Exploration of Automated Sonication and Direct Elution Approaches for the Analysis of Drugs in Dried Blood Spots and Other Matrices

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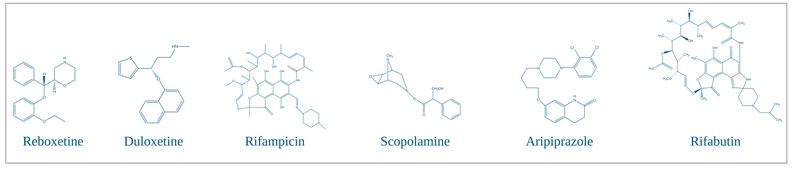
Introduction:

As detection sensitivity has improved substantially in recent years, collecting dried blood spots [1] has been reconsidered for broader application instead of collecting plasma or serum [2,3]. This paradigm shift was compelling for a variety of reasons mainly in the sample collection, transport and storage portions of the process. However, sample preparation and analysis productivity was impacted by reverting to manual processing and the need for additional method development. As recently as nine months ago, the only commercially available automated systems involved serialized punching of spots from cards. These instruments were format limited and judged to be not very fast or robust. As we began to explore the utility of dried blood spot analysis for Discovery PK applications, a decision was made to also explore two potential approaches for automating the processing of such samples.

A test set of drugs including reboxetine, duloxetine, rifampicin, scopolamine and aripiprazole were used to test two different automated approaches for the analysis of drugs in dried blood spots. In the first approach, a Tomtec® Quadra 4™ 96-channel pipettor was fitted with a custom depth sonication station. A 3mm diameter spot was suspended on the shoulder of the disposable tips, solvent was aspirated into the zone of the spot and the tip was immersed into the sonication reservoir. The solvent was then dispensed into a collection plate for LC/MS/MS analysis. Most of the work was performed using Whatman® FTA Classic™ filter paper although comparisons were also made with Ahlstrom® untreated filter paper and FTA Elute™. The second approach involved direct elution of the drugs from dried matrix spots placed inside a modified Tomtec® Elutrix™ instrument. The instrument secured the paper from both sides and applied a flow of solvent through the spot.



Experimental:

Sonication Approach Sample Preparation*

- Spotted 15 µL of blood onto the card, allowing it to dry for at least several hours
- Manually punched a 3 mm diameter spot, dropping it into the disposable tip
- Aspirated 100 µL of 25% MeOH chased by an air gap to elevate the solvent into the zone of the tip where the spot was suspended
- Lowered tip into sonication reservoir of water deep enough to immerse the tip above the level of the solvent inside, sonicating for 2 min
- Dispensed volume of solvent into a clean plate, repeating process for 3 cycles
- Slowly ran peristaltic pump to guard against any cross-contamination from the tips for second and third cycles
- Evaporated samples under heated N_2 at 40°C
- Reconstituted samples with 100 µL of 50% ACN
- Centrifuged for 5 minutes at 3400 rpm

Direct Elution Approach Sample Preparation*

- Spotted 25 µL of blood onto the card, allowing it to dry for at least several hours
- Centered the spot within a locator plate which was loaded by the Elutrix
- The instrument passed a 50% MeOH solution at a rate of 0.5 mL/min through a 5 mm diameter zone of the spot for 4 minutes collecting the effluent in a tube
- Samples were evaporated under heated N_2 at 40°C
- Reconstituted samples with 100 µL of 50% ACN
- Centrifuged for 5 minutes at 3400 rpm

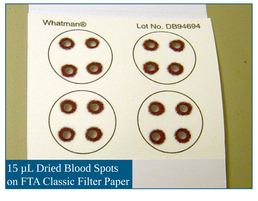
Control Vortexing Approach Sample Preparation*

- Spotted 15 µL of blood onto the card, allowing it to dry for at least several hours
- Manually punched a 3 mm diameter spot, dropping it into a deepwell plate
- Added 450 µL of 75% MeOH
- Vortexed plate for 1 hr on a multitube shaker
- Transferred 350 µL to a clean plate and evaporated under heated N_2 at 40°C
- Reconstituted samples with 100 µL of 50% ACN
- Centrifuged for 5 minutes at 3400 rpm

*For larger batch preparations, additional sample cleanup steps were introduced for several of the runs applying either an acetonitrile protein precipitation or a liquid-liquid extraction with ethyl acetate


Tips Submerged in Sonicator


Spot in Aspirated 100 µL of 25% MeOH



15 µL Dried Blood Spots on FTA Classic Filter Paper

Materials

- Whatman® FTA Classic™ filter paper
- Whatman® FTA Elute™ filter paper
- Ahlstrom® untreated 237 filter paper
- Bioreclamation® Sprague Dawley rat blood
- Bioreclamation® human blood
- Bioreclamation® human synovial fluid (osteoarthritis)
- Bioreclamation® mongrel dog plasma
- Bioreclamation® Sprague Dawley rat blood
- Gibco® Dulbecco's Phosphate Buffered Saline
- B6-DIO-Tac mouse liver samples (homogenized 5:1 with PBS)
- B6-DIO-Tac mouse fat samples (homogenized)
- PyMT oncomouse tumor samples (homogenized 8:1 with PBS)
- Sprague Dawley rat muscle samples (homogenized 7:1 with PBS)
- Sprague Dawley rat brain samples (homogenized 8:1 with PBS)
- Analytical Standards were obtained from Pfizer Research compound distribution (except for Rifampicin which was obtained from Sigma Aldrich®)

WORLDWIDE CLINICAL TRIALS

Drug Development Solutions
Bioanalytical Sciences

MS Conditions

- Instrument = Sciex API 4000
- Mode = ESI+
- Scan Type = MRM
- Turbo Ion Spray Voltage = 5000 V
- Temperature = 500°C

Compound	DP (V)	CE (V)	CXP (V)
Reboxetine	46	19	10
Duloxetine	46	9	14
Rifampicin	96	25	24
Scopolamine	66	31	12
Aripiprazole	91	37	12
Rifabutin	106	41	24
Phospholipids	120	52	12

Transitions	LC1 RT (min.)	LC2 RT (min.)
Reboxetine m/z 314 > 176	1.52	3.3
Duloxetine m/z 298 > 154	1.65	3.5
Rifampicin m/z. 823 > 791	1.78	3.8
Scopolamine m/z. 304 > 138	0.35	2.4
Aripiprazole m/z. 448 > 285	1.60	4.1
Rifabutin m/z. 848 > 815	1.75	4.4
Phospholipids m/z. 184 > 184	-	-

HPLC Conditions

Instrument 1	Instrument 2
Column = Discovery HS C18, 50 x 2.1 mm, 5 µm	Column = Discovery HS C18, 50 x 2.1 mm, 5 µm
Column Temperature = Ambient	Column Temperature = Ambient
Injection Volume = 10 µL	Injection Volume = 5 µL
Autosampler Temperature = 10°C	Autosampler Temperature = 10°C
Flow Rate = 0.5 mL/minute	Flow Rate = 0.6 mL/minute

Mobile Phases:

A = 0.1 % Formic Acid in Water	A = 10 mM Ammonium Acetate in Water
B = Acetonitrile	B = Acetonitrile

Gradient:

Time (min.)	%B	Time (min.)	%B
0.0	10	0.0	10
0.1	10	1.0	10
2.0	98	4.0	90
3.0	98	5.5	90
3.1	10	5.7	10
4.5	10	7.2	10

Autosampler Wash Solutions:

Organic Wash = 25% Isopropanol, 25% Methanol, 25% Acetonitrile, 25% Acetone, 1.5% Acetic Acid
Aqueous Wash = 0.1% Acetic Acid

Results:

Sonication Approach Parameter Sensitivity and Optimization

- % Organic and volume used; MeOH vs. ACN
- Spot size, location, stability, carryover
- Blood age, species
- Card type (Classic, Elute, untreated)
- Matrix type (Blood, plasma, water, various tissue homogenates)
- Sonication time and number of rinses

Exploring Different Parameters Related to Sonication of 3 mm Dried Blood Spots

Conc (ng/mL)	Matrix	Organic	Mixing	Card Type	Reboxetine Area Counts	% Recovery	Matrix Effect
100	Fresh Rat Blood	75% MeOH	Sonicate 5 min.	FTA Classic	21800	18	0.91
10	Fresh Rat Blood	75% MeOH	Sonicate 5 min.	FTA Classic	2807	23	0.90
100	**Fresh Rat Blood**	**50% MeOH**	**Sonicate 5 min.**	**FTA Classic**	**52300**	**40**	**0.80**
100	Fresh Rat Blood	75% ACN	Sonicate 5 min.	FTA Classic	16900	14	0.91
100	Fresh Rat Blood	75% MeOH	Sonicate 5 min.	FTA Elute	31167	28	0.83
100	Fresh Human Blood	75% MeOH	Sonicate 5 min.	FTA Classic	19200	18	0.77
100	1 mo. old Rat Blood	75% MeOH	Sonicate 5 min.	FTA Classic	17167	16	0.79
100	Fresh Rat Blood	75% MeOH	Sonicate 2 min.	FTA Classic	16433	13	0.93
100	Fresh Rat Blood	75% MeOH	Sonicate 8×8 min.	FTA Classic	19290	14	1.08
100	Fresh Rat Blood	75% MeOH	Sonicate 2×8 min.	FTA Classic	26473	20	0.99
100	Fresh Rat Blood	75% MeOH	Sonicate 5 min.	FTA Classic	20633	17	0.89
100	Fresh Rat Blood	75% MeOH	Vortex 25 min. post	FTA Classic	5046	4	0.89

Effect of Varying % Methanol on Response

Area Counts Summed from 6 × 2 min. Sonications (N = 3)

Card Type	FTA Classic	FTA Classic	FTA Classic	**FTA Classic**	FTA Classic	FTA Elute
% MeOH	100	75	50	**25**	0	50
Reboxetine	16330	51164	118182	**125901**	116860	70813
Duloxetine	2603	4744	6292	**9290**	9483	7178
Rifampicin	4843	13784	22967	**24631**	25980	5403
Scopolamine	20125	56779	126620	**135702**	127820	73437
Aripiprazole	14827	23958	23649	**27157**	26380	19645
No phospholipids observed						

Optimization Varying Volume, Duration and Repetitions of Sonication

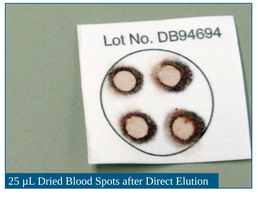
Reboxetine Normalized Area

	Volume	1 min.	2 min.	8 min.
25% MeOH 100 ng/mL	50 µL	104200	103416	116256
	100 µL	90984	**108049**	-
	200 µL	105585	-	-
25% meOH 10 ng/mL	100 µL	10319	-	-
	200 µL	10899	-	-
# Rinses to Recover > 90%		1 min.	2 min.	8 min.
25% MeOH 100 ng/mL	50 µL	3 to 5	4	2
	100 µL	4 to 5	**3**	-
	200 µL	4	-	-

Dried Blood Spot Analysis Sonication Approach – FTA Classic

Analyte	Curve Range (ng/mL)	Correlation Coefficient
Reboxetine	1 - 5000	0.9960
Duloxetine	1 - 5000	0.9949
Rifampicin	25 - 5000	0.9913
Scopolamine	5 - 5000	0.9957
Aripiprazole	1 - 5000	0.9961


Overview of Direct Elution Instrumentation



25 µL Dried Blood Spots after Direct Elution

Sonication Approach: Blood Components and Filter Paper Type Comparison

		Curve Range	Correlation Coefficient	Scopolamine Area (100 ng/mL)	Rifabutin IS Area	Concentration (ng/ML)	Accuracy %	Scopolamine Area (1000 ng/mL)	Rifabutin IS Area	Concentration (ng/mL)	Accuracy %
Sonication	Blood Classic	5 - 5000	0.9957	48101	78213	104	104	441010	74740	978	98
Sonication	Blood Untreated	5 - 5000	0.9949	22264	36812	103	103	192280	35324	909	91
Sonication	Plasma Classic	2 - 2500	0.9958	32304	39284	110	110	337650	53921	880	88
Sonication	Plasma Untreated	5 - 5000	0.9954	22035	11309	94	94	244320	10015	1170	117
Sonication	Water Classic	1 - 5000*	0.9946	16515	7724	86	86	191010	6688	1010	101
Sonication	Water Untreated	5 - 5000*	0.9966	21547	NR	86	86	270580	NR	1100	110
Vortex	Blood Classic	5 - 5000	0.9971	42808	140340	109	109	360830	122720	1070	107
ACN PPT	Blood	5 - 5000*	0.9947	40582	NR	114	114	311440	NR	878	88
NR denotes IS not reportable											
* denotes external standard curve											

Sonication Approach: Alternate Matrices Comparison

		Curve Range	Correlation Coefficient	Scopolamine Area (100 ng/mL)	Rifabutin IS Area	Concentration (ng/ML)	Accuracy %	Scopolamine Area (1000 ng/mL)	Rifabutin IS Area	Concentration (ng/mL)	Accuracy %
Sonication	Synovial Fluid Classic	1 - 5000	0.9920	54958	23768	94	94	511684	25409	866	87
Sonication	Liver Classic	1 - 5000*	0.9902	51380	2608	111	111	500906	5747	1090	109
Sonication	Tumor Classic	1 - 2500	0.9916	32620	7415	101	101	341201	7667	1030	103
Sonication	Brain Classic	1 - 5000	0.9948	33291	736190	118	118	251080	723390	952	95
Sonication	Muscle Classic	10 - 5000	0.9906	56055	1126900	116	116	546490	1293600	969	97
Sonication	Fat Classic	5 - 5000	0.9955	17142	758210	104	104	143100	751290	912	91
* denotes external standard curve											

Exploration of Sample Cleanliness with Alternate Extractions Following Sonication

		Curve Range	Correlation Coefficient	Aripiprazole Area (1000 ng/mL)	Rifabutin IS Area	Concentration (ng/mL)	Accuracy %	Aripiprazole Area (1000 ng/mL)	Rifabutin IS Area	Concentration (ng/mL)	Accuracy %
Sonication	Blood Classic	1 - 2500	0.9943	39491	632150	92	92	360050	557090	1090	109
ACN PPT				16067	218480	109	109	371780	580700	1080	108
				36481	609810	89	89	218710	380790	951	95
Sonication Ethyl Acetate LLE	Blood Classic	1 - 5000	0.9930	20036	558150	93	93	164770	516790	837	84
				29347	651160	117	117	217550	556690	1030	103
				21559	563960	99	99	216010	551190	1030	103

Optimization of Elutrix Direct Elution Parameters

Initial Design Card types: **FTA Classic** and FTA Elute
Concentrations: 0, 10 and 100 ng/mL for all 5 analytes
Collecting 3 x 1 minute rinses of 50% MeOH at 0.5 mL/min

Findings Substantial analyte response still present in third fraction
Carryover in Elutrix = 2%
Response proportional to concentration
Greater response with Classic card type

Second Design Duration of flow through spot: 1, 2, 3, **4** and 5 min
% MeOH: 0, 10 and **25**
Flow rate: 0.25 and **0.5** mL/min
Valve toggle (15sec delay after each minute): with and **without**

Findings
Maximum response flowing 25% MeOH at 0.5 mL/min
for 4 min with no valve toggle

Third Design % MeOH: 0, 25, **50**, 75 and 100
Duration: 3, **4**, 5, 6 and 8 min
Flow rate: **0.5**, 1 and 2 mL/min

Findings Maximum response flowing 50% MeOH at 0.5 mL/min for 4 min
Carryover 1-2%
Recovery was >80% (except for duloxetine at 35%)

Direct Elution Approach: Back-Calculated Standards

	Reboxetine		Duloxetine		Rifampicin		Scopolamine		Aripiprazole	
Correlation	0.9922		0.9928		0.9918		0.9946		0.9939	
Nominal Concentration	Concentration (ng/mL)	Accuracy %	Concentration (ng/mL)	Accuracy %	Concentration (ng/mL)	Accuracy %	Concentration (ng/mL)	Accuracy %	Concentration (ng/mL)	Accuracy %
2	2.0	100								
2	2.0	99								
5	5.4	109							5.1	103
5									5.3	106
10	8.7	87	9.9	99	9.3	93	10.2	102	8.3	83
10	9.9	99			11.6	116	9.7	97	10.0	100
25	26.7	107	24.3	97	22.9	92	27.5	110	21.6	87
25	27.0	108	26.4	106	24.4	98	25.5	102	28.7	115
50	56.6	113	54.4	109	50.7	101	54.3	109	50.1	100
50	44.4	89	49.7	99	39.7	79	42.3	85	48.6	97
100	90	90	103	103	93	93	87	87	106	106
100	81	81	82	82	87	87	86	86	94	94
250	273	109	298	119	260	104	257	103	291	116
250			206	82			232	93	253	101
500	447	89	491	98	640	128	526	105	560	112
500	420	84	447	89	452	90	501	100	513	103
1000	1010	101	957	96	1040	104	930	93	886	89
1000	1090	109	1060	106	1020	102	991	99	1110	111
2500	3120	125	2860	114	2570	103	2900	116	2380	95
2500					2810	112				
5000	4750	95	5010	100	4810	96	5220	104	4390	88
5000	5330	107			4810	96	5440	109	4790	96

Discussion

The approach of sonicating dried blood spots and quantitating drug concentrations by LC/MS/MS was demonstrated successfully for this test set of drugs. The experiments to optimize the sonication parameters on the basis of analyte response were summarized. Optimum recovery was attained at 25% methanol using 3 rinses of 100 µL of solvent with 2 minutes sonication. Duplicate standard curve data was collected for all 5 analytes in spots of blood, plasma, water, synovial fluid, as well as homogenized brain, liver, tumor, fat and muscle. The quantitative matrix effect ratios were close to unity for all of the analytes (> 0.8), however additional cleanup was desirable. Further data was presented probing the cleanliness of these samples by comparing results and recoveries after additional extraction steps to remove fibrous material from the filter paper as well as endogenous blood components resolubilized along with the drugs. The fibrous material appeared to be the greater concern. Other blood fractions and matrices were also spotted and sonicated to help better understand the properties governing resolubilization by sonication. Clear trends were presented comparing card types and blood fractions but more work is required to fully characterize the balance between recovery and sample cleanliness. Across the spectrum of matrices and card types, however, the results within a given set of conditions were found to be quantitative and reproducible despite using an internal standard (rifabutin MW=847 Da) that was structurally unrelated to most of the analytes in the test set.

The intent is that such a system would be paired with an automated device that would quickly and reliably punch the spots in parallel and load them into the tips described. The option of an automated sonication process lasting about 10 minutes in total rather than the current norm of up to an hour vortexing may be preferred, particularly if the conditions prove generic enough to reduce method development steps currently involved with solvent selection for the vortexing approach. Testing of these sonication parameters with more drugs is required to determine whether they are more broadly universal or limited to this test set.

Similar experiments working with a modified version of the Elutrix demonstrated the feasibility of directly eluting drugs from dried blood spots by flowing LC solvent through the filter paper. In these experiments, this process was performed separate from the final LC/MS/MS system. However, the intent would be to incorporate such an approach in an on-line mode of operation, trapping and focusing the eluate from the spot on a pre-column prior to switching it onto the LC/MS/MS. Further engineering development of a tool such as the Elutrix would be required to generate a robust, automated system capable of solubilizing larger batches of spotted samples. Such a system would need to reliably and quickly handle the source spotted cards, trap and remove any fibrous material eluting off the cards over time and minimize system carryover. The data presented here showed that such a flow through approach offered nearly complete analyte recovery in a quantitative manner for blood and other blood fractions. The optimum conditions were 0.5 mL/min of 50% MeOH for 4 minutes for all of the analytes. The eluate was collected and concentrated for analysis by LC/MS/MS. Standard curve data was collected for all 5 analytes in spots of blood, plasma and water on either treated or untreated filter paper.

Direct Elution Approach: Standard Curve Summary Statistics

Compound	Blood Range (ng/mL)	FTA Classic Paper Correlation Coefficient	Blood Range (ng/mL)	Untreated Paper Correlation Coefficient
Reboxetine	10 - 5000	0.9942	1 - 5000	0.9953
Duloxetine	5 - 5000	0.9936	100 - 5000	0.9924
Rifampicin	25 - 5000	0.9962	1 - 5000	0.9949
Scopolamine	5 - 5000	0.9910	25 - 5000	0.9869
Aripiprazole	1 - 5000	0.9921	5 - 5000	0.9911
Response from FTA Classic paper > Untreated paper				
Response from Blood > Plasma > Water				
Results were proportional within a given class				

Conclusions

- Sonication was demonstrated to be a feasible mechanism for solubilizing drugs in dried blood spots
- Direct elution by flowing LC solvent through filter paper was shown to be feasible
- Both techniques offer alternate paths to automate the quantitative analysis of drugs from dried blood spots

Acknowledgements

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