

End-to-End High-Throughput Biotransformation Workflow: Automated Data Acquisition and Processing of Sub-Second UPLC Peaks Using the Xevo™ Multi-Reflecting Time-of-Flight Mass Spectrometer and Dedicated Data-Mining Tools

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Abstract

Ultra-high performance liquid chromatography (UPLC™) coupled with high-resolution mass spectrometry (HRMS) has long been the standard for drug metabolite characterization. However, throughput is often limited by chromatographic peak dispersion, the need to balance mass resolution with scan speed, and the time required for data processing and interpretation. These factors, along with labor-intensive manual data review, slow down the delivery of actionable results.

To address these bottlenecks, a fully integrated, high-throughput data-dependent workflow has been developed

that combines next-generation UPLC with the Xevo MRT P10 MS multi-reflecting time-of-flight technology and dedicated data-mining software. This platform enables rapid, automated acquisition and processing of sub-second UPLC peaks, delivering part-per-billion mass accuracy (<0.7 ppm; <700 ppb RMS), scan speeds up to 100 Hz, and high mass resolution (100K FWHM).

Male beagle dogs received a single intravenous (i.v.) dose, and urine was collected both before and after drug administration. Samples were analyzed using reversed-phase UPLC coupled to a multi reflecting time-of-flight mass spectrometer. Data-dependent acquisition (DDA) was performed in positive mode of ionization (ESI+), and metabolites were identified using the dedicated MassMetaSite Software (Mass Analytica). Workflow improvements were assessed by comparing results obtained with shorter runtimes to those from a conventional UPLC method used by the contract research organization (CRO) that prepared the samples. This comparison focused on data quality, sample analysis times, and confidence in structural characterization.

Paracetamol and its metabolites were detected and characterized using the Xevo MRT P10 Mass Spectrometer (MS), coupled with a next generation UPLC. Multiple chromatographic gradient conditions were evaluated from 45 minutes with standard UPLC to just 90 seconds. The DDA was performed at 100 Hz, ensuring high-quality structural information and sufficient data points across the sub 1 second UPLC peaks generated, all within a rapid 90 second analysis. Across all detected metabolites, mass accuracy was maintained below 700 ppb (RMS).

Improved data quality enabled faster data interpretation and increased confidence in both expected and novel paracetamol metabolites, minimizing false identifications. Automated processing with MassMetaSite Software leveraged these unique data attributes, significantly reducing human bias and the need for extensive manual data interrogation. Overall, the workflow increased sample throughput while maintaining and, in key areas, improving data quality, delivering high-confidence results with reduced analytical compromise.

Benefits

- High-throughput and reliable results: Enhanced data quality, delivering greater confidence and reduced analytical compromise.
- Reliable results in less time: Excellent data quality achieved at fast acquisition rates.
- Mass accuracies: Root mean square (RMS) mass accuracies <0.7, increasing the confidence in identification.
- Automated processing with dedicated MassMetaSite Software: Reducing human bias and the need for extensive manual data interrogation.

- Increased productivity and reduced operating costs per sample: High-throughput workflow that boosts laboratory productivity by up to eight times while cutting organic solvent consumption by ~80% (on the bases of an average run time of 14 minutes and average flow rate of 0.4 mL/min).

Introduction

Acetaminophen (APAP; paracetamol) is a widely used analgesic and antipyretic and is among the most commonly used non-prescription drugs worldwide.¹ APAP is also used in veterinary practices alone or in combination with other analgesics by intravenous route (i.v.). APAP is metabolized through three primary pathways: glucuronidation, sulfation, and oxidation. In most species, APAP is mainly biotransformed to its glucuronide and sulphate conjugates; however, the relative contribution of each pathway can vary substantially between species, limiting its use to a small number of species.^{2,3} Oxidation via the hepatic cytochrome P450 system represents a minor yet important metabolic route in which APAP is converted into the reactive intermediate N-acetyl-p-benzonquinone imine (NAPQI). This metabolite is then detoxified by conjugation to glutathione (GSH) to form APAP-GSH, which is subsequently converted into the cysteine (APAP-Cys) and mercapturic acid (APAP-Mer) conjugates.^{1,4} At high dose, the hepatic content of GSH is depleted and the glucuronidation and sulfation pathways become saturated. When NAPQI accumulates, it binds to cellular macromolecules inducing hepatic lesions and necrosis.² In dogs, APAP toxicity is reported at doses greater than 200 mg/kg.^{2,4}

Traditional UPLC coupled to HRMS is the gold standard for drug metabolite characterization. However, throughput can be limited by peak dispersion, the need to balance mass resolution with scan speed, and the time required for data processing and interpretation. These factors, along with labor-intensive manual data review, slow down the delivery of actionable results. To address these challenges, high-throughput data dependent acquisition workflow combining a next generation UPLC with the Xevo MRT P10 MS and dedicated data-mining software was implemented.

In this study, two male beagles received a single intravenous dose of APAP (10 mg/kg). Urine samples were collected pre-dose and post-dose and analyzed using reversed-phase UPLC coupled to the Xevo MRT P10 MS. Metabolites were profiled under multiple chromatographic conditions using DDA and MassMetaSite Software. Using a 90-second gradient, DDA was performed at 100 Hz, providing high-quality structural information and

sufficient data points across sub-1-second UPLC peaks within the rapid analysis window.

Experimental

Sample Preparation

Male beagle dogs received a single i.v. dose of paracetamol (10 mg/kg). Urine was collected pre- and post-dose and stored at -20 °C. Samples were prepared fresh prior to analysis. Briefly, to 5 µL of urine samples, 95 µL of cold methanol were added. Samples were vortexed for 5 seconds and incubated at -20 °C for 10 minutes, then brought to room temperature, vortexed for a further 5 seconds, and centrifuged at 12,000 g for 10 minutes at 4 °C. 20 µL of supernatant was diluted with 480 µL of water. Finally, 2 µL of the diluted extract were analyzed by reversed-phase UPLC coupled to the Xevo MRT P10 MS, as described below.

LC-MS Conditions

LC system:	Waters ACQUITY™ Premier System
Analytical column:	ACQUITY UPLC HSS T3 Column 1.8 µm, 2.1 x 50 mm (p/n: 186003538)
Column temperature:	40 °C
Vials:	TruView™ pH Control LCMS certified Clear Glass Vial (p/n: 186005669CV)
Sample temperature:	6 °C
Injection volume:	2 µL
Flow rate:	0.6 mL/min

Mobile phase A: Water +0.1% HCOOH

Mobile phase B: Acetonitrile +0.1% HCOOH

LC Gradient Table

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0	0.6	95	5	Initial
0.25	0.6	95	5	Curve 6
0.90	0.6	70	30	Curve 6
1.00	0.6	5	95	Curve 6
1.40	0.6	5	95	Curve 6
1.45	0.6	95	5	Curve 6
1.70	0.6	95	5	Curve 6

MS Conditions

MS system: Xevo MRT P10 Mass Spectrometer

Ionization mode: ESI+

Mass range: m/z 50–1200

Acquisition mode: Data dependent acquisition (DDA)

Acquisition rates:	Survey scan time at 50 Hz, MS/MS scan rate at 100 Hz
Maximum simultaneous MS/MS acquisition:	5
MS/MS dynamic exclusion:	Acquire and then exclude for 1s
Lock mass:	Dual point lock mass using Leucine enkephalin (<i>m/z</i> 556.27658 and 120.08078)

Source Conditions

Capillary voltage:	0.8 kV
Cone voltage:	30 V
Source temperature:	120 °C
Desolvation temperature:	600 °C
Cone gas:	50 L/h
Desolvation gas:	1000 L/h

Collision Energy

Low mass ramp:	10–30 V
High mass ramp:	20–50 V

Software Tools

Data was acquired using the waters_connect™ Software Platform. Following acquisition, data sets were converted to mzML format using the DATA Convert Application within waters_connect Software. Data were centroided, and m/z precision was set to 64 bits. The resulting mzML files were then processed using Mass Analytica's MassMetaSite Software for metabolite identification.

Results and Discussion

Reducing the Total Runtime by Speeding the Chromatography

The analytical method provided by the testing accelerating laboratory consisted of a long chromatographic runtime of about 45 minutes. The analytical column was 150 mm x 4.6 mm, 3.5 μ m, and the chromatography was coupled to a scintillation detector. As this study did not use radioactivity, reducing the runtime was necessary to improve throughput. As a first step in the method transfer, alternative column dimensions with smaller particle size was evaluated. Two lengths were tested: 100 mm and 50 mm. Because metabolite separation was maintained with the 50 mm column (data not shown), the selected column was 50 x 2.1 mm, 1.8 μ m (details in the method section). Figure 1 illustrates the gradients used.

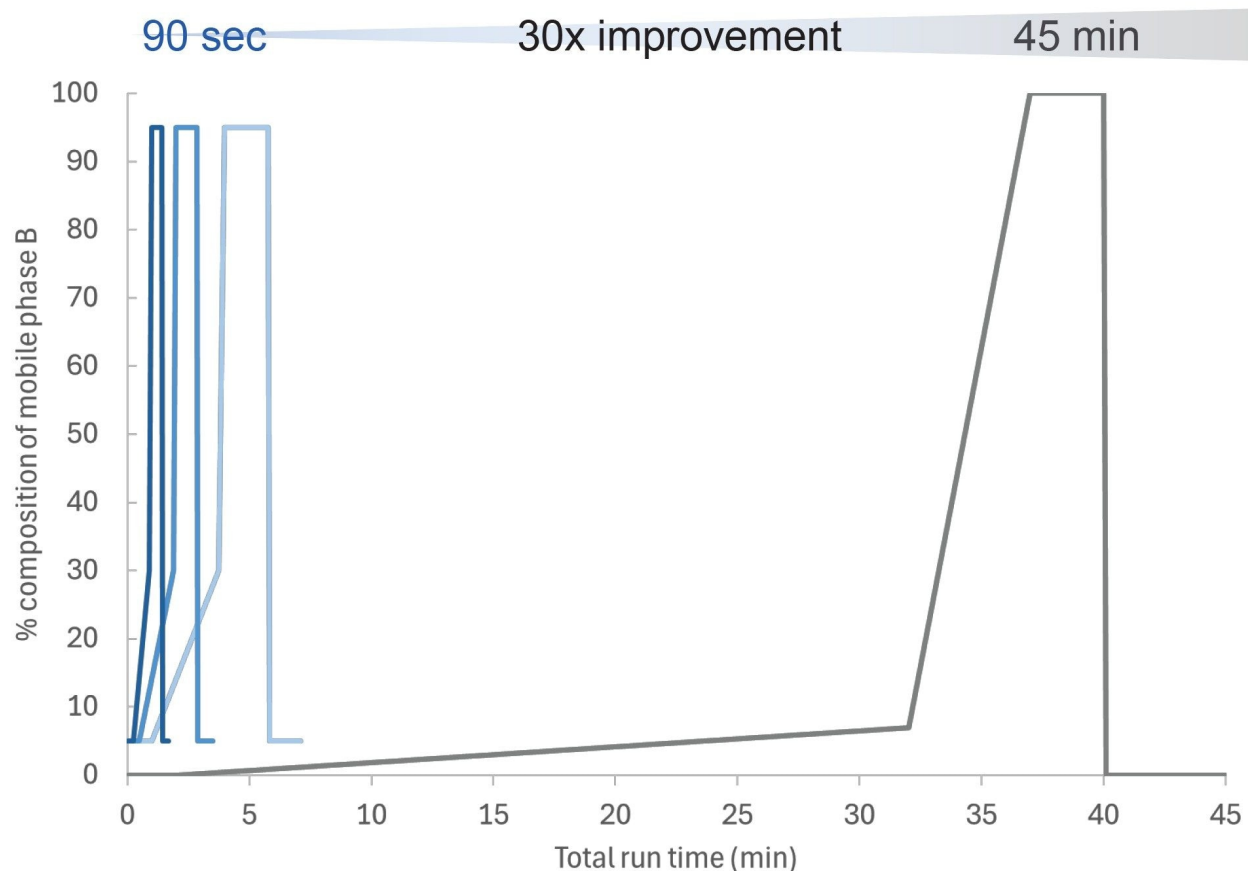


Figure 1. Comparison of the different gradients evaluated using the same column chemistry (HSS T3). The grey trace correspond to the initial chromatographic method using a 150 x 4.6 mm, 3.5 μ m column, and the blue traces correspond to the optimized method using a 50 x 2.1 mm, 1.8 μ m.

The grey traces in Figure 1 represent the gradient used by the CRO with a scintillation detector. The blue lines represent the gradients evaluated using the ACQUITY UPLC HSS T3 1.8 μ m, 2.1 x 50 mm column. By reducing the column dimensions and slightly increasing the flow rates, the total runtime was 30-fold reduced.

Identification of APAP's Metabolites in Urine 6 hours Post Administration

Urine extract collected pre-dose and 6 hours post i.v. administration of APAP, together with analytical standards, were analyzed under the different gradient conditions. Gradient acceptance criteria were based on the detection of all the expected APAP metabolites. To do so, DATA Convert, a waters_connect Software tool,

was used to convert the data into mzML. The resulting mzML files were then processed using Mass Analytica's MassMetaSite Software to identify APAP's metabolites. Figure 2 shows an overlay of the extracted ion chromatograms of all the identified metabolites in urine 6 hours post i.v. administration using a gradient of less than 90 seconds.

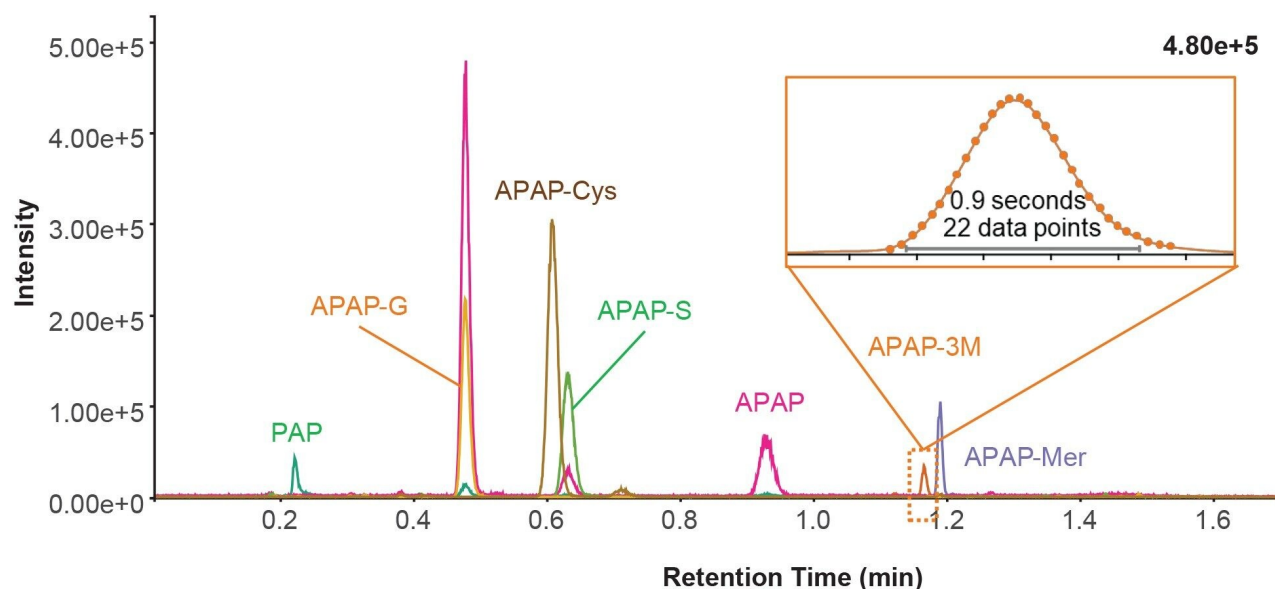


Figure 2. Extracted ion chromatogram (EIC) of identified metabolites from urine collected 6 hours post i.v. administration of paracetamol (APAP) obtained under a 90 second gradient in positive mode of ionization with data DDA at 100 Hz. APAP: Paracetamol (acetaminophen), PAP: 4-aminophenol, APAP-G: acetaminophen glucuronide, APAP-Cys: paracetamol cysteine conjugate, APAP-S: acetaminophen sulfate, APAP-3M: 3-Thiomethyl acetaminophen, APAP-Mer: acetaminophen mercapturic acid.

The boxed region in Figure 2 highlights 3-Thiomethyl Acetaminophen (APAP-3M). At an acquisition rate of 100 Hz and with a peak width of less than 1 second, at least 22 measurement points were collected across the peak.

All the metabolites were identified with a RMS of mass measurement accuracy of 0.697 ppm. Table 1 summarizes the observed m/z , the ion formula, mass measurement accuracy, and the isotopic similarity between the observed and theoretical compound.

Name	RT (min)	m/z obs.	m/z shift	Area %	Ion formula	m/z diff (ppm)	Iso. All Sim.
APAP-Cys	0.61	271.075	119	38.99	$[C_{11}H_{14}N_2O_4S+H]^+$	0.522	99.5
APAP-G	0.48	328.103	176	21.04	$[C_{14}H_{17}NO_8+H]^+$	0.921	99.2
APAP-S	0.63	232.028	80	18.44	$[C_8H_9NO_5S+H]^+$	0.613	99.4
APAP	0.93	152.071	0	11.15	$[C_8H_9NO_2+H]^+$	-0.388	
APAP-Mer	1.19	313.086	161	5.82	$[C_{13}H_{16}N_2O_5S+H]^+$	0.913	98.5
APAP 3M	1.17	198.059	46	1.95	$[C_9H_{11}NO_2S+H]^+$	-0.858	
PAP	0.48	110.060	-42	1.34	$[C_6H_7NO+H]^+$	0.875	100

Table 1. Paracetamol metabolites identified in urine 6 hours post i.v. administration in a 90 second gradient. All metabolites were identified with RMS mass measurement accuracy < 0.697 ppm.

APAP: Paracetamol (acetaminophen), PAP: 4-aminophenol, APAP-G: acetaminophen glucuronide, APAP-Cys: paracetamol cysteine conjugate, APAP-S: acetaminophen sulfate, APAP-3M: 3-Thiomethyl Acetaminophen APAP-Mer: acetaminophen mercapturic acid.

MassMetaSite Software performs the identification of metabolites and assigns chemical structures to automatically detected chromatographic peak based on the MS and MS/MS spectra of the substrate (APAP) and its metabolites. Figure 3 depicts the MS/MS spectra of the three main metabolites obtained using DDA acquisition at 100 Hz, with the structural assignment of each of the fragments. Boxed structures correspond to the proposed parent ion structure.

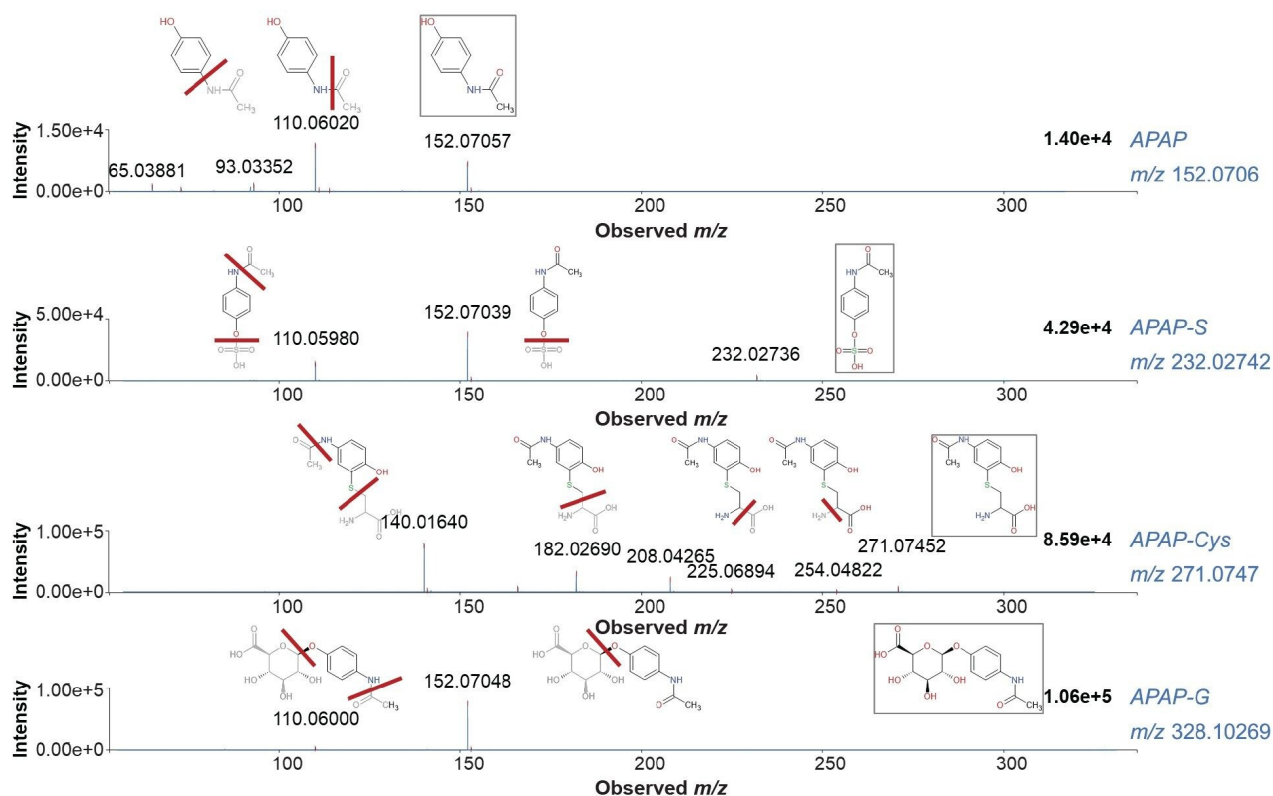


Figure 3. Fragmentation spectra for paracetamol (APAP) and three of its main metabolites detected in urine samples 6 hours post i.v. administration. Fragments were obtained using DDA acquisition method in a 90 second gradient. The acquisition rates were 50 and 100 Hz for survey and MS/MS, respectively. APAP: Paracetamol, APAP-G: acetaminophen glucuronide, APAP-Cys: paracetamol cysteine conjugate, APAP-S: acetaminophen sulfate.

Shorter gradients combined with higher flow rates produced narrower chromatographic peak widths; consequently, the acquisition rate was increased. The data presented above demonstrate that the acquisition rate was sufficient to collect multiple measurements across sub-second peaks (Figure 2, box, Figure 3). Importantly, overall instrument performance was maintained under these demanding conditions, with data quality matching that achieved using gradients ~30x longer and slower scan rates.

Conclusion

Excellent instrument performance under ballistic chromatographic conditions allowed reducing the total analytical run time at least by 8x times (on the bases of a total run time of 14 minutes). The Xevo MRT P10 MS maintained the high data quality, including mass accuracy and high quality of MS/MS spectra. The DDA mode ensured the correct assignment of the precursor and fragment ions. The end-to-end workflow from the data acquisition to the data interpretation using MassMetaSite was completed in less than 5 minutes, compared to an hour (for an average runtime of 14 minutes). This novel methodology achieved higher sample throughput while maintaining high data quality, delivering greater confidence and reduced analytical compromise.

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