

# Waters

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## METHODS

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 96-well sample preparation plate can be used to eliminate greater than 99% of residual PLs. The Ostro 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 prep 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 a 96-well phospholipid removal (PLR) plate.

Column:	ACQUITY UPLC® BEH C <sub>18</sub> , 2.1 x 50 mm, 1.7 µm			
Mobile Phase A:	0.1% NH <sub>4</sub> OH in H <sub>2</sub> O			
Mobile Phase B:	50/50 ACN/MeOH			
Flow Rate:	0.6 mL/min			
Gradient:	Time (min)	Profile %A	Curve %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

Injection Volume:	40.0 µL
Injection Mode:	Partial Loop
Column Temperature:	35 °C
Sample Temperature:	15 °C
Strong Needle Wash:	60:40 ACN: IPA + 2% HCOOH
Weak Needle Wash:	95/5 H <sub>2</sub> O/MeOH

**Waters Xevo™ TQ-S 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

	Precursor Mass	Fragment 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.*

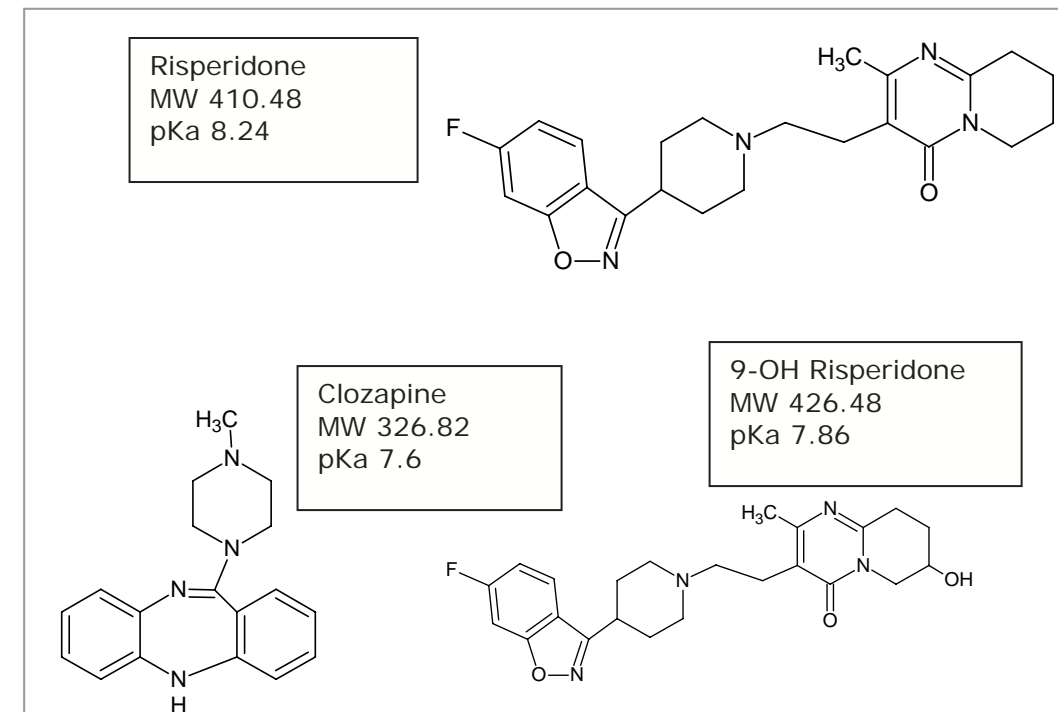


Figure 1. Structures, molecular weights, and pKa's of risperidone, 9-OH risperidone, and clozapine.



Figure 2. Whatman FTA DMPK Type C (untreated) dried blood spot cards.

**Sample Prep Conditions:**

Whole blood samples were spotted in 20  $\mu$ L aliquots onto Whatman DMPK-C untreated cards (*Figure 2*). The samples were allowed to dry for 2 hours at room temperature. 3 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 96-well plate. The traditional extraction was performed using 250  $\mu$ 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 96-well PLR plate was performed as a single step in-well extraction. 3 mm dried blood spots were added into the wells of the Ostro 96-well plate (*Figure 3*), 250  $\mu$ 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  $\mu$ L of water prior to direct injection on the LC/MS/MS system.

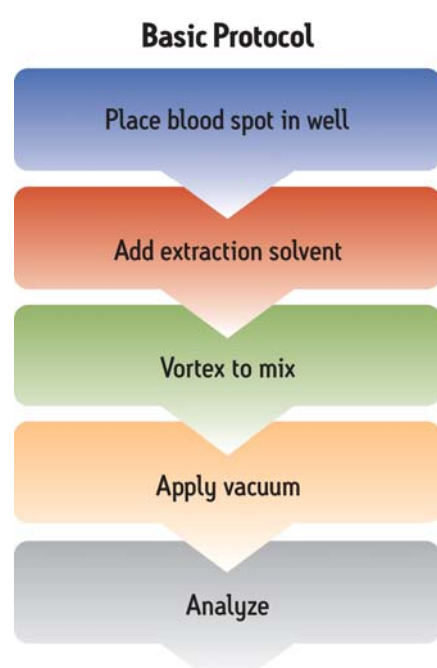


Figure 3. Protocol for extracting dried blood spots using the Ostro 96-well phospholipid removal plate.



## RESULTS/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 plate removed 99.9% of the phospholipids relative to the traditional DBS method (*Figure 4*). 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 5*). When Ostro sample prep 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 high percentage of organic solvent meant to prevent PL build-up (*Figure 6*).

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 7*) and the parent compound were achieved in whole blood. Standard curves were linear over 4 orders of magnitude, from 0.05 ng/mL to 50 ng/mL in whole blood (*Figure 8*).

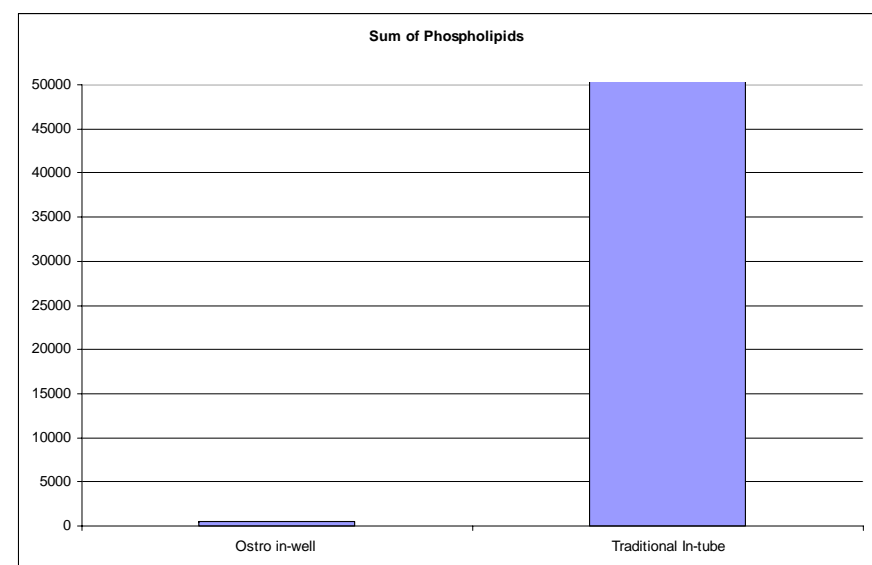


Figure 4. Comparison of the sum of phospholipid levels between DBS samples extracted in-well using the Ostro 96-well plate and DBS samples extracted using the traditional method in tubes, n=4 for each technique. Extraction solvent is 95/5 methanol/water in both cases. The 5 phospholipids summed had precursor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

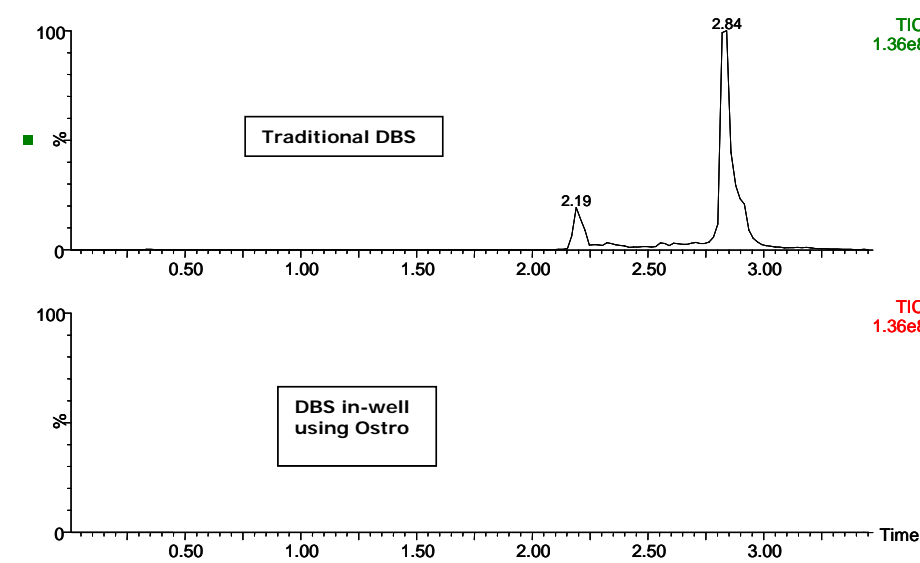


Figure 5. Representative chromatograms of the total ion current (TIC) of 5 individual phospholipids from traditional DBS extraction and DBS extraction in-well using Ostro.

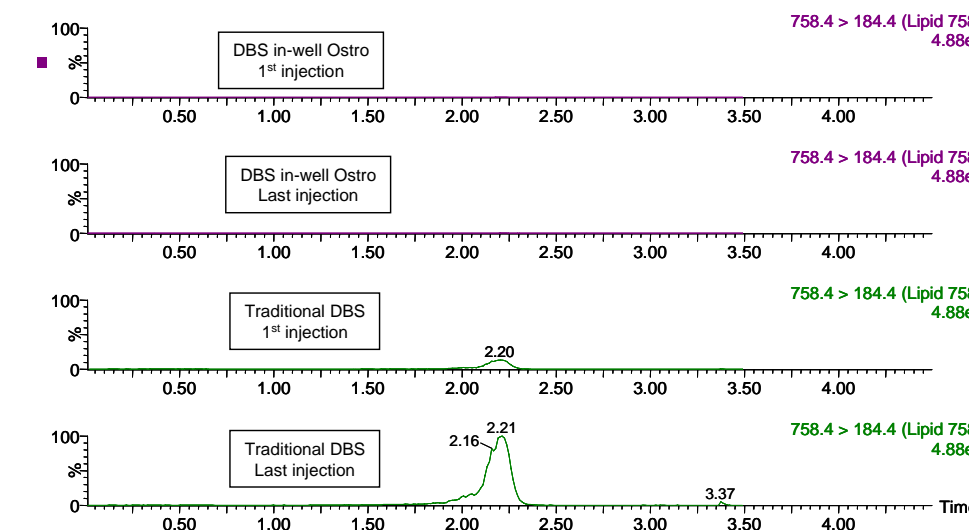


Figure 6. Chromatograms of the MRM transition 758>184 to demonstrate build-up of an individual PL over subsequent injections using traditional DBS and DBS extracted in-well using the Ostro plate. A gradient from 25-99.5% B in 2 minutes was used followed by a 1 minute hold at high organic.

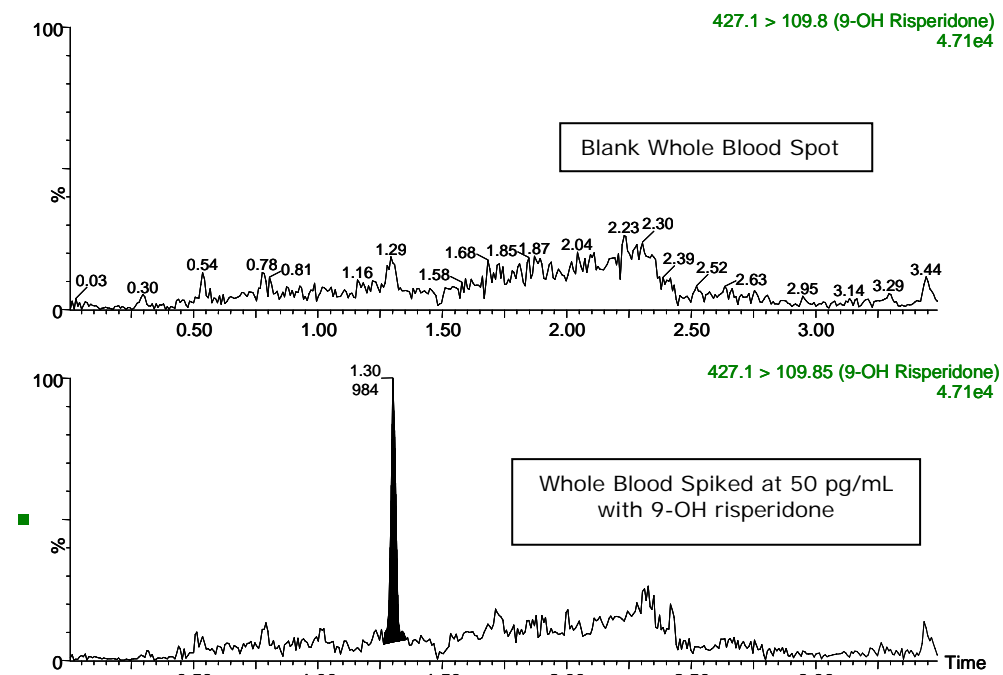


Figure 7. Representative chromatogram of blank DBS and 0.05 ng/mL 9-OH risperidone DBS extracted using Ostro 96-well plate and directly injected.

Name	Type	Std. Cond.	RT	Area	IS Area	Response	Cond.	%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	26760	38637	0.744	5.78	15.7
10_direct_inject_042011_001	Standard	10.00	1.52	57317	42903	1.335	10.49	15.9
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	286427	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	0.8
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 8. Representative standard and QC values for 9-OH risperidone in whole blood,  $R^2 = 0.991$  with  $1/x^2$  weighting.

- **Simple. single step sample prep method for DBS samples**
- **Removes 99.9% of residual phospholipids when compared to traditional DBS extraction technique**
- **Achieves highest sensitivity using the Xevo TQ-S mass spectrometer**
- **Eliminates build-up of phospholipids on LC columns**