THE APPLICATION OF SUB 2 µM POROUS CHROMATOGRAPHIC PARTICLES WITH OPERATING PRESSURES UP TO 15,000 PSI FOR ANALYSIS OF PHARMACEUTICAL COMPOUNDS IN BIOLOGICAL FLUIDS

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ABSTRACT

A new novel organo-silica hybrid 1.7 µm porous particle stationary phase has been developed for liquid chromatography. This material has been combined with a new liquid chromatography system with a 100 µL delay volume capable of operating at pressures up to 15,000 psi with enhanced diagnostics. The combination of these technologies has allowed a new region of chromatographic space to be exploited. With this new technology chromatographic resolution is improved by a factor of 1.7–3, increased sensitivity by a factor of 3–8 and throughput increased by a factor of 5–10. This technology, Ultra Performance Liquid Chromatography (UPLC™), has been combined with mass spectrometry for the analysis of biological fluids.

INTRODUCTION

One of the major challenges that face the scientific community in the 21st century is the identification and understanding of the interaction between human genetics, and risks of developing major diseases and the treatment of these diseases. This is a fundamental biological problem; predicting drug efficacy and idiosyncratic toxicity from human genome data is a major challenge. In order to resolve these "big biology" problems we will need to gain a much greater understanding of the underlying molecular processes. This requires the development of ever more powerful analytical chemistry techniques and data interpretation tools. The heart of any analytical process is the quality of the raw data. Thus if we can generate data of a higher quality, it will contain more information, hence improving our knowledge of the biological system under investigation.

LC/MS and LC/MS/MS have become the analytical tool of choice for proteomics, quantitative and qualitative bioanalysis and now the emerging science of metabonomics. Over the last 10 years, most of the advances in LC/MS have been focused on performance improvements in mass spectrometry, such as Q-Tof, FTMS and 2D ion trap technology, whilst the field of separation science has remained mainly unchanged. The advantages of using sub 2 µm particle sizes had been well defined by the early pioneers of chromatography. Chromatographic efficiency N is inversely proportional to particle d_p and the Parnel equation shows that resolution Rs is proportional to the square root of N, thus reducing d_p by a factor of 3 (5 µm → 1.7µm) give rise to a 1.7 fold increase in resolution. As peak width w is direct proportional to efficiency reducing the d_p by a factor of 3 will result in a 1.7 reduction in P_w and hence a increase in peak height P_h of a similar magnitude (as $P_h \propto 1/P_w$) and hence a 1.7 fold increase in sensitivity. As we can see from the flat nature of the van Deemter plot generated by these sub 2 µm phase it is also possible to operate these materials at significantly higher mobile phase linear velocities without compromising the column efficiency, Figure 1. Thus we can significantly increase the analytical throughput whilst maintaining the enhanced performance.

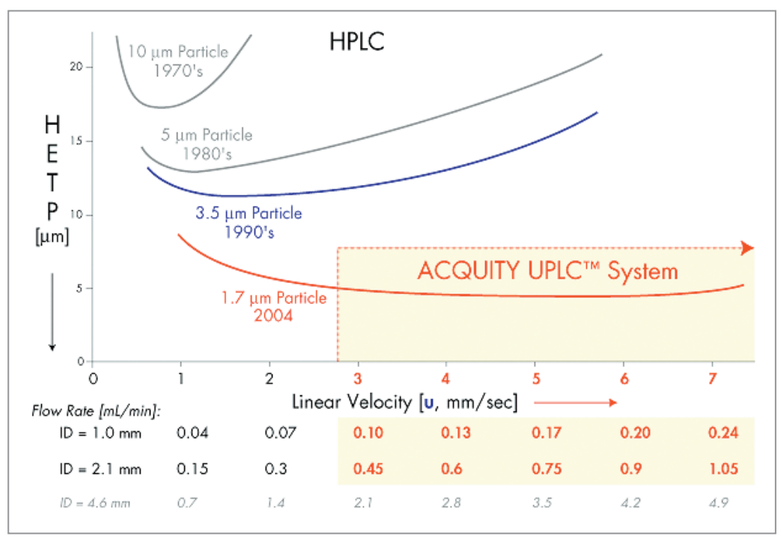


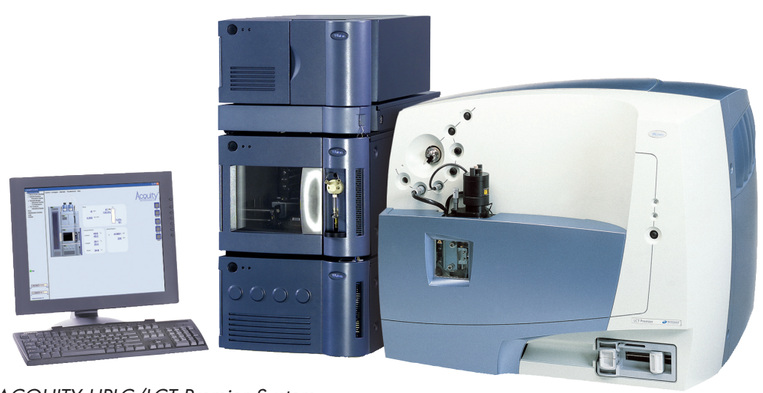
Figure 1. Van Deemter plot.

The column back pressure generated by these 1.7 µm stationary phases is inversely proportional to d_p^2 also the optimal velocity is inversely proportional d_p. Thus reducing d_p by a factor of 3 results in a 27 fold increase in back pressure. The instrument engineering required to cope with these increased back pressures generated by these small particles limited the exploitation of this technique being confined to academia [1].

In this paper we describe how UPLC has been applied to the area of drug metabolism and metabonomics. We will show how UPLC improve the chromatographic resolution, results in superior spectral information, increased sensitivity, greater throughput and increased biomarker identification.

EXPERIMENTAL

Mass spectrometry:	Waters® Micromass® LCT Premier™ or Q-Tof micro™
Ion mode:	ESi Positive ion
Data range:	50–850m/z, W optics DRE
Acquisition rate:	100 ms, inter-scan delay 100 ms
Lock-Spray:	Leucine enkephalin 25 pmol/µL
Cone:	60 V
Capillary:	3 kV
Desolvation temp:	250 °C
Source temp:	120 °C
Chromatography :	ACQUITY UPLC™
Column:	2.1 x 100 mm ACQUITY UPLC™ BEH C_{18} 1.7µm
Gradient:	A= 0.1% formic acid, B= acetonitrile, 0.1% formic acid. Either 0–95%B in 10 minutes @ 600 µL/min or 0–95%B in 30 mins @ 400 µL/min
Temp:	40 °C



ACQUITY UPLC/LCT Premier System

RESULTS AND DISCUSSION

DRUG METABOLISM

The rapid identification of drug metabolites in both discovery and development is a key part of bringing a new pharmaceutical compound to market. In this scientific field LC combined with tandem mass spectrometry or more recently hybrid quadrupole-TOF mass spectrometry is the technique of choice. The samples range in matrix complexity from the relatively clean in-vivo microsomal incubations to the complex matrices such as urine, plasma and bile. In these complex samples the numerous endogenous components often result in the ion suppression of the xenobiotics due to co-elution making detection and data interpretation difficult and time consuming. The extra resolution provided by UPLC significantly improved the data quality. The data in Figure 2 compares the LC/MS (TOF) and UPLC/MS (TOF) chromatograms of a rat bile sample following the oral administration of the anti-convulsant drug Midazolam. The HPLC was performed on a 2.1 x 150 mm 3.5 µm C_{18} column, eluted with a 0–95%B gradient over 30 mins at 250 µL/min; the UPLC separation was performed on a 2.1 x 100 mm 1.7 µm C_{18} ACQUITY UPLC™ column with the same gradient steepness at a flow rate of 400 µL/min. We can clearly seen from the TIC trace that UPLC results in sharper peaks, with a typical peak width of 5–6 seconds at the base, and hence more information. This extra clarity allows the scientist to more easily interpret the data and look for potential drug metabolite peaks. The UPLC system generated a back pressure of 7000psi.

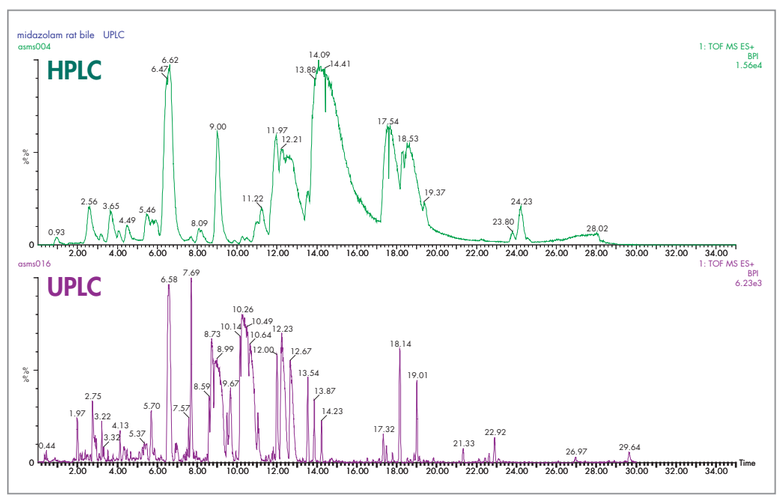


Figure 2. LC/MS (TOF) and UPLC/MS (TOF) chromatograms of a rat bile sample following the oral administration of Midazolam.

Two of the major metabolic transformations of Midazolam are hydroxylation followed by glucuronic acid conjugation. In this study two further drug related peaks were detected with a m/z= 548 corresponding to the double hydroxylation, O-methylation and conjugation with glucuronic acid of Midazolam. The XIC m/z= 548 of the HPLC/MS and UPLC/MS is given in Figure 3, here we can clearly see that in the HPLC chromatogram the two metabolites are unresolved whereas in the UPLC trace they are clearly separated. The extracted spectra of these UPLC and HPLC peaks are given in Figure 4, the resulting spectra demonstrates the advantage of UPLC. In the LC/MS trace the 548 ion is obscured in the background noise due to co-elution spectral overlap, whereas the UPLC spectra is much cleaner with the 548.1226 ion being dominant with a mass error of 1.2 ppm. There is also a significant increase in the intensity of the 548 ion in the UPLC spectra compared to that in the HPLC spectra, thus showing the increased sensitivity gained by employing UPLC.

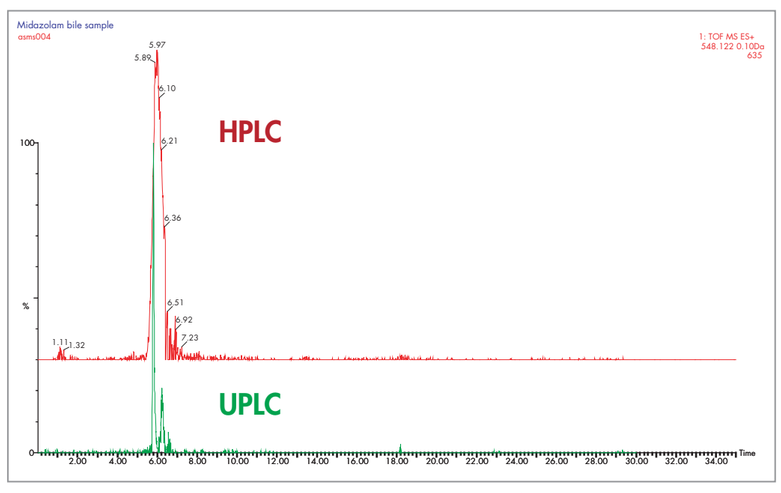


Figure 3. Extracted ion chromatograms (XIC m/z = 548) for the HPLC/MS and UPLC/MS analysis of rat bile following the oral administration of Midazolam.

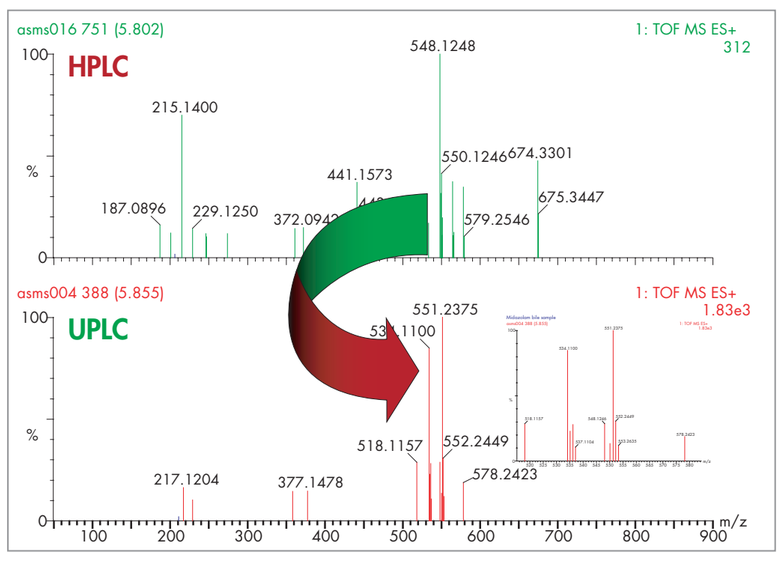


Figure 4. Mass spectra of HPLC/MS and UPLC/MS peaks shown in Figure 3.

The UPLC separation time was reduced to just 6 minutes by employing a flow rate of 750 µL/min, resulting in a back pressure of >13,000psi. The XIC m/z= 548 and extracted spectra for this separation is given in Figure 5. Here we can see that again the two glucuronides are clearly separated and that the 548 ion is prominent in the extracted spectrum.

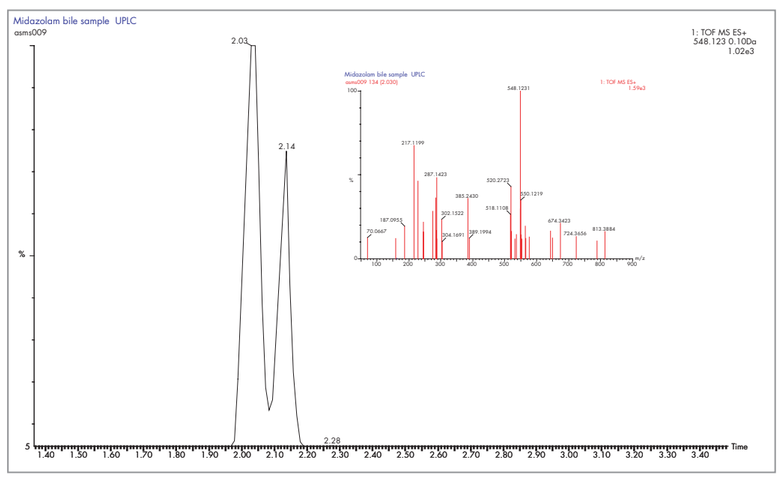


Figure 5. Extracted ion chromatogram for 6 minute UPLC separation.

In order to cope with the high back pressures generated in UPLC, up to 15,000 psi, it was necessary to develop a completely new stationary phase that was mechanically robust enough to with stand the stresses generated by these high pressures. This was achieved by extending the organic-silica hybrid particle technology to create a polyethoxysilane bridged hybrid, Figure 6. This material has not only great mechanical strength but is stable over a wide pH range 2–12, due to the incorporation of the ethane-silica bridge.

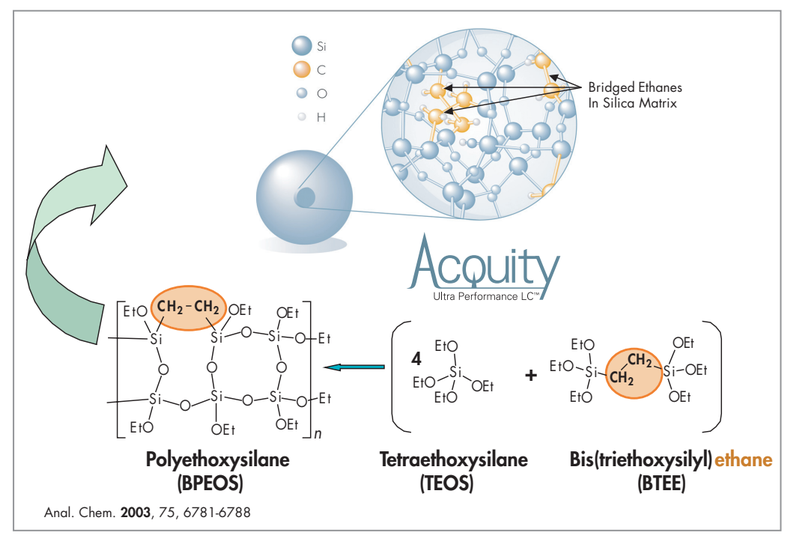


Figure 6. ACQUITY UPLC™ patented (Patent number 6,686,035 B2) hybrid particle technology.

METABONOMICS

In contrast to metabolite ID, where the endogenous components are merely interference to be removed allowing the xenobiotics to be characterized, in metabonomics it is the changes in these endogenous metabolites hold the key to identifying biomarkers of disease, efficacy or toxicity. Therefore increasing our understanding of the underlying metabolic pathway and hence the molecular biology, behind a disease state or toxic event. Thus the goal in metabonomics is to detect as many of the endogenous components as possible. As we have seen from the metabolite ID data the UPLC separation produces sharper LC peaks with reduced ion suppression and increased sensitivity allowing the detection of low concentration metabolites.

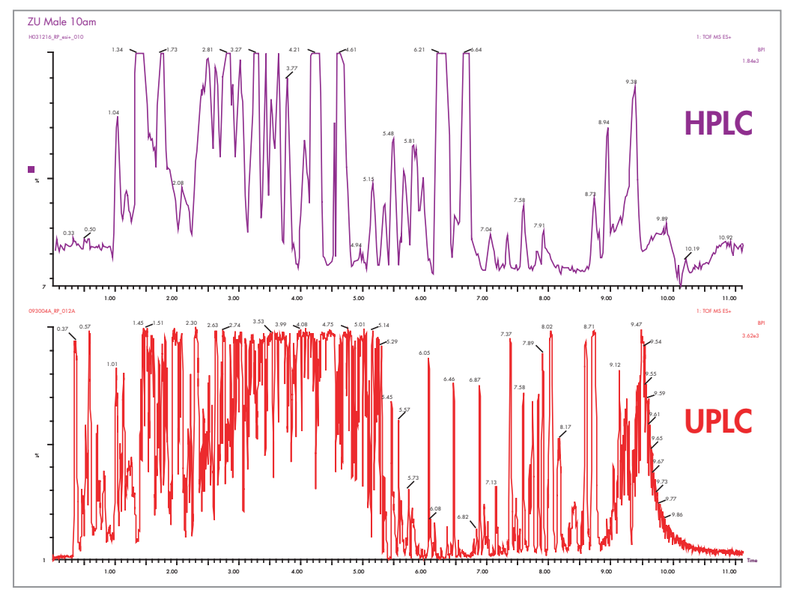


Figure 7. HPLC/MS-TOF and UPLC/MS-ToF chromatograms from the analysis of urine from Zucker ++ rats.

The data in Figure 7, show the chromatograms obtained from the HPLC/MS-TOF and UPLC/MS-TOF analysis of urine from Zucker ++ rats age week 18. The HPLC separation was performed on a 2.1 mm x 10 cm 3.5 µm column eluted with a 0–95%B linear gradient over 10 minutes at a flow rate of 500 µL/min, the UPLC separation was performed on a 2.1 mm x 10 cm 1.7 µm ACQUITY UPLC column eluted with a 0–95%B linear gradient over 8 minutes at a flow rate of 750 µL/min. It is clear from this data that the UPLC/MS-TOF gives significantly more information than the HPLC/MS-TOF. With the UPLC peaks having a width at the base (5%) in the region of 2 seconds, giving an average peak capacity of approximately 250 for an 8 minute separation. A total of 190 Zucker rat urine samples were processed by UPLC/MS-TOF, samples were taken on weeks 4–20 from -/-, +/- and +/+ animals. After peak integration and noise reduction using the MarkerLynx™ application manager the data was processed by Principal Components Analysis (using SIMCA, Umetrics, Sweden), the resulting multivariate analysis for the male pm animals is given in Figure 8. Here we can see clear differentiation between the -/-, +/- and +/+ samples as well as laboratory control male animals on week 20 (8a). The data in Figure 8b shows the trajectory of the Zucker +/+ animals over a 16 week period. It is very interesting to note how their position in metabolic space rapidly changes between weeks 12 and 14.

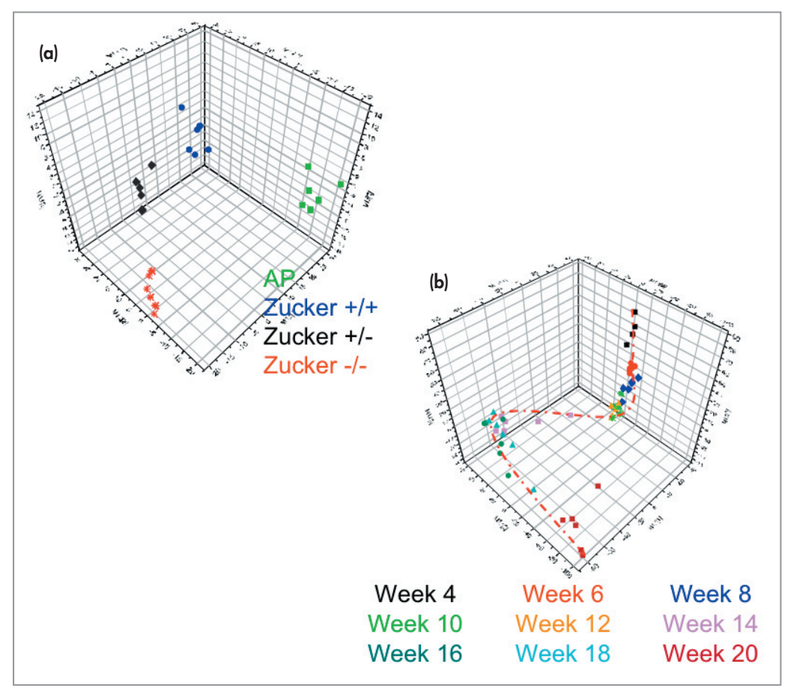


Figure 8. Multivariate analysis for the male pm animals.

CONCLUSION

UPLC is a major step forward in the science of chromatography generating much narrower peaks, improving resolution, sensitivity and throughput. Then combined with MS, UPLC facilitates:

- The detection of more peaks, as a result of enhanced resolution.
- Reduced spectral overlap, and hence superior MS data.
- Increased sensitivity, due to sharper peaks and the reduction in ion suppression.
- More throughput as a result of a wide chromatographic "sweet-spot."

In summary, UPLC is the ideal chromatographic interface for mass spectrometry.

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