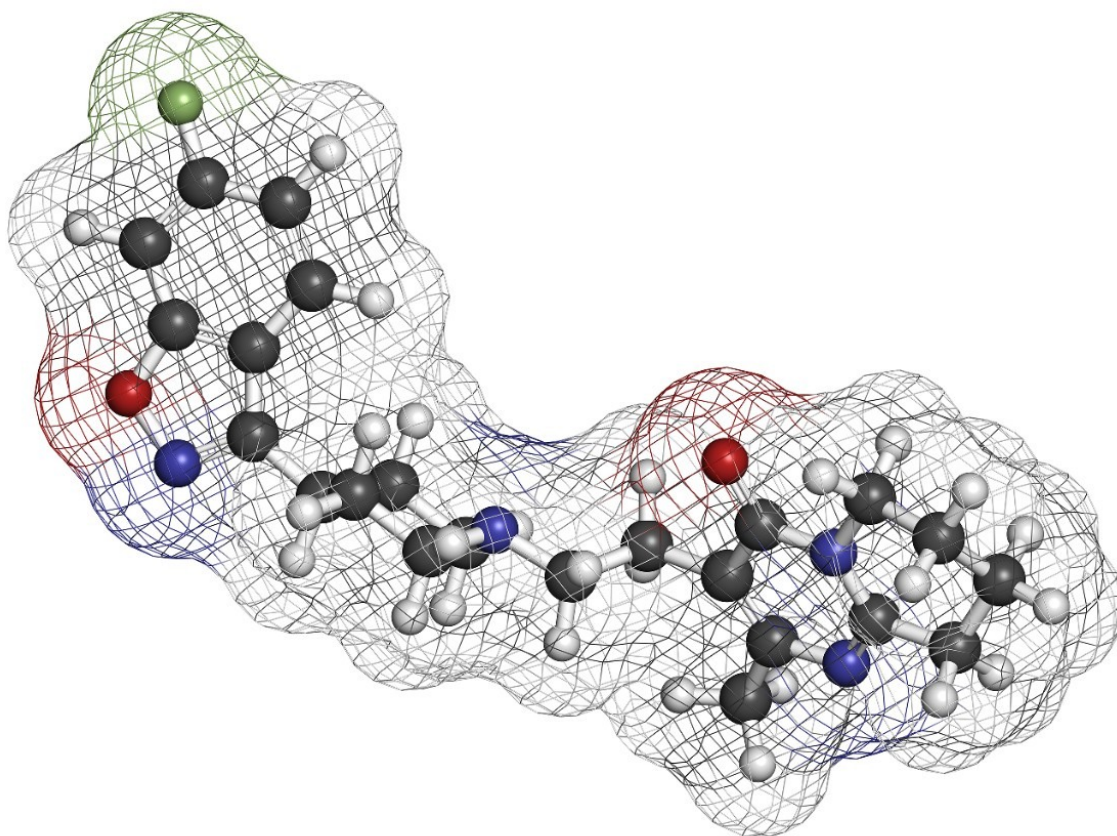


Improving the Cleanliness of DBS Extracts using the Ostro Pass-through 96-well Sample Preparation Plate and Single Step Method

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Abstract

In this study, whole blood samples containing risperidone, its hydroxylated metabolite, 9-OH risperidone, and the internal standard, clozapine were extracted in both the traditional manner and in-well using a 96-well Ostro sample preparation plate.

Benefits

- Simple, cleaner one-step DBS extraction with faster, in-well method
- Removal of 99.9% of residual phospholipids
- Improves robustness by eliminating build-up of PLs on LC columns

Introduction

Dried blood spot (DBS) analysis has been rapidly gaining momentum in the pharmaceutical industry. Economic and ethical issues surrounding laboratory animal usage and cost associated with shipping and storing biological samples has made DBS analysis an attractive option. DBS analysis can generate high analyte recoveries. However, this technique does little to eliminate endogenous interferences. Interferences, in particular residual phospholipids (PLs), are a major source of concern in bioanalysis. PLs build up in LC-MS/MS systems and are one of the major causes of matrix effects. Amongst other problems, matrix effects alter mass spectrometry response in an unpredictable manner, decrease method robustness, and add to method variability. The Ostro Pass-through 96-well Sample Preparation Plate can be used to eliminate greater than 99% of residual PLs. The Ostro Pass-through 96-well Plate further simplifies the DBS extraction process through the use of 96-well format plates which simultaneously extract analytes, reduce PLs and filter out the spot. DBS punches can be extracted in-well, diluted, and directly injected onto an LC-MS/MS system. This novel method significantly reduces sample preparation time and eliminates potential analyte losses due to extract transfer, dry down, and reconstitution. In this work, whole blood samples containing risperidone, its hydroxylated metabolite, 9-OH risperidone, and the internal standard, clozapine (Figure 1) were extracted in both the traditional manner and in-well using the Ostro Pass-through 96-well Plate.

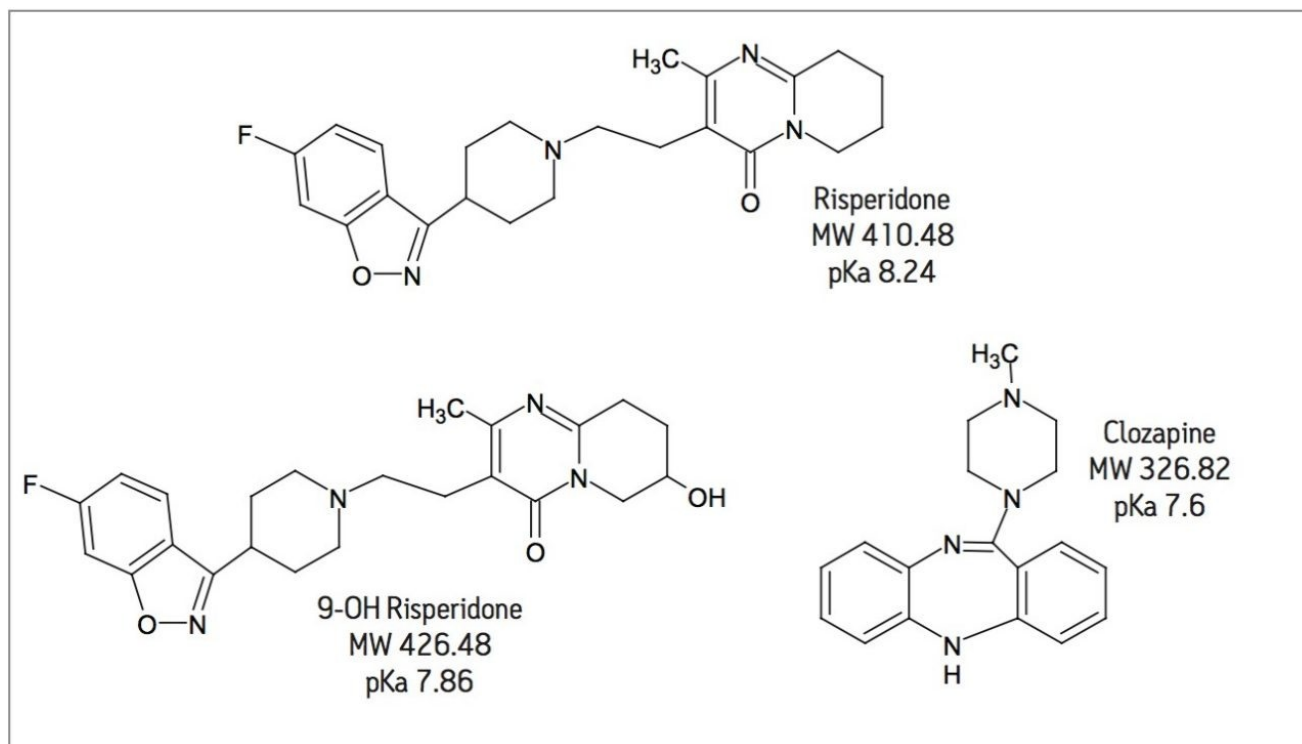


Figure 1. Structures, molecular weights, and pKa's for risperidone, 9-OH risperidone, and clozapine.

Experimental

ACQUITY UPLC Conditions

Column:	ACQUITY UPLC BEH C ₁₈ , 2.1 x 50 mm, 1.7 μ m
Mobile phase A	0.1% NH ₄ OH in H ₂ O
Mobile phase B	50/50 ACN/MeOH
Flow rate	0.6 mL/min
Injection volume	40 μ L
Injection mode	Partial Loop

Column temp.	35 °C
Sample temp.	15 °C
Strong needle wash	60:40 ACN: IPA + 2% HCOOH
Weak needle wash	95/5 H ₂ O/MeOH

Gradient:

Time(min)	Profile		Curve
	%A	%B	
0.0	75	25	6
2.0	0.5	99.5	6
3.0	0.5	99.5	6
3.1	75	25	6
3.5	75	25	6

Waters Xevo TQ-S MS Conditions, ESI+

Capillary voltage:	3.0 V
Desolvation temp.:	550 °C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	1000 L/Hr

Collision cell pressure: $2.6 \times 10^{(-3)}$ mbar

MRM transition monitored, ESI+: See Table 1

Compound Name	Parent Mass	Daughter Mass	Collision Energy (eV)	Cone Voltage (V)
Risperidone	411.1	190.9	28	46
9-OH Risperidone	427.1	109.8	38	42
Clozapine	327.0	269.7	22	42

Table 1. MRM transitions, collision energies, and cone voltages for risperidone, 9-OH risperidone, and clozapine

Sample Prep Conditions

Whole blood samples were spotted in 20 μ L aliquots onto Whatman DMPK-C untreated cards. The samples were allowed to dry for 2 hours at room temperature. Three-mm punches were taken using the Harris 3 mm micropunch and were placed in individual 1.5 mL Eppendorf tubes for traditional DBS extraction or directly into the wells of the Ostro Pass-through 96-well Plate. The traditional extraction was performed using 250 μ L of 5% water in methanol in 1.5 mL Eppendorf tubes, followed by vortexing for 1 minute, centrifuging for 1 minute at 3000 g, and finally transferring the extract to a 96-well collection plate. Extraction using Ostro Pass-through 96-well Plate was performed as a single-step in-well extraction. Three-mm dried blood spots were added into the wells of the Ostro 96-well plate (Figure 2), 250 μ L of 5% water in methanol was used as the extraction solvent, the plate was vortexed for 1 minute and vacuum was applied for 5 minutes. Eluates from both techniques were diluted with 250 μ L of water prior to direct injection on the LC-MS/MS system.

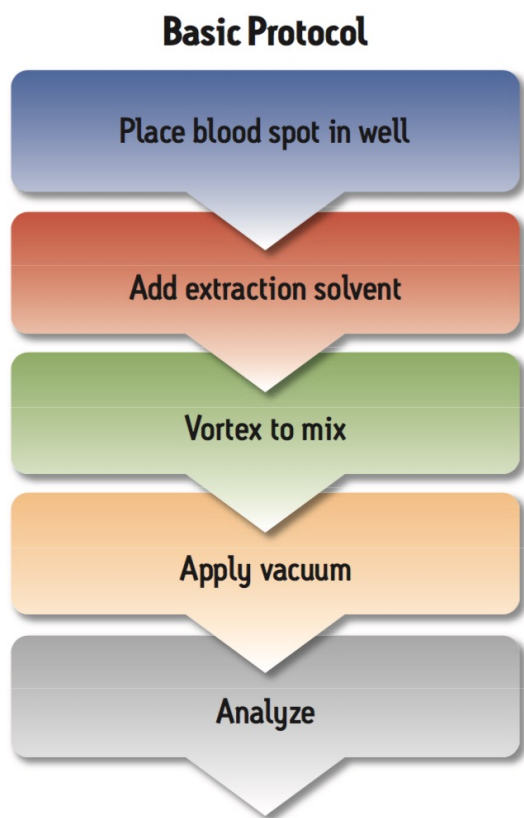


Figure 2. Protocol for extracting dried blood spots using the Ostro 96-well plate.

Results and Discussion

MS was performed in positive ion mode. As direct injection of DBS extracts typically results in very dilute samples, the Xevo TQ-S was used to significantly increase sensitivity. This facilitated the ability to directly inject the samples and skip potentially problematic evaporation and reconstitution steps.

The levels of PLs remaining after each sample preparation technique were compared. To compare PL levels, five PLs were quantified from DBS punches extracted in single tubes versus the DBS punches extracted in-well using the Ostro PLR plate. The Ostro Pass-through Plate removed 99.9% of the phospholipids relative to the traditional DBS method (Figure 3). To visually demonstrate remaining residual phospholipids, the total ion current (TIC) of 5 individual phospholipids is shown for both traditional DBS extraction and DBS extraction in well using Ostro (Figure 4). When the Ostro Pass-through Plate is used, the PL levels are negligible and no build-up occurs. When traditional DBS is used, a significant amount of PLs are present and accumulate throughout the run despite the 1-minute hold at a high percentage of organic solvent meant to prevent PL build-up (Figure 5).

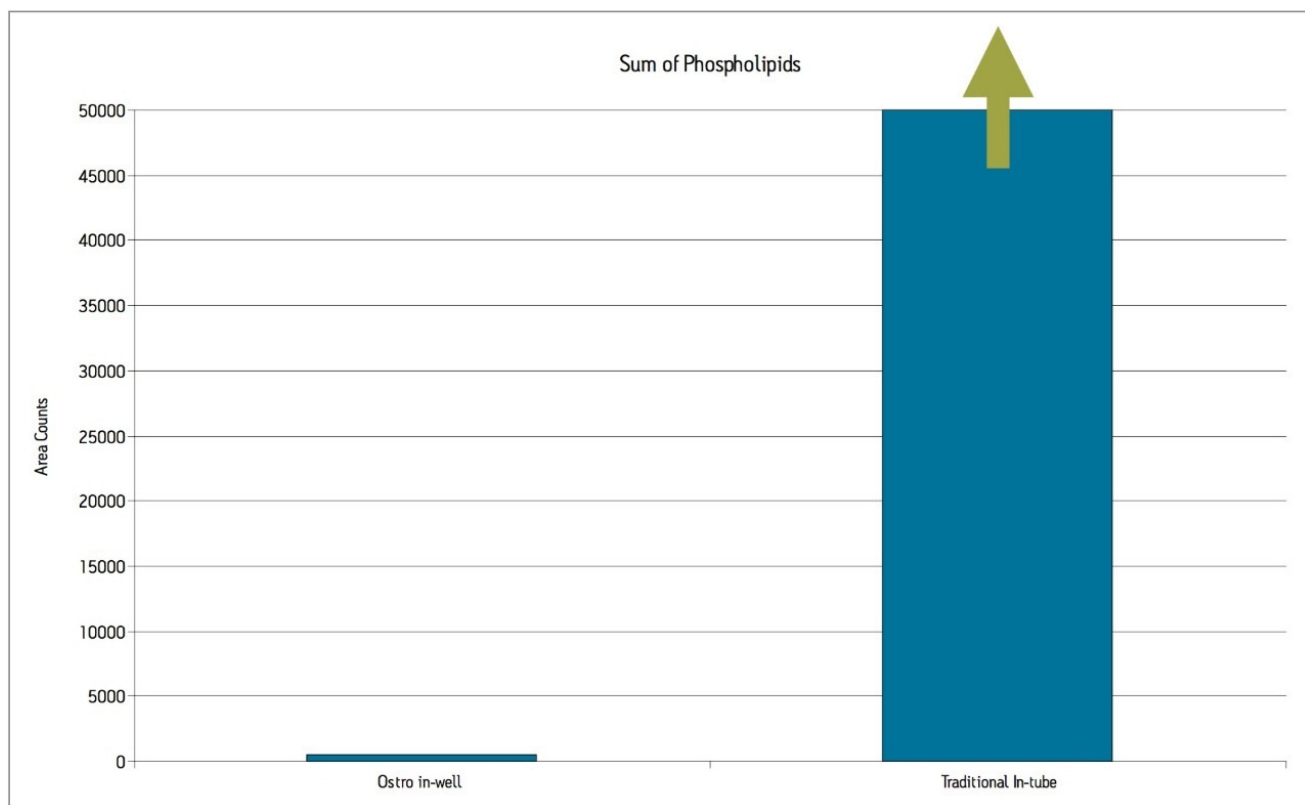


Figure 3. Comparison of the sum of area counts for phospholipid levels between DBS samples extracted in-well using the Ostro Pass-through 96-well Plate and DBS samples extracted using the traditional method in tubes, n=4 for each technique. Extraction solvent is 95/5 methanol/water in both cases. The 5 phospholipids summed had precursor masses of 496, 522, 704, 758, and 806.

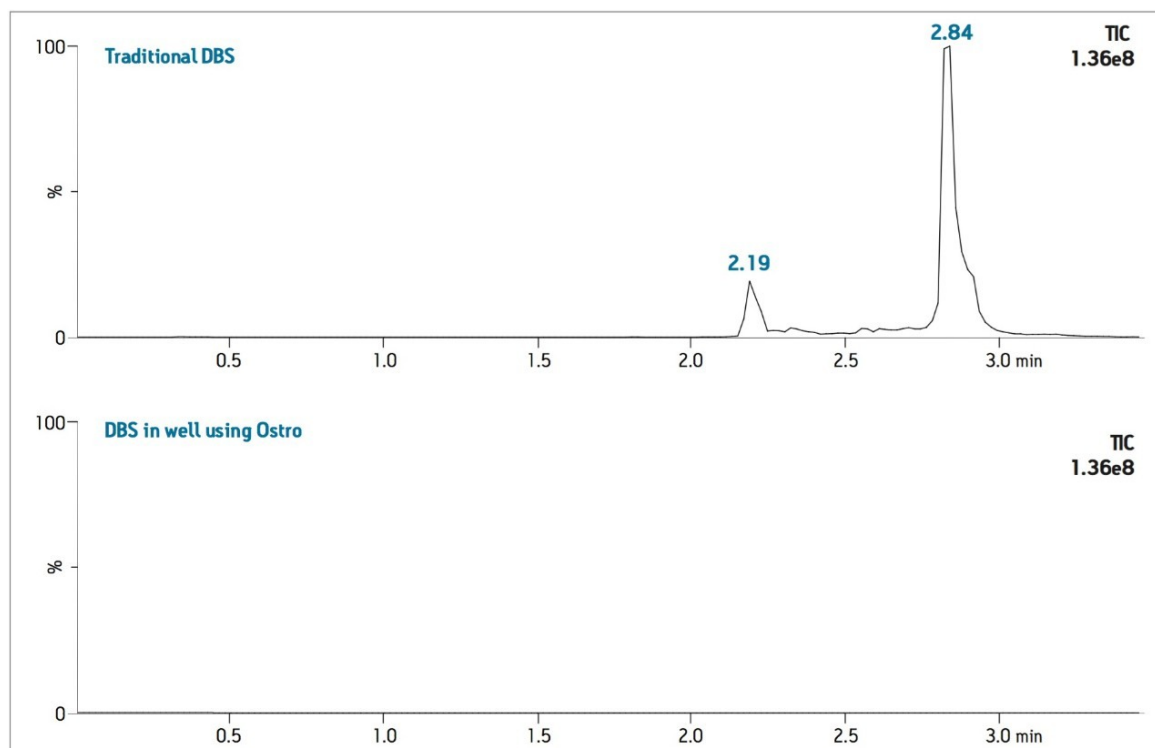


Figure 4. Representative chromatograms of the total ion current (TIC) of 5 individual phospholipids from traditional DBS extraction and DBS extraction in well using the Ostro Pass-through Plate



Figure 5. Chromatograms of the MRM transition 758>184 to demonstrate build-up of an individual PL over subsequent injections using traditional DBS and DBS extracted in-well using the Ostro Pass-through Plate. A gradient from 25 99.5% B in 2 minutes was used followed by a 1-minute hold at high organic.

Semi-validation was performed, and calculated QC sample concentrations were within 15% of expected, meeting regulatory criteria. LLOQ's of 0.05 ng/mL for 9-OH risperidone (Figure 6) and the parent compound were achieved in whole blood. Standard curves were linear over 3 orders of magnitude, from 0.05 ng/mL to 50 ng/mL in whole blood (Figure 7).

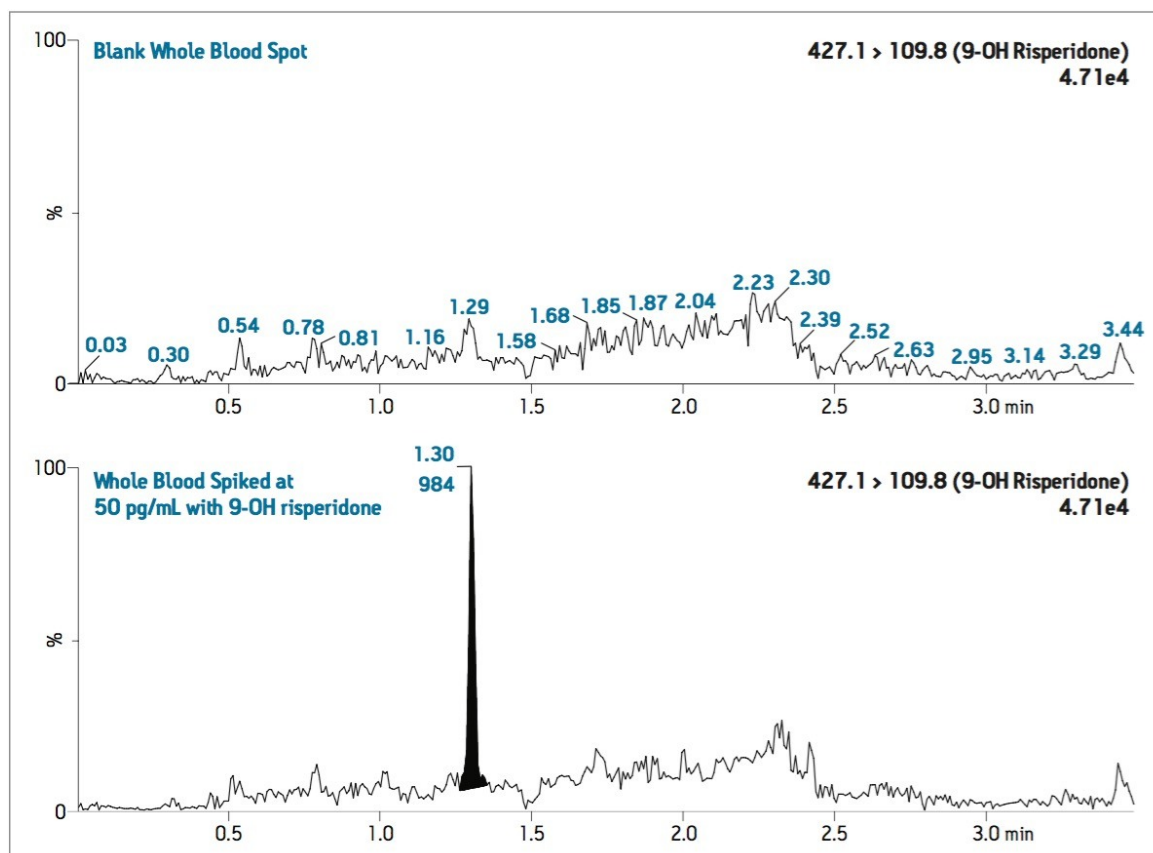


Figure 6. Representative chromatogram of a blank DBS and 0.05 ng/mL 9-OH risperidone DBS extracted using Ostro 96-well plate and directly injected.

Name	Type	Std. Conc.	RT	Area	IS Area	Response	Conc.	% Dev.
0_05_direct_inject_042011_001	Standard	0.05	1.53	945	37691	0.025	0.05	4.0
0_1_direct_inject_042011_001	Standard	0.10	1.52	1146	37531	0.031	0.10	-4.5
0_25_direct_inject_042011_001	Standard	0.25	1.53	1682	35798	0.047	0.23	-9.3
0_5_direct_inject_042011_001	Standard	0.50	1.53	3392	40329	0.084	0.52	4.5
1_direct_inject_042011_001	Standard	1.00	1.53	5114	39457	0.130	0.88	-11.5
5_direct_inject_042011_001	Standard	5.00	1.52	28760	38637	0.744	5.78	15.7
10_direct_inject_042011_001	Standard	10.00	1.52	57317	42923	1.335	10.49	4.9
25_direct_inject_042011_001	Standard	25.00	1.52	156098	51934	3.006	23.80	-4.8
50_direct_inject_042011_001	Standard	50.00	1.52	289427	45519	6.358	50.51	1.0
0_075_QC_direct_inject_042011_001	QC	0.075	1.52	1467	51641	0.028	0.08	4.8
0_75_QC_direct_inject_042011_001	QC	0.75	1.52	4473	44994	0.099	0.64	-14.1
7_5_QC_direct_inject_042011_001	QC	7.50	1.52	48110	49944	0.963	7.53	0.4
15_QC_direct_inject_042011_001	QC	15.00	1.52	96139	42200	2.278	18.00	20.0

Figure 7. Representative calibration curve and QC values for 9-OH risperidone in whole blood, $R^2 = 0.991$ with 1/x2 weighting.

Conclusion

- Simple, single step sample prep method for DBS samples
- Removes 99.9% of residual phospholipids when using Ostro Pass-through Sample Preparation Technique compared to the traditional DBS extraction technique
- Achieves highest sensitivity using the Xevo TQ-S Mass Spectrometer
- Eliminates build-up of PLs on LC columns

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