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Ultra Performance Liquid Chromatography (UPLC™) provides increased chromatographic resolution for improved detection of metabolic transformations.

High performance liquid chromatography (HPLC) and mass spectrometry (MS) have been used in bioanalytical laboratories for nearly 15 years. The use of these technologies to study metabolic transformations is critical for characterizing a drug's route of metabolism and identifying possible sites of toxicity. Low levels of metabolites, complex matrices such as bile, and metabolites with similar structures often complicate these assays. Despite early claims that MS, and particularly MS-MS, removed the need for good chromatography in these analyses, a poor LC assay will lead to issues of ion suppression and isobaric interferences that cannot be overcome by a mass spectrometer alone.

In this study, a novel approach to drug metabolism was taken using Ultra Performance Liquid Chromatography (UPLC™) coupled to a hybrid quadrupole orthogonal time of flight (Q-ToF™) mass spectrometer. UPLC is based on the theories and principles of HPLC, and takes advantage the relationship between linear velocity and plate height (column efficiency) defined by the Van Deemter equation. The use of sub-2- μm particles allows UPLC to push the limits of both peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities). With UPLC, sensitivity is also improved because chromatographic bands are more concentrated and elute as sharper peaks.

Experimental Conditions

Sample Preparation

A bile sample from a rat dosed with midazolam at a concentration of 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_{18} column, 2.1 \times 100 mm, 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, 5.25 min 5% A, 32 min 5% A, 32.1–32.5 min 100% A (curve 6)

Flow Rate: 400 $\mu\text{L}/\text{min}$

Injection Volume: 5 μL

*Similar gradient conditions were used for the HPLC comparison at the same flow rate using a Waters Symmetry® C_{18} column, 2.1 \times 100 mm, 3.5 μm particle size).

Mass Spectrometry Conditions

Mass Spectrometer: Waters Micromass® Q-ToF micro™

Ionization 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

Results and Discussion

Figure 1 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 presence of endogenous compounds. The peak

Maximizing Chromatographic Resolution of Metabolites Using Ultra Performance Liquid Chromatography (UPLC™)

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capacity in the UPLC separation compared to the HPLC analysis is greatly improved, resulting in greater differentiation among peaks. The increased peak capacity minimizes ion suppression resulting from the coelution of metabolites with bile salts or endogenous compounds.

The extracted ion chromatograms (Figure 2) for the glucuronide metabolites (m/z 548) of midazolam show that, with HPLC, just one peak is detected. By employing UPLC, it is evident that there are actually two separate glucuronides. Without UPLC, one of the glucuronides would have been missed even if MS-MS or MSⁿ 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.

Conclusions

When combined with exact mass Q-ToF MS, UPLC offers the chromatographic resolution necessary to differentiate and identify potential drug metabolites with greater confidence.

Acknowledgments

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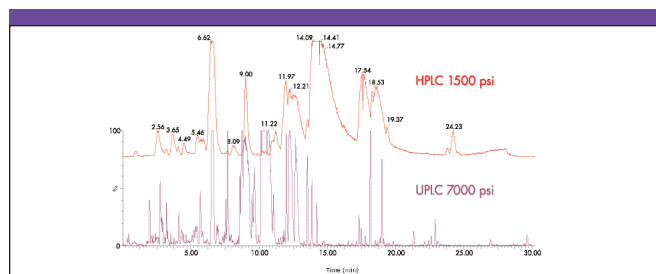


Figure 1: Comparison of HPLC/Q-ToF and UPLC/Q-ToF analysis for a bile sample collected from a rat dosed with midazolam.

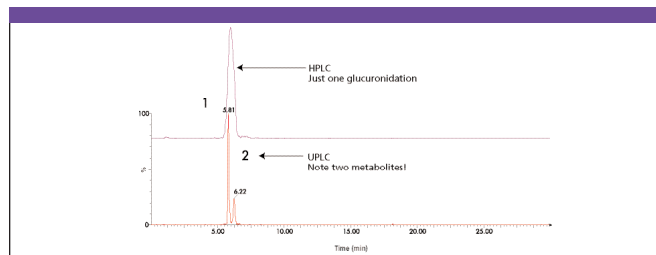


Figure 2: HPLC and UPLC extracted ion chromatograms for two glucuronidated di-oxidised-methylated metabolites of midazolam that have the same m/z values.

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