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Improving Drug Metabolite Identification in Biofluids with the ACQUITY Premier and Hybrid Organic Surface Technology: Increased Sensitivity and Reproducibility

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Abstract

Liquid chromatography coupled with electrospray tandem mass spectrometry is the primary platform for drug metabolite identification both *in vivo* and *in vitro*. Key to successful metabolite identification is the chromatographic resolution of the drug related analytes, both from each other and from endogenous components present in the matrix. Transition metals present in chromatography systems and columns can act as Lewis Acids interacting with analytes containing phospho- groups, uncharged amines, hydroxyls, and deprotonated carboxylic acids resulting in poor chromatographic peak shape or even severe analyte loss. The ACQUITY Premier Chromatography System and columns employs a hybrid organic surface technology to eliminate this type of non-specific binding. The analysis of the *in vivo* metabolites of gefitinib using the ACQUITY Premier Chromatography System and columns showed improved peak shape, increased signal response and cleaner MS/MS spectra.

Benefits

- Improved chromatographic resolution of drug metabolites
- Increased peak response
- Improved reproducibility

Introduction

Metabolite identification plays a critical role in the discovery and development process, allowing the identification of metabolic "soft spots", detection of reactive metabolites and confirmation of the fate of candidate drugs *in vitro* and *in vivo* in animal models and humans. LC-MS/MS has become the technology of choice for metabolite identification, especially with accurate mass spectrometry (MS), due to the specificity, sensitivity and structural information produced by the technology. Advances in mass spectrometry such as accurate mass and ion mobility have increased the ability of the DMPK scientist to "clean up" and filter the MS signal, improving the quality of the data acquired.[1]

Drug molecules can undergo extensive metabolism resulting in multiple metabolites or may be dosed at low levels resulting in extremely low circulatory concentrations of drug related molecules. In order to obtain a comprehensive understanding of the metabolic fate of the drug, all the drug metabolites present in the sample need to be both detected and resolved such that a clean MS and MS/MS spectra are obtained for subsequent interpretation. Transition metals used in chromatography systems and columns can act as Lewis Acids, interacting with analytes containing phospho- groups, uncharged amines, hydroxyls, and deprotonated carboxylic acids; resulting in in poor chromatographic peak shape or even severe analyte loss.[2] The ACQUITY Premier Chromatography System and columns employ a hybrid organic polymer surface technology to reduce/eliminate these interactions. The MaxPeak High Performance Surfaces (HPS) LC technology was specifically developed to address these unwanted interactions by providing a highly effective surface barrier to alleviate them. This MaxPeak HPS LC surface is comprised of a highly crosslinked layer, chemically similar to bridged-ethyl hybrid (BEH) silica, which provides a resilient barrier between the analytes and the metal surface but does not participate in the separation.

Gefitinib is an EGFR inhibitor used for the treatment of certain breast, lung and other cancers.[3] Gefitinib has an empirical formula of $C_{22}H_{24}ClFN_4O$ with a molecular mass of 446.91 g·mol^{-1}, it undergoes hepatic metabolism via CYP 3A4 to give eight major metabolites of which the O-desmethyl is the most significant, Figure 1. The major route of elimination is the faeces with a small percentage eliminated in the urine. Previous pharmacokinetic studies of gefitinib have shown that it is necessary to use a mobile phase containing a buffer such as ammonium acetate to prevent peak tailing and provide complete resolution of the peaks of interest from each other with an acceptable analysis time.[3-5] The presence of buffers such as ammonium acetate can reduce ionization efficiency, especially in negative ion mode, reducing analyte response and increasing the possibility of not detecting low concentration metabolites. In this application note we demonstrate the benefit of using the ACQUITY Premier Chromatography System for the analysis of metabolites of gefitinib in mouse urine.

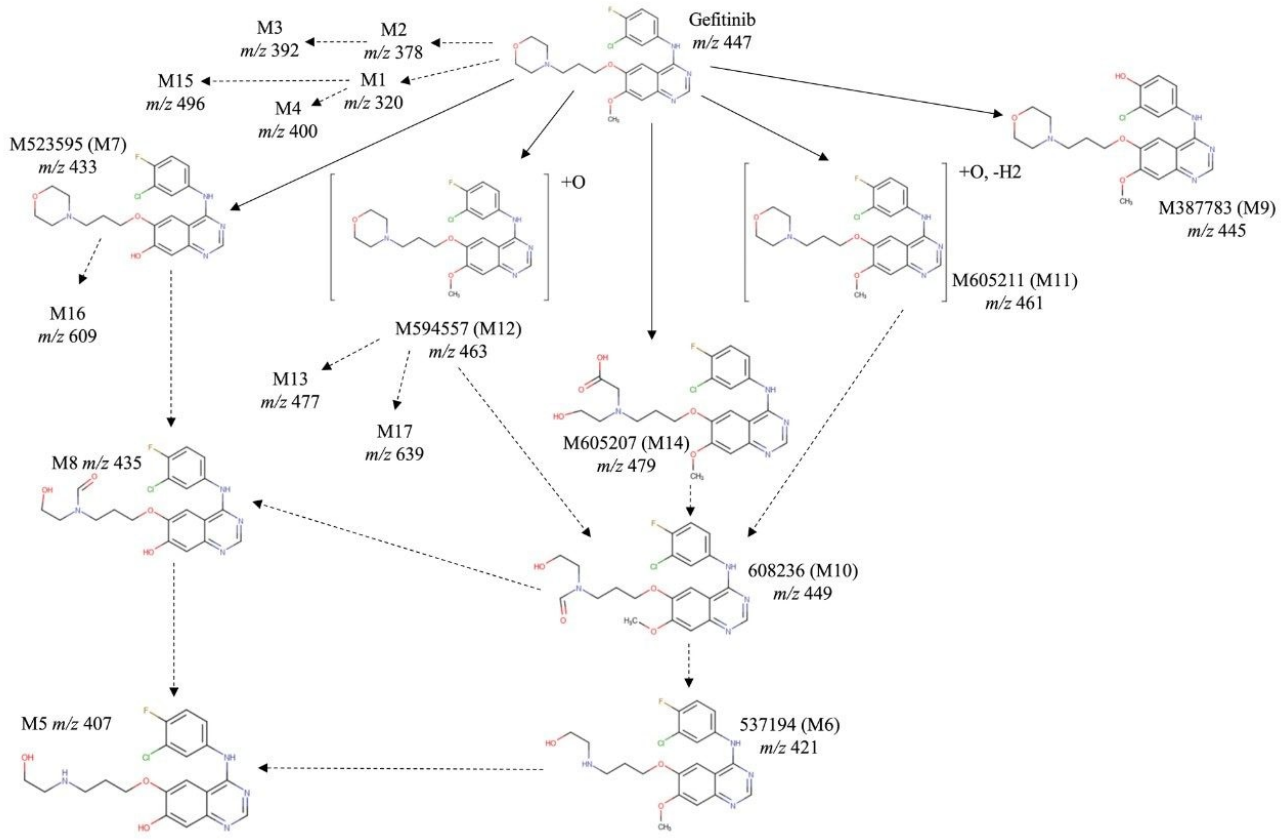


Figure 1. Schematic illustrating gefitinib and its associated metabolites.[2]

Experimental

Gefitinib and vehicle alone were dosed intravenously (IV) and orally (PO) to the mouse at 10 and 50 mg/Kg respectively. Urine samples were collected pre-dose, 0–3, 3–8, and 8–24hr post-dosing and stored on ice. Mouse urine samples were diluted 1:5 with water containing 0.1% (v/v) formic acid. The sample solution was vortex mixed and centrifuged at 25,000g for 5 mins. The resulting supernatant was removed and transferred to glass Max Recovery vials (600000670CV <https://www.waters.com/nextgen/in/en/shop/vials-containers--collection-plates/600000670cv-lcms-certified-clear-glass-12-x-32-mm-screw-neck-max-recovery-vi.html>) for analysis. The urine samples were analysed in triplicate by LC-MS^E.

LC Conditions

LC Conditions

LC system:	ACQUITY Premier or ACQUITY UPLC I-Class PLUS
Vials:	Waters Max Recovery
Column(s):	ACQUITY Premier HSS T3 2.1 x 100, 1.8 µm or ACQUITY UPLC HSS T3, 2.1 x 100, 1.8 µm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	3 µL
Flow rate:	600 µL/min
Mobile phase A:	0.1% Aqueous formic acid

Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	See below

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
0	0.6	99	1	Linear
1	0.6	99	1	Linear
3	0.6	85	15	Linear
6	0.6	50	50	Linear
9	0.6	5	95	Linear
10	0.6	5	95	Linear
10.1	0.6	99	1	Linear

MS Conditions

MS system:	SYNAPT XS
Ionization mode:	Electrospray (ESI) Positive Ion
Acquisition range:	*m/z* 50–1000
Capillary voltage:	2.8kV
Collision energy:	Linear CE ramp (19–45eV)

Cone voltage:	30V

Data Management

Chromatography software:	MassLynx v4.2
MS software:	MassLynx v4.2
Informatics:	Progenisis QI, Skyline

Results and Discussion

Biological fluids are, by their nature, complex mixtures containing analytes with a broad polarity range from highly polar small acids and base, amino acids to more lipophilic peptides and lipids. These analytes are also present over a wide dynamic range of concentrations from hormonal steroids and eicosanoids, which are present in trace quantities, to hippuric acid and taurine which are present at mMol concentrations. In order to accurately detect, identify, and quantify drug-related metabolites, it is critical that the chromatography system has the highest possible resolving power; allowing the drug metabolites to be separated, both each other and from the endogenous components in the sample.

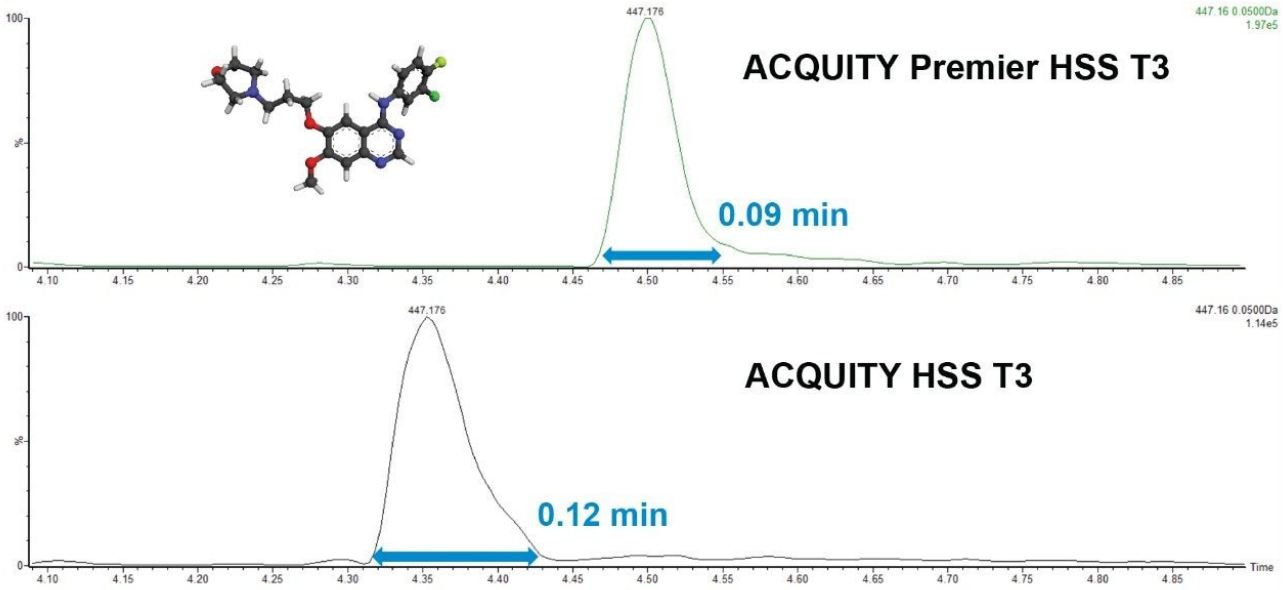


Figure 2. Chromatographic comparison between the ACQUITY Premier HSS T3 and ACQUITY HSS T3 columns for gefitinib. Improved peak width at base (Wb) is demonstrated for the ACQUITY Premier HSS T3.

The data displayed in Figure 2 compares the chromatographic performance of the ACQUITY Premier HSS T3 C_{18} Column with that of a standard ACQUITY column for the dosed compound gefitinib. The measured peak width at the base was determined to be 0.12 minutes (n=3) for the conventional ACQUITY HSS T3 C_{18} Column and 0.09 minutes (n=3) for the ACQUITY Premier HSS T3 C_{18} Column. The resulting chromatographic peak capacity was determined to be 111 and 83 for the ACQUITY Premier and conventional ACQUITY columns respectively.

This increased chromatographic performance obtained from the ACQUITY Premier Column resulted in a 72% increase in peak intensity from 1.14 e^5 (conventional column) to 1.97 e^5 (ACQUITY Premier Column). This improvement was also observed for the metabolite M523595, Figure 3, where the observed peak intensity was increased by a factor of two from 30 e^3 (conventional column) to 60 e^3 (ACQUITY Premier Column). The increase in peak response could result from the narrower peak shape observed with the ACQUITY Premier Columns or as a result of reduced analyte absorption to the exposed metal surfaces of the chromatography system. This improved metabolite response is critical for the detection of low concentration metabolites, especially for in low dosed cohorts.

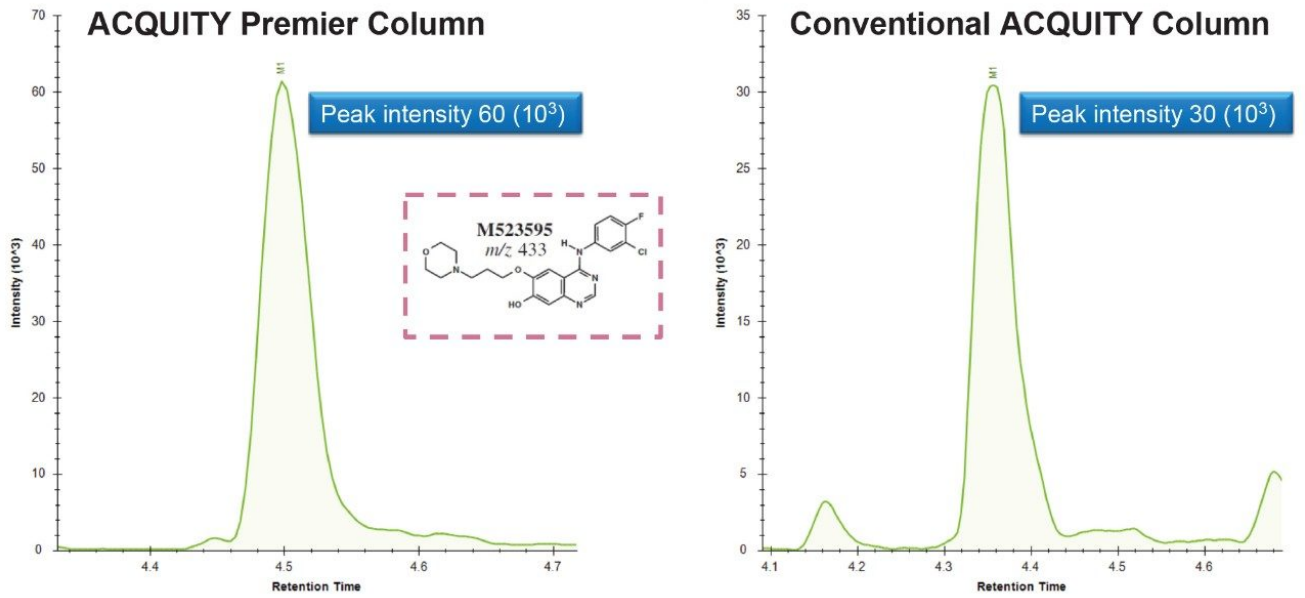
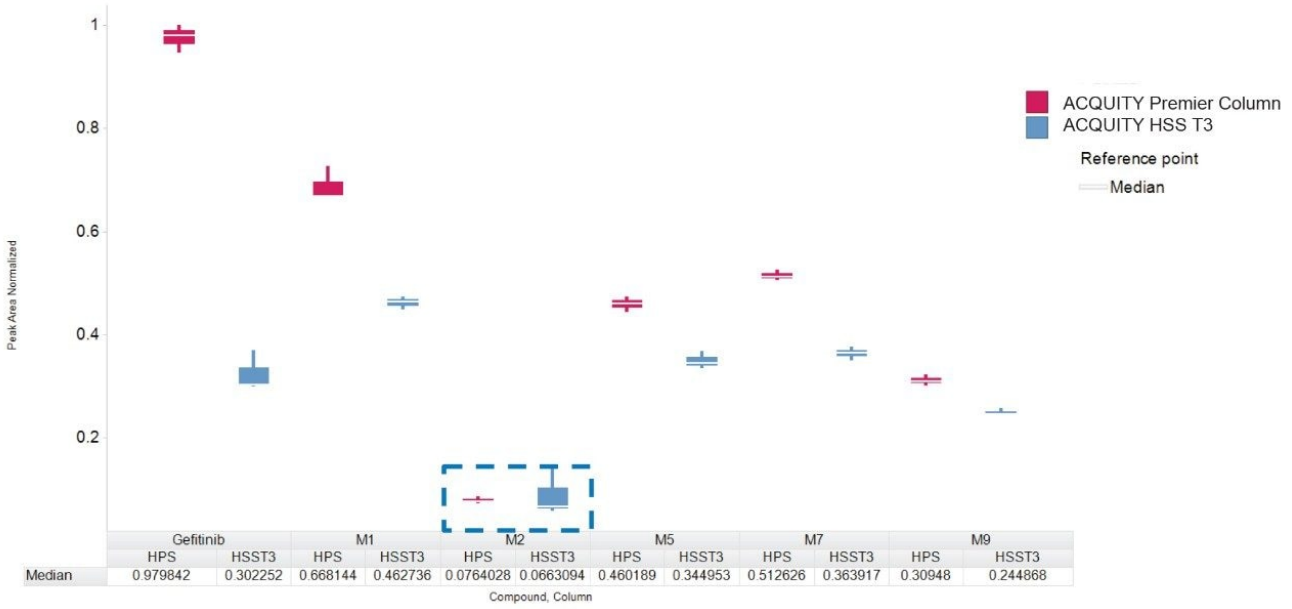


Figure 3. Comparison of peak intensity between the ACQUITY Premier HSS T3 and ACQUITY HSS T3 columns for the M1 gefitinib metabolite (M523595).

Understanding the degree of exposure to a metabolite can as important as determining its structure. Therefore, it is important to be able to reliably determine the concentrations of the drug metabolites, whether using an authentic standard, radiolabelled isotope or by comparing to the response to the parent compound response, especially at the terminal phase of the pharmacokinetic profile where the concentration is lowest. The increased peak response and peak shape observed, not only allows for superior peak intensity but greater reproducibility, which is illustrated by the results displayed in Figure 4. The data shows the metabolite peak response normalized against the gefitinib peak response. As can be seen from these results, the ACQUITY Premier Column delivers an increased peak response for each metabolite, while also showing lower variation in peak response for replicate injections of metabolites present at lower concentrations when the ACQUITY Premier Column is used. This is illustrated by the low concentration metabolite M2 (M605211), where the increased peak response ranges from 17–19 e^3 on the ACQUITY Premier Column compared to 12–14 e^3 on the conventional ACQUITY column.



	Gefitinib		M1		M2		M5		M7		M9	
	HPS	HSST3	HPS	HSST3	HPS	HSST3	HPS	HSST3	HPS	HSST3	HPS	HSST3
Median	0.979842	0.302252	0.668144	0.462736	0.0764028	0.0663094	0.460189	0.344953	0.512626	0.363917	0.30948	0.244868

Figure 4. Peak area response across replicates (n=3) for gefitinib and associated metabolites. Data corresponding with the ACQUITY Premier HSS T3 (red) and ACQUITY HSS T3 (blue) are shown.

The acquisition of high quality MS and MS/MS spectra is especially important for rapid and confident metabolite identification and structure elucidation. Fundamental to metabolite detection and identification is the chromatographic resolution of the analyte from other drug related components and interfering/co-eluting endogenous components in the sample. The extra chromatographic performance provided by the ACQUITY Premier System, narrower peaks, reduced peak tailing, allowing for greater peak intensity combined with enhanced resolution hence simpler characterization of metabolites. This is illustrated in the data displayed in Figure 5, representing the analysis of metabolite M7 (hydroxylation of gefitinib), as can be seen the peak intensity is significantly increased with the ACQUITY Premier System.

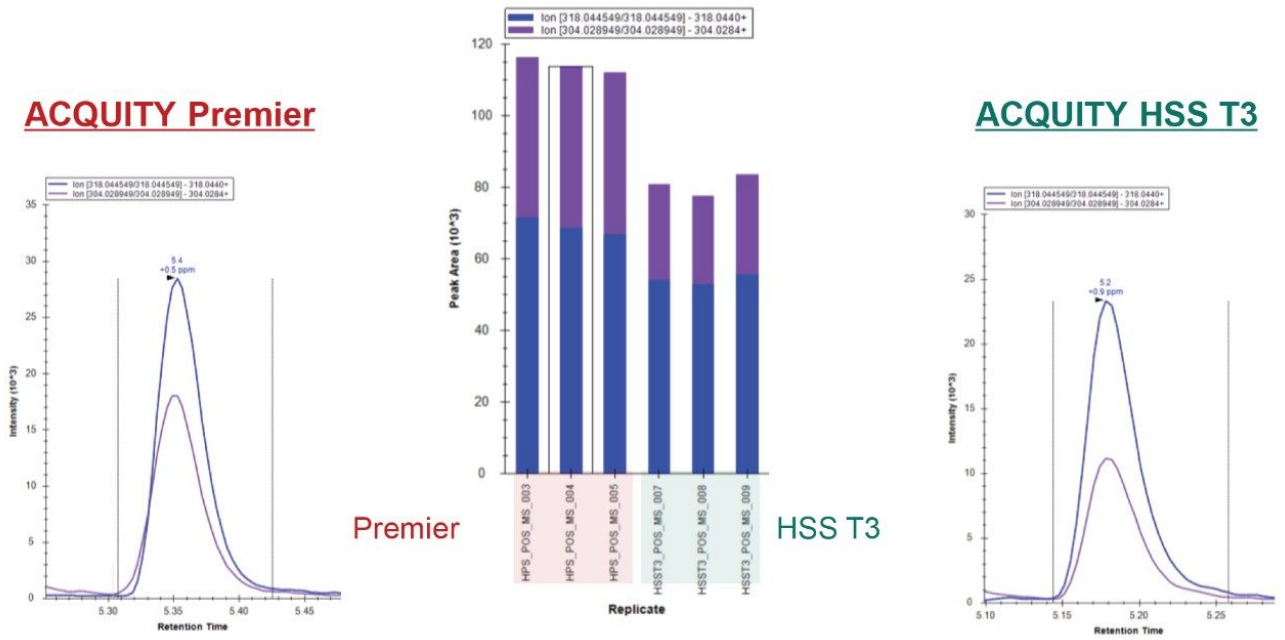


Figure 5. Improved fragment ion intensities demonstrated for gefitinib metabolite M7 (MQZP, m/z 378.1021). Increased ion intensities relating to transitions m/z 318.0440 and 304.0284 are shown to increase for the ACQUITY Premier HSS T3 Column.

Conclusion

The ACQUITY Premier System with hybrid organic polymer surface technology greatly reduces/attenuates the binding of many analyte molecules, such as phosphates, uncharged amines, hydroxyls, and deprotonated carboxylic acids, with the transition metal ions present on the metal surfaces in the chromatographic system. This results in improved peak shapes, greater sensitivity and improved reproducibility, delivering an average of 50% more peak intensity with the ACQUITY Premier Column when compared to the standard HSS T3 stainless steel column for analytes such as gefitinib and its metabolites. This is particularly important for the analysis of drug metabolites with low systemic concentrations, for example when the candidate drug is dosed at low levels or at the end of the elimination phase of the pharmacokinetic profile. This ability to detect metabolites at extremely low levels is critical in supporting early FTHM clinical studies, micro-dosing and micro-sampling,

enabling support of the 3R initiative to reduce, replace and refine the use of animals in such studies.

References

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