

Application Note

## A New Paradigm for Metabolism Studies: UPLC/Q-ToF

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## Abstract

This application note demonstrates a novel approach to drug metabolism using UPLC (Ultra Performance Liquid Chromatography) coupled to a hybrid quadrupole orthogonal time of flight (Q-Tof) mass spectrometer.

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## Introduction

Over the past 15 years LC-MS has become one of the most widely used technologies in bioanalytical laboratories worldwide. During all this time, there have been no major advances in HPLC technology apart from new column chemistries and smaller particle sizes (down to 3.5  $\mu\text{m}$ ). With the recent focus on advances in MS and MS/MS, it is easy to forget how important chromatography is when running bioanalytical assays, whether for quantitative or qualitative purposes.

Despite early claims that MS, and particularly MS/MS, removed the need for good chromatography, a poor LC assay will lead to issues of ion suppression and isobaric interferences that cannot be overcome by a mass spectrometer alone. These concerns often become apparent when you start asking questions such as: How fast do I need to run my analyses? What metabolite levels would I like to detect? How much do I know about my sample? What happens if two similar metabolites or impurities elute in the same chromatographic peak?

In order to generate the best quality bioanalytical data within the constraints imposed by throughput requirements, it is necessary to place equal emphasis on chromatographic separation and mass spectrometric detection. A good analytical separation will give better detection levels and improved MS data quality. With pressure on today's laboratories to increase throughput, there has been a tendency to neglect the importance of the chromatographic separation. However, the success of detecting and identifying metabolites depends upon having both LC and MS methods designed appropriately.

In order to address these issues, a novel approach to drug metabolism using UPLC (Ultra Performance Liquid Chromatography) coupled to a hybrid quadrupole orthogonal time of flight (Q-Tof) mass spectrometer will be described in detail in this paper.

UPLC leverages the theories and principles of HPLC and adds a new dimension to mass spectrometry. The heart of this technology lies in the particle size and new column chemistry. By using much smaller particles sizes, a new end point for the separation can be realized. The underlying principle to this approach is illustrated by the Van Deemter plot (Figure 1). The Van Deemter equation is an empirical formula that describes the relationship between linear velocity and plate height (column efficiency). It considers particle size as one of the variables that can be used to characterize performance at different linear velocities.

## The Promise of the van Deemter Plot

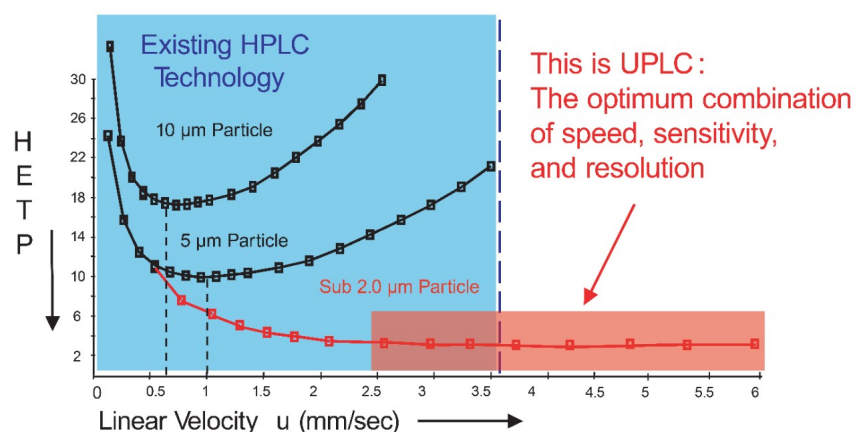


Figure 1. Van Deemter plot showing different particle sizes and advantages of using sub-2  $\mu\text{m}$  particle size.

From Figure 1, it can be observed that below a 2  $\mu\text{m}$  particle size, a new realm of chromatography is accessible. Sub-2  $\mu\text{m}$  particles offer the highest efficiency, and this higher efficiency is obtained at significantly higher linear velocities than with larger particles. Utilizing sub-2  $\mu\text{m}$  particles allows us to push the limits of both peak capacity (due to the higher efficiency) and speed of analysis (due to the higher linear velocities). In addition, sensitivity is improved because chromatographic bands are more concentrated and elute as sharper peaks.

The enhancement of chromatographic resolution and sensitivity is especially important when analyzing complex mixtures in biological samples. However, to achieve the benefits of operating at higher linear velocities, it is necessary to run at higher pressures— in the order of 10,000 psi. Waters ACQUITY UPLC systems and columns have been designed to operate effectively under these conditions.

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## Experimental

### Sample Preparation

A bile sample from a rat dosed with midazolam at a concentration 10 mg/kg was collected and diluted 1:10 with water prior to sample analysis.

### Chromatography Conditions

Solvent Delivery System:	Waters ACQUITY UPLC
Column:	ACQUITY UPLC BEH C <sub>18</sub> column, 2.1x100 mm, 1.7 μ m particle size
Mobile Phase A:	water + 0.1% formic acid
Mobile Phase B:	acetonitrile + 0.1% formic acid
Gradient for 30 minute run*:	0–0.25 min 100% A, 30.25 min 5% A, 32 min 5% A, 32.1–32.5 min 100% A (curve 6 for all)
Gradient for 6 minute run:	0–0.25 min 100% A, 5.25 min 5% A, 6 min 5% A, 6.1–6.5 min 100% A (curve 6 for all)
Flow Rate:	400 μL/min
Injection Volume:	5 μL

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*\* Similar gradient conditions were used for the HPLC comparison at the same flow rate using a Waters Symmetry C<sub>18</sub> column, 2.1x100 mm, 3.5 μm particle size.*

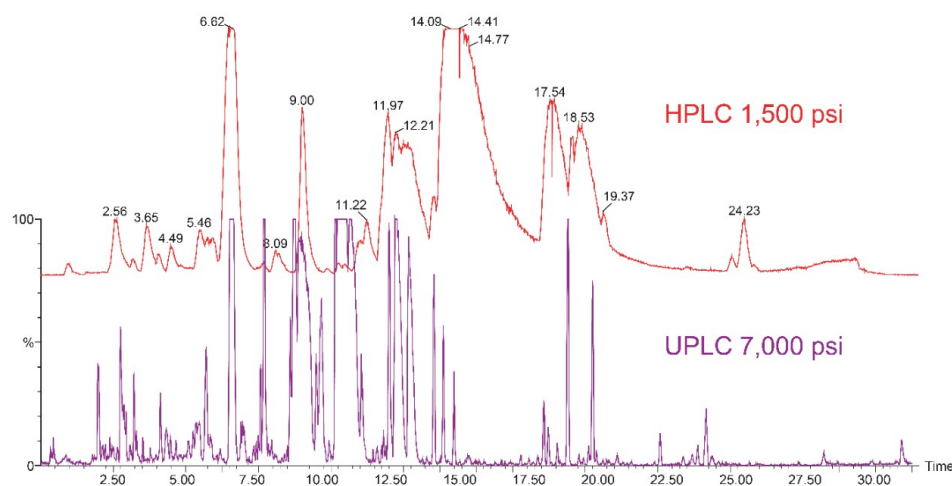
## Mass Spectrometry Conditions

Mass Spectrometer:	Micromass Q-Tof micro
Ionisation Mode:	Electrospray positive ion mode
Cone Voltage:	35 V
Capillary Voltage:	3.1 kV
Source Temperature:	120 °C
Desolvation Temperature:	300 °C
Lock Mass:	Leucine enkephalin <i>m/z</i> 556.2771, concentration 0.5 ng/μL

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## Results and Discussion

Figure 2 shows the *in vivo* metabolism of midazolam, an “anticonvulsant”, in rat liver bile. This is one of the most challenging separations faced in bioanalysis due to the high concentration of bile salts and endogenous compounds present.



*Figure 2. A comparison between HPLC/Q-ToF and UPLC/Q-ToF analysis, for a bile sample collected from a rat that was previously dosed with midazolam.*

As can be seen, the peak capacity in the UPLC chromatogram is greatly improved, resulting in greater differentiation among the peaks. For identification purposes, this is a tremendous leap forward because it means that less time will be spent ruling out false positives and redeveloping methods to improve the separation of metabolites. The impact from this is especially beneficial because in most cases, metabolite standards are not available for method optimization.

Moreover, increased peak capacity minimizes ion suppression resulting from the co-elution of metabolites with bile salts or endogenous compounds. Having a high peak capacity improves separations with complex matrices and limits the amount of coelution. More peaks can be resolved in the same run time.

The extracted ion chromatograms (Figure 3) for the glucuronide metabolites of midazolam show that, with HPLC, just one peak is detected. However, by employing UPLC, it is evident that there are in fact two separate glucuronides. Without UPLC, one of the glucuronides would have been missed even if MS/MS or MS<sup>n</sup> was employed. This would cause issues if this was a toxic metabolite and you were trying to change the chemistry to block the site of metabolism. It goes back to the core concerns: Am I sure about what I'm detecting? How much information am I missing?

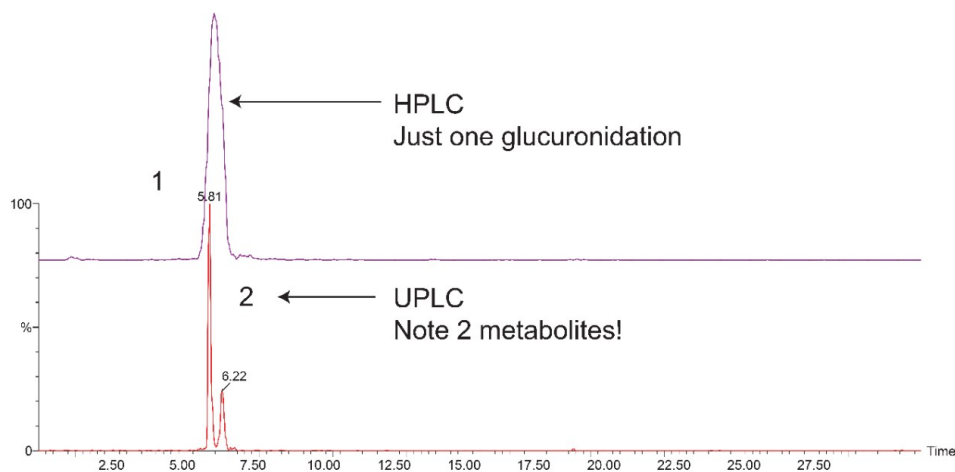


Figure 3. A comparison between HPLC and UPLC approaches for an extracted ion chromatogram containing two glucuronidated metabolites of midazolam with the same  $m/z$  values.

Drug metabolism cannot always be predicted and often produces unexpected biotransformations. Therefore, good data and accurate information is paramount in order to identify weak spots or possible sites of toxicity.

If the spectra of the two metabolite peaks resulting from HPLC and UPLC analysis (Figure 4) are examined, in the UPLC analysis there is a clear strong signal for the glucuronide at  $m/z$  548 whereas with HPLC, this peak is buried in the spectral noise due to co-eluting components. This makes the entire process of identification a lot more complex. In this case there is a point of reference, the isotopic signature of chlorine in midazolam, which can be used to eliminate false positives. But this is not the case for all compounds because there may not always be an isotopic descriptor to detect and identify putative metabolites. In both cases the mass measurement error is better than 3 ppm, showing that the Q-ToF is capable of generating good mass accuracy even across the narrow chromatographic peaks generated by UPLC.



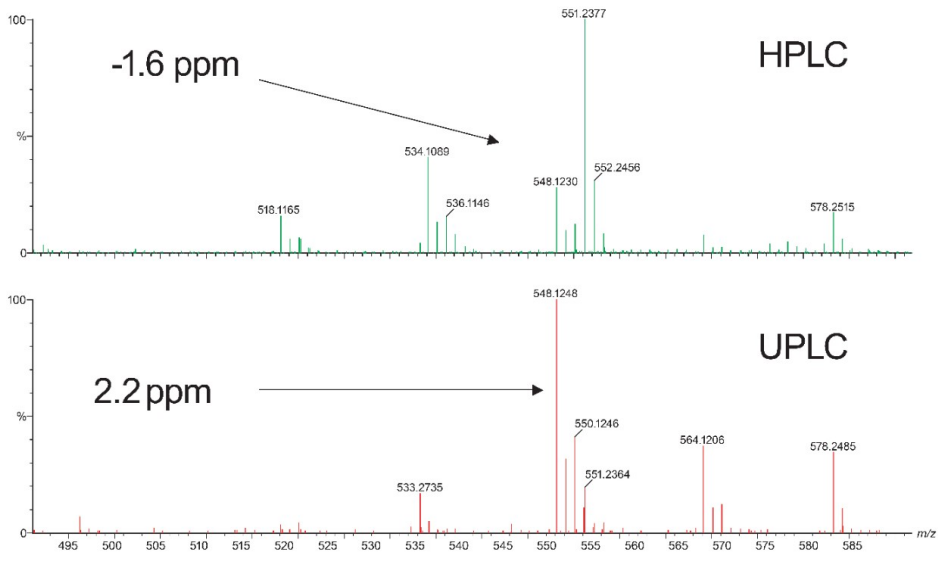


Figure 4. A spectral quality comparison for both HPLC and UPLC approaches when used in conjunction with the Q-ToF micro Mass Spectrometer for the glucuronidated metabolites of midazolam.

The data for both the HPLC and UPLC separations have been shown using a 30 minute gradient. What happens if an even shorter gradient, such as 6 minutes (Figure 5), is used with UPLC? Obviously the run time will be faster, but by shortening the gradient, resolution will be compromised compared to the 30 minute gradient.

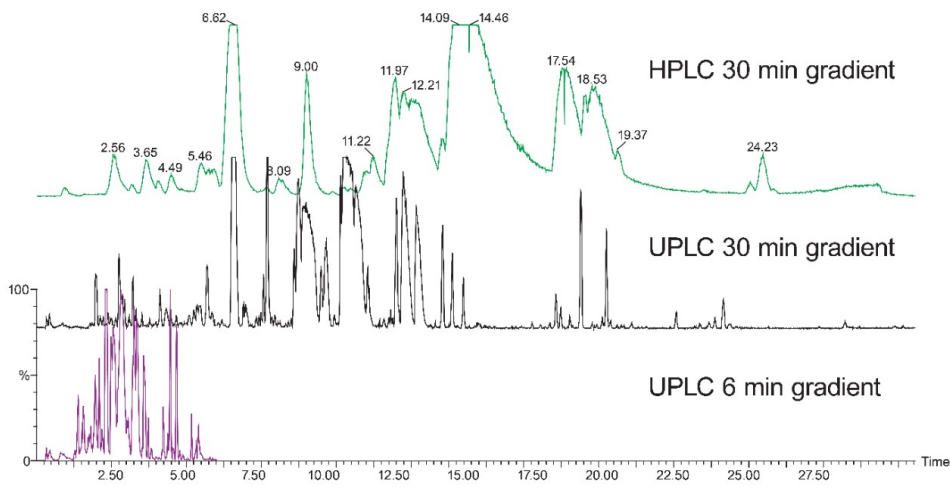


Figure 5. A comparison between HPLC and UPLC with different gradient times.

Even with the faster gradient, the two metabolites are still separated (Figure 6). Although the resolution is not as good as that achieved with the longer gradient, UPLC still produces significantly

increased data quality compared to the HPLC system. Gains in chromatographic resolution as well as analysis speed and sensitivity are achieved with UPLC. Having said that, for *in vivo* samples, a 30 minute run time will yield a much higher peak capacity, which is optimal for separating co-eluting metabolites and endogenous compounds to generate the best qualitative data possible.

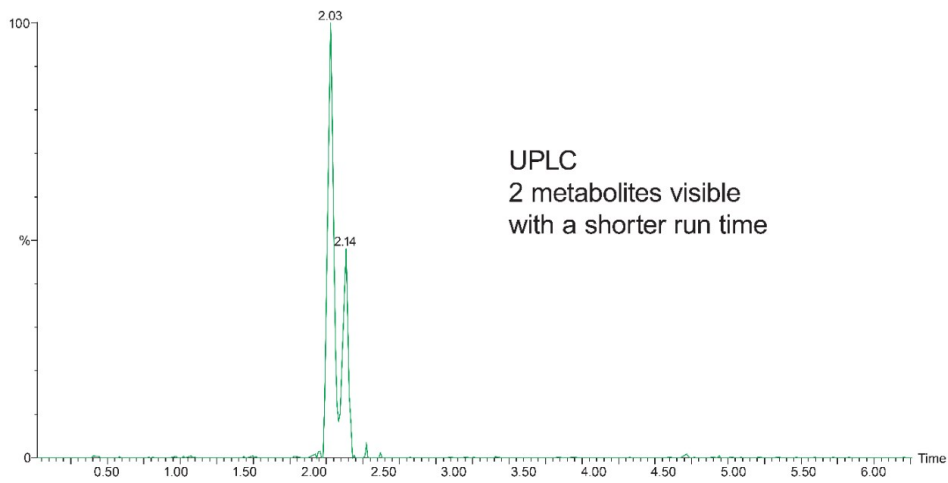


Figure 6. The extracted ion chromatogram corresponding to the conjugated metabolites of midazolam for the fast 6-minute gradient using UPLC/Q-ToF micro.

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## Conclusion

From the data presented, the advantages of using UPLC with Q-ToF MS for metabolism studies are obvious. As technology progresses, better levels of detection are obtainable not only via a more sensitive assay, but through better quality data. In a recent paper<sup>1</sup> Nicholson and Wilson postulated that metabolism is not logical but probabilistic, and all possible metabolites that can be created, will be created. Whether you see them or not depends on your analytical LC-MS strategy. Using UPLC with Q-ToF MS adds a new dimension to metabolism studies, enabling attainment of better detection limits, better throughput, and increased chromatographic resolution, which in turn will improve data quality from the mass spectrometer. It is a major leap forward, not just for this metabolism application but many areas in both qualitative and quantitative bioanalysis.

Finally, we all worry about how fast we can run samples and obtain good data. Too often, the bottleneck is merely shifted to the data processing step, which requires a considerable amount of time to sieve through the data to find potential metabolites. Better quality data facilitates this step and provides improved input for automated processing routines for detecting drug metabolites. Using UPLC is a novel approach that offers a platform on which we will be able to improve data quality and increase the knowledge of our samples.

"We only observe what we can detect and this limits our knowledge."

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## References

1. J.K. Nicholson and I.D. Wilson, *Nature Reviews Drug Discovery*, 2003, 2, 668–676.

## Acknowledgements

Special thanks to Iain Beattie, Andrew Wright and Karine Joncour from AstraZeneca, Loughborough UK, for providing the samples for this application note.

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## Featured Products

[ACQUITY UPLC System](#)

720000953, July 2004

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