to the advancement of DBS in this area of research.

Each of the papers is summarised and discussed in section 2. Citation numbers were collected on the 11th of May 2017 from Scopus.

See Appendix for supporting letters from lead authors and detail of the contributions made by this author to each of these published works.

2.1: Commentary paper 1

Matt Barfield, Neil Spooner, Rakesh Lad, Simon Parry, and Susan Fowles (2008). Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. Journal of Chromatography B, 870 (2008) 32–37.

Citations- 190

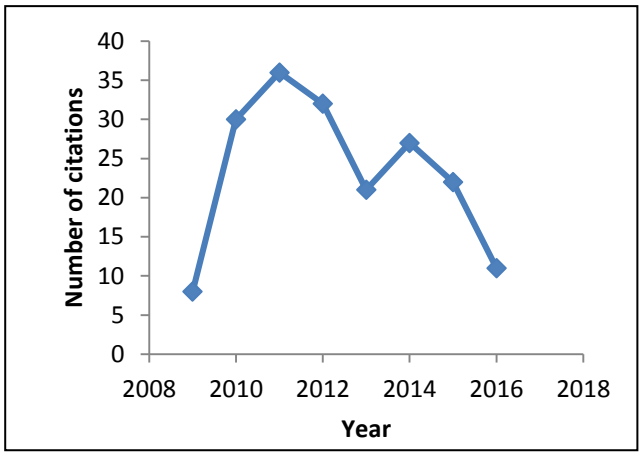


Figure 7. Paper 1: Number of citations per year

Overview of origin of citations
Chromatography
Bioanalysis
Analytical
Toxicology
Pharmaceutical & Biomedical
Clinical Biochemistry
Biosensors
Mass Spectometry
Clinical Chemistry
Antimicrobial/Chemotherapy
Clinical trials
Tropical medicines/hygiene
Peadiatrics
Drug Metabolism
Forensic
Therapeutic Drug Monitoring
Pharmacology
Animal Sciences
Colloids and Surfaces
Proteomics
Spectroscopy

Table 4. Paper 1: Origin of citations

2.1.1: Objectives

The novel use of DBS in support of a toxicokinetic study was described in this study with the following objectives:

1) To investigate the use of DBS as a means to significantly reduce sample volumes in TK studies
2) To conduct a full bioanalytical LC/MS/MS quantitative assay validation to recognized standards for conventional samples with additional experiments proposed for DBS samples
3) To compare TK parameters derived from conventional wet samples (50:50 blood:water) and DBS

Commentary paper 1 was the first to report the application of DBS for the analysis of samples obtained in a TK study, considering the advantages offered. Bioanalytical validations designed to meet these objectives by applying DBS are commonplace today across the industry. However, at the time this work was published in 2008 there was minimal information concerning preclinical validation employing DBS or other types of microsampling in support of TK. Publications prior to this focused on screening of in-born errors of metabolism (Guthrie, 1961), Therapeutic drug monitoring and pharmacokinetics (Beaudette and Bateman, 2004). Interestingly there was minimal work published around preclinical TK (Posyniak et al., 2002, Tawa et al., 1998). No work concentrated on the benefits that DBS or microsampling could offer in reducing and refining the use of animals and the many advantages that the technique could afford.

2.1.2: Experimental design

This paper described the validation of a DBS dog blood method for acetaminophen using 15µL of blood per sample. Dog and acetaminophen were chosen as there was an ongoing study which was utilized and saved running a dedicated study and using animals unnecessarily. A 3mm punch was taken and extracted with 100 µL of methanol containing internal standard ($[^2H_4]$-acetaminophen). Analysis was performed by means of LC-MS/MS using a reverse phase C18 chromatographic system and selected reaction monitoring.

Performance of the assay was assessed by demonstrating that validation parameters met predefined standards as discussed in section 1.8. Parameters investigated included linearity, selectivity, sensitivity, accuracy/precision, stability and matrix suppression.

To assess the application of DBS in support of toxicokinetic studies, a single daily oral gavage suspension of acetaminophen was dosed to one male and one female dog for 7 days at a target dose of 50 mg/Kg. Blood samples (1mL) were collected on days 1 and 7 at 0.5, 1, 2, 4, 8, 12 and 24 hours. For DBS samples, 15 µL of whole blood was

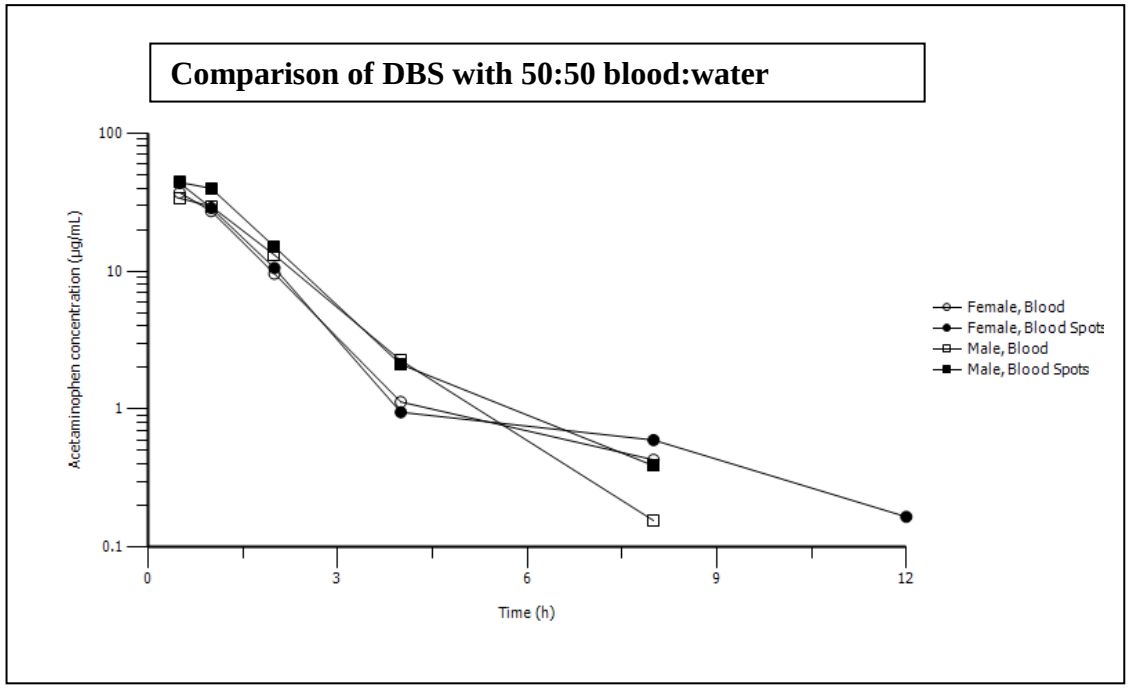
spotted onto DBS cards using a pipette and for blood samples, 50:50 blood:water samples were prepared by diluting 15 µL of blood with an equal volume of water.

Study Design:

- Validation of a quantitative DBS method in dog blood
- Comparison of DBS to conventional wet samples (50:50 blood:water)

Experimental:

- Validation
  - Linearity, selectivity and sensitivity
  - Precision and accuracy
  - Matrix suppression effects
  - Stability
  - Wet versus dry comparison
- Study
  - Oral dose of 50 mg/kg, sampling on day 1 and 7, both DBS and 50:50 blood:water collected



Conclusions:

- Successfully validated a quantitative method utilising DBS
  - Method proved to be selective
  - Linear range 0.1 – 50 µg/mL
  - Intra-day precision and bias less than 12%
  - Storage stability for at least 10 days
- DBS physiologically comparable with conventional wet samples

Figure 8. Schematic of study design for paper 1

2.1.3: Validation and toxicokinetic data output and impact

This study showed that a quantitative assay could be validated using DBS and used to support a TK study.

Using a 2.1 µL sample (3mm punch), a lower limit of quantitation of 0.1 µg/mL was achieved and linearity demonstrated up to 50 µg/mL. Precision and accuracy met acceptance criteria with a maximum accuracy and intra-day precision of 14.5% and 10.4%. Stability was acceptable, covering spotting and drying times in whole blood and 9 days of room temperature stability was generated to cover the length that cards would be stored for this study. The successful validation proved that robust assays could be developed using small sample volumes collected using DBS and at the time there was no other means to reduce blood volumes to these levels. This work at the time was considered radical and game changing.

The most important and pivotal output was the comparison between conventional wet sampling (50:50 blood:water) and that of dry (DBS). Comparison of **AUC** and **Cmax** from both techniques showed a less than 1.4 fold difference (Parry, 2014) and in TK terms this confirmed that DBS sampling was comparable with conventional.

This manuscript marked the start of the DBS microsampling revolution and focused bioanalysts, toxicologists and animal scientists to challenge the amount of sample required for each study (for TK purposes) and moved the industry forward in realising enhanced 3R benefits.

2.1.4: Technical considerations and reflections

This paper was designed to demonstrate that DBS (as a microsampling technique) had the potential to support preclinical studies and set about defining the advantages of a dried blood sample versus conventional samples. Acetaminophen was chosen as it was a marketed compound that was being used to support an investigative programme internally.

This paper focused the industry on microsampling and the 3Rs and the benefits that can be afforded by reducing the volume of samples collected.

As this was a novel application for DBS supporting TK and the community (Bioanalysis, Toxicology and regulators) as a whole (internal and external) was reserved, the publication was set out to present the data that was expected from the groups and to raise interest. Demonstrating that a full validation including precision, accuracy and stability could be achieved and obtaining an equivalent LLQ to previously published work using much larger volumes of liquid plasma was designed to raise interest within the bioanalytical and regulatory community. As the assay validation answered many of the questions potentially raised by the bioanalysts and regulators, the *in-vivo* data was designed to raise interest across the toxicology arena and also further enhance the technique to the bioanalysts and regulators.

This paper also opened up the discussion around which is the correct matrix to measure and really questioned why plasma was deemed appropriate when blood is actually what circulates.

A dry sample does offer different challenges. A good example is how to dilute samples when they are outside of the validated assay range. With a plasma method, control matrix is added to the sample but obviously a new approach was needed for DBS. The solution was to spot control matrix onto the relevant DBS card, allow to dry, punch and extract.

The main issue that was not considered at the time of the publication was the physical properties of blood. As an organization the matrix of choice in drug development had

always been plasma, and at that time, such experiments as the impact of **hemolysis** and **lipemic** plasma were not considered important; today these experiments are common place. Future investigations by GSK (Denniff and Spooner, 2010a) raised the concerns of varying hct and the impact on DBS. As discussed in 1.6 this is undoubtedly the largest concern facing DBS but was not understood at the time this work was conducted. Preclinically, hct is not as large an issue as with humans, as inbred animal strains have much smaller hct ranges. The greatest risk of varying hct is if the drug under evaluation causes the hct value to change or when evaluating disease states. Other factors not considered in this publication were homogeneity and recovery, both an issue with preclinical and clinical species and explored in 1.6.

2.2: Commentary paper 2

Neil Spooner, Rakesh Lad**, and Matt Barfield** (2009). Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method.
Anal. Chem. 2009**,** *81,* 1557–1563.
Citations – 277

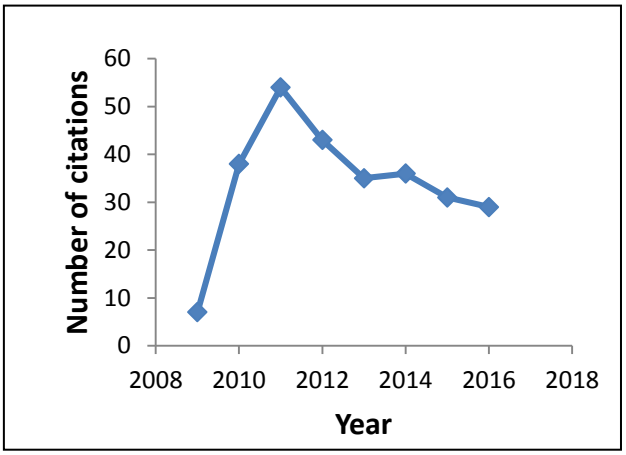


Figure 9. Paper 2: Number of citations per year

Overview of origin of citations

Chromatography
Bioanalysis
Analytical
Toxicology
Pharmaceutical & Biomedical
Clinical Biochemistry
Organic process
Mass Spectrometry
Clinical Chemistry
Antimicrobial/Chemotherapy
Clinical trials
Tropical medicines/hygiene
Peadiatrics
Drug Metabolism
Forensic
Therapeutic Drug Monitoring
Pharmacology
Animal Sciences
Colloids and Surfaces
Proteomics
Spectroscopy
ADME

Table 5. Paper 2: Origin of citations

2.2.1: Objectives

The novel use of DBS in support of a pharmacokinetics study was described in this study with the following objectives:

1) To explore validation experiments pertinent to the acceptability of DBS assays
2) To conduct a validation for Acetaminophen in human blood utilizing DBS
3) To support a DBS clinical study

This paper described the utility of DBS in the clinic and set out how to scientifically validate an assay, considering the characteristics of a dry sample. The industry had

many years of experience dealing with plasma samples in the clinical environment but the use of DBS in a quantitative manner was new to the industry.

The validation was designed to make the collection of the sample as straight forward for the site as possible. The author worked closely with the clinic to understand the pressures that face clinical staff and explore ways of building experiments into the validation that could help alleviate issues when sampling. One consequence of these interactions was to investigate the impact of the volume spotted. This was designed to ensure that when the sample was taken the volume did not need to be exactly 15 µL but instead a tolerance was built into the method.

2.2.2: Experimental design

This paper described the validation of a DBS human blood method for Acetaminophen using 15 µL of blood per sample. A 3mm punch was extracted using 100 µL of methanol containing internal standard ($[^2H_4]$-acetaminophen). Analysis was performed by means of LC-MS/MS using a reverse phase C18 chromatographic system and selected reaction monitoring.

Performance of the assay was assessed by demonstrating validation parameters met predefined standards as discussed in 1.8. Typical parameters investigated included linearity, selectivity, sensitivity, accuracy/precision, stability and matrix suppression. Also experiments to confirm the validity of the DBS were investigated, including stability of the analyte /metabolites in whole blood and as a DBS, volume spotted, spotting device and temperature of the blood spotted.

To assess the application of utilising DBS in support of clinical studies, a two period, single dose oral administration of acetaminophen at 0.5 and 1g was accessed. Blood samples were collected at 0.5, 1, 1.5, 3, and 5 hour, post-dose.

Study Design:

- Access relevant scientific experiments to validate a clinical DBS method
- Validation of a quantitative DBS method in human blood
- Support a clinical study utilizing DBS

Experimental:

- Typical validation experiments
  - Linearity, selectivity and sensitivity
  - Precision and accuracy
  - Matrix suppression effects
  - Stability
- Bespoke to DBS validation experiments
  - Stability of the analyte /metabolites in whole blood
  - Stability of the analyte /metabolites on card
  - Volume spotted
  - Spotting device
  - Temperature of the blood spotted
- Pilot study: Acetaminophen oral dose of 0.5 and 1g on two occasions

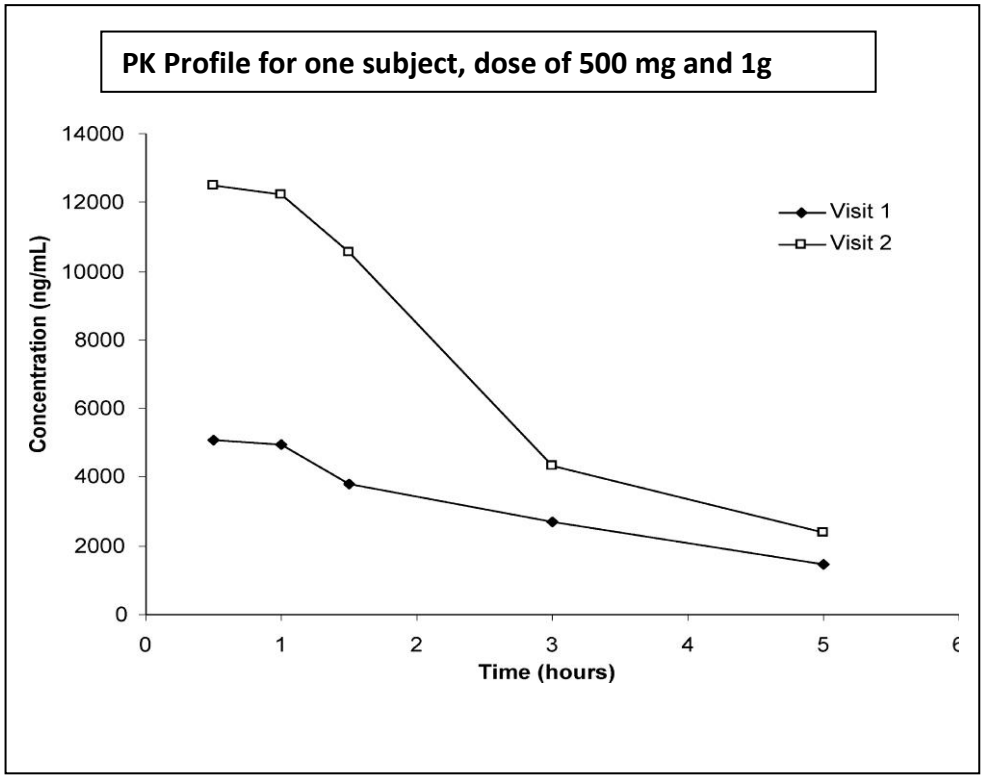


Figure 10. Schematic of study design for paper 2

Conclusions:

- Successfully validated a quantitative method utilising DBS
  - Method proved to be selective with <5% suppression
  - Linear range 25 – 5000 ng/mL
  - Intra-day precision and bias less than 11.2%
  - Storage stability for at least 113 days
- Experiments pertinent to DBS
  - Whole blood stability
  - No difference observed in spotting device (pipette:capillary)
  - No impact of the temperature of blood spotted (0°C, room temperature or 37°C)
  - No impact of spotting volume (10-20 µL)
- Pilot study supported with expected results

2.2.3: Validation and data output and impact

This paper demonstrated the use of DBS to support clinical PK and also explored validation experiments pertinent to a dry sample; a quantitative assay could be validated using DBS and used to support a clinical PK study. The ability to drive down sample volumes from typically >0.5 mL to 45 µL was so important to support paediatric investigations, reduce shipping costs and open up otherwise closed opportunities e.g. remote areas and home sampling.

Using a 3 mm punch, a lower limit of quantitation of 25 ng/mL was achieved and linearity demonstrated up to 5000 ng/mL. Precision and accuracy met acceptance criteria with a maximum accuracy and intra-day precision of -8.4% and 11.2%. Stability data was shown to cover spotting and drying times in whole blood and 113 days of room temperature stability was generated to cover the length that cards would be stored for this study. Investigations unique to a dry sample were explored and for this assay spotting volume (10-20 µL), temperature of the spotted blood (0^{o}C, room temperature and 37^{o}C) and spotting device (pipette and capillary) all had negligible impact (<15%).

The ability to support a clinical study was demonstrated and feedback from clinical staff, patients and analysts was extremely favourable, especially with its simplicity.

2.2.4: Technical considerations and reflections

This paper was pivotal to influencing the industry and showed DBS as a way of supporting PK and the potential to support paediatric studies and home sampling. It also provided an insight on the background investigations, and the thought process on how to deal with a dry sample from a clinical and bioanalyst's perspective. A dry sample is considerably different from a wet sample and experiments needed to be designed to prove the validity of the assay to support PK.

At the time GSK did not look at whole blood stability even though with plasma there is a short period (<1hour) when the sample is whole blood before being processed to plasma. With DBS, the blood sample is potentially still wet for approximately 2 hours (proven by constant weighing of the cards) and therefore the analyte(s) need to be proven to be stable for this period. As a result of this work it was actually demonstrated that GSK and the industry were lacking in their wet validation work and now whole blood stability is carried out for all new compounds regardless of matrix and technique.

Unique experiments detailed in this paper included stability, designed around a dry matrix. It was important to show that capillary sampling (to enable a less invasive sampling method for paediatrics and home sampling) was equivalent to a pipette. However, this experiment only showed the accuracies of the device and not the actual process of taking a finger prick using a capillary and comparing with an IV sample spotted using a pipette. This issue was investigated in commentary paper 3 as the technique was further developed.

The idea of including the spotting volume experiment to remove the need for accurate spotting volumes worked well, but this would still not highlight the hct impact or homogeneous nature of the sample. This investigation would require further development which is covered in commentary paper 7 and 8.

2.3: Commentary paper 3

Neil Spooner, Y Ramakrishnan, **M Barfield**, O Dewit & S Miller (2010). Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations.
Bioanalysis (2010) 2(8), 1515–1522.
Citations – 30

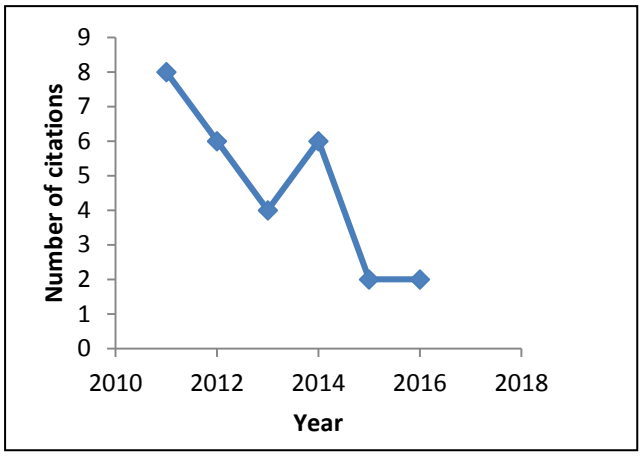


Figure 11. Paper 3: Number of citations per year

Overview of origin of citations
Bioanalysis
Mass Spectrometry
Analytical Chemistry
AAPS
Colloids and Surfaces
Analytical Methods
DBS – Applications & Techniques
British Journal, Clinical Pharmacology

Table 6. Paper 3: Origin of citations

2.3.1: Objectives

The use of DBS in support of a pharmacokinetics study was described in this study with the following objectives:

1) Practicalities of utilizing DBS in a clinical setting
2) Considerations of using finger pricks to obtain samples
3) Comparison of wet versus dry sampling and impact of collection site on concentration data

This paper set out to understand from both a clinical staff's perspective and a patient's view point the practicalities of using DBS to obtain PK samples in a clinical setting. It was critical to have a way of assessing the entire procedure in an unbiased way, which was achieved by employing a questionnaire to staff and patients. This was pivotal in progressing the use of DBS in the clinical environment.

A secondary objective was to compare concentrations in DBS samples, derived from finger pricks and venous cannula against conventional sampling.

2.3.2: Experimental design

Acetominophen was chosen as the work could easily be published and was administered orally at 500mg (first period) and 1g (second period) to 11 healthy subjects (male = 8, female = 3). Blood was sampled at 30, 60, 90, 180 and 300 minutes post dose. Finger prick samples (15 µL, n=3) were obtained by means of a single use lancing device, glass capillary collection and then spotted directly onto FTA filter paper. Blood from the cannula (6mL collected into EDTA tube) was split into two portions, one for spotting (15 µL, n=3) using a repeater pipette and the other to dilute 1:1 v/v with water for conventional analysis. Blood spots were allowed to dry for 2 hours under ambient conditions and then stored and shipped in desiccated bags. Wet samples were shipped and stored frozen until analysis.

For DBS analysis a 3 mm diameter central disc was extracted with methanol and blood:water samples (50 µL) were extracted by means of protein precipitation both followed by LC/MS/MS.

16 study staff completed a questionnaire relating to collecting samples and 11 patients answered questions relating to tolerability of sampling.

Study Design:

- Practicalities of DBS in a clinical setting
- Finger prick acceptance
- Wet vs dry samples and comparison of sampling sites

Experimental:

- Oral administration of Paracetamol to 11 patients at 500mg and 1g
- DBS samples and 50:50 blood:water samples taken at 30, 60, 90, 180 and 300 min
- Samping from both finger pricks and venus cannula
  Finger pricks for DBS and venus used for both DBS and 50:50 blood:water

Survey data – Pain scores

	Number of subjects responding				
Overall pain/discomfort	**None**	**Mild**	**Moderate**	**Severe**	**Extreme**
Finger-prick	5	15	1	0	0
Cannula	2	11	8	0	0

Conclusions:

- Finger pricking well tolerated by patients
- Staff found the use of DBS to be acceptable
- A small difference was seen when switching between 50:50 blood:water and DBS
- Differences were observed when comparing cannula sampling to finger prick, especially with low concentrations and early post-dose time points

Survey data – Staffs view of ease of DBS

	Number responding				
Staff rating of sample collection step	**Extremely easy**	**Easy**	**Neither**	**Difficult**	**Extremely difficult**
Taking blood by finger-prick	3	9	3	0	0
Taking blood by cannula	7	6	2	0	0
Blood spotting onto card	2	14	0	0	0

Figure 12. Schematic of study design for paper 3

2.3.3: Data output and impact

This paper showed the viability of DBS for both staff and patients in a clinical setting. For the continued development of DBS to support pharmacokinetics this was essential because the techniques employed needed to be accepted by the impacted groups.

Patients tended to prefer finger prick sampling (19 out of 21 occasions). Also at least two finger pricks per sample and six time points per study day were deemed acceptable. Staff who had received no more than 3 hours of familiarisation found taking samples *via* finger pricks and spotting onto cards to be generally easy.

The secondary outcome of the investigation was not as favorable but in the case of sampling site not surprising. When comparing measurement methods, only concentrations below 8000 ng/mL could be considered interchangeable. Comparison of sampling site showed concentrations at early time points and low concentrations to be significantly different (up to 4 fold higher using finger pricks). This observation has been well documented (Chiou, 1989) and forms the basis of modern requirements to perform bridging studies when switching sampling sites (Evans et al., 2015).

Differences between blood concentrations when comparing sampling sites (arterial and venous) are considered to be dependent on when the drug was dosed. Early time points show higher concentrations in arterial samples due to diffusion into the extravascular space via capillary membranes. Later time points show higher venous concentrations due to distribution of the drug into tissues which allows diffusion *via* capillary walls into the venous system. Expected concentrations for finger prick blood should be between arterial and venous blood concentrations.

2.3.4: Technical considerations and reflections

It was important to get an honest representation from the perspectives of both the clinical staff and patients. The feedback was extremely positive and important, especially around patient acceptance for finger pricks, which was encouraging for paediatrics.

Looking back at this work one of the largest assumptions was that the experiences of clinical staff in this study would be similar across other clinics. With this study the author spent time training face to face and was on hand during sampling occasions to help if any questions arose. Also this was a dedicated first time into patient clinic with well trained staff and well versed with the importance of accurate measurements. In reality across multi-sites clinical centers, training has to be remote. The training relies on individuals reading, watching videos and taking time to digest and understand the instructions. The author created a training video detailing the entire process. In reality clinics are a busy and often chaotic environment with time constraints. Often bank nurses are used for just a few hours and training can be missed. The majority of clinical staff will have seen DBS in the form of the Guthrie test where accuracy is not important and this does cause issues. Sometimes clinical staff feel that they don't require training and can ignore the materials provided, which can lead to unusable samples. This is obviously a serious cause for concern with all samples needing to be viable. This is further developed in commentary paper 9 where a solution to this issue is proposed.

However, the quality of the sample is not just limited to DBS and any accurate volumetric measurement needs to be carefully considered when moving to clinical centers. Experience has shown that even when taking 50:50 blood water samples, clinics have been known to ignore the provided accurate pipette and guess the measurements, utilizing plastic pastettes. It is the importance of the accurate measurement which can be missed and lost in the complexities of the studies. Techniques need to be as simple, logical and straightforward to be successful.

The fact that cannula samples did not match finger pricks means that careful consideration is required at an early stage for each compound. If switching between matrix type and/or sampling site the impact needs to assessed (by bridging studies) and ideally avoided if possible (Evans et al., 2015, Xu et al., 2013).

2.4: Commentary paper 4

Phillip E Turpin, Josephine EC Burnett, Lee Goodwin, Amanda Foster and **Matthew Barfield** (2010). Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories.
Bioanalysis (2010) 2(8), 1489–1499
Citations – 17

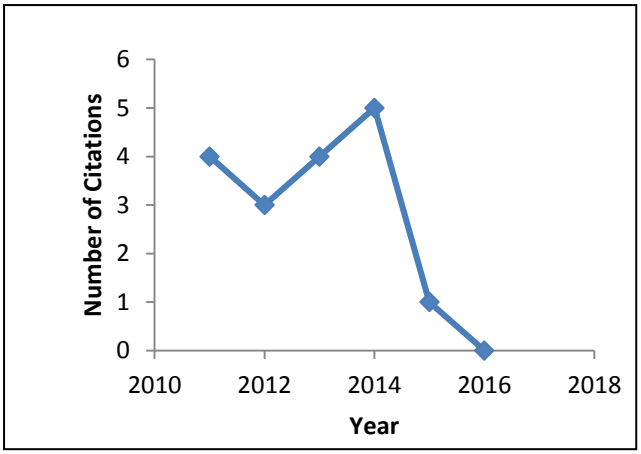


Figure 13. Paper 4: Number of citations per year

Overview of origin of citations

Analytical Chemistry
DBS-Applications and Techniques
Pharmacology & Toxicology
Bioanalysis
Mass Spectrometry
Drug Testing
Biomedical Chromatography
Pharmaceutical Analysis
Electrical Engineering
Toxicology

Table 7. Paper 4: Origin of citations

2.4.1: Objectives

With a trend for large pharmaceutical companies to reduce the size of internal Bioanalytical functions and to outsource large proportions of work to **Contract Research Organizations (CRO)**, it is essential that analytical assays are transferable between laboratories. This paper had the following objectives:

1) To support a Pioglitazone TK study utilizing DBS with analysis across two laboratories
2) To compare wet sampling (50:50 blood:water) with dry sampling (DBS)

At the time of authorship, transferring assays between labs was still not that common, whereas in today's world it is routine. Confidence is needed that any laboratory using the same or similar assay will provide equivalent data. Any change in PK or TK results must not be attributed to the Bioanalysis and any risk minimised by cross validation. Cross validation is usually carried out by a transfer of pooled samples or quality

controls and analysed at both sites. In today's world it is not uncommon to have three Bioanalytical centres for a single assay.

2.4.2: Experimental design

This paper described the validation of two different DBS methods across two laboratories to support a Pioglitazone study. In the originator laboratory a DBS method and a 50:50 blood:water method were developed and in the receiver laboratory only a DBS method was developed. The DBS method, in both instances, used 15µL of blood per DBS sample. A 3mm punch was extracted using 200µL of methanol containing internal standard ([$^2H_4$]-Pioglitazone) in the originator laboratory whereas the receiver laboratory used 100 µL. For the 50:50 blood:water samples, 20 µL of blood was extracted. Analysis was performed by LC-MS/MS using a reverse phase C18 chromatographic system (originator laboratory employed a gradient and receiver laboratory an isocratic) and selected reaction monitoring.

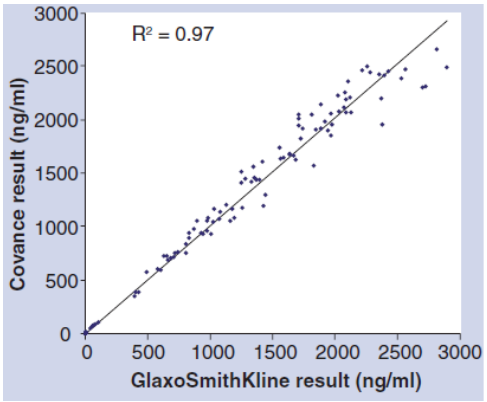
Performance of the DBS assay was assessed by demonstrating validation parameters met predefined standards as discussed in 1.8. Typical parameters were investigated and also bespoke tests included, specific to DBS e.g. effect of aliquot volume to remove the need to be very accurate when spotting.
For the 50:50 blood:water analysis an abbreviated validation was conducted and limited to intra-assay precision and accuracy, response, selectivity, room temperature stability, three freeze thaw cycles and processed extract.

For the investigation 12 rats received an oral (gavage) dose of 30mg/kg. The following time points were taken:
Rats 1-3; 0, 1, 2, 3.5, 5 and 7 hour (50:50 blood:water and DBS);
Rats 4-6; 0.5, 1.5, 2.75, 4.25, 6 and 24 hour (50:50 blood:water and DBS);
Rats 7-12; 0, 0.5, 1, 1.5, 2, 2.75, 3.5, 4.25, 5, 6, 7 and 24 hour (DBS only);
Rats 1-6 were used to generate composite profiles due to a larger blood volume being taken to accommodate the liquid blood samples. Rats 7-12 were used to generate serial profiles. All samples were taken from the lateral cordial vein. DBS samples (15 µL x 3) were obtained via a glass capillary.

Study Design:

- Evaluate transferability of DBS assays between laboratories
- Evaluate ability to switch between liquid and dry samples for Pioglitazone

Correlation between PK data across two laboratories



Blood concentration DBS profiles across two laboratories

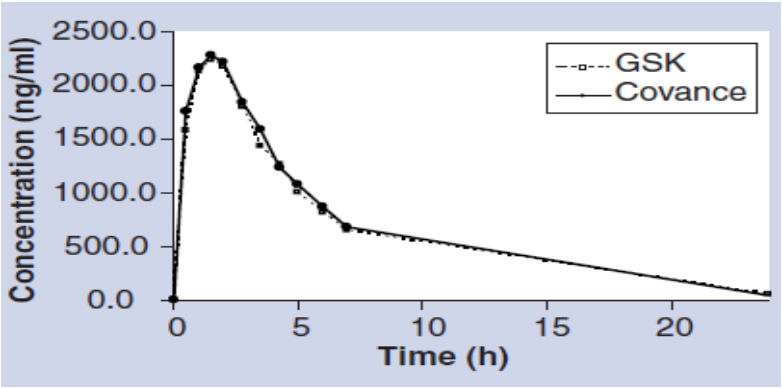


Figure 14. Schematic of study design for paper 4

Experimental:

- Validation of different methods for pioglitazone
  - Originator lab – DBS (full validation) and liquid blood (abbreviated validation)
  - Receiver lab – DBS (full validation)
- Bespoke DBS validation experiments
  - Volume spotted
- 30mg/kg oral dose to 12 rats
- Serial and composite sampling

Conclusions:

- Successfully validated two quantitative methods utilising DBS across two independent laboratories
  - Method proved to be selective with <3.5% suppression
  - Linear range 5 – 2500 ng/mL
  - Intra- and inter-day precision and bias acceptable
  - Recovery >75%
  - Reinjectability: minimum of 3 days and DBS room temperature stability up to 4 months
  - Variation of blood spot volume acceptable: 15-25 µL and 10-20 µL
  - Ability to dilute samples
- Abbreviated blood validation met all requirements
- Transferability of DBS analysis between laboratories showed excellent correlation (99% of results within 20%)
- Liquid blood and DBS samples gave excellent correlation

2.4.3: Validation and data output and impact

This paper demonstrated that different assays for Pioglitazone could be validated and then used to support analysis of equivalent DBS samples across two independent laboratories. This employed different analytical instrumentation, personnel, extraction and approach to the methodology. In reality this is common place with CROs redeveloping assays transferred from customers to fit their workflows and instrumentation. In the case of Pioglitazone, transferring between wet and dry samples was a viable option that is extremely important to aid projects and utilise paediatric studies in later phase studies. This was also the first time that the CRO had performed method development, a validation and sample analysis to support a DBS study.

A lower limit of quantitation of 5 ng/mL was achieved and linearity demonstrated up to 2500 ng/mL. Selectivity was proven and recovery proven to be >75%. Precision and accuracy met acceptance criteria of +/-15% and 20% at the LLQ. DBS stability was demonstrated at room temperature for up to 4 months, demonstrating one of the large benefits of the technique. Processed extract stability was also demonstrated for a minimum of three days. Accurate sample spotting volume was shown to not be critical with variances of size tested between 15-25 µL and 10-20 µL.

It was also shown that a 50:50 blood:water assay could be validated and this was used to compare DBS and wet samples. The correlation between the two was excellent allowing future changes of wet and dry samples moving forward.

However, the greater impact was the demonstration of the transferability of DBS analysis between laboratories with an excellent correlation (99% of results within 20%).

2.4.4: Technical considerations and reflections

The ability to support validations and sample analysis using different assays in different laboratories is essential. Acceptance criteria is 66.7% of the data sets (originator and receiver) should be within 20%. The results showed 99% correlation which in the author's experience, is very unusual and was very encouraging especially as this was a new and novel approach. Further to this the CRO had never been involved with the analysis of DBS showing that the technique was simple and robust. Cross validations do fail, and investigations into the reasons can be complex and time consuming. In some cases, the root cause cannot be identified. The majority of solutions when a cross validation fails has been to replicate the originators methodology and to not allow the freedom to alter the method. This can sometimes be challenging, with different equipment being utilized.

The comparison between blood:water (50:50) and DBS showed that for Pioglitazone the two could be inter-changeable. However, it is important to keep the sampling site consistent as described in commentary paper 3. This work was in the rat and at the time different sampling sites were investigated to minimize blood loss and make use of DBS. The work with DBS focused GSK to introduce a consistent approach to rat sampling across all sites (independent of sample collection technique) and encourage the industry to follow suit by presenting data and engaging the NC3Rs committee to publicise.

The greatest concern was actually ensuring that the blood:water (50:50) procedure was carried out correctly. In the past comparisons have failed due to the accuracy of the 50:50 measurement. One unexpected outcome from this work was to dispel the assumption that when dealing with blood as a liquid it had to be mixed (50:50) with water when freezing. This was a belief going back before the author joined the industry. This approach works well in discovery but is hard to ensure accurate volumes are dispensed as the complexities of studies progress. Work at the time showed that for some compounds, whole blood could be sub aliquoted after being frozen. This offered the benefit of reducing complexities and ensured high quality data when

dealing with blood. For all blood work now in development at GSK, whole blood is preferred over blood:water (50:50) if proven to be viable.

2.5: Commentary paper 5

Matthew Barfield, Sheelan Ahmad & Maria Busz (2011). GlaxoSmithKline's experience of incurred sample reanalysis for dried blood spot samples. *Bioanalysis* (2011) 3(9), 1025–1030
Citations – 10

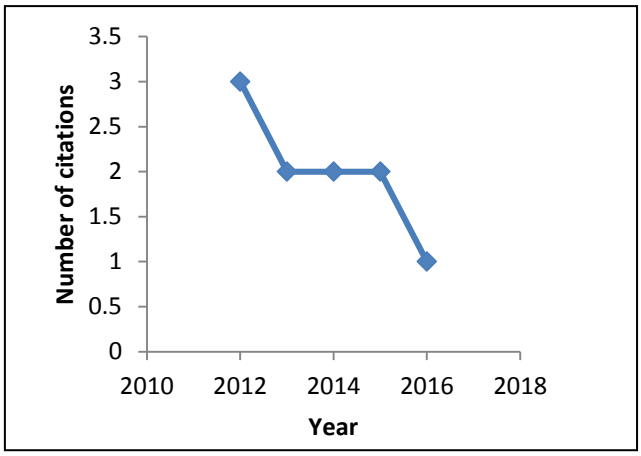


Figure 16. Paper 5: Number of citations per year

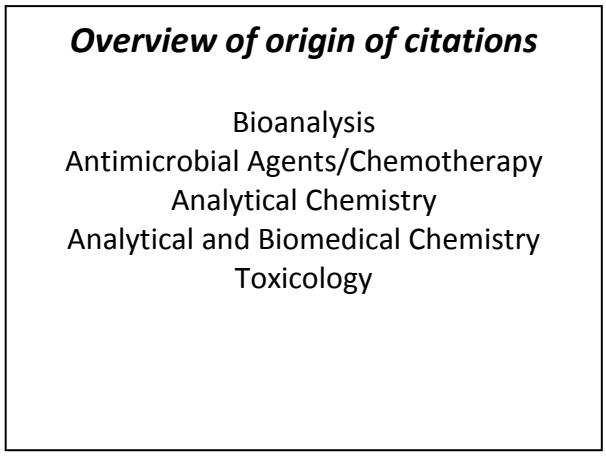


Figure 15. Paper 5: Origin of citations

2.5.1: Objectives

To critic the Incurred Sample Reproducibility (ISR) data generated during routine analysis within GSK and add to the growing evidence supporting the use of DBS within the industry for TK and PK analysis. The objectives were:

1) Review and compare all ISR studies conducted within GSK utilizing both DBS and blood
2) Explore reasons of concern
   - Differences between DBS sampling techniques
   - Card contamination
   - Explore any differences between liquid and DBS ISR

ISR is a regulatory requirement for TK and PK studies, originating from 1992 (Shah et al., 2000) and more recent regulations the 2008 AAPS/FDA (Fast et al., 2009). ISR involves the reanalysis of study samples to further validate the assay and has the

potential to highlight issues with metabolites or more commonly within-study user error e.g. incomplete mixing or sample ordering issues.

Whilst building confidence with DBS, routinely both DBS and liquid blood were used to support studies and this included ISR which allowed the opportunity to compare and contrast the two techniques.

2.5.2: Experimental design

The design was split into three areas of investigation in order to address concerns and possible issues with DBS when supporting TK and PK. Data were evaluated from 42 studies containing both liquid blood and DBS ISR.

1. Differences between DBS sampling techniques

   Unlike liquid blood which is collected into a tube and that is the sample, DBS has two possible approaches to sample collection. Either collecting the blood into a tube (wasteful of blood), mixing and then pipetting onto the DBS card, or using a capillary and taking the blood directly from the subject to the card. In the latter case it could be considered that each spot is a unique sample as 3 capillaries are required and each sample is separated by a small amount of time. To assess, studies containing both DBS cards spotted from a tube and capillary were evaluated.
2. Effect of card contamination

   DBS cards are more prone to contamination as they are relatively large and exposed to the environment (2 hour air drying time) than a simple blood tube with a cap. ISR data was scrutinized for impact of contamination.
3. Differences between liquid ISR and DBS ISR

   As with experiment 1, liquid ISR should actually give better ISR data due to a homogenous sample in a tube compared with potentially three discrete DBS samples from three capillaries. ISR data was explored to see if there was a marked difference or not.

Study Design:

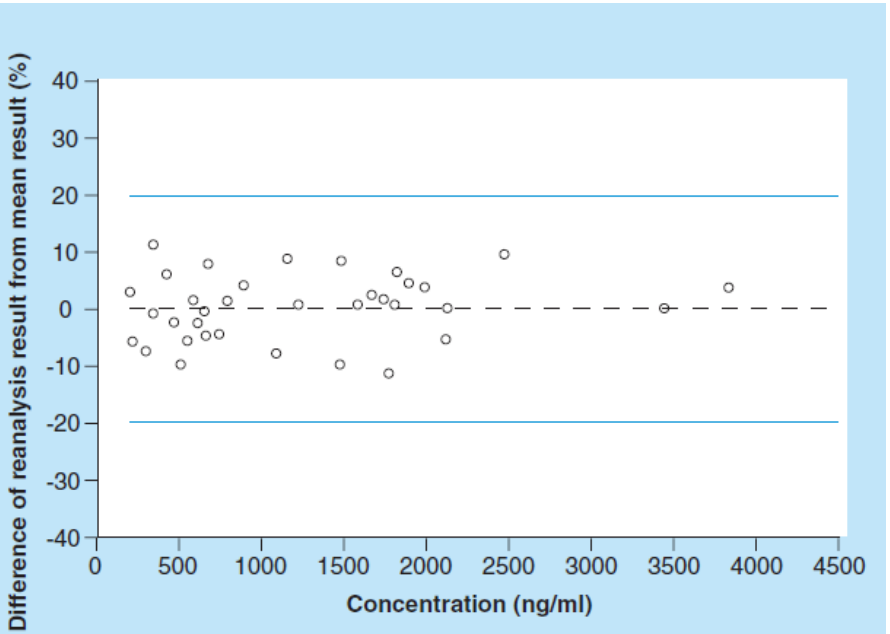
- Full data review of ISR studies for DBS and liquid blood studies
- Different sampling techniques
- Card contamination
- Differences between liquid and DBS ISR

Experimental:

ISR Protocol

- Run samples (DBS and 50:50 blood:water), review data and approve results
- After approval but as close to original analysis, re-extract 10% of the approved samples for ISR determination
- Acceptance criteria: >66.7% of incurred results need to be within +/- 20% of the mean result. This is the minimal requirement, also need to be aware of trends in data e.g. positive of negative bias
- Test multiple card types e.g. FTA DMPK A, FTA DMPK B and Alstrohm 226

ISR results for DBS study



Conclusions:

- Out of 42 DBS ISR investigations, two did not meet acceptance criteria
  - One was due to contamination. A portion of the study was repeated taking extra precautions and ISR was successful
  - One could not be explained. This has been observed with conventional ISR when the cause cannot be identified
- No differences were observed with DBS sampling techniques
- DBS and liquid blood ISR compared well with one exception. The ISR for one liquid blood study failed and the reason could not be explained. It wasn't due to stability even though a negative bias of 40% was seen

Figure 17 Schematic of study design for paper 5

2.5.3: Data output and impact

This data built on the confidence that DBS was a reliable means of collecting, storing and shipping samples for TK and PK analysis.

42 studies containing ISR for both DBS and liquid blood were reviewed with two not meeting the acceptance criteria. One concern around DBS was contamination and this was observed with one of the failures. Contamination of a large proportion of the cards was observed to the extent that fur was visible on some cards. With the addition of extra precautions to minimize contamination for this study a portion of the study was rerun which was successful, including the ISR. Precautions included using different laboratories to sample, spot and dry and different personnel to dose and spot. However, this is not a routine practice and typical precautions are generally employed and no issues are observed.
The second failure was never resolved even though an investigation did take place. The ISR results were generally 40% higher than the original analysis. Contamination was ruled out as this would have been less consistent. The author has observed other situations using conventional methods of sample collection where ISR has failed and even after extensive investigation the cause has never been determined.

No difference was observed from DBS sampling from a tube or individual capillaries and the argument of capillary sampling being 3 individual samples was found to be unfounded.

Liquid blood ISR and DBS ISR showed excellent correlation apart from one instance when liquid ISR failed showing a negative bias of more than 40%. Originally this was thought to be an issue of stability but stability testing disproved this theory. The cause was once again never answered.

2.5.4: Technical considerations and reflections

When ISR first became a regulatory requirement, it was felt as a tick box, and an experiment that would never fail or add quality. Now with 1000s of ISR experiments performed at GSK it has proven a valuable tool to give confidence of the validity and robustness of a bioanalytical assay. It is now used as one of the decision factors when considering contracting the method. However, when the reason for a failure is unknown and extensive investigation leads to no conclusions, it does pose serious questions to the data. There is always the question of which data set is correct and if any is actually valid.

From a recent review of over 1000 ISR studies (all sample types) from 2007 to 2017, 3 % of the studies reviewed have had ISR issues. From these 3 % of studies, only 4 studies have resulted in non-reportable data. The majority of failures can actually be associated with user error or stability when there is too greater time difference between original and re-analysis. User error is usually associated with insufficient mixing of the sample. From the author's experience these errors are associated with conventional plasma samples where samples are frozen and then thawed and this builds greater opportunities for non-homogenous samples and stability issues. As discussed previously DBS should show greater stability (D'Arienzo et al., 2010, Heinig et al., 2010, Bowen et al., 2010) and the freeze thaw process is negated. Scientifically DBS samples should offer greater ISR acceptance as long as recovery from the DBS card is consistent.

With capillary bleeds becoming more common place with increased use of microsampling, this data review has been invaluable in confirming that for one-time point, 3 capillaries taken one after the other does not impact the data. This has allowed for new and exciting developments in microsampling which are explored in chapter 3.

2.6: Commentary paper 6

Matthew Barfield and Robert Wheller (2011). Use of Dried Plasma Spots in the Determination of Pharmacokinetics in Clinical Studies: Validation of a Quantitative Bioanalytical Method.
Anal. Chem. 2011, *83,* 118–124
Citations – 48

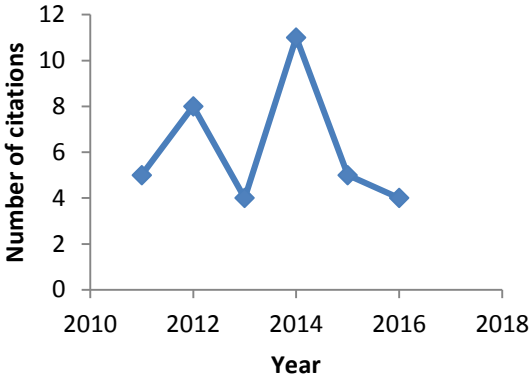


Figure 18. Paper 6: Number of citations per year

Overview of origin of citations
Bioanalysis
Mass Spectrometry
Analytical Chemistry
Drug Testing
Science and Nutrition
Biomedical Chromatography
Pharmaceutical Analysis

Table 8. Paper 6: Origin of citations

2.6.1: Objectives

The novel use of DPS in support of a pharmacokinetics study was described in this study with the following objectives:

1) To explore validation experiments pertinent to assess the acceptability of DPS assays
2) To conduct a validation for Paroxetine in human blood utilizing DPS
3) To evaluate DPS in support of a clinical study
4) To investigate the potential to interchange between wet and dry plasma samples
5) To investigate the use of indicating papers to simplify the visual identification of plasma spots

Since historically the matrix of choice for TK and PK was plasma, the ability to switch from liquid plasma to dry plasma could afford some advantages demonstrated with DBS. A dry sample is simpler and cheaper to ship and store (requiring no freezers or

dry ice shipments) than a wet plasma sample. It also lends itself to the rapidly upcoming techniques focusing on card samples and removing the need to punch samples making the extractions more efficient and less time consuming e.g. CAMAG (Abu-Rabie and Spooner, 2009, Abu-Rabie and Spooner, 2011, Fingerhut et al., 2014) and DART (Crawford et al., 2011, Wagner et al., 2016, Wang et al., 2013).

The author used the strong relationships that were built with the paper manufacturers during the early work on DBS to have a new indicating paper developed. When plasma is spotted onto paper the sample is visible, however upon drying the plasma spot is almost invisible to the naked eye. Working with GE Healthcare (formerly Whatman) a new paper was developed that was purple in colour and then when plasma was spotted and dried the sample was easily identifiable (plasma turns the paper pink). This was unique and never attempted before for plasma even though others had experimented with non-commercial options (Christianson et al., 2010).

2.6.2: Experimental design

The paper included three sample types, liquid plasma, DPS on Ahlstrom 226 (plain cellulose paper), and DPS on indicating FTA DMPK (cellulose paper impregnated with chemicals that lyse blood cells and has colour changing properties). This design allowed the comparison of liquid versus dry and conventional papers compared with an indicating paper. The paper described the validation of the three paroxetine methods. For DPS, 20 µL of plasma was used per sample and a 6mm punch was extracted using 100µL of 70:30 methanol-water (v/v) containing internal standard ([$^2H_6$]- Paroxetine). For liquid plasma a 50 µL aliquot was used per sample and extracted using 100µL of acetonitrile containing internal standard ([$^2H_6$]- Paroxetine). Analysis was performed by means of LC-MS/MS using a reversed phase C18 chromatographic system and selected reaction monitoring to provide a highly selective method.

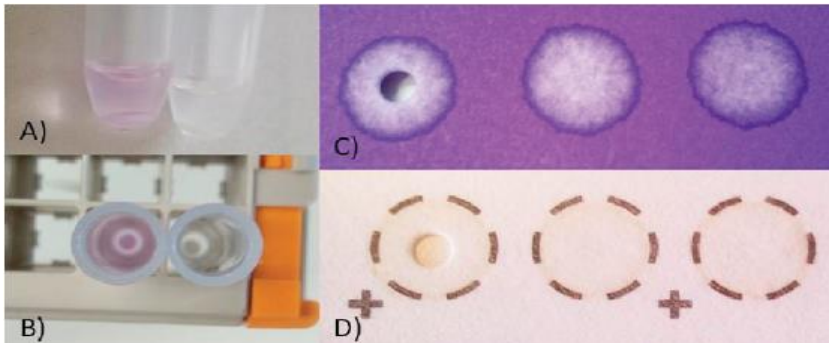
Performance of the assays were assessed by demonstrating validation parameters met predefined standards as discussed in chapter 1.8.
Typical parameters investigated included linearity, selectivity, sensitivity, accuracy, precision, stability and matrix suppression, ability to dilute. Also experiments to confirm the validity of the DBS were also investigated including volume spotted.

To assess the application of utilising DPS in support of clinical studies, a two period, single dose oral administration of paroxetine at 37.5 mg was accessed. Venous blood samples were collected at 0, 2, 4, 6, 8, 10, 12, 15, 18, 24, 32, 48, 72, 120, and 168 hours. This was not a dedicated study and old remaining samples were pooled from an existing study to generate profiles for testing.

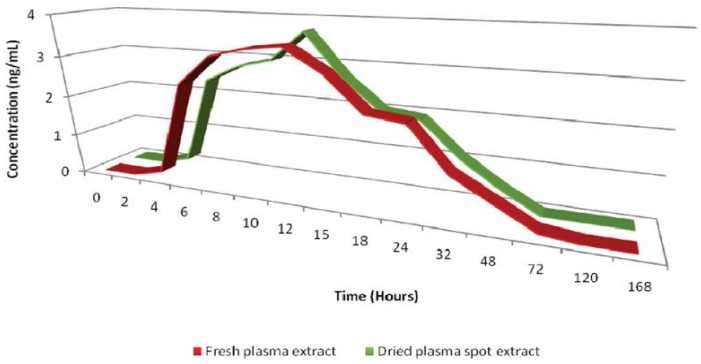
Study Design:

- Access relevant scientific experiments to validate a clinical DPS method
- Validation of quantitative DPS methods and liquid plasma method in human plasma
- Support a clinical study utilizing DPS
- Assess the use of indicating papers
- Assess the ability to change sampling

A) and B) Extracted sample indicating/non indicating
C) Indicating papers D) non indicating



PK profiles comparing wet plasma and DPS



Experimental:

- Validation experiments
  - Linearity, selectivity and sensitivity
  - Precision and accuracy
  - Matrix suppression effects
  - Stability
  - Ability to dilute
- 37.5 mg oral dose
- Serial sampling but pooled profile for analysis

Conclusions:

- Successfully validated a quantitative method utilising DPS
  - Method proved to be selective
  - Linear range 0.2 - 200 ng/mL
  - Storage stability for at least 35 days on paper
  - No impact of spotting volume (15-25 µL)
- Liquid plasma validation successful as was abbreviated indicating paper validation
- Excellent comparison between liquid plasma and DPS
- Advantages of indicating papers demonstrated i.e. Plasma spot easily identifiable even when dry and extracted sample also coloured to aid manual procedures

Figure 19. Schematic of study design for paper 6

2.6.3: Validation and data output and impact

This paper demonstrated the use of DPS to support clinical PK. As virtually all late stage clinical assets utilize plasma, the data was important to show the ease of transition between a wet and dry sample. This was designed to allow the benefits of a dry sample to be utilised until DBS transitioned into late stage clinical. The investigation of a new development grade of indicating paper showed no detrimental impact (proven by **post column infusion**) to the data generated but had significant advantages. The ability to easily identify the plasma spot is essential and not easily possible with conventional papers. Simple recognition also allows automation to be developed and used to reduce the manual extractions carried out at the bioanalytical laboratory. An unexpected advantage was the colouring of the extracted sample making it easier to track extracted samples through the bioanalytical phase.

Using a 20 µL sample and a 6mm punch, a lower limit of quantitation of 0.2 ng/mL was achieved and linearity demonstrated up to 200 ng/mL for both DPS and wet plasma. Assay spotting volume was also investigated for a 15, 20 and 25 µL spot and the accuracy differences between the three volumes was less than 9.2%. Essential evidence to allow for inaccuracies in spotting volume and ensuring a representative sample can be achieved.

Comparing DPS with liquid plasma in a pooled PK profile showed interconversion of the two techniques to be possible with PK parameters (AUC, T_{max} and C_{max}) being calculated to within 10% across the two techniques.

2.6.4: Technical considerations and reflections

The ability to switch from a plasma sample to a dry sample between studies at the time was an interesting idea. It takes approximately 20 years to develop a drug which equates to 20 years to have all suitable assets supported by DBS. DPS was seen as a way of transitioning and gave certain benefits offered by a dry sample. It was not considered as a substitute for DBS, due to the wastage of sample (creating plasma) and specialist equipment required (centrifuges).

One major advantage is the removal of the hct effect compared with DBS, which is the main issue associated with the technique. Hct would only be an issue if the plasma sample was hemolysed, which is an issue with any plasma sample, be it wet or dry. Although hct may not be an issue, investigations into the properties of plasma would be required. Varying levels of lipid, albumin and glycoproteins may have a similar impact as varying hct levels for DBS.

The regulatory acceptance of DPS would be a concern with the continued drive to support all studies with both conventional sampling and dry sample (Emmons and Rowland, 2010, Evans et al., 2015). The cost associated with this is unacceptable on a long term basis.

From this investigation the use of indicating papers would be strongly advised. The papers are more expensive (20%) and it is adding another chemical into the sample, but the advantages in the author's opinion are considerable. The ability to visually see the sample throughout the process is essential and this is true for manual and automatic processing. The indicating paper would also lend itself well to other matrices, such as saliva and sweat. These matrices are becoming more important in areas such as home sampling and forensics (Meesters and Hooff, 2013).

2.7: Commentary paper 7 and 8

Papers 7 and 8 have been combined as both papers are fundamentally based on a consortium approach.

Paper 7: Ronald de Vries, Matthew Barfield, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Zoe Cobb, Steve White & Philip Timmerman. The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium.
Bioanalysis (2013) 5(17), 2147–2160
Citations – 54

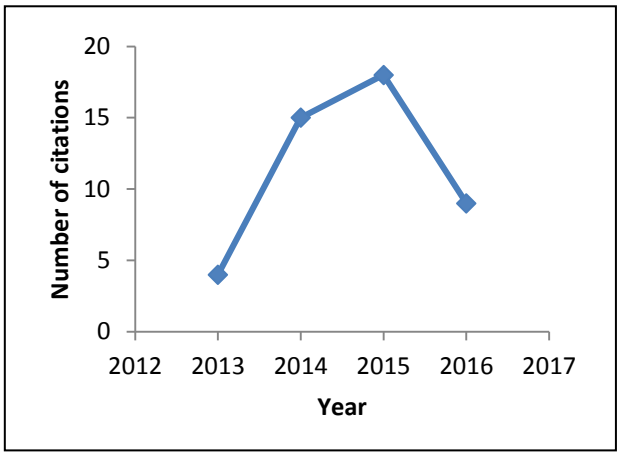


Figure 20. Paper 7: Number of citations per year

Overview of origin of citations

Bioanalysis
Pharmaceutical & Biomedical Analysis
Mass Spectrometry
Analytical Chemistry
Clinical Biochemistry
Chromatography
Drug Metabolism and Toxicology
AAPS

Table 9. Paper 7: Origin of citations

Paper 8: Zoe Cobb, Ronald de Vries, Neil Spooner, Stephen Williams, Ludovicus Staelens, Mira Doig, Rebecca Broadhurst, **Matthew Barfield**, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Steve White & Philip Timmerman. In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium."
Bioanalysis (2013) 5(17), 2161–2169
Citations - 30

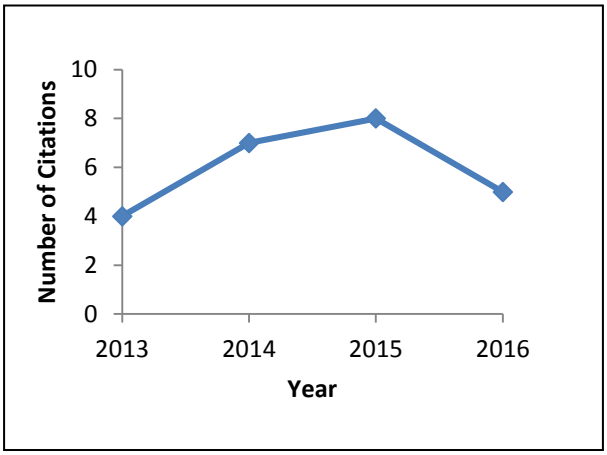


Figure 21. Paper 8: Number of citations per year

Overview of origin of citations

Bioanalysis
Pharmaceutical & Biomedical Analysis
Mass Spectrometry
Tropical Diseases
Analytical Chemistry

Table 10. Paper 8: Origin of citations

2.7.1: Objectives

Paper 7: To use a consortium approach to explore the impact of hct on DBS:

1) To explore relationship between hct and the following:
    - Response
    - Spot homogeneity
    - Spot size
    - Extraction recovery
    - Card type
2) To explore relationship between age of wet blood and the result
3) To explore relationship between age of blood spot and the result

These objectives were pivotal to the continued success of DBS. The advantage of using a consortium was to give a view from the industry rather than just one company's perspective. Also the diverse portfolio across six companies gave access to a vast array of different compounds covering a broad chemical space and different experience and knowledge to design a robust protocol.

The author was a key player in instigating the European Bioanalytical Forum consortium and bringing likeminded companies to a general consensus.

Paper 8: To bring the learnings' from two EBF DBS-microsampling consortium Working Groups, focusing on homogeneity of DBS samples. Two methods to investigate homogeneity were chosen:

1) Nonradiolabeled approach
2) Radiolabeled approach

So as not to have too many work streams the consortium initially had four areas of focus.

1. Effect of hct (Paper 7)
2. Addition of I.S. (Van Baar et al., 2013)

3. Stability of blood and cards (Timmerman et al., 2013)
4. Mechanism for dilution of samples (Timmerman et al., 2013)

The teams investigating hct and stability both included experiments to investigate homogeneity and it was these data that made up the content of this paper.

2.7.2: Experimental design

Paper 7: This paper described experiments to best answer the impact of a changing hct. Generally hct in preclinical species is constant, however in human patient populations differences can be large e.g. neonates at birth 31-67% (Denniff and Spooner, 2010a)

Twelve compounds were selected with a broad spectrum of chemical properties e.g. log P and pKa.

Multiple papers were tested selecting from the main manufacturers. Ahlstrohm 226 ™, BondElut DMS ™, GE DMPK-A ™, and GE DMPK-B ™ and the following hct tested for each experiment.

Spot size and analyte response on Ahlstrohm paper using fresh blood (less than 48 hours old): 20, 30, 35, 40, 45, 50, 60, and 70%. Aged blood (was spotted onto all cards and tested at 20, 45 and 70% hct). 25 µL was spotted and a 3-3.3 mm punch was sampled.

Recovery and Spot homogeneity: 20, 45 and 70% on all papers in fresh blood, with aged blood tested at 45% hct. For recovery, 5 µL was spotted and a 6 mm punch was sampled. For spot homogeneity 25 µL was spotted and two 1 mm punches were sampled, one from the centre and the other from the perimeter.

All results were expressed as peak area ratios.

Hct preparation: It was important to have a consistent way of preparing the samples across all 12 sites to allow for a fair test. The hct of fresh blood was first tested in duplicate and then the volume of plasma to be added or taken away calculated to obtain the required hct level. To decrease the hct plasma was added. To increase the

hct the blood was centrifuged, the required plasma removed and then the resulting blood roller mixed to obtain a homogenous sample and the hct levels checked in duplicate.

Paper 8: The experimental design for the nonradiolabeled investigation has already been described in paper 7.

The radiolabeled approach used ^{14}C-lacosamide and ^{14}C-deoxy-D-glucose and the work was carried out across two companies (limited due to availability of equipment). Fresh blood (heparin and EDTA) was spiked at 2200 and 1400 ng/mL with one site focusing on lacosamide mouse blood and the second site, deoxyglucose in human and rat blood.

The same DBS papers were tested as paper 7 and both 15 and 30 µL volumes were spotted on day 1. For lacosamide, samples were also spotted on days 2, 3, 4, 5, 8, 10 and 15. Spots were allowed to dry for 2 hours and the radioactivity across the spots measured with a scanner.

Study Design:

- 6 companies testing 12 compounds of varying chemical space
- Identical experiments following the same protocol
- 4 card types tested with varying hct values spanning the minimum and maximum values possible across patient populations (20-70%)

Experimental:

- Varying hct tested for fresh and aged blood tested at the following levels
  20, 30, 35, 40, 45, 50, 60, and 70% (not all levels tested for each variable)
  Hct levels prepared by either removal of plasma (>hct) or addition of plasma (<hct)
- Hct experiments included:
  Spot size and analyte response - 25 µL spot / 3-3.3 mm punch
  Recovery / Spot homogeneity - 5 µL spot / 6 mm punch
  Spot homogeneity - 25 µL spot / two 1 mm punches (one center punch and second from the perimeter)
- All results were expressed as peak area ratios with internal standard correction

Spot size measurements with varying HCT

HCT	1	2, 3	4	5	6	7, 8, 9	10, 11, 12	Average	%CV
226									
20	62	65	75	78	70	73	74	70.9	7.8
30	56	64	63	66	70	66	68	64.9	7.2
35	54	61	67	68	67	64	68	64.1	8.2
40	54	64	64	61	65	63	66	62.5	6.4
45	54	60	62	63	64	65	65	61.8	6.4
50	50	59	62	58	63	61	63	59.4	7.6
60	49	55	64	59	64	59	61	58.8	8.7
70	47	52	52	51	58	57	58	53.5	7.9
DMPK-A									
20	54	63	69	75	65	71	71	66.8	10.4
45	52	57	69	64	58	62	63	60.6	8.7
70	49	51	58	49	52	58	58	53.5	7.8
DMPK-B									
20	66	62	76	71	69	139	102	83.7	33.0
45	58	58	74	66	65	136	129	83.8	40.3
70	56	52	60	55	57	118	95	70.5	36.0
BondElut									
20	22	29	38	35	14	32	38	29.5	30.2
45	30	39	48	41	19	37	40	36.4	25.4
70	34	41	47	43	25	40	42	38.8	19.2

Conclusions:

- Absolute response is impacted by hct
- Higher hct gives a higher viscosity resulting in a smaller spot and higher concentration from a partially punched spot
- Hct impacts recovery and homogeneity and is compound dependent
- Aged blood can lower the recovery
- During validation the risks associated with changing hematocrit should be accessed

Figure 22. Schematic of study design for paper 7

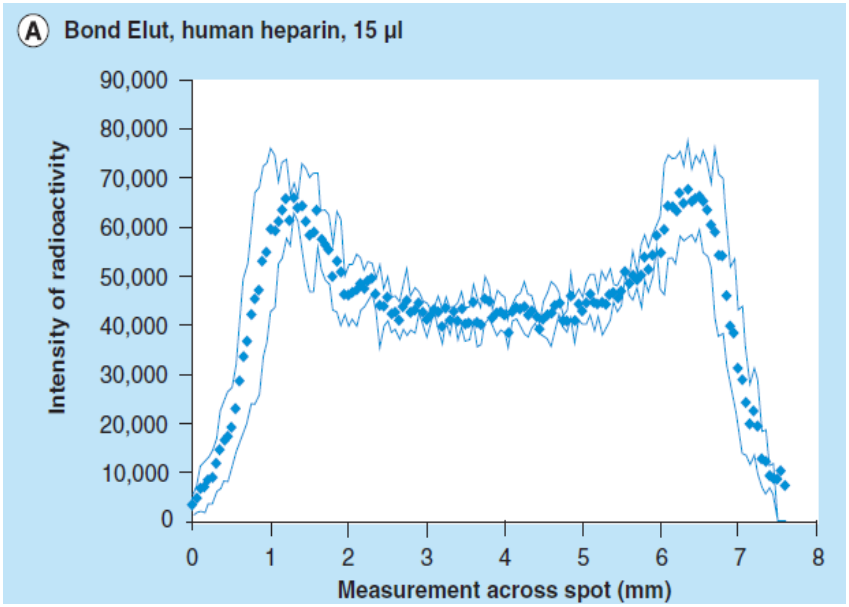
Study Design:

- Two methods assessed to study homogeneity:
  Non radiolabeled approach
  Radiolabeled approach

Experimental:

- Non radiolabeled – covered in paper 7
- Radiolabeled approach
  ^{14}C-lacosamide and ^{14}C-deoxy-D-glucose tested
  DMPK-A, DMPK-B, DMPK-C, Ahlstrom 226 & Bond Elut
  Concentrations tested – 2200 and 1400 ng/mL
  Spotting volumes – 15 and 30 µL

Radioactivity across DBS on Bond Elute



Conclusions:

- Radiolabeled and nonradiolabeled investigations into homogeneity provide complimentary data
- Homogeneity of DBS is impacted by
  Compound
  Card type
  Hct levels
- Data produced by both methods supports results reported in the literature
- Homogeneity must be investigated during validation to ensure accuracy of the measurement

Figure 23. Schematic of study design for paper 8

2.7.3: Data output and impact

Paper 7: The data confirmed the literature data going back to the first paper published on hct (Denniff and Spooner, 2010a).

As the hct levels increase, viscosity of the blood increases causing the blood to flow less freely across the card. This results in a smaller more concentrated spot and when taking a partial punch, larger concentrations of analyte are observed.

Hct can impact the extract recovery of the analyte from DBS samples. The age of the spot can reduce the extract recovery and this can sometimes be associated with hct. Spot homogeneity is also impacted by hct and is compound dependant.

Varying hct can cause increased variability exceeding the acceptance criteria for regulated bioanalysis. The impact should be greatest with the widest spread of hct values e.g. neonates, but results show that even normal hct variances in healthy humans could push the assay variances beyond acceptance criteria.

Careful validation is required to fully understand the impact of hct for each assay and negate the impact of hct and the negative impacts that it plays on DBS.

Paper 8: Radiolabeled and nonradiolabeled investigations into homogeneity provide complimentary data and also agree with previously published work (O'Mara et al., 2011) .

For DMPK-A, DMPK-C, Ahlstrom 226 and Bond Elut a volcano effect in concentration distribution was observed, meaning that there was a higher concentration of analyte at the perimeter of the spot compared with the centre (not seen with DMPK-C in nonlabeled approach). DMPK-B showed the opposite, with higher central concentrations compared with the perimeter.

From the results homogeneity can be impacted by:

1. Card type
2. Compound
3. Hematocrit

DMPK-A although still showed nonhomogeneity it was the least impacted by compound and hct levels.

Nonhomogeneity is a major concern when using DBS to support TK and PK analysis. The assay can be severely impacted especially if a partial punch is taken. During method development and validation homogeneity should be carefully assessed to ensure careful control and understanding.

2.7.4: Technical considerations and reflections

The results from these experiments for both hct and homogeneity were predicted before the work was carried out. Previously published work was not in doubt. Both publications combined all issues associated with hct and homogeneity into two discrete publications from an industries perspective. At the time GSK were concerned that they may be viewed as pushing the technology for the companies benefit and this was not the case. Engagement with the EBF had begun many years earlier with the conception of a microsampling team and the first symposium in 2010 (Connecting Strategies on Dried Blood Spots). The author has been heavily involved from the conception of the team. The EBF consortium was pivotal in having a single voice from the industry and helps the understanding across other disciplines e.g. regulatory perspective.

Working in consortia as always has advantages and challenges. The final output is extremely powerful and influential, but careful management is essential. Choosing individual's to represent their company's views that have relevant expert knowledge is essential. The view of all companies has to be taken into account and not just the companies that have the strongest opinions. Planning experiments in a forum can be a lengthy experience with agreement necessary from all parties. From the author's experience, gaining commitment from all parties, with the understanding of what is expected, and delivering milestones to strict timelines is essential for the success of any consortium. The author's role included setting up the group, agreeing a common protocol, ensuring everyone had the correct supplies, gathering the data together and interpreting it, helping prepare the manuscript and sharing data at meetings.

This work and previous hct work has encouraged the industry to seek opportunities to combat the hct effect. Published options include: whole spot analysis, special filter substrates, measure or predict hct, and DPS (Leuthold et al., 2015, De Kesel et al., 2014, De Kesel et al., 2013, Capiau et al., 2013).

2.8: Commentary paper 9

Tina Panchal, Neil Spooner & Matthew Barfield. Ensuring the collection of high-quality dried blood spot samples across multisite clinical studies.
Bioanalysis (2017) 9(2), 209-213
Recently published so no citation data available.

2.8.1: Objectives

To design and implement a simple way of training and verifying DBS spotting across multi-site clinical studies worldwide:

1) To develop a surrogate for blood
2) To design and develop a dried blood spot training kit
3) Monitor the success rate of spotting using the new training regime

What is felt simple and routine in a bioanalytical laboratory can be considered challenging to others who are unfamiliar. Simple written instructions and DVDs can be ignored or misunderstood. Sample quality is key, without ensuring the quality of sample collection, PK and TK data can be compromised with potentially dangerous consequences to patient safety. Studies comprising of a single site centre are simpler to manage and ensure adequate training, however as compounds progress and patient groups need to be accessed, the number of sites increases as do the complexities. The author compiled written training material and a simple video to help train individuals remotely. However, in the first GSK multisite study over 30% of the DBS samples were unusable, which is not acceptable.

This paper set out how to best train and validate the quality of personnel involved with DBS spotting.

2.8.2: Experimental design

Due to the impracticalities of sourcing blood worldwide a suitable surrogate was designed. Water and milk containing a red die were initially tested to produce the most realistic blood surrogate for test spotting. The author developed a test kit which was shipped to sites containing all the necessary equipment and instructions for the test.

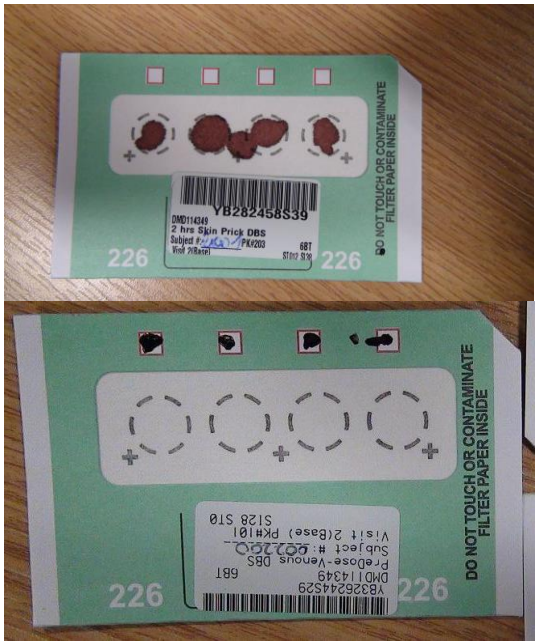
The kit was initially tested on two people from GSK who had never been involved in DBS before to assess its usage. These were non-scientific individuals who were not used to laboratory working. The test proved to be easy to follow and provided encouraging results.

The first multi-site study tested consisted of 15 sites across the US and Germany. Before actual sample collection all those involved had to complete the training and send pictures of spotting with the surrogate matrix for approval of the technique.

Study Design:

- Multi-site clinical centre across 15 sites
- Remote training via DBS test kit
- Evaluation of training

Examples of poor spotting from multi-site studies



Experimental:

- Screen blood surrogates concentrating on easily accessible reagents e.g. milk and water. Must behave as blood when spotted
- Design a test kit containing all equipment necessary to facilitate training and validation of spotting
- Evaluation of kit by local inexperienced staff to validate procedure
- Kits provided to study sites. Use material to self-train and send pictures of spotting for validation

Conclusions:

- Mixture of condensed milk powder, water and red die gave best blood like properties
- Local testing validated the test kit using novice DBS users before testing in a clinical environment
- 15 clinical centres supporting one study were successfully trained remotely using the test kit
- Subsequently 2 further multisite centres were supported. All study centres had no DBS samples that could not be analysed

Figure 24. Schematic of study design for paper 9

2.8.3: Validation and data output and impact

The paper demonstrated that the ability to practice blood spotting prior to the collection of study samples is essential for quality sampling. Utilising traditional training techniques, such as written instructions and DVDs, is not sufficient and can lead to samples of unacceptable quality which can result in jeopardizing entire studies.

The ability to train individuals prior to spotting real samples is valuable and coupled to peer review; this has proven to be a reliable means of obtaining quality DBS samples. Historically failure rates of greater than 30% were typical. Using the test kit and peer review, failure rates are now down to 0%, across three multi-site clinical studies utilizing DBS.

2.8.4: Considerations and reflections

The idea of actually validating individuals is key to success. One development that has evolved during the work is the difficulties to ship what was first thought an innocuous material, milk powder. As this is a food product for some countries it is not a straightforward process. At the time local sourcing was envisaged, but this also proved problematic. The final solution which can be sourced locally, shipped across borders and does not require refrigeration is red quality control glucose solution. This is used across the world as StatStrip™. For future training this will be the product used.

The lessons learned around training have been extremely valuable and can be applied to many areas. Especially of interest are new microsampling devices that are continually being developed. An important factor for any new device has to be the simplicity of use and ease of training. Any training needs to be tailor made to the product and needs to have a way of checking the quality of the end product before committing to study samples.

3: Impact of the work

The initial two publications made the largest impact on the industry (190 and 277 citations) with the subsequent publications exploring the practicalities and science behind the technique. At the height of impact of DBS, a publications titled "Bioanalysis Using Dried Blood Spots: The Biggest Advancement in Bioanalysis Since LC–MS-MS?" (Paul D. Rainville, 2010) was released. In a way this was true with the interest being widespread and perceived as an exciting development in the history of Bioanalysis. The impact can also be seen with the number of publications on the technique (Figure 25) and also the global spread of publications (Figure 26)

At the time of the initial publications, data on GSK compounds and novel techniques was not encouraged, as it could divulge sensitive information and destroy a possible competitive advantage. DBS did offer GSK many possible competitive advantages. However, the advantages to animal welfare (reduction and refinement), paediatrics and home sampling overweighed any risks and the work was allowed to be shared with the industry as a whole.

A publication strategy was developed to enable others to help develop the technique and probe deeper into the underlying science. Also presentations around the globe were made to spread the word with most of the large relevant meetings having dedicated DBS/microsampling sessions. A list of presentations made by the author are included in appendix 1.

The impact of these works can be measured as a factor of:

- Economic – including the money saved with less animal usage, less compound dosed, ambient shipping and storage, to the economy created from a microsampling industry, developing jobs and revenue in this area.
- Knowledge – the in-depth scientific understanding of DBS. Also brought together clinicians, toxicologists, animal technicians, bioanalysts etc. This enabled better understanding for each other's processes and constraints, which led to better processes e.g. unified rodent sampling, increased sample quality.

- Social – from the opportunity for less invasive sampling, potential to develop medicines that can be used for paediatrics, near patient sampling and the ability to access developing and 3rd World countries.
- Regulatory – Regulators verbally encourage DBS and microsampling due to its ethical advantages. FDA draft guidelines (Bioanalytical Method Validation, 2013) set out requirements to validate a DBS assay. ICH SA3 Focus on Microsampling and ICHM10 (industry and all main regulator) are currently discussing an overall guidance for method validation and this will cover microsampling.
- Health – the ability to home sample and access to paediatrics.

GSK invested heavily into the technique and also investigating its limitations. GSK found the hct effect, which ultimately changed the industry's views on DBS. Initially it was seen as a replacement for conventional sampling; however it is now viewed as a niche technology, only used in certain applications e.g. paediatric studies and home sampling. The search for a solution to microsampling is continuous, and the author and GSK have encouraged and challenged the industry to find future microsampling solutions. As a result, an entire industry has been built around new microsampling techniques (figure 27 and table 11) with the largest drivers being 3Rs, paediatric support, and remote sampling.

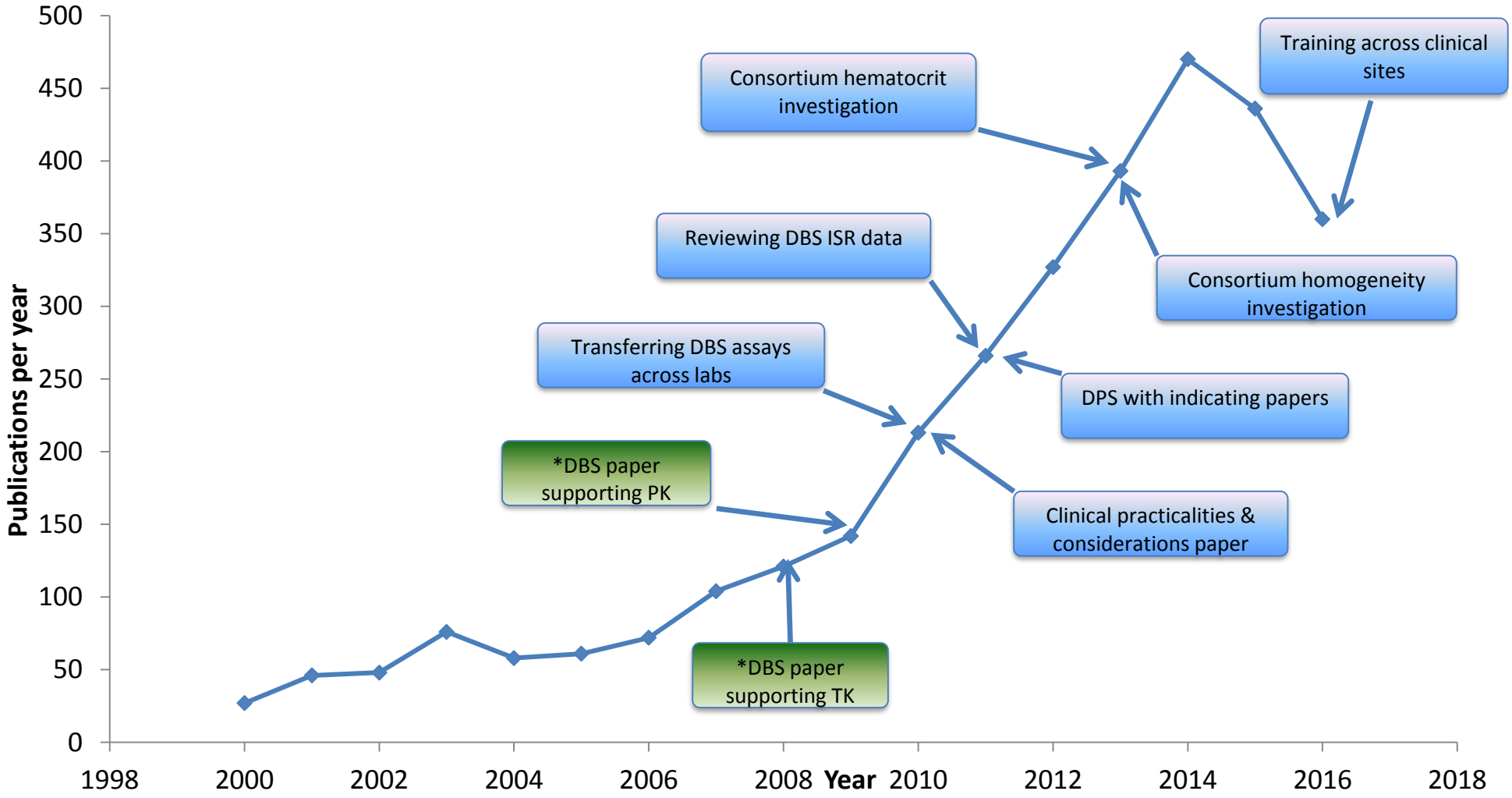


Figure 25. The number of publications per year that contain "Dried Blood Spot". Source – Scopus (Elsevier)

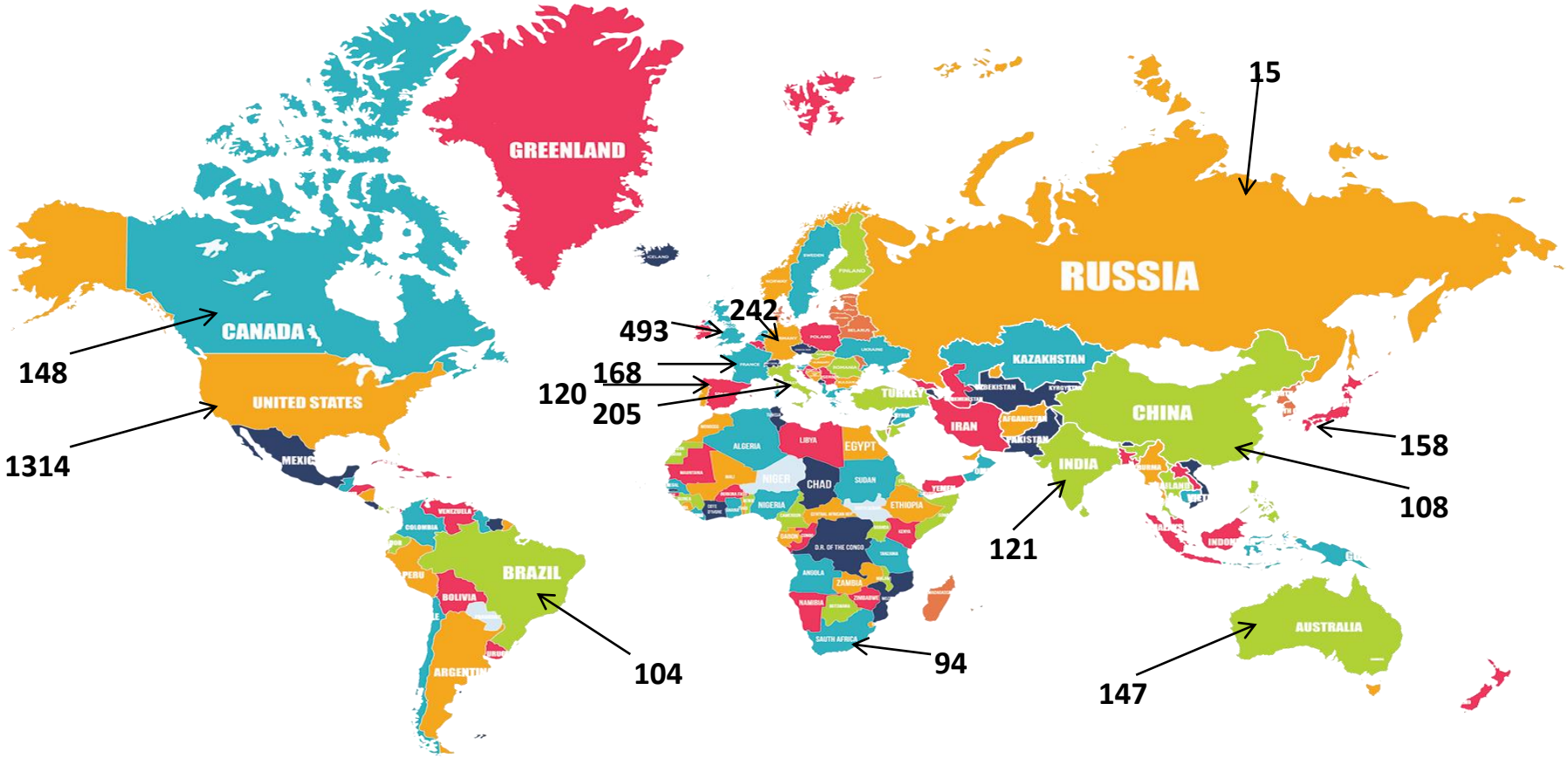


Figure 26. The number of publications across the World that contain "Dried Blood Spot". Source – Scopus (Elsevier) (capped at 94 and showing Russia for interest)

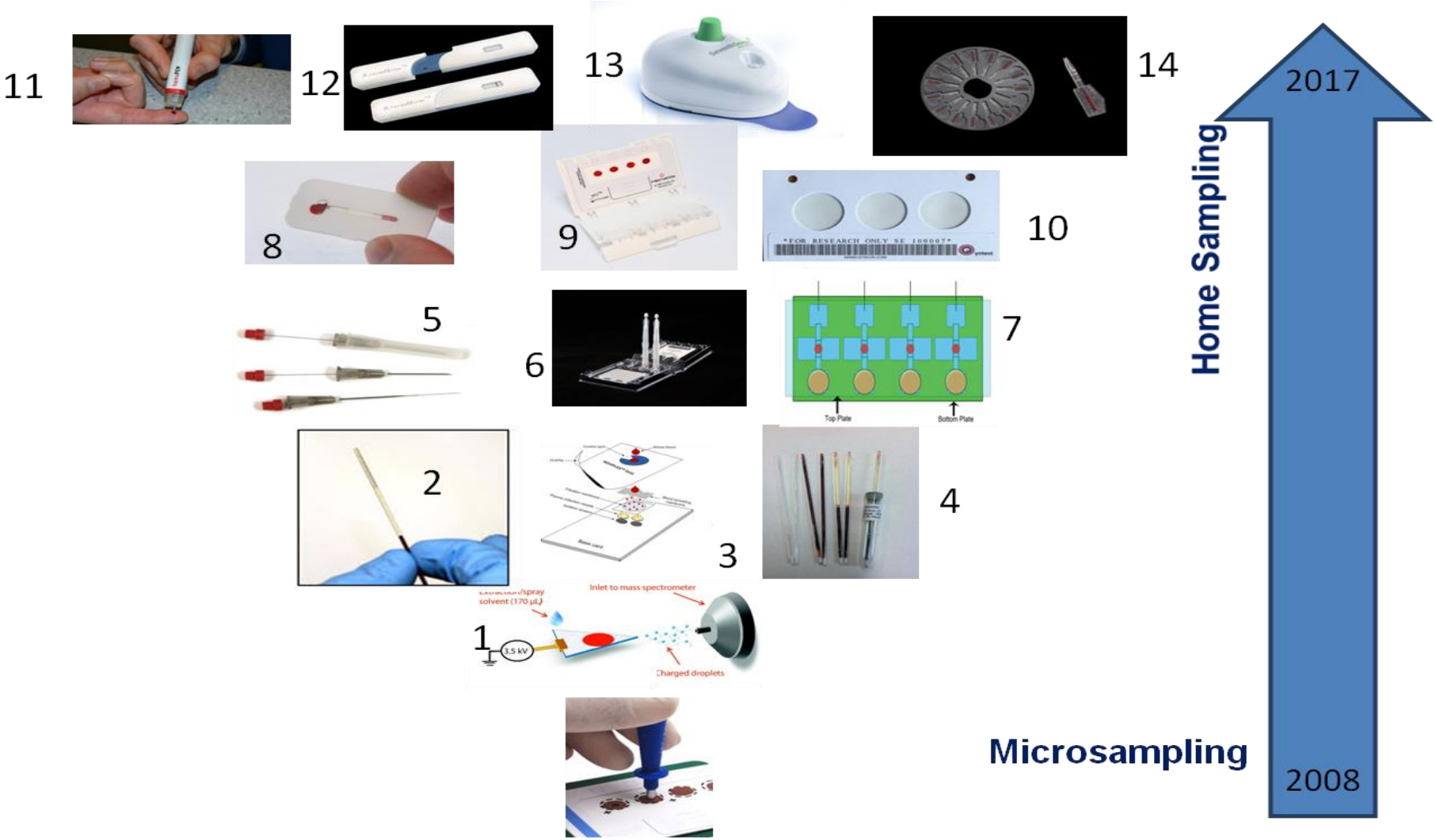


Figure 27. Novel microsampling techniques since the conception of DBS for PK and TK analysis

Date introduced	Technique	Description	Pros	Cons
1: 2011 (Liu et al., 2014, Manicke et al., 2016, Yannell et al., 2017)	Paper Spray (PS)	Blood sample applied to an integrated paper mounted device. This is a total solution, sample collection, storage, shipping and direct analysis	Complete solution	-The device could be expensive -Dry vs wet comparison may be needed in all studies for regulatory acceptance -Still in development -Accurate volume of blood required - Effect of changing hct on signal
2: 2012-2013 (Nilsson et al., 2013)	Plasma Capillary Microsampling (AZ method)	Blood sample collected into a capillary. Plasma and blood separated by centrifugation and the capillary cut and the blood portion discarded. An accurate plasma volume is collected in a second capillary, which is placed in a tube and frozen ready for shipment and analysis.	Analysis of a wet sample so mirrors established process. No regulatory concerns	-Approximately 50% of the sample is wasted -Requires freezers, cold storage and shipment -Time consuming to process samples and hard to automate, generally only used in preclinical -Not simple
3: 2012-2013 (Li et al., 2012, Kim et al., 2013)	Plasma separation cards. Blood filtration devices (e.g. Yorktest, Noviplex & Novilytics	Blood sample (25-50µL) is applied to a membrane based filter card. The blood cell component is separated producing a DPS. Sample is dried and ship/stored at ambient	Hct issues for a dry sample negated (accurate volume and plasma) Cost savings: Ambient shipping/storage Produces a RBC free sample	-Dry vs wet comparison may be needed in all studies for regulatory acceptance -Still in development Endogenous levels of plasma components (lipids,glycoprotein) may have a similar effect as varying haematocrit -Approximately 50% of the sample is wasted -What is the sample? Is it plasma?
4: 2013 (Bowen et	Plasma capillary Microsampling	Blood sample collected into a capillary (75µL),	Analysis of a wet plasma sample so mirrors	-Approximately 50% of the sample is wasted -Requires freezers,

al., 2013)	(Drummond)	containing a thixotropic gel which acts as an interface between the blood and plasma fractions after centrifugation. A Wiretrol™ device is used to expel the plasma from the capillary into a micro sample tube, and frozen. Aliquots can be taken for analysis.	established process. No regulatory concerns	cold storage and shipment -Time consuming to process samples and hard to automate
5: 2013 (Ahmad et al., 2015, Ahmad, 2013)	Solid Phase Micro Extraction (SPME)	Small fibre coated with an extracting phase inserted into the animal or human. The fibre is left in the circulation for 1 minute to allow equilibrium to be reached and then can be shipped in a tube at ambient temperature	No sample wastage and actually no blood loss. Potential to use fibre as a direct ionisation tool Simple to use, ambient shipping and storage	-Totally new concept, so industry and regulatory acceptance required -Fibre is physically inserted into the subject which may be hard to implement for humans
6: 2014 (Denniff and Spooner, 2014)	VAMS/MITRA ™ (Phenomenex)	Device has a tip that absorbs a fixed volume of blood. Based around a pipette tip. Dry sample technology	-No sample wastage (blood) -Simple -Fixed volume so no issues with dry sample and hct -Cost savings:Ambient shipping/storage	-Dry vs wet comparison may be needed in all studies for regulatory acceptance -Little data available -Hard to automate
7: 2014 (Fobel et al., 2014)	Digital Microfluidics (DMF)	Blood sample is applied to an integrated chip. The device is a collection device, used for storage, shipping and analysis	-Direct analysis, simple processing	-Dry vs wet comparison may be needed in all studies for regulatory acceptance -Still in development -Accurate volume required
8: 2015 (Lenk et al., 2015)	CapiTainer™	Uses dissolvable membranes to apply an accurate volume of blood to a card (DBS)	Accurate volume of blood, remove hct issues -Simple	- Apart from hct any other issues associated with DBS -Still in development
9: 2015 (Leuthold et al., 2015)	DBS System: HemaXis™	Incorporates a microfluidic platform linked to a DBS card	-Accurate volume of blood, remove hct issues -Simple	-Apart from hct any other issues associated with DBS -Still in development

10: 2015 (Mengerink et al., 2015)	Qyntest™ by Qynion	Polmer card with a layered design to minimise hct and homogeneity issues	-Potentially overcomes hct and homogeneity issues -Benefits as DBS	-Dry vs wet comparison may be needed in all studies for regulatory acceptance -Hard to punch and still in development
11: 2016 (Capiau, 2015)	Trajan - hemaPEN™	Utilises capillaries for accurate blood volume measurement and DBS technologies	-Accurate volume of blood, remove hct issues -Simple	-Expensive - Apart from hct any other issues associated with DBS -Untested, in development
12: 2016	Boston Microfluidics (bmf)	Uses microfluidics to accurately dispense blood on a DBS card	-Accurate volume of blood, remove hematocrit issues	-Untested -Dry vs wet comparison may be needed in all studies for regulatory acceptance -Untested, in development
13. 2017	TAP™ – Seventh Sense	A device to take pain free blood samples. Designed for home sampling and in the future will be coupled to DBS	-Accurate volume of blood, remove hct issues -Simple	-Expensive - Apart from hct any other issues associated with DBS -Untested, in development
14. 2017	Wing device™	Device that collects blood which is then centrifuged and the tips can be broken off to give an accurate volume of plasma	Very simple Analysis of a wet sample so mirrors established process. No regulatory concerns	-Approximately 50% of the sample is wasted -Hard to automate -Untested, in development

Table 11. Descriptions of new microsampling techniques with pros and cons associated

4: Conclusions

This thesis sets out to detail the development of DBS in support of both TK and PK, through the author's published work and brings together learnings, spanning the last 12 years. The published works demonstrates fundamental understanding, scientific advancement of DBS as an application, and the investigation of best practice. DBS has focused the industry on microsampling and the major benefits that can be gained, specifically in animal savings and ability to support previously unattainable clinical studies e.g. paediatric and remote areas. The author has been pivotal from conception, planning and executing experiments, reporting and influencing the community.

The major contributions to the knowledge detailed in the commentary were: (1) the first TK study supported by DBS which focused on the possible advantages; (2) the first PK study supported by DBS which focused on the possible advantages; (3) practical considerations of supporting clinical studies; (4) transferability of DBS assays across laboratories; (5) utilising ISR data to further understand the technology; (6) using paper technologies to support plasma; (7) investigating the impact of hct; (8) homogeneity of the sample; (9) procedure to train remotely.

One area that has not been explored is the area of change management. Introducing a new technology is not a straightforward process. Scientists are generally thought as innovative and keen to explore new possibilities. From the author's experience this is not always the case and change management is a key to the introduction of any new technology or process. The author's role was to engage across GSK and externally, describe in detail the potential advantages and issues associated and deliver scientific data concisely that was understandable for all. Also to seek individuals and teams who were keen to implement and try new technologies and further push the scientific understanding.

Not only have advantages been reviewed but also a critical evaluation of the technique, detailing its limitations especially in a highly regulated environment. DBS is now one of the many microsampling tools and is still currently in use at GSK and across the industry supporting late phase and niche studies. With future advances in both paper technologies and/or accurate simple volumetric sampling, DBS could have the potential to be the primary sampling method for TK and PK. DBS has started a microsampling industry and the impact has been across multiple disciplines.

An excellent example is a recent publication (Verhaeghe, 2017) which shows the elimination of TK satellites from GLP studies, with the first reference being commentary paper 3. This demonstrates the continued influence the author's work has had. One certainty is that microsampling, born from DBS is routine and expected from both the industry and regulators.

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6. Appendices

Appendix 1: Invited speaker at International Conferences:

- NC3Rs Conference, London, 2008
- DMDG DBS Meeting, Manchester, 2008
- DMDG (Drug Metabolism Discussion Group), Cambridge, UK, 2008
- LASA (Laboratory Animal Science Association), Scotland, 2008
- TKDG (Toxicokinetics Drug Discussion Group), Scotland, 2008
- House of Lords NC3Rs, London, 2009
- GSK DBS Day (International attendance), Ware, UK, 2009
- Reid Bioanalytical Forum, Guildford, UK, 2009
- DMDG, Cambridge, UK, 2009
- Syngenta DMPK Forum, London, 2009
- Unilever DMPK Forum, London, 2009
- 58th ASMS Conference, Salt Lake City, USA, 2010
- EBF Focused Workshop, Brussels, 2010
- EBF (Microsampling Consortium), Brussels, 2011
- Reid Bioanalytical Forum, Guildford, UK, 2011
- DMDG (organising committee for scientific programme, chaired a session titled 'Novel Solutions in Bioanalysis'), Cambridge, UK, 2012
- JPAG (Joint Pharmaceutical Analysis Group)/DMDG (Organiser), London, 2013
- EBF Open (chaired microsampling session), Barcelona, 2016

Appendix 2: Summary of Contributions by Matthew Barfield to each of the Commentary papers

Commentary paper no.	Title	Contribution by Matthew Barfield
1	Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies	• Lead Author • Designed and conducted experiments • Champion in establishment DBS Capability at GSK
2	DBS as a sample collection technique for the determination of pharmacokinetics in clinical studies.	• Designed and conducted experiments • Experimental data manipulation • Contributing author
3	Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations.	• Participated in research design • Provided analytical input and resources • Contributing author
4	Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories.	• Contributing author • Participated in research design • Provided analytical input and resources
5	GlaxoSmithKline's experience of incurred sample reanalysis for DBS samples.	• Lead Author • Designed and conducted experiments

6	Use of dried plasma spots in the determination of pharmacokinetics in clinical studies: validation of a quantitative bioanalytical method.	• Lead Author • Designed and conducted experiments
7	The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium.	• Lead in bringing consortium together • Contributing author Participated in research design • Provided analytical input and resources
8	In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium	• Lead in bringing consortium together • Contributing author Participated in research design • Provided analytical input and resources
9	Ensuring the collection of high quality dried blood spot samples across multisite clinical studies	• Contributing author • Mentor junior author • Participated in research design • Provided analytical input and resources

Appendix 3: Letters from lead authors and GSK Position on common practice for authorship

Provided here are letters from lead authors of the papers upon which this Commentary is based, confirming Matthew Barfield's input to these papers and the work which they report. These are for Commentary papers 2, 3, 4, 7, 8 and 9; Matthew Barfield was lead author on papers 1, 5 and 6. Also included in this Appendix is a document to provide background to the GSK common practice for contribution to works and authorship of papers.

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To whom it may concern,

This statement is designed to explain the situation around authorship of publications from industry to assist The University of Lincoln and other interested parties in assessing the works included in the PhD Commentary by Matthew Barfield. All publications authored or co-authored by Matthew Barfield have been produced whilst he has been employed by GlaxoSmithKline Research and Development Ltd. (GSK).

It is common practice in industry to have varied contributors for published papers as the work being published often arises through collaborations across multiple representative groups and drug development projects. The list of co-authors is then dictated by a number of influences and may not necessarily be all inclusive or indeed reflect, for example, the magnitude of the contribution. That said, there are strict guidelines concerning justification for inclusion of an individual as an author, which are adhered to *[GSK ensures that authorship selection is consistent with Good Publication Practice (GPP2) and the International Committee of Medical Journal Editors (ICMJE) guidelines (www.icmje.org)].*

For all papers where Matthew Barfield has not been the main author, approval from the main author has been granted. Written confirmation has been included and details that for each paper:

"I confirm that Matthew Barfield contributed significantly to the experimental design, support of the work performed and publication preparation as detailed in the paper"

Only papers that Matthew Barfield has had a proven significant role, will be used to support his PhD by publication

Yours faithfully,

Dr. Scott summerfield, PhD. [Industrial Supervisor for Matthew Barfield]

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I confirm that Matthew Barfield contributed significantly to the experimental design, support of the work performed and publication preparation as detailed in the paper --

Ronald de Vries, Matthew Barfield, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Zoe Cobb, Steve White & Philip Timmerman. " The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium."

Bioanalysis (2013) 5(17), 2147–2160

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Phillip E Turpin, Josephine EC Burnett, Lee Goodwin, Amanda Foster and Matthew Barfield.
"Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories."
Bioanalysis (2010) 2(8), 1489–1499.

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I confirm that Matthew Barfield contributed significantly to the experimental design, support of the work performed and publication preparation as detailed in the paper –

Neil Spooner, Y Ramakrishnan, M Barfield, O Dewit & S Miller. " Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations." *Bioanalysis* (2010) 2(8), 1515–1522

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01 Sep 16

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Neil Spooner, Rakesh Lad, and Matt Barfield. " Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method."
Anal. Chem. 2009, *81,* 1557–1563.

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I confirm that Matthew Barfield contributed significantly to the experimental design, support of the work performed and publication preparation as detailed in the paper –

Zoe Cobb, Ronald de Vries, Neil Spooner, Stephen Williams, Ludovicus Staelens, Mira Doig, Rebecca Broadhurst, Matthew Barfield, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij , Ben van Baar, Steve White & Philip Timmerman. "In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium"

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Dr Z. Cobb, PhD
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Appendix 4: Ethics approval

University of Lincoln approval reference: UID CoSREC289

GSK approval:

Ethics statements relating to PhD Commentary on Published Works by Matthew Barfield BAR15620627:

All of the work sponsored or conducted by GlaxoSmithKline, detailed in the papers included in this Commentary, complied with the following:

All GSK-sponsored studies were conducted in accordance with the guiding principles of the Declaration of Helsinki and in compliance with good clinical practices and local regulatory guidelines. The protocols and informed consent forms were approved by an institutional review board or independent ethics committee at each study site before any subject was enrolled or study procedure performed. All human biological samples were sourced ethically and, where applicable, patient consent was obtained for their use for research. Additionally subject anonymity was maintained through use of study assignment randomisation codes.

All GSK-sponsored animal studies were conducted in accordance with the relevant GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee (or similar at the time of conduct) either at GSK or by the ethical review process at the institution where the work was performed.

Dr Scott Summerfield FRSC

Senior Director, Global Head of Bioanalysis, GlaxoSmithKline Research and Development Ltd,

Appendix 5: Reproduced principle papers

1. Reprinted with permission from **Matt Barfield**, Neil Spooner, Rakesh Lad, Simon Parry, Susan Fowles, 2008. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies: Journal of Chromatography. Elsevier, Copyright 2008.

2. Reprinted with permission from Neil Spooner, Rakesh Lad, and **Matt Barfield**. DBS as a sample collection technique for the determination of pharmacokinetics in clinical studies: Anal Chem. Copyright 2009, American Chemical Society.

3. Neil Spooner, Y Ramakrishnan, **M Barfield**, O Dewit & S Miller. Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations: Bioanalysis, 2010.
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4. Phillip E Turpin, Josephine EC Burnett, Lee Goodwin, Amanda Foster & **Matthew Barfield**. Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories: Bioanalysis, 2010.
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5. **Matthew Barfield**, Sheelan Ahmad & Maria Busz. GlaxoSmithKline's experience of incurred sample reanalysis for DBS samples: Bioanalysis, 2011.
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6. Reprinted with permission from **Matthew Barfield** and Robert Wheller. Use of dried plasma spots in the determination of pharmacokinetics in clinical studies: validation of a quantitative bioanalytical method: Anal Chem. Copyright 2011, American Chemical Society.

7. Ronald de Vries, **Matthew Barfield**, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Zoe Cobb, Steve White & Philip Timmerman. The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium: Bioanalysis, 2013.
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8. Zoe Cobb, Ronald de Vries, Neil Spooner, Stephen Williams, Ludovicus Staelens, Mira Doig, Rebecca Broadhurst, **Matthew Barfield**, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij , Ben van Baar, Steve White & Philip Timmerman. In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium: Bioanalysis, 2013.

9. Tina Panchal, Neil Spooner, **Matthew Barfield**. Ensuring the collection of high quality dried blood spot samples across multisite clinical studies. Bioanalysis 2017.

Appendix 6: Papers in chronological order

Journal of Chromatography B, 870 (2008) 32–37

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies

Matt Barfield, Neil Spooner*, Rakesh Lad, Simon Parry, Susan Fowles

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ABSTRACT

A reversed phase HPLC-MS/MS method has been developed and validated for the quantitative bioanalysis of acetaminophen in dried blood spots (DBS) prepared from small volumes (15 μL) of dog blood. Samples were extracted for analysis with methanol. Detection was by positive ion TurboIonSpray™ ionisation combined with selected reaction monitoring MS. The analytical concentration range was 0.1–50 μg/mL. The intra-day precision and bias values were both less than 15%. Acetaminophen was stable in DBS stored at room temperature for at least 10 days. The methodology was applied in a toxicokinetic (TK) study where the data obtained from DBS samples was physiologically comparable with results from duplicate blood samples (diluted 1:1 (v/v) with water) analysed using identical HPLC-MS/MS conditions. This work demonstrates that quantitative analysis of a drug extracted from DBS can provide high quality TK data while minimising the volume of blood withdrawn from experimental animals, to an order of magnitude lower than is current practice in the pharmaceutical industry. This is the first reported application of DBS analysis to a TK study in support of a safety assessment study. The success of this and similar, related studies has led to the intent to apply DBS technology as the recommended analytical approach for the assessment of pharmacokinetics (PK)/TK for all new oral small molecule drug candidates, which have previously demonstrated a successful bioanalytical validation.

1. Introduction

Traditionally, the toxicokinetics (TK) of exposure to new chemical entities in preclinical safety studies is reported in plasma, due to the ease of sample collection and storage. In order to derive the appropriate volume of plasma required for quantitative bioanalysis, relatively large volumes of blood (between 100 and 500 μL) need to be taken from the animals. Due to the physiological and ethical limitations of obtaining multiple serial plasma samples from individual animals, especially small or juvenile rodents [1], composite sampling is often chosen. This leads to an increased number of animals being utilised and may result in lower quality TK data.

Regulatory authorities have long acknowledged that blood is an acceptable biological matrix for the measurement of drug exposures [2,3]. However, due to practical difficulties with shipping and storing blood samples, plasma is usually selected as the matrix of choice. Filter paper blood sampling is an established technique for the screening of in-born errors of metabolism [4,5]. There have also been a number of reports on its use in humans for therapeutic drug monitoring and pharmacokinetic (PK) studies [6–13]. Dried blood spots (DBS) offers the advantage of less invasive sampling, simpler matrix preparation and transfer (no centrifugation to produce plasma) and easy storage and shipment to laboratories (no requirement for freezers and dry ice). It is notable that there are very few reports of application of this technology to preclinical PK assessments [14–16]. For these studies, in addition to the advantages associated with the clinical use of DBS, the small blood volumes used for DBS facilitates increased PK data quality, through the ability to take serial bleeds from the same animal, particularly when coupled with sensitive and selective HPLC-MS/MS techniques. This benefit becomes even more significant as the pharmaceutical industry focuses on the FDA requirement to evaluate juvenile animals as part of the Safety Evaluation phase for drugs for paediatric use [17]. A further important consideration is that the use of DBS in pre-clinical rodent TK studies for the safety assessment of new chemical entities has the potential to significantly reduce the number of animals required, giving significant ethical and cost benefits.

This paper describes the development and validation of a method for the quantification of acetaminophen in dog blood, as a DBS sample. The results of these tests were evaluated against internationally used acceptance criteria described by Shah et al. [18].

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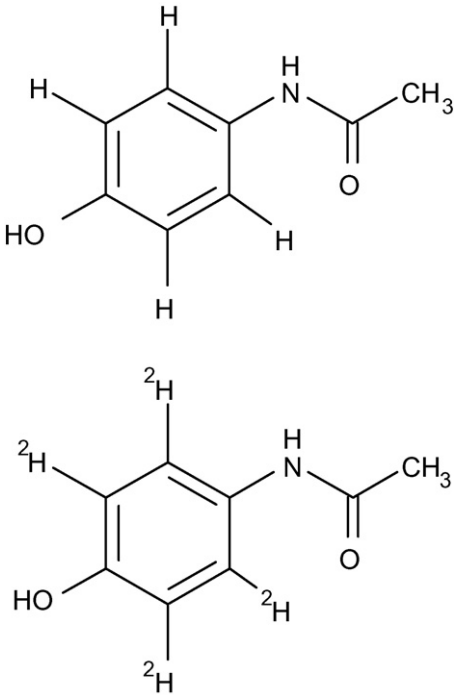

Fig. 1. Chemical structures of acetaminophen and [$^2H_4$]-acetaminophen.

The subsequent use of the method for the analysis of samples taken from dogs in a routine TK dose range finding toxicology study following the oral administration of acetaminophen is described. This work was conducted in support of a current GSK drug development programme. The methodology was validated in dog as this species is to be used in the development of the molecule. Comparison of the DBS data obtained from analysis of replicate whole blood samples (diluted with water (1:1, v/v)) using the same HPLC-MS/MS conditions was used to validate the new technology. The outcome demonstrates the potential advantages of further application of DBS in pre-clinical and regulated drug development.

2. Experimental

2.1. Chemicals and reagents

Methanol and water were of HPLC gradient grade and were obtained from Fischer (Loughborough, UK). All other chemicals were of AnalaR grade, supplied by VWR International Limited (Poole, UK). Dog (Beagle) blood was supplied by Harlan (Hull, UK). Acetaminophen (Fig. 1) was obtained from Aldrich (Poole, UK). The stable isotopically labelled internal standard, [$^2H_4$]-acetaminophen (Fig. 1) was produced by Isotope Chemistry, GlaxoSmithKline (Stevenage, UK).

2.2. Equipment

FTA® Elute blood spot cards were supplied by Whatman (Sanford, USA). Sample tubes were obtained from Micronics (Platinastraat, The Netherlands). Centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat was supplied by Whatman (Sanford, USA). Plastic bags for the storage of blood spot cards were supplied by Fischer (Loughborough, UK). Sachets of desiccant were obtained from Sud-Chemie (Northwich, UK).

2.3. Preparation of standard stock and working solutions

Primary stock solutions of acetaminophen (in duplicate) and [$^2H_4$]-acetaminophen (internal standard) were prepared in dimethylformamide (1 mg/mL). Working standard solutions of acetaminophen in H_2O:acetonitrile (1:1, v/v) were prepared from the primary stocks at concentrations of 100 and 10 μg/mL. Internal standard working solutions (0.5 μg/mL) were prepared from the primary stock using methanol for blood spot analysis and acetonitrile for blood/water analysis. All the solutions were stored at 4 °C and brought to room temperature before use.

2.4. Preparation of calibration standards and quality control (QC) samples

Calibration standards were prepared fresh on the day of analysis (for both validation and TK study sample analysis) by diluting the appropriate working solutions with blank whole dog blood for DBS analysis and blank dog blood/water (1:1, v/v) for blood/water analysis. The concentrations for both DBS and blood/water calibrants were 0.1, 0.2, 0.5, 2, 5, 20, 40 and 50 μg/mL whole blood.

QC samples for all analyses were prepared from a separate stock solution to that used for the calibration standards. QC samples for the DBS validation were prepared by diluting the appropriate working solutions with blank dog matrix to give concentrations of 0.1, 0.3, 5, 40 and 50 μg/mL whole blood. For the analysis of TK study samples, QC's were prepared with blank whole dog blood (DBS) and blank dog blood/water (1:1, v/v) for blood/water analysis at concentrations of 0.3, 5, 40 μg/mL whole blood.

For DBS analyses 15 μL aliquots of calibration standards and QC samples were spotted onto FTA® Elute cards and allowed to dry at room temperature for at least 2 h prior to analysis. When required, the DBS QCs were stored at room temperature in a sealed plastic bag containing desiccant until analysis.

For blood/water analyses, 30 μL aliquots were transferred to a clean tube, prior to analysis. When required, the blood/water QCs were transferred to sample tubes for storage at −20 °C until analysis.

2.5. Sample preparation

For DBS analyses, a 3 mm diameter disk was punched from the centre of the DBS into a clean tube. Methanol (100 μL) containing internal standard ([$^2H_4$]-acetaminophen) was added and the tube vortex mixed for approximately 30 s. The tube was centrifuged for 5 min at 3000 × *g* and the supernatant transferred to a clean tube and a portion injected onto the HPLC-MS/MS system.

For blood/water analyses, a 30 μL aliquot was extracted with 300 μL acetonitrile containing internal standard ([$^2H_4$]-acetaminophen). This was vortex mixed for approximately 30 s and then centrifuged for 5 min at 3000 × *g* and a portion of the supernatant injected onto the HPLC-MS/MS system.

2.6. HPLC-MS/MS analysis

The HPLC-MS/MS system consisted of a CTC HTS PAL autosampler (Presearch, Hitchin, UK) with fast wash, an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven and divert valve, and a Polaris Amide C18, 3 μm, 50 mm × 3.2 mm i.d. HPLC column (Varian Limited, Oxford, UK). The post column flow was diverted to waste for first 0.5 min of each chromatographic run. During this time, flow (0.25 mL/min, methanol:water (1:1, v/v)) was provided to the MS by a Knauer pump (Presearch, Hitchin, UK).

The chromatographic separation was achieved using a solvent gradient employing the mobile phases 10 mM ammonium formate

containing 0.3% ammonia (A) and methanol (B). Following sample injection (3 μL) the mobile phase was held at 98% A for 0.2 min. A ballistic gradient to 5% A at 0.3 min, was followed by an isocratic period at 5% to 0.8 min. The mobile phase was then returned to 98% A by 0.9 min and was held at this composition until 1.5 min, before the injection of the next sample. The flow rate was 1 mL/min, the column was maintained at 40 °C. The HPLC effluent was split approximately 1:3 before entering the ion source.

MS detection was by a Sciex API-3000 (Applied Biosystems/MDS Sciex, Canada) equipped with a TurboIonspray™ ion source. The source temperature was 450 °C with a turbo gas flow of 7 L/min (N_2) and a nebuliser gas setting of 10 (N_2). The curtain gas and collision gas settings were 10 and 6, respectively (both N_2). The characteristic precursor $[M+H]^+$ to product ions transitions, m/z 152–110 and 156–114 were consistent with the structures of acetaminophen and the internal standard (loss of CH_2CO), respectively, and were used as selected reaction monitoring transitions to ensure high selectivity. A dwell time of 200 ms was used for both transitions. The pause time was 5 ms.

HPLC-MS/MS data were acquired and processed (integrated) using Analyst software (v1.4.1 Applied Biosystems/MDS Sciex, Canada). Concentrations were determined from the peak area ratios of analyte to internal standard using the in-house laboratory information management system, SMS2000 (v1.6, GlaxoSmithKline, UK).

2.7. Assessment of matrix suppression effects

To assess the suppression of HPLC-MS/MS detector response due to matrix components associated with DBS, the peak areas of the internal standard in extracts of replicate DBS QC samples at 0.3, 5 and 40 μg/mL whole blood, were compared to those of the same concentration of internal standard spiked directly into methanol.

2.8. Application of the DBS assay to a toxicokinetic study

One male and one female dog received a single daily oral gavage administration of a suspension of acetaminophen at a target dose of 50 mg/kg for 7 days. Blood samples (1 mL) were collected by jugular venepuncture into EDTA tubes on days 1 and 7 at 0.5, 1, 2, 4, 8, 12 and 24 h after administration.

For DBS analyses, replicate ($n=3$ for each time point) 15 μL aliquots from each blood sample were spotted onto FTA® Elute paper and allowed to dry at room temperature for 2 h. These were then stored and shipped at room temperature in a sealed plastic bag containing desiccant before analysis.

For blood/water analyses, a further 15 μL of blood from each sample was aliquoted into a tube containing 15 μL of HPLC grade water. The tubes were mixed by inversion and frozen immediately over carbon dioxide. The diluted blood samples were stored and shipped at −20 °C before analysis.

3. Results and discussion

3.1. Method validation

3.1.1. Linearity, selectivity and sensitivity

A calibration plot of analyte/internal standard peak area ratio versus the nominal concentration of acetaminophen was constructed and a weighted $1/x^2$ linear regression applied to the data. Linear responses were observed for DBS over the range 0.1–50 μg/mL dog blood. This is represented by the following linear regression equation: $y = 1.56 \times 10^{-5}x + 7.68 \times 10^{-4}$, $r^2 = 0.9947$, where y represents the peak area ratio of acetaminophen to that of IS and x represents the concentration of acetaminophen in μg/mL.

The selectivity of the blood spot method was established by the analysis of samples of control dog blood from six individual beagle dogs extracted from FTA® Elute paper. No unacceptable interferences, i.e. those with peak areas of >20% of that observed for the LLQ, at the retention times of acetaminophen or its internal standard were observed in any of these lots. A representative chromatogram of a control DBS double blank is shown in Fig. 2.

The lower limit of quantification (LLQ) for the analysis of DBS sample extracts was 0.1 μg/mL. This was defined as the lowest concentration that gave accuracy within 15% of nominal and precision not exceeding 15% for $n=6$ replicates. Representative mass chromatograms of blank and LLQ samples are shown in Figs. 3 and 4, respectively. The suppression of the response for the stable isotope labelled internal standard when analysing DBS extracts by HPLC-MS/MS was 76%.

3.1.2. Accuracy and precision

The accuracy (%bias) and precision (%CV) of the DBS method was evaluated using QC samples at five concentrations analysed against calibration standards prepared from a separate stock solution. Each QC was analysed in replicate ($n=6$) in a single validation batch. Accuracy was assessed by calculating the percent deviation from the theoretical concentration. Precision was determined by calculating the coefficient of variation for intra-day replicates. Table 1 shows a summary of the individual QC concentration data obtained during the validation. The maximum accuracy and intra-day preci-

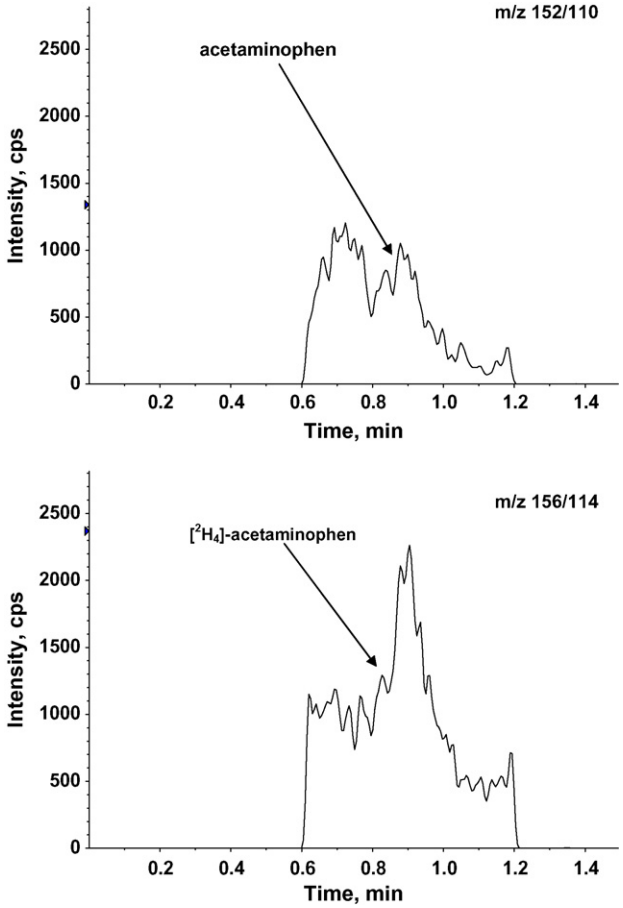

Fig. 2. Representative HPLC-MS/MS chromatograms of a double blank dried dog blood spot sample.

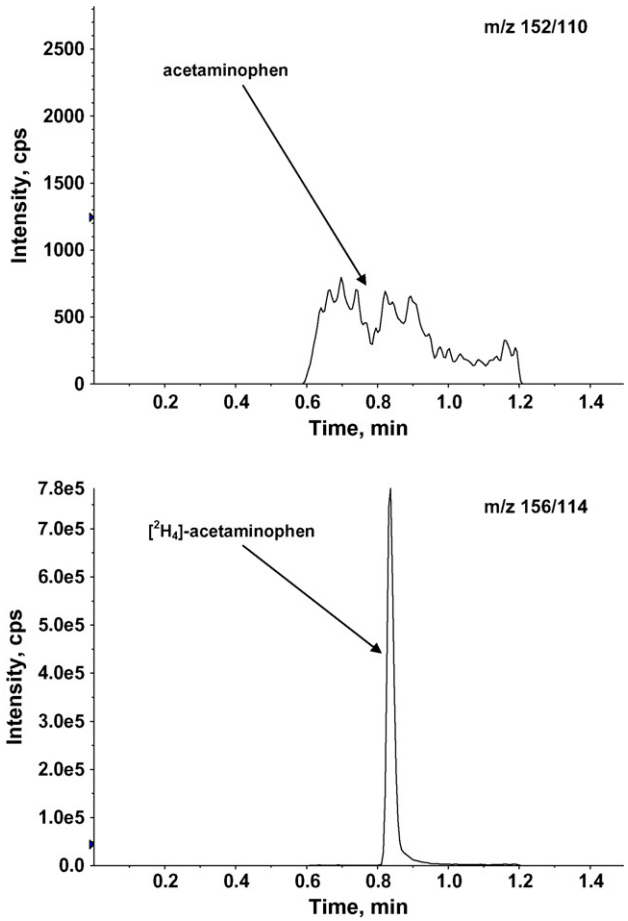

Fig. 3. Representative HPLC-MS/MS chromatograms of a blank dried dog blood spot sample.

sion values observed were 14.5% and 10.4%, respectively, which are well within internationally recognised acceptance criteria for assay validations [11].

3.1.3. Stability

The stability of acetaminophen in matrix during the process of collecting the blood sample, and spotting and drying on the paper, was assessed by comparing peak area ratios of replicate ($n = 6$) dog blood samples spiked at 40 μg/mL and stored for 4 h at 37 °C, with those of the fresh samples. Samples (30 μL) were extracted by the addition of acetonitrile (300 μL) containing IS. The difference was 2.2% (Table 2), indicating no acetaminophen instability under the conditions of storage.

The stability of acetaminophen in blood dried on the FTA® Elute paper for the maximum period a sample was likely to be stored before being analysed, was assessed by comparing concentrations of replicate ($n = 6$) dog blood samples spiked at 0.3 and 40 μg/mL after storage of the DBS at room temperature for 7 days, with those of the same samples extracted immediately after initial spotting and drying. The difference was less than 15% (Table 3), indicating no acetaminophen instability under the conditions of storage.

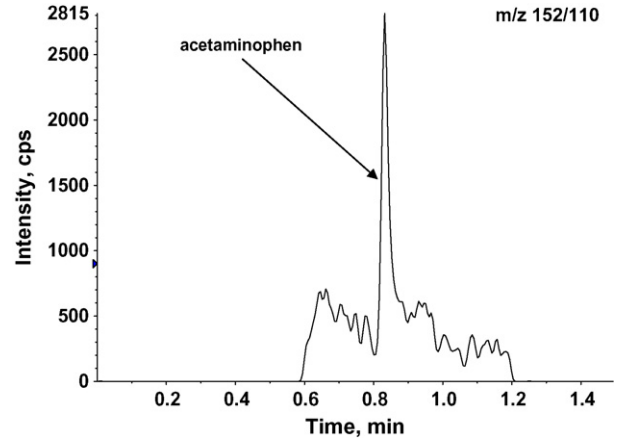

Fig. 4. Representative HPLC-MS/MS chromatogram of a dried dog blood spot sample at the LLQ (0.1 μg/mL acetaminophen).

Table 1
Accuracy, precision and individual validation sample concentrations for acetaminophen in dried dog blood spot samples

Nominal concentrations (μg/mL)	QC 1	QC 2	QC 3	QC 4	QC 5
	0.1	0.3	5	40	50
	0.099	0.350	5.828	34.886	44.264
	0.096	0.391	5.298	42.138	45.588
	0.107	0.301	6.145	38.045	44.223
	0.105	0.345	5.432	40.821	47.384
	0.126	0.348	5.103	40.954	42.852
	0.117	0.326	4.903	37.009	45.682
Mean	0.108	0.344	5.452	38.976	44.999
S.D.	0.011	0.030	0.463	2.785	1.565
Precision (%CV)	10.4	8.6	8.5	7.1	3.5
Accuracy (%bias)	8.3	14.5	9.0	−2.6	−10.0

Table 2
Stability of acetaminophen in whole dog blood stored at 37 °C for 4 h

	Peak area ratio	
	Fresh	4 h at 37 °C
	0.973	0.962
	0.995	0.988
	0.903	0.905
	0.943	0.928
	0.879	0.990
	0.915	0.959
Mean	0.935	0.955
S.D.	0.044	0.033
Precision (%CV)	4.7	3.5
Difference (%)		2.1

Table 3
Stability of acetaminophen in dried dog blood spots on Whatman FTA® Elute paper stored at room temperature for 7 days

	Nominal concentrations			
	0.300 μg/mL		40 μg/mL	
	Fresh	Stored	Fresh	Stored
	0.293	0.299	38.947	36.503
	0.314	0.289	40.548	35.584
	0.295	0.272	37.431	35.241
	0.267	0.228	38.772	37.062
	0.307	0.232	40.625	33.492
	0.328	0.225	39.247	35.280
Mean	0.301	0.258	39.262	35.527
S.D.	0.021	0.033	1.200	1.233
%CV	7.0	12.9	3.1	3.5
%Bias	0.2	−14.2	−1.8	−11.2
%Difference	−14.4		−9.5	

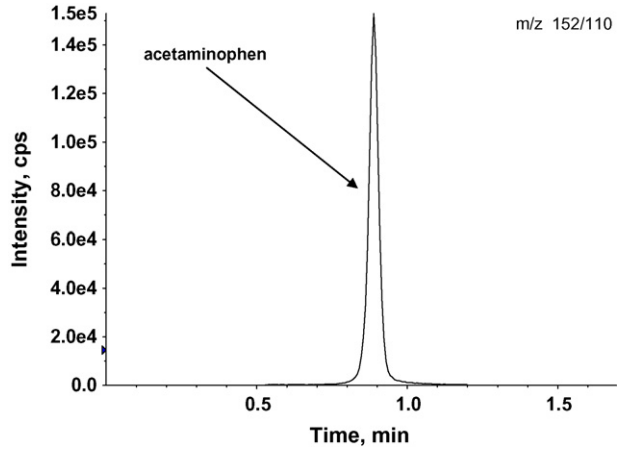

Fig. 5. Representative HPLC-MS/MS chromatogram of a dried dog blood spot sample obtained 0.5 h following daily oral administration of 50 mg/kg acetaminophen to a male dogs for 7 days (corresponding to the maximum observed peak concentration (C_{max})).

3.2. Application of the DBS assay to a toxicokinetic study

A representative mass chromatogram of a DBS sample extract, corresponding to a C_{max} sample is shown in Fig. 5. TK analysis of the DBS and blood/water concentration data obtained was performed by non-compartmental pharmacokinetic analysis using WinNonlin™, Enterprise Edition Version 4.1. The systemic exposure of acetaminophen was determined by calculating the area under the concentration–time curve (AUC) from the start of dosing to the last quantifiable time point (AUC_{0-t}) using the linear-logarithmic trapezoidal rule. The maximum observed peak concentration (C_{max}) was determined by inspection of the observed data. The AUC and C_{max} results obtained are presented in Table 4. As only one male and one female dog were used in the TK study, it is inappropriate to employ statistical methods to compare the data obtained from DBS and blood/water samples. However, data generated from such small group sizes over many years in our laboratories for multiple new chemical entities suggest that TK data can vary by up to two-fold and still be considered comparable. Hence, the <1.4-fold variability in data obtained from DBS and blood/water samples in this study demonstrates that TK exposure parameters for acetaminophen by DBS analysis are physiologically comparable to those obtained from direct analysis of blood/water. This is also illustrated graphically in the blood concentration–time plots shown in Fig. 6.

Table 4
Toxicokinetic parameters obtained from the analysis of dried blood spot and blood/water (1:1, v/v) samples obtained following daily oral administration of 50 mg/kg acetaminophen to dogs for 7 days

Period	Sex	AUC (μg h/mL)		C_{max} (μg/mL)	
		Blood/water	Blood spot	Blood/water	Blood spot
Day 1	Male	58.8	71.3	29.9	37.9
	Female	55.9	60.4	31.4	41.5
Day 7	Male	65.7	81.6	33.8	44.0
	Female	57.6	64.7	37.1	43.5

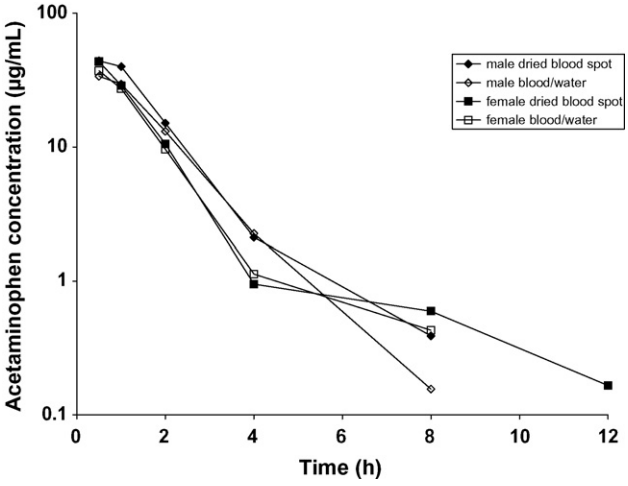

Fig. 6. Blood concentrations of acetaminophen obtained from the analysis of dried blood spot and blood/water (1:1, v/v) samples obtained following daily oral administration of 50 mg/kg acetaminophen to dogs for 7 days.

4. Conclusion

DBS has been reported to be a convenient matrix, readily amenable to measurement of biomarkers and drugs like the anti-malarials, in blood for both clinical and pre-clinical studies. However, this is the first reported application of DBS analysis for the assessment of TK in a pre-clinical safety assessment study with all the potential advantages this technology allows. This paper describes the validation of an acetaminophen method in dog blood by combining extraction of the drug from DBS and HPLC-MS/MS quantification. The method was selective for acetaminophen and linear over the concentration range 0.1–50 μg/mL using only 15 μL of blood, with intra-day precision and bias of less than 12%. The stability of acetaminophen in dog blood spotted onto the FTA Elute paper and stored at room temperature was demonstrated for at least 10 days. The methodology has been successfully employed in a TK study following daily oral administration of 50 mg/kg acetaminophen to dogs for 7 days. The resultant TK data from DBS samples are physiologically comparable with that obtained from duplicate blood/water samples analysed using identical analytical conditions. This work demonstrates the capability of DBS analysis to provide high quality TK information using significantly smaller volumes of blood than are conventionally required. Further, the reduction in blood volume leads to a decrease in animal numbers used, through serial rather than composite sampling regimes, giving ethical benefits and an increase in data quality. In addition, if continued through the life-cycle of a new drug, the technology offers the advantage of simpler sample collection, storage and shipment for both preclinical and clinical study samples, leading to notable ethical and financial benefits. The success of this and similar, related studies has led to the intent top apply DBS technology as the recommended analytical approach for the assessment of PK/TK data for all new oral small molecule drug candidates, which have previously demonstrated a successful bioanalytical validation.

Acknowledgement

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Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method

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A novel approach has been developed for the quantitative determination of circulating drug concentrations in clinical studies using dried blood spots (DBS) on paper, rather than conventional plasma samples. A quantitative bioanalytical HPLC-MS/MS assay requiring small blood volumes (15 μL) has been validated using acetaminophen as a tool compound (range 25 to 5000 ng/mL human blood). The assay employed simple solvent extraction of a punch taken from the DBS sample, followed by reversed phase HPLC separation, combined with selected reaction monitoring mass spectrometric detection. In addition to performing routine experiments to establish the validity of the assay to internationally accepted criteria (precision, accuracy, linearity, sensitivity, selectivity), a number of experiments were performed to specifically demonstrate the quality of the quantitative data generated using this novel sample format, namely, stability of the analyte and metabolites in whole human blood and in DBS samples; effect of the volume of blood spotted, the device used to spot the blood, or the temperature of blood spotted. The validated DBS approach was successfully applied to a clinical study (single oral dose of 500 mg or 1 g acetaminophen).

Conventionally, plasma has been used for the determination of exposures and pharmacokinetics (PK) of new chemical entity drugs in clinical trials because of its ease of handling, shipping, and storage compared to whole blood. However, it is notable that regulatory authorities acknowledge that blood is an acceptable biological matrix for the measurement of drug exposures.[1,2] The collection of blood samples on paper, known as dried blood spots (DBS), is an established technique for the screening of in-born errors of metabolism.[3,4] There have also been a number of reports on its use in humans for therapeutic drug monitoring and PK studies.[5−27]

The collection of whole blood samples as DBS for PK studies offers a number of advantages over conventional plasma sampling. The small blood volumes required for DBS samples (less than 100 μL, compared to >0.5 mL blood which are usually obtained for conventional plasma analyses) make this a particularly suitable approach for the collection of blood samples for pediatric studies. In addition, it offers the advantage of less invasive sampling (finger or heel prick, rather than conventional venous cannula) which enables recruitment of subjects for clinical studies. Further, the simpler matrix preparation and transfer (no refrigerated centrifugation to produce plasma) and easy storage and shipment to analytical laboratories (no requirement for freezers and dry ice) offer further benefits. In addition, these requirements lead to notable environmental benefits. The transport and storage of samples is further simplified by the antimicrobial properties of the DBS sample, removing the requirements for special biohazard arrangements.[28] The significant reduction in blood volume required for DBS, allows for the simplification of current approaches to the determination of drug exposure (toxicokinetics (TK)) in pre-clinical animal studies and leads to significant benefits in the 3Rs (reduction, refinement, and replacement) for animal use in drug development.[29]

For these reasons, GlaxoSmithKline (GSK) is currently utilizing DBS sampling as the technique of choice for the evaluation of TK and PK for all new oral small molecule drugs selected as drug development candidates, which have previously demonstrated a successful validation of the bioanalytical method.

This manuscript describes the development and validation of a method for the quantification of drugs in human blood, as a DBS sample, using acetaminophen as a tool compound. The results of these tests were evaluated against internationally used acceptance criteria described by Shah et al.[30] A similar approach has been previously described for the abbreviated validation of a

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method for determination of acetaminophen TK in a safety assessment dose range finding (non GLP) dog study.[29] This manuscript describes a full clinical validation of a DBS method and in addition, describes a number of important experiments not included in current guidances,[30] which the authors consider should be performed to assess factors specifically associated with the use of DBS for the measurement of the circulating concentrations of new chemical entities in clinical trials (i.e., influence of volume of blood spotted, influence of device used for spotting, influence of whole blood temperature, analyte stability in DBS, stability of acetaminophen sulfate and glucuronide metabolites in DBS and whole blood). The validated method was subsequently used for the analysis of samples taken from a clinical study designed to investigate the practicalities of the use of DBS samples in human drug trials, further details of which are published elsewhere.[31]

EXPERIMENTAL SECTION

Chemicals and Reagents. Methanol and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd. (Loughborough, U.K.). All other chemicals were of AnalaR grade, supplied by VWR International Limited (Poole, U.K.). Control human blood was supplied by GSK volunteers in accordance with current GSK policies on informed consent and ethical approval. Acetaminophen (Figure 1) was obtained from Aldrich (Poole, U.K.). [$^2H_4$]-Acetaminophen (Figure 1) was produced by Isotope Chemistry, GSK (Stevenage, U.K.). Glucuronide and sulfate metabolites of acetaminophen were both obtained from Sigma (Poole, U.K.).

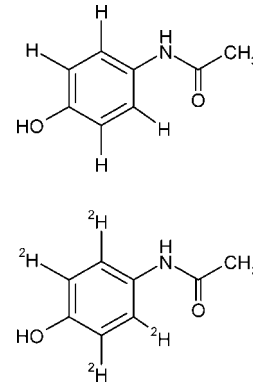

Figure 1. Chemical structures of acetaminophen and [$^2H_4$]-acetaminophen.

Equipment. FTA blood spot cards were supplied by Whatman (Sanford, U.S.A.). Sample tubes were obtained from Micronics (Platinastraat, The Netherlands). Centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat was supplied by Whatman (Sanford, U.S.A.). Plastic bags for the storage of blood spot cards were supplied by Fisher Scientific Ltd. (Loughborough, U.K.). Sachets of desiccant were obtained from Süd-Chemie (Northwich, U.K.). The repeater multipipette used for spotting blood was obtained from Eppendorf (Cambridge, U.K.). EDTA coated capillaries were from Sarstedt (Leicester, U.K.).

Preparation of Standard Stock and Working Solutions. Primary stock solutions of acetaminophen (in duplicate) and [$^2H_4$]-acetaminophen (internal standard) were prepared in dimethylformamide (DMF, 1 mg/mL). Working standard solutions of acetaminophen in H_2O-acetonitrile (1:1, v/v) were prepared from the primary stocks at concentrations of 100, 10, and 1 μg/mL. Internal standard working solutions (0.25 μg/mL) were prepared from the primary stock using H_2O-methanol (1:1,v/v) for blood spot analysis and acetonitrile for blood/water analysis.

Primary stock solutions of acetaminophen glucuronide and sulfate metabolites were prepared in DMF (1 mg/mL). Working solutions of both metabolites at 100, 10, and 1 μg/mL were individually prepared in H_2O-acetonitrile (1:1, v/v) from the primary stock solutions.

All the solutions were stored at 4 °C and brought to room temperature before use.

Preparation of Calibration Standards and Quality Control (QC) Samples. Calibration standards were prepared fresh on the day of analysis by diluting the appropriate working solutions with blank whole human blood. The concentrations of calibrants were 25, 50, 100, 500, 1000, 2000, 4000, and 5000 ng/mL whole blood.

QC samples for all analyses were prepared from a separate stock solution to that used for the calibration standards. QC samples were prepared by diluting the appropriate working solutions with blank whole human blood to give concentrations

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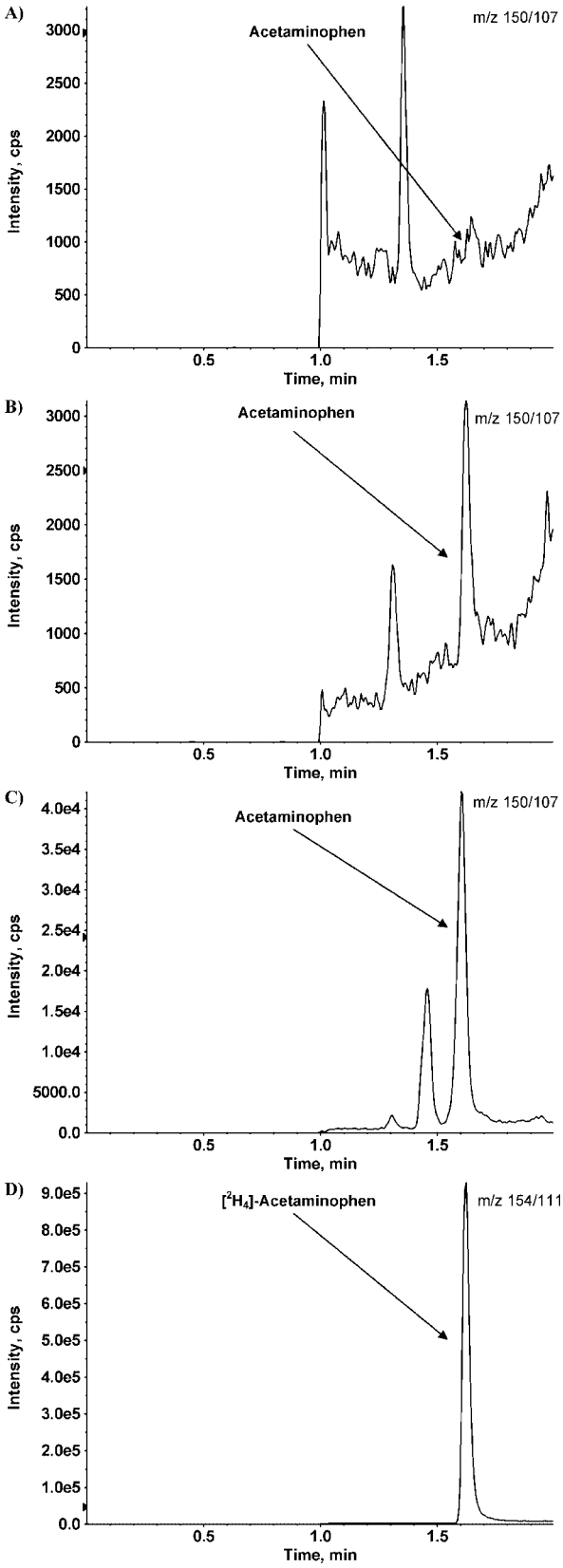

Figure 2. Representative HPLC-MS/MS selective reaction monitoring chromatograms of (A) a blank human DBS sample, (B) a human DBS sample spiked with acetaminophen at the LLQ (25 ng/mL human blood), (C) a human DBS sample obtained 1.5 h following a single oral administration of 500 mg acetaminophen to a volunteer (corresponding to the maximum observed peak concentration (C_{max})), and (D) the internal standard ([$^2H_4$]-acetaminophen).

of 25, 100, 1000, 4000, and 5000 μg/mL whole blood. For the analysis of study samples, QC's were prepared at concentrations of 100, 1000, and 4000 ng/mL whole blood.

Blood Spotting. Aliquots (15 μL) of calibration standards and QC samples were spotted onto FTA cards with a repeater pipet and allowed to dry at room temperature for at least 2 h prior to analysis. When required, the QCs were stored at room temperature in a sealed plastic bag containing desiccant until analysis.

DBS Sample Processing. A 3 mm diameter disk was punched from the center of the DBS into a clean tube. This was then extracted by the addition of 100 μL internal standard working solution, followed by vortex mixing for approximately 20 s. The tube was centrifuged for 1 min at 3000 $\times$ *g*, and the supernatant transferred to a clean tube for analysis by HPLC-MS/MS.

HPLC-MS/MS Analysis. The HPLC-MS/MS system consisted of a CTC HTS PAL autosampler (Presearch, Hitchin, U.K.) with fast wash, an Agilent 1100 binary pump (Palo Alto, CA, U.S.A.) with integrated column oven and divert valve, and a YMC-Pack ODS AQ, 3 μm, 50 mm $\times$ 4.0 mm i.d. HPLC column (YMC Europe, GmbH). The post column flow was diverted to waste for the first 1.0 min of each chromatographic run. During this time, flow (0.25 mL/min, methanol−water (1:1, v/v)) was provided to the MS by a Knauer pump (Presearch, Hitchin, U.K.).

The chromatographic separation was achieved using a solvent gradient employing the mobile phases ammonium acetate (10 mM; adjusted to pH 10 with ammonia) (A) and methanol (B). Following sample injection (5 μL) the mobile phase was held at 100% A for 0.08 min. A ballistic gradient to 0% A at 1.08 min was followed by an isocratic period at 0% A to 1.25 min. The mobile phase was then returned to 100% A by 1.26 min and was held at this composition until 2.0 min, before the injection of the next sample. The flow rate was 0.8 mL/min, and the column was maintained at room temperature.

MS detection was by a Sciex API-5000 (Applied Biosystems/MDS Sciex, Canada) equipped with a TurboIonspray ion source. The source temperature was 700 °C. The gas 1 and gas 2 settings were 50 and 40 psi, respectively (both N_2). The curtain gas and collision gas settings were 25 and 5, respectively (both N_2). The characteristic precursor $[M - H]^-$ to product ions transitions, *m/z* 150 to 107 and 154 to 111 were consistent with the structures of acetaminophen and the internal standard (loss of CH_3CO), respectively, and were used as selected reaction monitoring transitions to ensure high selectivity. A dwell time of 150 msec was used for both transitions. The pause time was 5 msec.

HPLC-MS/MS data were acquired and processed (integrated) using Analyst software (v1.4.1 Applied Biosystems/MDS Sciex, Canada). Concentrations were determined from the peak area ratios of analyte to internal standard using the in-house laboratory information management system, SMS2000 (v2.0, GSK, U.K.).

Validation Procedures. *Linearity.* Calibration standards were prepared in duplicate for each analytical run. Calibration plots of the analyte peak area ratio to that of the internal standard versus the nominal concentration in blood were constructed.

Selectivity and Matrix Suppression Effects. Total blank and blank DBS's were derived from 15 μL aliquots of control blood from 6 individual subjects and from matrix pools. Total blank samples

Table 1. Intra- and Inter-Assay Performance Data for Acetaminophen in Human DBS Samples (n = 6 for Each Concentration Level in Each Individual Run)

nominal concentration (ng/mL)		25	100	1000	4000	5000
		Individual (Intra-Run) Statistics				
run 1	mean concentration (ng/mL)	24.2	98.0	950.6	3959.0	4955.1
	SD.	2.6	2.0	30.7	54.7	174.1
	precision (%CV)	10.9	2.1	3.2	1.4	3.5
	accuracy (% bias)	−3.1	−2.0	−4.9	−1.0	−0.9
run 2	mean concentration (ng/mL)	22.9	99.5	940.1	4049.3	4952.2
	SD.	2.6	5.4	14.0	81.3	70.9
	precision (%CV)	11.2	5.4	1.5	2.0	1.4
	accuracy (% bias)	−8.4	−0.5	−6.0	1.2	−1.0
run 3	mean concentration (ng/mL)	25.6	103.4	1041.9	4019.1	4884.5
	SD.	2.1	6.6	70.7	273.2	189.1
	precision (%CV)	8.2	6.4	6.8	6.8	3.9
	accuracy (% bias)	2.5	3.4	4.2	0.5	−2.3
		Overall (Inter-Run) Statistics				
	mean concentration (ng/mL)	24.2	100.3	977.6	4009.1	4930.6
	SD.	2.6	5.3	63.4	162.1	148.4
	overall precision (%CV)	10.6	5.3	6.5	4.0	3.0
	average accuracy (% bias)	−3.0	0.3	−2.2	0.2	−1.4
	average intra-run precision (%)	10.1	5.1	4.6	4.2	3.1
	inter-run precision (%)	Negligible	Negligible	5.4	Negligible	Negligible

Table 2. Influence of the Volume of Human Blood Spotted on FTA Paper on the Precision and Accuracy of the Assay at Two Concentration Levels (n = 6 Replicates)[a]

nominal concentration	100 ng/mL			4000 ng/mL		
volume of human blood spotted onto FTA paper	10 μL	15 μL	20 μL	10 μL	15 μL	20 μL
mean concentration (ng/mL)	100.3	99.5	104.8	3852.8	3959.0	4129.9
SD.	9.7	5.4	7.1	110.8	54.7	207.0
precision (%CV)	9.6	5.4	6.8	2.9	1.4	5.0
accuracy (% bias)	0.3	−0.6	4.8	−3.7	−1.0	3.2
difference from 15 μL Spot (%)	0.9		5.4	−2.7		4.3

[a] Concentrations were measured against a calibration line prepared with 15 μL human blood.

Table 3. Influence of Device Used to Spot 15 μL Human Blood onto FTA Paper on the Precision and Accuracy of the Assay at Three Concentration Levels (n = 6 Replicates)

	spotting device					
	pipette			glass capillary		
nominal concentration (ng/mL)	100	4000	10000[a]	100	4000	10000[a]
mean concentration (ng/mL)	103.0	4358.6	10158.5	103.6	4081.0	10160.4
SD.	3.3	128.4	677.2	6.6	65.7	583.2
precision (%CV)	3.2	2.9	6.7	6.4	1.6	5.7
accuracy (% bias)	3.0	9.0	1.6	3.6	2.0	1.6

[a] Sample extract diluted 1/10 with extract of blank human DBS.

(n = 1 for each subject or pool) were processed as outlined above, in the absence of internal standard.

To assess the suppression of HPLC-MS/MS detector response due to matrix components associated with DBS, the peak areas of the internal standard in extracts of replicate DBS QC samples at 100, 1000, and 4000 ng/mL whole blood were compared to those of the same concentration of internal standard spiked directly into methanol−water (1:1, v/v).

Accuracy and Precision. The intra- and inter- assay accuracy and precision of the method were determined by assaying six replicates of each of the five concentrations of validation QC samples on three separate occasions. Concentrations were determined from the appropriate calibration plot within each analytical run. Accuracy was assessed for each concentration by calculating the percent deviation from the theoretical (nominal) concentration. The precision was determined for each concentration by calculating the percent coefficient of variation (%CV; relative standard deviation) for each set of replicates.

Stability in Stock Solutions. The HPLC-MS/MS peak area ratios (analyte to internal standard) of replicate (n = 6) 1 mg/mL samples stored for 109 days at 4 °C were compared with those of the fresh samples. When analyzing the samples, a 25 μL aliquot was diluted to 50 mL with a 500 ng/mL solution of internal standard ([$^2H_4$]-acetaminophen) in H_2O-methanol (1:1, v/v).

Stability in Whole Blood. The HPLC-MS/MS peak area ratios (analyte to internal standard) of replicate (n = 6) control whole human blood samples at 100 ng/mL and 4000 ng/mL stored for 24 h at room temperature were compared with those of aliquots of the same samples when fresh. Immediately prior to extraction, samples (25 μL) were diluted with an equal volume of water. The entire diluted sample was then extracted with 150 μL acetonitrile containing internal standard. This was vortex mixed for approximately 20 s and then centrifuged for 10 min at 3000 × g. An aliquot (50 μL) of the supernatant was transferred to a clean tube containing 100 μL H_2O and vortex mixed for approximately 5 s.

Stability in Dried Blood Spot on FTA Paper. Replicate (n = 6) 15 μL human blood samples at 100 and 4000 ng/mL were spotted

Table 4. Influence of the Temperature of Blood Spotted onto FTA Paper on the Precision and Accuracy of the Assay ($n = 6$ Replicates)

	blood storage conditions								
	room temperature			on ice			37 °C		
nominal concentration (ng/mL)	100	4000	10000[a]	100	4000	10000[a]	100	4000	10000[a]
mean concentration (ng/mL)	99.5	3811.6	10239.1	103.8	3854.9	9975.9	108.7	4125.4	9680.5
SD.	6.5	138.3	815.5	4.2	148.6	769.3	7.0	227.8	265.1
precision (%CV)	6.5	3.6	8.0	4.0	3.9	7.7	6.5	5.5	2.7
accuracy (% bias)	−0.5	−4.7	2.4	3.8	−3.6	−0.2	8.7	3.1	−3.2

[a] Sample extract diluted 1/10 with extract of blank human DBS.

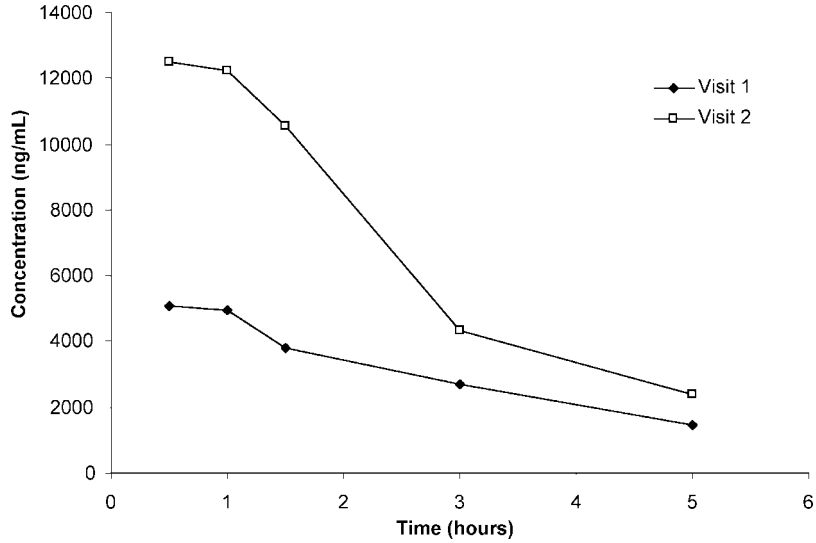

Figure 3. PK profiles of acetaminophen obtained from human DBS samples taken from a subject after a single oral administration of 500 mg (visit 1) and 1 g (visit 2) acetaminophen on two separate occasions.

on FTA paper and stored desiccated at room temperature for 113 days. The measured concentrations were compared to those of the same samples extracted and analyzed immediately after initial spotting and drying.

Stability in Processed Samples. Extracts from a DBS validation run were reinjected after storage at room temperature for 24 h, against freshly prepared calibration standard extracts.

Stability of Metabolites. Control human blood samples containing either the glucuronide or the sulfate metabolites of acetaminophen were prepared at 100 and 4000 ng/mL. Aliquots (15 μL) of the blood were spotted onto FTA paper and left for 24 h at room temperature. These samples were then processed and analyzed for acetaminophen. Further aliquots (25 μL) of the whole blood samples were kept for 6 h at room temperature. These samples were diluted with water (1:1 v/v) prior to extraction with 150 μL acetonitrile containing internal standard. Extracts were qualitatively analyzed for the presence of the metabolites and acetaminophen using HPLC-MS/MS.

Dilution of Samples Above the Higher Limit of Quantification (HLQ). Control human blood was spiked at 10000 ng/mL (2 × HLQ) and six replicate 15 μL aliquots were spotted onto FTA paper. After drying at room temperature for 2 h, a 3 mm diameter punch was taken from each spiked DBS sample. Each punch was individually extracted with 100 μL of methanol−water (1:1, v/v), containing internal standard. Diluent was prepared by individually extracting 10 × 3 mm punches taken from replicate spots of blank human DBS's on FTA paper with 100 μL aliquots of methanol−water (1:1, v/v) containing internal standard. These blank diluent extracts were then pooled. A 10 μL aliquot of each individual spiked sample extract was then diluted with 90 μL of pooled blank extract diluent. Concentrations of acetaminophen in these individual extract dilution samples were determined by LC-MS/MS and corrected for the dilution factor.

Blood Spot Size. Replicate ($n = 6$) 10, 15, and 20 μL aliquots of control human blood at 100 and 4000 ng/mL were spotted onto FTA paper. After drying, a 3 mm punch was taken from the center of each sample for extraction and analysis. Concentrations of acetaminophen were determined from a calibration line consisting of standards derived from 15 μL DBS.

Influence of Spotting Device. Replicate ($n = 6$) 15 μL aliquots of single 1000 μL pools of control human blood at 100, 4000, and 10000 ng/mL were spotted onto FTA paper using either a multipipette or a capillary (a fresh tip being used for each sample and a fresh capillary being used for each spot, respectively). Concentrations of acetaminophen were determined in DBS extracts versus a calibration line spotted with a pipet. The 10000 ng/mL DBS sample extracts were diluted as described above.

Influence of the Temperature of Blood Spotted. Control human blood was spiked at 100, 4000, and 10000 ng/mL. Replicate 1000 μL pools were maintained for 30 min at either room temperature, on wet-ice, or at 37 °C. Replicate ($n = 6$) 15 μL aliquots of each were then spotted onto FTA paper. Concentrations of acetaminophen were determined in DBS extracts versus a calibration line

spotted with standards at room temperature. The 10000 ng/mL DBS sample extracts were diluted as described above.

Application of the DBS Assay to the Analysis of Samples from a Clinical Study. Blood spot samples were obtained from a two-period, single dose, single center, open label study designed to evaluate the practicalities of using DBS technology for blood sample collection at multiple time-points in the context of a phase I clinical trial in healthy human volunteers.[31] The study design was reviewed and approved by the local Ethics Committee, and informed consent was obtained from the subjects prior to the study.

Acetaminophen was administered to the volunteers in two doses; 500 mg in the first session and 1 g in the second session. Blood samples from the peripheral venous cannula were collected at 0.5, 1, 1.5, 3, and 5 h post-dose. Replicate ($n = 3$) 15 μL aliquots were spotted onto FTA cards with a repeater pipet and allowed to dry at room temperature for at least 2 h prior to shipping to the analytical site. Dilution of samples above the HLQ of the assay was performed with extract of blank human DBS as outlined above, that is, individual sample extracts were diluted with pooled extract of blank blood spots.

RESULTS AND DISCUSSION

Method Validation. *Linearity, Selectivity and Matrix Suppression Effects.* Calibration plots of analyte/internal standard peak area ratio versus the nominal concentration of acetaminophen in blood were constructed and a weighted $1/x^2$ linear regression applied to the data. Linear responses were observed for DBS over the range 25 to 5000 ng/mL human blood. This is represented by the following linear regression equation (average of all 3 validation runs): $y = 8.49 \times 10^{-5}\, x + 2.98 \times 10^{-4}$, $r^2 = 0.9972$ where y represents the peak area ratio of acetaminophen to that of IS and x represents the concentration of acetaminophen in ng/mL.

The selectivity of the method was established by the analysis of blank and double blank DBS samples of control human blood from both six individual subjects and from pooled samples. No unacceptable interferences, that is, those with peak areas of >20% of that observed for the lower limit of quantification (LLQ), at the retention times of acetaminophen or its internal standard were observed in any of these samples. A representative mass chromatogram of a control DBS blank sample is shown in Figure 2.

The suppression of the response for the stable isotope labeled internal standard when analyzing DBS extracts by HPLC-MS/MS was negligible (less than 5%).

Accuracy, Precision, and Sensitivity. The intra- and inter-run performance data are summarized in Table 1. All values obtained were well within internationally recognized acceptance criteria for assay validations[11] and were within the pre-defined 15% limits required.

The LLQ for the analysis of DBS sample extracts was 25 ng/mL human blood. This was defined as the lowest concentration that gave accuracy and precision values within the pre-defined limits of 15%. A representative mass chromatogram of a LLQ sample is shown in Figure 2.

Stability. The difference in peak area ratios between a stock solution after storage for 109 days and the freshly prepared solution was −0.3%, indicating that acetaminophen is stable in solutions of DMF stored at 4 °C for at least 109 days.

To determine whether there were any issues with the stability of acetaminophen between the collection of the blood sample and spotting an aliquot onto FTA paper, a comparison was made between whole blood samples stored for 24 h at 37 °C and the fresh samples. The differences in peak area ratios were −5.0% and 3.0% at 100 and 4000 ng/mL, respectively, indicating that acetaminophen is stable in whole human blood for this length of time.

To determine the stability of acetaminophen in DBS samples on FTA paper, a comparison was made between samples stored with desiccant for 113 days at room temperature and the fresh samples. The differences were −1.8 and 0.4% at 100 and 4000 ng/mL human blood, respectively, indicating that acetaminophen is stable under the conditions of storage.

The accuracy, precision, and sensitivity of processed human DBS validation samples were found to be acceptable (precision and accuracy within the pre-defined 15% limits) on re-injection with freshly prepared calibration standards, after storage at room temperature for 24 h. This demonstrates that processed samples were stable when stored under these conditions.

DBS and whole blood samples spiked individually with acetaminophen glucuronide and sulfate metabolite standards showed no detectable formation of acetaminophen after storage for 24 and 6 h at room temperature, respectively. Further, a qualitative assessment of the peak areas for the metabolites showed no notable decrease with time. This indicates that these metabolites are stable in DBS on FTA paper and as whole blood samples and will therefore not interfere with the quantification of acetaminophen. Further, chromatographic separation of acetaminophen (1.5 min) from the sulfate (1.4 min) and glucuronide (0.7 min) metabolites was obtained to ensure that any in-source fragmentation of the metabolites did not contribute to an enhancement of the acetaminophen signal during HPLC-MS/MS analyses.

Dilution of Samples Above the HLQ. The ability to dilute samples containing acetaminophen at concentrations above the HLQ was demonstrated by performing replicate 10-fold dilutions of extracts of human DBS samples with extracts of blank human DBS. The bias and within-run precision values were less than the pre-defined acceptance limits of 15% and were therefore acceptable. It is important to note that when diluting DBS samples for analysis by HPLC-MS/MS, the diluent used should be obtained by the extraction of blank matrix from the same species as the sample to be diluted. If neat solvent, or an extract of the same matrix from another species is used, there is a risk that suppression effects (of either the analyte or the internal standard) during HPLC-MS/MS analysis would give the incorrect concentration value. The effects can be minimized by the use of isotopically labeled internal standards and/or adequate chromatography. In the case of this study, no such suppression effects were observed.

Blood Spot Size. The accuracy and precision of quantitation of acetaminophen extracted from a 3 mm diameter punch taken from a DBS sample derived from 10, 15, and 20 μL aliquots of human blood are presented in Table 2. For all blood sample volumes examined, the within-volume precision and accuracy values for calculated concentrations were less than or equal to the pre-defined acceptance criteria of 15% and are therefore acceptable. Further, the differences between the accuracy values for extracts from 10 and 20 μL spots compared to those from 15 μL were all

less than 5.5%. This indicates that there is no notable difference in distribution of analyte and blood across spots derived from blood volumes between 10 and 20 μL, when a 3 mm punch is taken from the center of the blood spot.

Influence of Spotting Device. The accuracy and precision data for DBS samples derived from spotting blood with either a pipet or capillaries are presented in Table 3. For all concentrations examined, the parameters were within the pre-defined 15% acceptance criteria, demonstrating that the spotting device does not have an influence on the concentration determined. This is an important consideration for studies where accurate pipettes may not be readily available at the Study Centre, and EDTA coated capillaries offer an affordable and convenient alternative.

Influence of the Temperature of Blood Spotted. The accuracy and precision data for DBS's derived from acetaminophen spiked blood stored for 30 min at either, room temperature, on-ice, or at 37 °C prior to spotting are presented in Table 4. For all concentrations examined, the parameters were within the pre-defined 15% acceptance criteria, demonstrating that the temperature of the blood spotted does not have an influence on the concentration determined. This allows for flexibility in local practice for storage of blood samples at different Study Centers and in the analytical laboratory.

Application of the DBS Assay to the Analysis of Samples from a Clinical Study. PK profiles obtained for acetaminophen using DBS samples taken from human volunteers after the administration of a single dose of 500 mg and 1 g acetaminophen on two occasions is shown in Figure 3.

CONCLUSION

A simple and rapid assay has been developed and validated for the determination of acetaminophen in human DBS samples by HPLC-MS/MS. The validated method was demonstrated to be accurate, precise, and robust and complied with the most recent regulatory guidelines.[30] In addition to the factors normally tested under these guidelines, a number of other parameters associated exclusively with the utility of DBS samples were also validated, leading to greater confidence in the robustness and utility of the technique. The stability of the analyte in whole blood provides reassurance that there is no degradation of acetaminophen from the time of collection of the blood sample to it being dried on the blood spot paper. Data demonstrating that there is no quantitative difference between DBS samples derived from samples stored on-ice, at room temperature, or at 37 °C, or on the spotting device used, or of the size of spot obtained allows for flexibility in sample processing procedures between different Study Centers. This data also indicates that obtaining blood samples from a finger, or heel prick, rather than venous cannula is also feasible. The stability of acetaminophen in DBS samples stored desiccated at room temperature for 113 days demonstrates that samples in this form can be stored for long periods of time before analysis, without the need for refrigeration, thus reducing costs and increasing flexibility in selection of Study Centers and bioanalytical laboratories. The authors suggest that performing these experiments be considered by others when developing and validating quantitative bioanalytical methods for the measurement of drugs in pre-clinical and clinical studies using dried blood spots as the sample collection media.

The validated method for analysis of acetaminophen in DBS samples has been applied to the support of a study to investigate the utility of this sample format for the determination of exposure and PK in clinical studies.[31] The outcome of this and related pre-clinical studies[29] and the advantages afforded by the use of DBS (small blood volumes, simplified sample processing, storage, and shipment, etc.) has led to the recommendation that DBS samples be used as GSK's preferred analytical approach for the assessment of PK and TK for all new oral small molecule drug candidates, which have previously demonstrated a successful bioanalytical validation. At the time of publication, quantitative bioanalytical methods using DBS samples have been validated and used for study support for more than 50 different molecules in our laboratories.

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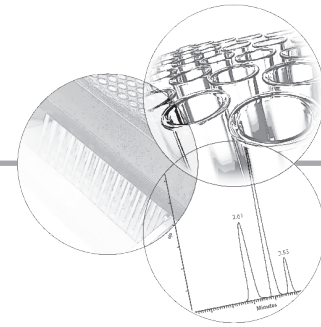

Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations

Background: A clinical investigation was performed into the practicalities of the collection of blood samples for the determination of drug exposures on filter paper, known as dried blood spot (DBS) sampling using a two-period, single-dose, open-label trial conducted in 11 healthy volunteers who received a single oral dose of paracetamol. Questionnaires relating to the blood sampling and spotting process and tolerability were completed by staff and volunteers. Paracetamol concentrations in DBS samples obtained by venous cannula (DBS-Can) were compared against those from fingerprick (DBS-FP) and fresh whole blood obtained from a cannula (WB-Can). **Results:** The questionnaires demonstrated that FP and blood spotting was easy to perform and well tolerated and compared favorably with cannula sampling. Paracetamol concentrations in DBS-Can were greater than those in WB-Can (positive bias) except below 8000 ng/ml when both were interchangeable. When comparing DBS-FP to DBS-Can, both the bias and variability differed significantly across the five sampling time points. **Conclusion:** The study has shown that the DBS technique is practical in the context of a clinical trial. Interchangeability of drug concentrations between blood sampling site and mode of blood collection has to be checked and taken into account when designing pharmacokinetic studies for other compounds.

Conventionally, drug exposures in preclinical studies and clinical trials have been determined from plasma samples. However, it is notable that measuring drug concentration from whole blood (WB) is acknowledged by the regulatory authorities to be a suitable alternative [1,2]. Dried blood spot (DBS) samples are recognized as a convenient technique for the collection and storage of WB. The approach was first documented in the early 1960s for the detection of phenylketonuria in newborns [3] and has since been widely used for the detection of a number of inborn errors in metabolism. More recently, DBS sampling has been used in a variety of applications, including the quantitative determination of circulating drug concentrations for therapeutic drug monitoring and clinical trials, particularly in remote areas, for example antimalaria therapies [4–6]. The technique involves spotting the blood sample onto a filter card, which are available in a variety of formats. The particular format of card used for this study is shown in **Figure 1**. After spotting, the card is then dried at room temperature and later analyzed. The current practice in our studies is to apply three replicate blood spots (i.e., one drop of blood in each circle on the DBS card) for each time point onto a single card, with one spot being used for the primary analysis in singlicate and the other two being available for repeat analysis if required.

Dried blood spot samples offer a number of advantages over conventional blood and plasma samples, specifically:

- Small sample volumes for DBS (10–20 µl blood per spot, compared with more than 0.5 ml blood for conventional plasma analyses) make this particularly useful for the determination of **pharmacokinetics** (PK) in clinical studies where the availability of blood volume may be limited, for example pediatric studies and studies in critically ill patients;
- Minimal blood volume requirements enable reductions in animal numbers and refinements in their use, for toxicokinetic assessments during safety assessment studies in drug development [7];
- Simplified blood sampling, for example by finger-prick (FP) rather than conventional cannula;
- Simpler matrix preparation at the site of collection, as refrigerated centrifugation to produce plasma and subsequent transfer of the supernatant is not required;

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Key Terms

Dried blood spot samples: A technique for the collection, transport and storage of blood on cellulose-based 'filter' papers.

Pharmacokinetics: Study of the drug dose–circulating concentration relationship within the body over a period of time.

Paracetamol: Also called acetaminophen. Drug used to relieve mild headache or muscle and joint pain and to reduce fever.

- Easy storage and shipment to analytical laboratories, as there is no requirement for freezers and dry ice and the DBS samples are considered safe to ship without recourse to specialized shipping considerations [101]. This offers particular advantages for multicenter studies in Phases I, II and III.

The logistics and acceptability to staff and subjects of collecting and spotting WB samples from clinical studies, especially in the context of a study with numerous time points, should be evaluated before DBS technology can be used reliably in clinical trials, and this was the primary objective of this study. We investigated this by the use of questionnaires for all staff and subjects involved in the study. The secondary objective was to compare **paracetamol** concentrations in DBS samples (derived from FP and venous cannula) against samples obtained by more conventional means, for example fresh WB obtained from cannula.

The compound tested was paracetamol, because its safety profile is known and its plasma PK and metabolism are well characterized in the literature [8]. The drug is completely excreted in 1 day and is not known for high interindividual variability. Furthermore, the recommended clinical dose reaches a high circulating blood concentration, enabling the use of a simple method for the quantitative determination of drug concentration in the limited sample volumes (15 µl) obtained for each sample in the study.

A particular consideration of the study was that the DBS technology involves the measurement of concentrations in WB fixed on filter paper. There was therefore a requirement to measure paracetamol concentrations in conventional WB samples not spotted on filter paper, to determine the validity of the spotting approach. However, unlike plasma, we have found that WB cannot be readily manipulated (e.g., subaliquotted) after frozen storage. The only way we have found to readily store WB samples and still be able to work with them is to dilute them with water (1:1 v/v) prior to freezing.

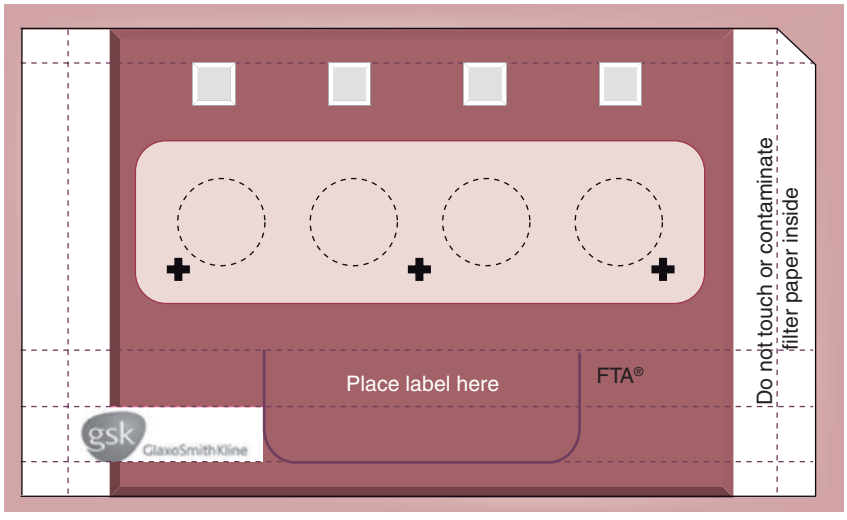

Figure 1. Whatman FTA® blood spot card used in the study.

Experimental

Clinical study design

A two-period, single-dose, open-label trial using paracetamol was conducted in 11 healthy male (n = 8) and female (n = 3) volunteers (one subject completed period 1 only and was replaced). This study design was reviewed and approved by the local Cambridge 3 Ethics Committee and informed consent was obtained from the subjects prior to the study.

Paracetamol was administered orally; 500 mg in the first period and 1 g in the second period (~1 week apart). These two different doses were given in order to achieve a wider range of concentrations for comparison between measurement methods, rather than to compare one dose to the other. Blood was sampled at 30, 60, 90, 180 and 300 min post-dose from both FP (three drops of 15 µl) and cannula (6 ml into an EDTA tube). FP samples were taken with a single-use lancing device. Once an adequate sample of blood was formed on the skin, this was sampled using a 20-µl EDTA-coated capillary marked with a line for approximately 15 µl, attached to a holder and bulb. The blood from FP was spotted onto FTA filter paper (DBS-FP) directly from the collection capillary. Blood from the cannula (Can) was split into two groups: collection on filter paper (three aliquots of 15 µl spotted using a repeater pipette [DBS-Can]) and the remainder diluted 1:1 (v/v) with deionized water (to facilitate freezing of WB [WB-Can]). DBS samples were allowed to dry at room temperature for at least 2 h prior to shipping to the analytical site in sealed plastic bags (all the samples from a single subject in a single bag) containing a sachet of desiccant. No particular precautions were taken to control the temperature of the DBS samples during their shipment (~30 km). They were stored at laboratory temperature and humidity until analysis. WB samples were shipped frozen on dry ice to the analytical site, where they were stored at -20°C until analysis. Quality control samples were prepared and stored with the study samples within 1 week of their receipt.

■ Questionnaires

In order to assess the acceptability and practicalities of collecting PK samples by DBS, on each study day, study staff (n = 16) completed a questionnaire relating to the ease of the blood spotting, blood sampling and blood dilution procedures, and volunteers (n = 11) answered questions relating to the tolerability of blood sampling procedures. A copy of which is available at **www.future-science.com/doi/suppl/10.4155/bio.10.105/suppl_file/suppl_questionnaire_1.pdf.**

■ Analytical methods

Concentrations of paracetamol in DBS and blood water samples were determined in singlicate using quantitative bioanalytical methods validated to internationally accepted criteria [9,10]. DBS samples were analyzed in singlicate for paracetamol using a validated method based on extraction of a 3-mm diameter disc taken from the center of the DBS, by addition of methanol, followed by **HPLC–MS/MS** analysis [11]. Human blood/water (1:1 v/v) samples (50 µl) were analyzed for paracetamol using a validated analytical method based on protein precipitation, followed by HPLC–MS/MS analysis [GlaxoSmithKline, Unpublished Data]. For both assays, the LLOQ was 25 ng/ml and the HLOQ was 5000 ng/ml. Both assays were linear over the analytical range, with acceptable precision and accuracy being observed over the entire analytical range. Samples above the HLOQ of the assay were analyzed after dilution with blank matrix extract for DBS, or human blood/water (1:1 v/v) for blood/water samples.

■ Statistical analysis

Questionnaires of the relative merits of the sample collection methods were analyzed qualitatively and using simple summary statistics. No formal comparisons between methods were made owing to low power as a result of relatively small subject numbers.

Paracetamol concentrations were compared in a pair-wise manner (DBS-Can versus WB-Can; DBS-FP versus DBS-Can) using an extension of the approach of Bland and Altman [12,13]. Data were log-transformed for analysis because differences were observed to increase proportionally with the mean, as is usually the case with concentration measurements. Results were subsequently back-transformed, so differences are expressed as ratios or percentage differences, averages as geometric-means and standard deviations as coefficients of variation (CVs). For each pair-wise comparison, an analysis of covariance model was built with the ratio of the two concentrations as the dependent variable, the geometric mean concentration as a covariate, time point and dose as categorical factors and using variance components to account for the repeated measurements on the same subject. Each model was then simplified by dropping terms that did not contribute significantly (e.g., $p < 0.05$) to the model. This approach allowed quantification of the bias (e.g., systematic difference between the methods) and CV (i.e., inconsistent/noisy difference between the methods) and identified factors which influenced these quantities. Subsequently, limits of agreement were calculated (bias ± 1.96 CV), that provide a range by which one method can be expected to give a result above or below the other. Results were also displayed graphically plotting ratio against geometric mean and overlaying the estimated limits of agreement. All statistical analysis was carried out in SAS (Windows V9.1; SAS Institute Inc., NC, USA).

The gender balance (three female, eight male) was not considered appropriate to draw conclusions regarding any gender differences. No formal pharmacokinetic analysis of paracetamol was carried out (e.g., estimation of C_{max} or AUC).

■ Materials

Paracetamol tablets were obtained from John Bell, Croyden. The Unistik single-use lancing device was obtained from Owen Mumford Ltd (Oxford, UK). EDTA-coated capillaries (20 µl) were obtained from Sarstedt Ltd (Leicester, UK) and the capillary holders and bulbs from Brand GMBH & Co. (Wertheim, Germany). Sample tubes were obtained from Micronics BV (Platinastraat, The Netherlands). FTA® filter paper cards were sourced from Whatman (Maidstone, UK). The particular format of DBS collection cards is also available from Whatman in a nonproprietary format as FTA DMPK A [102]. Plastic bags for storage and shipping of blood spot cards were supplied by Fischer Scientific Ltd (Loughborough, UK). Sachets of desiccant were obtained from Süd-Chemie Ltd (Cheshire, UK). The repeater multipipette was obtained from Eppendorf UK Ltd (Cambridge, UK).

Key Term

HPLC–MS/MS: Analytical methodology routinely used for the detection and quantification of circulating drug concentrations.

Results & discussion

■ Questionnaires

Of the 21 occasions when samples were collected from the 11 subjects, the subjects preferred FP 19 times, cannula once and on one occasion

Table 1. Degree of pain/discomfort recorded by subjects during blood sampling via either finger-prick or venous cannula.

	Number of subjects responding				
Overall pain/discomfort	***None***	***Mild***	***Moderate***	***Severe***	***Extreme***
Finger-prick	5	15	1	0	0
Cannula	2	11	8	0	0

Questionnaires were completed by 11 subjects after the single oral administration of paracetamol at two doses (500 mg and 1 g) on two occasions.

no preference was expressed. **Table 1** shows the overall pain/discomfort reported for the two collection methods. All subjects reported that they would tolerate at least two FPs per time point and at least six time points in any given session (study day).

Table 2 shows how staff who carried out each step rated the ease of sample collection by the two methods. It is notable that staff required minimal training (2–3 h familiarization) to perform blood spotting (labeling blank card, spotting on paper, drying blood spot and storing the card into the bag). None of the staff had training prior to this brief practical session. The fact that those with limited experience with the DBS technique could then perform the technique in the clinical setting suggests that this technique can be learnt rapidly.

■ Concentration–time profiles

Typical concentration–time profiles for an individual subject after the single oral administration of paracetamol at two doses (500 mg and 1 g) on two occasions are shown in **Figure 2**.

■ Statistical analysis

Data from all 11 subjects from both dosing periods and all sampling time points were combined for statistical analysis. The majority (79%) of measured concentrations were in the range 1000–10,000 ng/ml, with 11% of values over 10,000 ng/ml (generally at the 1- and 1.5-h time points) and 10% less than 1000 ng/ml (generally at the 0.5-h time point). Conclusions are therefore more robust for concentrations in the 1000 to 10,000 ng/ml range.

A Bland–Altman plot comparing DBS concentrations to those in WB, where both samples were taken from a peripheral venous cannula, is shown in **Figure 3**. The final model included mean concentration as a covariate, but found no effect of dose or time point. A bias was observed such that DBS-Can gave a value 6% higher than WB-Cannula at concentrations of 1000 ng/ml and this bias increased slightly with concentration to 16% for concentrations of 10,000 ng/ml. There was no significant change in variability across the range of concentrations, with a constant CV of 11.8% (95% CI for CV: 10.3–13.8%). As observed from **Figure 3**, there was increasing bias when comparing DBS-Can concentrations to those in WB-Can. The combination of increasing bias with constant CV meant that the lower and upper 95% limits of agreement increased from (-15% and +32%) for a concentration of 1000 ng/ml to (-7% and +44%) for a concentration of 10,000 ng/ml. When validating bioanalytical methods for the determination of circulating drug concentrations, a limit of ±15% for bias and 15% for CV is considered acceptable [9,10]. Taking these limits into account, only concentrations below 8000 ng/ml could be considered interchangeable between the two measurement methods. Exploration of factors such as the temperature of the blood spotted, the pipetting device used, the method used to dilute samples into the analytical range and the stability of paracetamol metabolites, did not provide explanations for the bias [11]. Other factors, such as the process of diluting the WB samples with water prior to storage and analysis, or any changes in viscocity with sampling time could have had an

Table 2. Staff response to ease of performing processes associated with taking blood samples.

	Number responding				
Staff rating of sample collection step	***Extremely easy***	***Easy***	***Neither***	***Difficult***	***Extremely difficult***
Taking blood by finger-prick	3	9	3	0	0
Taking blood by cannula	7	6	2	0	0
Blood spotting onto card	2	14	0	0	0

Questionnaires were completed by 16 study staff after the single oral administration of paracetamol at two doses (500 mg and 1 g).

effect on the results for these samples. However, these factors were not investigated as part of this study.

In the analysis comparing DBS concentrations from samples collected by FP to those taken by cannula, the time point at which the sample was taken was found to significantly influence the ratio of concentrations and the relationship of this ratio to the mean concentration (i.e., an interaction), but no effect of dose was found. A series of Bland–Altman plots demonstrating this are shown in **FIGURE 4**, with a separate panel for each time point. The 0.5-h samples gave concentrations up to fourfold higher by DBS-FP than DBS-Can, with the largest biases at the lower concentrations. The 1- and 1.5-h samples also showed positive bias (33 and 14%, respectively, at a concentration of 3000 ng/ml), while the subsequent 3- and 5-h samples showed negative bias (-17 and -10%, respectively). The CV also varied across the sampling time points: 36.2, 22.8, 19.3, 20.7 and 11.6%, at 0.5, 1, 1.5, 3 and 5 h, respectively.

When comparing DBS-FP to DBS-Can, both the bias and CV varied across the five sampling time points. The finding that blood concentrations of paracetamol vary with sampling site, that is, between capillary and venous blood, is consistent with data from blood samples also collected by FP and cannula presented by Mohammed and co-workers [14]. In that study, paired FP and venous blood samples were taken at 0, 15, 30 and 60 min following a 1 g oral dose of paracetamol, from eight adult subjects. Paracetamol concentration was determined using HPLC and UV detection with a LLOQ of 2200 pg on column. At 15 min post-dose, there was a significant difference between the paracetamol concentrations obtained from FP and venous samples. At 30 min post-dose the FP paracetamol concentration was approximately 20% greater than the venous concentration. However, by 60 min post-dose any significant differences had disappeared. It was demonstrated that for paracetamol, the two sampling methods do not give comparable results and may not be used interchangeably until 60 min post-dose.

This discrepancy between venous and FP samples is not borne out in clinical studies with tacrolimus [15] and cyclosporine [16]. However, Chiou has described significant or marked blood sampling site dependence in concentration for at least 42 compounds in animal and human studies [17]. Reasons outlined for the differences between arterial and venous concentrations include:

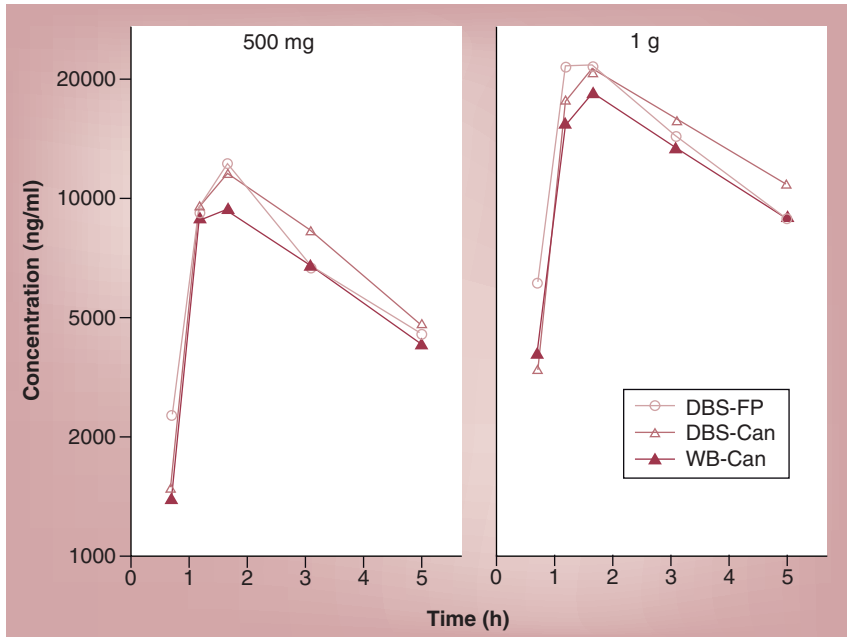

Figure 2. Paracetamol concentration–time profiles for a typical subject after the single oral administration of paracetamol at two doses on two occasions. DBS samples were obtained by either FP or venous Can.
Can: Cannula; DBS: Dried blood spot; FP: Finger prick; WB: Whole blood.

- Differences in the early phase after administration may be due to diffusion of the drug into the extravascular space through the capillary membrane, giving higher arterial than venous concentrations for early time points;

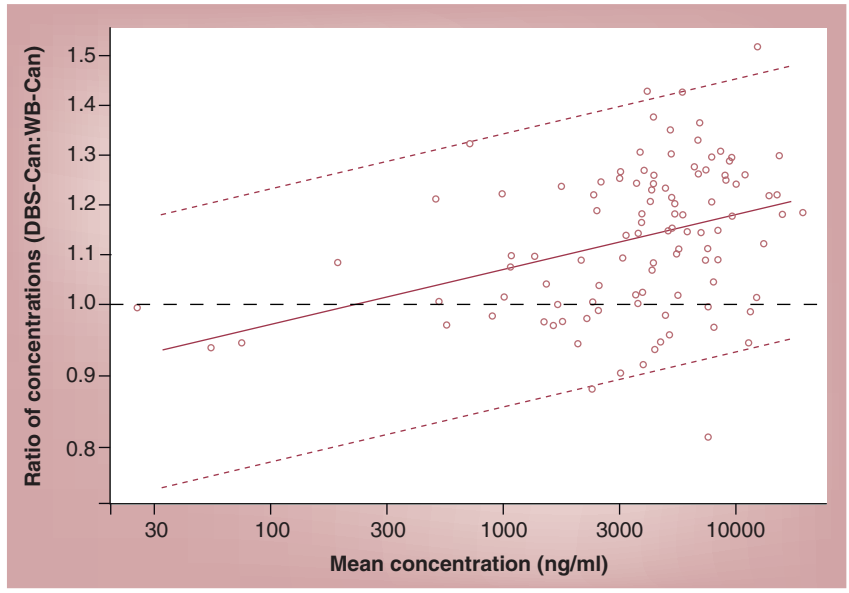

Figure 3. Bland–Altman plot comparing concentrations of paracetamol in DBS to WB samples, both obtained from a venous Can. Bias is seen to increase with concentration (solid line). Limits of agreement increase in parallel with bias (dotted line). The line of identity (ratio = 1) is also shown (dashed line).
Can: Cannula; DBS: Dried blood spot; WB: Whole blood.

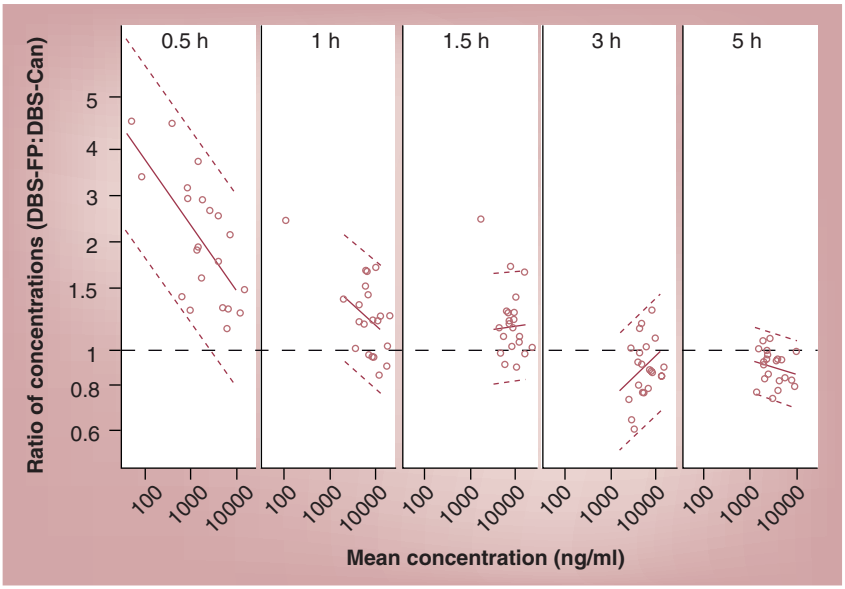

Figure 4. Bland–Altman plot comparing concentrations of paracetamol in DBS samples obtained from FP to DBS obtained from venous Can at each sampling time point. Bias is positive at time points up to 1.5 h and negative subsequently (solid lines). Limits of agreement (dotted lines) and the line of identity (ratio = 1) are also shown (dashed line).
Can: Cannula; DBS: Dried blood spot; FP: Finger prick.

- Differences in the late (terminal) phase after administration may be attributed to a decrease in the arterial concentration due to elimination and distribution into other extravascular tissues, whilst drug retained by tissues may begin to diffuse through the capillary wall into the venous system, giving higher venous than arterial concentrations for late time points;
- Concentrations for capillary blood should lie between the concentrations of arterial and venous blood.

The above may help to explain the high bias of DBS-FP (capillary) versus DBS-Can (venous) for early time points and the opposite for later time points **(Figure 4)**.

Conclusion

We have demonstrated in staff and volunteers that the procedures involved in FP sampling combined with blood spotting are an acceptable method for the collection of blood samples and that this approach compares favorably with more conventional cannula WB collection. Furthermore, we have demonstrated that sufficient samples can be collected by FP to enable the performance of PK studies.

The study data showed that quantitative drug exposure data can be generated using DBS sampling. However, when collecting venous samples from a cannula, switching the sample type from WB to DBS can make a small difference in the measured concentrations. Furthermore, when using DBS samples, switching the collection technique from cannula to FP makes a major difference on the low concentrations of paracetamol and the early post-dose time points.

The lack of interchangeability observed between both sampling site (FP and cannula) and sample type (DBS and WB) may not be limited to paracetamol. This should therefore be taken into account when designing PK studies and may need to be investigated as part of the study design.

Future perspective

The DBS sample format has been demonstrated to be a viable approach for the collection of samples for the determination of circulating drug concentrations in animal and clinical studies in place of plasma samples. This and the numerous advantages over current practice will inevitably lead to the more widespread adoption of the technique for clinical trials and therapeutic drug monitoring. However, many factors still require investigation before this novel approach will be fully accepted by all involved in the drug development process. These factors include, but are not limited to, the potential effects of

Executive summary

- Dried blood spots (DBS) sampling is a viable approach for the determination of clinical drug pharmacokinetics.
- The technique is acceptable to both staff and study subjects.
- Differences in drug concentrations can be observed between blood storage methods for samples taken from the same sampling site (diluted whole blood versus DBS from venous cannula).
- Differences in drug concentrations can be observed between DBS samples taken from different sampling sites (finger prick versus venous cannula).
- This lack of interchangability needs to be taken into account when designing the pharmacokinetic aspects of clinical studies.

hematocrit, homogeneity of the blood spots, variability of drug recovery, and the stability of the drug and/or metabolites during the collection, drying, transport and storage of DBS samples. Another aspect is that the DBS approach is generally considered to be more complex for the bioanalytical laboratory, compared with conventional plasma assays; for example, multiple paper substrate types and extraction solvents need to be investigated as part of method development, and assay sensitivity is limited by the smaller sample volumes employed. However, recent progress in the direct analysis of DBS samples [18,19] without the need for offline punching and extraction of the sample prior to analysis is pointing the way to a more simplified analytical process in the future.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

This study was supported by GlaxoSmithKline (GSK) Research and Development. All authors were employees of GSK at the time the study was performed and are eligible for GSK stock options and have stock ownership. Yujay Ramakrishnan is currently an employee of the British National Health Service.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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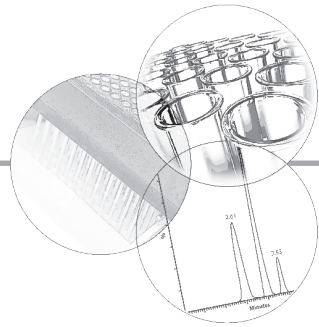

Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories

Background: There are little published data on either the comparison of liquid blood and dried blood spots (DBS) analyses or the ability to generate comparable DBS data at different analytical laboratories. We assess the comparative results of samples stored as liquid blood and DBS. We also determine the transferability of DBS samples by comparing the analysis at two laboratories. **Results:** Bioanalytical methods for the analysis of pioglitazone in DBS and liquid blood samples were validated to US FDA guidelines. Pharmacokinetic data generated from DBS and liquid blood samples demonstrated area under the time-concentration profile (0–24 h) values within 3% of each other and maximum plasma concentration values within 7% of each other. Comparing DBS sample results at different laboratories showed more than 99% of results agreeing within 20%. **Conclusions:** The results indicate that comparable concentration results are obtained from DBS and whole blood samples within the same laboratory, indicating that changing between the two matrices is viable. The comparable results of DBS samples analyzed at two laboratories using different analytical methodologies demonstrate that the technique is robust and transferable.

Pioglitazone is a prescription drug with hypoglycemic action from the thiazolidinedione class of compounds used in the treatment of diabetes mellitus (Type 2) and related diseases. Pioglitazone is administered orally at a dosage of 15 or 30 mg/day, with titration to 45 mg/day if necessary [1]. The compound is absorbed quickly from the gastrointestinal tract and shows bioavailablity of greater than 80%, giving maximum plasma concentrations (C_{max}) within 2 h (t_{max}) of dosing [2]. A variety of methods have been established for the quantitative bioanalysis of pioglitazone in plasma and serum to determine ng/ml levels of the compound [3–6].

The **dried blood spots** (DBS) approach to toxicokinetic (TK) and pharmacokinetic (PK) sampling has recently received considerable attention owing to the inherent advantages of the technique. Preclinical DBS sampling has ethical advantages in the reduction and refinement of animal use; less blood is sampled at each timepoint than for plasma or serum analysis meaning that serial, rather than composite, TK profiles can be obtained from individual animals [7]. Another advantage of the DBS approach is the potential to utilize finer needles to sample the blood, thereby causing less distress to the animals. The use of DBS in the clinical arena has the advantage that it may eliminate the requirement for sampling by venous cannula as it is feasible to sample peripheral blood (e.g., from a finger-prick) [8]. Less invasive, simpler sampling would be of great benefit when conducting pediatric studies and would also allow home sampling by patients taking part in clinical trials. When compared with plasma or serum, the approach has a low biohazard risk, minimal post-collection processing with lower cost sample transport and storage [9–11] making PK sampling far easier in remote locations.

Bioanalytical methods for the analysis of compounds in DBS have been published for a number of drugs including acetaminophen, metformin, piperaquine, rifampicin, tacrolimus and dextromethorphan [7,12–16]. Considerations when developing a DBS assay are the type of card to utilize (different card materials and chemical treatments), the spot diameter to be sampled from the card, the solvent to remove the analyte of interest from the paper, on-card stability of the analyte of interest during storage and how to dilute samples into the assay range, if required.

In this study, LC–MS/MS assays for the quantitative analysis of pioglitazone in DBS in the range 5 to 2500 ng/ml were established in two laboratories. Both assays were validated to conform to internationally recognized acceptance criteria [17]. A consideration when assessing the accuracy and precision of bioanalytical results of unknown samples is whether the

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Key Terms

C_{max}: Maximum observed concentration in a pharmacokinetic or toxicokinetic profile.

t_{max}: Time point at which C_{max} occurred.

Dried blood spots: Means of storing blood samples in a dried, rather than the traditional liquid, format.

Transferability: Ability to transfer samples between laboratories and/or assays and achieve comparable results.

analysis procedure is having an effect on the results. As there is no evidence in the literature that incurred DBS samples give comparable results when assayed by different methods, we assessed the **transferability** of DBS samples by analyzing the sample set at two laboratories. The two sets of results were compared to determine interlaboratory comparability of the DBS sampling and analysis technique when using different bioanalytical methods on the same study, as advised in the regulatory guidance [18]. The ability to directly compare results from liquid and dried blood samples was considered important as larger sample volumes may be necessary when developing clinical assays to reach the required limits of quantification. Achieving a larger sample volume by changing the matrix to plasma is not considered suitable as the blood cell binding factor means that results from blood and plasma are not directly comparable. An additional comparison was made to determine whether dried and liquid blood samples gave comparable results by using two sampling techniques (DBS and blood:water 1:1 v/v) for harvesting PK blood samples following a single oral (gavage) administration of pioglitazone to the rat.

Experimental

■ Chemicals & reagents

Pioglitazone **(FIGURE 1)** and stable isotopically labelled internal standard (IS) [$^2H_4$]-pioglitazone, were obtained from Toronto Research Chemicals (Toronto, Canada). Acetone, methanol and 2-propanol were of HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, UK) and Sigma-Aldrich (Poole, UK). Acetonitrile was of HPLC grade and obtained from Fisher Scientific (Loughborough, UK) and Sigma-Aldrich. Water was of ultra-pure grade produced in-house using a Millipore Super-Q™ osmosis system (MA, USA) and Chromasolv®Plus for HPLC was obtained from Sigma-Aldrich. All other chemicals were of Analar grade and were supplied by Sigma-Aldrich. Rat blood (sodium heparin) was supplied by Harlan Laboratories (Shardlow, UK) and B&K Universal Ltd (Hull, UK).

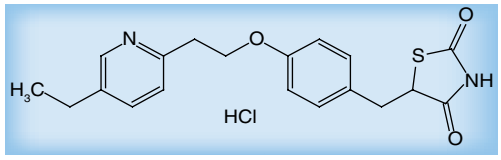

Figure 1. Pioglitazone (HCl salt).

Equipment

■ Laboratory 1 (Covance)

FTA® Elute blood spot micro cards, sachets of desiccant, Harris Uni-Core 3.0-mm punches and Harris cutting mats were supplied by Whatman (Maidstone, UK). CoStar 96-well sample plates were obtained from Corning (NY, USA). Centrifuge (model Rotanta 460R) was supplied by Hettich (Tuttingen, Germany). Flexus liquid handling system was supplied by Anachem (Luton, UK). Disposable glass 20-µl capillary pipettes of containing sodium heparin were supplied by Hirschmann Laborgerate (Eberstadt, Germany).

■ Laboratory 2 (GlaxoSmithKline)

FTA® Elute blood spot cards, sachets of desiccant, Harris Uni-Core 3.0-mm punches and Harris cutting mats were supplied by Whatman. Alphanumeric 1.4-ml U-bottom sample tubes were obtained from Micronic (Lelystad, The Netherlands). Centrifuge (model 5810R) was supplied by Eppendorf (Cambridge, UK). Hamilton liquid handling system was supplied by Hamilton (Bonaduz, Switzerland).

HPLC–MS/MS analysis

■ Laboratory 1

The HPLC–MS/MS system consisted of an Acquity UPLC system (PA, USA) coupled with a Sciex API-4000 (Applied Biosystems/MDS Sciex, Canada). Chromatographic and MS conditions are detailed in **TABLES 1 & 2**, respectively. HPLC–MS/MS data were acquired and processed using Analyst 1.4.2 software (Applied Biosystems). The chromatographic conditions utilized gradient chromatography with separation achieved on an Acquity BEH C18 (50 × 2.1 mm, 1.7 µm) column with an aqueous mobile phase of 0.1% formic acid in 10 mM ammonium acetate and an organic mobile phase of 0.1% formic acid in methanol. The retention time of pioglitazone was 2.0 min.

■ Laboratory 2

The HPLC–MS/MS system consisted of an Acquity UPLC system coupled with a Sciex API-5000. Chromatographic and MS conditions are detailed in **TABLES 1 & 2**, respectively. HPLC–MS/MS data were acquired and processed using Analyst 1.4.2 software. The chromatographic conditions utilized isocratic chromatography with separation achieved on an Acquity BEH C18 (50 × 2.1 mm, 1.7 µm) column with a mobile phase of 70/30 0.1%

Table 1. Chromatographic conditions at laboratories 1 and 2.

Laboratory 1			
Analytical column	Acquity BEH C18, 50 x 2.1 mm, 1.7 µm		
Column oven temperature (°C)	Nominal 40		
Mobile phase A	0.1% formic acid in 10 mM ammonium acetate		
Mobile phase B	0.1% formic acid in methanol		
Time	***A (%)***	***B (%)***	***Flow rate (ml/min)***
0.0	70	30	0.6
0.2	70	30	0.6
1.5	50	50	0.6
2.1	50	50	0.6
2.2	2	98	0.8
4.5	2	98	0.8
4.6	70	30	0.6
Autosampler weak wash	0.1% formic acid in 10 mM ammonium acetate:methanol (80:20 v/v)		
Autosampler strong wash	Methanol:acetone:water:trifluoroacetic acid (50:40:10:0.1 v/v/v/v)		
Run time (min)	5		
Injection volume (µl)	10		
Laboratory 2			
Analytical column	Acquity BEH C18, 50 x 2.1 mm, 1.7 µm		
Column oven temperature (°C)	Nominal 60		
Mobile phase A	0.1% formic acid in 10 mM ammonium acetate		
Mobile phase B	Acetonitrile		
Isocratic settings A/B	70:30 at 0.8 ml/min		
Weak wash	0.1% formic acid		
Strong wash	Acetonitrile:propan-2-ol:water:formic acid (40:30:30:0.1 v/v/v/v)		
Run time (min)	1.2		
Injection volume (µl)	2		

Key Term

1:1 blood:water (v/v): Means of storing frozen blood samples whilst keeping them in a liquid and homogeneous state.

formic acid in 10 mM ammonium acetate/acetonitrile (v/v). The retention time of pioglitazone was 0.8 min.

Validated DBS & 1:1 blood:water (v/v) analysis procedures

DBS methods were validated at both laboratories to US FDA guidelines [17] to include determination of precision and accuracy on three occasions, on-card stability assessment, determination of assay specificity and extract re-injection viability.

The analytical method for **1:1 blood:water (v/v)** was validated at laboratory 1 to include determination of precision and accuracy on one occasion, freeze–thaw and room temperature stability assessment, determination of assay specificity and extract re-injection viability.

■ Laboratory 1

Stock solutions of pioglitazone were prepared in methanol (0.25 mg/ml) IS stock solutions of [$^2H_4$]-pioglitazone (0.1 mg/ml) were prepared in methanol. All the solutions were stored at 4°C and brought to room temperature before use.

Calibration standards were prepared fresh on the day of analysis by diluting the stock solutions into appropriate concentration working solutions (with methanol), then spiking into blank whole rat blood to give a calibration line with standards at 5, 10, 20, 100, 250, 1000, 2000 and 2500 ng/ml, ensuring that the volume of the working solution remained less than 5% of the total standard volume. QC samples for all analyses were prepared from a second stock solution and were prepared by diluting the stock solution into appropriate concentration working solutions (with methanol), then spiking into blank whole rat blood to give QCs at 5 (validation only), 15, 125, 1800 and 12500 (validation only) ng/ml. For DBS analyses, 20 µl of calibration standards and QC samples were spotted onto FTA Elute cards and allowed to dry at room temperature for at least 2 h prior to analysis.

Table 2. MS/MS conditions at laboratories 1 and 2.

Laboratory 1: mass spectrometer parameters API 4000				
Mode of operation		APCI (positive ion; MS/MS)		
Collision gas setting (arbitrary units)		9		
Curtain gas setting (arbitrary units)		40		
Ion source gas 1 (arbitrary units)		35		
Nebuliser current (µA)		3.0		
Temperature (°C)		500		
Pause time (ms)		5		
Collision gas		Nitrogen		
Compound name	***Ions monitored***	***Dwell time (ms)***	***Declustering potential (V)***	***Collision energy (V)***
Pioglitazone	357.2 → 134.1	100	85	45
$[^2H_4]$-pioglitazone	361.2 → 134.1	100	85	45
Laboratory 2: mass spectrometer parameters API 5000				
Mode of operation		Turbo ion spray (positive ion; MS/MS)		
Collision gas setting (arbitrary units)		7		
Curtain gas setting (arbitrary units)		15		
Ion source gas 1 (arbitrary units)		50		
Ion source gas 2 (arbitrary units)		50		
Ion spray Voltage (V)		1500		
Temperature (°C)		750		
Pause time (ms)		5		
Collision gas		Nitrogen		
Compound name	***Ions monitored***	***Dwell time (ms)***	***Declustering potential (V)***	***Collision energy (V)***
Pioglitazone	357.4 → 134.2	150	100	38
$[^2H_4]$-pioglitazone	361.4 → 138.2	100	100	38

A 3.0-mm diameter disk was punched from the center of the DBS into a 2-ml 96-well plate and 200 µl of methanol containing 50 ng/ml IS ($[^2H_4]$-pioglitazone) was added. The plate was gently vortex mixed for approximately 15 min then centrifuged for 5 min at 3000 × g. A 20-µl aliquot of supernatant was transferred to a clean 1.2-ml 96-well plate using a Flexus robot and 180 µl of 0.1% formic acid in 10 mM ammonium acetate/methanol (80/20 v/v) added. A 10-µl volume was then injected onto the HPLC–MS/MS system.

The ability to switch from DBS analysis to analysis of whole blood at a later date of the drug development process was also investigated. The blood analysis involved taking 20 µl of 1:1 blood:water (v/v) and adding 200 µl of methanol containing 50 ng/ml IS ($[^2H_4]$-pioglitazone). The samples were vortex mixed followed by centrifugation. A 20 of µl aliquot of the supernatant was transferred to a second 96-well plate to which 180 of µl 0.1% formic acid in 80:20 10 mM ammonium formate:methanol (v/v) was added. The samples were vortex mixed followed by centrifugation and submitted for HPLC–MS/MS analysis.

■ Laboratory 2

Stock solutions of pioglitazone were prepared in dimethyl formamide (1 mg/ml). IS stock solutions of $[^2H_4]$-pioglitazone (1 mg/ml) were prepared in dimethyl formamide. All the solutions were stored at 4°C and brought to room temperature before use.

Calibration standards were prepared fresh on the day of analysis by diluting the stock solutions into appropriate concentration working solutions (with 50:50 acetonitrile:ultra-pure water v/v), then spiking into blank whole rat blood to give a calibration line with standards at 5, 10, 20, 100, 250, 1000, 2000 and 2500 ng/ml, ensuring that the volume of the working solution remained less than 5% of the total standard volume. QC samples for all analyses were prepared from a second stock solution and

were prepared by diluting the stock solutions into appropriate concentration working solutions (with 50:50 acetonitrile:ultra-pure water [v/v]), then spiking into blank whole rat blood to give QCs at 5 (validation only), 20, 250, 2000, 2500 (validation only) and 5000 (validation only) ng/ml. For DBS analyses, 15 µl of calibration standards and QC samples were spotted onto FTA Elute cards and allowed to dry at room temperature for at least 2 h prior to analysis.

A 3.0-mm diameter disk was punched from the center of the DBS into a 1.4-ml Micronics tube and 100 µl of methanol containing 100 ng/ml IS ([$^2H_4$]-pioglitazone) was added. The plate was gently vortex mixed for approximately 30 min then centrifuged for 5 min at 3000 × g. A 50-µl aliquot of supernatant was transferred to a clean 1.4-ml Micronics tube using a Hamilton robot and 150 µl of 50:50 acetonitrile:water (v/v) added. A 2-µl volume was then injected onto the HPLC–MS/MS system.

In vivo test system

Female rats (six per group; HsdHan: WIST strain) were housed in a single, exclusive room, air-conditioned to provide a minimum of 15 air changes/h. Female rats were specifically selected to assess any effects of the sampling procedures on smaller animals. The temperature and relative humidity ranges were maintained in the specified ranges of 19–25°C and 40–70%, respectively. Fluorescent lighting was controlled automatically to give a cycle of 12 h light (0600 h –1800 h) and 12 h dark and the animals were housed in groups of three.

Throughout the study the animals had access *ad libitum* to SQC Rat and Mouse Maintenance Diet No 1, Expanded, (Special Diets Services Ltd, Witham, UK) and mains water was provided *ad libitum* via water bottles. Bedding was provided on a weekly basis to each cage by use of clean Aspen wood chips (Datesand Ltd, Manchester, UK) or European softwood bedding (BetaBed Grade 6, Datesand Ltd Manchester, UK). The diet, water and bedding was analyzed for specific contaminants and the results confirmed no contaminants were present at levels that might have interfered with achieving the objective of the study.

The animals were individually identified by electronic implant as follows:

- Group 1F: animals one to six
- Group 2F: animals seven to 12

Oral pioglitazone formulation at 30 mg/kg was administered at a dose volume of 10 ml/kg and was selected to provide measurable blood concentrations of pioglitazone, but was not expected to result in any toxicity. It was formulated as a suspension in 0.6 (w/w) gelatine and 0.9% sodium carboxymethylcellulose in purified water and stored at 2–8°C in a sealed container. The formulation was stirred continuously before and throughout dosing.

Blood sampling procedures

A single oral (gavage) dose at 30 mg/kg was administered to Group 1F. After dosing, DBS samples (three × 20 µl; three separate samples were taken at each timepoint with less than 30 s between the start of sample one and the end of sample three) were collected from the lateral caudal vein of each animal over a 24 h period. Each DBS sample was collected via a disposable 20-µl glass capillary tube, onto a labelled FTA Elute sample card, allowed to air dry for a minimum of 2 h before being stored in sealed polypropylene bags/boxes containing desiccant sachets, these were kept at room temperature. In addition, whole blood samples (0.1 ml) were also collected from Group 1F over a 24 h period into tubes containing lithium heparin. Samples (50 µl) of whole blood were added to purified water (50 µl), mixed gently by hand and placed on a cooled rack prior to storage at -20°C.

A single oral (gavage) dose at 30 mg/kg was administered to Group 2F. After dosing, DBS samples (three × 20 µl; three separate samples were taken at each timepoint with less than 30 s between the start of sample one and the end of sample three) were collected from the lateral caudal vein of each animal over a 24-h period and processed as described above.

Blood was sampled at the following timepoints:

- Group 1F: animals one to three at 0, 1, 2, 3.5, 5 and 7 h post dose (liquid blood and DBS);
- Group 1F: animals four to six at 0.5, 1.5, 2.75, 4.25, 6 and 24 h post dose (liquid blood and DBS);
- Group 2F: animals seven to 12 at 0, 0.5, 1, 1.5, 2, 2.75, 3.5, 4.25, 5, 6, 7, 24 h post-dose (DBS).

The data from Group 1F was used to generate composite PK profiles. The data from Group 2F was used to generate **serial PK profiles**.

Key Term

Serial profile: Pharmacokinetic or toxicokinetic profile composed of samples at all timepoints in single individuals.

Results & discussion

■ DBS validations

All intra- and inter-assay precision and accuracy data at both laboratories were within the predefined 15% limits (20% at the LLOQ) and are presented in **TABLES 3 & 4**.

■ Effect of aliquot volume

Variation of the blood aliquot volume of up to ±25% of the 20-µl target volume in laboratory 1 and ±33% of the target 15-µl target volume in laboratory 2 did not affect the quantification of pioglitazone, indicating that the blood spot volume did not significantly affect the amount of analyte in the punched blood spot disk (**TABLES 3 & 4**).

■ Response

The response of the detector to pioglitazone was linear for both laboratories methods over the concentration range 5 to 2500 ng/ml with a weighted $1/x^2$ linear regression applied to the data (mean R^2 value 0.9968 and 0.9981 at laboratories 1 and 2, respectively). The analyte response (peak height) at the LLOQ was greater than five-times the blank blood spot response. Representative chromatograms are presented in **FIGURE 2** (laboratory 1) and **FIGURE 3** (laboratory 2) demonstrating an excellent signal-to-noise ratio at the LLOQ and lack of significant interferences at the retention time of pioglitazone.

■ Selectivity

Neither method displayed significant interferent peaks (>20% of the mean utilized LLOQ) in matrix blanks in the retention window of pioglitazone and IS, respectively. There were no significant interferent peaks (>20% of the mean utilized LLOQ) detected in the six individual blank samples in the retention window of pioglitazone and IS, respectively. Matrix effects causing suppression and enhancement were determined to be less than 3.5% when comparing peak areas of post-spiked QC extracts (n = 6) to pure standards (n = 6) of theoretical equivalent concentrations. Recovery was calculated at more than 75% when comparing peak areas of extracted QCs (n = 6) to post-spiked QC extracts (n = 6) of theoretical equivalent concentrations.

Table 3. Intra- and inter-assay precision and accuracy quality control performance data for pioglitazone in rat dried blood spots and effect of blood aliquot volume on precision and accuracy of QC sample data at laboratory 1.

Intra-assay	***QC 5 ng/ml (LLOQ QC)***	***QC 15 ng/ml (LQC)***	***QC 125 ng/ml (MQC)***	***QC 1800 ng/ml (HQC)***	***QC 12500 ng/ml (DiQC)†***	***QC 2500 ng/ml (DiQC)‡***
Mean (ng/ml)	4.86	14.1	126	1730	12100	11200
Accuracy (%)	97.2	94.0	100.8	96.1	96.8	89.6
RSD (%)	4.3	6.2	3.4	4.2	3.8	2.1
n	6	6	6	6	6	6
Inter-assay	***QC 5 ng/ml (LLOQ QC)***	***QC 15 ng/ml (LQC)***	***QC 125 ng/ml (MeQC)***	***QC 1800 ng/ml (HQC)***		
Mean (ng/ml)	5.19	14.4	122	1720		
Accuracy (%)	103.8	96.0	97.6	95.6		
RSD (%)	9.7	6.9	3.8	3.8		
n	18	18	18	18		
Quality control sample data						
Concentration (ng/ml)	125					
Blood spot size (µl)	15	25				
Spot size deviation from 20 µl assay volume (%)	-25	25				
Mean observed concentration (ng/ml)	115	126				
Standard deviation (n - 1)	3.54	6.68				
RSD (%)	3.1	5.3				
Accuracy (%)	92.0	100.8				
n	6	6				

†Analyzed after tenfold dilution with extracted blank rat blood spots.
‡Analyzed after tenfold dilution post-extraction with internal standard.
DiQC: Diluted QC; HQC: High QC; LQC: Low QC; MQC: Medium QC; RSD: Relative standard deviation.

Table 4. Intra- and inter-assay precision and accuracy quality control performance data for pioglitazone in rat dried blood spots and effect of blood aliquot volume on precision and accuracy of QC sample data at laboratory 2.

	QC 5 ng/ml (LLOQ QC)	QC 20 ng/ml	QC 250 ng/ml	QC 2000 ng/ml	QC 2500 ng/ml (HQC)	QC 5000 ng/ml (DiQC)†
Intra-assay						
Mean (ng/ml)	4.35	19.8	262	2010	2440	5150
Accuracy (%)	87.1	98.9	104.8	100.3	97.4	103.0
%CV	7.8	6.6	4.7	2.7	3.1	2.7
n	6	6	6	6	6	6
Inter-assay						
Mean (ng/ml)	4.67	19.5	255	1900	2330	
Accuracy (%)	93.4	97.5	102.0	95.0	93.2	
%CV	7.7	6.5	5.1	6.1	6.7	
n	18	18	18	18	18	
Quality control sample data						
Concentration (ng/ml)	QC 20		QC 2000			
Blood spot size (µl)	10	20	10	20		
Spot size deviation from 15 µl assay volume (%)	-33	33	-33	33		
Mean observed concentration (ng/ml)	18.2	20.6	1810	1960		
Standard deviation (n - 1)	0.42	0.82	54.1	77.6		
%CV	2.3	4.0	3.0	4.0		
Accuracy (%)	91.0	103.0	90.5	98.0		
n	6	6	6	6		

†*Analyzed after tenfold dilution with extracted blank rat blood spots.*
CV: Coefficient of variation; DiQC: Diluted QC; HQC: High QC.

■ Processed sample viability

Viability of pioglitazone in the extracts produced via laboratory 1 was found to be acceptable when stored at nominal 4°C and reinjected 3 days after initial injection (precision and accuracy within the predefined 15% limits). The viability of pioglitazone in the extracts from laboratory 2 was acceptable when stored at room temperature and reinjected 96 h after the initial injection (precision and accuracy within the predefined 15%).

■ Storage stability of pioglitazone in DBS

Pioglitazone exhibits acceptable stability in DBS when stored at room temperature for up to 4 months (**Table 5**).

■ Validation of 1:1 blood:water (v/v) (laboratory 1)

A partial validation of a method for the determination of pioglitazone in 1:1 blood:water (v/v) was performed to assess intra-assay precision and accuracy (single batch), response, selectivity, room temperature stability in matrix, freeze–thaw (three cycles) stability in matrix and processed extract viability. Intra-assay precision and accuracy (single batch) data were within the predefined 15% limits (20% at the LLOQ).

■ Response

The response of the detector to pioglitazone was linear over the concentration range

Table 5. Long-term stability of pioglitazone in dried blood spots stored at room temperature (data generated at laboratory 1).

Quality control level (ng/ml)	Baseline stability samples	1.5 months (46 days) storage		4 months (120 days) storage	
	Mean observed concentration (ng/ml)	*Mean (ng/ml)*	*Difference from nominal (%)*	*Mean (ng/ml)*	*Difference from nominal (%)*
15	14.6 (±5.9%)	15.9 (±1.8%)	6.0	16.5 (±7.8%)	10.0
1800	1710 (±4.3%)	2000 (±2.1%)	11.1	1940 (±2.6%)	7.8

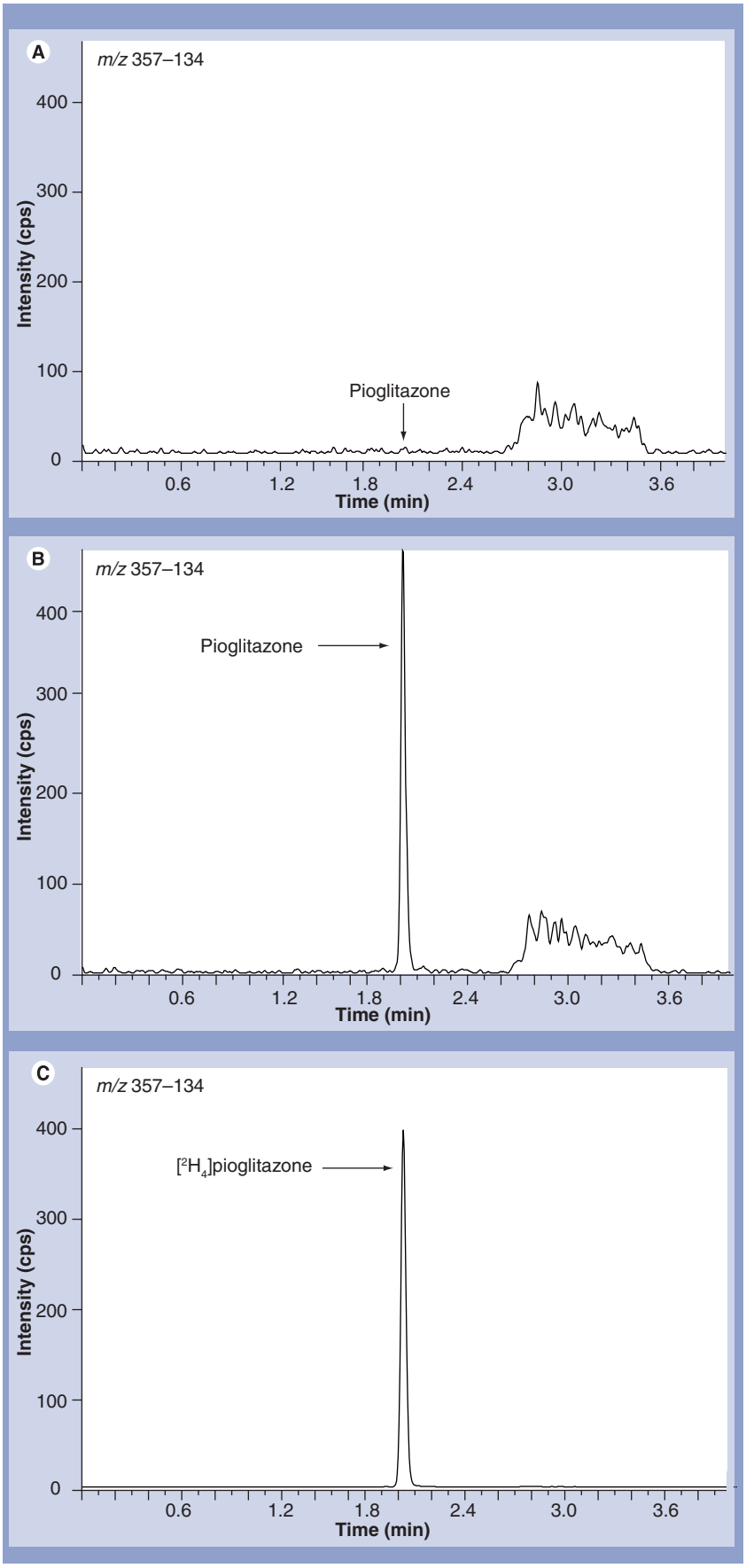

Figure 2. Representative HPLC–MS/MS selective reaction monitoring chromatograms. Data from Laboratory 1 of **(A)** a blank plus internal standard rat dried blood spots (DBS) sample, **(B)** a rat DBS sample spiked with pioglitazone at the LLOQ (5 ng/ml) and **(C)** a rat DBS sample spiked with the internal standard ([$^2H_4$]-pioglitazone).

5–2500 ng/ml. The analyte response (peak height) at the LLOQ was greater than five-times the blank 1:1 blood:water (v/v) response.

■ Selectivity

There were no significant interferent peaks (>20% of the mean utilized LLOQ or >5% of IS response) detected in reagent, matrix blanks and ULOQ calibration standard (without IS) in the retention window of pioglitazone and IS, respectively. There were no significant interferent peaks (>20% of the mean utilized LLOQ or >5% of IS response) detected in the six individual blank samples in the retention window of pioglitazone and IS respectively. The accuracy values of each of the six spiked individual matrix samples were within 85–115% of the theoretical value. Therefore, individual matrix samples do not affect the quantification of pioglitazone.

■ Room temperature stability of pioglitazone in matrix

The data indicate that pioglitazone has acceptable stability in 1:1 blood:water (v/v) stored at room temperature for up to 25 h (precision and accuracy within the predefined 15% limits).

■ Freeze–thaw stability of pioglitazone in matrix

The data indicate that pioglitazone has acceptable stability in 1:1 blood:water (v/v) when exposed to up to three additional freeze/thaw cycles (precision and accuracy within the predefined 15% limits).

■ Processed sample viability

The viability of pioglitazone in the extracts was found to be acceptable when stored at nominal 4°C and reinjected 69 h after the initial injection (precision and accuracy within the predefined 15% limits).

■ PK results (laboratory 1)

Blood concentrations of pioglitazone were quantifiable in samples after dosing from all animals in Groups 1F and 2F, confirming systemic exposure; the PK parameters are presented in **TABLE 4**.

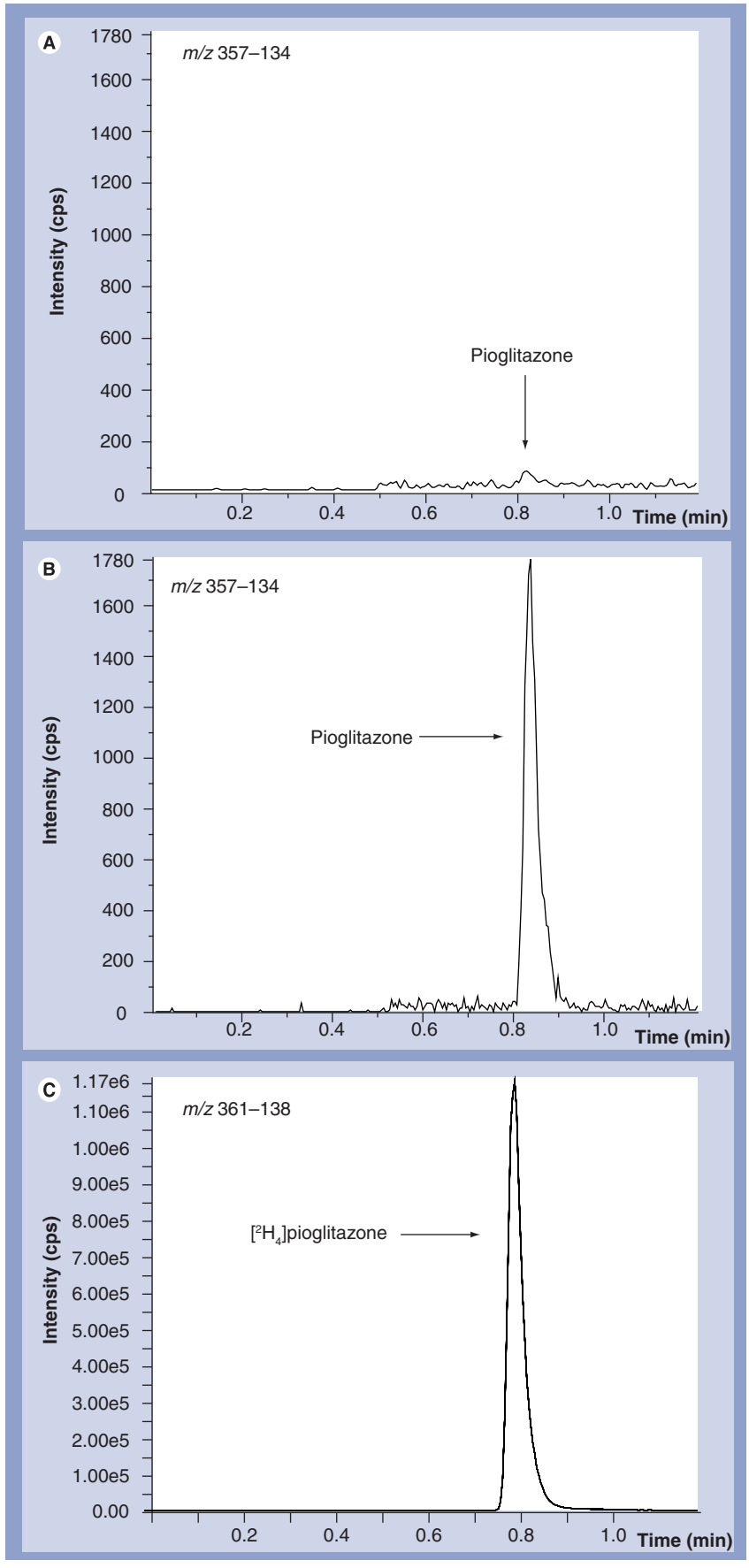

Figure 3. Representative HPLC–MS/MS selective reaction monitoring chromatograms. Data from laboratory 2 of **(A)** a blank plus internal standard rat dried blood spots (DBS) sample, **(B)** a rat DBS sample spiked with pioglitazone at the LLOQ (5 ng/ml) and **(C)** a rat DBS sample spiked with the internal standard ([$^2H_4$]-pioglitazone).

Following a single oral (gavage) administration of pioglitazone, blood concentrations increased rapidly to reach maximum levels at sampling times between 1 and 2 h post-dose. The systemic exposure of pioglitazone was independent of sampling technique with $AUC_{(0\text{-}t)}$ and C_{max} values being similar for both blood spot and 1:1 blood:water (v/v) samples (**Figure 4 & Table 6**). **$AUC_{(0–24\ h)}$** values for dried and liquid blood samples were within 3% and C_{max} values were within 7%.

■ Between-laboratory analysis of PK samples

There was a good correlation ($R^2 = 0.97$) between DBS PK profile data generated at the two laboratories from the same samples (**Figures 5 & 6**). The correlation highlights the stability of pioglitazone in DBS samples as they were stored at room temperature for over 3 months between analyses at the two laboratories. The acceptance criteria for demonstrating that an assay is adequate in terms of incurred sample reproducibility (ISR), where the same samples are analyzed on different occasions with the same assay, is that two thirds of results should be within 20% of each other. As different occasions and laboratories with different methods and analysts for this work we are not demonstrating ISR, however, we demonstrate that the data generated at the two laboratories shows more than 99% of results are within 20% of each other, proving excellent correlation on reanalysis of DBS samples. The correlation is well within that deemed acceptable for ISR despite having far more variables.

Conclusion

A quantitative procedure for the determination of pioglitazone in DBS over the concentration range 5 to 2500 ng/ml is demonstrated. Stability investigations carried out demonstrate that pioglitazone is stable in DBS for up to 4 months when stored at room temperature, enabling blood spot samples to be collected and stored prior to transportation. Blood spot volume does not need to be accurate as long as the

Key Terms

$AUC_{(0-24\ h)}$: Area under the time–concentration profile from t = 0 – 24 h.

Composite profile: Pharmacokinetic or toxicokinetic profile composed of samples at different timepoints in different individuals.

same size core is taken from the spot, thereby allowing flexibility of blood spot preparation at the PK sampling stage allowing a capillary to be used rather than a calibrated pipette.

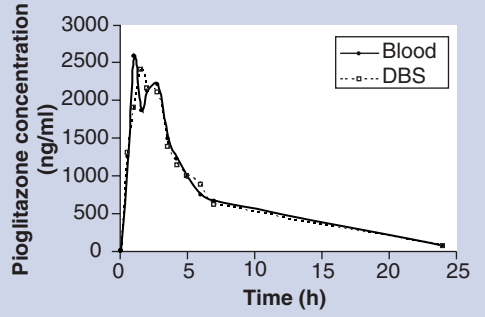

Figure 4. Correlation between pharmacokinetic profile data for DBS and whole blood sampling techniques analyzed at laboratory 1.
n = 6 rats.
DBS: Dried blood spots.

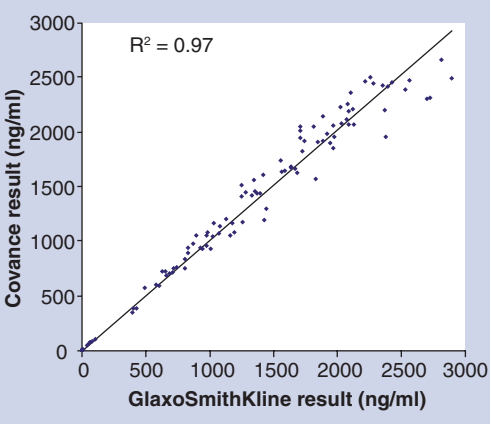

Figure 5. Correlation between pharmacokinetic profile data analyzed at two laboratories (n = 6 rats).

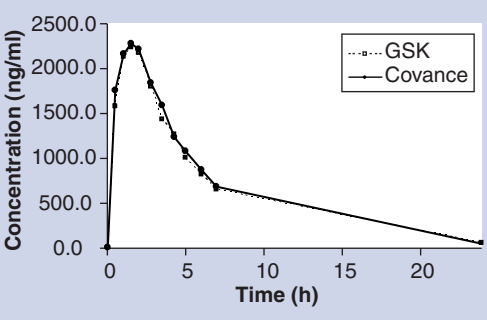

Figure 6. Blood concentration–time profiles following a single, 30 mg/kg oral dose of pioglitazone as determined by dried blood spots methodology in two separate laboratories.

Collection of DBS samples from dosed animals enabled up to 12 samples to be taken from a single rat in a 24-h period, with considerably less distress to the animal as smaller sample volumes are required. The technique was shown to be a viable alternative to the current procedures used for PK/TK sampling from rats. Using DBS, an entire PK/TK profile can be obtained from a single rat, rather than **composite profiles**, which should improve the quality of the data obtained and also reduce the number of rats used in a study.

The data presented indicate a good correlation between DBS PK profile data analyzed at two laboratories, successfully demonstrating the analytical inter-laboratory reliability of the DBS sampling and analysis technique when using different bioanalytical methods on the same study.

Future perspective

Pharmacokinetic data generated gives a good correlation between blood concentration data from the two sample types DBS and liquid blood, demonstrating that DBS analysis is a suitable alternative to the more traditional liquid sample type and that DBS samples provide comparable exposure data to conventional blood samples. Therefore, if required at a later stage in the drug development process (e.g., moving from preclinical to clinical sample analysis and the associated lower concentration levels) switching DBS sampling to whole blood sampling is possible. The good correlation between the DBS PK data generated at the two laboratories indicates that ISR should not cause issues when using the DBS approach.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Table 6. Pharmacokinetic parameters for pioglitazone.

Dose level (mg/kg)	Matrix	Profile type	$AUC_{(0-24\ h)}$ (ng.h/ml)	C_{max} (ng/ml)	t_{max} (h)
30 (Group 1F)	1:1 blood:water (v/v)	Composite	15900	2580	1
30 (Group 1F)	Blood spot	Composite	15500	2410	1.5
30 (Group 2F)	Blood spot	Serial (n = 6)	16200 (±20.2%)	2330 (±9.7%)	1.5

Group 1F sampled by liquid blood and dried blood spots over a 24-h period.
Group 2F sampled by dried blood spots over a 24-h period.

Executive summary

- Methods were determined at two laboratories to determine pioglitazone in dried blood spots (DBS) over the range of 5–2500 ng/ml.
- A method was also determined at one laboratory to determine pioglitazone in 1:1 blood:water (v/v) over the range of 5–2500 ng/ml.
- All methods displayed results within regulatory acceptance criteria.
- Serial profiles were able to be constructed for individual rats using the DBS method.
- Accurate blood sampling was shown to be unnecessary.
- Storage stability of pioglitazone in DBS was shown for at least 4 months.
- The transferability of DBS analysis between laboratories using different analytical instrumentation, personnel and methodology showed excellent correlation with more than 99% of results within 20% of each other.
- Pharmacokinetic data generated by liquid blood and DBS sampling analyses gave excellent correlation.

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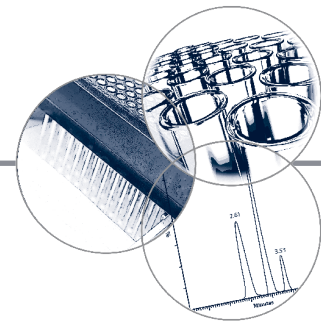

GlaxoSmithKline's experience of incurred sample reanalysis for dried blood spot samples

Dried blood spots are becoming a popular alternative to plasma for many different applications. This has been driven by animal ethics but also by ease of use and cost savings. Recent regulatory guidance now has a requirement for incurred sample reanalysis. This article details three examples of incurred sample reanalysis using dried blood spot samples.

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The use of **dried blood spot** (DBS) samples for the determination of drug concentrations in **pharmacokinetic** (PK) and toxicokinetic (TK) studies is still a new technique compared with conventional plasma analysis and the methodology continues to evolve and develop [1–7]. The industry's experience of incurred sample reanalysis (ISR) for plasma samples is extensive, but ISR for DBS samples is still a much debated subject owing to the infancy of the technique and its relatively limited use across the industry. GlaxoSmithKline (GSK) is rapidly gaining more experience with ISR data for DBS samples and has built up a large dataset from a host of different study designs and across a varied range of compounds from different chemical space. This article sets out to detail some of these experiences, and with this information we continue to build on the confidence that DBS is a valuable technique to quantify drug concentrations in whole blood as long as good practice is observed.

GSK's ISR strategy

Incurred sample reanalysis is not a new validation criteria and has been requested as far back as 1992 following the publication of the Shah paper [8]. This has been even more routine with bioavailability and bioequivalence (BE) studies and for these requests GSK would historically use pooled samples to conduct the analysis. The US FDA became interested in ISR as a result of observing widely different concentrations from original results when PK repeats were carried out. These differences were sometimes noted as being as much as 300%. Many publications have covered the subject of ISR [9–11] and a very recent publication from the European Bioanalytical Forum gives an excellent account and makes sound recommendations [12]. GSK has followed the recommendations from the 2008 AAPS/FDA ISR guidance [13].

GSK's strategy is to perform ISR for all clinical studies and one study in each preclinical species. The acceptance criteria are; more than 66.7% of the incurred sample results must be within the limits of ±20% of the mean of the reanalysis result and its corresponding original result.

It is important to note that the acceptance criteria are only a guide and scientific judgement must be used. A particular example of this is taking note of the bias and, even if the acceptance criteria is met, if a dataset shows a directional bias we would investigate further.

Incurred sample reanalysis is carried out as soon as is practical after the original analysis has been accepted. The time between original analysis and ISR is kept to a minimum because it is ISR that is measured and not **incurred sample stability** (ISS).

Advantages of DBS compared with conventional techniques

The collection of whole blood samples as DBS for TK and PK studies offers a number of advantages over conventional plasma sampling. The small blood volumes required for DBS samples (between 30 and 80 µl, compared with >0.5 ml blood as is usually obtained for conventional plasma analyses) makes this a particularly suitable approach for the collection of blood samples from preclinical animals and pediatric studies. For nonclinical studies, the reduction in sampled blood volume leads to a significant benefit in the reduction, refinement and replacement (3Rs) for animal use in drug development. Animals need no prewarming, PK samples can be taken directly from the safety animals meaning a reduction in animal use, a direct correlation with safety and PK as well as cost savings. In addition, the smaller sample volume reduces the need

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Key Terms

Dried blood spot samples: A technique for the collection, transport and storage of blood on cellulose-based 'filter' papers.

Pharmacokinetics: Study of the drug dose–circulating concentration relationship within the body over a period of time.

Incurred sample stability: The performance of confirmatory reanalysis of incurred samples to demonstrate that the assay is reproducible.

HPLC–MS/MS: Analytical methodology routinely used for the detection and quantification of circulating drug concentrations.

Quantification: Measuring the quantity of a compound.

for composite sampling, meaning better data quality. For pediatric studies, DBS offers the advantage of less invasive sampling (finger or heel prick, rather than conventional venous cannula), which enables recruitment of subjects for clinical studies. Furthermore, the simpler matrix preparation and transfer (no refrigerated centrifugation to produce plasma) and easy storage and shipment to analytical laboratories (no requirement for freezers and dry ice) offer further benefits, primarily for late-stage clinical studies.

As with all techniques still in their relevant infancies, there are and will always be disadvantages and issues to evaluate (e.g., hematocrit), but to date the advantages continue to push the DBS technique forward.

Methodology

One of the many advantages of DBS lies in its simplicity. There are typically two ways to collect a sample and both processes work in the preclinical and clinical arena. The first involves collecting blood into a blood tube, mixing and then spotting using a pipette. The second method involves collecting the blood sample into an EDTA-coated capillary and then using the capillary to spot directly onto the card. Typically three 15 µl samples of whole blood are spotted onto the card, but this volume is dictated from the assay validation. A variety of cards are available. The examples used in this paper utilized either Whatman (now part of GE Healthcare) FTA or FTA elute (now called FTA DMPK A and B, respectively), which were both designed for nucleic acid analysis, or Alstom 226, an untreated card. The blood spot is allowed to dry at ambient temperature (10–30°C) away from direct sunlight and in controlled laboratory conditions for at least 2 h and then typically a 3 mm diameter disk is punched from the center of the DBS into a clean tube. The punched spot is then extracted by the addition of internal standard working solution in methanol (e.g., 100 µl), followed by vortex mixing for approximately 20 s. The tube is centrifuged for 1 min at 3000 × g, and the supernatant transferred to a clean tube for analysis by means of **HPLC–MS/MS**. Further detail of the entire process can be found in [6–7].

50:50 blood:water samples are processed by taking an aliquot (25 µl), and extracted by the addition of internal standard working solution in acetonitrile (e.g. 75 µl), followed by vortex mixing for approximately 20 s. The tube is centrifuged for 1 min at 3000 × g, and the supernatant transferred to a clean tube for analysis by means of HPLC–MS/MS.

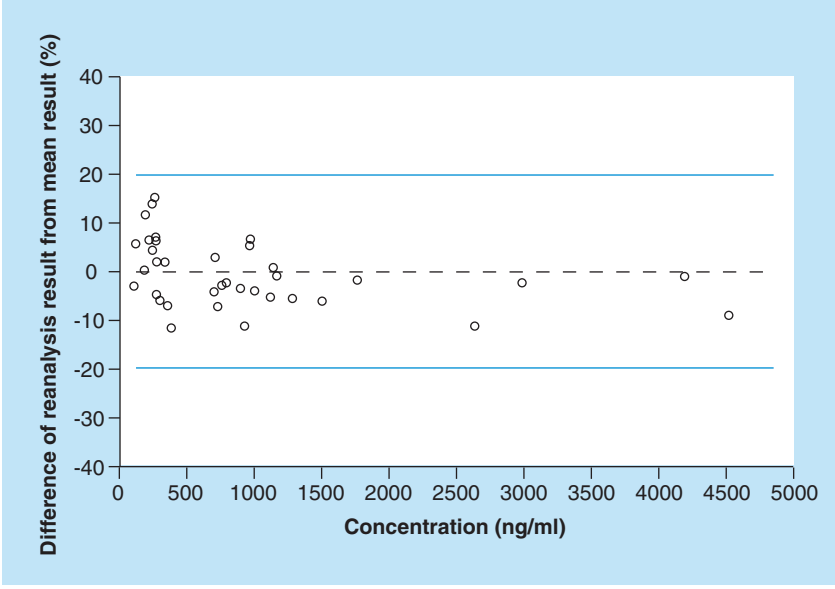

Figure 1. Percentage difference of reanalysis result versus mean result for a study utilizing dried blood spots derived from EDTA-coated capillaries.

Fundamental differences between DBS ISR & conventional liquid matrix ISR

There are many differences when dealing with any liquid sample and a DBS sample, and as a result the different sample types have different issues and areas of concern.

- For DBS there are two ways to collect a sample, which could lead to differences, whereas with a liquid samples there is only one;
- For liquid samples, if ISR is not carried out on the same day as the original sample analysis a further freeze–thaw is required. As DBS transportation and storage is at ambient temperature freeze–thaw is never an issue;
- A liquid sample, if treated correctly, will always be a single homogenous sample, whereas a DBS sample is normally three discrete samples (spots). It is possible to take one larger spot, but this can make the spotting process harder and could lead to homogeneity issues;
- The sample volume accuracy for a liquid sample is dependent on a pipette, a punch dictates the volume accuracy for a DBS sample;

- Contamination of the DBS card is more likely than a liquid sample as a lid is used to protect the liquid sample but a card is only sealed from the atmosphere after a 2 h drying period;

- With rodent studies, and especially juveniles or transgenic animals, the liquid plasma sample volume is not sufficient for repeat analysis, as the analytical method typically requires 25–100 µl of plasma. This can sometimes lead to only sufficient sample for a single analysis. For DBS, three samples are always supplied due to the limited amount of matrix required (15 µl for each spot).

The following three examples attempt to answer some of the concerns between liquid and DBS samples.

Difference between sampling techniques

A concern with using capillary sampling is that for each time point, three individual capillaries are used and each sample is separated by a small amount of time. As every spot is only sampled once, it is necessary to take three samples to allow for any analytical or PK repeats and ISR experiments. Some would argue that this could have an effect and each sample could give a different result. The alternative approach is to sample into a sampling tube, mix and then spot using a pipette. The second approach should be more accurate but leads to some wastage of matrix since excess is needed when pipetting. During validation, variable spot size is investigated, when sampling 15 µl it is investigated whether a 10 and 20 µl spot gives the same result within ±15% so variance in volume should not be an issue [5,6]. ISR would immediately show if in fact there is a difference between spots. Looking at recent data generated and comparing studies using both sample collection methods there is actually no difference in the quality of ISR. The examples given (FIGURES 1 & 2) show a dataset for the capillary collection and a dataset for the tube collection. Both methods show excellent comparison with extremely tight data and no results outside the 20% cut off.

Effect of card contamination highlighted by ISR

Initially this example looked like the assay was unreliable, but further investigation actually proved gross contamination at the sample collection stage. During analysis some of the cards were noted to have traces of fur on them. In addition, when reviewing the data, many of the controls had quantifiable levels well above the lower limit of **quantification**. ISR analysis failed (FIGURE 3) with a wide spread of data, and differences observed being greater that 150%. On investigation and because of controls and physical contamination seen on the cards, samples were taken from unspotted regions of the cards to see if actual compound

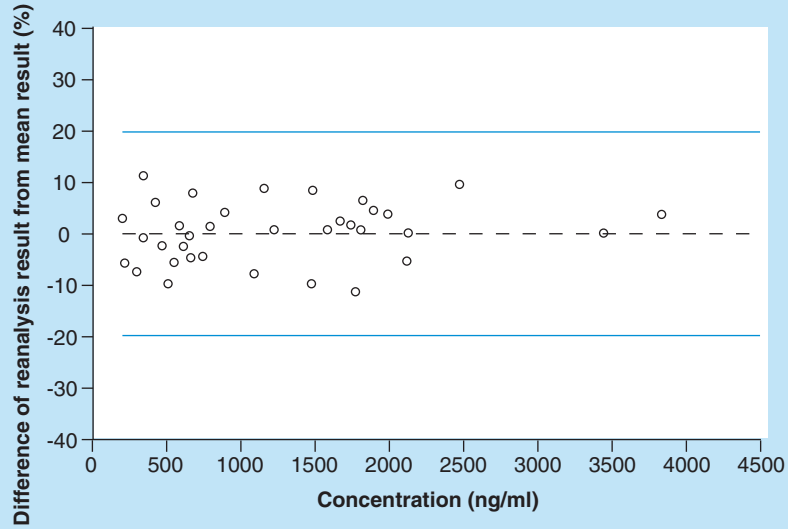

Figure 2. Percentage difference of reanalysis result versus mean result for a study utilizing dried blood spots derived from tubes.

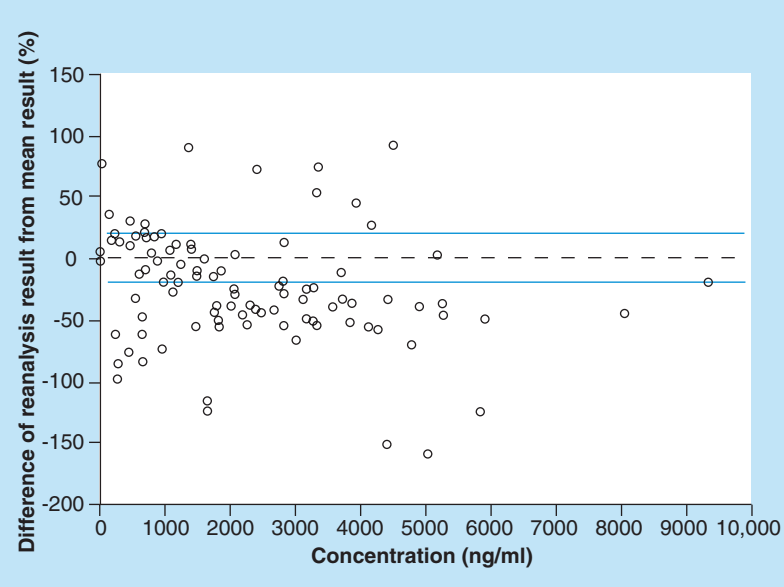

Figure 3. Percentage difference of reanalysis result versus mean result for a study with contamination of the dried blood spot cards.

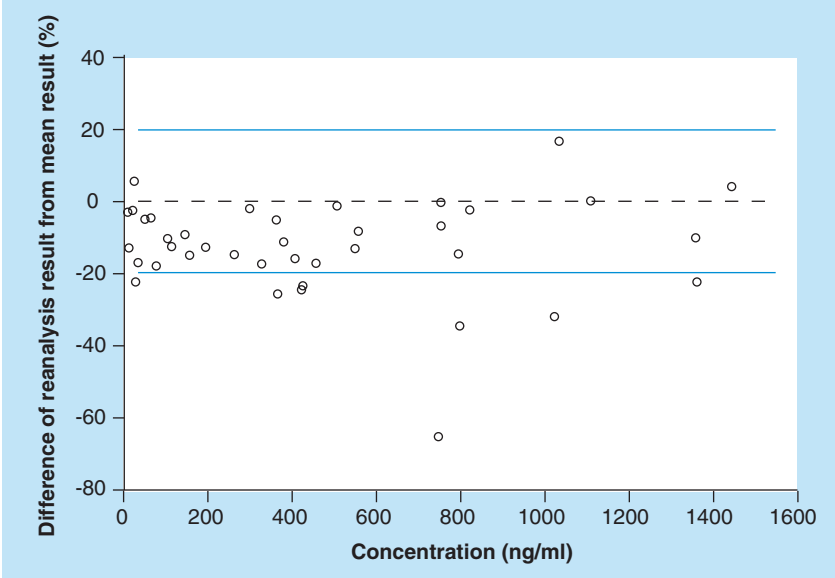

Figure 4. Percentage difference of reanalysis result versus mean result for a study with sampling, spotting and drying conducted in separate areas.

had contaminated the whole of the card. This confirmed that many of the cards had been contaminated, which resulted in a portion of the study being repeated.

For the new study extra precautions when sampling animals and spotting were put into place. These included using three different laboratory areas to sample, spot and dry. Different people were used to sample and spot. All these extra precautions led to a successful study, with clean controls and acceptable ISR data (Figure 4).

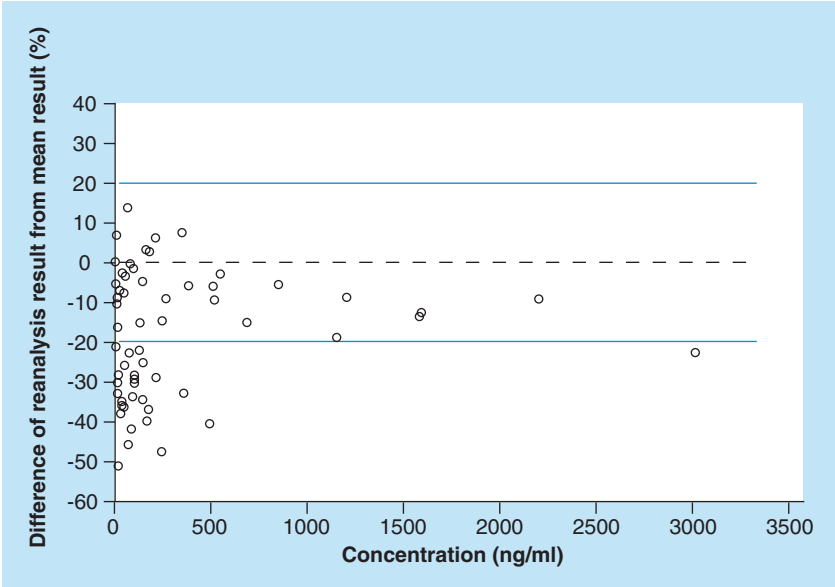

Figure 5. Percentage difference of reanalysis result versus mean result for a 50:50 blood:water study showing a large negative bias.

Differences seen between liquid ISR & DBS ISR

Before DBS had become fully accepted as a routine means of collecting, shipping and storing of whole blood samples, to gain experience and build confidence both DBS and 50:50 blood:water samples were routinely taken for each time point. The liquid samples were then used to validate the DBS methodology. ISR in these cases was conducted on both types of sample and in one case the 50:50 blood: water sample actually showed a large negative bias, with more than 40% of the results differing by more than 20%, (Figure 5), which was not observed with the DBS samples (Figure 6). Due to recent publications [14–16] showing greater stability of DBS samples compared with liquid samples, the immediate investigation was to access the stability of 50:50 blood:water. Instability proved not to be an issue and 100 days storage at -20°C was obtained for both DBS and 50:50 blood:water. Other investigations included looking at different sources of blood and also the types of tubes used, but these proved to be nonconclusive. Further work to solve this issue is still ongoing.

Conclusion

GlaxoSmithKline have carried out 42 DBS ISR investigations to date, two of which have failed. One was due to contamination, but resolved when extra precautions were employed, and the second is currently under investigation. From the data obtained, no observed differences between collection types have been seen and this is compelling evidence to conclude that both collection by tube or directly into capillaries are acceptable and valid for obtaining DBS samples.

Unfortunately, no conclusions can be made as to why in one instance for the same method DBS ISR passed whereas ISR for 50:50 blood:water failed. It can only be concluded that this was not due to stability, tubes or a matrix effect. When an issue occurs with an ISR study and the investigation is nonconclusive, it places in question which data to believe and this is true for any ISR study independent of matrix.

Any assay, be it liquid or on card, is only as good as the sample provided and all possible precautions should be included with every study design. ISR not only tests the rigidity of the assay, but also that of the samples and acts as a further tool to enhance and probe at the quality of both TK and PK analysis.

Future perspective

Application of DBS in the industry is still in its infancy, but is rapidly growing in favor and its use is becoming more widespread throughout the pharmaceutical industry. The vast majority of benefits associated with DBS are easily obtainable and are not just limited to DBS, but can be applied to any liquid sample that can be collected on a paper format. The future of dry matrix samples is dependent on solving issues such as the effect of hematocrit for DBS and, also, building confidence throughout the industry with good quality data. The use of direct elution tools within the bioanalysis laboratory will also help to embed the technique, simplifying the process for the analyst. ISR is now an expected part of the regulated bioanalysis package and as such its future is a forgone conclusion and should always be a necessary requirement.

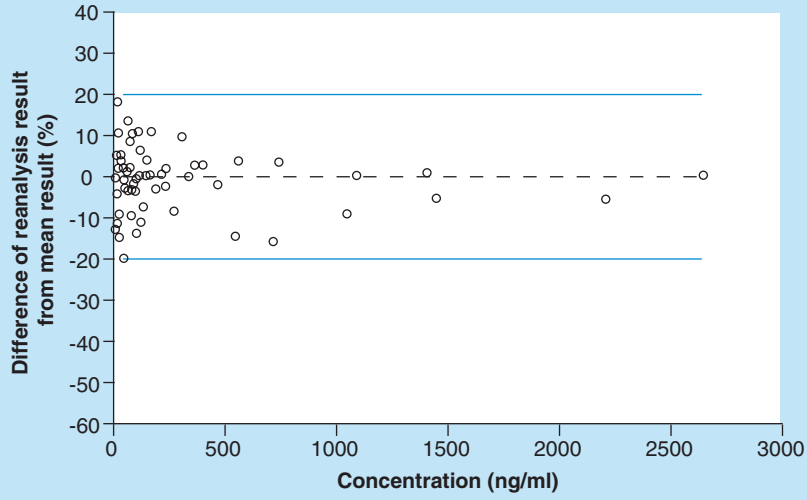

Figure 6. Percentage difference of reanalysis result versus mean result for a dried blood spot study where for the same study 50:50 blood:water showed a large negative bias.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Executive summary

- 2008 AAPS/US FDA incurred sample reanalysis (ISR) guidance set out the industry standard for ISR acceptance and study conduct, this is the approach GlaxoSmithKline has adopted.
- Advantages of dried blood spots (DBS) include: ethical advantages due to smaller sample volumes (refinement and reduction); improved quality of data; simplified sample collection, which aids pediatric studies; and, in late-stage studies, reduced costs.
- DBS pros and cons
  - Two different ways of collecting DBS samples; freeze–thaw is never an issue with DBS; accuracy is dependent on a punch; contamination is more likely if good practice is not followed.
- Liquid samples pros and cons
  - When mixed, liquid samples are always homogenous; accuracy is dependent on a pipette; larger sample volumes can lead to a lack of sample for analysis.
- No difference (utilizing ISR data) can be observed between sampling techniques.
- Contamination is more of an issue with DBS, but by following simple rules, contamination issues can be rectified.
- ISR not only tests the rigidity of the assay but also that of the samples and acts as a further tool to enhance and probe the quality of both toxicokinetic and pharmacokinetic analysis.

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Anal. Chem. **2011,** *83,* 118–124

Use of Dried Plasma Spots in the Determination of Pharmacokinetics in Clinical Studies: Validation of a Quantitative Bioanalytical Method

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A novel approach has been developed for the quantitative determination of circulating drug concentrations in clinical studies using dried plasma spots (DPS) on paper substrates, rather than conventional plasma samples. A quantitative bioanalytical high-pressure liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) assay has been validated using paroxetine as a tool compound (range 0.2−200 ng/mL human plasma). The assay employed simple solvent extraction of a punched disk taken from the DPS sample, followed by reversed phase HPLC separation, combined with multiple reaction monitoring mass spectrometric detection. In addition to performing routine experiments to establish the validity of the assay to internationally accepted criteria (precision, accuracy, linearity, sensitivity, selectivity), experiments are included to assess the effect of the volume of plasma spotted and the use of an indicating paper. The validated DPS approach was successfully applied to a clinical study utilizing pooled samples and a direct comparison of DPS and plasma was made (single oral dose of 37.5 mg of paroxetine).

Conventionally, plasma has been used for the determination of exposures and pharmacokinetics (PK) of new chemical entity drugs in clinical trials. The collection of plasma samples on paper, known as dried plasma spots (DPS), is an established technique for viral load determination, e.g., HIV-1v.[1−3] More recently, the use of dried blood spot (DBS) samples for toxicokinetic (TK) and PK analysis is rapidly becoming the method of choice, as it offers significant advantages over conventional plasma analysis.[4−7] However, many compounds in development or marketed already utilize plasma. Unfortunately, blood and plasma data cannot be readily interconverted without prior detailed knowledge of distribution in these two fluids. Demonstrating that dried plasma spots (DPS) work for the determination of PK parameters would give many of the benefits of DBS sampling for quantitative bioanalysis.

The collection of whole plasma samples as DPS for TK and PK studies offers a number of advantages over conventional plasma sampling. Considerable cost savings in late phase studies are due to easy storage and shipment to analytical laboratories (no requirement for freezers and dry ice). In addition, these requirements lead to notable environmental benefits. The transport and storage of samples is further simplified by the antimicrobial properties of the DPS sample which is also a benefit of DBS,[8] removing the requirements for special biohazard arrangements. The ability to utilize direct analysis tools such as a thin layer chromatography MS interface (CAMAG),[9−12] direct elution,[13,14] desorption electrospray ionization (DESI),[15−18] and direct analysis in real time (DART)[19,20] is also presented, in turn generating an increased market for these tools, hence encouraging manufacturers to develop new interfaces. These advantages benefit all areas of drug discovery through development. For these reasons, GlaxoSmithKline (GSK) is currently investigating DPS sampling as a technique for the evaluation of TK and PK for late stage compounds currently utilizing plasma methods.

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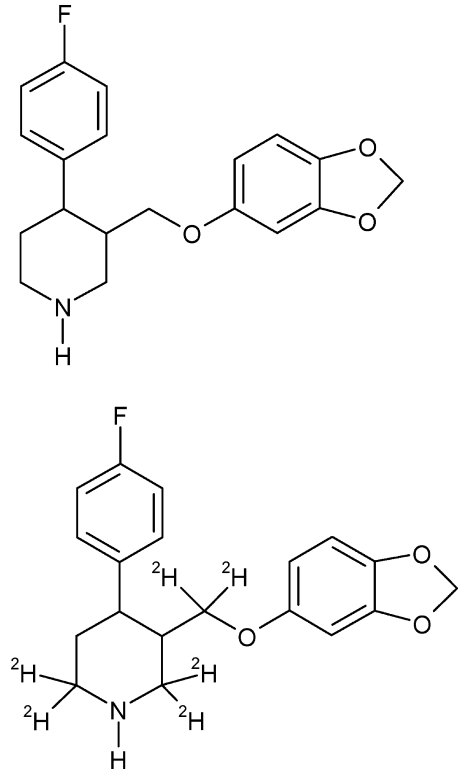
Scheme 1. Chemical Structures of Paroxetine and [$^2H_6$]-Paroxetine



Because of the color of blood, DBS samples are easily distinguishable on paper. However plasma is not so highly colored, which could lead to problems in distinguishing the DPS on paper. A developmental grade of untreated FTA DMPK paper containing a colored indicator has been evaluated to counter this concern.

This manuscript describes the development and validation of a method for the quantification of drugs in human plasma, as a DPS sample, using paroxetine which historically has been quantified by means of conventional plasma analysis.[21] The results of these tests were evaluated against internationally used acceptance criteria.[22,23] Further to this, samples from an ethically approved clinical trial were prepared using the DPS and a validated plasma method and the PK parameters were compared. Details of the validated plasma method are also presented.

Chemicals and Reagents. Methanol and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd. (Loughborough, U.K.). All other chemicals were of AnalaR grade, supplied by VWR International Limited (Poole, U.K.). Control human blood was supplied by GSK volunteers in accordance with current GSK policies on informed consent and ethical approval. Paroxetine (Scheme 1) was obtained from Aldrich (Poole, U.K.). [$^2H_6$]-paroxetine (Scheme 1) was produced by Isotope Chemistry, GSK.

Equipment. Ahlstrom grade 226 paper for blood spots was supplied by ID Biological Systems (Abbots Langley, U.K.) and the developmental grade of untreated indicating FTA DMPK (indicating FTA DMPK) paper was supplied by GE Healthcare (formally Whatman) (Kent, U.K.). Sample tubes were obtained from Micronics (Platinastraat, The Netherlands). The centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). The Harris punch and cutting mat was supplied by GE Healthcare (formally Whatman) (Sanford). Plastic bags for the storage of blood spot cards were supplied by Fisher Scientific Ltd. (Loughborough, U.K.). Sachets of desiccant were obtained from Sud-Chemie (Northwich, U.K.). The repeater multipipet used for spotting blood and plasma was obtained from Eppendorf (Cambridge, U.K.).

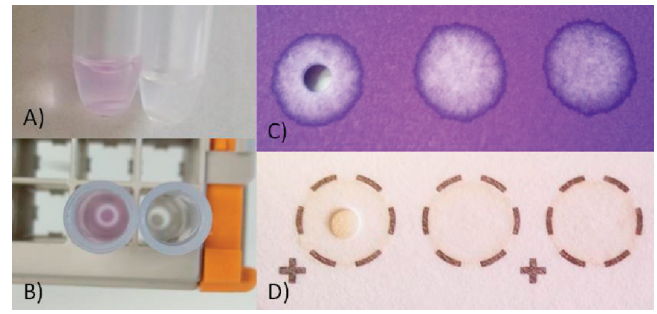


Figure 1. Illustrations of (A) a side on and (B) a top down view of two extracted DPS samples, indicating FTA DMPK paper on the left and 226 paper on the right using 100 μL of 70:30 methanol−water (v/v); (C) three 20 μL dried plasma spots on indicating FTA; and (D) three 20 μL dried plasma spots on 226 paper.

Preparation of Standard Stock and Working Solutions. Primary stock solutions of paroxetine (in duplicate) and [$^2H_6$]-paroxetine (internal standard) were prepared in dimethylformamide (DMF, 1 mg/mL). Working standard solutions of paroxetine in H_2O−acetonitrile (50:50, v/v) were prepared from the primary stocks at concentrations of 10 000, 1 000, 100, and 10 ng/mL. Internal standard working solutions (2.0 ng/mL) were prepared from the primary stock using 70:30, methanol−water, (v/v) for plasma spot analysis and acetonitrile for plasma analysis. All the solutions were stored at 4 °C and brought to room temperature before use.

Preparation of Calibration Standards and Quality Control (QC) Samples. Calibration standards were prepared fresh on the day of analysis by diluting the appropriate working solutions with blank whole human plasma. The concentrations of calibrants were 0.2, 0.4, 0.8, 2, 8, 20, 160, and 200 ng/mL whole plasma.

QC samples for all analyses were prepared from a separate stock solution to that used for the calibration standards. QC samples were prepared by diluting the appropriate working solutions with blank whole human plasma to give concentrations of 0.2, 0.8, 10, 160, and 200 ng/mL whole plasma. For the analysis of study samples, QCs were prepared at concentrations of 0.8, 10, and 160 ng/mL whole plasma. The volume of organic solution added to each calibration standard and QC was less than or equal to 5% of the total volume.

Plasma Spotting. Aliquots (20 μL) of calibration standards and QC samples were spotted onto 226 and indicating FTA DMPK paper with a repeater pipet and allowed to dry at room temperature for at least 2 h prior to analysis (see Figure 1). When required, the QCs were stored at room temperature in a sealed plastic bag containing dry desiccant until analysis.

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DPS Sample Processing. A 6 mm diameter disk was punched from the center of the DPS into a clean tube. This was then extracted by the addition of 100 μL internal standard working solution, followed by vortex mixing for approximately 20 s. The tube was centrifuged for 1 min at 3000*g*, and the supernatant was transferred to a clean tube for analysis by high-pressure liquid chromatography−tandem mass spectrometry (HPLC−MS/MS).

Plasma Sample Processing. 50 μL of sample was added to a clean tube. This was extracted with 150 μL of acetonitrile containing 2.0 ng/mL internal standard, followed by vortex mixing for 20 s. The tube was centrifuged for 10 min at 3000*g*, and the supernatant was transferred to a clean tube for analysis by HPLC−MS/MS.

HPLC−MS/MS Analysis. The HPLC system consisted of an Acquity (pump, autosampler, and column oven) (Waters, Watford, U.K.), and a Phenomenex Synergi, 4 μm, 50 mm × 3.0 mm i.d. HPLC column (Phenomenex, Macclesfield, U.K.). The postcolumn flow was diverted to waste for the first 0.6 min of each chromatographic run. During this time, flow (0.25 mL/min, 50:50, methanol−water, (v/v)) was provided to the MS by a Knauer pump (Presearch, Hitchin, U.K.).

The chromatographic separation was achieved using an isocratic system at a composition of 61:39 A−B employing the mobile phases water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.5% (v/v) formic acid (B). The flow rate was 0.8 mL/min, the column was maintained at 60 °C, and 5 μL of extract was injected.

MS detection was by a Sciex API-5000 (Applied Biosystems/MDS Sciex, Canada) equipped with a TurboIonspray ion source. The source temperature was 700 °C. The gas 1 and gas 2 settings were 60 and 60 psi, respectively (both N_2). The curtain gas and collision gas settings were 25 and 4, respectively (both N_2). The characteristic precursor $[M + H]^+$ to product ions transitions, m/z 330 to 192 and 336 to 198 were consistent with the structures of paroxetine and the internal standard, respectively, and were used as selected reaction monitoring transitions to ensure high selectivity. A dwell time of 100 ms was used for both transitions. The pause time was 5 ms.

HPLC−MS/MS data were acquired and processed (integrated) using Analyst software (v1.4.2 Applied Biosystems/MDS Sciex, Canada). Concentrations were determined from the peak area ratios of analyte to internal standard using the in-house laboratory information management system, SMS2000 (v2.2, GSK, U.K.). PK data interpretation was achieved using WinNonLin (Enterprise v4.1, Pharsight).

Validation Procedures. *Selectivity and Matrix Suppression Effects.* Total blank and blank DPS and plasma samples were derived from respective 20 and 50 μL aliquots of control plasma from 6 individual subjects and from matrix pools. Total blank samples (n = 1 for each subject or pool) were processed as outlined above, in the absence of internal standard.

To assess the suppression of HPLC−MS/MS detector response due to matrix components and/or paper substrate components, six separate sources of blank matrix 226 paper and plasma samples were extracted and spiked with paroxetine at 0.8, 10, and 160 ng/mL. These extracts were compared to those of the same concentration spiked directly into 70:30, methanol−water (v/v) for DPS or acetonitrile for plasma.

A postcolumn infusion was utilized to assess the difference in suppression effects caused by injecting three blank extracted samples from 226 paper, indicating FTA DMPK paper, and plasma. This was investigated by infusing paroxetine at a concentration of 50 ng/mL and a flow of 0.1 mL/min into the LC flow after the HPLC column[24] and injecting the three extracted blanks.

Accuracy and Precision. The intra- and inter-assay accuracy and precision of the method were determined by assaying six replicates of each of the five concentrations of validation QC samples on three separate occasions using 226 paper and plasma and on one occasion using indicating FTA DMPK paper. Concentrations were determined from the appropriate calibration plot within each analytical run. Accuracy was assessed for each concentration by calculating the percent deviation from the theoretical (nominal) concentration. The precision was determined for each concentration by calculating the percent coefficient of variation (% CV; relative standard deviation) for each set of replicates.

Stability in Stock Solutions. A stock solution of 1 mg/mL stored for 183 days at 4 °C was compared to a freshly prepared stock. From each stock, replicate dilutions (n = 6) in acetonitrile at a concentration of 200 ng/mL were prepared prior to comparing the HPLC−MS/MS peak area ratios (analyte to internal standard) of the samples.

Stability in Dried Plasma Spot on 226 Paper. Replicate (n = 6) 15 μL human plasma samples at 0.8 and 160 ng/mL were spotted onto 226 paper and stored desiccated at room temperature for 35 days. The measured concentrations were compared to those of the same samples extracted and analyzed immediately after initial spotting and drying.

Freeze/Thaw Stability in Plasma. The stability of paroxetine in spiked human plasma samples after three freeze−thaw cycles from −20 °C to room temperature was assessed at 0.8 and 160 ng/mL (in replicates of 6) by comparing the mean concentrations against those of the freshly prepared spiked samples.

Stability in Human Plasma. The stability of paroxetine in spiked human plasma stored at room temperature was assessed at 0.8 and 160 ng/mL (in replicates of 6) by comparing the mean concentrations of samples extracted after storage for 24 h against those of the samples extracted immediately upon spiking.

Stability in Processed Samples. Extracts from a validation run on 226 paper and in plasma were reinjected after storage at room temperature for 24 and 72 h, respectively, against freshly prepared calibration standard extracts.

Dilution of Samples above the Higher Limit of Quantification (HLQ). Control human plasma was spiked at 400 ng/mL (2 × HLQ), and six replicate 20 μL aliquots were spotted onto 226 paper. After drying at room temperature for 2 h, a 6 mm diameter punch was taken from each spiked DPS sample. Each punch was individually extracted with 100 μL of 70:30 methanol−water, (v/v) containing an internal standard. Diluent was prepared by individually extracting 10 × 6 mm punches taken from replicate spots of blank human DPSs on 226 paper with 100 μL aliquots of 70:30 methanol−water (v/v) containing internal standard. These blank diluent extracts were then pooled. A 10 μL aliquot of each individual spiked sample extract was then diluted with 90 μL of

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Table 1. Linear Regression Equations for the Three Validation Runs on 226 Paper and in Whole Plasma and the Single Validation Run on Indicating FTA DMPK Paper[a]

	linear regression equation		
	226 paper	whole plasma	indicating FTA DMPK paper
validation run 1	$y = 0.0127x + 0.000\,373$, $r = 0.9962$	$y = 0.0375x + 0.000\,402$, $r = 0.9987$	$y = 0.0135x + 0.000\,815$, $r = 0.9975$
validation run 2	$y = 0.0128x + 0.000\,689$, $r = 0.9945$	$y = 0.0376x + 0.000\,597$, $r = 0.9989$	
validation run 3	$y = 0.0125x + 0.000\,245$, $r = 0.9951$	$y = 0.0381x + 0.000\,817$, $r = 0.9994$	

[a] y represents the peak area ratio of paroxetine to that of IS, and x represents the concentration of paroxetine in nanograms/millilter.

pooled blank extract diluent. Concentrations of paroxetine in these individual extract dilution samples were determined by LC−MS/MS and corrected for the dilution factor. The ability to dilute plasma samples at concentrations above the HLQ was demonstrated by performing 6 replicate 10-fold dilutions of human plasma samples spiked at 200 ng/mL.

Volume of Plasma Spotted. Replicate ($n = 6$) 15, 20, and 25 μL aliquots of control human plasma at 0.8 and 160 ng/mL were spotted onto 226 substrate. After drying, a 6 mm punch was taken from the center of each sample for extraction and analysis. Concentrations of paroxetine were determined from a calibration line consisting of standards derived from 20 μL of DPS.

Recovery. Replicate ($n = 6$) 4 μL human plasma samples at 0.8, 10, and 160 ng/mL were spotted on 226 paper and the whole spot punched using a 6 mm punch, while replicate ($n = 6$) 50 μL aliquots were spiked at the same concentrations for whole plasma extraction. Samples were extracted according to their respective methods and the measured concentrations were compared against matrix blank samples spiked after extraction with analyte at the same concentrations.

Application of the DPS Assay to the Analysis of Samples from a Clinical Study. Plasma samples were obtained from an open-label, randomized, single dose, two-period crossover study which was ethically approved. Paroxetine was administered to the volunteers as a single 37.5 mg oral dose, and venous blood samples were collected at 0, 2, 4, 6, 8, 10, 12, 15, 18, 24, 32, 48, 72, 120, and 168 h and transferred to tubes containing 2.4 μg/mL EDTA. Samples were centrifuged at 1500*g* for 10 min under chilled conditions (approximately +4 °C), and the resultant plasma was transferred into appropriately labeled plastic 1.4 mL matrix tubes and stored frozen. Samples were transferred on dry ice to the analytical site for analysis. Because of the nature of this study, a pooled profile was constructed, consisting of five pooled samples from individual subjects per time point. For DPS, 20 μL of plasma was spotted onto 226 paper with a repeater pipet and allowed to dry at room temperature for at least 2 h prior to analysis; this process was carried out at the analysis site. Both DPS and plasma samples were extracted as per their respective validated methods and analyzed with their respective calibrants and QCs on the same day.

RESULTS AND DISCUSSION

Method Validation. *Linearity, Selectivity, and Matrix Suppression Effects for 226 Paper.* Calibration standards were prepared in duplicate for each analytical run for each type of paper substrate and whole plasma. Calibration plots of the analyte peak area ratio to that of the internal standard versus the nominal concentration in plasma were constructed.

Calibration plots of analyte/internal standard peak area ratio versus the nominal concentration of paroxetine in plasma were constructed and a weighted $1/x^2$ linear regression applied to the data. Linear responses were observed for DPS and whole plasma over the range 0.2−200 ng/mL human plasma. Regression equations for the three validation runs using 226 paper and whole plasma as well as the single validation run using indicating FTA DMPK paper are presented in Table 1.

The selectivity of the method was established by the analysis of blank and double blank 226 paper and whole plasma samples from both six individual subjects and from pooled samples. No unacceptable interferences at the retention times of paroxetine or its internal standard were observed in any of these samples. An unacceptable interference would constitute a peak with an area of >20% of that observed for the lower limit of quantification (LLQ). A representative mass chromatogram of a control 226 paper blank sample is shown in Figure 2. Blank chromatograms from indicating FTA DMPK paper and plasma samples were comparable.

Postcolumn infusions of paroxetine at a concentration of 50 ng/mL and a flow of 0.1 mL/min into the LC flow after the HPLC column[24] while injecting extracted blank samples showed little difference between 226 paper, indicating FTA DMPK paper, and plasma. No unacceptable interferences were seen at the retention time of paroxetine or its internal standard. The data is shown in Figure 3.

Matrix effects from using the stated DPS extraction method on 226 paper when monitoring for the paroxetine MRM transition at concentrations of 0.8, 10, and 160 ng/mL contributed between 37.2% and 41.7% suppression. Matrix effects in plasma were monitored at the same concentrations with suppression values between 2.8% and 24.6%. Precision values calculated for each concentration in DPS or plasma did not exceed 15% and therefore met predefined acceptance criteria.

Accuracy, Precision, and Sensitivity. The intra- and inter-run performance data are summarized in Tables 2−4. All values obtained were within internationally recognized acceptance criteria for assay validations[22,23] and within the predefined 15% limits required.

The LLQ for the analysis of whole plasma and DPS extracts on 226 and indicating paper was 0.2 ng/mL human plasma. This was defined as the lowest concentration that gave accuracy and precision values within the predefined limits of 15%. A representative mass chromatogram of an LLQ sample on 226 paper is shown in Figure 2. The retention time for indicating FTA DMPK paper samples was identical with comparable sensitivity. The extracted plasma samples eluted 3 s earlier than the DPS samples. The reason for this difference in elution time was due to the higher organic content of the plasma samples, consisting of 100%

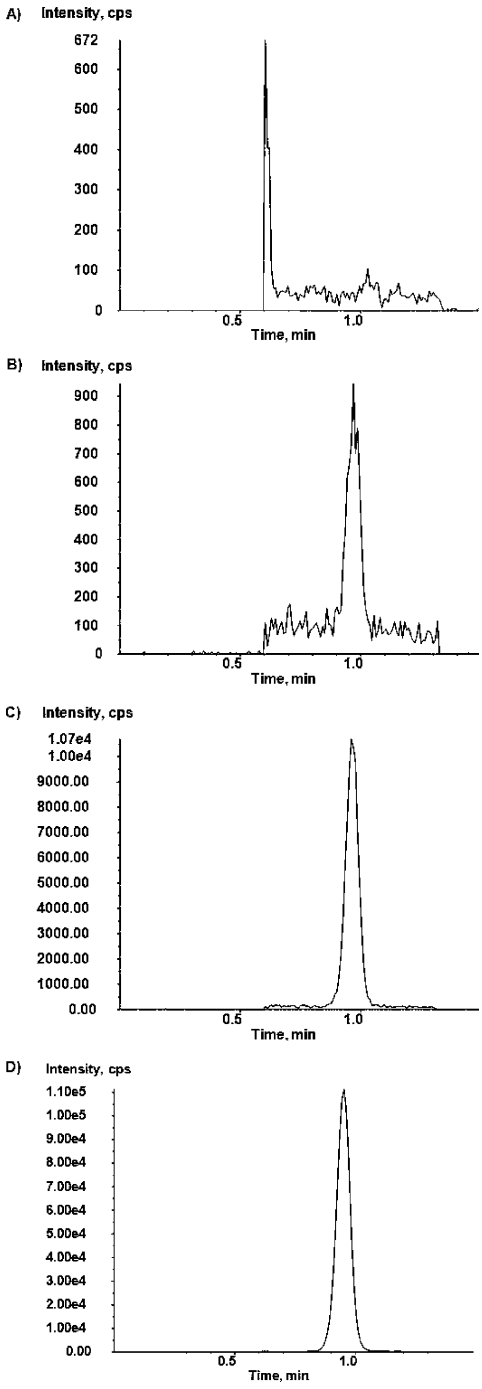

Figure 2. Representative HPLC−MS/MS multiple reaction monitoring (MRM) chromatograms on 226 paper of (A) a blank human DPS sample, (B) a human DPS sample spiked with paroxetine at the LLQ (0.2 ng/mL human plasma), (C) a human DPS sample obtained 12 h following a single oral administration of 37.5 mg of paroxetine to a volunteer (corresponding to the maximum observed peak concentration (C_{max})), and (D) the internal standard ([$^2H_6$]-paroxetine).

acetonitrile, whereas the DPS samples consisted of 70:30, methanol−water, (v/v).

Characteristics of Plasma Spots on Paper Substrates. Plasma samples when spotted onto 226 paper are initially visible when wet; however, when the card dries the definition of the spot becomes less clear, and this could cause a potential issue with study samples because if a sample is incorrectly spotted, either in the wrong position or the wrong volume is used, the analyst

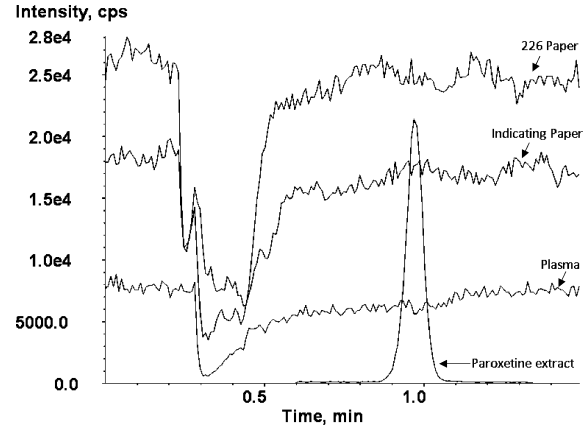

Figure 3. HPLC−MS/MS chromatograms of blank samples extracted from 226 paper, indicating paper, and plasma while running a postcolumn of 50 ng/mL paroxetine at 0.1 mL/min. HPLC−MS/MS chromatogram of an extracted plasma QC at 160 ng/mL overlaid to provide retention time reference, in order to assess possible ion suppression or enhancement.

Table 2. Intra- and Inter-Assay Performance Data for Paroxetine in Human DPS Samples on 226 Paper (n = 6 for Each Concentration Level in Each Individual Run)

	nominal concentration (ng/mL)	0.2	0.8	10	160	200
	Individual (Intra-Run) Statistics					
run 1	mean concentration (ng/ml)	0.202	0.826	9.57	170	217
	SD	0.030	0.066	0.77	9.2	4.3
	precision (% CV)	14.6	8.0	8.0	5.4	2.0
	accuracy (% bias)	0.8	3.3	−4.3	5.9	8.3
run 2	mean concentration (ng/ml)	0.226	0.848	9.41	169	212
	SD	0.023	0.070	0.77	11	5.4
	precision (% CV)	10.1	8.2	8.2	6.3	2.6
	accuracy (% bias)	13.1	6.0	−5.9	5.4	5.9
run 3	mean concentration (ng/ml)	0.226	0.748	9.61	164	210
	SD	0.031	0.081	0.54	14	6.2
	precision (% CV)	13.7	10.9	5.6	8.7	2.9
	accuracy (% bias)	13.0	−6.5	−3.9	2.3	4.7
	Overall (Inter-Run) Statistics					
	mean concentration (ng/mL)	0.218	0.807	9.53	167	213
	SD	0.029	0.081	0.67	11	5.9
	overall precision (% CV)	13.2	10.1	7.0	6.7	2.8
	average accuracy (% bias)	9.0	0.9	−4.7	4.5	6.3
	average intrarun precision (%)	12.9	9.0	7.4	6.9	2.5
	inter-run precision (%)	3.8	5.4	<0.1	<0.1	1.4

may not identify the issue from visual inspection and proceed to punch an area of the paper that may not be completely saturated with plasma. This issue could be negated through the use of indicating FTA DMPK paper, which initially is purple in color; however, when plasma is spotted it turns a lighter shade making the sample easily distinguishable when dry. The viscosity of plasma is lower than blood and, as such, plasma samples disperse onto a larger area of paper than spotted blood samples using the same aliquot volume. With the use of the 226 paper, a 15 μL aliquot of plasma gave a 9 mm diameter spot, while a 20 μL aliquot was 12 mm and a 25 μL aliquot was 14 mm. In comparison, a 15 μL aliquot of blood gave a 7 mm diameter, 20 μL aliquot gave an 8 mm diameter, and a 25 μL aliquot gave a 10 mm diameter.

Stability. The difference in peak area ratios between a stock solution after storage for 183 days and the freshly prepared solution was −1.2%, indicating that paroxetine is stable in solutions of DMF stored at 4 °C for at least 183 days.

Table 3. Intra- And Inter-Assay Performance Data for Paroxetine in Human Whole Plasma Samples (n = 6 for Each Concentration Level in Each Individual Run)

	nominal concentration (ng/mL)	0.2	0.8	10	160	200
	Individual (Intra-Run) Statistics					
run 1	mean concentration (ng/mL)	0.209	0.826	10.8	166	202
	SD	0.010	0.027	0.34	4.4	6.1
	precision (% CV)	4.8	3.2	3.1	2.6	3.0
	accuracy (% bias)	4.7	3.2	7.6	3.6	0.9
run 2	mean concentration (ng/mL)	0.206	0.814	10.7	163	199
	SD	0.0062	0.023	0.24	3.3	7.1
	precision (% CV)	3.1	2.8	2.3	2.0	3.6
	accuracy (% bias)	2.9	1.7	6.6	1.9	−0.5
run 3	mean concentration (ng/ml)	0.199	0.823	10.2	168	212
	SD	0.012	0.017	0.22	2.8	12
	precision (% CV)	5.9	2.0	2.2	1.7	5.9
	accuracy (% bias)	−0.3	2.8	2.2	5.1	6.2
	Overall (Inter-Run) Statistics					
	mean concentration (ng/mL)	0.205	0.821	10.5	166	204
	SD	0.010	0.022	0.35	4.0	10
	overall precision (% CV)	4.9	2.6	3.4	2.4	5.1
	average accuracy (% bias)	2.4	2.6	5.2	3.5	2.2
	average intrarun precision (%)	4.7	2.7	2.7	2.2	4.4
	inter-run precision (%)	<0.1	<0.1	<0.1	<0.1	<0.1

Table 4. Intra-Assay Performance Data for Paroxetine in Human DPS Samples on Indicating Paper (n = 6 for Each Concentration Level in Each Individual Run)

	nominal concentration (ng/mL)	0.2	0.8	10	160	200
	Individual (Intra-Run) Statistics					
run 1	mean concentration (ng/mL)	0.179	0.882	9.60	162	200
	SD	0.011	0.052	0.63	9.1	11
	precision (% CV)	6.4	5.9	6.5	5.6	5.6
	accuracy (% bias)	−10.7	10.2	−4.0	1.1	−0.2

To determine the stability of paroxetine in DPS samples on 226 paper, a comparison was made between samples stored with desiccant for 35 days at room temperature and the fresh samples. The differences were −8.1 and 7.0% at 0.8 and 160 ng/mL human plasma, respectively, indicating that paroxetine is stable under the conditions of storage.

The accuracy, precision, and sensitivity of processed human validation samples in whole plasma and on 226 paper were found to be acceptable (precision and accuracy within the predefined 15% limits) on reinjection with freshly prepared calibration standards, after storage at room temperature for 72 and 24 h, respectively. This demonstrates that processed samples were stable when stored under these conditions.

The freeze−thaw stability of paroxetine was determined from spiked human whole plasma samples after three freeze−thaw cycles from −20 °C to room temperature. The difference of stored samples compared to fresh samples was −1.6% and 1.4% at 0.8 and 160 ng/mL, respectively.

Table 5. Influence of the Volume of Human Plasma Spotted on 226 Paper on the Precision and Accuracy of the Assay at Two Concentration Levels (n = 6 Replicates)[a]

nominal concentration	0.8 ng/mL			160 ng/mL		
volume of human plasma spotted onto 226 paper	15 μL	20 μL	25 μL	15 μL	20 μL	25 μL
mean concentration (ng/mL)	0.756	0.832	0.760	167	173	168
SD	0.061	0.076	0.042	6.4	7.8	13
precision (% CV)	8.130	9.127	5.586	3.8	4.5	7.8
accuracy (% bias)	−5.5	4.0	−5.0	4.4	8.0	4.8
difference from 20 μL spot (%)	−9.2		−8.7	−3.4		−3.0

[a] Concentrations were measured against a calibration line prepared with 20 μL human plasma.

The stability of paroxetine in spiked human plasma stored at room temperature was assessed. Differences of −3.9% and −2.0% at 0.8 and 160 ng/mL were evident when comparing stored samples against fresh samples.

Dilution of Samples Above the HLQ. The ability to dilute samples containing paroxetine at concentrations above the HLQ was demonstrated by performing replicate 10-fold dilutions of extracts whole human plasma as well as human DPS samples on 226 paper. The bias and within-run precision values were less than the predefined acceptance limits of 15% and were therefore acceptable. It is important to note that when diluting DPS samples for analysis by HPLC−MS/MS, the diluent used should be obtained by the extraction of blank matrix from the same species as the sample to be diluted. If neat solvent or an extract of the same matrix from another species is used, there is a risk that suppression effects (of either the analyte or the internal standard) during HPLC−MS/MS analysis would give the incorrect concentration value. The effects can be minimized by the use of isotopically labeled internal standards and/or adequate chromatography. In the case of this study, no such suppression effects were observed.

Volume of Plasma Spotted. The accuracy and precision of quantitation of paroxetine extracted from a 6 mm diameter punch taken from a DPS sample on 226 paper derived from 15, 20, and 25 μL aliquots of human plasma are presented in Table 5. For all plasma sample volumes examined, the within-volume precision and accuracy values for calculated concentrations were less than or equal to the predefined acceptance criteria of 15% and are therefore acceptable. Further, the differences between the accuracy values for extracts from 15 and 25 μL spots compared to those from 20 μL were all less than 9.2%. This indicates that there is no notable difference in distribution of analyte and plasma across spots derived from plasma volumes between 15 and 25 μL, when a 6 mm punch is taken from the center of the plasma spot, and thus allowing for inaccurate spotting.

Recovery. Recovery of paroxetine from DPS using the stated extraction method was carried out on 226 paper at concentrations of 0.8, 10, and 160 ng/mL and yielded recoveries of between 60.2% and 76.7%. Plasma recoveries were calculated at 0.8 and 160 ng/mL and were 93.8% and 92.6%, respectively. Precision values calculated for each concentration in DPS or plasma did not exceed 15% and are therefore acceptable.

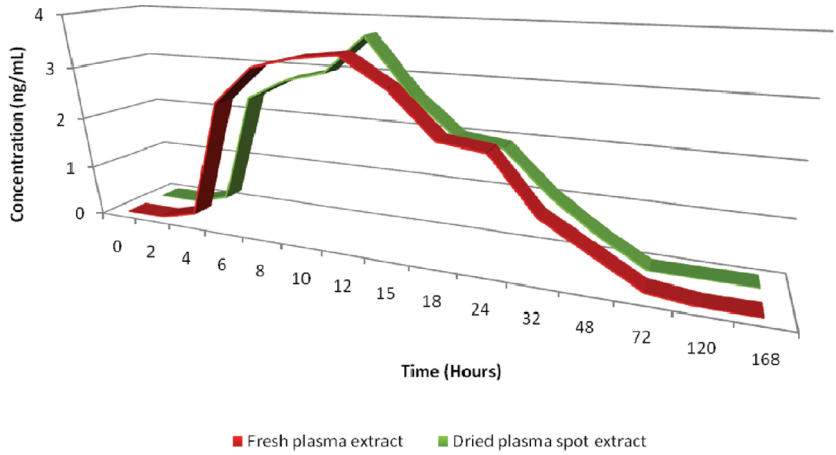

Figure 4. PK profiles of paroxetine obtained from extraction of human DPS and plasma pooled samples, consisting of five individual samples per time point after a single oral administration of 37.5 mg of paroxetine.

Table 6. PK Parameters Obtained from the Analysis of Extracted Plasma and DPS Pooled Profiles Using a Linear/log Trapezoidal Model with WinNonLin

	type of sample	
	fresh plasma	dried plasma spot
AUC (ng h/mL)	83.52	76.56
C_{max} (ng/mL)	3.51	3.75
T_{max} (h)	12	12

Application of the DPS and Plasma Assay to the Analysis of Samples from a Clinical Study. The pooled plasma and DPS profile samples prepared as previously outlined were extracted according to their respective extraction methods and analyzed, resulting in a PK profile for each sample type shown in Figure 4. The profiles were interpreted with WinNonLin using a linear/log trapezoidal model, and the parameters generated are shown in Table 6.

The total exposure shown by calculating the area under curve (AUC) for the DPS profile fell within 8.3% of the fresh plasma profile, the C_{max} (maximum calculated concentration) for DPS was within 6.4% of plasma, and the T_{max} (time point at C_{max}) was identical at 12 h for both profiles.

CONCLUSIONS

A simple and rapid assay (less than 4 min combined extraction and run time) has been developed and validated for the determination of paroxetine in human DPS samples by HPLC−MS/MS, comfortably being able to reach an LLQ of 0.2 ng/mL using a 6 mm punch size from a 20 μL spot. The validated method was demonstrated to be accurate, precise, and robust and complied with the most recent regulatory guidelines.[22,23] In addition to the factors normally tested under these guidelines, a number of other parameters associated exclusively with the utility of dried matrix samples on a paper substrate were also validated, leading to greater confidence in the robustness and utility of the technique. Data demonstrating that there is no quantitative difference between the size of the spot obtained allows greater flexibility in sample processing and spotting procedures between different study centers. The stability of paroxetine in DPS samples stored desiccated at room temperature for 35 days demonstrates that samples in this form can be stored for moderately long periods of time before analysis, without the need for refrigeration, thus reducing costs and increasing flexibility in selection of study centers and bioanalytical laboratories. The comparison of DPS to fresh plasma extraction through analysis of a pooled PK profile has shown for this work that interconversion of these techniques is possible. This is initially evident from the visual tight fit of the various time points and reinforced through the calculation of PK parameters, which all fall within 10%. The use of indicating FTA DMPK paper has been assessed for DPS extraction and it offers the benefit of an easily recognizable dried spot, so allowing spotting errors to be easily flagged. It makes the sample simple to track during manual processing and lends itself well to automated processing due to the contrast in coloration from the spot to the surrounding paper. For this particular work, no experimental issues were identified with the use of the indicating FTA DMPK paper. The excellent comparison of DPS and conventional plasma analysis demonstrates the simple transition between DPS and plasma and opens up many of the benefits of card sampling to late stage clinical, drug discovery groups using plasma and direct elution techniques and compounds that are not applicable to DBS.

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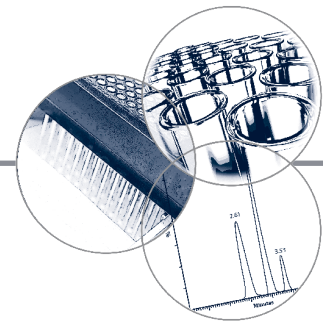
Research Article
Mini Focus Issue: Dried blood spots



The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium

Background: The European Bioanalysis Forum dried blood spots (DBS)/microsampling consortium is reporting back from the experiments they performed on further documenting the potential hurdles of the DBS technology. This paper is focused on the impact of hematocrit changes on DBS analyses. **Results:** The hematocrit can have an effect on the size of the blood spot, on spot homogeneity and on extraction recovery in a compound-dependent manner. The extraction recovery can change upon aging in an hematocrit-dependent way. Different card materials can give different outcomes. **Conclusions:** The results from the conducted experiments show that the issues of DBS in regulated bioanalysis are real and that the technology will need improvements to be ready for use as a general tool for regulated bioanalysis.

Dried blood spot sampling has been used since the 1960s for newborn screening [1]. Dried blood spots (DBS) offers a number of advantages over conventional blood sampling, such as the use of a simplified and less invasive sampling method (heel or finger prick), simpler sample handling, shipment and storage (no centrifuges and freezers are required) and a reduction in sample volume (typically 10–20 µl per spot) [2]. Over the past few years, the technology has been applied by more and more companies as a tool for microsampling in drug research and development in support of TK and PK studies. As a result, the pharmaceutical industry is gaining a better insight into the strengths but also into potential weaknesses and hurdles in application of the technique. Hurdles such as changes in the size of the blood spot as a function of **hematocrit**, inhomogeneous distribution of analytes across the blood spot and hematocrit-dependent extraction recovery, as detailed below, are unique to DBS and not applicable to more traditional wet matrices, such as plasma and serum.

The recent recommendation on DBS from the European Bioanalysis Forum (EBF) states that "*Hematocrit is currently identified as the single most important parameter influencing the spread of blood on DBS cards, which could impact the validity of the results generated by DBS methods*" [3]. The hematocrit of a blood sample influences the spread of blood on dried blood spot cards due to the fact that the viscosity of blood increases with increased hematocrit [4]. As a result, in general a linear inverse relationship between spot area and blood hematocrit is observed [5,6]. Thus, when a fixed blood spot punch is taken, the punch contains more blood for a high-hematocrit sample than for a low-hematocrit sample. Also, it has been reported that the analyte does not distribute evenly across the spot, as demonstrated by center/perimeter (C/P) concentration ratios not being equal to one. The C/P ratio can also vary depending on the hematocrit [6,7]. Finally, hematocrit can have an influence on the extraction recovery [8,101].

In 2011, the EBF initiated the composition of several teams to design and execute experiments to further document the scientific relevance of the (real or perceived) issue with respect to the acceptability of DBS in regulated bioanalysis [9–11,102]. The experiments conducted by the hematocrit team are described in the current article, together with results, observations and conclusions. The results from other teams on spot homogeneity [12] and IS addition [13] were also published, and the results from all teams has also resulted in an update of the EBF recommendation for the use of DBS in regulated bioanalysis [14].

Building on data on the subject that was already available in the peer-reviewed literature, we designed and executed an experimental plan to try to provide an answer to a number of key questions around hematocrit and DBS as listed below:

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Key Term

Hematocrit: Percentage of a blood sample that consists of red blood cells, measured after the blood has been centrifuged and the cells compacted.

- What is the relationship between hematocrit and response (peak area ratio)?
- What is the relationship between hematocrit and spot homogeneity?
- What is the relationship between hematocrit and the actual spot size?
- What is the relationship between hematocrit and extraction recovery?
- Does the age of the wet blood used have an impact on the results?
- Does the age of the blood spot used have an impact on the results?
- What is the influence of different card types (treated, nontreated, cellulose-based or noncellulose based) on the results obtained at different hematocrit?
- Which parameters are compound dependent?

Details of the experiments and results are provided in the next sections.

Experimental

Analytes

Twelve compounds were selected covering a wide chemical space with a variety of log P and pKa values. The compounds that were included in the tests are listed in Table 1, together with some of their physicochemical parameters.

Blood samples

A standardized way of preparing blood with varying hematocrit values was used by all participating companies. A fresh blood sample was taken, mixed and the hematocrit was measured in duplicate. Then the volume of plasma to be added or removed to obtain each of the target hematocrit values (see below) was calculated. Subsequently, the required plasma volume was added to blood samples for which the hematocrit needed to be decreased. The blood that required plasma removal to increase the hematocrit was spun for 10 min at 1300 g and the required plasma volume was removed. The blood samples prepared in this way were gently roller-mixed for 30 min. The hematocrit of each of the samples was finally measured in duplicate to confirm that the target hematocrit was obtained. The blood samples at each hematocrit value were spiked with one or multiple analytes at the concentrations as shown in Table 2.

Card materials

The Ahlstrohm 226™ (ID Biologicals, SC, USA), BondElut DMS™ (Agilent, CA, USA), GE DMPK-A™ (formally FTA, Whatman, GE Healthcare, UK) and GE DMPK-B (formally FTA-Elute, Whatman, GE Healthcare) were selected as card types for the experiments. The DMPK-A and DMPK-B cards are treated cards and according to the vendor, contain proprietary chemical mixtures that lyse cells and denature degrading enzymes. The other two card types are untreated. All cards are cellulose-based, except for the BondElut DMS. Of note, virtually all studies conducted and published in literature on effect of hematocrit are from filter paper (cellulose-based) dried blood spot cards.

Spotting

For the regular analyses, 25 µl of spiked blood was spotted onto the cards using a volumetric pipette. For recovery determination, a smaller volume of 5 µl was spotted. All cards were dried for at least 2 h at ambient temperature and subsequently transferred to plastic bags containing desiccant. The spotted cards were analyzed as soon as practical (within 1 week for most compounds; see Table 3) and additional spots were put aside (stored at room temperature) for analysis at a later time point (after 2 months for one compound, after 6–6.5 months for most compounds; see Table 3). In this way, the influence of the age of the blood spot itself on the results could be assessed.

Analysis

All experiments were conducted at one analyte concentration. Analyte concentrations for each analyte are detailed in Table 2. As QC, one appropriate test sample was injected in triplicate at the beginning, middle and end of the run. The coefficient of variation over the nine values had to be ≤15%, and the mean of the last injections versus the mean of the first injections had to differ by no more than 15%.

The experimental details are summarized in Table 2. The table contains details on the extraction solvents and volumes, the IS and the equipment used. For all of the assays an IS was used. For eight assays this was a stable-isotope-labelled IS, and for four assays a structural analog was used. All results presented in this paper are based on peak area ratios, so these data include correction by the IS. The age of the blood spots at the initial analysis and at analysis after aging is listed in Table 3.

■ Spot size & response dependence on hematocrit

For the investigations on spot size and analyte response dependence on hematocrit, spiked fresh blood was tested at eight different hematocrit values (Hct = 20, 30, 35, 40, 45, 50, 60 and 70%) on the Ahlstrohm 226 card. Both fresh and stored spiked blood were tested at three hematocrit values (Hct = 20, 45 and 70%) on all four card types. It was decided to test over a hematocrit range from 20 to 70% to be sure that the clinically relevant range of hematocrits was covered, including the higher hematocrit values that are in particular relevant in dried blood spot applications for neonate blood samples [5,15,16].

The spotted blood volume was 25 µl and the punch size was 3–3.3 mm to reflect common practice used in bioanalytical laboratories for dried blood spot analyses. Spot size measurements were in mm^2 and were determined using Image J software [103]. The same software was used across all test sites for consistency in the spot size measurements. Analyte response was expressed as peak area ratio. For all analytes an IS (structural analog or stable-isotope-labeled) was used. Analyses were measured in singleton on three individual blood spots per hematocrit per card type, both for fresh and for stored blood.

■ Recovery dependence on hematocrit

For the investigations on recovery dependence on hematocrit, spiked fresh blood was tested at three hematocrit values (Hct = 20, 45 and 70%) on all four card types. Stored blood was spiked and tested at one hematocrit value (Hct = 45%) on all four card types. For these experiments, duplicate 6-mm punches were taken from 5-µl blood spots, which means that the whole spot was punched. Duplicate blank spots were also included; these were punched, extracted and thereafter spiked so that the recovery could be calculated. Recovery was calculated as:

Response of blood spot spiked before spotting of the blood/response of blank blood spot where analyte was spiked to the extraction solvent post-extraction × 100%

EQUATION I

For the endogenous compounds in the test set, recovery was determined using stable isotope-labeled or structural analogs and calculated as:

Table 1. Physicochemical parameters of compounds included in the tests.

Compound ID	1	2	3	4	5	6	7	8	9	10	11	12
cLogD (pH 7.4)	4.4	2.1	2	0.4	3.5	>4.4	5.4	4.7	2.1	-0.4	~20	6.9
pKa	4.4†; 8.1‡	2.4; 8.5	9.2	5.2†	12‡	2.5; 5.2	5.6‡	1‡; 9‡	3.1‡; 8.2‡	2.2 (COOH), 9.1 (NH_2), 10.1 (OH)	>20	4.5†
Blood/plasma distribution	0.61	0.7	0.8	0.67	1.0	Unknown	0.68	0.7	0.72	Unknown	Unknown	Unknown
PPB	98	Unknown	Unknown	97	89–98	Unknown	99.7	Unknown	90	Unknown	Unknown	Unknown
Molecular weight	442	470	450	270	854	750	366	555	410	181	884	304

†Acidic group.
‡Basic group.
PPB: Plasma protein binding.

Table 2. Extraction conditions and equipment used for the different assays.

Compound ID	Extraction method	IS	Instrument	Concentration
1	Methanol containing IS – 500 µl (recovery experiments) or 200 µl (all other experiments)	Stable isotope	HPLC–MS/MS (API5500 Triple Quad™)	100 ng/ml blood
2	20/80 methanol/(10 mM ammonium formate/methanol [95/5] v/v) + 0.1% formic acid (v/v) 450 µl (recovery experiments) or 300 µl (all other experiments)	Structural analog	HPLC–MS/MS (API5000 Triple Quad™)	200 ng/ml blood
3	20/80 methanol/(10 mM ammonium formate/methanol [95/5] v/v) + 0.1% formic acid (v/v) 450 µl (recovery experiments) or 300 µl (all other experiments)	Structural analog	HPLC–MS/MS (API5000 Triple Quad)	200 ng/ml blood
4	200 µl methanol, including IS After extraction dilute with 200 µl water	Stable isotope	HPLC–MS/MS (API3000 Triple Quad™)	6.25 µg/ml blood
5	200 µl water; 25 µl IS solution in methanol; 1 ml TBME; evaporate and redissolve	Structural analog	HPLC–MS/MS (API3000 Triple Quad)	250 ng/ml blood
6	200 µl of a mixture of methanol and water (80/20, v/v), containing the IS. Vortex mixed for 2 h	Stable isotope	HPLC–MS/MS (Thermo Vantage™)	15 ng/ml blood
7	20/80 water/methanol (v/v) – 500 µl (recovery experiments) or 200 µl (all other experiments)	Stable isotope	HPLC–MS/MS (API4000 Triple Quad™)	200 ng/ml blood
8	20/80 water/methanol (v/v) – 500 µl (recovery experiments) or 200 µl (all other experiments)	Stable isotope	HPLC–MS/MS (API4000 Triple Quad)	200 ng/ml blood
9	20/80 water/methanol (v/v) – 500 µl (recovery experiments) or 200 µl (all other experiments)	Stable isotope	HPLC–MS/MS (API4000 Triple Quad)	200 ng/ml blood
10	Extract 1: 20 µl water, after 5 min 80 µl methanol Extract 2: 100 µl isopropanol – combine extract 1 and 2	Stable isotope	HPLC–HRMS (Orbitrap™)	Native (~75 µmol/l range)
11	Extract 1: 20 µl water, after 5 min 80 µl methanol Extract 2: 100 µl isopropanol – combine extract 1 and 2	Structural analog	HPLC–HRMS (Orbitrap)	Native (~10 µmol/l range)
12	Extract 1: 20 µl water, after 5 min 80 µl methanol Extract 2: 100 µl isopropanol – combine extract 1 and 2	Stable isotope	HPLC–HRMS (Orbitrap)	Native (~20 µmol/l range)

Response of blood spot spiked with stable isotope or structural analog before spotting/response of blank blood spot spiked with stable isotope or structural analog post-extraction × 100%

EQUATION 2

Table 3. Age of the blood spots at initial analysis and after aging.

Compound ID	Age of blood spots at initial analysis (weeks)	Age of blood spot at re-analysis after aging (months)
1	0–1	2
2	0–1	6
3	0–1	6
4	0–1	Not analyzed
5	0–1	Not analyzed
6	0–1	5
7	0–1	6.5
8	0–1	6.5
9	0–1	6.5
10	7	Not analyzed
11	9	Not analyzed
12	9	Not analyzed

■ Spot homogeneity dependence on hematocrit

For the investigations on spot homogeneity dependence on hematocrit, spiked fresh blood was tested at three hematocrit values (Hct = 20, 45 and 70%) on all four card types. Stored blood was spiked and tested at one hematocrit value (Hct = 45%) on all four card types. For these experiments, two 1-mm punches were taken from the same 25-µl blood spot, one from the center and one near the perimeter of the spot. This experiment was carried out on three individual blood spots per hematocrit per card type, both for fresh and for stored blood.

Results & discussion

From published hematocrit ranges in human blood, it is apparent that these can vary over a wide range. Hematocrit values across different patient populations as reported from a 1-year data set from Ghent University Hospital (Ghent, Belgium) range from 19 to 63% [15], and published typical human hematocrit levels for different age groups range from 28

to 67% [5]. For neonates, the hematocrit can vary over a relatively broad range (i.e., 42–64% at birth and 31–67% when less than 1-month old) [16], whereas for healthy adults the range of hematocrits is much smaller (i.e., 36–44% for adult females and 41–50% for adult males). It was decided to focus on human blood only, and to test over a hematocrit range from 20 to 70% to be sure that the clinically relevant range of hematocrits was covered, including the higher hematocrit values that are in particular relevant in dried blood spot applications for neonate blood samples.

For practical reasons, it is very useful if human blood can be used for a prolonged period of time for preparation of calibration curves and QC samples. Therefore, both fresh human blood (≤1-day old) and stored human blood (14-day old, blood stored refrigerated until use) were included in the tests to determine whether 14-day-old control (wet) blood could still be used reliably for preparing calibration standards and QC samples.

Spot size dependence on hematocrit

The results of the spot size measurements as obtained by Image J are shown in Tables 4 & 5. For the three cellulose-based cards (226, DMPK-A and DMPK-B), the spot size decreases with increased hematocrit. This can be explained by the fact that the viscosity of the blood increases at higher hematocrit, thus reducing the radial travelling speed of the blood on the card and resulting in a smaller spot. Comparable findings were reported in various other papers [5,17]. The results obtained at the different laboratories from the team members were similar, although not always completely identical. There was some variation across laboratories despite the fact that the same cards and the same spot size measurement technique were used. This implies that spotting of the same blood volume at the same hematocrit on the same card type does not always result in exactly the same size of the resulting DBS. For 226 and DMPK-A cards, the %CV of the spot size measurements over the different laboratories was between 6 and 10%, for DMPK-B and BondElut the %CV was higher.

For the BondElut cards, the spot size is much smaller than on the other card types, and the spot size increases with increased hematocrit. This is probably related to the material used for the cards. The exact composition of the material used was not disclosed by the vendor, but is mentioned to be noncellulose based. The

Table 4. Results of spot size measurements (in mm²) as obtained using Image J.

HCT	1	2, 3	4	5	6	7, 8, 9	10, 11, 12	Average	%CV
226									
20	62	65	75	78	70	73	74	70.9	7.8
30	56	64	63	66	70	66	68	64.9	7.2
35	54	61	67	68	67	64	68	64.1	8.2
40	54	64	64	61	65	63	66	62.5	6.4
45	54	60	62	63	64	65	65	61.8	6.4
50	50	59	62	58	63	61	63	59.4	7.6
60	49	55	64	59	64	59	61	58.8	8.7
70	47	52	52	51	58	57	58	53.5	7.9
DMPK-A									
20	54	63	69	75	65	71	71	66.8	10.4
45	52	57	69	64	58	62	63	60.6	8.7
70	49	51	58	49	52	58	58	53.5	7.8
DMPK-B									
20	66	62	76	71	69	139	102	83.7	33.0
45	58	58	74	66	65	136	129	83.8	40.3
70	56	52	60	55	57	118	95	70.5	36.0
BondElut									
20	22	29	38	35	14	32	38	29.5	30.2
45	30	39	48	41	19	37	40	36.4	25.4
70	34	41	47	43	25	40	42	38.8	19.2

HCT: Hematocrit.

Table 5. Comparison of spot sizes obtained by spotting fresh blood and by spotting 14-day-old wet blood†.

HCT	1	2, 3	4	5	6	7, 8, 9	10, 11, 12	Average	%CV
226									
20	97.9	95.2	97.3	92.9	ND	97.5	ND	96.2	2.2
45	109.5	97.0	104.5	96.8	ND	95.8	ND	100.7	6.0
70	96.4	95.6	55.2	103.6	ND	99.6	ND	90.1	22.0
DMPK-A									
20	95.8	105.1	104.3	NR	ND	98.9	ND	101.0	4.4
45	103.0	98.9	100.0	NR	ND	99.4	ND	100.3	1.8
70	103.0	94.1	94.7	NR	ND	99.6	ND	97.9	4.3
DMPK-B									
20	117.2	99.8	109.6	112.4	ND	100.5	ND	107.9	7.0
45	116.9	96.2	96.1	105.6	ND	88.5	ND	100.7	10.9
70	118.0	98.2	95.7	99.2	ND	83.1	ND	98.9	12.6
BondElut									
20	96.6	102.6	98.6	94.8	ND	72.2	ND	93.0	12.9
45	92.1	85.5	86.4	96.7	ND	92.8	ND	90.7	5.2
70	105.3	86.7	105.2	102.6	ND	91.4	ND	98.2	8.8

†Spot sizes in the table are expressed as (spot size of spotted 14-day-old wet blood/spot size of spotted fresh blood x 100%).
Spot sizes were obtained as a mean of one (compound 1), two (compound 2) or four (compound 4, 5, 6, 7/8/9, 10/11/12) singleton measurements on individual blood spots.
HCT: Hematocrit; ND: Not determined; NR: No result.

spot size measurements for the DMPK-B cards was more difficult to conduct, as for these cards a notable halo is present around the blood spot as shown in Figure 1. The halo was included in the spot size measurements for this card type. The size of the halo was quite large for two of the participating companies, leading to partial blending of the adjacent spots via the halos. This explains the much higher spot areas found for these two participating companies. The reason why the size of the halo varied remains unclear.

The spot sizes found when 14-day-old blood was spotted were within 90–110% of the spot sizes found when fresh blood was spotted, for all tested card types and hematocrits for the majority of the measurements. Apparently, the spot size is not significantly affected by the changes that may occur in whole blood when stored for up to 14 days at 4°C.

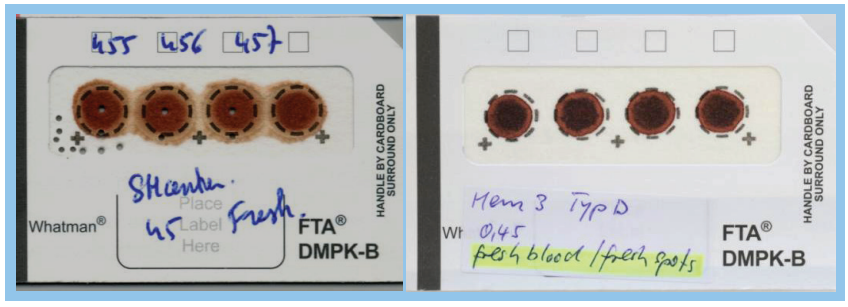

Figure 1. Variation in shape of the halo on DMPK-B cards obtained in two different laboratories.

Results of the spot size measurements can be seen in Table 4. Spot sizes are expressed as mm^2. Some compounds were co-spiked in one spot, therefore only one set of spot size measurements was available for compounds 2/3, 7/8/9 and 10/11/12. Spot sizes were obtained as a mean of one (compound 1), two (compound 2) or four (compound 4, 5, 6, 7/8/9, 10/11/12) singleton measurements on individual blood spots.

■ Response dependence on hematocrit

The response (peak area ratio) was expressed relative to the response obtained at Hct 45%. The results are shown in Table 6. For the majority of the compounds, an increase in response is seen with increased hematocrit on all card types. This correlates with the decreased spot size (and thus larger blood volume punched) with increasing hematocrit as observed for the 226, DMPK-A and DMPK-B cards. However, the BondElut card shows an increase in spot size with increasing hematocrit, so the increase in response cannot be explained by a decrease in spot size for this card type.

A large variation in results between different compounds was observed. For instance, on the 226 cards, the relative response at Hct 20% varied between 51 and 95%, and the relative response at Hct 70% varied between 81 and 145% of that at Hct 45%. Some of the compounds (2/3, 7/8/9 and 10/11/12) were

co-spiked into the same blood spot, but even then for some compounds a variation in results was observed. For instance, when compound 7 and 8 are compared on the 226 card, the results differ significantly. Thus, it can be concluded that different compounds behave differently on the cards. Therefore, the change in response as a function of hematocrit cannot solely be attributed to a change in spot size.

The consequence of these findings for the use of DBS in regulated bioanalysis is that the accuracy and precision at different hematocrits in QC samples needs to be assessed over the range of hematocrits for which the dried blood spot assay is validated. In these experiments, the hematocrit of the blood used for the calibration curve should be held constant. The range of hematocrits within which the normal QC acceptance criteria can be met will vary depending on the intrinsic accuracy and precision the assay already has when run at equal hematocrit for curve and QCs combined with the hematocrit/response relationship on the chosen card type for the compound for which the assay is validated [18,104].

■ Response dependence on age of the wet blood

The results of the influence of the age of the wet blood used on the results are shown in Table 7. For most analytes, no large differences in response were observed when spiked to fresh (<1-day old) or spiked to stored (14-day old) wet blood. However, for individual compounds there are differences, indicating that for some compounds the use of 14-day-old wet blood for preparation of calibration and QC samples would not be acceptable. For instance, for compound 4 at Hct 45 the result in fresh and 14-day-old wet blood is the same, whereas at Hct 20 and 70 the response in 14-day-old wet blood is much lower than in fresh blood. It has been reported that clots were seen in blood after 5–8 days of refrigerated storage [12], indicating that the wet blood samples are physically changing over this storage period.

The consequence of these findings for the use of DBS in regulated bioanalysis is that if stored wet blood is used for preparation of calibration curves or QC samples in DBS, it should be demonstrated that the results obtained in aged wet blood over the hematocrit range used are comparable with the results obtained when fresh wet blood is used. It is advised that the wet blood is used before it starts to change

Table 6. Relative response (hematocrit 45 normalized at 100%) as a function of hematocrit[†].

HCT	1	2	3	4	5	6	7	8	9	10	11	12	Average	%CV
226														
20	67	79	84	64	76	77	66	77	69	86	95	51	74	16
30	87	95	98	80	90	93	79	81	79	96	80	68	85	11
35	88	94	97	85	89	93	92	94	87	91	85	74	89	7
40	95	94	98	90	96	102	114	106	103	98	96	95	99	7
45	100	100	100	100	100	100	100	100	100	100	100	100	–	–
50	94	98	101	103	105	117	121	109	110	104	128	103	108	9
60	99	113	115	120	110	111	140	109	118	108	94	111	112	10
70	112	117	120	119	135	126	145	103	125	129	81	84	116	16
DMPK-A														
20	85	85	92	84	NR	83	74	84	83	87	80	88	84	5
45	100	100	100	100	NR	100	100	100	100	100	100	100		
70	89	107	111	116	NR	114	108	95	111	114	133	124	111	11
DMPK-B														
20	54	78	79	63	133	121	58	123	68	50	170	44	87	46
45	100	100	100	100	100	100	100	100	100	100	100	100		
70	87	77	78	106	84	100	124	131	127	143	105	96	105	21
BondElut														
20	85	69	69	95	87	79	90	118	91	79	105	78	87	16
45	100	100	100	100	100	100	100	100	100	100	100	100		
70	287	142	136	274	116	134	170	183	178	135	97	116	164	37

[†]Each result is the mean of three measurements.
HCT: Hematocrit; NR: No result.

Table 7. Response in fresh wet blood/response in stored wet blood × 100%†.

HCT	1	2	3	4	5	6	7	8	9	10	11	12	Average	%CV
226														
20	105	126	120	54	105	105	98	97	98	NC	NC	NC	101	20
45	100	113	115	100	93	105	117	112	111	NC	NC	NC	107	8
70	107	103	100	103	107	91	109	117	98	NC	NC	NC	104	7
DMPK-A														
20	97	108	99	49	NR	87	105	109	102	NC	NC	NC	95	21
45	100	102	102	94	NR	108	96	106	102	NC	NC	NC	101	5
70	115	113	112	53	NR	94	111	120	107	NC	NC	NC	103	21
DMPK-B														
20	93	97	111	58	111	63	111	101	102	NC	NC	NC	94	21
45	100	87	103	110	99	73	102	142	106	NC	NC	NC	102	18
70	90	97	117	68	93	67	101	112	97	NC	NC	NC	94	18
BondElut														
20	88	112	108	46	101	110	108	121	111	NC	NC	NC	101	22
45	100	123	119	103	106	96	115	132	112	NC	NC	NC	112	10
70	102	105	104	70	109	124	93	99	87	NC	NC	NC	99	15

†Each result is the mean of three measurements.
HCT: Hematocrit; NC: Not conducted; NR: No result.

significantly physically, and at least before clots start appearing, which could be within 5 days after collection [12].

■ Recovery dependence on hematocrit

There are some indications in the literature that the extraction recovery can vary as a function of hematocrit. For verapamil, for example, extraction recoveries at Hct 70 and Hct 30% were 68 and 92%, respectively, and the recovery at Hct 70% further decreased to 58% upon aging of the blood spot [101]. In a company perspective paper, it was mentioned that for some compounds the recovery from high hematocrit blood is reduced relative to lower hematocrit levels [19]. In a paper about perforated DBS [8], a decrease in extraction recovery was observed for lansoprazole with increasing hematocrit. After further optimization of the extraction conditions, this effect was no longer observed. The individual results of the experiments conducted in our team are shown in Table 8.

The recovery as a function of hematocrit varied considerably between different compounds and also between different card types. For some compounds, the recovery remained more or less constant across the range of tested hematocrit values (e.g., for compounds 7 and 9 on all card types). For other compounds, the recovery either increased or decreased with increasing hematocrit. For one and the same compound, the behavior on different card types can be different. For instance, for compound 1 on 226 cards the recovery at Hct 70% was only 49%, whereas the recovery at Hct 20% was 105%. For the same compound, on DMPK-B cards, the recovery at Hct 70% was 59%, whereas the recovery at Hct 20% was 39%. Thus, for this compound on 226 cards, the recovery decreases with increasing hematocrit, whereas on DMPK-B, the recovery increases with increasing hematocrit. For compound 5, on 226 cards the recovery was more or less constant at different hematocrits, whereas on DMPK-B cards, the recovery at Hct 70% was approximately one third of the recovery at Hct 20%.

It is possible that the matrix effect can also vary as a function of hematocrit. For methods in which a stable-isotope-labeled IS is used, such as the assay for compound 1 in the above example, it may be assumed that the IS will compensate for differences in matrix effect at the different hematocrit values. However, for compounds where a structural analog was used as IS, such as the assay for compound 5 in the above example,

the observed variation in absolute recovery as a function of hematocrit may be (partially) attributed to a difference in matrix effect as a function of hematocrit or may be attributed to a combination of both matrix effect and recovery.

No relationship was found between the absolute recovery and the difference in recovery as a function of hematocrit. Thus, a low absolute recovery at Hct 45% does not necessarily result in a large variation in recovery as a function of hematocrit and vice versa.

For most compounds, the absolute recovery in fresh and 14-day-old wet blood was approximately the same.

The consequence of these findings for the use of DBS is that the absolute recovery needs to be assessed and optimized to be more or less constant across the range of hematocrits for which the assay is validated, both for freshly spiked blood spots and for aged blood spots (see next section). In wet matrix assays, such as plasma or serum, the IS is typically added to the wet matrix prior to extraction. In this way, it is able to compensate for variations in extraction recovery across different samples. For dried blood spot analysis however, the IS does not compensate for variations in extraction recovery when added to the punched spot together with the extraction solvent. For this reason, it is much more important to have a constant extraction recovery for DBS than for wet matrix assays. It is likely to achieve this for most assays, but it does require additional method development efforts, since recovery needs to be assessed at multiple hematocrits both in fresh and aged blood spots [13].

■ Recovery in aged blood spots at different hematocrits

DBS will usually not be analyzed immediately after the samples have been taken. There could be weeks, months or sometimes maybe even a year between sampling and the actual analysis, depending on the type of study. Therefore, it is important to demonstrate that the aging of the spots does not impact the analysis result. During storage, analytes can theoretically degrade on the card and the extraction recovery could change upon storage.

The experiments on aged blood spots were conducted for compounds 2, 3, 6, 7, 8 and 9. In Table 9, the relative recovery (expressed as the ratio of recovery in aged spots/fresh spots × 100%) is shown at the different hematocrits.

For compound 8, the absolute recovery dropped significantly on all card types, the 226 card being

Table 8. Absolute recovery as a function of hematocrit.

HCT	1	2	3	4	5	6	7	8	9	10	11	12	Average	%CV
226														
20	105	85	79	44	73	81	88	78	87	91	85	87	82	17
45	65	104	101	43	77	75	90	64	84	91	117	98	84	24
70	49	84	73	56	84	62	79	41	82	67	120	78	73	28
DMPK-A														
20	91	87	83	51	NR	83	86	75	83	NR	116	110	86	21
45	82	95	93	43	NR	78	87	70	86	NR	111	114	86	24
70	79	80	85	55	NR	69	88	59	85	NR	79	94	77	16
DMPK-B														
20	39	81	80	42	108	79	89	88	95	72	NR	NR	77	28
45	50	117	93	43	49	72	92	92	91	75	NR	NR	77	31
70	59	89	91	57	35	67	96	88	82	74	NR	NR	74	26
BondElut														
20	106	83	72	42	95	95	84	84	87	92	90	99	86	19
45	111	85	85	44	92	79	93	82	90	90	95	93	87	18
70	106	95	89	54	93	72	82	69	84	78	100	100	85	18

HCT: Hematocrit; NR: No result.

Table 9. Recovery in aged spots versus fresh spots as a function of hematocrit.

HCT	1	2	3	4	5	6	7	8	9	10	11	12	Average	%CV
226														
20	NC	113	119	NC	NC	88	99	31	75	NC	NC	NC	88	36
45	NC	82	84	NC	NC	90	87	36	77	NC	NC	NC	76	26
70	NC	94	97	NC	NC	103	71	43	81	NC	NC	NC	82	27
DMPK-A														
20	NC	77	74	NC	NC	97	85	55	79	NC	NC	NC	78	18
45	NC	99	88	NC	NC	131	74	58	76	NC	NC	NC	88	29
70	NC	106	104	NC	NC	106	80	67	80	NC	NC	NC	91	19
DMPK-B														
20	NC	85	92	NC	NC	92	96	59	79	NC	NC	NC	84	16
45	NC	70	107	NC	NC	101	78	78	88	NC	NC	NC	87	17
70	NC	76	83	NC	NC	105	70	57	81	NC	NC	NC	79	20
BondElut														
20	NC	69	77	NC	NC	78	74	40	59	NC	NC	NC	66	22
45	NC	89	89	NC	NC	81	90	55	75	NC	NC	NC	80	17
70	NC	98	96	NC	NC	98	68	49	69	NC	NC	NC	80	26

HCT: Hematocrit; NC: Not conducted.

the most extreme. For the 226 card, the recovery for 6.5-month-old blood spots was only 31–43% of the recovery in fresh spots. In human plasma, compound 8 was found to be stable for at least 723 days in the freezer at -20°C and for at least 72 h at room temperature. Therefore, it is likely that the decrease found is due to a lower recovery rather than due to instability on the card.

A hematocrit-dependent decrease in recovery was also observed. For instance, for compound 3 on DMPK-A, the recovery at Hct 20% was 74% of the recovery in fresh spots, whereas at Hct 70% the recovery was 104% of the recovery in fresh spots.

■ Spot homogeneity dependence on hematocrit

When blood is spotted on a dried blood spot card, the analyte can show a chromatographic or distribution effect on the card material due to interaction of the blood and/or the analyte with the dried blood spot card. This may result in a nonhomogeneous distribution of the analyte across the blood spot. A way to investigate spot homogeneity is to take two small punches from one blood spot, one at the center and one at the perimeter, so near the edge of the spot. The C/P concentration ratio will then demonstrate the homogeneity of the spot. A C/P ratio of 1 is expected for a homogeneous spot, whereas a C/P ratio <1 would be an indication for a preferential distribution of the analyte to the perimeter of the spot rather than to the center. Significant differences in measured analyte concentrations between central and peripheral areas within a blood spot have been reported [6,7,17,20]. The C/P ratio can differ depending on the compound, card type and hematocrit, with a more homogeneous blood spot at higher hematocrit reported in literature [7].

For the evaluation of the results on spot homogeneity, the C/P response ratio was determined at different hematocrit values, as shown in Table 10. For the comparison of the results obtained in fresh blood versus stored 14-day-old wet blood, response ratios were calculated and presented in Table 11.

The C/P response ratio was <1 for most of the compounds and hematocrits on the 226, DMPK-A and BondElut cards, demonstrating that for these card types most (but not all) compounds have a preference to migrate towards the perimeter of the spot. Thus, these data confirm the 'volcano effect' that has been described previously [20]. A C/P ratio of <1 was

Table 10. Center/perimeter response ratios.

HCT	1	2	3	4	5	6	7	8	9	10	11	12	Average	%CV
226														
20	0.52	1.04	1.01	0.58	0.83	1.05	0.73	0.68	0.73	0.84	NR	0.54	0.78	25
45	0.44	0.94	0.95	0.53	0.76	1.22	0.60	0.71	0.70	0.86	NR	0.76	0.77	28
70	0.93	0.96	0.92	0.98	0.81	0.96	0.91	0.93	0.89	0.96	NR	0.90	0.92	5
DMPK-A														
20	0.70	0.95	0.93	0.81	0.88	0.97	0.77	0.79	0.86	0.80	NR	0.75	0.84	11
45	0.74	0.86	0.88	0.83	0.87	0.83	0.73	0.91	0.77	0.84	NR	0.83	0.83	7
70	0.80	0.94	0.94	0.91	1.01	0.90	0.94	1.08	0.91	0.97	NR	0.99	0.94	7
DMPK-B														
20	0.93	0.88	0.88	1.06	1.14	1.25	2.02	10.83	1.95	1.17	NR	1.28	2.13	137
45	1.09	1.03	1.04	1.76	1.14	1.12	3.68	6.41	3.79	1.44	NR	2.60	2.28	75
70	1.05	1.16	1.13	1.45	1.23	0.97	5.88	5.74	5.47	1.46	NR	6.89	2.95	83
BondElut														
20	0.60	0.81	0.84	0.34	0.99	1.01	0.53	0.48	0.46	0.78	NR	1.00	0.71	34
45	0.86	1.00	1.04	0.36	1.07	0.85	0.63	0.45	0.64	0.96	NR	1.03	0.81	31
70	0.87	1.09	1.12	0.80	1.03	0.96	0.96	0.77	0.84	0.93	NR	0.98	0.94	12

HCT: Hematocrit; NR: No result.

Table 11. Comparison of center/perimeter response ratios in spots obtained from 14-day-old and fresh wet blood[†].

HCT	Card type	1	2	3	4	5	6	7	8	9	10	11	12	Average	%CV
45	226	141	94	93	144	114	94	126	108	108	NC	NC	NC	114	17
45	DMPK-A	89	100	101	117	NR	157	122	94	113	NC	NC	NC	112	19
45	DMPK-B	300	103	102	107	99	120	112	80	94	NC	NC	NC	124	54
45	BondElut	69	92	90	105	102	127	86	111	90	NC	NC	NC	97	17

[†]Center/perimeter response ratios in the table are expressed as (response in center punch from blood spot obtained from 14-day-old wet blood/response in perimeter punch from the same blood spot obtained from 14-day-old wet blood)/(response in center punch from blood spot obtained from fresh wet blood/response in perimeter punch from the same blood spot obtained from fresh wet blood) × 100.
HCT: Hematocrit; NC: Not conducted; NR: No result.

also reported by O'Mara *et al.* for 226, DMPK-A and DMPK-C cards [7], thus our results are in line with their findings. The DMPK-A card showed the smallest variation in C/P response ratio across compounds and hematocrits.

For the 226 and BondElut cards, the variation in C/P response ratio across different compounds was smallest at Hct 70%, but these card types showed quite a large variation in C/P response ratio across different compounds at hematocrits Hct 45 and 20%.

On DMPK-B cards, the C/P response ratio was >1 for most of the compounds and hematocrits, with a few compounds having a rather extreme value for the C/P response ratio, thus showing a large preference of the compounds not to migrate to the perimeter. Also these findings are in line with O'Mara *et al*'s findings [7] who also reported C/P ratios >1 for DMPK-B cards. In contrast with the other card types, DMPK-B cards form a halo around the main blood spot. For compounds 1–6, the perimeter punch was done close to the edge of the main blood spot, whereas for compounds 7–12, the perimeter punch was done in the halo surrounding the spot. Most of the compounds punched from the halo showed a very high C/P ratio, indicating that not much of the compound is migrating into the halo. However, compound 10 was an exception with a C/P ratio of 1.2–1.5, thus showing significant migration into the halo. This result is not surprising, as compound 10 is the most water soluble of all compounds tested and does not significantly bind to plasma proteins. It is expected that compounds with these characteristics follow the migration of water in the blood spot.

For some compounds and card types, there is a noticeable difference between the C/P response ratios when stored 14-day-old wet blood is used instead of freshly obtained blood. For some compounds a decrease is observed, but more common is an increase in the C/P response ratio, which implies that the compound has a decreased tendency to move towards the perimeter of the spot obtained from 14-day-old wet blood as compared with fresh wet blood.

The consequence of these findings for the use of DBS in regulated bioanalysis is that spot nonhomogeneity increases the variability of a dried blood spot assay. In order to limit the variability of the assay as much as possible, investigation of spot homogeneity on different card types over a range of hematocrit levels during the development of a DBS assay can be considered. The card type showing a C/P ratio closest to 1 and with the most consistent C/P ratio across different hematocrits could be chosen. Also, it is advised to use a punch size that is large enough and punches the majority of the spot. A way to completely eliminate the influence of spot nonhomogeneity on the assay would be to punch the complete spot [6], but this approach would require sufficient control over the accuracy of the spotted blood volume. This is a risk, particularly for large multisite clinical studies.

Conclusion

Our experiments confirm literature data that the hematocrit changes affect the absolute response found for the spiked compounds when a partial punched spot is presented for bioanalysis. The sample viscosity is higher at higher hematocrit, thus influencing both the spot size and the actual blood volume punched. The hematocrit can also influence extraction recovery and spot homogeneity, and is compound-dependent. The extraction recovery can decrease with aging of spots, sometimes in a hematocrit-dependent way.

As a result, an increased variability in the assay performance is observed, sometimes beyond the current applicable acceptance criteria for validated assays in regulated bioanalysis [18,104]. The largest impact is expected for assays that need to cover a large range of hematocrits, such as for blood spots obtained from neonates where hematocrit values can vary over a broad range (31–67%) [16]. However, many of the observed fluctuations still remain significant, and are thus also relevant for hematocrit values from 36–50% that are more typical for a healthy adult female and male population.

It has to be noted that the experiments did not account for inter-patient variation of the components in blood that could possibly also influence spot size to some degree (e.g., due to variation in total protein content and white blood cell count). Also, it has to be noted that the general use of DBS, such as in newborn screening, is complicated further by the fact that sometimes not single spots but multiple, nonconcentric drop spots are submitted. This is a further complication to the use of DBS in clinical trials.

The hematocrit could also have an effect on measured analyte concentrations in wet matrices such as plasma and serum, in particular for compounds with a blood/plasma ratio significantly different from 1. The hematocrit effect in plasma assays is not a bioanalytical issue though, as the accuracy of the plasma assay as

such is not dependent on the hematocrit. Still, this effect is important and PK and TK scientists should be aware of this effect. On the other hand, for dried blood spot assays the hematocrit does affect the accuracy of the assay.

During development of a dried blood spot assay, sample extraction conditions can be optimized to obtain a consistent recovery at different hematocrits, both on freshly prepared blood spots and on aged blood spots. Various card types can be tested for spot homogeneity, extraction recovery and accuracy and precision at different hematocrits, and the card type that shows most consistent results across the range of hematocrits required for the assay could be selected.

In order to eliminate the influence of the hematocrit on the amount of blood analyzed if only part of the spot is punched, a constant accurately pipetted amount of blood could be spotted for each sample and the whole blood spot could subsequently be punched [6]. However, this would require sufficient control over the accuracy of the blood volume spotted. While this may be achievable for the majority of preclinical studies, it will be difficult to assure in most clinical studies, especially in case of multicenter patient trials or in case of therapeutic drug monitoring. In case of punching the entire blood it still needs to be assured that the absolute recovery is the same at different hematocrits in both fresh and aged DBS.

We recommend that in the validation of dried blood spot assays, multiple additional parameters are evaluated beyond what is required for the validation of an assay of a liquid sample. It is advisable to test the accuracy and precision of the method at different hematocrit values over the range of hematocrit values for which the assay is intended to be used. This experiment will show the combined influence of spot size, spot homogeneity and absolute recovery at different hematocrits on accuracy and precision. Also, it is advised to assess long-term stability at different hematocrits as absolute recovery can decrease in aged DBS in a hematocrit-dependent way.

Finally, we look forward to the development of easy-to-use tools that could guarantee the accuracy of spotted blood volumes in areas where sampling of accurate volumes is less obvious, such as multicenter patient trials. In addition, further innovations in the card technology that would decrease or even eliminate the hematocrit issue on DBS would be very welcome. These, and potentially other innovations and improvements, will be needed to allow DBS to develop towards a routine technology for regulated bioanalysis.

Future perspective

The effect of hematocrit is a significant hurdle for the use of DBS for regulated bioanalysis. During development of a dried blood spot assay, sample extraction conditions can be optimized to obtain a consistent extraction recovery at different hematocrits, both on freshly prepared and on aged blood spots. Still, the hematocrit has an effect on the size of the blood spot that affects the response, unless the whole blood spot is punched. Whole blood spot punching requires the spotting of an accurate blood volume. For the moment, the use of DBS methods may be limited until manufacturers are able to develop easy-to-use tools that can guarantee the accuracy of spotted blood volumes, which would allow punching of the whole blood spot. Nevertheless, also in the latter case, additional consideration is needed on the compound dependent effects of hematocrit and aging of the spots on extraction recovery variability. Going forward, the EBF considers DBS as a developing technology, and awaits further innovations and improvements to better balance the advantages of the technique versus its current limitations.

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Disclaimer

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Ethical disclosure

For investigations involving human subjects, informed consent has been obtained from the participants involved. The participants gave written approval to use the donated blood for the research conducted and described in the current paper.

Executive summary

- The relationship between hematocrit and response, spot homogeneity, spot size, extraction recovery and card type was investigated for 12 different compounds.
- The relationship between hematocrit and the age of the wet blood, as well as the age of the dried blood spot itself, was also investigated.
- Hematocrit changes affect spot size, and as a consequence also the absolute response (in case of partial spot punching).
- Hematocrit can also influence extraction recovery and spot homogeneity in a compound-dependent way.
- The extraction recovery can decrease with aging of spots, and the decrease can be hematocrit-dependent.
- As a result, increased variability in assay performance of dried blood spot assays is observed in comparison with wet matrix (plasma) assays, sometimes beyond the current applicable acceptance criteria for validated assays in regulated bioanalysis.

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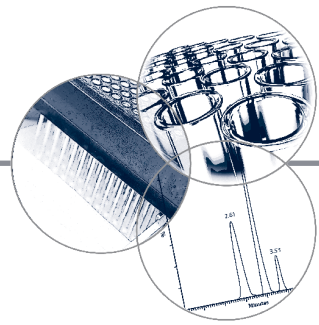
RESEARCH ARTICLE
MINI FOCUS ISSUE: DRIED BLOOD SPOTS



In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium

Background: At the start of their work, the European Bioanalysis Forum dried blood spots microsampling consortium did not form a dedicated team to investigate the spot homogeneity. However, two teams performed experiments that produced results relating to sample homogeneity. **Results:** The data, which were produced via two different approaches (a radiolabeled and a nonradiolabeled approach), are highly complementary and demonstrate clear effects on sample inhomogeneity due to the substrate type, compound and hematocrit levels. **Conclusion:** The results demonstrate that sample inhomogeneity is a significant hurdle to the use of dried blood spots for regulated bioanalysis that should be investigated further in the method establishment phase if the whole spot is not sampled.

The European Bioanalysis Forum (EBF) dried blood spots (DBS) microsampling consortium was formed in June 2011 with the aim of pooling the resources of pharmaceutical companies and contract research organizations interested in the development and understanding of DBS technologies. Specifically, the aim of the consortium was to enable research into the four major hurdles, identified in the EBF recommendation paper published in August 2011 [1] to be barriers to the implementation and use of DBS in regulated quantitative bioanalysis. The four major hurdles identified were: effect of hematocrit; addition of IS; stability of blood and cards; and mechanisms for dilution of samples. For each, a cross-company team was formed to design and conduct experiments that would enable a deeper understanding of each of these hurdles and hopefully lead to recommendations for overcoming them. The consortium recognized sample homogeneity as an additional hurdle, but as the experiments conducted in relation to two of the aforementioned hurdles would also unveil spot inhomogeneity, a dedicated team was not established to investigate this. Both the hematocrit and the stability team designed experiments that encompassed an element of investigation into the homogeneity of a dried blood sample. It is these data that will be discussed in this article.

For any quantitative bioanalytical technique to be considered accurate, it is important that the portion of sample removed for analysis is representative of the entire sample. For standard liquid biological samples this involves careful mixing and removal of an accurate volume of sample with a pipette. One of the major perceived benefits of DBS is the simplicity of the sample collection compared with plasma. The simplicity of this technique relies on the DBS sample being homogeneous in terms of both the spotted blood and the analyte contained within it, as well as that the blood spot area is directly proportional to the volume of blood spotted on the card with no change in the density of the blood or analyte per unit area. This enables the spotting of an approximate volume of blood onto a card followed by punching out a portion of the sample for analysis. This process is highly advantageous over the alternative, which includes spotting an accurate volume and extracting the entire spot. Spotting accurate volumes probably requires significant training and risk, particularly for a multisite clinical study. However, to achieve this subsampling approach, it is important that the DBS sample is homogeneous.

It has been observed during the formation of a DBS sample that the wet blood that is spotted onto the substrate and the analytes contained within it may undergo a chromatographic effect as the blood adsorbs or spreads onto the substrate. This can lead to a nonhomogeneous sample and it has been documented in the literature on a number of occasions [2–5] that the measured analyte concentrations can, in some cases, be significantly different between the central and peripheral areas of a DBS sample.

The homogeneity experiments reported in this manuscript were conducted by the two

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Table 1. Physicochemical parameters of compounds included in the nonradiolabelled assessment.

Property	Compound number											
	1	2	3	4	5	6	7	8	9	10	11	12
cLogD (pH 7.4)	4.4	2.1	2	0.4	3.5	>4.4	5.4	4.7	2.1	-0.4	~20	6.9
pKa	4.4†; 8.1‡	2.4; 8.5	9.2	5.2†	12‡	2.5; 5.2	5.6‡	1‡; 9‡	3.1‡; 8.2‡	2.2 (COOH), 9.1 (NH_2), 10.1 (OH)	>20	4.5†
Blood/plasma distribution	0.61	0.7	0.8	0.67	1.0	Unknown	0.68	0.7	0.72	Unknown	Unknown	Unknown
PPB	98	Unknown	Unknown	97	89–98	Unknown	99.7	Unknown	90	Unknown	Unknown	Unknown
MW	442	470	450	270	854	750	366	555	410	181	884	304

†*Acidic group.*
‡*Basic group.*
PPB: Plasma protein binding.

multisite teams. The two experiments were designed with different objectives in mind, and as a result different methods of measuring the homogeneity were used. However, the data are highly complementary and provide a greater understanding of the effect of hematocrit, the age of control whole blood and homogeneity of DBS samples across a range of different substrates (card types) with a range of analytes with varying physiochemical properties.

The aim of the hematocrit team was to determine whether the hematocrit levels in a blood sample affected the distribution of a drug within the DBS sample. To do this, the team used well-established, validated or qualified conventional LC–MS/MS methods to measure nonradioactive compounds, referred to in this article as the nonradiolabelled approach. Homogeneity was assessed by comparing the measured concentrations of analyte in punches taken from the central and peripheral areas of the DBS sample [6]. This assessment was performed over a wide chemical space; molecular weights varying from 181 to 854 Da; a pKa ranging from 1 to >20; blood:plasma ratios ranging from 0.61 to 1.0; and Log P values ranging from -0.4 to approximately 20.

The stability team set out to establish a range of recommendations for storage, drying and age of the control (wet) blood to enable users to differentiate what might be genuine stability issues from sample handling issues. As a result, they determined how long control (wet) blood could be stored for and still used reliably for preparing calibration standards and QC samples (the data for which will be included in a later publication). As part of this investigation, they performed an experiment to assess the homogeneity of DBS samples with increased age of control (wet) blood. The team used a nonconventional approach, referred to in this paper as the radiolabelled approach, where radiolabelled compounds were spiked into control blood, spotted and the distribution of radioactivity measured across each spot.

The data obtained from both of these experiments are presented and discussed in this article.

Materials & methods

Analytes

For the nonradiolabelled approach, 12 small-molecule compounds were selected covering a wide chemical space with a variety of log P and pKa values. The compounds that are included in

the tests are listed in Table 1, together with some of their physicochemical parameters.

For the radiolabelled approach, two radiolabelled compounds, ^{14}C-lacosamide and ^{14}C-deoxy-D-glucose, were selected. Both compounds have a blood plasma ratio close to one. The structures and physicochemical parameters are represented in Figure 1 and Table 2, respectively.

Matrix

Control blood was obtained by each site according to local rules on ethics and informed consent.

Substrates

Both teams used the following DBS cards: Ahlstrohm 226 (Perkin Elmer, MA, USA); Bond Elut DMS (Agilent, CA, USA); FTA DMPK-A (formally FTA, GE Healthcare, UK); and FTA DMPK-B (formally FTA-Elute, GE Healthcare). In addition, the radiolabelled approach was also tested on the FTA DMPK-C substrate (GE Healthcare). The DMPK-A and DMPK-B cards are treated substrates and according to the vendor contain proprietary chemical mixtures that lyse cells and denature degradative enzymes. The other three card types are untreated substrates.

Nonradiolabelled approach

A standardized way of preparing control blood with varying hematocrit values was used by all participating companies. A fresh blood sample was taken, mixed and the hematocrit was measured in duplicate. Following this, the volume of plasma to be added or removed to obtain each of the target hematocrit values (20, 45 and 75%) was calculated. Subsequently, the required plasma volume was added to blood samples for which the hematocrit had to be decreased. The blood that required plasma removal to increase the hematocrit was spun for 10 min at 1300 g and the required plasma volume was removed. The blood samples prepared in this way were gently roller-mixed for 30 min. The hematocrit of each of the samples was finally measured in duplicate to confirm that the target hematocrit was obtained. The blood samples at each hematocrit value were spiked with one or multiple analytes [6].

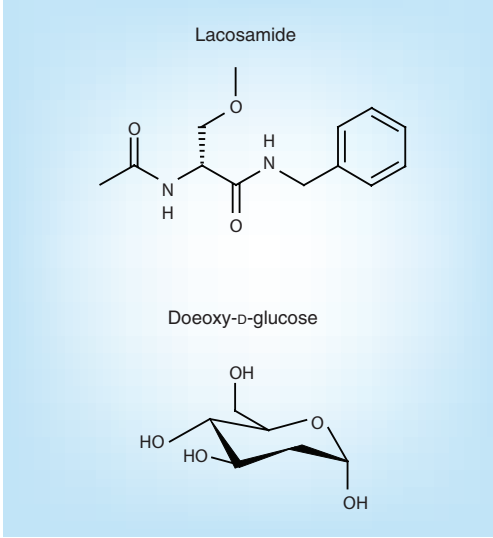

Figure 1. Compounds included in the radiolabelled assessment.

To prepare the samples, human whole blood at 20, 45 and 70% hematocrit was spiked with one or more of the 12 compounds. The amount of solvent added to the blood during spiking consisted of <2% of the blood volume. Spiked blood (25 µl) was spotted onto DMPK-A, DMPK-B, Ahlstrom 226 and Bond Elut cards, and a 1-mm diameter punch was taken from the center and the perimeter of the spot. The spots were then extracted and analyzed by LC–MS/MS and the concentration, peak area or peak area ratio data are used as appropriate.

The experiments were performed at six different sites with each site producing data for at least one or more compounds. All experiments were conducted at a single concentration with n = 3 spots analyzed per card type for each compound [6]. As a QC, one appropriate test sample was injected in triplicate at the beginning,

Table 2. Physiochemical parameters of compounds included in the radiolabelled assessment.

Compound name	Molecular properties				
	cLogD (pH 7.4)	pKa	Blood/plasma distribution	PPB	Molecular weight
Lacosamide	0.91	~14	~1	Unknown	250
Deoxy-D-glucose	0.73	~15	~1	<15%	164

PPB: Plasma protein binding.

Table 3. Center/perimeter response ratios of dried blood spot samples with variation of hematocrit for 12 analytes on four substrate types for 12 analytes analyzed at one of six laboratories[†].

HCT%	1	2	3	4	5	6	7	8	9	10	12	Average	%CV
226													
20	0.52	1.04	1.01	0.58	0.83	1.05	0.73	0.68	0.73	0.84	0.54	0.78	25
45	0.44	0.94	0.95	0.53	0.76	1.22	0.60	0.71	0.70	0.86	0.76	0.77	28
70	0.93	0.96	0.92	0.98	0.81	0.96	0.91	0.93	0.89	0.96	0.90	0.92	5
DMPK-A													
20	0.70	0.95	0.93	0.81	0.88	0.97	0.77	0.79	0.86	0.80	0.75	0.84	11
45	0.74	0.86	0.88	0.83	0.87	0.83	0.73	0.91	0.77	0.84	0.83	0.83	7
70	0.80	0.94	0.94	0.91	1.01	0.90	0.94	1.08	0.91	0.97	0.99	0.94	7
DMPK-B													
20	0.93	0.88	0.88	1.06	1.14	1.25	2.02	10.83	1.95	1.17	1.28	2.13	137
45	1.09	1.03	1.04	1.76	1.14	1.12	3.68	6.41	3.79	1.44	2.60	2.28	75
70	1.05	1.16	1.13	1.45	1.23	0.97	5.88	5.74	5.47	1.46	6.89	2.95	83
Bond Elut													
20	0.60	0.81	0.84	0.34	0.99	1.01	0.53	0.48	0.46	0.78	1.00	0.71	34
45	0.86	1.00	1.04	0.36	1.07	0.85	0.63	0.45	0.64	0.96	1.03	0.81	31
70	0.87	1.09	1.12	0.80	1.03	0.96	0.96	0.77	0.84	0.93	0.98	0.94	12

[†]*Center/perimeter ratio was determined by comparing the analyte response by LC–MS/MS for a 1-mm-diameter punch taken from the center and periphery of the dried blood spot sample derived from 25 µl of human blood.*
No data are available for compound **11**.
HCT: Hematocrit.

middle and end of the run. The coefficient of variation over the nine values was required to be ≤15%, and the mean of the last injections versus the mean of the first injections were required to differ by no more than 15%.

■ Radiolabelled approach

A radioactive compound (^{14}C-lacosamide or ^{14}C-deoxy-D-glucose) was added to the control (wet) blood at 2200 and 1400 ng/ml (prepared by adding 10 µl of radiolabelled solution to 2 ml of blood), respectively, on the day of blood collection. Lacosamide was added to mouse blood, while a second site added deoxyglucose to human and rat blood. Both sites compared heparin and EDTA anticoagulants. Then, four replicates of 15 or 30 µl of blood was spotted onto DMPK-A, DMPK-B, DMPK-C, Ahlstrom 226 and Bond Elut cards, on day 1. The spiked mouse blood was also spotted (n = 4 per card type) on day 2, 3, 4, 5, 8, 10 and 15. Once dry (2 h at room temperature) the radioactivity across the spots was measured. The measurement of ^{14}C-lacosamide was performed every 0.425 mm across the diameter of the spot using a Fuji BAS-5000 with a Fuji SR 2025 phosphorus screen (Japan). ^{14}C-deoxy-D-glucose was measured on a GE Healthcare Typhoon FLA7000 scanner at 50-µm resolution following exposure to phosphor screens (BAS-SR, Fuji Photo Film, Japan) for approximately 24 h. The hematocrit levels were not measured for any of the control matrices, however the human blood was obtained from a healthy volunteer and the rat and mouse hematocrit levels are expected to be typical for hematocrit concentrations in healthy laboratory rats and mice.

Results & discussion

■ Nonradiolabelled approach

The data obtained via the nonradiolabelled approach, was carried out across six different sites, using a total 12 different compounds from a variety of chemical classes, with at least one or more compounds assessed at each site.

The homogeneity between the center and the perimeter of each spot has been plotted against the blood hematocrit level for each individual compound, on each card type (Table 3). Homogeneity was calculated from the analyte response (peak area ratio of the analyte to its corresponding IS) of the center punch, divided by the analyte response of the perimeter punch (C/P ratio). Values below one indicate that the analyte was observed at a higher concentration

in the perimeter punch. Values greater than one indicate that the analyte was observed at a higher concentration in the center punch.

For most compounds at all three levels of hematocrit, the C/P ratio on DMPK-A, 226 and Bond Elut cards is less the one. This indicates that in the majority of cases these cards types result in analyte concentrations that are higher in the peripheral punch, than the central punch. Of these three card types, DMPK-A shows the smallest variation in the data obtained from the 12 different compounds and between the three different hematocrit levels, indicating that the hematocrit and compound type have less effect on the homogeneity of the sample than the card itself. Both the 226 and Bond Elut cards show extensive variation (>50%) between the 12 different compounds demonstrating that, for these cards both the card type and the compound itself have a notable effect on the homogeneity of the DBS sample. The level of hematocrit also affects the homogeneity for these card types with an observed improvement in homogeneity being seen on both cards for nearly all compounds when there is 70% hematocrit, which is supported in the literature [2].

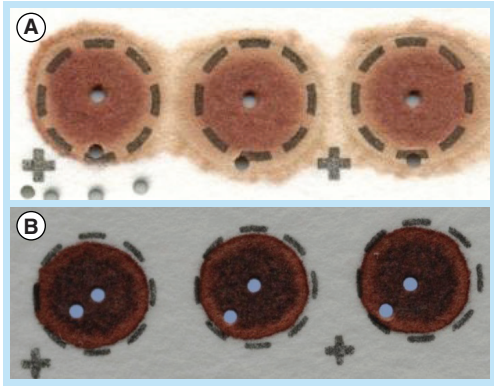

Figure 2. Photographs showing the location of the 1-mm-diameter punches taken from dried blood spot samples of 25 µl of human blood on FTA DMPK-B substrate. (A) Peripheral punch taken from the halo; and **(B)** peripheral punch taken from the main body of the spot.

The homogeneity data obtained for DMPK-B has C/P ratios notably greater than one. There is also extensive variation in homogeneity between the different compounds investigated. Values greater than one indicate that analyte concentrations are higher in the central punch than the peripheral punch. For some compounds

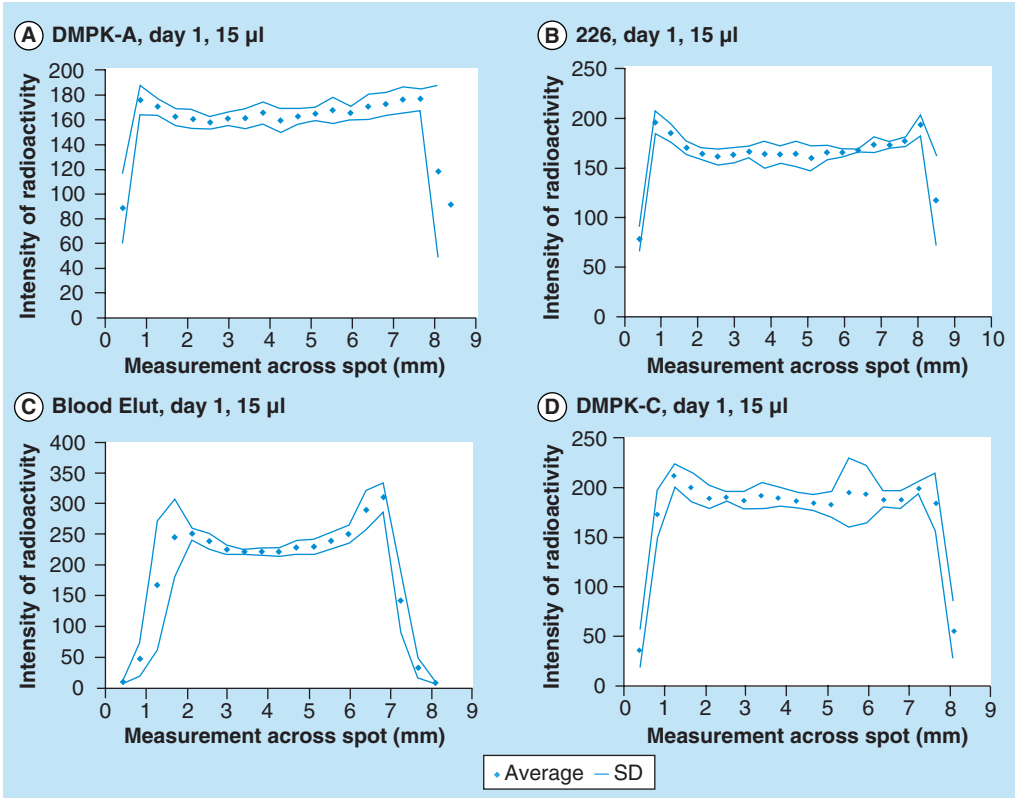

Figure 3. Average measure of radioactivity from four dried blood spot samples for 14C-lacosamide, in 15-µl dried blood spot samples derived from mouse blood. (A) DMPK-A substrate; **(B)** 226 substrate; **(C)** Bond Elut substrate; and **(D)** DMPK-C substrate.

Table 4. Average measure of intensity of radioactivity across the diameter of four dried blood spot samples on each substrate.

Distance across spot (mm)	DMPK-A	DMPK-C	226	Bond Elut	DMPK-B
0.425	0.0	0.0	0.0	0.0	7.8
0.85	0.0	0.0	78.2	0.0	30.5
1.275	88.6	36.2	196.4	9.4	66.2
1.7	175.8	173.9	185.5	47.0	77.2
2.125	170.3	212.5	170.5	167.2	92.0
2.55	162.3	200.0	164.4	244.4	100.7
2.975	160.6	190.3	161.3	250.4	107.6
3.4	157.6	190.8	163.2	238.2	111.6
3.825	160.8	186.9	166.5	224.4	115.4
4.25	161.1	191.9	164.1	220.9	117.7
4.675	165.6	190.6	163.6	221.4	119.3
5.1	159.5	187.6	164.5	221.1	118.9
5.525	162.7	184.9	159.9	228.0	119.7
5.95	164.8	182.8	166.0	229.5	121.6
6.375	167.7	194.8	165.6	239.0	117.9
6.8	165.4	193.1	168.3	250.1	116.8
7.225	170.3	188.2	173.8	289.5	113.1
7.65	172.8	188.1	173.4	310.1	106.3
8.075	176.2	200.0	177.3	141.6	96.8
8.5	176.5	185.3	193.6	32.1	83.1
8.925	118.2	55.0	117.1	7.6	69.2
9.35	91.3	0.0	15.8	0.0	35.0
9.775	0.0	0.0	0.0	0.0	10.0
10.2	0.0	0.0	0.0	0.0	4.3

and hematocrit levels there is a large difference in analyte response between the central and peripheral punch locations, such that in one case, a peripheral punch, yields a tenfold less concentrated sample than the center punch.

The formation of a halo (a paler area around the outside edge of the DBS sample) is the most notable difference between spots obtained on DMPK-B cards and spots obtained on all other cards. Of the six sites conducting these experiments, two sites sampled the peripheral punch from the halo (Figure 2A; Table 1; 1–6) while four sites sampled the peripheral punch from the outer edge of the main body of the spot (Figure 2B; Table 1; 7–12). By sorting the compounds based on the location of the peripheral punch, it can be noted that those compounds, for which the peripheral punch was taken from within the main body of the spot, have a reasonably consistent homogeneity between the different hematocrit levels. The exception to this is compound 4, which has a tendency towards a slightly higher concentration of analyte in the central punch. For those compounds where the peripheral punch was taken from the halo, the results are the most variable, clearly demonstrating that in general (with one exception) compounds do not distribute homogeneously between the main body of the spot and the halo. Compound 10, a small and highly polar compound, is the exception to this observation. This result is not surprising as this compound is the most water soluble of all compounds tested and does not significantly bind to plasma proteins. It is possible that compounds with these characteristics follow the migration of water in the blood spot into the halo.

It is also interesting that three of the four compounds that do not distribute homogeneously into the halo, have increased homogeneity when the hematocrit level is 20%.

The nonradiolabelled assessment was performed over a wide chemical space, as detailed in Table 1. However, the data are too variable on the majority of cards to be able to link the observed homogeneity to the physicochemical properties of the compounds used. It is clear from these data that the distribution of the spiked compound across the blood spot is not homogeneous and that the card substrate, compound and hematocrit level can greatly affect the extent of the homogeneity. This confirms sample homogeneity in DBS as a major hurdle for which methods of overcoming must be examined in greater detail.

■ Radiolabelled approach

A typical example of the data obtained from the radiolabelled assessment is represented in Figure 3A. The plot shows average data from four blood spots, prepared by spiking fresh control wet mouse blood with ^{14}C-lacosamide and spotting 15 µl onto a DMPK-A card. The enclosing lines represent the standard deviation seen within the four sets of data. It can be clearly observed from this profile that a 'volcano'-shaped graph is obtained, with lower amounts of radioactivity measured in the center than the edge of the spot. Therefore, ^{14}C-lacosamide concentrations are lower in the center of the spot than the periphery. These data confirm the finding from the nonradiolabelled approach and that reported in the literature [2,4]. Both the 226 and Bond Elut cards show similar trends with a higher concentration at the perimeter of the spot than the center (Figure 3B & C). However, the dip in the center of the volcano is more pronounced on these two substrates, which may be the cause of the extensive variation seen between the different

molecules on these cards in the nonradiolabelled assessment. DMPK-C was also seen to produce a volcano effect using the radiolabelled assessment. The numerical data used to prepare the graphs in Figure 3 are presented in Table 4, along with the data for the DMPK-B cards.

A similar pattern of distribution was observed for DMPK-A, DMPK-C, 226 and Bond Elut substrates when ^{14}C-deoxy-D-glucose was added to freshly obtained rat and human wet blood, with 15 or 30 µl spotted onto the cards. With this analyte there did appear to be some slight changes in homogeneity for different species, blood spot volume or anticoagulant on DMPK-A, DMPK-C and 226 cards. There was also a much more pronounced volcano effect for rat blood than human blood (Figure 4) on the Bond Elut cards, demonstrating that in some cases the species of control matrix, in addition to the substrate and analyte, can have a significant effect on the homogeneity.

The distribution of ^{14}C-lacosamide across the DMPK-B cards is notably different to those of the other card types, with analyte distribution being almost Gaussian across the spot diameter (Figure 5). Higher concentrations of this analyte were observed at the center of the spot than the perimeter, potentially due to a strong interaction with the substrate, which prevents the analyte from spreading. This also supports the data from the nonradioactive approach and from O'Mara *et al.* [3]. A photograph of the spots used to obtain this data is presented in Figure 6. The data outside the two vertical lines in Figure 5 are representative of the halo, while anything within the lines can be considered as the main body of the spot. In this case approximately five- to six-fold more ^{14}C-lacosamide is measured in the center of the spot than the in halo. In view of these data, it is possible that compound-to-compound differences seen in the nonradioactive data could be as much due to the location of the peripheral punch as the compound itself.

A similar pattern of distribution was observed for the DMPK-B substrate, when ^{14}C-deoxy-D-glucose was added to freshly obtained rat and human wet blood and 15 or 30 µl spotted onto the cards. It was possible to observe small changes in the ratio of the radioactivity measured at the center and periphery of the spots, for different anticoagulants in human DBS samples (Figure 7).

For the ^{14}C-lacosamide data no difference in homogeneity was observed, when spotting 15 or 30 µl, or when using heparin or EDTA as the anticoagulant, for any of the substrates types.

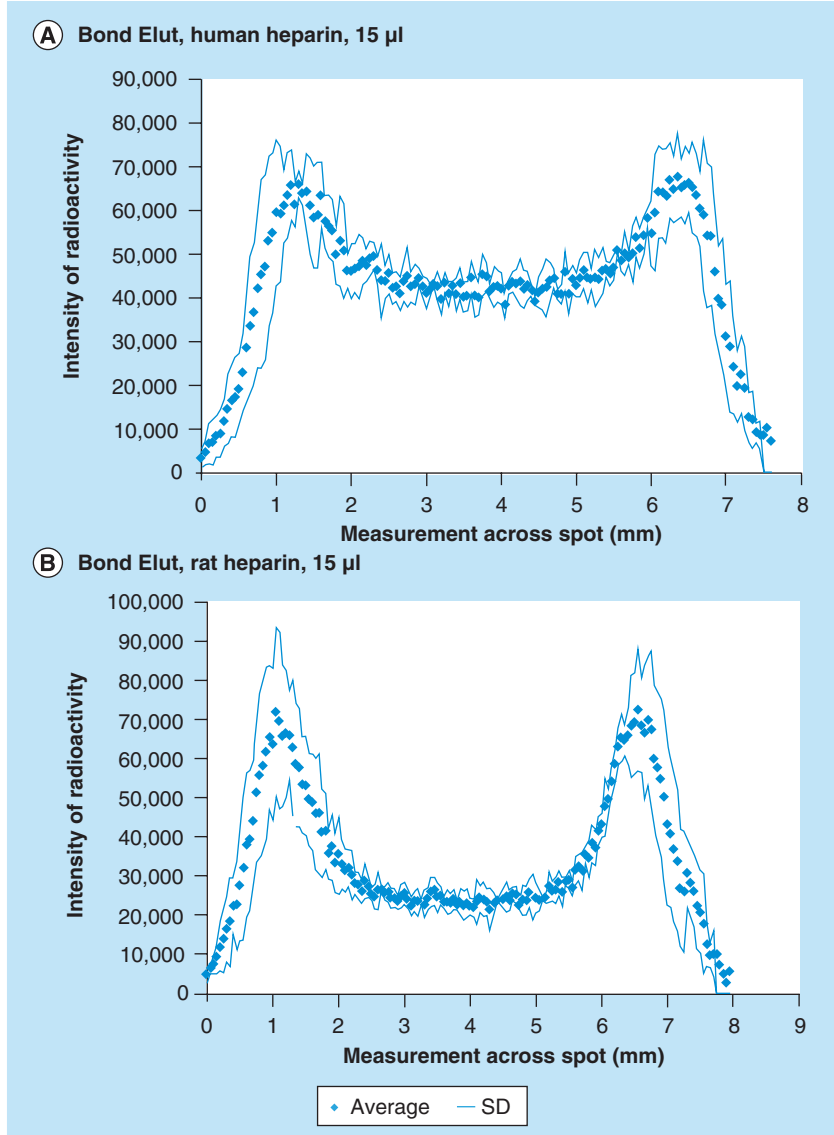

Figure 4. Measure of radioactivity across four 15-µl dried blood spot samples on a Bond Elut substrate, using 14C-deoxy-D-glucose as the analyte spiked in blood. (A) Human heparin blood; and **(B)** rat heparin blood.

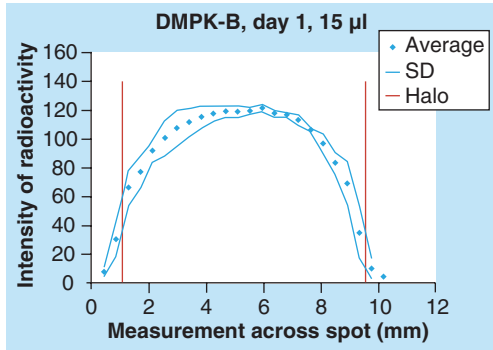

Figure 5. Average measure of radioactivity from four dried blood spot samples for ^{14}C-lacosamide, in 15-µl dried blood spot samples derived from mouse blood on DMPK-B substrate.

As for the nonradiolabelled approach, it is clear that the distribution of the spiked compound across the blood spots is not homogeneous and that the card substrate in particular affects the extent of the homogeneity; again confirming sample homogeneity in DBS as a major hurdle.

Figure 6. Photograph of corresponding DMPK-B dried blood spots for Figure 4.

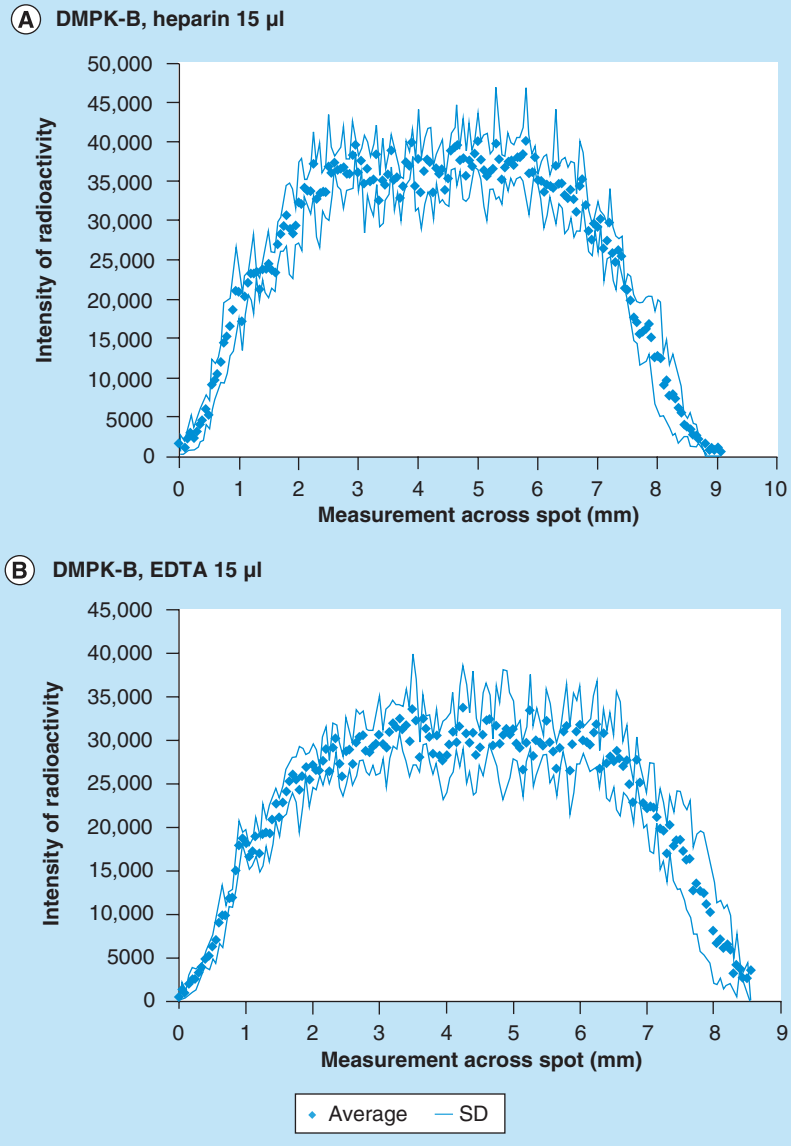

Figure 7. Measure of radioactivity across four 15-µl DBS samples on a Bond Elut substrate, using ^{14}C-deoxy-D-glucose as the analyte spiked in blood. (A) Human heparin blood; and **(B)** human EDTA blood.

Conclusion

In conclusion, radiolabelled and nonradiolabelled approaches to measuring the homogeneity of DBS samples have produced highly complementary data, which also supports the results reported in the literature. A volcano effect in distribution of the analyte (lower analyte concentrations in the center of the spot than at the edge) was observed for DMPK-A, DMPK-C, 226 and Bond Elut cards using the radiolabelled approach. A similar effect was observed for DMPK-A, 226 and Bond Elut cards using the nonradiolabelled approach. Both methods also illustrated that DMPK-B cards exhibit higher analyte concentrations at the center of the spot.

It can be concluded from these data that the degree of nonhomogeneity across a DBS sample is affected by a number of factors, including, but not limited to: card substrate type, compound and hematocrit levels; in control blood. DMPK-A card types appear to be less affected by compound and hematocrit levels than the other cards, there is still a degree of nonhomogeneity observed across this card type that will need to be managed appropriately during method establishment, to allow the method to provide reliable and reproducible concentration values.

It is also worth noting that these assessments of homogeneity have been performed in analytical laboratories where it is possible to generate perfect spots due to the pipetting of control blood directly onto the card substrate. In a clinical setting it may not be possible to prepare these 'perfect' spots, which may also have an additional impact on the sample homogeneity.

In light of the fact that DBS samples are generally nonhomogeneous, for use in regulatory bioanalysis going forward, it will be necessary to investigate the homogeneity for any particular analyte, perhaps using a range of hematocrit levels, during method development, to ensure that these issues do not exist. Where nonhomogeneity exists, there is an expectation that the inaccuracy due to homogeneity, in addition to the other sources of inaccuracy in the method, should not add to the imprecision of the method beyond what is accepted as standard bioanalytical acceptance criteria. Approaches that may limit the effect of nonhomogeneity include punching the whole spot and taking it through the extraction procedure, although it is important to note that this has severe implications for the sample collection procedure that will require significant training and is a risk, particularly for large multisite clinical studies, as the sampling

must be accurate. Another approach is to take one punch per spot; punching from the same location in every sample. However this leads to a question of how the bioanalyst could ensure and prove that the punch is being taken from the same location in every sample. Another possibility might be to take a larger diameter punch, which may be a compromise between the two alternatives without the need for accurate spotting of the sample in a clinical setting.

Finally, our data demonstrate that spot nonhomogeneity can significantly affect the performance of a DBS method, particularly if the whole spot is not taken through the extraction. As such, the effect of any nonhomogeneity should be assessed during the method establishment.

Future perspective

DBS sample homogeneity may be a significant hurdle for the use of DBS for regulated bioanalysis and it should be investigated during the method establishment to ensure that the affect is overcome or compensated for where DBS methods that do not use the whole spot are employed. Going forward, EBF considers DBS as a developing technology and awaits further innovations and improvements to better balance the advantages of the technique versus its current limitations. In order to realize the full benefits of DBS, we require manufacturers to develop substrates that enable the formation of a homogeneous DBS.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Dried blood spot sample homogeneity was investigated using two different techniques that provided highly complementary data.
- Sample homogeneity was demonstrated to be primarily affected by the compound, card substrate and hematocrit levels.
- Sample homogeneity was also affected (to a lesser extent) by species and anticoagulant.
- Sample inhomogeneity is confirmed as a hurdle to the use of dried blood spots for regulated bioanalysis, which may be overcome or limited by taking the whole spot through the extraction procedure.
- As spot inhomogeneity could potentially affect the performance of a dried blood spot method, the sampling approach taken for analysis of dried blood spot samples should be assessed to ensure it compensates for any inhomogeneity observed, or the whole spot should be analyzed.

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▪▪ **Supporting paper investigating the hematocrit effect.**

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Bioanalysis

Ensuring the collection of high-quality dried blood spot samples across multisite clinical studies

Aim: The quality of quantitative analytical measurements is dependent on the quality of the sample collected, and dried blood spots (DBS) are no exception. As the use of DBS has matured into late-stage clinical drug-development studies, it has become apparent that a simple and straightforward approach in a controlled single-site, first-time-into-human clinic, does not always translate into multicenter clinical studies. Using synthetic blood, a method of training and assessing clinical laboratory staff has been developed to ensure the quality of sampling.**Methods:** A test kit comprising of synthetic blood, a pipetting aid, blank blood spot card, drying rack and training manual was sent to each clinical site for each technician to assess and approve prior to spotting PK samples. **Results:** The development of a DBS training kit along with a step-by-step guide has been successfully implemented. **Conclusion:** The training kit has been 100% successful across three large multisite clinical studies.

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Keywords:clinical bioanalysis • data quality • dried blood spot samples • training

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The use of the dried blood spot (DBS) sampling technique in the drug development process has been highlighted in recent years as an alternative to plasma [1,2], the preferred matrix for toxicokinetic and pharmacokinetic (PK) studies. The evolution from its initial use in neonatal screening [3,4] of DBS has been a combined effort of many scientists across the pharmaceutical industry, all focusing on a common goal and using their expertise to overcome the challenges faced, leading to a better understanding of its application in the drug development process [5]. This technology offers multiple advantages; preclinically, the most recognized advantage is ethical and relates to the 3Rs (replacement, reduction and refinement) in animal use, while clinically the reduction in sample volumes allows for pediatric PK assessments, simplified blood sampling, enrollment of critically ill patients and facilitates the collection of samples in remote locations and at home. Logistically, the use of DBS sampling delivers cost savings through ambient sample shipment and storage, particularly for multisite clinical trials. DBS does have many advantages, but also there are limitations especially with changing hematocrit for certain compounds and patient populations [6].

One of the issues we have observed during the performance of clinical studies using DBS sampling is the quality of the spotting (regardless of sampling technique i.e., heel prick, finger prick or venous cannula) particularly for late-phase multisite studies. Historically, DBS multisite studies were provided training materials in the form of a digital video disk (DVD) and an illustrated textual guide (Figure 1) [7]. In the first study supported by our laboratories where DBS samples were collected at multiple sites and across different continents, training materials were provided in the form of a DVD and an

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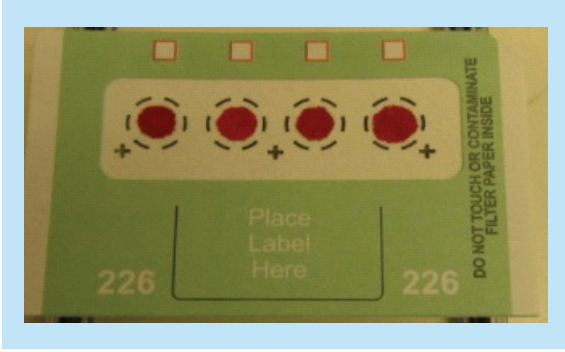

Figure 1. Valid DBS sample – correct spotting technique used.

illustrated textual guide. In that study, approximately 2150 DBS samples were supplied for analysis. However, due to the incorrect spotting technique (i.e., incorrect volume spotted, irregular spots outside the specified area and puncturing of the filter paper during spotting) used by the site technicians, approximately 700 of the samples could not be analyzed, equating to >30% of the study samples (Figure 2).

This study highlighted that even the simple instructions provided could be readily misinterpreted and that the current training procedure was inadequate. In many cases, the first attempt to spot blood by a healthcare provider was with the actual study sample, which is a major study risk. Sending analysts to train hospital staff all across the world is not a viable solution, so an alternative approach was sought to ensure higher quality samples were obtained in a reliable manner. It was identified that in addition to a guide on blood spotting, the clinical sites also needed to be able to practice their spotting technique and that an approval process was required for each technician, prior to spotting real samples to ensure that high-quality spots were received. Further, as control blood is not readily available to perform this practice, we developed a suitable surrogate blood matrix. This manuscript highlights that process.

Materials & methods/experimental

Falcon tubes (20 ml) were supplied by Corning Science (Mexico, SA). Condensed milk powder and red dye were supplied by Sigma Aldrich (Dorset, UK). Synthetic blood was spotted using 15 µl EDTA capillary tubes, supplied by Bibate limited (Daventry, UK) utilizing a microcap dispensing device (referred to as a pipette aid), supplied by Drummond Scientific Co (PA, USA). Blank blood spot cards (type dependent on validated method). Those illustrated were provided by Whatman (GE Healthcare, Maidstone, UK; FTA™ DMPK-A) and Perkin Elmer (MA, USA; 226). Drying racks were supplied by Whatman (GE Healthcare).

The DBS training kit

The kit is illustrated in Figure 3 and comprised a 20 ml falcon tube containing 1 g condensed milk powder and 0.5 g red dye (to make the synthetic blood), 250 × 15 µl EDTA capillary tubes, 1 microcap dispensing device (referred to as a pipette aid), three blank blood spot cards (filter paper type dependent on validated method), one drying rack and PK blood spotting instructions incorporated into the Biopacket/sample procedure manual. The training manual sent to clinical sites is included in Supplementary material.

Results & discussion

In developing the synthetic blood test solution, water and milk were initially tested. However, the consistency of the solution did not adequately mimic blood when spotted. It was found that using condensed milk powder with the addition of water provided the best match for blood and had the most similar characteristics. Red dye was also added to the milk powder to ensure the synthetic blood solution once spotted was visible. This DBS training kit was tested and validated internally by two colleagues who had never performed DBS sampling, to see if the kit was adequate and would provide sufficient instructions to a 'new' DBS user. Both users found the instructions easy to follow and were able to produce four acceptable spots per card. This demonstrated and gave confidence that this tool was a successful method to train and confirm blood spotting technique of new users.

The kits were subsequently used for multisite clinical trials where the sampling procedure was identical to that used previously. The initial pilot study consisted of 15 sites, spanning across the USA and Germany and involved multiple technicians. Blood sampling in all cases was by venous cannula of 250 µl into a 1-ml EDTA vacutainer tube and 4 × 15 µl aliquots accurately spotted utilizing glass capillaries onto the card. All sites received the DBS training kits prior to study's start and each technician was assessed and approved prior to spotting PK samples. All samples received from this study (2158 samples) were of good enough quality for analysis. Subsequently, the technique has been used for two further studies each with one site, which have also demonstrated high-quality spots (1890 samples). Notably, no technicians failed the evaluation across all three studies across 17 sites. Further, successful incurred sample reproducibility was obtained for the assay from these samples (Figure 5) [7]. This experiment is required by regulators for quantitative drug bioanalytical data and involves the repeat analysis of a proportion of samples and demonstration of the concordance of the repeated samples with that of the original. The results of this experiment clearly demon-

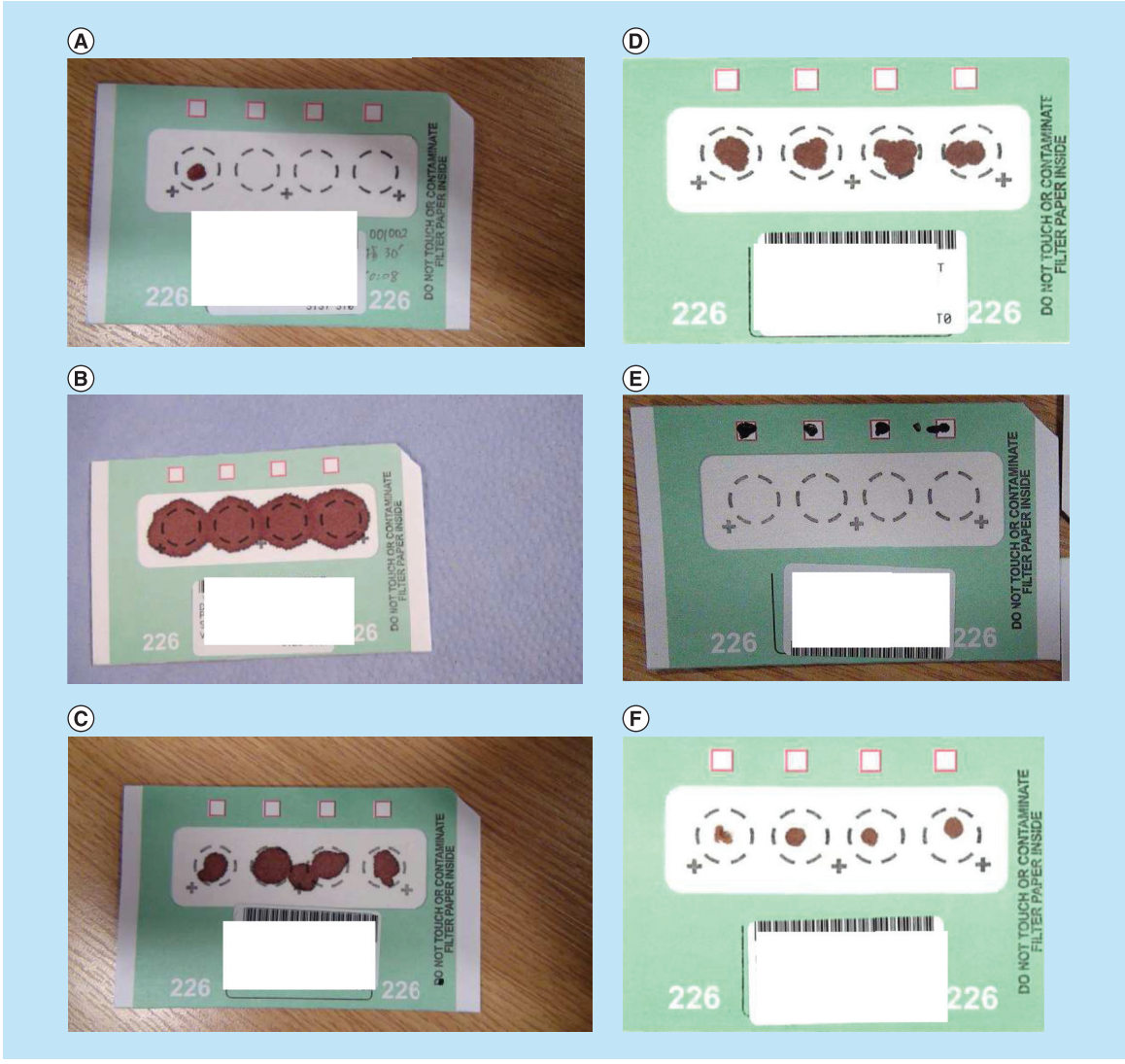

Figure 2. Invalid DBS samples. (A) Spot too small, likely incorrect volume spotted. Only one spot supplied, four spots are required per sample time point. **(B)** Spots too large hence overlapping and merged with the next spot. **(C)** Irregular spots and additional spot outside the marked area. **(D)** Irregular spots made up of multiple spots. **(E)** Spots placed in incorrect area **(F)** Card punctured during spotting and spots too small.

strate consistency between the replicate spots for the same sample on the same card. The use of a test kit has shown that technicians in different geographical locations can all receive the same training to ensure that high-quality PK blood spots are obtained when supporting large clinical multisite studies. Without this procedure in place, the use of DBS for late-stage clinical development would not be a viable option. Figure 4.

There is a possibility that DBS and other dried blood sampling approaches may facilitate the collection of blood samples in the home setting, rather than a central clinical facility, either by the patient themselves, or a healthcare provider. This would offer increased patient convenience and potential cost savings. Further, it may enable the collection of samples from patients in remote locations, or at times not currently possible with centralized testing. However, there is a risk that the quality of such samples may not be sufficient. Therefore, if this approach is to be used, it is imperative that the patient and/or healthcare provider receive/s high-quality training and if possible demonstrate their competence in the collection of high-quality samples using approaches similar to that outlined in this manuscript.

Conclusion

The use of DBS to produce high-quality concentration data for toxicokinetic and PK studies in drug development is highly dependent on the quality of the DBS provided for analysis. Previous experience with single-center studies had not highlighted the training of staff responsible for spotting the samples as one of

Figure 3. DBS training kit.

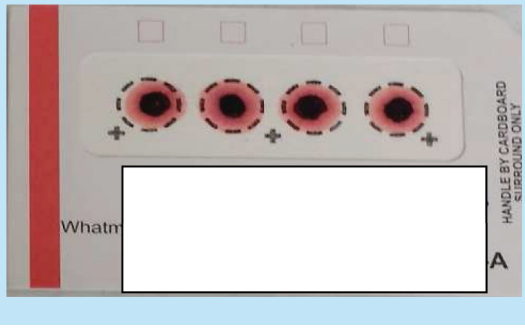

Figure 4. Scanned test card from site technician.

the challenges to using DBS sampling. However, our experience with an initial multicenter study demonstrated considerable issues with sample quality. We therefore developed a blood spot training kit using synthetic blood and a peer review process to assess and approve the quality of the spots produced. To date, the implementation of a synthetic blood spot training kit in three large multisite clinical trials has demonstrated that the test-site technician's blood spotting technique can be assessed prior to handling PK blood samples, ensuring the PK sample is of adequate quality. Since, successfully deploying this strategy for all new DBS studies within GSK, it has been demonstrated that technicians across different geographical locations who have followed the training kit have produced high-quality DBS samples, with no spots which were unable to be analyzed. The implementation of this procedure now allows the confident use of DBS for large multisite clinical studies and the benefits that are associated with this technique to be realized.

Future perspective

The shipment of condensed milk powder across different continents is not as straightforward as initially perceived, due to import laws. Currently, investigation work is being performed to find a suitable alternative which can be shipped at room temperature to various continents. One possibility is a red quality control glucose solution for StatStrip glucose monitors, which does not need to be shipped refrigerated. In addition, an 'off the shelf' DBS kit would be the ideal solution moving forward, which could be supplied and controlled by the healthcare groups which help pharmaceutical companies run their clinical trials. For studies which run for a long duration, a monitoring process

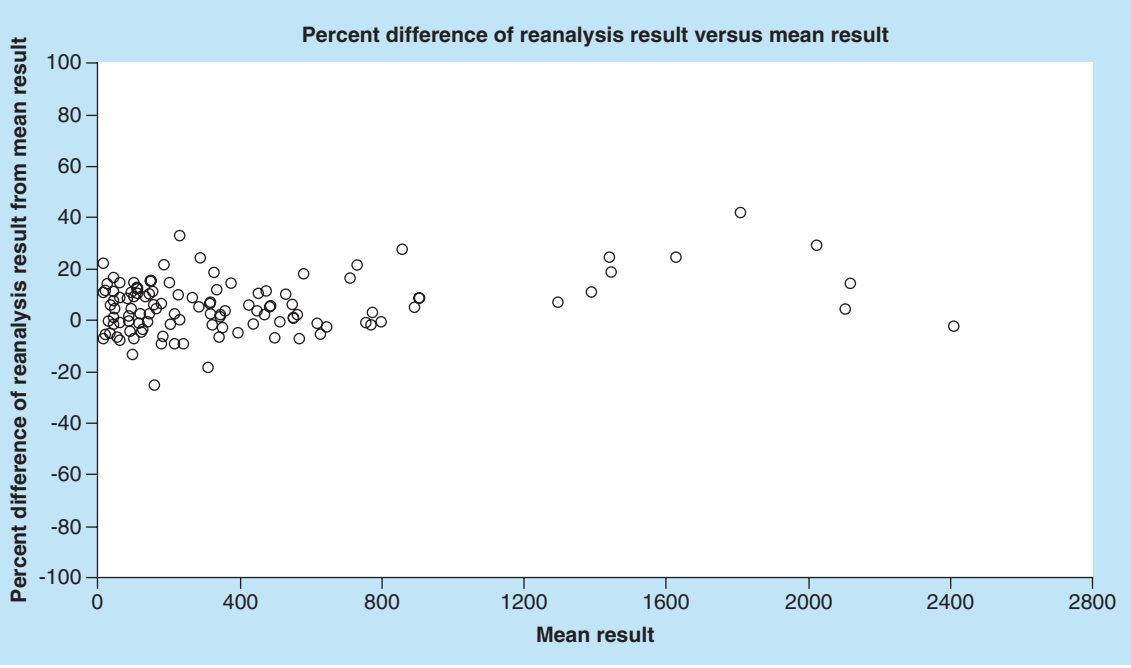

Figure 5. Incurred sample reproducibility in dried spots of human blood study samples.

is required to ensure all new technicians continue to receive adequate training, prior to handling PK blood spot samples.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.2016.0189

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The quantification of drugs in dried blood spot samples is dependent on the quality of the initial sample collected.
- Without sufficient training to the test site technicians, poor-quality spots can lead to samples that cannot be analyzed, which is not acceptable.
- The use of a dried blood spot training kit has been demonstrated as a valid approach to assess and approve the test site technicians, prior to handling pharmacokinetic blood samples.
- Implementation of the training kit has shown to be successful in three large clinical multisite studies.

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