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DRIED MATRIX SPOTING - AN INNOVATIVE SAMPLE PREPARATION TOOL IN BIOANALYSIS

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ABSTRACT: Bioanalysis is a sub-discipline of analytical chemistry covering the quantitative measurement of xenobiotics (drugs and their metabolites and biological molecules in unnatural locations or concentration) and biotics (macromolecules, proteins, DNA, large molecule drugs, metabolites) in biological system. The focus of bioanalysis in the pharmaceutical industry is to provide as quantitative measure of the active drug and/or its metabolite(s) for the purpose of pharmacokinetics, toxicokinetics, bioequivalence and exposure response (pharmacokinetics / pharmacodynamics studies). Over the past several years dried matrix spot (DMS) sampling technique has emerged as a pertinent method in both qualitative and quantitative bioanalysis context. There are many types of DMS techniques such as dried blood spot (DBS), dried plasma spot (DPS), dried urine spot (DUS) and dried breast milk spot (DBMS). The most commonly used technique is DBS wherein the blood sample is directly soaked on to a paper (with or without treatment) and after drying it can be analyzed by modern analytical, immunological or genomic detection systems. Several advantages of DMS techniques such as low sample requirement, transportation and storage without special treatment, better analytes stability, enhanced clinical cooperation in clinical trials and reduced unforeseeable exposure of biohazard to analysts, make it the most appropriate sampling technique for bioanalysis. This review illustrates the available information on DBS, DPS, DUS and DBMS methods which may serve for investigators in the field of bioanalysis. Further, the proficiency and appliance of DMS method in pharmacokinetic (PK), therapeutic drug monitoring (TDM), toxicokinetic (TK), metabolomic and disease diagnosis is explored.

INTRODUCTION: Bioanalysis was traditionally thought of in terms of measuring small molecule drugs. However, the past twenty years has seen an increase in pharmaceuticals (*e.g.* Proteins and peptides) which has been developed to address many of the same diseases as small molecules.

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These large biomolecules have presented their own unique challenges to quantification. Techniques commonly used in bioanalytical studies include:

i. Hyphenated techniques like liquid chromatography - mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), liquid chromatography- diode array detector (LC-DAD), capillary electrophoresis-mass spectrometry (CE-MS).

ii. Chromatographic techniques like high pressure liquid chromatography (HPLC), gas chromato-

graphy (GC), ultra pressure liquid chromatography (UPLC), Supercritical fluid chromatography (SFC).

iii. Electrophoresis

iv. Ligand binding assays like dual polarization interferometer, enzyme linked immune sorbent assay (ELISA), magnetic immunoassay (MIA), radio immunoassay (RIA).

Of all the above listed techniques, frequently used is LC-MS/MS, for bioanalysis of small molecules and ELISA, for bioanalysis of macromolecules.

Sample Preparation and Extraction: Bioanalysis deals with complex biological samples containing the analyte alongside a diverse range of chemicals that can have an adverse impact on the accurate and precise quantification of analyte. As such, wide range of techniques is applied to extract analyte from its matrix. These includes

- Protein Precipitation
- Liquid-Liquid Extraction
- Solid phase Extraction

Automated sample preparation methods and liquid handling robots are commonly employed to increase efficiency and reduce cost. Bioanalytical methods are not intended for elucidating quality parameters (*e.g.* identity, purity) of a biological sample; they are intended to determine the quantity of drug (or the presence of induced antibodies) in biological samples. For that reason, technique used to perform bioanalytical methods vary according to molecular entity's nature [1].

Dried Matrix Spotting (DMS): DMS methods often employ a color indicating process that enhances the ability to analyze these mostly transparent fluids when spotted onto collection paper. The color indicating dye allows the analyst to visually confirm the location of the dried sample spot.

Other benefits of using color indicating dye include improved method accuracy and precision because the process of addition allows for the concurrent addition of the internal standard prior to sample addition and extraction. To date, matrices that have been analyzed using DMS include saliva, tears, urine, milk and plasma [2, 3].

Dried Blood Spot (DBS): In DBS method, blood sample is directly soaked on to a paper (with/ without treatment) and after drying it can be analyzed by modern analytical immunological or genomic detection system. Implementation and effectuation of dried blood spot (DBS) technology in the realm of therapeutics and health sciences had simplified the conventional blood collection and analysis process. DBS is anticipated to be a promising surrogate of established liquid bio matrices (plasma / serum) for pharmacokinetic (PK) and toxicokinetic (TK) studies and can even surpass them. Ivar Christian Bang, the father of modern clinical microchemistry, pioneered the DBS method for quantification of blood sugar. After half a century of Bang's DBS application, Guthrie and Susi in 1963, reported DBS method for the analysis of phenylketonuria in neonates and there after DBS method gained popularity in bioanalysis.

Blood is the most preferred and regulatory acknowledged biological sampling matrix for *in-vivo* concentration assessment in variety of species. Following the collection of blood drops on a paper, drying and transportation, the blood spot is extracted and analyzed in the laboratory. DBS cards are considered to be less bio hazardous in comparison to plasma samples in view of a fact that blood is in dried form; all the proteins, pathogens and enzymes get inactivated on card and bacterial growth is prevented. The less invasive nature of DBS and use of micro-volume blood samples have made it a very useful sampling method for neonatal and juvenile subjects. DBS reduces a number of steps preceding drug analysis. By using DBS technique, public health laboratories screened more than 95% of all newborns in the United States of America (USA) for inborn metabolic disorders. For PK and TK studies in small animals such as rats or mice, DBS method can facilitate rich sampling and these samples are stable at a wide range of temperatures. This technique has been reported to be suitable for drugs which are susceptible for photo-degradation [4]. It is being widely utilized in the pharmaceutical industries, hospitals and research centers, particularly where blood or plasma sample volumes are low, difficult to collect, store, process or transport. Tools and techniques involved in DBS are as follows:

Selection of Paper: The DBS cards are composed of non-cellulose or cellulose (filter paper) matrix of specific pore size and thickness. Commercially, Whatmann 903 cards are used in newborns screening, FTA DMPK type A, B, C cards are used in PK/TK studies and FTA Elute cards are intended mainly for collection and purification of DNA for downstream analysis. All types of DMPK cards are available in two forms: regular and indicating. Indicating types of cards are useful for colorless samples like urine, plasma, synovial fluid and cerebrospinal fluid. US food and drug administration (FDA) has approved three DBS cards namely, Ahlstrom 226-K062932, Whatmann 903 and PerkinElmer 226 under 21 CFR 862.1675 as medical device for blood specimen collection.

Non-cellulose DBS cards (Bond Elut DMS Card, Agilent Technologies, USA) are also commercially available for DMPK research. They are claimed to be superior in form of improved mass spectrometry (MS) signal, less effort in punching and hematocrit independent spot homogeneity. In-house treatment of card has also been reported for compound specific stability. Guowen *et al.,* used citric acid solution on DBS card to stabilize their drug candidate (KAI-9803) from thiol-disulfide exchange. Irrespective of unknown blood volume spotting on conventional DBS card, Gabriel *et al.* developed a disposable chip. It has self-actuated dissolvable valves which meter and transfer exact volume of the blood in microliters.

Specimen Collection: For therapeutic drug monitoring (TDM) and diagnosis of diseases in humans, whole blood sample is collected from a finger, toe or heel prick with the aid of sterile disposable lancet or surgical blade. For PK and TK studies in rat and mouse, blood can be collected from the caudal vein. For qualitative purpose, DBS specimen can be prepared by cautiously applying a few freshly drawn blood drops from a finger/toe prick on card. When objective is quantitative analysis, a measured amount of blood volume is gently applied on DBS card with the aid of pipette or capillary tubes. In such cases, use of an anticoagulant (Heparin, EDTA) is perquisite for appropriate spotting, EDTA is used most widely. However, EDTA may cause interference in MS but it has advantages over heparin in its mixing and drying abilities along with calcium dependent phospholipases and ester hydrolases inhibition. Use of anticoagulants significantly affects the results in telomere length measurements in quantitative polymerase chain reaction (qPCR) based analysis in DBS.

Volume of blood to be spiked on DBS card depends on the sensitivity of bioanalytical method or instrumentation facility. The pipette tip should be held just above the card allowing drop formation and soaking onto the surface. The pipette tip should not be touched repeatedly to the surface of the card as it may damage the paper leading to sample in homogeneity. Multi layered DBS spot formation may occur due to repeated application of blood drops in the same collection circle.

All these should be avoided as they make specimen invalid and lead to misinterpretation of results. As described by Ren *et al.,* autoradiography may be a solution for visualization of uneven distribution of analyte(s) on DBS spots. Hematocrit value, which represents the measure of packed cell volume in blood, affects the size of DBS and is usually main cause of the bias in results. Also, there are few other factors such as the type of card material and the environment of spotting which contribute to uneven distribution of analyte on DBS cards. Several approaches are tried to overcome the effects of above mentioned factors. Blood samples can be spotted for a precapiau *et al*., reported a method for determining the hematocrit value in any DBS by measuring potassium in DBS extract using a routine clinical chemistry analyzer. This add-on technique can assess the hematocrit dependent intra-individual variability and further facilitate the non volumetric DBS sampling.

Drying of Card: After sample collection, the DBS cards are generally dried horizontally for 2 - 3 h on a card rack at room temperature or under nitrogen flow and controlled humidity. Cards should not be stacked or allowed to touch other surfaces during drying. Exposure of DBS specimen to any milieu like direct sunlight, dust or flying insects should be avoided as that may compromise its integrity. Drying of the card is the crucial step for unstable analytes. Various modifications like changes in pH, temperature and humidity are recommended. Heat stabilization can also be a solution for meta-bolically unstable drugs.

However, according to Blessborn *et al.*, enzymatic activity of blood can be reduced by drying DBS card at 95 °C for 30 sec. They found that oseltamivir, cefotaxime and ribavirin are stable after heat stabilization but artemether and dihydroartemisinin were unstable due to their decomposition at 60 °C.

Storage and Transportation: In contrast to conventional biological matrices, DBS provides huge simplification in the arena of storage and transportation. Unlike plasma / serum samples, DBS neither requires freezers for storage nor bulky volumes of dry ice for shipping which are costly and need special care. Baring humidity factor which has significant influence on specimen stability and elevates the chances of bacterial growth, DBS cards can be shipped and stored at ambient temperature. For protection from environmental humidity, DBS cards are advised to be carefully wrapped and packed in sealable heavy duty plastic bags with adequate desiccant and humidity indicator to find out at what time desiccant has to be replaced. Properly labeled DBS cards packets, which clearly convey the bio hazardous nature of the content inside package to transportation personnel and other employees, can be shipped to analytical laboratories through mail, courier or express mail delivery services.

For establishing sample integrity and safety from occupational exposure of hazardous blood sample, basic triple packaging technology is used for DBS card shipment. Triple package comprises of primary container, secondary container and a third covering of high quality paper envelope with an affix or print of the international biohazard symbol. If long term stability of certain analytes at room temperature is not established on DBS cards, the packed DBS cards with desiccant can be stored in laboratory freezers until analysis to minimize analyte degradation.

Extraction and Analysis: Dried cards can be punched out with various available diameters punching tools (manual) semi automated and automated). Punched dried cards can be used directly (by micro fluidics) or by extraction of analytes with suitable extraction solvent. Extraction solvent should be optimized as per the solubility profile of the analyte(s) with consideration of minimizing extraction of interfering endogenous impurities. After extraction, samples are subjected to analysis using a suitable analytical technique.

Automation: Commercial instruments are available for fully automated online DBS sampling and analysis like ABS2; Instech Solomon, Pennsylvania, USA and Culex; BASi, Indiana, USA. Deglon *et al.*, have innovated an online analytical method in which sample can be analyzed by online desorption of DBS spot in an inox cell by column switching mode with assurance of purification and separation of samples. This online method paves a way to high throughput by reducing various steps of bioanalysis like centrifugation, extraction, vortexing, drying or reconstitution.

Calculation and Correlation with Plasma / Serum Results: Blood level of drug is the sum of levels of drug in red blood cell (RBC) and plasma. Often due to differential binding of drug to specific blood components, concentration in plasma and RBC varies; hence considering whole blood concentration as equivalent to plasma concentration becomes inappropriate. Before comparing the plasma/serum levels with DBS levels, parameters like hematocrit (H) and RBC to plasma partitioning ($K_{RBC/plasma}$) or blood - plasma partitioning ($K_{Blood/plasma}$) should be considered as DBS results may differ from serum or plasma results. Plasma concentration can be calculated from DBS results by using the following equation:

$$\text{Calculated plasma concentration} = \frac{\text{Analyte concentration in DBS}}{[(1-H) + H^* K_{RBC/plasma}]}$$

If, $K_{RBC/plasma}$ or $K_{Blood/plasma}$ is;

> 1, DBS will have better PK/PD correlation
= 1, DBS can be an alternate for plasma
< 1, DPS (dry plasma spot) method can be applied with two layered polymer film which separates plasma from whole blood without centrifugation [4].

Advantages of DBS:

i. Low blood volume requirement.
ii. Transportation and storage without special treatment.
iii. Better analytes stability.

iv. Enhanced clinical cooperation in clinical trials.
v. Reduced unforeseeable exposure of biohazard to analysts.
vi. Most appropriate blood sampling technique.

Limitations of DBS:

- Can only be coupled with highly sensitive analytical techniques.
- Inadequate for air sensitive or volatile analytes.
- Capillary blood analyte concentration may vary from venous blood.
- Unable to calculate accurate bioavailability.
- Not suitable for compounds having minimal blood cell uptake ($K_{blood/plasma} \leq 0.55$).
- Spot homogeneity and compound degradation.

DBS Applications: Application of DBS technology has gained momentum in various fields including neonatal metabolic screening, therapeutic drug monitoring (TDM), preclinical and clinical pharmacokinetics (PK), toxicokinetics (TK), forensic, biological and immunological sciences.

Preclinical Pharmacokinetic Studies: Serial blood micro-sampling is possible with DBS in rodents without changing their hemodynamic balance. By implementing DBS method, animal requirement can be reduced to the extent of at least 60%. Preclinical pharmacokinetic (PK) studies in rodents require sampling of blood with 1 - 2 ml per sample at multiple time points. Therefore, the studies are done by sparse sampling. Thus, the low sampling volumes required for DBS also facilitates serial sampling from the same animal, offering improved data quality over composite sampling paradigms from multiple study animals.

Additionally, it will reduce the cost of experiment by reducing number of animals especially in case of transgenic or knockout model studies. Also, it eliminates the harvesting step of serum/plasma and reduces the quantity of new chemical entities (NCE) required during drug discovery and development programme. For the compounds with high red blood cells to plasma partition coefficient using plasma as analytical matrix leads to significant errors in pharmacokinetic - pharmaco-dynamic (PK-PD) calculation due to hemolysis of blood. These errors can be overcome by replacing plasma with DBS.

Therapeutic Drug Monitoring: Therapeutic drug monitoring is the quantification of drug(s) concentration in patients' blood with time to optimize dose regimen. It helps clinician as well as patient regarding drug safety, as dose optimization can be achieved without compromising the efficacy of therapeutically potent molecules. TDM may help to minimize the incidences of drug resistance as antibiotic or antiviral drug concentration levels can be maintained within the therapeutic window. DBS method is especially suitable for TDM in neonates.

Population Disease Control and Clinical Pharmacokinetic Studies: Multi-centric clinical investigations can be studied easily with the help of DBS technique. Congenital or genetic disorders in neonates are being investigated by DBS method. Many diseases can be screened by using DBS sampling technique. The toe or finger prick is always better than venipuncture as it can improve volunteer recruitment in clinical studies. DBS is always advantageous for multiple sampling in remote areas where transportation, cold chain facilities or intravenous blood sampling may reduce the efficiency of mass policy implementation. DNA based diagnosis of infectious and genetic disorders can be performed through DBS coupled polymerase chain reaction tests such as human cytomegalus virus (HCMV) detection, diagnosis of HIV, T-cell receptor excision circle (TREC), Fabry disease mutation and spinal muscular atrophy (SMA) diagnosis, *etc*. In USA, DBS method was evaluated and validated by centers for disease control and prevention for new born screening tests. Conduction of a clinical trial is always a costly affair. In case of worldwide multi-centric clinical study, cold chain (freezer or dry ice) supported transportation of biological samples from clinical facility to analytical laboratory involves huge capital investment along with stability issues. After drying, spotted DBS can be transported at ambient temperature. The proficiency of DBS can reduce the overall clinical trial budget up to 50% [4].

Other Applications: DBS is applicable to most of the blood related qualitative or quantitative analysis. Doping analysis of banned substances can easily be performed with the help of DBS technology. Thomas *et al.*, analyzed 26 prohibited compounds in a single analysis using DBS coupled with LC-MS/MS consisting of a quadrupole mass

filter, higher collision dissociation (HCD) cell and an orbitrap detector. DBS is not just a technique for whole blood analysis; it can also be used for analysis of banned substances in urine or saliva and implemented for dope testing. DBS method is equally applicable for plasma or serum analysis in medico-legal and forensic applications. Kong *et al.*, performed metabolomic profiling of 695 detectable and 137 identifiable markers through DBS using GC/MS and recommended it as a substitute of plasma for metabolomic studies.

Lal *et al.*, used DBS method for PK drug- drug interaction study of centchroman and its metabolite with carbamazepine, indicating its applicability in PK drug-drug interaction study. Wijnen *et al.*, applied DBS method for DNA isolation in pharmacogenetics and found that the DNA isolation from DBS technique is faster, cheaper and easier than commercially available DNA isolation kits. LC-MS/MS method developed and validated to determine the reference intervals of cortisol, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxy-cortisol, androstenedione, corticosterone, and 11-deoxycorticosterone simultaneously in 453 DBS samples. The samples were from Korean subjects stratified by age group (78 full-term neonates, 76 premature neonates, 89 children, and 100 adults) [5]. Blood extraction method for inherited metabolic disorder screening since 1960's. With introduction of LC-MS/MS, not only DBS could be used to analysis drugs in small blood volume, but in various fields, such as toxicology, drug therapeutic monitoring, drug diagnostic screening, and illicit drugs. In toxicology field, many drugs (*e.g.* benzodiazepines, acetaminophen and small molecule drugs) have been tested with DBS. We optimized the DBS procedure and LC-MS/MS conditions for 18 benzodiazepines, seven benzodiazepine metabolites, and one z-drug (zolpidem) analysis in blood. 30 mL of whole blood was spotted on FTA DMPK card C and dried for 2 h in desiccators [6].

HbA1c was measured in duplicates/triplicates in whole venous blood (WB), capillary blood (capDBS) and venous blood placed on the matrix paper (venDBS), by turbidimetric inhibition immuno-assay. Intra-assay coefficients of variation (CV) were calculated. DBS values were compared to WB results using linear regression, Bland-Altman plots and cross-validation models [7]. When capDBS values were applied to equations derived from regression analyses, results approached those of WB values. A cross-validation model showed that capDBS results on D0, D4 and D7 were close to the WB results, with prediction intervals that were narrow enough to be clinically acceptable. The measurement of HbA1c from DBS samples provided results that were comparable to results from WB samples, if measured up to seven days after collection. Intra-assay coefficients of variation were low, results were in agreement with the gold-standard, and prediction intervals were clinically acceptable. The measurement of HbA1c through DBS sampling may be considered in situations where traditional venipuncture is not available.

Literature reports on the applications of DBS in the different types of quantitative analysis of drugs are compiled in **Table 1.**

Other Matrices:

Dried Urine Spot (DUS): Nowadays dried urine spot technique is applied in both qualitative and quantitative bioanalysis context. Dried urine spot have been reported to provide a simple screening tool for congenital cytomegalovirus (CMV) infection. Two applications of 20 µL urine remained within the rim of the filter paper disc and were used to determine the analytical performance of Towne CMV spiked into urine and applied on the discs. In this case DBS technique have been reported to lack sensitivity in a large US study (2010), but a recent European study suggested an improved sensitivity (2011) and report from Japan suggest dried urine spot (DUS) could also provide a highly sensitive screening tool for congenital CMV infection by simply inserting a filter paper into the infant's diaper [40].

Electromembrane extraction coupled with liquid chromatography-tandem mass spectrometry (LC–MS/MS) was developed for determination of ten volatile organic compound metabolites in dried urine spot samples. Aliquots of urine were spotted onto Bond Elut DMS cards and dried at room temperature. After drying, the punched out dried urine spot was eluted with water. Volatile organic compound metabolites were extracted from the sample through a supported liquid membrane into an alkaline acceptor solution inside the lumen of a

hollow fiber with the help of an electric potential. The optimum extraction conditions were determined by using design of experiments (fractional factorial design and response surface methodology). An analytical method based on the use of electromembrane extraction (EME) for the determination of VOC metabolites in dried urine spot (DUS) was developed for the first time [41, 42].

TABLE 1: APPLICATIONS OF DBS FOR INVESTIGATION OF DRUGS

S. no.	Drug name	Application	Analytical technique
1	Acetaminophen	TK studies	LC-MS/MS [8]
2	Actinomycin-D	PK studies	LC-MS/MS [9]
3	Amodiaquine, chloroquine and chlorthalidone	Preclinical PK studies	LC-MS/MS [10]
4	Bisoprolol, ramipril and simvastatin	TDM	LC-HRMS [11]
5	Busulfan	TDM	LC-MS/MS [12]
6	Caffeine	PK studies	LC-MS/MS [13]
7	Chloroquine and desethylchloroquine	PK studies	HPLC-UV [14]
8	Cyclosporin A and tacrolimus	TDM	LC-MS/MS [15]
9	Darunavir, etravirine, raltegravir and ritonavir	TDM	LC-MS/MS [16]
10	Dextromethorphan and dextrorphan	Enantioselective method	LC-MS/MS [17]
11	Diazepam	PK studies	LC-MS/MS [18]
12	Efavirenz	TDM	RP-HPLC-UV [19]
13	Ethyl glucuronide (EtG) and ethyl sulfate (EtS)	Diagnosis of recent alcohol uptake	LC-MS/MS [20]
14	Fluoxetine, norfluoxetine, reboxetine and paroxetine	TDM, toxicological analysis and PK studies	NICI-MS-MS [21]
15	Gabapentine	Preclinical and clinical PK studies	LC-MS/MS [22]
16	Gemifloxacin	Preclinical PK studies	HILIC with fluorescence detector [23]
17	Linezolid	TDM	LC-MS/MS [24]
18	Losartan	Preclinical PK studies	LC-MS/MS [25]
19	Methadone	TDM	HPLC [26]
20	Metoprolol	Preclinical, clinical PK and doping studies	LC-MS/MS [27]
21	Metronidazole	PK/PD studies in neonatal patients	HPLC [28]
22	Nevirapine and Efavirenz	TDM	LC-MS/MS [29]
23	Nifedipine	Photo-degradation experiments	LC-MS/MS [30]
24	Opiates, cocainics and amphetamines	Illicit drugs determination in suspected cases of driving under the influence of drugs	LC-MS/MS [31]
25	Paclitaxel	Preclinical PK studies	LC-MS/MS [32]
26	Propranolol	PK studies in neonates or young infants	LC-MS/MS [33]
27	Quinine and 3-hydroxyquinine	Clinical PK studies	HPLC with fluorescence detection [34]
28	Ranitidine	TDM in pediatrics samples	LC-MS/MS [35]
29	Sulfadoxine and pyrimethamine	TDM and epidemiological studies	HPLC-UV [36]
30	Tacrolimus, sirolimus, everolimus and cyclosporin A	TDM	LC-MS/MS [37]
31	Vincristine	Clinical pharmacological studies	LC-MS/MS [38]
32	Zidovudine	Monitoring of zidovudine therapy among HIV-infected pregnant mothers and their newborns	RIA [39]

Filter Paper used in DUS: Whatmann 903 neonatal cards with 13 mm diameter discs and Whatmann no. 903 filter paper are used in DUS. These filter papers can be shipped and tested in well equipped laboratories.

Advantages of DUS:

i. Eliminated disadvantages of liquid urine collection.
ii. Non- invasive.
iii. Simple collection procedure.

Disadvantages of DUS:

i. Problem with 24 h urine collections: Inaccuracy in all urine - missed collections.
ii. Inaccuracy in determining volume of urine collected during 24 h.
iii. Up to 40% of 24 h urine collections are done improperly.
iv. Results altered by liver or kidney disease.

v. Inconvenient for assessment of monthly variations.
vi. Expensive and advanced testing technology required for better accuracy.

Applications:

i. Measures total daily output of steroids.
ii. Measures steroid metabolites.

Dried Plasma Spot (DPS): Dried plasma spot were employed as an alternative sample collection technique for the quantitative determination of drug in human plasma using an automated LC-MS/MS technique. Dried plasma spot specimens especially applied to pharmacokinetic studies, where plasma sampling procedure becomes rapid and required plasma volumes negligible [43]. An evaluation of plasma micro sampling for DPS has been conducted for the first time using ritonavir as a model compound orally administered to dogs. For this evaluation, an LC-MS/MS method was developed and validated according to the current health authorities' guidance and industry practice for the analysis of ritonavir in DPS samples.

The measured ritonavir concentrations in the DPS samples prepared using SAFE-TEC devices and directly from the conventional wet plasma using standard pipette were compared with each other and against those of conventional wet plasma. Both DPS results correlated well with each other and were comparable to those of the wet plasma. Good incurred sample reanalysis results were obtained for the two sets of DPS samples and wet plasma as well. The current plasma micro sampling for DPS can serve as an alternative to DPS sampling via standard pipetting and wet plasma in *in-vivo* studies [44]. Validated methods were applied for the determination of pregabalin levels in dried blood and plasma samples obtained from patients with epilepsy, after oral administration of commercial capsules.

Comparison of drug level in blood and plasma, as well as correction steps undertaken in order to overcome hematocrit issue, when analyzing DBS, are also given. The potential displacement of plasma by DBS relies on overcoming hematocrit issue and correlation of pharmacokinetics data from blood to plasma. An alternative strategy for overcoming the hematocrit effect was presented and it is based on application of simple mathematical equation that correlates correction factor with hematocrit values. This is the first step in establishing of DBS, which already constitute "the state of the art" in modern bioanalysis, as the routine procedure in the coming years, after addressing main concerns during their implementation [45].

Cards used in DPS: Noviplex TM plasma preparation cards are used in this technique. These cards are simple powerful micro sampling tools used to collect volumetric samples of plasma from non-volumetric applications of whole blood in just minutes.

Advantages:

i. Accepted as gold standard testing method by the conventional medical community.
ii. Wide range of hormone tests available: ideal for testing peptide hormones.
iii. Automated with FDA - approved methodology.
iv. Familiarity with established levels and ranges.

Disadvantages:

- Invasive
- Inconvenient to patient-requires driving blood draw station.
- Processing of specimen - centrifugation.
- Shipment- Biohazard labeling and requires cold packs.
- Normal ranges are too wide.
- Difficult to measure multiple times during day due to collection logistics.
- Usually measures total and not bioavailable fraction of hormone in bloodstream.
- Cannot be used to measure hormone metabolites.
- Limitations with testing of sex hormones: no distinction between bound and free hormone.
- Not a valid method for measuring hormones delivered topically.

Applications:

i. For quantitative determination of gabapentin [46].
ii. Evaluation of plasma micro-sampling for dried plasma spots (DPS) in quantitative LCMS/MS bioanalysis using ritonavir as a model compound [47].

Dried Breast Milk Spot (DBMS): Recently dried breast milk spot is also used for quantitation of drugs. Dried breast milk spots were prepared by spotting 30 mL of human breast milk on each circle of Whatmann 903 Protein Saver cards. The dried breast milk spot method is simple, robust, accurate and precise, and can be used in settings with limited resources.

Application:

i. Development, validation and clinical application of a novel method for the quantification of efavirenz in dried breast milk spots using LC-MS/MS.

Methods: Dried breast milk spots were prepared by spotting 30 mL of human breast milk on each circle of Whatman 903 Protein Saver cards. Chromatographic separation was achieved on a reverse-phase C18 column with 1 mM ammonium acetate in water / acetonitrile using a solvent gradient at a flow rate of 400 mL / min and detection was by TSQ quantum access triple quadrupole mass spectrometer equipped with a heated electrospray ionization source. The method was applied to characterize the breast milk pharmacokinetic profile of efavirenz in HIV-positive nursing mothers receiving regimens containing 600 mg of efavirenz once daily [48].

ii. The validation and clinical application of an LC–MS/MS method for the quantification of nevirapine in dried blood spots (DBS) and dried breast-milk spots (DBMS) are presented.

Methods: DBS and DBMS were prepared from 50 and 30 mL of nevirapine-spiked whole blood and human breast milk, respectively. Chromatographic separation was achieved on a reverse-phase C_{18} column with 0.1% formic acid in water / acetonitrile using a solvent gradient programme at a flow rate of 400 mL/min, and detection was by a TSQ quantum access triple quadrupole mass spectrometer.

The clinical application was evaluated in HIV positive nursing mothers and their breastfed infants. These methods further extend opportunities for conducting clinical pharmacokinetic studies in nursing mother - infant pairs, especially in resource-limited settings [49].

Dried Cerebrospinal Fluid Spot (DCSFS): New DMS method for cerebrospinal fluid represents an interesting alternative that increases the quality and efficiency in preanalytics. This should enable the better exploitation of Aβ analytes for Alzheimer's diagnosis. The use of dried matrix spot (DMS) overcame preanalytical problems and allowed the determination of Aβ concentrations that were highly commutable (Bland-Altman) with those obtained using CSF in classical tubes. Moreover, we found a positive and significant correlation (r^2 = 0.83, Pearson coefficient p = 0.0329) between the two approaches.

Dried Serum Spot (DSS): A newly developed dried serum spot (DSS) vitamin B12 assay compares well with a conventional reference serum vitamin B12 microbiological assay (rs0.97, ns161) and demonstrates adequate within (CV% -6) and between assay (CV% -10) reproducibility. These qualities underline the suitability of the DSS matrix for epidemiological screens of serum vitamin B12 levels by obviating the need for costly refrigeration and specialized handling of serum samples and allowing economic transportation using the basic postal service. The consistency of long-term vitamin B12 assay performance was supported and validated using a reconstituted International reference serum (IRR 81/563) stored at y708C as both whole serum aliquots and as DSS. The inclusion of such reference sera also allows accurate comparisons to be made with data from other laboratories and studies. The DSS matrix displays excellent characteristics of pre-analytical serum vitamin B12 stability at ambient temperatures with less than 5% loss of activity occurring at 4 ºC, 20 ºC and 37 ºC after 7 days of storage in the dark [50].

CONCLUSION: The analysis of blood spotted and dried on a matrix (*i.e.*, "dried blood spot" or DBS) has been used since the 1960's in clinical chemistry; mostly for neonatal screening. Since then, many clinical analytes, including nucleic acids, small molecules and lipids, have been successfully measured using DBS. Although this preanalytical approach represents an interesting alternative to classical venous blood sampling, its routine use is limited. Here, we review the application of DBS technology in clinical chemistry, and evaluate its future role supported by

new analytical methods such as mass spectrometry. One interesting aspect of DBS is the possibility of simplified "self/home blood sampling". The patient will be able to independently and safely collect a blood sample. The DBS will then be sent to the laboratory by mail.

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