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Advantages and challenges of dried blood spot analysis by mass spectrometry across the total testing process

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ABSTRACT

Introduction

Through the introduction of advanced analytical techniques and improved throughput, the scope of dried blood spot testing utilising mass spectrometric methods, has broadly expanded. Clinicians and researchers have become very enthusiastic about the potential applications of dried blood spot based mass spectrometric applications. Analysts on the other hand face challenges of sensitivity, reproducibility and overall accuracy of dried blood spot quantification. In this review, we aim to bring together these two facets to discuss the advantages and current challenges of non-newborn screening applications of dried blood spot quantification by mass spectrometry.

Methods

To address these aims we performed a key word search of the PubMed and MEDLINE online databases in conjunction with individual manual searches to gather information. Keywords for the initial search included; "blood spot" and "mass spectrometry"; while excluding "newborn"; and "neonate". In addition, databases

were restricted to English language and human specific. There was no time period limit applied.

Results

As a result of these selection criteria, 194 references were identified for review. For presentation, this information is divided into: 1) clinical applications; and 2) analytical considerations across the total testing process; being pre-analytical, analytical and post-analytical considerations.

Conclusions

DBS analysis using MS applications is now broadly applied, with drug monitoring for both therapeutic and toxicological analysis being the most extensively reported. Several parameters can affect the accuracy of DBS measurement and further bridge experiments are required to develop adjustment rules for comparability between dried blood spot measures and the equivalent serum/plasma values. Likewise, the establishment of independent reference intervals for dried blood spot sample matrix is required.

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INTRODUCTION

A century ago, for the very first time, *Ivar Bang* described a dried blood matrix as an unconventional sampling method (1). Later, *Robert Guthrie* in 1963 introduced the dried blood spot technique for screening. Guthrie's application of the dried blood spot, and his personal crusade to utilise this approach to screen intellectually disabled children, heralded the introduction of newborn screening. Although the particular assay is now defunct, the term "Guthrie card" remains to colloquially describe the dried blood spot collection technique which still underpins today's newborn screening programs worldwide (2).

The original semi-quantitative bacterial inhibition test developed by Guthrie to screen for phenylketonuria was highly sensitive but had a low analytical throughput (3). Through the introduction of advanced analytical techniques, that have expanded testing options and improved throughput, the scope of newborn screening blood spot sample applications were extended; this included screening applications for congenital hypothyroidism and cystic fibrosis in many centres (4). Further to this expansion was the introduction of tandem mass spectrometry for newborn screening dried blood spot analysis in the 1990s (5).

Mass spectrometry is now the most common technique reported in the literature for dried blood spot analysis (6-9). Dried blood spot analysis offers the advantage of collecting a small sample volume, which is easily transported. However, this also means that the concentration of the target analyte is potentially quite low (*e.g.* less than 1 ng/L), requiring a sensitive and specific assay for detection and quantification. These considerations, coupled with the expansion of mass spectrometry into clinical laboratories, have led to a surge in the utilisation of this sampling method outside of the scope of newborn screening in the published literature.

Clinicians and researchers have become optimistic about the potential applications of dried blood spot based mass spectrometric applications and it has been used for a range of clinical utilities including drug toxicology and sports doping screening. Scientists and technical analysts on the other hand face challenges regarding how to ensure optimal sensitivity, reproducibility and overall accuracy of dried blood spot quantification. In this review, we aim to bring together the clinical and analytical facets to discuss the advantages and current challenges of non-newborn screening applications of dried blood spot quantification by mass spectrometry.

To address these aims, we performed a key word search of PubMed and MEDLINE online databases in conjunction with individual manual searches to gather information. Keywords for the initial search included; "blood spot" and "mass spectrometry"; while excluding "newborn"; and "neonate". In addition, databases were restricted to English language and human specific. No time period limit was applied. As a result of this selection criteria, 194 references were identified for review. For presentation, this information is divided into clinical applications and analytical considerations across the total testing process. It is not our intention in this review to highlight all analytical aspects related to quality mass spectrometric analyses (as this has been covered extensively elsewhere) but rather to address the analytical aspects pertinent to the dried blood spot matrix.

CLINICAL APPLICATIONS

The first application of mass spectrometry (MS) to dried blood spot (DBS) analysis was reported 40 years ago (in 1976) for fatty acid determination by direct chemical ionisation (10). By the mid-1980s, when gas chromatography (GC) was the technique of choice for separation and analysis of volatile small molecules, derivatized fatty acids were measured from DBS samples using GC-MS (11). In the 1990s, when electrospray ionization became commercially available, liquid chromatography - tandem mass spectrometry (LC-MS/MS) began to be incorporated into the analytical tools for newborn screening laboratories, leading to the significant expansion of screening applications with phenylalanine and tyrosine being two of the early markers (5, 12). Today dried blood spot based mass spectrometric (DBS-MS) applications are the workhorse for many newborn screening (NBS) laboratories worldwide; and additional tests are continually being added to the repertoire. Outside of NBS, an epidemiological study analysing benzoylecgonine was the first reported DBS-MS application (13).

Now, many potentially reliable and compatible MS detection methods are available across a wide range of disciplines (14). Our literature search identified 97 references encompassing 121 distinct biomarkers determined from DBS samples utilising MS technology beyond its application in NBS. Notably, the role of DBS analysis by MS now encompasses translational research and clinical diagnostic analytes in the areas of therapeutic drug monitoring (TDM); pharmakinetics); toxicokinetics; forensic; endocrinology and metabolism; and other areas of bio-analysis. Table 1 provides a list of these biomarkers.

Therapeutic and toxicological drug analyses are the most extensively reported DBS-MS applications in the literature (Table 1). These DBS-MS applications, (encompassing both LC-MS/MS and GC-MS techniques) are particularly fit for population-based studies of multiple biomarkers (15, 16). Similarly, DBS-MS applications are now applied in sport related doping tests for the detection of anabolic, ergogenic and masking agents (17-19).

Irrespective of the clinical application, there are specific analytical considerations. Several parameters can impact on the accuracy of DBS measurement (10). The following sections of this review will focus on important considerations for DBS-MS quantification in the pre-analytical, analytical and post-analytical phases.

PRE-ANALYTICAL

The pre-analytical phase of testing incorporates the following processes: 1) blood collection from the patient; 2) its application onto the filter paper; 3) drying; and 4) transport and storage of the DBS sample. In addition to the pre-analytical variables identified for routine blood collection, DBS faces additional challenges, including the quality of the DBS sample (which is subject to

sample collection and spotting variations), choice of collection card, collection (bleeding and blotting), transport and storage. Moreover, biological factors such as sample viscosity, haematocrit level and the nature of the target analyte, may lead to variation in sample quality.

Abbreviations for Table 1 (in alphabetical order)

2D: *two dimensional*
AnalTech: *analytical technique*
APTDCI-MS: *atmospheric pressure thermal desorption chemical ionization mass spectrometry*
CE: *chemical exposure*
D: *day*
DAA: *drug abuse athletics*
Dab: *drug abuse*
Dad: *drug adherence*
ESI: *electrospray ionisation*
FIA-ESI-MS/MS: *flow Injection analysis-electrospray ionisation tandem mass spectrometry*
FT-ICR-MS: *fourier transform ion cyclotron resonance mass spectrometry*
GC: *gas chromatography*
GC-HRMS: *gas chromatography–high resolution mass spectrometry*
HILIC-MS/MS: *hydrophilic Interaction chromatography tandem mass spectrometry*
ID: *illicit drug*
IDES-MS/MS: *isotope-dilution electrospray tandem mass spectrometry*
LC: *liquid chromatography*
LC-HRMS: *Liquid chromatography–high resolution mass spectrometry*
LLOQ: *lower limit of quantitation*
M: *month*
MetV: *method validation*
MI: *metabolic intermediate*
MS: *mass spectrometry*
NR: *not reported*
PD: *pharmaceutical drugs*
PS-MS: *paper spray mass spectrometry*
PK: *pharmakinetics*
Ref: *reference*
RepU: *reporting unit*
Stab: *stability*
TDM: *therapeutic drug monitoring*
TK: *toxcicokinetics*
TLC-MS: *thin-layer chromatography mass spectrometry*
W: *week*
Y: *yes*

Table 1 The list of biomarkers determined from dried blood spot samples utilising mass spectrometry technology, beyond its application in newborn screening studies

No	Analyte	AnalTech	LLOQ	RepU	Stab	MetV	Ref
Metabolic intermediate							
1	Acylcarnitines	APTDCI-MS/MS	NR	µmol/L	NR	NR	(110)
		ESI-MS/MS	NR	µmol/L	NR	NR	(111)
2	Adrenal steroids	LC-ESI-MS/MS	0.75-6.3	nmol/L	NR	Y	(112)
3	Amino acids profile	LC-MS/MS	0.25	µmol/L	NR	Y	(113)
4	Bile Acids	ESI-MS/MS	1	µmol/L	10d	Y	(114)
5	Carnitine	IDES-MS/MS	1	µmol/L	NR	Y	(115)
		ESI-MS/MS	NR	µmol/L	NR	Y	(111, 116)
6	Creatine	FIA-ESI-MS/MS	0.25-3.57	µmol/L	NR	Y	(117)
7	Creatinine	LC-MS/MS	116	µmol/L	7d	Y	(118-120)
8	CYP450enzymes	LC-MS/MS	0.1	ng/ml	4w	Y	(121)
9	Cystathionine	LC-MS/MS	0.1-2.5	µmol/L	14d	Y	(122)
10	F2-isoprostanes	LC-MS/MS	6	pg/mL	NR	Y	(123)
11	Gamma-butyrobetaine	ESI-MS/MS	NR	µmol/L	NR	NR	(116)
12	Glucosylceramide	LC-MS/MS	NR	µg/mL	NR	Y	(124)
13	Guanidinoacetate	FIA-ESI-MS/MS	0.25-3.57	µmol/L	NR	Y	(117)
14	Haemoglobin peptides	LC-MS/MS	NR	Ratio	NR	NR	(125)
15	Haemoglobin variants	MS	NR	NR	NR	NR	(64)
16	Haemoglobins α- and β-chains	FT-ICR-MS	NR	%	NR	NR	(126)

17	Homocysteine	LC-MS/MS	1	µmol/L	3m	Y	(127)
		LC-MS/MS	0.1-2.5	µmol/L	14d	Y	(122)
18	Insulin-like growth factor-1	LC-MS/MS	50	ng/ml	8d	Y	(19)
19	Methotrexate polyglutamates	LC-MS/MS	5	nmol/L	NR	Y	(128)
20	Methylmalonic acid	LC-MS/MS	10	nmol/L	8w	Y	(129)
21	Methylmalonyl-carnitine	LC-MS/MS	0.025	µmol/L	NR	Y	(130)
22	Nucleoside profile	LC-MS/MS	NR	µmol/L	NR	Y	(131)
23	Orotic acid	HILIC-MS/MS	0.18	µmol/L	NR	Y	(132)
24	Protein profile	LC-MS/MS	NR	NR	NR	NR	(133)
25	Proteomics	LC-MS/MS	NR	µmol/L	NR	NR	(134)
26	Peptide profile	LC-MS/MS	NR	µg/ml	10d	NR	(135)
27	Stroles	APTDCI-MS	NR	Ratio	NR	NR	(110, 136)
28	Succinylacetone	LC-MS/MS	0.67	µmol/L	NR	Y	(137)
		LC-MS/MS	0.25	µmol/L	NR	Y	(113)
29	Succinyl-carnitine	LC-MS/MS	0.025	µmol/L	NR	Y	(130)
30	Sulfatides	LC-MS/MS	NR	µg/mL	NR	NR	(6)
31	Vitamin D	LC-MS/MS	4.8	nmol/L	>20y	Y	(38, 138-141)
Pharmaceutical drugs							
32	Acetaminophen (Paracetamol)	LC-MS/MS	50	ng/ml	NR	NR	(142)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
		TLC-MS	5.0-50	ng/ml	NA	NR	(143)
33	Amitriptyline	PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
		LC-MS/MS	20	µg/L	1m	Y	(144)

34	Amprenavir	LC–MS	11.7	ng/ml	3m	Y	(145)
35	Paroxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
		LC–MS	11.7	ng/ml	3m	Y	(145)
36	Atazanavir	LC-MS/MS	0.1	mg/L	7d	Y	(146)
37	Atenolol	LC-HRMS	25	ng/ml	2m	Y	(147)
38	Benzodiazepines	LC-MS/MS	NR	ng/mL	NR	NR	(148)
39	Benzethonium chloride	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
40	Bisoprolol	LC-HRMS	0.5-5.0	ng/ml	12w	Y	(149)
41	Bosentan	LC-MS/MS	2	ng/ml	5w	Y	(150)
42	Busulfan	LC-MS/MS	50	ng/ml	NR	Y	(151)
43	Citalopram	PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
44	Clarithromycin	LC-MS/MS	0.05-0.15	mg/L	2m	Y	(152)
45	Clomipramine	LC-MS/MS	20	µg/L	1m	Y	(144)
46	Cyclosporin A	LC-MS/MS	116	µmol/L	7d	Y	(118, 120, 153-155)
47	Darunavir	LC-MS/MS	0.1	mg/L	7d	Y	(156)
		LC–MS	11.7	ng/ml	3m	Y	(145)
48	Dasatinib	LC-MS/MS	2.5-50	µg/L	28d	Y	(157)
49	Dexamethasone	LC-MS	15	ng/ml	28d	Y	(158)
50	Efavirenz	LC-MS/MS	41-102	ng/ml	NR	N	(145)
		LC-MS/MS	0.1	mg/L	7d	Y	(156)
51	Emtricitabine	LC-MS/MS	2.5	ng/ml	6 d	Y	(159)
52	Endoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
53	Ertapenem	LC-MS/MS	0.2	mg/L	30d	Y	(54)

54	Etravirine	LC-MS/MS	50	ng/ml	7d	Y	(160)
		LC–MS	11.7	ng/ml	3m	Y	(145)
55	Everolimus	LC-MS/MS	116	µmol/L	7d	Y	(118, 120, 153, 155, 161)
56	Fluoxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
57	Flurbiprofen	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
58	Gamma-hydroxybutyric acid	GC-MS	1	µg/ml	15d	Y	(67, 163)
59	HIV protease inhibitors	LC-MS/MS	0.025-10	µg/ml	7d	Y	(164)
60	Ibuprofen	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
61	Imatinib	LC-MS/MS	20.5-50	µg/L	28d	Y	(157)
62	Imipramine	LC-MS/MS	20	µg/L	1m	Y	(144)
63	Linezolid	LC-MS/MS	0.4	mg/L	1m	Y	(165)
64	Loratadine	LC-MS/MS	0.2	ng/ml	271d	Y	(166)
65	lopinavir	LC–MS/MS	0.1	mg/L	7d	Y	(156)
		LC-MS	11.7	ng/ml	3m	Y	(145)
66	Losartan	LC-MS/MS	1	ng/ml	30d	Y	(167)
67	Mefloquine	LC-MS/MS	2.5	nmol/L	3w	Y	(168)
68	Midazolam	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
69	MK-1775 (Wee1 inhibitor)	HILIC-MS/MS	2	ng/ml	14m	Y	(169)
70	N-desmethyltamoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
71	Nevirapine	LC-MS/MS	41.102	ng/ml	NR	N	(156)
		LC-MS/MS	0.1	mg/L	7d	Y	(53)
72	Nilotinib	LC-MS/MS	2.5-50	µg/L	28d	Y	(157)
73	NIM811 (cyclophilin inhibitor)	LC-MS/MS	10	ng/ml	24h	Y	(170)

74	Nitisinone	LC-MS/MS	0.1	µmol/L	NR	Y	(171)
		LC-MS/MS	0.25	µmol/L	NR	Y	(113)
75	Norfluoxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
76	Nortriptyline	LC-MS/MS	20	µg/L	1m	Y	(144)
77	O-desmethylvenlafaxine	LC-MS/MS	20	µg/L	NR	Y	(172)
78	Omeprazole	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
79	Oseltamivir	LC-MS/MS	5	ng/mL	7d	Y	(173)
80	Paclitaxel	LC-MS/MS	0.2	ng/ml	45d	Y	(174)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
81	Paroxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
		LC–MS	11.7	ng/ml	3m	Y	(145)
82	Phenobarbital	LC-MS/MS	1	mg/L	10d	Y	(175)
83	Phenytoin	LC-MS/MS	0.3	mg/L	30d	Y	(176)
84	Posaconazole	LC-MS/MS	5	ng/ml	13d	Y	(177)
85	Proguanil	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
86	Propranolol	LC-MS/MS	2.5	µg/L	30d	Y	(178)
87	Raltegravir	LC-MS/MS	0.125	µg/L	7d	Y	(179)
88	Ramipril	LC-HRMS	0.5-5.0	ng/ml	12w	Y	(149)
89	Reboxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
90	Ribavirin	LC-MS/MS	0.05	ng/ml	140d	Y	(180)
91	Rifampicin	LC-MS/MS	0.05-0.15	mg/L	2m	Y	(152)
92	Rifapentine	LC-MS/MS	51	ng/ml	11w	Y	(181)
93	Rifaximin	LC-MS	0.1	ng/ml	30d	Y	(182)
94	Ritonavir	LC-MS	11.7	ng/ml	3m	Y	(145)

95	Rosiglitazone	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
96	Saquinavir	LC–MS	11.7	ng/ml	3m	Y	(145)
97	Simvastatin	LC-HRMS	0.5-5.0	ng/ml	12w	Y	(149)
		TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
98	Sirolimus	LC-MS/MS	116	µmol/L	7d	Y	(118, 120, 153, 155)
99	Sitamaquine	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
100	Sunitinib	PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
101	Tacrolimus	LC-MS/MS	1	µg/L	20d	Y	(8, 60, 118-120, 153, 155, 183)
102	Tamoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
103	Tenofovir	LC-MS/MS	2.5	ng/ml	6d	Y	(159)
104	Topiramate	LC-MS/MS	10	µg/ml	194d	Y	(184)
105	Valproic acid	LC-MS	10	µmol/L	42d	Y	(185)
		GC-MS	5	µmol/L	21d	Y	(69)
106	Vemurafenib	LC-MS/MS	1	µg/ml	NR	Y	(186)
107	Venlafaxine	LC-MS/MS	20	µg/L	NR	Y	(172)
108	4-nitrophthalic acid	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
109	4-hydroxytamoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
Illicit drugs							
110	Amphetamines	ESI-MS/MS & GC-MS	2.3-11	ng/ml	NR	Y	(187)
		2D-LC-MS/MS	5	ng/ml	6 m	Y	(188)

111	Caffeine	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
112	Cocainics	ESI-MS/MS & GC-MS	2.3-11	ng/ml	NR	Y	(187)
		2D-LC-MS/MS	5	ng/ml	6 m	Y	(188)
113	Novel psychoactive substances	LC-ESI-MS/MS	1.0-10	ng/ml	1w	Y	(189)
114	Opiates	2D-LC-MS/MS	5	ng/ml	6m	Y	(188)
		ESI-MS/MS & GC-MS	2.3-11	ng/ml	NR	Y	(187)
115	Δ9-tetrahydrocannabinol	LC-MS/MS	100	ng/ml	3m	Y	(190)
Drug abuse athletics							
116	Anabolic steroid esters	LC-MS/MS	0.1	ng/ml	28d	Y	(18)
117	Centchroman metabolites	LC-MS/MS	1.5-4.5	ng/ml	3m	Y	(191)
118	Testosterone glucuronides	GC-MS	NR	pg/mL	NR	NR	(17)
119	Therapeutic proteins	LC-MS	NR	ng/mL	2w	NR	(192)
Chemical exposure							
120	Benzene oxide-Hb	GC-MS	NR	pmol/g	NR	NR	(193)
121	Polybrominated diphenylethers	GC-HRMS	0.05	ng/ml	30d	Y	(194)

To support the quality management of the pre-analytical phase, there are defined recommendations for positive patient identification (20), sample collection of capillary blood (21), choice of filter paper (22), application of the sample onto the filter paper (23) and shipment of the DBS sample (24). An example of a standardised protocol for the DBS pre-analytical process is provided in Table 2.

Sample collection

The sample collection technique is important for accurate analysis of the DBS. Capillary blood collection is a common approach, as it usually requires less sample volume and is more patient friendly, compared to venepuncture. Both the World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) certify the quality of DBS samples and maintain healthcare workers safety through the provision of guidance manuals and standard operating procedures for DBS sample collection (25, 26). Due to the likelihood of significant sources of artefact formation, specific DBS collection training points are highlighted, including the choice of prick point, lancet type/size, prick depth, pressure rate during blood drop collection, dropping size/speed

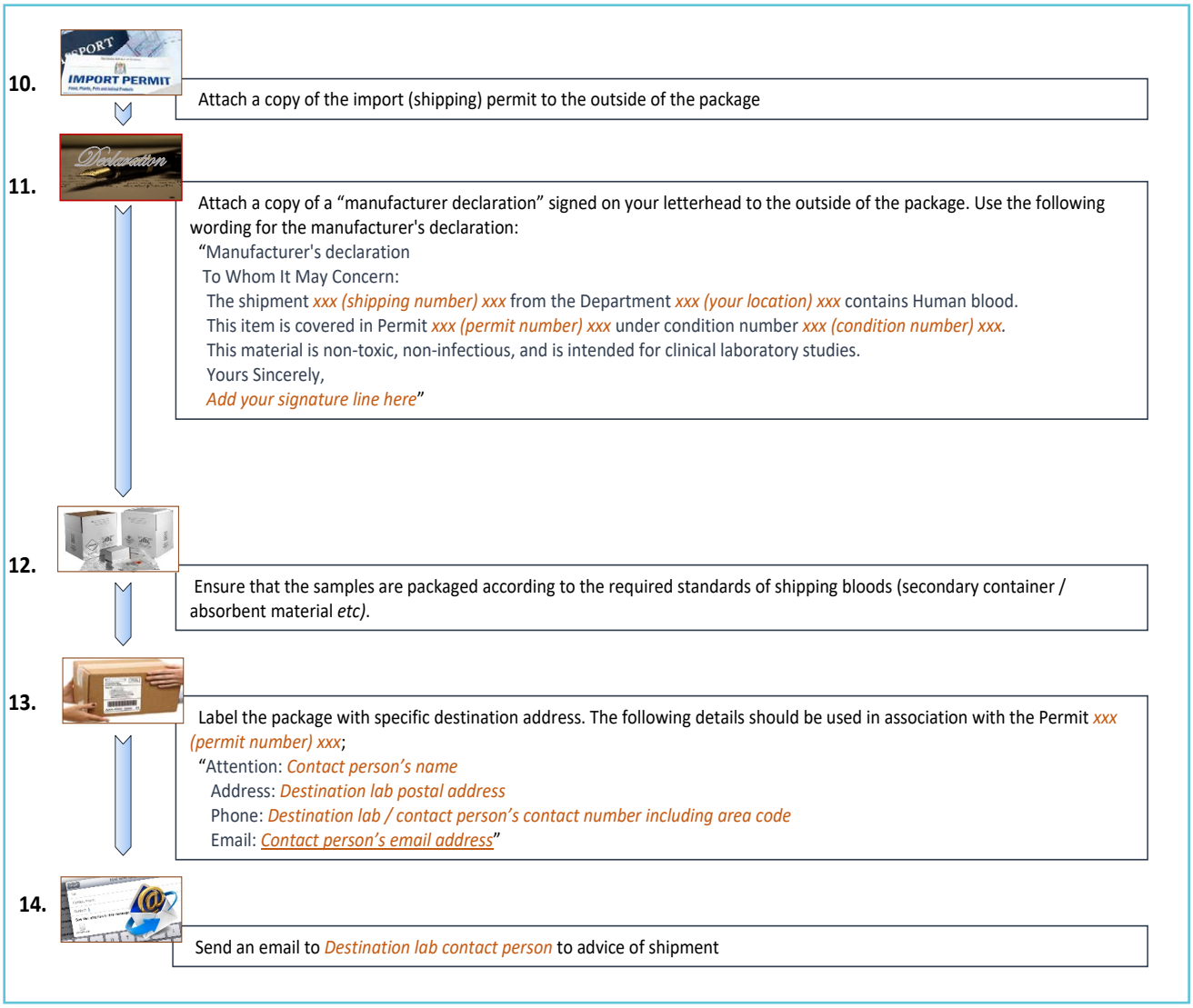
consistency, uniformity of sample diffusion onto the collection card and DBS contamination due to extended air or light exposure during the drying process. Importantly, WHO highlight that "working with DBS, whole blood or plasma requires the same biohazard safety precautions as whole blood" (26).

Selection of filter paper

Collection of DBS samples is onto one of two types of untreated solid support: pure cotton filter paper and glass microfiber paper. The choice of paper, including its thickness and density, influences the rate of adsorption and dispersion. As an example, the rate of membrane non-specific analyte adsorption is reduced in glass microfibre paper (27). Accordingly, dissimilarities in these solid supports may induce variations in the DBS sample attributes leading to potential differences in analyte stability, commutability, volume per area, and analytical effects (covered later in this review) (28). These DBS specific pre-analytical variables require standardisation and the Clinical and Laboratory Standards Institute (CLSI) offer a guideline, NBS01-6, to support DBS collection (22).

Table 2 Example dried blood spot sample collection and transport protocol

1.		Positive patient identification
2.		From a capillary collection, allow one drop of blood to penetrate each position of the filter paper. This should be one large drop of blood approx. 20µl
3.		Repeat for all positions
4.		Record at least two (prefer 3) identifier on the card (*eg.* Name, date of birth, study number, Health record number)
5.		Record sample collection date and time
6.		Record the following information in the logbook; a) Participants first name and surname, b) Participants date of birth, c) Study number, d) Card bar code number, e) Date of collection, f) Any other relevant information
7.		Allow samples to air-dry for a minimum of 2 hours (longer may needed in humid or cold environment)
8.		Store in Biohazard / Specimen Transport Bag in dry place at room temperature until shipment to the destination lab
9.		Send dried blood spot sample cards following the protocol to the destination lab with a copy of the logbook entries



The CLSI guideline recommends the use of two specific collection cards: the Whatman 903 and Ahlstrom 226. Both filter papers are approved by the Food and Drug Administration (FDA), Newborn Screening Quality Assurance Program (NSQAP) and also the Centers for Disease Control and Prevention (CDC) (23).

Sample application to filter paper

Both the Whatman 903 and Ahlstrom 226 cards have a target collection area printed on the paper to indicate and ensure the 50-75 µl sample volume limits (23). The whole blood drop should be spotted onto the mid-point of the collection area to allow for radial dispersion to the designated edge of the spot. This lateral distribution is uneven; as clearly evaluated by autoradiography (29). Usually, the concentration of biomarkers is decreased along the edge of the DBS, while the middle is affected by the volcano effect (paper chromatography effect) and occasionally has a speckled pattern (30). The physical characteristics of the DBS sample are also potentially affected by the patient's haemoglobin and haematocrit level; which also influences the spreading area of the blotted blood.

Variations in haematocrit will affect the relative plasma percentage of the spot. This is important

for analytes predominately found in serum/plasma, as the relative amount of plasma in a disk punched from different spots can vary; and particularly exaggerated when the haematocrit is extremely high or low (31). Whole blood samples with a higher haematocrit tend to distribute to a lesser extent across the filter paper (*i.e.* smaller blood spot diameter), and consequently the target analyte diffusion distance is shorter. The inverse applies for lower haematocrits. As a result, the determined concentration compared with the "normal" haematocrit sample would be over or under estimated due to the change in the analyte's distribution and infusion pattern across the blood spot (32, 33). Hence, for accurate quantitation, ideally the patient's haematocrit needs to be determined, by either a separate capillary drop/sample collected at the time of the DBS collection or directly from the DBS card. Figure 1 provides a visual demonstration of the effect of haematocrit on diffusion.

Analytes stability (storage and transport condition)

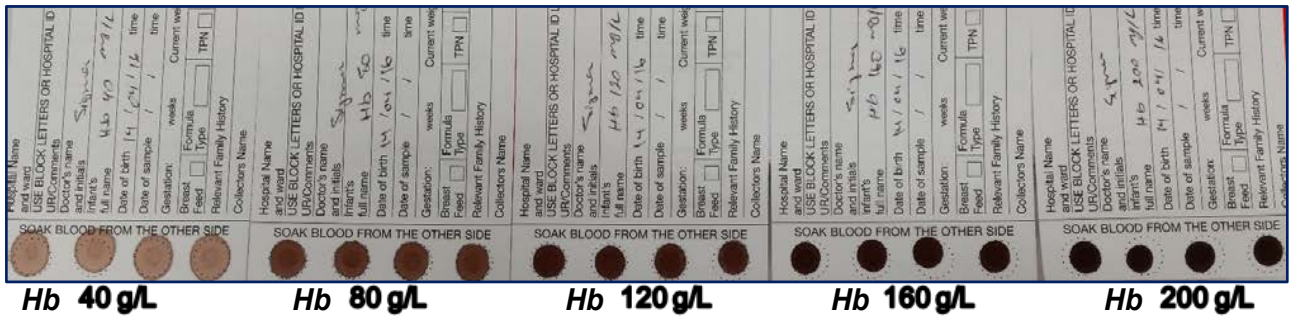
DBS samples should be allowed to completely dry before transport and/or storage. It has been demonstrated that rapid drying and storage in low humidity conditions improves the stability of DBS samples (16). The length of time required for air-drying will depend on the local environmental conditions such as air conditioning, room temperature and humidity. DBS drying usually takes from 90 minutes to approximately 4 hours and ideally, the DBS sample should not be left exposed to direct strong sunlight during this period.

The appropriately dried sample can then be placed in an envelope or similar container for the logistically simple and cost effective transport process (16). As the low volume of the dried specimen significantly reduces the risk of infection transmission compared to the other biological samples (34, 35), it can be transported in small lightweight packages that do not require temperature regulation. This negates the often cumbersome and expensive processes associated with transport of liquid biological samples (16).

Once the DBS samples are received at the destination, the size and properties of the DBS samples make storage relatively easy as minimal space is required; and they can often be stored at room temperature.

The stability of the DBS sample does require consideration, as the relevant stability for different analytes on DBS is quite variable. The stability of numerous blood biomarkers on blotting paper at

Figure 1 **Whole blood samples with different levels of haemoglobin/haematocrit (Hb/Hct) do not diffuse similarly on the collection card**



For a fixed punch size, a high level of Hb/Hct results a higher amount of blood on the punch thus the measured target analyte is falsely elevated. This figure is a visual demonstration of the effect of haematocrit on diffusion.

room temperature has been confirmed for a minimum of 7 days (27), several months (pharmaceutical drugs, opiates and nucleic acids) (36, 37), and even more than 20 years (vitamin D) (38). Freezing at -20°C or -80°C has been shown to further increase the stability of DBS samples; often extending the stability from days/months to years (39-41). Further, some reports show that some metabolites have better stability in the form of card-blotted whole blood compared to plasma (42).

Despite these exciting reports of long-term stability, there are many recognized potential influences, in addition to storage time, which could affect stability; including the type of filter paper, light exposure, temperature, humidity as well as the nature of the target analyte. Examples of changes to the collection process to improve stability include: 1) rapidly lowering the pH of the spotted blood sample to extend the stability time of some structurally unstable drugs which degrade during the drying process; and 2) use of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant to preserve and stabilise DBS samples for enzyme activity determination (43, 44). As a result of variations in stability, analyte specific protocols need to be administered for the collection and storage of DBS samples. Table 1 includes a guide to the stability of DBS samples for different analytes found in the literature.

ANALYTICAL CONSIDERATIONS

The solid form of the DBS sample is not compatible with most analytical techniques and requires elution of the sample from the filter paper. Accordingly, the testing process commonly includes three main stages: primary sample preparation, sample pre-treatment and sample analysis. Analysis can be further divided into chromatographic separation and mass spectrometric filtration and detection. The overall quality of this analytical process needs consideration. In this section of the review, we will discuss each of these aspects in turn. Figure 2 provides a general summary of the DBS-MS process.

Primary sample preparation

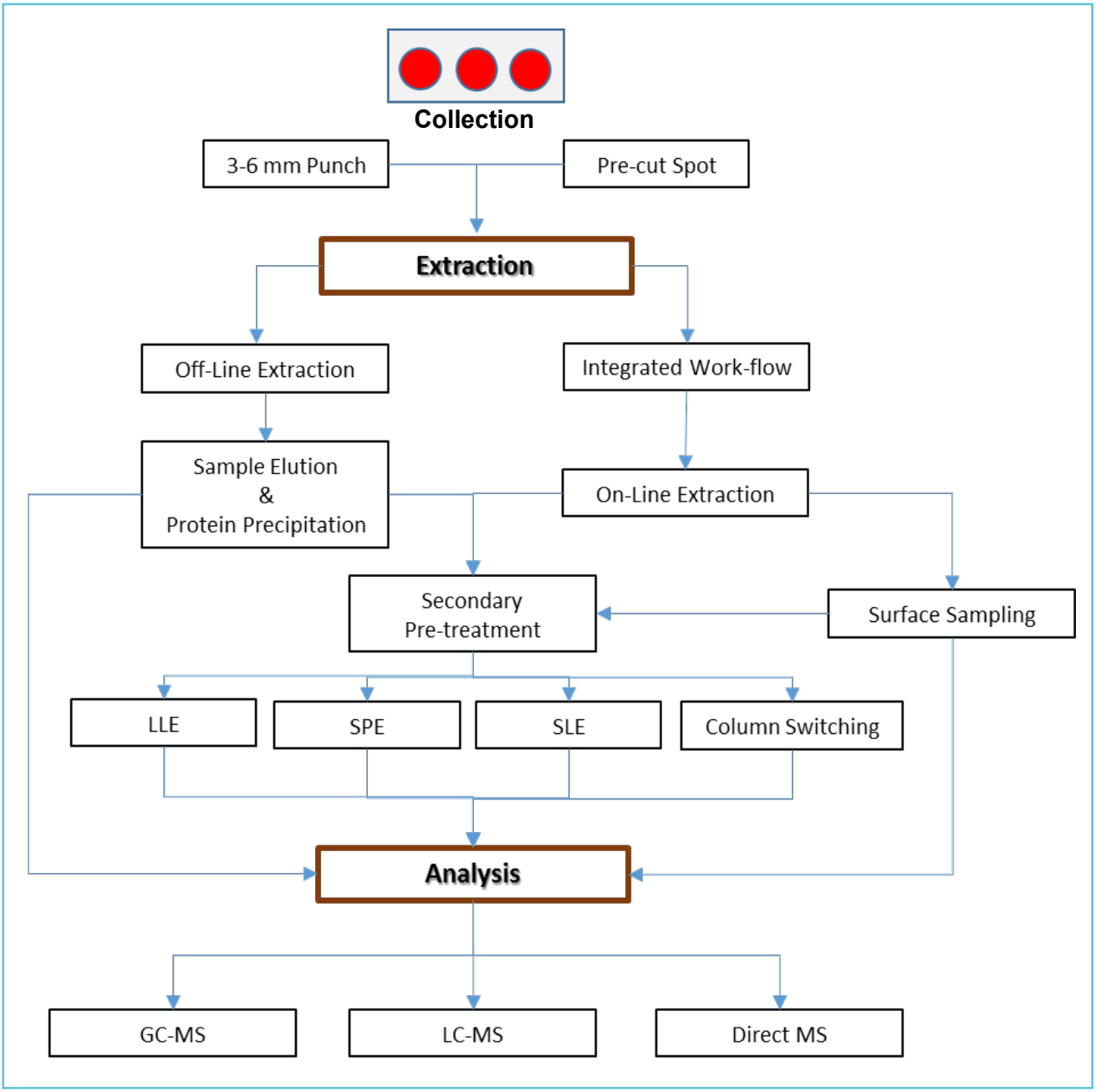
The punch

Sample preparation usually starts with deportation of a segment of the DBS from the blotter using a manual or automated puncher. Commonly, to minimise the assay bias due to punch location, it is recommended to consistently take the DBS punch either from the centre or close to the outer edge (45). The punch size may vary from 3 – 6 mm to the whole spot, depending on the method.

Techniques have been developed to overcome the variations in haematocrit and also minimize the labour associated with the sample preparation process. Strategies to overcome the haematocrit effect include:

1. Pre-cutting or perforating the filter paper as part of the DBS handling procedure to recover the haematocrit effect and eliminate the chance of carry-over between the punches;
2. Blotting of less whole blood volume (*e.g.* 10 µl) on the smaller pre-cut disk (3 or 6 mm) and analysis of the whole disk to disregard the haematocrit effect and improve the assay bias, (46-49);
3. A two-layered polymeric membrane to form a separated secondary dried plasma spot from the whole blood sample to be analysed following solid phase extraction (50);
4. Development of a novel collection card for DBS sampling, which generates a volumetric plasma sample (2.5 or 5.0 µL) from a non-volumetric application of whole blood sample. The purported advantages of this collection matrix includes enhanced assay reproducibility and selectivity, with a simplified sample extraction procedure and elimination of the haematocrit effect (51).

Figure 2 Dried blood spot sample analysis process flowchart for mass spectrometric analysis



Elution

For analysis, the analyte of interest firstly requires elution out of the filter paper along with the whole blood matrix by using appropriate extractor buffers. The efficient elution of analytes from the DBS is challenging and there is always a chance of analyte loss due to ineffective extraction; poor sample elution is due to either incomplete extraction or analyte degradation. Hence, the choice of optimal extractor materials may vary from one compound to the other. As an example, pure methanol is considered a

generic solvent for drugs of abuse extraction of the blood spot sample (52). Water on the other hand impairs the interaction between cellulose and the target analyte's hydroxyl groups and the partial addition of water prior to the organic extraction advances the efficiency in certain cases (*eg.* antivirals) (53). To achieve effective analyte recovery with maximum extraction efficiency, the extraction parameters, including extractor solution mix, duration, temperature and application of additional solvation energy (sonication), need to be optimised for each individual target metabolite (31, 54).

Sample pre-treatment

A variety of sample preparation approaches have been suggested, with selection depending on the molecular characterisation of the target compound. Incorporation of sample pre-treatment methods, either in combination with each other or in isolation, include the classic sample preparation process of: 1) protein precipitation (PPT); 2) liquid-liquid extraction (LLE); 3) solid phase extraction (SPE); 4) supported liquid extraction (SLE); and/or 5) derivatization.

Extraction and derivatization procedures applied manually (or offline) are considerably time consuming and laborious. Whilst derivatization is not required for many plasma based analytes using LC-MS/MS, it is required for many DBS analysis to improve the sensitivity; offsetting the small sample volume. However, as the derivatization process prolongs the overall analysis time it is considered to be a limiting factor and has been a driver for the development of on-line extraction techniques to facilitate the DBS sample pre-analytical treatment.

Automation of sample preparation directly coupled with the LC-MS/MS system has been introduced to improve turn-around time and run cost. PPT is a simple and popular method for automation that has been utilised for TDM (55). However, following a single PPT procedure, salts and other endogenous analytes are still present which may cause ion suppression in the MS process. SPE-LC-MS/MS set-up is designed to facilitate online sample desorption and is a time and cost effective method for DBS analysis (37, 56-59). Compared to PPT, SPE presents an improved sample clean-up (60). There are specific challenges with on-line extraction approaches, in comparison with the off-line extraction methods which may be a significant source of assay bias, including; non-homogenous mixture of internal standard (ISTD) with the analyte in the extract; sample dilution then band broadening in chromatography separation; and/or inadequate focusing of the extract onto the analytical column (10). Accordingly, as part of the method development process, certain strategies are required to eliminate these issues.

Technology has been developed that allows for the direct sampling of the DBS, without the need for a change to liquid or elution. As it is described by the manufacturer, "Liquid Microjunction Surface Sampling Probes (LMJ-SSP) are self-aspirating devices where liquid is pumped to and aspirated away from a surface of interest to a mass spectrometer for integrated extraction and ionization" (61). By utilising the LMJ-SSP technology, the analyte of interest could be directly extracted from the different surfaces and detected by a mass spectrometer in a short time frame with minimum sample handling (62). The LMJ-SSP device coupled with the MS has been utilised for the determination of proteins in the DBS sample (63), direct tandem mass spectrometer for detection of haemoglobin (64), as well as therapeutic drugs (65, 66). Likewise, novel "on spot" direct derivatization approaches provide a time and cost effective alternative sample preparation procedure; a technique introduced to determine thiorphan drug (67, 68).

Sample analysis

Gas chromatography is known as a cheaper and faster separation technique compare to the LC, utilising long and compact capillary columns that enhance resolution. GC-MS analytical techniques often offer a higher separating power and efficient reproducibility compared with LC-MS. Accordingly, GC-MS is still utilised for the determination of volatile biomarkers with low molecular weight and heat resistance fragments. Applications include, steroids, metabolomics and therapeutic drug monitoring studies using DBS samples (17, 67, 69-71) (Table 1). However, the application of GC is limited to gas soluble, volatile and heat resistance small molecules (often derivatization is required to turn non-volatile molecules to volatile), which are gas soluble (72, 73).

LC is preferred for analysis of heat sensitive analytes, with no molecular size restriction. Additionally unlike GC, target compounds co-interact with both mobile phase and solid phase which results in better selectivity (73). The choice of GC or LC instruments depends on the required sensitivity and target analyte characteristics. Despite the fact that the GC-MS provides selectivity, sensitivity and robustness for many DBS analytes, the literature demonstrates that, it is not as popular as LC-MS/MS (Table 1). This is likely to be due to the improved specificity and sensitivity afforded by LC-MS/MS for blood spot analysis; associated with a significantly faster and usually more cost effective process compared to GC-MS (74, 75).Furthermore, advanced UHPLC technology has boosted the resolution of peak separation (even more effective than GC) (72, 73).

By introduction of the two dimensional chromatography (2D-C) tools (applicable on both GC and LC), the separation efficiency, analytical sensitivity, quantitation accuracy and precision have been improved. This 2D-C process has reduced the DBS matrix and carry-over effects, with reports suggesting improved imprecision and bias (76, 77). With the further addition of on-line extraction joined to the 2D-C system, sensitivity and specificity is maximised when coupled with either a triple-quadrupole tandem mass spectrometer or a high-resolution quadrupole time of flight mass spectrometer (QTOF-MS) (60, 78).

The advances in ion source technology have enhanced sensitivity for both polar and non-polar analytes from DBS samples (79-81). Selective/multiple reaction monitoring (SRM/MRM) modes in MS/MS detection, focusing on specific transitions, have advanced the assay specificity remarkably along with improving linearity and limits of detection (10, 82).

Negating the pre-analytical clean-up and chromatographic front end separation, direct MS methods and surface sampling techniques coupled with MS have been used for DBS samples (83). Desorption electrospray ionisation (DESI), direct analysis in real time (DART) and direct electro spray ionisation mass spectrometry (ESI-MS) methods have been utilised in order to generate ions from the surface, thus avoiding purification or derivatization processes (83-90). However, elimination of this primary sample purification and separation may result in loss of sensitivity and precision due to the disintegrated metabolite interferences (89).

Thus, the application of non-paper blotting matrices and online SPE in conjunction with the direct MS methods is recommended to enhance the sensitivity and measurement precision (75, 91). Ultimately, the gains of time efficiency and throughput need to be balanced with achieving the desired method performance.

Quality considerations

There are some important considerations in relation to the method validation and acceptance criteria for DBS analysis. Accordingly, the European bio-analysis forum (EBF) has described

the details of the DBS sample analysis methodology to provide specific recommendations for validation of DBS methods (92). The EBF recommendations document includes specific concepts of; collection card variability, sample-to-sample variability, DBS homogeneity, punch point effect, sample stability, blood physical parameters effect, matrix effect, extraction recovery, IS application and internal quality control (IQC) and calibrator preparation. In addition to the detail provided in this document, traceability to the liquid matrix sample, fitness for clinical purpose and reference intervals/decision limits for interpretation require consideration.

Collection cards

To avoid issues of inter-card variability, calibrator and control material should be prepared using identical collection card type/manufacture as the patient samples. If multiple type/manufacturer of cards are used, then a method comparison is required to determine the comparative card stability, extraction recovery and matrix effect (93).

Haematocrit effect

As mentioned earlier, the physical behaviour of blotted whole blood is influenced by different parameters such as; haematocrit level, degree of haemolysis and anticoagulant type (if it is applied). Currently the haematocrit is recognised as the most significant parameter affecting blood spot characteristics (drying time, diffusion and homogeneity) and assay reproducibility. The Haematocrit effect is more substantial when a sub-sample disk punch is analysed, rather than the whole DBS sample. Hence, method validation studies for DBS sample applications also need to include investigations of the impact of haematocrit variation on measurement and assay performance (94).

Application of internal standard

The incorporation of the ISTD to the DBS sample processing is an important step and ideally should occur early in the process. 1) Collection cards pre-treated with the ISTD can be prepared prior to the spotting of the blood. This ensures both the ISTD and nominated compounds have undergone the same matrix and extraction effect. However, this approach logistically might not be practicable when dealing with multiple studies. 2) Commonly manual extraction methods utilise the approach of integrating the ISTD into the DBS elution reagent/extraction solvent. In this method, the ISTD is co-extracted along with the target analyte. 3) Addition of the ISTD into the sample along with extraction/preparation process is another simple alternative. However, as the ISTD is not fully incorporated with the paper matrix, variations in elution recovery are not accounted for. 4) Using on-line DBS sample preparation technology, the ISTD is sprayed on the blood spot before the extraction using the Touch-spray technique (95-97).

Carry-over

Carry-over is a significant issue for DBS-MS analysis. Carry-over may have different sources including: physical card to card contact during storage; spot to spot originated from the puncher head and post-preparation initiated from the instrument (*e.g.* auto-sampler and analytical column) (92). As the puncher head is re-used, contamination and sample carry-over are notable concerns. To overcome this issue, either a clean-up step or a blank-card punch in between the samples is recommended (98). To investigate the instrumentation carry-over, two injections of sequential blank DBS extracts should be performed after an injection of a sample with the upper limit of quantitation concentration. The response for the first and second blank matrix should not exceed 20% and 5% respectively of the mean response of the lower limit of detection of the analyte of interest (99).

Internal quality control

IQC spiked samples preparation for DBS analysis requires special considerations compared to liquid phase biologic samples. The main challenge of internal QC is keeping the matrix consistent with that of patient's blood spot sample. Both sample dilution and saturation may occur in spiked sample preparations (93). Ideally fresh non-haemolysed whole blood samples, with a closely matched haematocrit level to the study group, should be chosen for spiking (99). These IQCs should be spotted onto the filter paper and eluted along with the patient samples.

External quality assurance

External quality assurance (EQA) programs are considered essential tools in evaluating the reliability and traceability of the analytical assay as well as monitoring the quality of the laboratory performance. The United Kingdom National External Quality Assessment Service (UK-NEQAS), the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) and the CDC (NSQAP) provide a variety of schemes for DBS-NBS testing. However, there is no further EQA program available to assure the accuracy of DBS analysis outside of NBS. Hence, for most DBS analytes discussed in this review we do not have a peer review process to fully gauge laboratory performance. This represents a gap in harmonisation of analytes measured in this matrix.

Calibration

The preparation of standards for DBS quantitative analysis includes whole blood fortification (replacement of certain amounts of plasma with the artificial plasma containing a known concentration of target analyte) with a set of commercial or in-house calibrator materials before spotting. The percentage of non-aqueous components replaced with plasma needs to be minimised to prevent solvent effects creating inconsistency between spiked samples (calibrators) and patient samples in terms of spot formation (92). Most DBS analytes currently measured (Table 1) are small molecular weight well defined compounds and therefore, in principle, full standardisation with traceability should be achievable. However, in practice the DBS matrix adds an extra level of complexity to the traceability chain and commutability needs to be determined.

POST-ANALYTICAL

Post-analytical concerns of the testing process phase mainly include; result reporting and interpretation, assay total error management and turnaround time (100). The key for the introduction of DBS analysis as a diagnostic tool is the cross validation of the method to a reference plasma/serum based assay. However, it is essential to take into account the fact that the concentration of the biomarkers in whole blood may vary from serum/plasma.

To turn the numerical result generated from the DBS analysis into a clinical meaningful result, a reference interval (RI) or decision point needs to be established. As such DBS specific RI have been developed for many analytes (101-106). For analytes routinely measured in liquid whole blood comparative RI can often be transferred to DBS samples. However, it is often more challenging for DBS analytes that require a comparison to serum for their clinical interpretation.

Some DBS analytes, such as vitamin D, require a conversion to their serum equivalent concentration for interpretation. This requires the development of a robust relationship between the measured analyte in the blotted whole blood and the equivalent serum sample. For this, we need to be able to estimate the equivalent blood volume in the blood spot punch. Two main approaches have been described to

evaluate the corresponding serum volume; the application of chemical tracers and geometric calculation (10).

Chemical tracers, such as ^{125}I-albumin, ^{51}Cr-haemoglubin and ^{125}I-L-thyroxin, have been used to estimate the equivalent serum volume of the blotted whole blood punch. In this process, the serum volume is determined by comparing radiochemical counts of the blood spot punch with the known volume of whole blood samples in the liquid phase (29). A geometric calculation to evaluate the ratio of the punched disk to the known volume of the entire spotted whole blood with the pre-measured haematocrit has been applied (107, 108). However, application of this approach does not fully take into account the chromatographic effect of the blood distribution on the filter paper, and still raises issues of analytical bias for the final measurement (109); this is why it is recommended that the punch is taken from the centre of the spot. Considering the fact that these common methods are not applicable to archived DBS samples, direct estimation of haemoglobin concentration on the DBS punch is an alternative, but is currently not described in the literature.

CONCLUSIONS

The initial widespread application of dried blood spot was utilised for newborn screening. More recently, it has been applied more broadly and mass spectrometric based applications are the dominant techniques, with liquid chromatography separation being more popular than gas chromatography. Drug monitoring (therapeutic and toxicology) and pharma-toxico-kinetics studies are the major application groups outside of newborn screening. For many analytes, method validation and further bridge experiments are required to develop adjustment rules to convert the results obtained from the dried blood spot analysis to the equivalent serum/plasma values. Likewise, establishment of robust reference intervals or decision limits is essential for dried blood spot analytes. It is envisaged, with the inherent advantages of the alternative dried blood spot sampling technique compared to the classic plasma based strategies, in future micro-sampling based assays will certainly play a substantial role for analysis of biomarkers.

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