Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Rapid, Reliable Metabolite Ratio Evaluation for MIST Assessments in Drug Discovery and Preclinical Studies

Jonathan Danaceau, Erin Chambers, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Removal of plasma phospholipids
- Rapid analysis times
- Low system back pressure
- Quantitative accuracy

WATERS SOLUTIONS

Ostro™ Pass-through 96-well Plates

XBridge® BEH C_{18} ***XP*** Column

Xevo® TQ-S Mass Spectrometer

ACQUITY UPLC® System

KEY WORDS

Phospholipid removal, metabolite identification and safety testing (MIST), metabolite quantification

INTRODUCTION

The U.S. Food and Drug Administration (FDA) recommends, in their Metabolites in Safety Testing (MIST) guidelines, that the safety of drug metabolites be evaluated if they are present in human plasma at concentrations greater that 10% of the parent drug (or API) at steady state.[1] For this reason, it is important to be able to accurately quantify drug metabolites during the discovery stage and preclinical assessments of candidate drugs. In addition, the need to rapidly evaluate multiple, diverse drug candidates and metabolites in a discovery or preclinical setting requires methodologies that are generic, fast, and robust. This application note highlights the use of Ostro Pass-through 96-well Plates and XBridge BEH e***X***tended ***P***erformance (***XP***), 2.5 µm columns for accurate metabolite ratio determination. Ten pairs of drugs and metabolites were accurately analyzed within a 2.5-minute analysis time, with almost no phospholipid buildup, and column back pressures of less than 3500 psi, allowing the flexibility to use ***XP*** columns on any HPLC, UHPLC, or UPLC® system.

EXPERIMENTAL

Method Conditions

LC Conditions

LC system:	ACQUITY UPLC
Vials:	96-well plate with deactivated 700-µL glass inserts (p/n 186000349DV)
Column:	XBridge BEH C_{18} ***XP*** 2.1 x 50 mm, 2.5 µm (p/n 186006029)
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	500 µL/min
Mobile phase A:	Milli-Q water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid

The initial gradient conditions were 95:5, A:B. The percentage of mobile phase B (MPB) was increased to 98% over 1.5 min, and held for 0.5 min. The percentage of MPB was then reduced back to initial conditions (5%) over 0.1 min, and held at that proportion for the remaining 0.4 min of the gradient. The entire cycle time was 2.5 min.

MS Conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI positive
Acquisition range:	Optimized by component
Capillary voltage:	1.0 kV
Collision energy:	Optimized by component, see Table 1
Cone voltage:	Optimized by component

Data Management

Chromatography:	MassLynx® Software
Quantification:	TargetLynx™ Software
Optimization:	IntelliStart™ Software

Sample description

Plasma samples were prepared using Ostro Pass-through Plate (p/n 186005518). 100 µL of sample was added to the plate, followed by 300 µL of acetonitrile (ACN) containing 1% formic acid (FA). The plasma/ACN mixture was aspirated four times, and eluted into a 96-well plate containing 700-µL glass inserts. Five µL of the resulting solution was injected onto the analytical system. For samples prepared by protein precipitation (PPT) only, 300 µL of ACN containing 1% formic acid was added to 100 µL of plasma. Samples were vortexed for 30 seconds, and spun in a microcentrifuge at 16,000 rcf for 5 min. The supernatant was then transferred to the same collection plate as the Ostro prepared samples.

Stock and working solutions were prepared in methanol. Plasma samples containing parent drugs and metabolites were prepared by adding 20 µL each of working solutions of parent drugs and metabolites to 1 mL of plasma. All parent drugs were spiked at a concentration of 20 ng/mL, with the exception of testosterone, which was spiked at 100 ng/mL. Metabolites were spiked at 100%, 30%, 10%, and 0% of parent drug concentrations.

RESULTS AND DISCUSSION

Chromatography

The compounds and metabolites used in this study, molecular formulae, the MRM transitions used for quantification, and their retention times are listed in Table 1. MS conditions and MRM transitions were optimized using IntelliStart Software. Representative chromatograms for the parent drugs and their metabolites are displayed in Figure 1. All peak widths (at 5% height) were under 2.5 seconds at base. System pressure was also monitored throughout the analytical run, and reached a maximum of 3300 psi. This combination of peak capacity and low system back pressure allows maximum flexibility in the choice of chromatographic systems.

Compound	Formula	Transition	Retention time
Risperidone	$C_{23}H_{27}FN_4O_2$	411.2 → 191.2	0.84
9-OH-Risperidone	$C_{23}H_{27}FN_4O_3$	*427.2 → 207.2*	*0.84*
Clozapine	$C_{18}H_{19}ClN_4$	327.1 → 192.1	0.88
N-desmethylclozapine	$C_{17}H_{17}ClN_4$	*313.2 → 192.0*	*0.82*
Bupropion	$C_{13}H_{18}ClNO$	240.1 → 184.1	0.89
Hydroxybupropion	$C_{13}H_{18}ClNO_2$	*256.1 → 238.1*	*0.81*
Quetiapine	$C_{21}H_{25}N_3O_2S$	384.2 → 253.1	0.93
Norquetiapine	$C_{17}H_{17}N_3S$	*296.1 → 210.1*	*0.90*
Midazolam	$C_{18}H_{13}ClFN_3$	326.1 → 291.1	0.96
α-OH Midazolam	$C_{18}H_{13}ClFN_3O$	*342.1 → 324.1*	*0.94*
Fentanyl	$C_{22}H_{28}N_2O$	337.3 → 88.2	0.96
Norfentanyl	$C_{14}H_{20}N_2O$	*233.2 → 177.2*	*0.75*
Amitryptyline	$C_{20}H_{23}N$	278.3 → 233.3	1.09
Nortryptyline	$C_{19}H_{21}N$	*264.3 → 233.2*	*1.08*
Terfenadine	$C_{32}H_{41}NO_2$	472.3 → 436.4	1.26
Fexofenadine	$C_{32}H_{39}NO_4$	*502.3 → 466.3*	*1.06*
Testosterone	$C_{19}H_{28}O_2$	289.3 → 109.2	1.27
DHT	$C_{19}H_{30}O_2$	*291.3 → 255.3*	*1.41*
Clopidogrel	$C_{16}H_{16}ClNO_2S$	322.1 → 212.1	1.43
Clopidogrel carboxylic acid	$C_{15}H_{14}ClNO_2S$	*308.2 → 198.1*	*0.84*

Table 1. Compounds and metabolites.

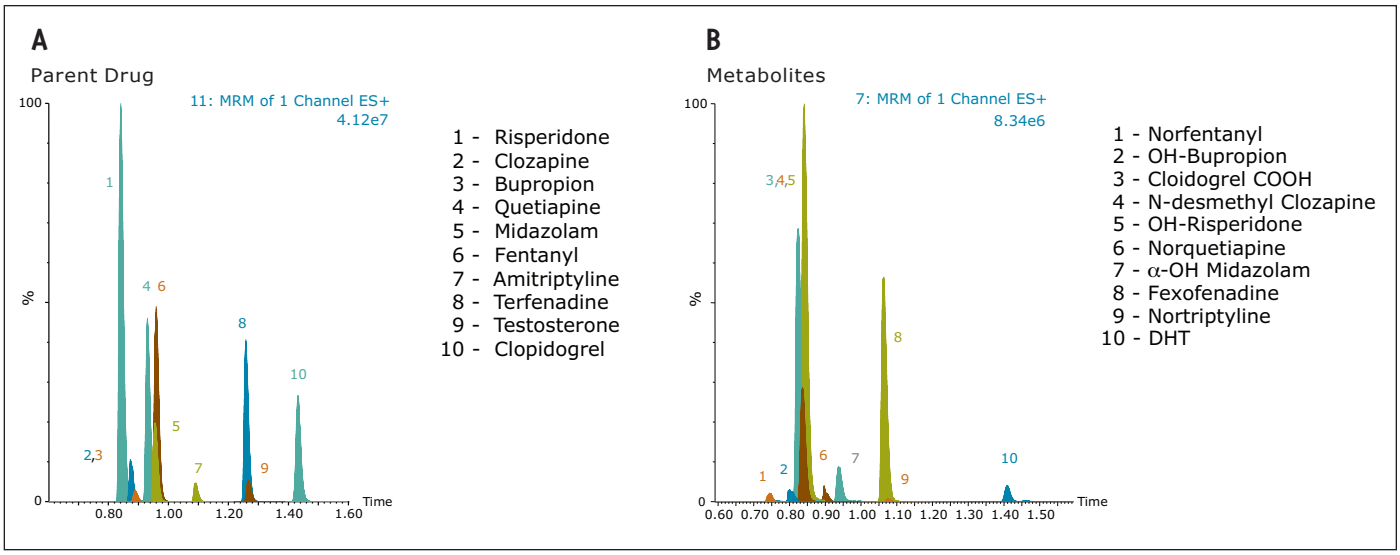

Figure 1. Representative chromatograms for the parent drugs and their metabolites.

Phospholipid removal

Phospholipid (PL) transitions of 496.3›184.3, 524.4›184.3, 703.6›184.3, 758.6›184.3, and 806.6›184.3 were monitored, according to the protocols in Chambers *et al.*[2] The MRM transition of 184.3›184.3 was also monitored, so that the approximate abundance of all phosphatidylcholine-containing PLs could be monitored. Combined phospholipid traces from the initial and final PPT injections of an analytical batch are plotted in Figure 2. Figure 2B also includes the earliest and latest eluting parent compounds superimposed over the PL trace. This figure demonstrates the dramatic increase in PLs eluting in the early portions of the chromatogram as they accumulate on the column, as well as the co-elution of the analytes with PLs remaining in PPT samples. Both traces also reveal the elution of a significant PL peak at the end of the re-equilibration of the solvent gradient, which could prevent the use of rapid gradient cycles and increase batch analysis time.

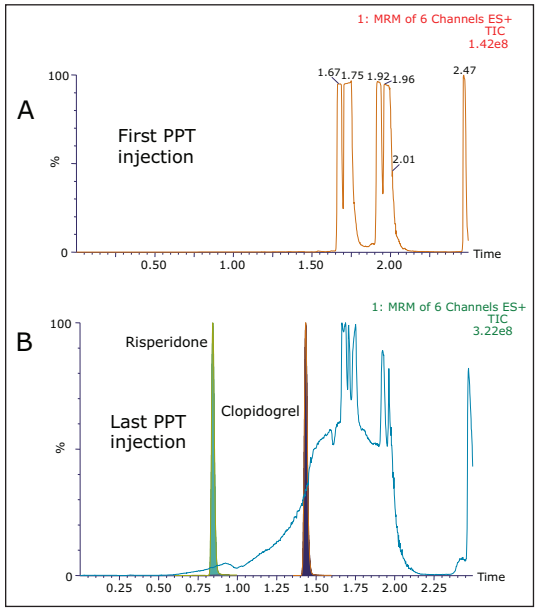

Figure 2. Total phospholipid chromatograms – PPT samples.

Figure 3 shows the accumulation of phosphatidylcholine-containing PLs during an analytical batch. The red and blue markers represent the total abundance (measured by area) of PLs in the Ostro prepared samples and the PPT prepared samples, respectively. The accumulation of PLs on the column from the PPT prepared samples is quite evident. The increase in total PL abundance demonstrates that, after approximately six injections, the column becomes saturated with PLs. By contrast, in the Ostro prepared samples, minimal PLs were detected, and no evidence of accumulation on the column was seen. The mean area of PLs in the Ostro prepared samples was 0.26% of those seen in the PPT prepared samples. In actuality, the proportion of PLs in the Ostro prepared samples is most likely an overestimate, as the signal for many of the individual PLs from the PPT samples saturated the MS detector.

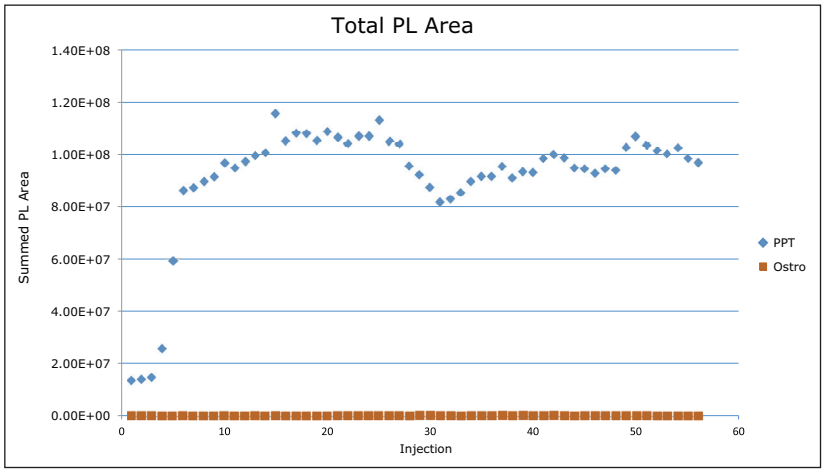

Figure 3. Phospholipid accumulation.

Quantitative accuracy

The FDA requirement of testing the safety of drug metabolites that exceed 10% of the steady state concentration of the parent compound makes accurate quantification of early drug candidates and their metabolites a necessity. In order to assess the accuracy of quantification for each metabolite, individual sets of samples were analyzed with drug metabolites spiked at 100%, 30%, and 10% of the concentration of the parent drugs. The response ratios of the samples spiked at 10% and 30% of the parent concentration versus those spiked at 100% of the parent concentration were then calculated. Figure 4 shows that the response ratios of all metabolites spiked at 10% of the parent drug concentration were determined to be between 10% and 13%, while those spiked at 30% ranged between 28% and 32%. For example, the metabolite N-desmethyl clozapine was spiked at 20, 6, and 2 ng/mL, representing 100%, 30%, and 10% of the parent drug concentration. The ratio of the response of the 6 ng/mL samples to the 20 ng/mL samples should be 0.3 or 30%. This "expected response" is indicated by the red line on the graph in Figure 4B. The actual measured ratio between the two samples was 0.305 or 30.5%, and is indicated by the bars on the graph. There was no significant difference in the ratios calculated from the Ostro prepared samples versus the PPT prepared samples, indicating that there is no appreciable analyte loss or interaction with the Ostro Sorbent during processing.

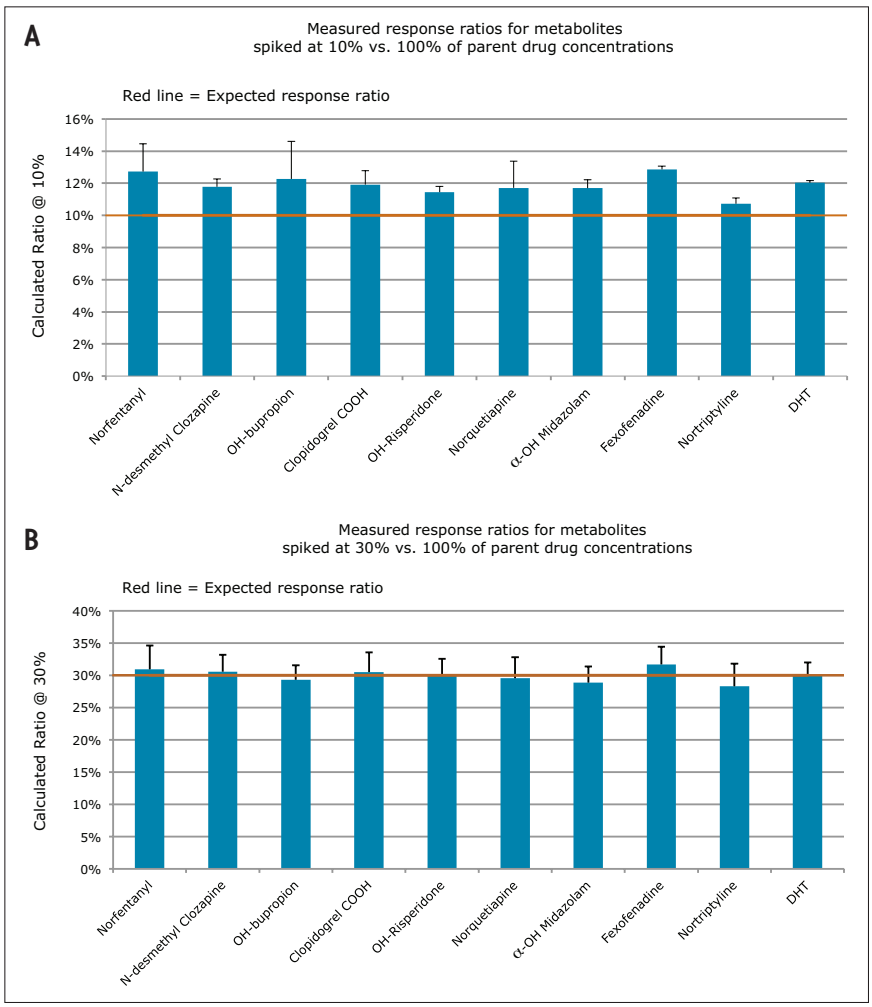

Figure 4. Response ratios of all metabolites.

CONCLUSIONS

The use of Ostro Pass-through 96-well Plates, and XBridge *XP* 2.5 µm columns represents an attractive solution for assessment of drug metabolite concentrations during discovery and preclinical studies. Sample preparation, using Ostro Pass-through Plate, is sufficiently generic for use with a variety of compounds. The removal of phospholipids prevents PL accumulation on the analytical column, and minimizes the risk of matrix effects due to PLs. The removal of PLs also allows for rapid cycle times, without the need for extended re-equilibration. Expected responses from analytes spiked at different concentrations are very close to theoretical targets, allowing for accurate quantification. Finally, *XP* columns provide excellent performance and peak capacity at back pressures that enable their use on any LC system, at analysis speeds conducive to high throughput production.

Acknowledgement

The authors would like to thank Hongbin Yu of Boehringer Ingelheim for this research idea and for his valuable technical input.

References

1. FDA: Guidance for Industry Safety Testing of Drug Metabolites. Dept of Health and Human Services. 2008.
2. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography* B. 2007; 852 (1-2): 22-34.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com