Dried Blood Spotting Sample Volume Variability

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ASMS 2011
ThP 114

Agilent Technologies

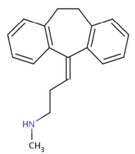
Introduction

Dried blood spotting for bioanalytical analysis in clinical and preclinical pharmaceutical testing has gained in popularity in recent years. As drug studies require quick high quality toxicokinetic and pharmacokinetic data, dried blood spotting offers a good alternative to traditional plasma extraction and analysis.

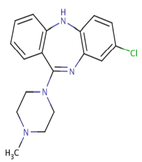
Blood is taken in clinical trials using a capillary rather than a syringe tube. 10-20 µL of blood can then be dropped on to a card, dried for storage & transport. A potential problem is variable sample volume. Anywhere from 10-20 µL may be spotted but the analytical results need to be consistent regardless of sample volume. Analysts overcome this obstacle by consistently punching a small portion of the spot, thereby normalizing the volume taken for analysis The blood dispersion in the spot needs to be homogenous and spread evenly, vertically and horizontally .

A study is undertaken to compare variable spot volumes in a non-cellulose blood spotting device. Four compounds chosen due to their readily available deuterated analogues.

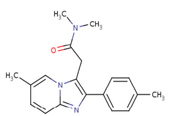
Analytes



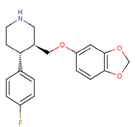
Nortriptyline
LogP: 4.5 pKa: 10.1



Clozapine
LogP: 3.2 pKa: 7.5



Zolpidem
LogP: 2.6 pKa: 6.9



Paroxetine
LogP: 3.95 pKa: 9.0

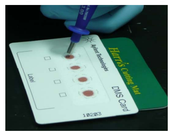
Standards were ordered at a concentration of 1.0 mg/mL in methanol (from Cerilliant) and diluted to the appropriate concentration for sample spiking

Experimental

Figure I – Blood Spotting Technique

Human blood was spiked to a concentration 5.0 ng/mL of Zolpidem, Clozapine, Paroxetine and Nortriptyline. 10 µL, 15 µL, and 20 µL spots were dried on a non cellulose Agilent Bond Elut DMS (Dried Matrix Spotting) card. Each spot was allowed to dry overnight.

Figure II – Spot punching



A 3 mm disk was punched and placed into a 96 well plate. 300 µL of 0.1% formic acid in 80% methanol with 0.066 ng/mL deuterated Internal standard mix was added to each well and vortexed. The samples were evaporated to dryness and reconstituted in 100µL of mobile phase.

LC-MS Conditions –

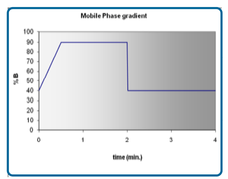
Agilent 1290 LC / 6460 QQQ

Column – Pursuit XRs ultra C18 50 mm x 2.0mm
Mobile Phase –
A: 0.1% Aqueous Formic Acid B: MeOH
Pump Program

Flow rate 200 µL/ min.

t_0	A: 60%, B: 40%
$t_{0.5-2.0}$	A: 10%, B: 90%
$t_{2.01-4.00}$	A: 60%, B: 40%

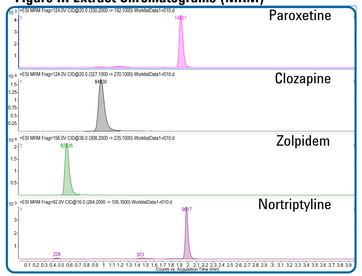
Run Time = 4:00 minutes.
Gas Temp: 350 °C
Gas Flow: 10 l/min
Nebulizing: 20 psi
Pol: Pos



Compound	Q1 ion	Product ion	CE
Zolpidem	308.2	235.1	36 V
Zolpidem-D6	314.2	235.0	36 V
Clozapine	327.1	270.1	20 V
Clozapine-D4	331.2	272.1	20 V
Paroxetine	330.2	192.1	20 V
Paroxetine-D6	336.2	198.1	20 V
Nortriptyline	264.2	105.1	16 V
Nortriptyline-D3	267.2	233.0	8 V

Results and Discussion

Figure III Extract Chromatograms (MRM)



Baseline separation was achieved for all four compounds. Good peak shape and good signal obtained for a 5.0 ng/mL blood spot extract

In order to verify a good analytical method was being used, a calibration curve was analyzed from 0.2 ng/mL to 500 ng/mL. A first order regression was applied and the correlation coefficient was better than 0.995 for all four analytes.

Figure IV – Calibration Curves

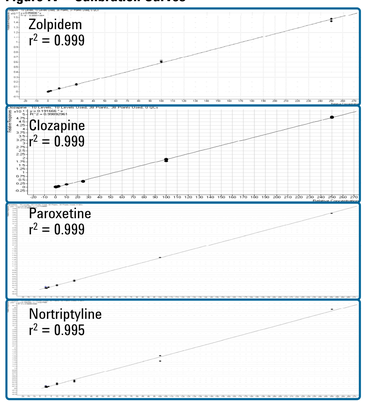
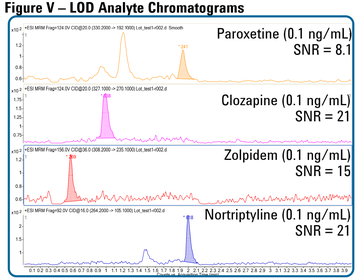


Figure V – LOD Analyte Chromatograms



SNR calculated using peak height with RMS times 5.

Good analyte response was achieved at the 0.1 ng/mL LOD with signal to noise rations better than twenty to one.

Figure VI – Accuracy and Precision Results (Relative Recoveries)

Zolpidem Accuracy and Precision (n=6)			
Concentration	**Avg result**	**Accuracy**	**RSD**
5.0 ng/mL	5.08	102%	5%
500 ng/mL	524	105%	4%

Clozapine Accuracy and Precision(n=6)			
Concentration	**Avg result**	**Accuracy**	**RSD**
5.0 ng/mL	4.89	98%	5%
500 ng/mL	549	110%	4%

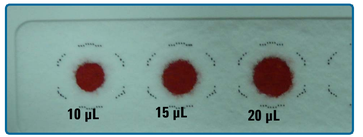
Paroxetine Accuracy and Precision(n=6)			
Concentration	**Avg result**	**Accuracy**	**RSD**
5.0 ng/mL	5.09	102%	6%
500 ng/mL	552	110%	6%

Nortriptyline Accuracy and Precision(n=6)			
Concentration	**Avg result**	**Accuracy**	**RSD**
5.0 ng/mL	4.45	89%	7%
500 ng/mL	462	92%	7%

Accuracy and precision studies were analyzed to verify curve fit and reproducibility. Six replicates at 5.0 ng/mL and 500 ng/mL were spotted, extracted and analyzed. The results were calculated based on linear fit of analyte the relative response

Results and Discussion

Figure VII – Blood Spot Volumes



Blood spots sizes for corresponding blood volumes. Area increases with volume spotted

Figure of 3 mm punch taken out of each spot

In order to verify the sample volume is consistent the spot area was measured and compared to punch area to determine the actual sample volume being analyzed

Figure VIII – Blood Spot Areas

Spot Volume	Radius (mm)	Area (mm²)
10 µL	2.32	16.9
15 µL	2.80	24.6
20 µL	3.32	34.6
3 mm punch	1.5	7.07

By determining the ratio of the area of the punch to the area of the spot we can determine the volume taken from a 3 mm punch

10 µL sample volume $\frac{7.07\ mm^2}{16.9\ in^2} = \frac{volume}{10\ \mu L}$ = 4.2 µL

15 µL sample volume $\frac{7.07\ mm^2}{24.6\ mm^2} = \frac{volume}{15\ \mu L}$ = 4.3 µL

20 µL sample volume $\frac{7.07\ mm^2}{34.6\ mm^2} = \frac{volume}{20\ \mu L}$ = 4.1 µL

The actual blood volume sampled is consistent regardless of the amount of volume spotted on the card

Figure IX - Analyte MS Response for Each Volume

5 ng/mL in human blood		10 µL	15 µL	20 µL
Zolpidem	Average Response (n=4)	944	912	931
	CV (from 15 µL)	3.4%	--	2.0%
Clozapine	Average Response (n=4)	716	736	763
	CV (from center)	-2.7%	--	3.7%
Paraoxetine	Average Response (n=4)	1034	1041	1094
	CV (from center)	-0.7%	--	4.8%
Nortriptyline	Average Response (n=4)	1674	1672	1757
	CV (from center)	0.1%	--	5.0%

The 15 µL spot was used as the benchmark and the 10 and 20 µL spots compared as a reference. Variability in analyte recoveries was less than 5 % for either the 10 or 20 µL spots.

Conclusions

- An accurate and reproducible method was developed for the desorption and analysis of four compounds in a dried blood spot. (Figure I&II)
- Good chromatography (Figure III) with baseline separation was achieved and linear calibration curves each having a correlation coefficient better than 0.995 (linear fit)for all four compounds (Figure IV)
- Good signal to noise at 0.1 ng/mL (> 8) was achieved for all compounds (Figure V)
- Analyses was accurate and reproducible (Figure VI). All results were within 11% of the true value of the spiked standard. Six replicates were analyzed at each level with RSDs-equal or less than 7% for all compounds.
- Actual volume of blood sampled was approximately 4 µL regardless of the volume spotted on the card (Figure VIII).
- Analyte response remained consistent and reproducible regardless of volume (Figure IX)
- Removing sample volume variability is critical to minimize potential human error factors from a sensitive assay.