A NOVEL ULTRA-HTS METHOD BY UPLC-MS FOR METABOLITE DETECTION AND IDENTIFICATION

Waters

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ABSTRACT

In drug metabolism, remove and especially in discovery, speed of analysis and identification of putative metabolites is very important. LC/MS has played a pivotal role for compound screening in DMPK. Whilst mass spectrometers have been constantly developed to obtain better sensitivity, faster scanning, better resolution and exact masses, HPLC has been relatively untouched in terms of development. HPLC is a major part of the LC/MS system and one which can help to reduce 'ion suppression', separate isomers and help to obtain lower levels of detection. Improving chromatographic resolution will allow us to obtain superior separations with high peak capacity, thus reducing coelution of metabolites and also enhance the sensitivity in the MS system. To investigate this we have employed an Ultra Performance Liquid Chromatography (UPLC™) system utilizing 1.7 µm particles giving an average column pressure of around 10,000 psi. The main advantage of running with such a unique particle size is the separation, where we can separate closely eluting metabolites. This instrument has been coupled to a hybrid quadrupole-Tof mass spectrometer. In order to show the potential of this particular development, we will show the results from the analysis of a number of marker compounds in both *in vitro* and *in vivo* matrices. Major routes of metabolism were identified for all compounds analysed in both *in vitro* and *in vivo* samples. By this approach, we were able to either cut typical run times by up to a factor of 3 or increase the peak capacity by a factor of 2–4. A significant increase in signal intensity and improved chromatographic resolution will be demonstrated in conjunction with accurate mass measurement better than 3 ppm RMS. This, we believe is a major leap forward for the future HTS for Drug Metabolism using LC/MS.

INTRODUCTION

Over the past 15 years LC/MS has become one of the most widely used technologies in many laboratories worldwide. LC/MS is a very well accepted and established technique. 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 µm. With the recent focus on advances in MS and MS/MS it is easy to forget how important HPLC is when running bioanalytical assays, whether it is for quantitative or qualitative purposes. The role of the separation step has been taken for granted. There is a famous saying "What you put in you get out" and this is very true for LC/MS. 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 the mass spectrometer cannot overcome. 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.

Ion suppression is one of the major topics of discussion within the mass spectrometry community. How fast do you need to go? What levels would you like to detect? How much do you know about your sample? In the case of metabolism or impurity analysis, what happens if two similar metabolites or impurities elute in the same chromatographic peak? MS/MS will not help.

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 the novel chemistry utilised in this particular approach. By utilising much smaller particles sizes, a new end point for the separation can be realised. 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 and it can be used to characterise performance at different linear velocities. From Figure 1 it can be observed that below a 2 µm particle size[1,2] a realm of chromatography opens up. Sub 2-µm particles offer the highest efficiency, and this higher efficiency is obtained at significantly higher linear velocities than with larger particles. Utilizing sub-2 µm particles will allow us to push to the limits of both peak capacity due to the higher efficiency and speed of analysis, due to the higher linear velocities. In order to achieve these higher linear velocities it is necessary to run at higher pressures (in the order of 10,000 psi) and the Waters ACQUITY UPLC™ instrumentation and columns have been designed to operate effectively under these conditions.

In addition to improvements in speed of analysis and chromatographic resolution which is paramount especially when running complex mixtures in biological samples, UPLC offers improved sensitivity since chromatographic bands are more concentrated, resulting in sharper peaks.

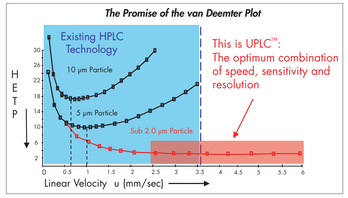

Figure 1. Van Deemter plot showing different particle sizes and advantages of using sub 2 µm particle size.

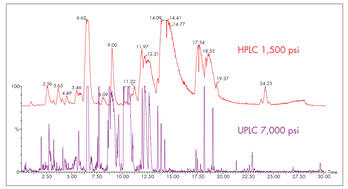

Figure 2. Shows a comparison between HPLC and the UPLC approach for a bile sample collected from rat which was previously dosed with Midazolam.

EXPERIMENTAL 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 m/z 556.2771, concentration 0.5 ng/µL
Solvent Delivery System:	ACQUITY UPLC™
Column:	ACQUITY UPLC™ BEH C_{18} column, 100 x 2.1 mm id, 1.7 µm particle size
Mobile Phase A:	water + 0.1% formic acid
Mobile Phase B:	acetonitrile + 0.1% formic acid
Gradient:	0–0.25 min 100% A, 30.25 min 5% A, 32 min 5% A, 32.1–32.5 min 100% A
Flow rate:	400 µL/min
Injection volume:	5 µL

** Similar gradient conditions were used for the HPLC comparison at the same flow rate using a C_{18} Symmetry® 100 x 2.1 mm id, 3.5 µm column.*

Sample details: Rat bile sample dosed with Midazolam at a concentration 10 mg/kg. The sample was diluted 1:10 prior to sample analysis.

RESULTS

- Figure 2 shows the *in vivo* metabolism of Midazolam, "an anticonvulsant", in rat liver bile. This is one of the most challenging separations we face due to the high concentration of bile salts and endogenous compounds present.
- As can be seen, the peak capacity is greatly improved. For identification purposes this is a tremendous leap forward because this means that we spend less time ruling out false positives.

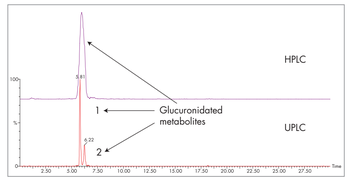

Figure 3. Shows a comparison between HPLC and the UPLC approach for an extracted ion chromatogram for the glucuronide conjugation metabolite of Midazolam at m/z 548.

- Having a high peak capacity enables us to obtain better separations with complex matrices and limit the amount of co-eluting peaks. We can resolve more peaks in the same run time. Also, note the difference in system backpressures for HPLC (1,500 psi) and UPLC (7,000 psi), allowing us to work in a separations space that has not previously been attainable.
- If we observe the extracted ion chromatograms (Figure 3) for the glucuronide conjugated di-oxidised, methylated metabolites of Midazolam we can see that with HPLC just one peak is detected, however by employing UPLC we see that there are in fact two separate conjugated metabolites.
- Without UPLC this information would be erroneous even if we employ MS/MS or MS^n.
- We have seen data showing the 30 minute gradient with both the HPLC and UPLC systems. What if we go for an even shorter gradient such as 6 minutes? (Figure 4).
- Even with the faster gradient we can see that we can still separate the two metabolites (Figure 5).
- Although this is not as good as the longer gradient, it is still producing significantly increased data quality compared with the HPLC system, because we not only gaining in chromatographic resolution but also speed of analysis and sensitivity.
- For *in vivo* samples a 30 minutes run time will yield a much higher peak capacity separating co-eluting metabolites, and endogenous compounds, generating the best qualitative data possible.
- If we now examine the spectra of these two peaks corresponding to HPLC and UPLC (Figure 6) we can see that for UPLC 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.
- In this case we have a point of reference, which is the isotopic signature of chlorine in Midazolam and we can use this to eliminate false positives. But, this is not the case for all compounds because we may not have an isotopic descriptor to detect and identify putative metabolites.

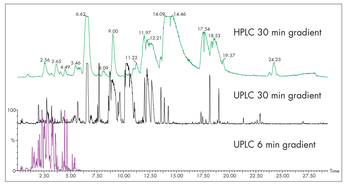

Figure 4. Shows a comparison between HPLC and UPLC with different gradient times.

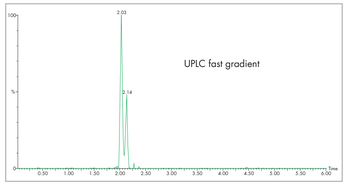

Figure 5. Shows the extracted ion chromatogram corresponding to the conjugated metabolites of Midazolam for the fast 6 minutes gradient using UPLC/QTof micro.

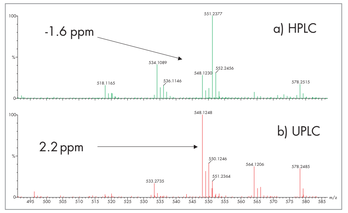

Figure 6. Spectra for glucuronidated metabolite using HPLC (a) and UPLC (b).

CONCLUSION

From the data presented the advantages of using this novel UPLC-TOF-MS approach for metabolism studies are obvious. As technology progresses, we are obtaining better levels of detection not only via a more sensitive assay, but through better quality data. Using UPLC-TOF-MS adds a new dimension to metabolism studies enabling us to obtain better detection limits, better throughput and increased chromatographic resolution, which in turn will improve the data quality from the mass spectrometer. It is a major leap forward, not just for this application but many others in both qualitative and quantitative bioanalysis.

Finally, we all worry about how fast we can run samples and obtain good data and, too often, the bottleneck is merely shifted to the data processing step, which takes a considerable amount of time sieving 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. In order to show this more work will follow along these lines. In summary, this novel approach is a platform on which we will be able to improve data quality and increase the knowledge of our samples.

REFERENCES

1. Anton D. Jerkovich, J. Scott Mellors, and James W. Jorgeson, The use of Micrometer-Sized Particles in Ultrahigh Pressure Liquid Chromatography, LCGC North America Volume 21, Number 7 (2003).
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