Dried Blood Spot Analysis - Consistent Spot Homogeneity with Variable Spot Punch Locations

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Introduction

Dried Blood Spot (DBS) or Dried Matrix Spot (DMS) technology combined with the analytical capability of modern mass spectrometers (LC-MS/MS) has recently emerged as an important method for the quantitative bioanalysis of small molecules. It is increasingly being looked at as a microsampling approach for preclinical and clinical pharmacokinetic/toxicokinetic (PK/TK) studies[1]. The primary advantage of DBS is the significant reduction in blood volume requirements, leading to cost and ethical benefits (3Rs implications - reduction, refinement, and replacement) for animal use, facilitating pediatric studies, and offering simplified sample collection[2]. It also facilitates reduction in processing, sample shipping, and storage costs under ambient conditions.

As a relatively new technique in bioanalysis, it is essential to investigate the impact of variables that may affect the overall efficiency of the technique. Possible effects of varying the position of the punch, and varying hematocrit levels were studied.

Table 1. Basic drugs screened - Structures, and general information.

Compound	Structure	Log P	pKa	Therapeutic Use
Zolpidem		2.60	6.9	Hypnotic
Clozapine		3.23	7.5	Antipsychotic
Paroxetine		3.95	9.0	Antidepressant
Nortriptyline		4.51	10.1	Antidepressant

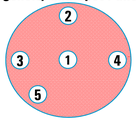
Standards were ordered at a concentration of 1.0 mg/mL in methanol (from Cerilliant) and diluted to the appropriate concentration for sample spiking.

Experimental

Spot Homogeneity

Fresh human whole blood was spiked with a mix of four pharmaceutical drugs (zolpidem, clozapine, paroxetine, and nortriptyline) at a concentration range of 20 ng/mL. After vortexing, 20 µL spiked blood was spotted on Agilent Bond Elut DMS (Dried Matrix Spotting) cards, with a new type of spotting media, which is non-cellulose in nature. Cards, once spotted, were left overnight for drying. 1.5 mm disks were punched from the center, top, left, right, and bottom left edge and placed in 2 mL vials. Each spot was dissolved in 300 µL desorption solvent (80% methanol with 0.1% formic acid containing 0.066 ng/mL deuterated internal standard mix), and vortexed. Spots were left to soak in desorption solvent for ~ 2 hours, samples were then removed and put in conical vials, followed by evaporation to dryness. Samples were reconstituted in 100 µL of mobile phase, vortexed, and subjected to LC-MS/MS analysis.

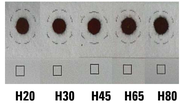
Figure 1. Spot Homogeneity and Spot Punch Locations



Hematocrit

Hematocrit levels were adjusted by adding or removing plasma from whole blood. The cell volume of the original blood sample was measured at 45. Samples were diluted or concentrated to HCT levels of 20, 30, 45, 65, 80. Two analytes, paroxetine and nortriptyline, with their deuterated equivalents serving as internal standards were selected. 15 µL of a 20 ng/mL spiked blood sample from different HCT levels were spotted onto Agilent Bond Elut DMS cards. A 3 mm disk was punched from each dried spot and placed into a 96 well collection plate. 300 µL desorption solvent comprising of 0.1% formic acid in 80% methanol (with 0.066 ng/mL of deuterated internal standard mix) was added to each well, vortexed, and left to soak for ~2 hours. The samples were evaporated to dryness and reconstituted in 100 µL of mobile phase.

Figure 2. Hematocrit levels investigated



Experimental

LC-MS Conditions

a) Spot Homogeneity

Column:	Zorbax SB-C18 Rapid Resolution HT, 1.8µm, 50 x 2.1 mm
Mobile Phase:	A: 0.1% Formic Acid in H_2O, B: 0.1% Formic Acid in MeOH
Flow rate:	400 µL/ min
Gradient:	t_0 A: 75%, B: 25% $t_{2.0}$ A: 30%, B: 70% $t_{2.1}$ A: 10%, B: 90% $t_{2.1\text{-}2.5}$ A: 10%, B: 90% $t_{2.6}$ A: 75%, B: 25% $t_{2.6\text{-}4.0}$ A: 75%, B: 25%
Run Time:	4:00 min
Gas Temp:	325°C,
Gas Flow:	10 L/min
Nebulizer:	20 psi
Sheath Gas Temp:	400°C,
Gas Flow:	11 L/min
Polarity:	Positive
Instrument:	Agilent 1290 LC / 6460 QQQ

b) Hematocrit

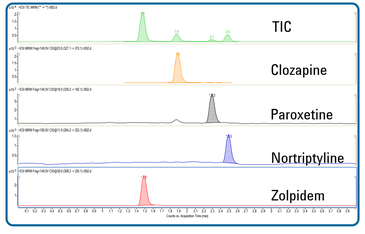
Column:	Poroshell 120 EC-C18, 2.7 µm, 50 x 4.6 mm
Mobile Phase:	A: 0.1% Aqueous Formic Acid, B: MeOH
Flow rate:	400 µL/ min
Gradient:	t_0 A: 40%, B: 60% $t_{2.0\text{-}2.1}$ A: 20%, B: 80% $t_{2.01\text{-}3.0}$ A: 40%, B: 80%
Run Time:	3:00 min
Gas Temp:	350°C,
Gas Flow:	10 L/min
Nebulizer:	20 psi
Sheath Gas Temp:	275°C,
Gas Flow:	8 L/min
Polarity:	Positive
Instrument:	Agilent 1290 LC / 6460 QQQ

Compounds

Compound	Q1 ion	Product ion	CE
Zolpidem	308.2	235.1	39 V
Zolpidem-D6	314.2	235.1	39 V
Clozapine	327.1	270.1	23 V
Clozapine-D4	331.2	272.1	27 V
Paroxetine	330.2	192.1	19 V
Paroxetine-D6	336.2	198.2	19 V
Nortriptyline 264.2	233.1	11 V	
Nortriptyline-D3	267.2	233.1	11 V

Results and Discussion

Figure 3. LC-MS/MS chromatogram of 20 ng/mL spiked blood after DMS work-up on Zorbax SB-C18 RRHT column.



Baseline separation was achieved for all four compounds within 3 min. The Rapid Resolution High Throughput column provides fast separations with maximum resolution. Good peak shape is observed for all analytes.

Table 1. Spot Diameter Consistency from Lot-to-Lot.

Lot No.	1	2	3	4	Avg.	RSD
80062	0.214″	0.218″	0.212″	0.211″	0.214″	1.4%
81121	0.224″	0.214″	0.219″	0.209″	0.217″	3.0%
80862	0.217″	0.205″	0.216″	0.210″	0.212″	2.6%

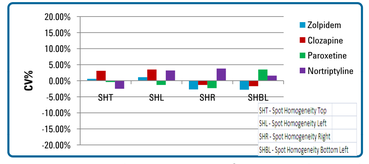
RSD values for spot diameters for spots taken from three different lots is 3.0% or lower.

Table 2. Consistent Spot Responses with Variable Spot Punch Locations.

		SHC	SHT	SHL	SHR	SHBL
Zolpidem	Average Response (n=4)	11113	11641	11233	10807	10802
	CV (from center)	--	0.6%	1.1%	-2.7%	-2.8%
Clozapine	Average Response (n=4)	6122	6462	6511	5972	5933
	CV (from center)	--	3.1%	3.5%	-1.3%	-1.7%
Paroxetine	Average Response (n=4)	1480	1474	1462	1446	1532
	CV (from center)	--	-0.4%	-1.3%	-2.3%	3.5%
Nortriptyline	Average Response (n=4)	5233	5100	5398	5431	5318
	CV (from center)	--	-2.5%	3.2%	3.8%	1.6%

Results and Discussion

Figure 4. CV% of Response of different punch locations relative to the center.



Upon comparing the responses of punches taken at different locations to the center punch, CV% was within 5% implying that consistent spot recoveries are obtained irrespective of punch location.

Hematocrit studies

Table 3. Spot Area measurements for Non-Cellulose and Cellulose media for different HCT values.

Agilent Bond Elut DMS Non-cellulose media: Blood Spot Radius

H20	H45	H80
0.1076	0.1169	0.1128
0.1051	0.1134	0.1109
0.1083	0.1122	0.1100
0.1068	0.1090	0.1150
0.1074	0.1055	0.1156
0.1102	0.1022	0.1158
0.1094	0.1076	0.1164
0.1117	0.1109	0.1158

Average Area

H20	H45	H80
0.0369 in^2	0.0378 in^2	0.0409 in^2
CV = -3%		CV = 8%

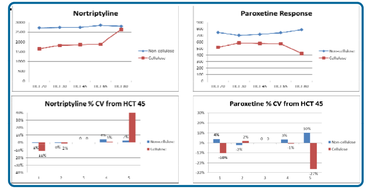
Competitive Cellulose media: Blood Spot Radius

H20	H45	H80
0.1542	0.1504	0.1349
0.1575	0.1455	0.1334
0.1550	0.1466	0.1385
0.1552	0.1383	0.1313
0.1514	0.1402	0.1330
0.1567	0.1441	0.1300
0.1513	0.1479	0.1230
0.1553	0.1411	0.1295

Average Area

H20	H45	H80
0.0750 in^2	0.0653 in^2	0.0550 in^2
CV = 15%		CV = -16%

Figure 5. Comparison of analyte response and % CV vs. different HCT values on non-cellulose and cellulose-based cards.



On a non-cellulose card, the variation in area of the blood spots going from H20 to H80 is ~11%, while a cellulose card deviates 31% over the same HCT range. Decreased variability in spot area translates to less variability in the corresponding analytical result. Recoveries on the non-cellulose membrane remained constant throughout, sample response % CV were 10% or less. Cellulose membrane exhibited a much greater variability, up to 50% for nortriptyline. Potential cause for this increased variability could be attributed to possible chromatography occurring on the card affecting flux and diffusion properties of blood spotted on paper.

Conclusions

- Spot diameter was consistent across three different lots of cards tested with RSD values of 3% or lower.
- Consistent spot responses are obtained regardless of the position of the punch. Responses of punches taken at the top, left, right, and bottom left when compared to that of the center yielded CV% within 5%.
- In hematocrit studies, going from HCT 20 to HCT 80, the variation in spot area is much less on the non-cellulose based card compared to its cellulose-based counterpart. Decreased variability in spot area on non-cellulose membranes (11%) implies decreased variability in analytical results (CV% of response is 10% or less). In contrast, cellulose-based membranes showed a 31% variation resulting in much greater variability in analyte response (CV% up to 50%).

References

1. Spooner, N.; Lad, R.; Barfield, M.; *Anal. Chem.* 2009, *81*, 1557–1563.
2. Barfield, M.; Spooner, N.; Lad, R.; Parry, S.; Fowles, S. *J. Chromatogr. B* 2008, *870*, 32–37.