The Development of Dry Plasma Spot Analysis and a Comparison with Dry Blood Spots and Conventional Plasma Bioanalysis using Methylene Blue

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The frenzy of activity in dried blood spots has overshadowed the potential of dry sample storage procedures for use with plasma. An investigation has been performed using Methylene Blue to assess the possibility of converting a wet plasma assay to a dry plasma assay. This has been run in parallel with a comparison group using dry blood spot samples.

Introduction

Anyone reviewing the hottest buzzwords in bioanalysis over the last few years could not escape the phrase 'Dry Blood Spot' or DBS. The advantages of the technique have been tirelessly championed by Matt Barfield and Neil Spooner of GSK and dry blood spots have been the subject of countless scholarly articles, conference presentations and will even be the subject of its own dedicated conference [1] when the European Bioanalysis Forum hold the EBF workshop "Connecting Strategies on Dried Blood Spots" in Brussels on the 17 - 18 June 2010.

The use of dried blood spots on filter paper blood sampling is a well established technique for the screening of in-born errors of metabolism [2,3]. The physics of blood dispersion on the filter paper limit the amount of matrix that can be practicably stored and sub-sampled using a dried blood spot. For many years this limitation prohibited the use of dried blood spots by the bioanalytical community for the development of pharmaceuticals. This was primarily because the required assay detection levels could not be achieved. The benefits of DBS are well documented in numerous publications [4,5,6]; in recent years the relentless improvements in separation and detection techniques have allowed DBS to be considered by bioanalysts as a practical alternative to 'wet' plasma. The technique is rapidly becoming established in bioanalysis, especially in Europe, although it is still in its infancy for pharmaceutical development. Significant developments in workflow automation are still required.

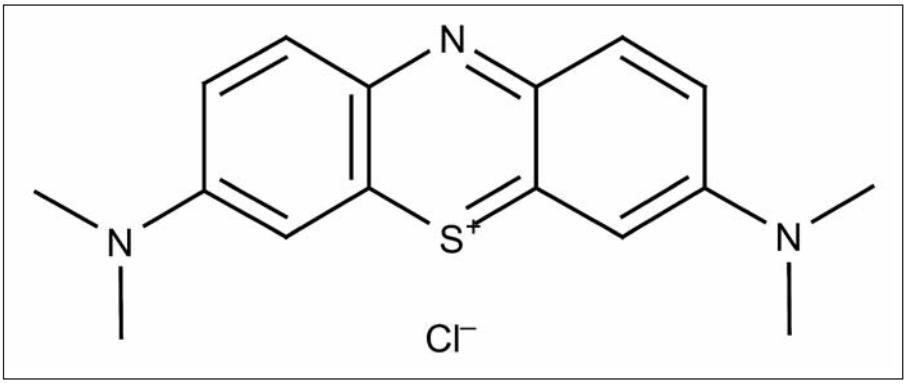

Figure 1 - Methylene Blue

The principles of developing an assay for dry matrices are not significantly different to those applied to any other matrices; the only exception being that you require some method of getting the analytes out of the dry paper and into a form suitable for analysis (usually by LC-MS/MS). This is typically achieved by re-suspending the analytes in a suitable solution containing the internal standard. Once in solution, the analytes are subjected to further extraction/analysis procedures that have been optimised to achieve the intended bioanalytical objective. This aspect is no different to normal bioanalysis and can vary from a simple injection of the spot supernatant to other sample extraction techniques such as solid phase extraction (SPE) or post column derivatisation [7]. A major advantage of dry spotted samples is that they take direct advantage of the improvements in separation and detection techniques mentioned above. The amount of matrix in a spot is so low that the majority of published methods are able to use a direct injection of the supernatant without significant additional (and costly) sample workup.

One of the widely reported benefits of DBS is that the pharmacokinetics are assessed using the circulating fluid, i.e. whole blood. However, this also presents a problem, as directly comparing data obtained from dry blood spots to data obtained from plasma is complicated by the level of blood cell association with the analyte of interest [4]. Therefore once a drug development program is instigated using a given matrix, it must stay in that matrix unless considerable effort and cost is incurred to bridge the data across the different matrix formats.

This means that the benefits of DBS [4,5,6] are potentially denied to any drug development program that has been instigated using plasma as the preferred matrix. Looking beyond DBS, it was hypothesised that the concept of dry sample storage could be applied to programs using plasma in the

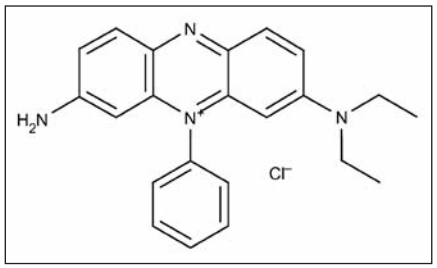

Figure 2 - Methylene Violet 3-RAX

form of Dry Plasma Spots (DPS). This would be of particular use in clinical trials with samples being generated across a variety of worldwide sites requiring costly global shipments to analytical laboratories.

Experimental

To test the hypothesis of dry plasma spot suitability as an alternative matrix to wet plasma, a research study was performed using Methylene Blue (Figure 1). Methylene Blue was chosen for a number of reasons; it is cheap, highly sensitive to MS/MS detection (due to its permanent positive charge), readily available and is well characterised with publicised stability in human blood and plasma to at least 3 freeze/thaw cycles [8]. A suitable analogue internal standard (Methylene Violet 3-RAX, Figure 2) was also readily available [8].

Methylene Blue has strong absorbance maxima at 609 and 668 nm [9] and is known to demonstrate extensive adsorption to different surfaces [8] including binding to mammalian cells. Overall, Methylene Blue was considered to be ideally suited as a test compound to show up any deficiencies in the DPS hypothesis and the analysis of dry samples in general.

The developed methods were not intended for validation according to accepted standards [10, 11]. In order to minimise differences between the methods used for the 3 different matrices under investigation, the methods were developed to use only rudimentary sample preparation techniques. The DBS and DPS method was a direct injection method for the analysis of 3mm sample disks of dry blood/dry plasma. The plasma method was a slightly modified version using dilution and direct injection of wet plasma samples for comparison purposes.

During the development phase, untreated paper was found to give a lower recovery for Methylene Blue and Whatman DMPK B cards were selected for the blood/plasma comparison. The assay was developed to take advantage of the high sensitivity of MS/MS detection with Methylene Blue to allow a high level of extract dilution combined with a gradient chromatography system offering a high k' value using uHPLC/semi-UPLC [12, 13, 14] chromatography. This minimised the risk of matrix effects biasing the observed results obtained from the 3 different matrices. The analytical range was set at a nominal 2-500 ng/mL, affording a signal to noise of well over 10:1 at the lower limit of quantification (LLOQ).

The testing process included a single batch validation in each matrix consisting of an assessment of linearity, specificity, matrix effects, equipment carryover, accuracy and precision at the LLOQ, low, mid and high levels. The nominal acceptance criteria for each of the above tests were consistent with current bioanalytical accepted standards [10, 11].

Equipment

The analytical system consisted of an Applied Biosystems API4000 triple quadrupole mass spectrometer with heat assisted electrospray ionisation (ESI) source, a Perkin Elmer Series 200 micro binary LC system (optimised for low volume and using a Waters Acquity high pressure mixer), a CTC HTC PAL with cheminert valve and a Perkin Elmer Series 200 column oven. The chromatographic and detector parameters were as shown in Table 1 and 2, respectively. This used a conventional microbore column utilised in a uHPLC/semi-UPLC [12, 13, 14] format to achieve a high linear velocity and high efficiency on conventional (highly optimised) HPLC column and equipment.

Results and Discussion

The results for the assay linearity, accuracy and precision and matrix effects for the different matrices are presented in Tables 3 – 5.

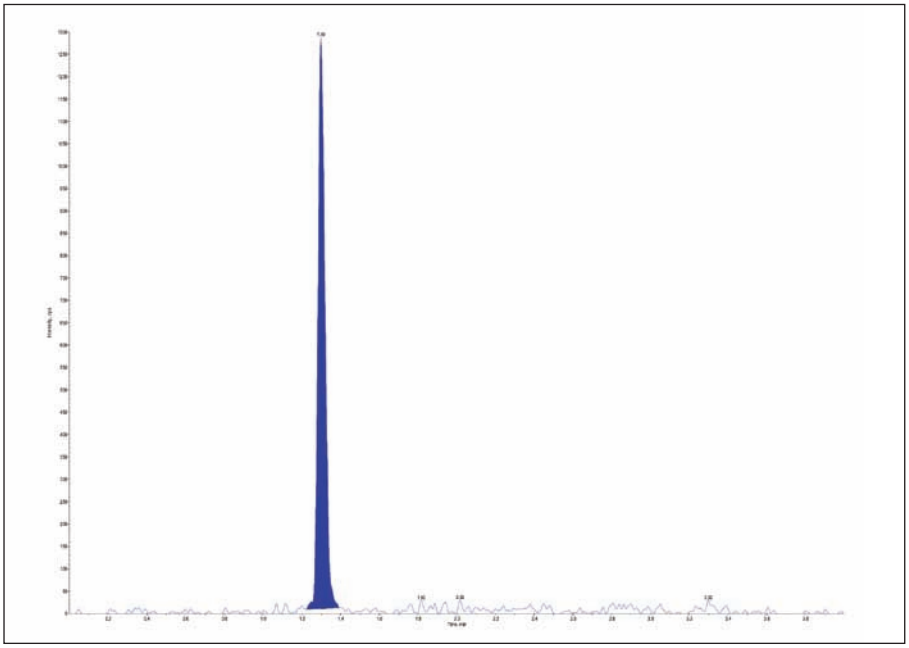

Figure 3 – Example Chromatograms of wet and dry plasma extracts at the LLOQ

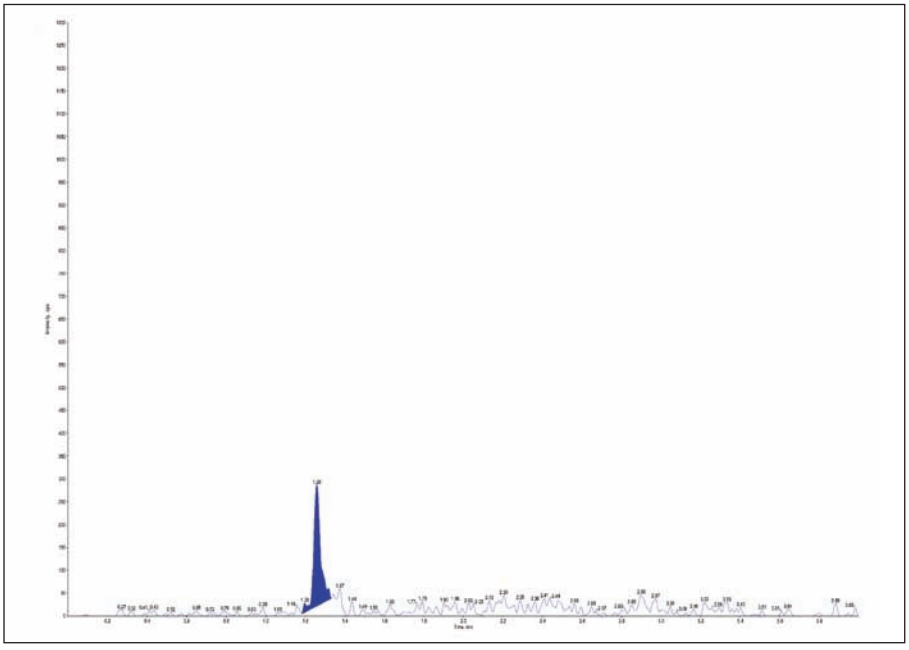

Figure 4 – Example Chromatogram of a dry plasma extract at the LLOQ

Table 1 - Chromatographic Parameters

Analytical Column:	ACE 3AQ 30 x 2.1 mm, 3 µm
Guard Column:	Anachem PreFrit Filter, 0.5 µm, 0.062 x 0.25 in.
Mobile Phase A:	Methanol/Acetic acid (100/0.1, v/v)
Mobile Phase B:	Water/Acetic acid (100/0.1, v/v)
Flow Rate:	1000 µL/min
Column Temperature:	60°C (please ensure pre-heater is used)
Autosampler Temperature:	ca 4°C
Injection Volume:	1 - 20 µL, (depending on instrument sensitivity)
Needle Wash 1:	Acetonitrile/Water/Isopropanol (5/1/1, v/v/v) containing 5% Trifluroacetic acid
Needle Wash 2:	Acetonitrile/Water/Isopropanol (5/1/1, v/v/v) containing 5% Triethylamine
Retention Times:	ca 1.3 min, Methylene Blue ca 1.7 min, Methylene Violet 3-RAX
Run Time:	3.0 minutes

Gradient - High Pressure Mixing

Time (min)	Flow rate (µL/min)	Curve	A (%)	B (%)
0.0	1000	0	95	5
0.5	1000	1	95	5
2.0	1000	0	35	65
2.2	1000	0	0	100
2.4	1000	0	95	5
2.6	1000	0	0	100
2.8	1000	0	95	5
3.0	1000	0	0	100
4.0	1000	0	95	5

Table 2 - Detector Parameters

Ionisation Mode	TurboIonSpray, positive
Ion Spray Temperature:	Hot as possible without sensitivity loss. 750°C used
Ion Spray Voltage:	2000V used. (Due to permanent positive charge. High ion spray potentials will increase background noise with no discernable benefit to signal intensity).
Q1 Resolution	Unit
Q3 Resolution	Unit
Settling Time	10 ms
MR Pause	10 ms
Probe Position	5 mm (X) and 5 mm (Y)
Gases	Standard API4000 (Nitrogen and Air)
Ions Monitored	284.2→268.3 (±0.5, Methylene Blue) 343.1→299.6 (±0.5, Methylene Violet 3-RAX) dwell time: 100 msec per transition

These showed that the assay was acceptable for all tests when Methylene Blue and Methylene Violet 3-RAX were extracted from dry blood spots and wet plasma samples. Although the precision and accuracy of measurement of methylene blue from dry plasma spots was acceptable, comparison of peak areas from dry plasma spots were found to be approximately 10-15% of those observed in equivalent samples obtained from wet plasma. The comparison of absolute peak areas between wet and dry samples cannot be compared directly as the absolute amount of sample taken is dependent upon the dispersion characterirtics of the blood. A punch from a 3mm disk is equivalent to ~5 µL of wet plasma. Example LLOQ chromatograms obtained from wet plasma and dry plasma extracts are shown in Figure 3.

The theoretical void time of the column used (Table 1) was 60 µL [15] with approximately 20 column volumes passing through the column prior to the elution of Methylene Blue.

The reason for the low observed peak areas obtained from the dry plasma spots was determined to be poor recovery from the spot as follows:

The non-extracted (solvent sample) data demonstrated that the sensitivity of the equipment had not drifted during the run.

The matrix effect data demonstrated that there were no significant ion suppression or enhancement effects across n=6 individual sources of each matrix.

The same bulk plasma samples had been used for both the dry and wet plasma experiments.

The observed stability of Methylene Blue peak areas in wet plasma was consistent with the literature data.

Methylene Blue was stable in whole blood during the fortification and drying procedure.

An equivalent drop in the peak areas of the internal standard was not observed between wet and dry plasma samples.

It is postulated that the poor recovery is being caused by the Methylene Blue binding to the paper surface and not resolublising in the internal standard solution. In blood the Methylene Blue is able to bind to the blood cells before spotting and can be liberated from the blood cells back into the internal standard solution for analysis.

Conclusions

Methylene Blue was chosen for this investigation because it was considered to have characteristics that would highlight any deficiencies in the hypothesis of dry plasma spot suitability as an alternative matrix to wet plasma. The results described have demonstrated that it is unsafe to assume that an assay can be converted from wet plasma samples to dry plasma samples without an appropriate amount of assay development and validation. This will ensure that the resultant dry plasma assay is fit for the intended purpose. Despite this, the concept that the dry plasma sample could conceivably be substituted for the traditional wet plasma sample is worthy of further investigation.

The next stage of this investigation is to examine ways of optimising the dry plasma assay to increase recovery to a point where the observed results are consistent with the dry blood spot and wet plasma assay. This is

Table 3 – Assay Linearity

All results expressed as percentage accuracy

Concentration (ng/mL)	Dry Blood Spot	Wet Plasma	Dry Plasma Spot
2	107.3	98.8	-
3	93.3	99.3	-
5	93.5	107.0	97.8
10	117.7*	98.2	109.9
20	99.1	95.4	75.4*
30	96.4	95.0	86.9
50	105.9	99.3	91.2
100	90.8	100.4	103.3
200	104.1	97.4	101.8
300	104.6	100.9	98.1
430	103.4	100.6	105.5
500	101.7	107.8	105.5

- Peaks considered too small. Values not calculated

* Excluded from the linear regression

Table 4 - Assay Accuracy and Precision (n=6 replicates)

All results expressed as a percentage

QC Level	Dry Blood Spot		Wet Plasma		Dry Plasma Spot	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
LLOQ	109.0	10.5	103.3	5.0	-	-
LOW	93.8	12.6	108.7	5.2	96.4	5.3
MED	105.8	3.4	103.6	7.4	101.5	6.1
HIGH	104.7	4.4	104.6	2.3	96.2	8.4

- Peaks considered too small. Values not calculated

Table 5 - Assay Matrix Effects (n=6 matrix sources)

All results expressed as a percentage of the mean response of the non-extracted (solvent) samples

QC Level	Result	Dry Blood Spot		Wet Plasma		Dry Plasma Spot	
		Analyte	IS	Analyte	IS	Analyte	IS
Low	Average	108.2	105.0	90.7	98.8	89.2	102.5
	Min	102.5	103.2	87.2	95.0	83.0	96.4
	Max	114.6	106.0	97.5	104.1	97.1	110.9
High	Average	100.6	103.7	94.8	102.3	95.4	96.8
	Min	97.9	100.4	83.4	90.1	82.7	86.0
	Max	103.1	107.1	100.6	110.6	110.4	108.8

necessary because, as explained previously, there are sensitivity limitations with any type of dry sample due to the low sample volumes that can be spotted without introducing paper chromatography effects.

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