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Dried Blood Spot Sampling Analysis: Recent Advanced and Applications

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ABSTRACT

New drug development stages are major affected by bioanalytical method because it is important to understand the pharmacokinetics of any drug and/or its metabolites. Advanced analysis techniques like Dry Blood Spot(DBS) is very important role in global clinical trials. Current advances in DBS method development is due to advancement of analytical instruments and extraction techniques. This article reviews recent progresses by utilizing hyphenated techniques like DBS and various instruments related to the DBS. This article also gives information regarding the cost reduction & reduction in Blood loss of animals & human subjects in various preclinical & clinical trials. It also reviews some spotting methods like dry blood spotting (DBS) and its extraction.

Keywords: Dry Blood Spot(DBS), DBS Applications, Advancements in Instruments of DBS.

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INTRODUCTION

Clinical trials in patients often require a large amount of sampling from the participating volunteers. Prior to inclusion in the study, served blood samples are taken to determine whether or not the volunteering patient's condition complies with the criteria specified in the protocol. Often samples are also drawn to achieve baseline values of a number of parameters. During the trial, patients visit their physician for further examination and it is not uncommon to draw a number of blood samples during those visits. Thus Depending on the phase of the program and the type of drug under development, the number of blood samples collected during a clinical study may vary from a few hundred to many thousands. As a rule, all these samples need to be shipped to specialized laboratories to be analyzed. The amount of work involved around sample collection, shipment and analysis is quite substantial and accounts for a significant time and cost factor in regulated drug development. The concept of dried blood spot (DBS) sampling, i.e collecting a capillary blood sample from pricking the heel or a finger, was introduced in Scotland in 1963 by Robert Guthrie to enable screening for metabolic diseases in large populations. From late sixties neonatal screening was implemented in many countries in the western world. While originally it only concerned testing for phenylketonuria alone, later the list of tests has been expanded to included hypothyroidism, sickle cell disorders and HIV. Alongside neonatal screening there are an increasing number of tests made available for ambulant patients. This concerns measurements of, for example, a number of hormone levels as well as therapeutic drug monitoring. DBS has the potential to play a major role in all these testing. Recently, DBS was on the agenda of a number of recent meetings concerned drug development. So far, it mostly concerns animal pharmacokinetic and toxicokinetic studies; the advantages of DBS here are in the refinement and reduction of animal experiments. DBS in patient trials has only or mainly been in the area of antimalarial drugs, although its Application for pharmacokinetic (PK) objectives in human volunteer studies has recently been reported. [1]

DBS Procedure

The DBS method, utilizing whole blood spotted onto a DBS card, is well established for a number of diagnostic tests and for drug monitoring [2, 3]. The DBS cards are made from specially manufactured filter paper designed to allow the blood to saturate the paper, creating a “blood spot” whilst giving an even and reproducible spread. DBS samples are then dried at room temperature for 2 hours prior to storage/shipment at ambient temperature, or analysis. DBS samples and whole blood are by validated solvent extraction and protein precipitation methods, respectively, before being analysed by LC-MS/MS.

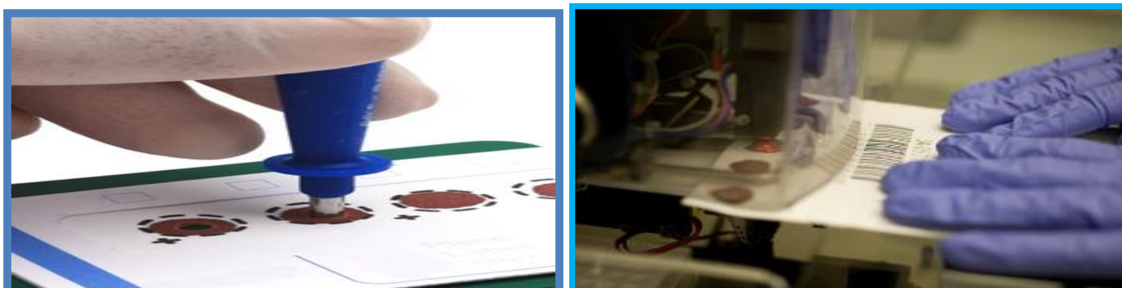

Figure A: Dry Blood Spot Paper[4]

Blood spotting: Five microliters of real or spiked whole blood is spotted [4] (**fig: A**) on a filter paper card, or Whatman paper, using a volumetric micropipette. The blood spots are allowed to dry at room temperature for 2 hrs and then packed in a sealable plastic bag containing desiccant before analysis. They are stored in the dark at ambient temperature except for short-term stability experiments, in which a variety of temperatures are tested.

Figure B: Dry Blood Spot[5]

Sample preparation for DBS Analysis

The standard sample preparation **(fig: B)** approach for DBS analysis consists of punching out a disk from the card that contains the DBS, followed by extraction of the analyte. The punched sample disks typically range in diameter from 3 to 8 mm and are extracted with an organic solvent, or a mixture of aqueous and organic solvent. Internal standard (IS) is usually added (5ml) to the extraction solvent. The extract is then analyzed by LC–MS/MS [5]. This standard approach (extraction of analytes providing a liquid sample for LC–MS/MS analysis) is likely to be suitable for the vast majority of compounds. However, performing this process manually is time consuming and labor intensive. There is an emerging need for greater efficiency, either through the automation of the sample preparation process or use of alternative approaches that require minimal or no sample preparation, such as direct analysis of DBS.

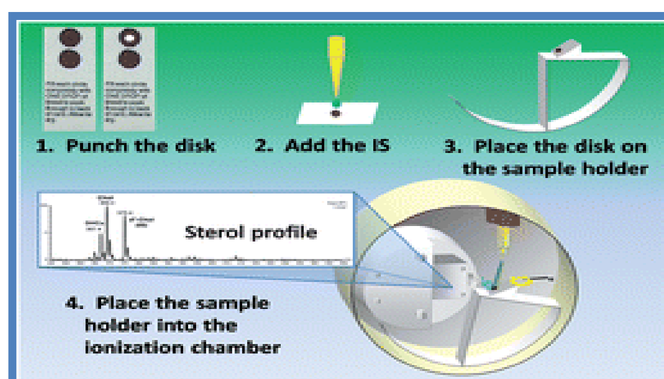

Figure C: Procedure for DBS analyses[6]

Direct desorption of DBS samples:

Direct desorption of DBS samples can be achieved by ambient-ionization MS techniques [6, 7] such as desorption electro spray ionization (DESI) [8] and direct analysis in real time (DART) [9]. DESI was developed by Cooks *et al.* in 2004 and is marketed commercially by Prosolia [10]. DART was developed in 2005 by Cody and Laramee and is marketed commercially by Ion Sense [11]. For analysis by DESI, DBS cards are cut into strips

and secured onto a microscope glass slide whereas blood is spotted directly on a glass slide for analysis by DART. The surface is moved in one direction at constant velocity so that an ion spray in a DESI source or metastable gas phase species (helium) in a DART source desorbs analytes directly from the surface of the DBS without prior sample preparation. These direct desorption techniques greatly increase the through put of DBS sample analyses by eliminating the extraction step and by utilizing short analysis times (from seconds to a minute). However, they also suffer from the absence of chromatography resulting in poor sensitivity due to ion suppression and possible interference from decomposition of metabolites.

DBS in newborn screening

This blood test is used to screen all newborn babies for some rare metabolic and congenital disorders. These include congenital hypothyroidism, phenylketonuria (PKU), cystic fibrosis (CF), galactosaemia, and several conditions affecting the breakdown of fats (fatty acid oxidation defects) and proteins (amino acid metabolism disorders). Many of these conditions are genetic, and inherited from two healthy parents each carrying the abnormal gene. For the test, a few drops of blood are taken from the baby's heel and collected onto four small spots on a specially prepared absorbent paper card. [12]

General Application

Surveillance of HIV type-1, drug resistance

DBS was first successfully applied to detect markers of human immunodeficiency virus type-1 (HIV-1) infection during post-natal serology-surveillance studies in the late 1980s [13]. More recently, the application of HIV/DBS testing has been broadened to include the following laboratory assays: nucleic acid detection [14-15], viral load determination by quantitative real-time PCR techniques [16-17], env V3 loop serotyping [18], anti-HIV antibody detection by gelatin particle agglutination [19], p24 antigen detection [20] and, drug resistance genotyping [21-22], Blood spot technology offers a number of advantages over standard diagnostic testing procedures. First, it circumvents the requirement for trained phlebotomists, given the simplicity of sample collection in the field. Secondly, DBS can be safely transported from the place of collection to the laboratory at ambient temperature, via standard postal systems. This permits remote testing without the requirement of specialist low temperature shipping, as is required for plasma RNA testing. Taken together, DBS offers a simple, safe and cost-effective method for monitoring HIV-1 drug resistance in resource-poor settings. However, this approach has some potential disadvantages compared with standard genotyping methodologies: DBS testing approaches may lack sensitivity when compared with plasma RNA-based methods and the contribution of HIV proviral DNA may also misrepresent historically archived sequences [21], and this may skew interpretation of the drug resistance genotype of the viraemic strain. The recent innovation of several DBS-based genotyping assays shows global interest in the development and implementation of such tests [21-23] however, no consensus as to optimal collection matrices, storage conditions, and nucleic acid extraction and amplification strategies has yet been determined.

National Social Life, Health, and Aging Project (NSHAP)

DBSs are one of the direct biological measures (bio measures) collected in the (NSHAP). These measures, which also include weight, waist circumference, height, blood pressure, smell, saliva collection, taste, a self administered vaginal swab for female respondents, " Get Up and Go, " distance vision, touch, and oral mucosal transudate HIV test, were collected for analysis of measures associated with physiological functioning. Biomarkers for analysis from DBSs in NSHAP were selected based on several criteria, including availability of a valid assay, cost, and relative value of the measure with respect to the objectives of the survey. Analytes quantified in NSHAP DBS samples include C-reactive protein (CRP), antibodies to the Epstein - Barr virus (EBV), haemoglobin, and HbA1c. In most cases, there was more sample collected than used, allowing for the potential future use of these DBSs for more analyses. The consent process was designed with this in mind, and participants were asked for permission to store their DBS samples for future use. However, length of storage and stability of a new analyte must be considered before any future analyses [24].

Assessment of Vit A status in population groups

The need for simpler and more affordable laboratory methods for assessment of vitamin A status of population groups is widely recognized. Next, the current high-performance liquid chromatography (HPLC) method for serum or plasma retinol is expensive, time consuming, and difficult to use in developing countries because of cumbersome handling, preservation, and transportation of specimens, complex laboratory instrumentation requiring specialized skills, and expensive equipment. Two alternative methods have apparently reached a sufficiently advanced stage of development and field validation to merit examination of their potential as surrogates for HPLC serum/plasma retinol: a dried blood spot (DBS) on filter Paper HPLC method by Craft Technologies, and a retinol-binding protein (RBP) assay in serum using an enzyme-immunoassay (EIA) method and portable plate reader by the Program for Appropriate Technologies in Health (PATH). The International Vitamin A Consultative Group (IVACG) recommends serum retinol as a legitimate measure of the vitamin A status of populations [8].

Indeed, serum vitamin A both Craft and PATH have strong track records in the development and use of methodologies for assessing micronutrient status and other health-related parameters (such as immunological parameters) in infants from at-risk populations.

DBS in Drug development

Among the different applications, pharmacokinetic studies represent an interesting approach for the evaluation of the DBS automated system because multiple samples have to be collected over a short period. In a pharmacokinetic study of flurbiprofen (FLB) and its metabolite 4-hydroxyflurbiprofen (OHFLB) was performed on human volunteers to assess the activity of cytochrome P450 2C9 (CYP2C9). This enzyme is responsible for phase I (oxidative) metabolism of widely prescribed xenobiotics, such as non steroidal anti-inflammatory (NSAI) or anticoagulant drugs, and represents an important inter-individual variability which can lead to severe side effects when such drugs are administered[5,25,26].

The use of FLB, an NSAI drug, is commonly used for the phenotyping of CYP2C9 [27] by determining the ratio between the phase I metabolite and the parent drug [28, 29].

Toxicokinetic (TK) investigations supporting non-clinical toxicology studies have been performed using plasma samples, which require centrifugation and separation. Hence, relatively large volumes of blood, often up to 0.5 mL, are needed. For TK studies in rodents, the volume of blood that can be sampled is limited and this results in use of large numbers of satellite animals used only for the purpose of TK blood sampling. Furthermore, it is usually not possible to obtain sufficient samples from a single animal for a full TK profile and composite results from different animals have to be used. TK results obtained from whole blood samples have been acceptable to regulatory authorities for many years. Indeed, they can be advantageous in removing the need for assumptions about possible drug binding to the cellular fraction of the blood. The routine use of whole blood samples, however, is hampered by storage and transportation issues. Recent publications [30] have advocated the use of a dried blood spot (DBS) technique in toxicokinetic and pharmacokinetic studies. The DBS method is well established for a number of diagnostic tests in newborn infants and for drug monitoring and trials in remote areas. It involves applying a spot of blood to a specially designed collection card, which lyses the cells and denatures proteins. The blood spots can then be stored and transported at room temperature.

Thus, DBS is being recommended as the 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 [31]. As the benefits of DBS are not restrained to preclinical studies, a DBS strategy can be considered for clinical phase-I and IIa studies as well. As in the case of TK studies, nurses and other study personnel will have to be trained, but feedback from the staff is generally favourable to DBS based sampling. Another interesting area is therapeutic drug monitoring (TDM), where circulating drug concentrations need to be monitored, typically for drugs with a narrow therapeutic window and/or large inter-subject variability. This area often involves clinical laboratories, and it is interesting to see that DBS has been used longer in TDM than in TK and PK studies. Examples include the monitoring of metformin in diabetic patients, which reportedly has been done with DBS [3] or the immunosuppressant everolimus in transplant patients [32]. Eurofins Global Central Laboratory advanced further on the path of immunosuppressants and offers therapeutic drug monitoring based on DBS for combined LC/MSMS analysis of everolimus, tacrolimus and cyclosporine A.

Direct PCR without DNA extraction

Polymerase chain reaction (PCR) is commonly used when evaluating Guthrie card blood spots. Unfortunately, amplification of DNA from dried Guthrie card blood spots present considerable technical difficulties due to the presence of natural PCR inhibitors, e.g., protein, heavy metals, heme, and heme degradation products [4], and the fact that the amount of genetic material is limited. A number of specialized protocols have been developed to effectively recover DNA from Guthrie card blood spots [33, 34], although they still prove to be time consuming, cumbersome, and expensive, especially for high throughput studies. The Failsafe PCR System [35] enabled direct PCR amplification using whole blood that had been preserved under various conditions, without prior purification of

the DNA template. This reports a fast and easy way to obtain consistent, reproducible PCR amplification directly from dried blood spots collected on Guthrie cards or glass slides, without the need for DNA extraction.

Measurement of Glycated Haemoglobin A1c

Measurement of glycated haemoglobin A1c (A1C) in human blood is an indicator for long-term control of glycemic state in diabetes patients and is an important marker in the diagnosis and treatment of diabetes. The significance of A1C has been emphasized by the report of the Diabetes Control and Complications Trial [36]. There are currently four principal A1C assay techniques (ion exchange chromatography, electrophoresis, affinity chromatography, and immunoassay) that measure different glycated products and report different units. For large-scale epidemiological evaluation of A1C, especially to assess the impact of intervention programs, storage and transportation of samples at -20 °C or lower temperatures poses a challenge in developing countries like India. A method that circumvents the need to store and ship the samples at a low temperature would be highly desirable, and assays using dried blood appear to be a viable alternative. Several community-based studies have shown dried blood to be convenient and reliable for evaluation of a number of analytes [37]. Measurement of A1C in dried blood by colorimetry [38], affinity chromatography [39], and ion exchange chromatography [40] have been reported and found to be useful. The immunoturbidimetric inhibition method for measurement of A1C is a relatively recent technique [41].

Recent trends

The European Bioanalysis Forum is a non-profit organization comprising of European pharmaceutical companies (25 members to date). Their activities focus on bringing together managers and scientists in the broad field of bioanalysis to discuss topics related to science, process and regulations. There has been much interest over the past few years in the potential application of dried blood spots as an alternative to traditional plasma collection in pharmacokinetic studies. The success of the technique has been highlighted by several companies. Seven of the European Bioanalysis Forum member companies are using dried blood spots intensively and 22 then are using it or plan to use it very soon, initially in non regulated studies.

However, most companies have less than one year of experience with dried blood spots and, beyond the scientific merit, it is still not clear how the technique is perceived by key client groups, such as toxicology, clinical and regulatory authorities. Hence, a symposium was to organized bring together representatives from all of these client groups as well as a broad bioanalytical audience to discuss the various perspectives on DBS and to try to provide answers to unresolved and potential. The symposium included sessions on dried blood spots in the non regulated environment, toxicology and regulatory/QA perspectives, clinical use and bioanalytical applications and tools. There was plenty of time for discussion within the program in tutorials; poster and break-out sessions and the degree of delegate participation reflected the high level of engagement with the topic. A total of 190 delegates attended from more than 80 organizations.[42]

The Major Highlights of the Symposium are mentioned below:

Bert Ooms (Spark Holland) discussed 'Online DBS-SPE-MS/MS-feasibility of a concept for rapid DBS analysis without punching'. Both online DBS extraction with a loop interface and online DBS extraction with a SPE interface along with whole blood online SPE, using a high-pressure solvents dispenser were investigated.

The reported mention uses of whatman protein saver 903 cards and is capable of analysing a mixture of four drugs at a rate of 20 samples/hrs. Spark also has a new device at the concept stage with a fully automated card selection from a stacker, ID, inspection and online extraction.

Gary Harland (Waters) described 'the use of an integrated microfluidic LC-MS/MS device for DBS assays as and approach to increased sensitivity'. In the contrast of inhaled and biopharmaceutical products.

Waters used TRIZIAC^{TM}, was fabricated ceramic tile, which can be a UPLC or HPLC device (1.7-µm particles). It has low system volumes with decreased band broadening and higher sensitivity. The performance is similar to a capillary column. Another devices, Xevo TQS has a new, off-axis ion source technology, known as StepWave^{TM}, which has greater sensitivity than the Xevo^{TM} TQ. It also features ScanWave collision cell technology to provide enhanced spectral LC-MS/MS data acquisition capabilities, and Rader - an information -rich acquisitions approach that allows the collection of highly specific quantitative data for target compounds while providing the ability to visualize all other components.

Waters coupled the TRIZIAC and the Xevo TQS, and compared compounds run with UPLC on the API 5000. Silamiquine and extendin (large molecules) both showed large gains in sensitivity, with analysis times as low as 3 min.

John Dinan (BSD Robotics) brought the DBS advantage into laboratory by introducing advantages and application of 226 paper/cards. BSD robotic units BSD 600, BSD 600 (Dute) and BAD 700. These are much faster than manual handling and allow positives identification of the punches in the plate wells. They have a versatile dual punch system, a series 3 quick-release punch, and utilize a barcode reader and plate maps with easy-to-use software. The BSD 1000 - a unit with minimal operator involvement, positive identification of the spot and integration with other systems. Storage of three magazines is possible with 100 cassetted cards/ magazine. The tray carousel has a capacity for six plates. the systems automatically scans cards and determines the best positions to punch. BSD is working with Watson LIMS to ensure compatibility. [42]

Bioanalytical Application

The ethical, financial and organizational advantage of the application of DBS within the development process is well recognized. However, for the bioanalyst, DBS is challenging because switching from plasma to blood adds more complexity. It also requires a change in the well established method validation and sample analysis procedures. With a focus on

automated, high-throughput analysis the direct analysis of DBS samples would be preferred over the currently established semi-automated extraction procedures. Direct analysis can be achieved either by various direct desorption technique such as direct analysis in real time, desorption electrospray, laser electrospray, paper spray analysis or by direct elution.

DBS bioanalysis has been especially useful in the high sensitive LC-MS/MS quantification of corticosteroids using. Inhalation drugs usually require a LLOQ in the low pg/ml range, which cannot be achieved using DBS and a common LC-MS/MS system. Moreover inhalation drugs, such as budesonide have a rather wide dynamic range due to high C_{max} values but short-half life of the analyte. To cope with these requirements, developed a new integration algorithm entitled Signal Finder^{TM} has been developed, which corrects saturation effects at the upper limit of quantification (ULOQ) and therefore allows a wider liner range. By increasing the liner range, the need for sample dilution is reduced, and dilution integrity issues discovered with DBS can be overcome. The required sensitivity can be achieved using a UPLC system coupled to a high-sensitivity triple quadrupole mass spectrometer.

The cast benefit of dry spots technology. To date, 231 DBS methods have been validated for 105 compounds. A total of 168 nonclinical GLP studies have been performed using DBS cards (20357 samples and 85 different analytes analyzed in these studies), 86 nonclinical GLP studies (18829 samples and different analytes analyzed in these studies) and nine clinical studies (4027 samples and 15 different analytes analyzed in these studies). GSK estimated cost savings of GBP£5-8 million a year if all toxicokinetic and clinical studies were performed suing DBS instead of 'conventional' plasma samples. The cost benefit offered by the card technology could also be used for dry plasma spot (DPS) and dry urine spot (DUS) because feasibility studies performed at GSK showed that plasma and urine may also be spotted on the filter cards. DPS may therefore be an attractive option for compounds already in a later phase of development where a switch in matrix would not be sensible.

Roche has been working towards applying DBS to stabilize ester prodrug and glucuronide metabolites. Four labile compounds that require the addition of inhibitors as well as cooled storage/processing in conventional plasma analysis were chosen as model compounds. Two compounds were ester prodrugs, one was a glucuronide and the last was known to hydrolyze rather easily. The outcomes of the feasibility study was that the use of DBS samples insted of plasma or whole blood samples generally improved the stability of the model compounds in the short term. Long-term stability was compound dependent. Generally, the stability was strongly dependent on the type of card used for the experiment. Fast drying as well as storage as low temperature seemed to be beneficial with respect to stability enhancement. The study concluded that the coating of treated cards may stop enzymatic activity but may not stop chemical degradation. [42]

Advantages of DBS

Investigation work indicates that a 40 µL blood spot is sufficient for 4 replicate analyses. This represents at least an order of magnitude smaller volume than the volume required for traditional plasma analysis. It has substantial benefits for reduction in animal numbers in preclinical studies. The reduced blood volume also makes it possible to take

several serial samples from individual animals; even mice. This removes the need for composite TK profiles using results from more than one animal and should improve the quality of TK data. From a clinical view point, the potential advantages for samples from paediatric trials, which may be taken by finger or heel prick, are obvious. Additional major advantages are realised in transportation and storage. The DBS cards are easily shipped and/or stored at room temperature resulting in fewer issues if transport delays occur and has substantially lower costs. The cost reductions possible in large clinical trials using this technique could be very significant. Procedures are simplified due to the absence of the plasma preparation step and the need for frozen storage.[43]

Disadvantages of DBS

The main perceived disadvantage of the DBS technique relates to the historical use of plasma analysis. Clearly, plasma and whole blood results are not directly comparable due to the concentration effect of plasma separation, so it is best to use consistent analysis techniques throughout the drug development programme.[44] For programmes already underway using plasma analysis it may not be efficient to change to the DBS method (although potential cost savings in transport and storage of clinical samples should be taken into account). For investigational new drugs embarking on non-clinical studies to support first-in-man trials, serious consideration should be given to developing and validating the bioanalytical methods using DBS. [3]

CONCLUSION

Our conclusion is that clinical trials may see substantial savings in costs and an increase in overall quality when a number of PK or biomarker sample scan be collected and thus analyzed on DBS cards rather than as a plasma sample. For a complicated trial requiring multiple and frequent dry ice shipments and/or cooled centrifuges and/or-80°C freezers on site, the costs savings can easily add up to multiples of €100,000. A second aspect is that we expect improvements in quality in most of the processes related to sampling. An increased quality is in the benefit of all: sponsor, investigator and especially for the patient. Finally, we see improvements in safety. Not only is a DBS card a far less hazardous sample than plasma or full blood, there is also the benefit of no longer needing to manipulate the sample with dry ice.

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