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

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Introduction

Traditional ultra-high performance liquid chromatography (UPLC™ System) paired with high-resolution mass spectrometry (HRMS) has long been the standard for drug metabolite characterization, but its throughput is limited by peak dispersion, the need to balance mass resolution with scan speed, data processing and rationalization. These factors, along with labor-intensive manual data review, slow down the delivery of actionable results.

To overcome these bottlenecks, we introduce a fully integrated high-throughput data dependent workflow combining a UPLC system with multi reflecting time-of-flight technology (Xevo™ MRT P10 MS) and dedicated data-mining software. This platform enables rapid, automated acquisition and processing of sub-second UPLC system peaks, achieving part-per-billion mass accuracy (727 ppb RMS), scan speeds up to 100 Hz, and high mass resolution (100K FWHM).

Experimental

Male beagle dogs received a single intravenous (i.v.) dose of 10 mg/kg of paracetamol, and urine was collected before and after dosing. After a protein precipitation step, urine samples were analyzed using reversed-phase UPLC system coupled to multi reflecting time of flight mass spectrometer.

Data dependent acquisition mode was used, and metabolites were identified in positive electrospray ionization mode, utilizing Xevo MRT P10 MS and a dedicated MassMetaSite software (Figure 1).

Xevo MRT P10 MS

Ethics and Conflict of interest

This study was performed by Pharmaron UK Ltd., after full management and ethical review. The study was conducted in accordance with the appropriate Test Facility Standard Operating Procedures and with due regard to and in the spirit of GLP as per Pharmaron Uk Ltd company policy.

Hania Khoury-Hollins, Richard Lock, David Pickles, Robert Plumb are employees of Waters Corporation, Ismael Zamora and Fabien Fontaine are employees of Mass Analytica, Professor Ian Wilson has been a paid consultant of several companies including Waters.

Experimental

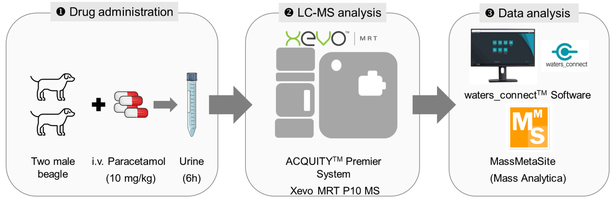

Figure 1. Schematic representation of the experimental design. Urine samples were collected before and after the i.v administration of paracetamol. After a protein precipitation step using methanol, the supernatant was diluted with water. 2 µL of the extracts were analyzed using reversed-phase UPLC system coupled to Xevo MRT P10 MS.

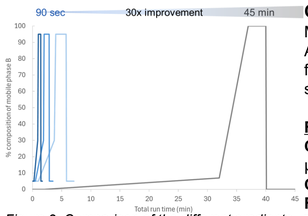

Figure 2. Comparison of the different gradients evaluated

Chromatographic parameters:
Multiple gradients were evaluated. A dramatic reduction in total runtime from 45 minutes with standard UHPLC system to just 90 seconds (Figure 2).

Final Condition:
Column: ACQUITY UPLC HSS T3 1.8 µm, 2.1x50mm (P/N 186003538).
Column Temp: 40°
Flow rate: 0.6 mL/min
Total run time: 90 seconds

MS system:	Xevo MRT P10 MS
Ionization mode:	ESI+
Mass range:	m/z 50–1200
Acquisition mode:	DDA
Source Conditions:	
Capillary voltage:	0.8 kV
Cone voltage:	30 V
Source temp.:	120°C
Desolvation temp.:	600°C
Cone Gas:	50 L/h
Desolvation gas:	1000 L/h
Source Offset:	30 V
Transmission tune settings:	
StepWave RF:	150 V
Body gradient:	10 V
Lock mass:	Leucine enkephalin (m/z 556.27658, 120.08078)
Acquisition rate:	**Survey at 50 Hz (0.02s), MS/MS at 100 Hz (0.01 s)**
Top N:	5 ions
Start MS/MS threshold:	25000
Stop MSMS:	either TIC falls below 10000, or after 2 scans
Dynamic Exclusion:	Exclude after 1 seconds
Dynamic Exclusion tolerance:	5 ppm
Collision energy	Low mass: ramp 10 – 30 V
	High Mass: ramp 20 – 50 V

MS acquisition:
Using faster gradients reduced the chromatographic peak width. To maintain sufficient datapoints across the chromatographic peak, the acquisition rates were increased.

Final DDA parameters:
Survey scans at 50 Hz and MS/MS at 100 Hz ensured high-quality structural information and sufficient data points across the sub 1 second UPLC system peaks generated, all within a rapid 90 second analysis.

Results

Detection of Paracetamol and its metabolites

DDA data was processed using MassMetaSite (MMS) software tool for drug metabolite identification. Six metabolites were detected and identified with RMS of mass measurement accuracy 0.727 ppm. Table 1 lists all the identified metabolites and Figure 3 depicts the extracted ion chromatogram (EIC) of the identified paracetamol metabolites in urine 6h after i.v. administration.

Name	RT (min)	*m/z* obs.	*m/z* shift	Area %	ion formula	*m/z* diff (ppm)	Iso. All Sim.
APAP-Cys	0.61	271.0749	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	232.0276	80	18.44	$[C_8H_9NO_5S + H]+$	0.613	99.4
APAP	0.93	152.0707	0	11.15	$[C_8H_9NO_2 + H]+$	-0.388	
APAP-Mer	1.19	313.0856	161	5.82	$[C_{13}H_{16}N_2O_5S + H]+$	0.913	98.5
APAP 3M	1.17	198.0585	46	1.95	$[C_9H_{11}NO_2S + H]+$	-0.858	
PAP	0.48	110.0599	-42	1.34	$[C_6H_7NO + H]+$	0.875	100

Table 1: Paracetamol' s metabolites identified in urine 6 hours post i.v. administration in a 90 second chromatography. All metabolites were identified with RMS mass measurement accuracy 0.727 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 mercapturinc acid.

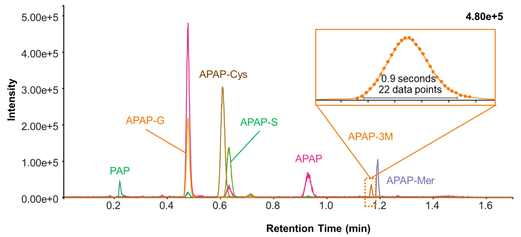

Figure 3. Extracted ion chromatogram of all the identified metabolites in urine samples 6 hours post i.v administration of acetaminophen analyzed using 90 seconds gradient in positive mode of ionization using data depended acquisition mode. APAP: Paracetamol (acetaminophen), PAP: 4-aminophenol, APAP-G: acetaminophen glucuronide, APAP-Cys: paracetamol cysteine conjugate, APAP-S: acetaminophen sulfate, APAP-3M: 3-Thiomethyl Acetaminophen APAP-Mer: acetaminophen mercapturinc acid.

Results

MS/MS spectrum of metabolite at 100 Hz - (90 sec total run time)

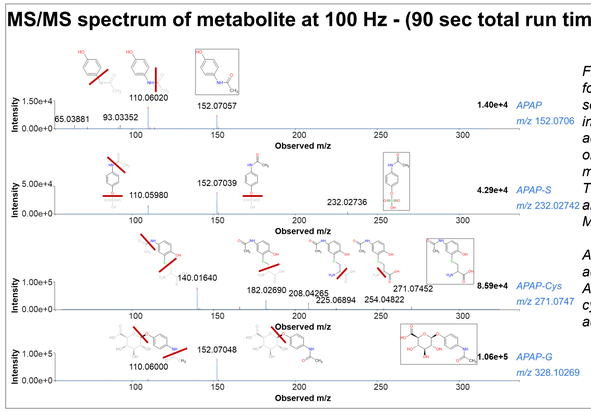

Figure 4. Fragmentation spectra for paracetamol (APAP) and some of its metabolites detected in urine samples 6 h post i.v administration. Fragments were obtained using DDA acquisition method for a 90 sec runtime. The acquisition rates were 50 and 100 Hz for survey and MS/MS, respectively.

APAP: Paracetamol, APAP-G: acetaminophen glucuronide, APAP-Cys: paracetamol cysteine conjugate, APAP-S: acetaminophen sulfate.

Conclusions

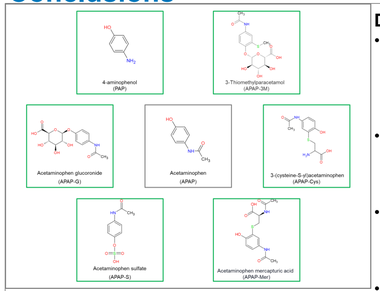

Figure 5. Structures of the paracetamol and its identified metabolites using DDA acquisition mode at 100 Hz in a 90 seconds total runtime chromatography.

Drug metabolites in urine

- Data dependent mass spectrometry acquisition was performed at 100 Hz, ensuring high-quality structural information and sufficient data points across the sub 1 second UPLC system peaks generated, all within a rapid 90 second analysis.
- The improved data quality, enabled fast data rationalization and increased confidence in both expected and novel paracetamol metabolites, minimizing false detections.
- Automated processing with dedicated MassMetaSite software leveraged these unique data attributes, markedly reducing human bias and the need for extensive manual data interrogation.
- This workflow achieved higher sample throughput alongside enhanced data quality, delivering greater confidence and reduced analytical compromise. Figure 5 illustrates the structures of the 6 identified metabolites