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A Fast Screening Method for Metabolite ID With ACQUITY UPLC, Quattro Premier XE, and MetaboLynx

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Abstract

The use of the UPLC-MS/MS provides a very fast and effective approach to metabolite identification in discovery laboratories. Coupling the groundbreaking performance advantages ACQUITY UPLC System with a fast-scanning instrument such as the Quattro Premier XE Mass Spectrometer delivers excellent levels of detection and speed of analysis. Automated processing of the large data sets typically generated by these studies with the MetaboLynx Application Manager further increases productivity and ensures easy detection of even low-level metabolites

Introduction

In vitro metabolism studies have become an integral part of the drug discovery and development process. The information from these studies is often used to make critical decisions about the fate of a compound early in the

discovery process (*e.g.*, allowing the medicinal chemist to successfully modify a compound for improved pharmacokinetic parameters). Rapid turnaround of results is essential, and for this reason HPLC coupled to mass spectrometry has evolved as the analytical platform of choice in most DMPK labs. However, since the advent of the Waters ACQUITY UltraPerformance LC (UPLC) System, a new dimension in speed of analysis, chromatographic resolution, and sensitivity is possible to outpace and outperform HPLC methodologies.

UPLC is based on 1.7 µm particle size column innovations. Since chromatographic theory states that increasing column efficiency increases resolution, reducing the stationary phase particle size has been the standard approach to achieving this goal for the last 40 years. The use of sub-2 µm particles require systems to run at pressures well above the current limits of 400 bar (6,000 psi). Therefore, it is necessary to have a solvent delivery system that can sustain this elevated pressures and possess a much smaller system volume that will not compromise gradient performance. The ACQUITY UPLC System has been designed with these critical features in mind.

The chromatographic peaks eluting from UPLC may only be a few seconds wide, so the detector must be capable of acquiring data very rapidly. The Quattro Premier XE Mass Spectrometer is designed for rapid rates of acquisition (5,000 amu/sec scan speeds), generating large amounts of data in short timeframes. This results in a need to process the data with novel software that automates interpretation.

The MetaboLynx Application Manager for MassLynx Software detects putative biotransformations for expected and unexpected metabolites. MetaboLynx automatically runs UPLC-MS/MS sample sets and processes the resulting data. The samples may be run singly or in a batch mode. Results are reported via a Data Browser view that enables a rapid review of both the chromatographic and mass spectroscopic data for each automated metabolic assignment.

MetaboLynx operates by comparing and contrasting each metabolized sample with a control sample, though metabolite searching may still be performed in the absence of a suitable control. Samples from *in vitro* incubations or *in vivo* dosing experiments can be quickly analyzed by UPLC-MS/MS, followed by a multi-dimensional data search which correlates retention time, *m/z* value, intensity, and components from alternative detection technologies (*e.g.*, photodiode array UV or radiochemical detectors). Comparison of analyte data with the control sample data allows for filtering of matrix-related peaks, which would otherwise produce an unmanageable list of false metabolite peaks. For this study, rat microsomal incubation samples using a series of

substrates at 20 µM levels were analyzed.

Waters ACQUITY UPLC System with the Quattro Premier XE Mass Spectrometer.

Experimental

The purpose of this study is to demonstrate the utility of the ACQUITY UPLC with the Quattro Premier XE and MetaboLynx for rapid identification of metabolites generated by *in vitro* studies in drug discovery.

Sample Preparation

In vitro metabolism: Propanolol and Diazepam were incubated using rat liver microsomes at 20 µM levels. The incubation period was 60 minutes at 37 °C, in a solution of 50 mMol Potassium Phosphate (adjusted to pH 7.4 with NADPH). The reaction was then terminated with 2 volumes of cold acetonitrile to 1 volume of sample. The resulting samples were stored frozen at -80 °C prior to UPLC-MS analysis.

UPLC Conditions

LC system:	ACQUITY UPLC System
System column:	ACQUITY UPLC BEH C_{18} 2.1 x 100 mm, 1.7 µm
Mobile phase:	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow rate:	0.6 mL/min

Gradient Table

Time (min)	Profile		Curve
	%A	%B	
Initial	100	0	1
2.00	20	80	6
3.00	20	80	6
3.10	100	0	6

MS Conditions

MS system:	Quattro Premier XE Mass Spectrometer
Ionization mode:	Electrospray, positive ion

Capillary voltage:	3 kV
Cone voltage:	35 V
Source temp:	120 °C
Desolvation temp:	320 °C
Acquisition range:	150–400 amu
Total scan time:	0.16 sec

Results and Discussion

Figure 1 illustrates the speed of analysis and chromatographic resolution that can be achieved with UPLC-MS/MS, providing dramatic increases in sample throughput.

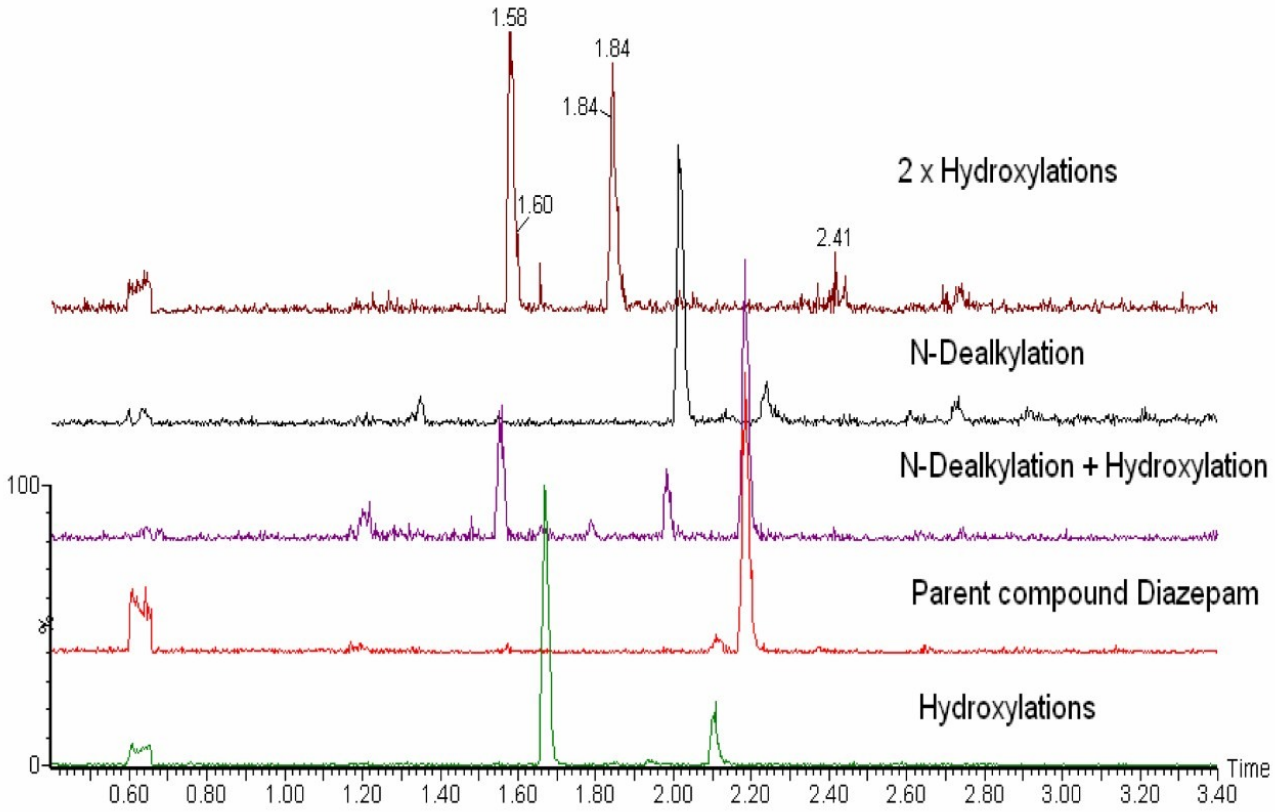

Figure 1. Extracted ion chromatograms for the microsomal incubation of diazepam and its metabolites analyzed by UPLC-MS/MS.

Frequently, the same biotransformation, such as hydroxylation, will occur at a number of different sites on the same candidate molecule. Some of these isobaric metabolites will co-elute and can create a problem when acquiring MS/MS data to identify the site of biotransformation. In order to resolve this situation, the chromatographic method must be re-optimized to achieve separation of the isobaric co-eluting metabolites. However, as developing uniquechromatographic methods for each compound isunfeasible at this stage of development, generic methods are ideal for the screening process. The strengths of fast generic gradients with UPLC are clearly illustrated in the method described in this work. We were able to achieve a rapid, robust separation of the isobaric hydroxyl metabolites of propanolol (Figure 2).

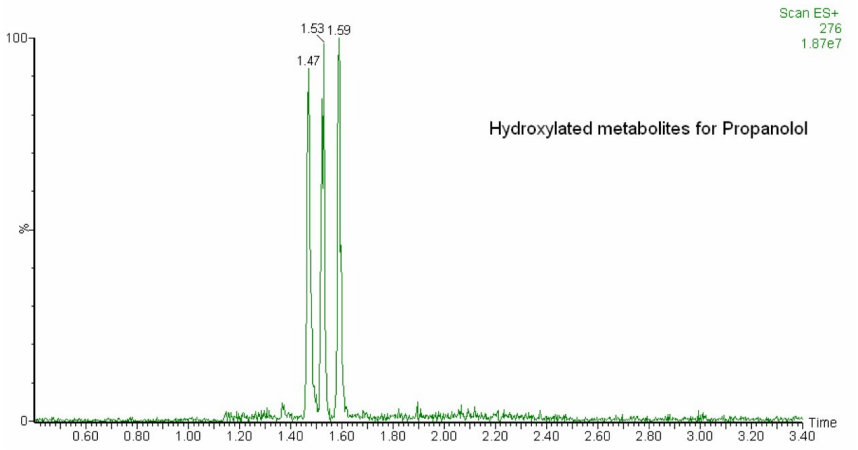

Figure 2. The separation obtained by UPLC-MS/MS for the hydroxlylated metabolites of propanolol

One of the biggest bottlenecks in metabolism studies is the data processing step. A great deal of time is required to mine data and eliminate false positives. The new features of MetaboLynx enable users to filter the data by the use of peak area thresholds and retention time windows after the data has been processed (Figure 3). These filters can be switched on and off, ensuring that the original data is retained. As a consequence, the need to generate multiple processing methods is eliminated, simplifying the entire metabolite identification process.

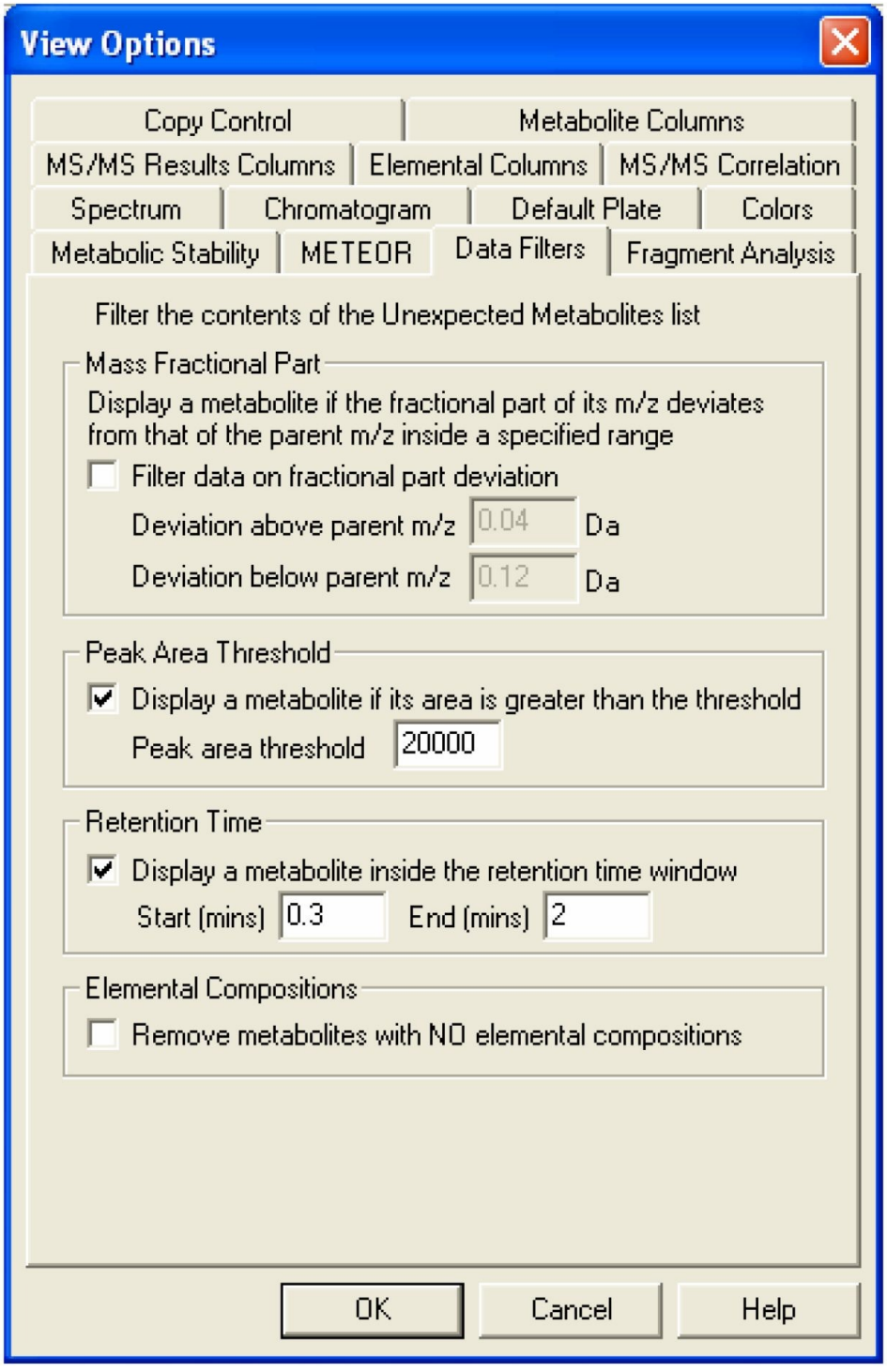

Figure 3. Data filters used for the elimination of false positives in MetaboLynx.

Figure 4 shows the MetaboLynx data browser with all the automatically detected metabolites of diazepam. The browser provides easy access to all information obtained from this experiment. A very useful feature is the chromatogram that shows all detected metabolites. The information generated by this single combined metabolic trace is linked to the spectra and extracted ion chromatograms obtained for each metabolite and the parent compound in the expected and unexpected tables. This powerful browser feature allows the user to quickly verify the results of the automated analysis and generate a complete metabolism report.

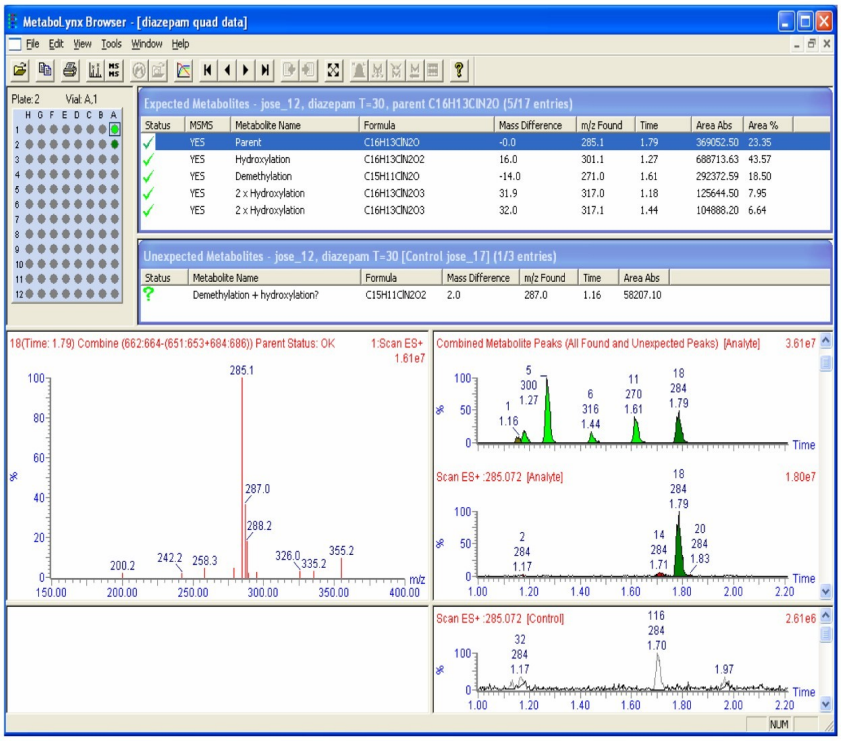

Figure 4. The MetaboLynx data browser for Diazepam and its metabolites in vitro.

Conclusion

The use of the UPLC-MS/MS provides a very fast and effective approach to metabolite identification in discovery laboratories. Coupling the groundbreaking performance advantages ACQUITY UPLC System with a fast-

scanning instrument such as the Quattro Premier XE Mass Spectrometer delivers excellent levels of detection and speed of analysis. Automated processing of the large data sets typically generated by these studies with the MetaboLynx Application Manager further increases productivity and ensures easy detection of even low-level metabolites.

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720001599, April 2006