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应用纪要

Exploring the Impact of Part Per Billion Mass Accuracy for Metabolite Identification Using Multi Reflecting Time-of-Flight MS With UPLC™ Part A

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Abstract

A non-targeted data independent (DIA) screening assay for small molecules analysis has been performed. The UPLC-MS (ultra-performance liquid chromatography mass spectrometry) accurate mass measurement specificity attained using a SELECT SERIES™ Multi Reflecting Time-of-Flight (MRT) mass spectrometer has been assessed.

Multiple therapeutic xenobiotics and corresponding metabolites have been identified at an acquisition rate of 10 Hz by UPLC-MS^E ES+ with a system mass resolving power >200,000 FWHM.

Precursor and fragment ion part-per-billion (ppb) accurate mass measurements were acquired routinely. The attained mass accuracy enables more stringent post detection data processing tolerances to be applied, which reduces false detection rates, and simultaneously enhances confidence in small molecule analyte identification.

System mass accuracy performance was monitored over a 24-hour period for a urinary screening analysis. An RMS error of 549 ppb was determined for 2651 detections of parent drugs and metabolite identifications, demonstrating excellent system robustness.

The ppb mass accuracy achieved can be used to improve data analysis efficiency and identification confidence in small molecule application research.

Benefits

Ultimate compound identification confidence and reduced false detection rates

- Enhanced analysis efficiency
- Uncompromised chromatographic peak fidelity using 10 Hz UPLC-MS^E ES+ using system resolving power >200,000 FWHM
- Routine MRT ppb mass accuracy for precursor and DIA fragment ions in complex sample analysis
- Enhanced identification confidence for small molecule therapeutic drugs and metabolites
- Fine isotope structure, ion selectivity, and ppb mass accuracy facilitate identification of unknowns

Introduction

The SELECT SERIES MRT (Figure 1) is a state-of-the-art hybrid quadrupole MRT mass spectrometer. It provides a unique combination of high resolving power (>200,000 FWHM), and routine ppb mass accuracy, independent of acquisition speed. High resolution mass spectrometers (HRMS) such as quadrupole time of flight mass analyzers (Q-Tof), have become more prevalent as screening tools in clinical, forensic toxicology, and metabolite identification, where the constituents of interest are present in complex biological matrices such as urine. Using non-targeted "wide-scope" data acquisition, thousands of detections can be made in a single analysis and can be followed by retrospective targeted data analysis. A high mass resolving power enhances ion selectivity and subsequently the detection of analytes in complex matrices. MRT routine ppb mass accuracy performance generates high quality mass spectrometry data, facilitating unequivocal determination of analyte elemental compositions determined in non-targeted screening workflows. The enhanced mass accuracy specificity can be utilized to improve identification confidence in research involving small molecules, such as metabolite identification.

The purpose of a screening method is to rapidly detect and identify target compounds in the sample under investigation, with the aim to minimize false detection rates. Typically measured properties of a compound, such as the accurate mass, isotope pattern, and fragment/product ion spectrum, are used with appropriate data processing filters to determine the presence of a compound in a sample.[1] The resolution achievable with MRT of >200,000 FWHM affords the opportunity to also exploit fine isotope structure (FIS) as an identification confirmation criterion. For complex analyses, the mass resolution that can be achieved using the Q-MRT allows matrix interferences and analytes of interest to be distinguished and results in sub ppm (part-per-million) mass accuracy for precursor and fragment ions providing greater identification specificity, helping to mitigate false detections.

Publication of the FDA MIST (metabolites in safety testing) guidelines in 2008 initiated major changes in experimental strategies employed within the pharmaceutical industry, to identify, quantify, and improve metabolite

coverage.[2] For drug candidates brought forward in pharmaceutical research, formation of reactive and potentially toxic metabolites during metabolism is a continuing challenge. In some cases, the formation of acyl or ester glucuronidation and an association with drug toxicity, has led to instances of drug withdrawal.[3] The timing of clinical/nonclinical studies to generate this information with certainty is critical.

Metabolite identification is an important part of the drug development process where the metabolic fate of a drug molecule is investigated. This requires mass spectrometry techniques with high specificity for structural elucidation. Here we describe the use of an ACQUITY Ultra Performance LC™ coupled with a SELECT SERIES MRT, for the LC-MS analysis of drug metabolites. A urinary screen of a healthy human volunteer was undertaken to identify therapeutic drugs and metabolites. A metabolite identification workflow using LC-MS (system resolution >200,000 FWHM) has been implemented.[4]

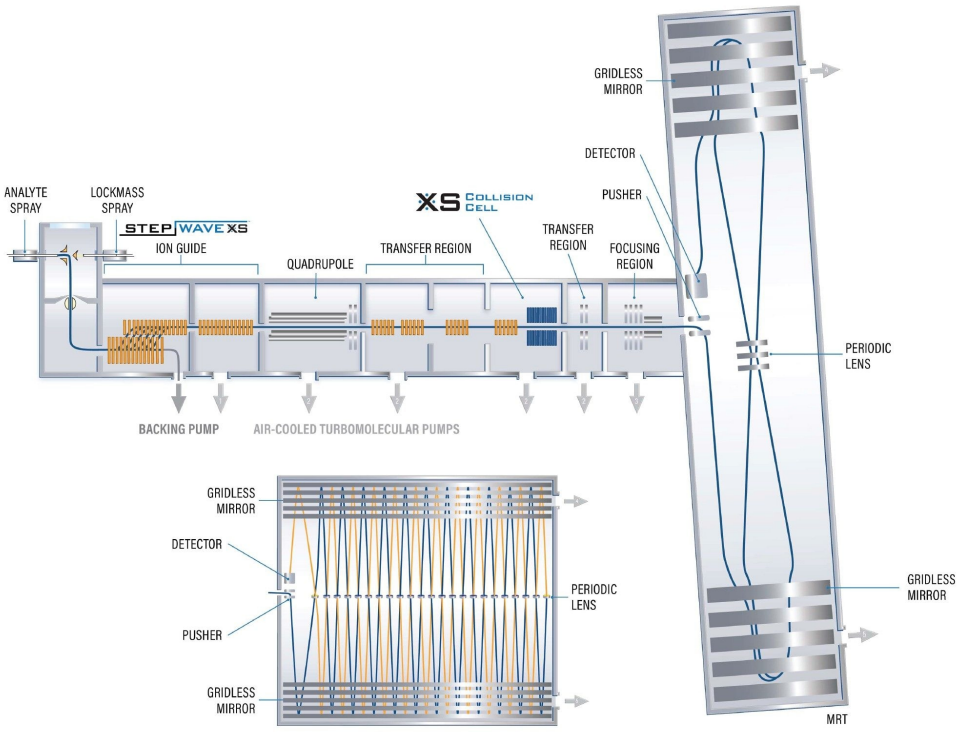

Figure 1. SELECT SERIES MRT instrument schematic.

Experimental

Sample Description

Human urine sample diluted:	10:1 (H_2O)
Carbamazepine dosage:	2 x 200 mg tablets
Acetaminophen dosage:	2 x 500 mg tablets
Naproxen dosage :	1 x 500 mg tablet

Sample time points:	0, 2, 4, and 6 hours after medication was administered

LC Conditions

LC system:	Waters™ ACQUITY UPLC I-Class Premier Chromatograph
Column:	ACQUITY UPLC HSS T3 C_{18} (100 mm X 2.1 mm, 1.8 µm) Column
Column temperature:	40 °C
Sample temperature:	4 °C
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Mobile phase A:	Water (containing 0.1% formic acid *v/v*)
Mobile phase B:	Acetonitrile (containing 0.1% formic acid *v/v*)

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.5 mL/min	99	1	initial
1.0	0.5 mL/min	99	1	6
3.0	0.5 mL/min	85	15	6
6.0	0.5 mL/min	50	50	6
9.0	0.5 mL/min	5	95	6
10.0	0.5 mL/min	5	95	6
10.1	0.5 mL/min	99	1	6
12.0	0.5 mL/min	99	1	6

MS Conditions

Acquisition:	ES+
Capillary voltage:	0.5 kV
Desolvation temperature:	500 °C
Source temperature:	120 °C
Cone voltage:	20 V
Collision energy ramp:	15-45 eV
Mass Range:	*m/z 50–2400*
MS^E Acquisition rate:	10 Hz
Acquisition/processing software:	MassLynx™ v4.2 SCN1026 and waters_connect™ 3.1.0.243

Results and Discussion

To explore the impact of high-resolution mass spectrometry (HRMS) mass accuracy upon small molecule identification, LC-MS urinary screening of a healthy volunteer patient has been performed at time course points of 0, 2, 4, and 6 hours post dose. Figure 2 (I) shows an example of an MRT-LC-MS ES+ chromatographic separation obtained at 10 Hz for the complex human urine sample analyzed. Using the extracted mass chromatogram of carbamazepine as an example, 18 data points are obtained across the chromatographic peak (3.6 second base peak width). The corresponding continuum mass spectrum is shown in Figure 2 (II), where mass resolution >170,000 FWHM was obtained for the monoisotopic ion at *m/z* 237.1. The example shown, illustrates high resolution DIA can be performed across the mass range, where at low *m/z,* duty cycle is not compromised for high mass resolution and chromatographic fidelity to be attained.

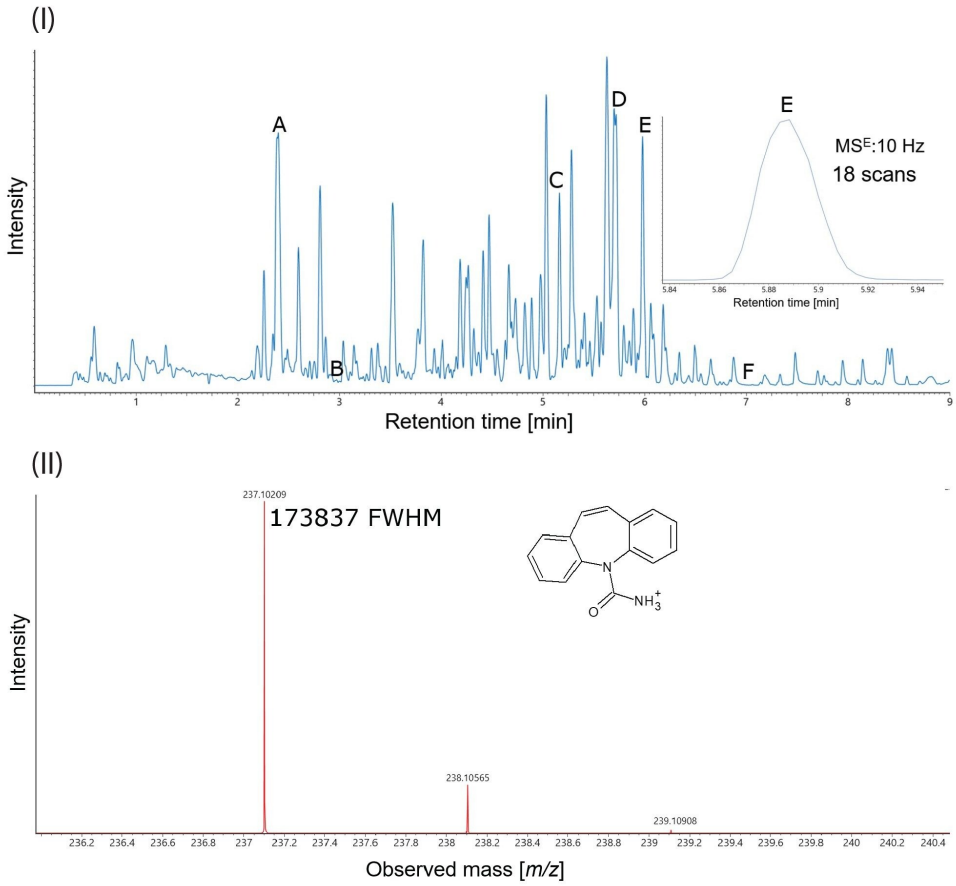

Figure 2. MRT-LC-MS ES+ base peak ion chromatogram, for the analysis of therapeutic xenobiotics and metabolites identified in the urine of a healthy volunteer patient. I) a) acetaminophen glucuronide, b) acetaminophen, c) carbamazepine-N-glucuronide, d) naproxen glucuronide, e) carbamazepine, and f) naproxen. Inset expanded extracted mass chromatogram of carbamazepine. II) m/z 237 $[M+H]^+$ high resolution mass spectrum of carbamazepine.

Following a metabolite identification workflow, xenobiotic therapeutics, and metabolites of acetaminophen, naproxen, and carbamazepine have been identified. The primary route of metabolism for acetaminophen is in the liver to sulphate and glucuronide conjugates (ninety percent), at therapeutic doses, which are excreted in the urine. As shown in Table 1, RMS mass measurement errors for acetaminophen (444 ppb), acetaminophen sulphate

(527 ppb) and acetaminophen glucuronide (538 ppb) have been attained over a 24-hour period for repeat analysis of the human urine time course point samples demonstrating excellent reproducibility.

Confidence in therapeutic drug xenobiotics and metabolite identification, is further enhanced where DIA fragment ions are generated using MS^E and mass resolved from matrix interferences. In Figure 3 an example of UPLC MRT ES+ precursor and fragment ion spectra obtained for [acetaminophen glucuronide + H]$^+$ acquired at 10 Hz is presented with measurement error between -88 ppb and 118 ppb.

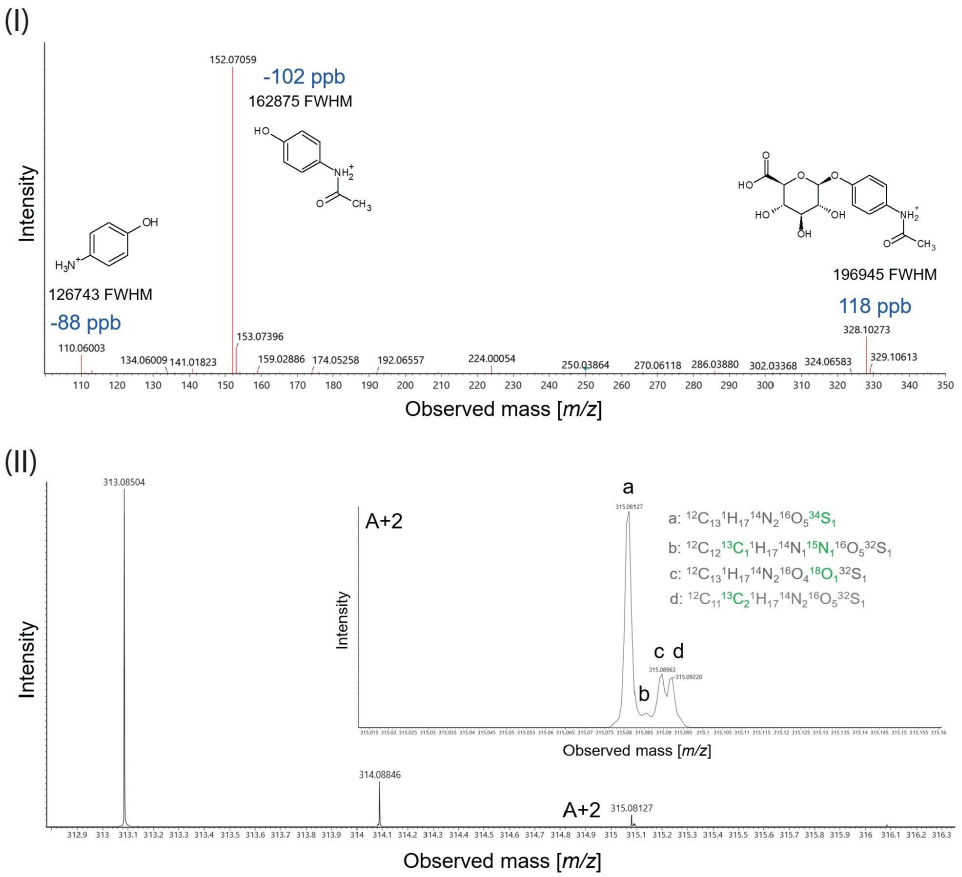

Figure 3. 10 Hz UPLC MRT ES+ precursor and fragment ion spectra obtained for [acetaminophen glucuronide +H]+. II) Fine isotope structure (FIS) observed for acetaminophen-acetyl-S-cysteine conjugate.

In humans the nonsteroidal anti-inflammatory drug naproxen is metabolized to 6-0- desmethyl naproxen. Both parent drug and metabolites are conjugated as acyl glucuronides and excreted in urine. In the UPLC MRT ES+ assay performed, desmethyl naproxen was not identified and naproxen is only observed at low intensity (approximately 1% of the dose is excreted as naproxen and 6-O-desmethyl-naproxen, respectively).[5] However, the acyl glucuronides of naproxen and 6-0-desmethyl naproxen were identified. Although the glucuronide metabolites ionize efficiently in ES- mode, in each case for the ES+ analysis, the sodiated adduct has been observed. The

sodiated adduct of a 6-0-desmethyl naproxen glucuronide metabolite may be a convoluted identification. Observed at t_r 4.83 min, precursor ion mass *m/z* 415.09995 was determined with an RMS mass measurement error of 503 ppb, with corresponding fragment ion mass accuracy *m/z* 171.08045 (-316 ppb) and *m/z* 217.08592 (192 ppb), see Figure 4. Additionally, the observed time course response profile is indicative of a true metabolite identification (see Figure 7), where detection is not determined in solvent or matrix blanks. Fine isotope structure also provides additional identification criterion, as shown for acetaminophen-acetyl-S-cysteine conjugation in Figure 3 (II). In the case of 6-0 desmethyl naproxen glucuronide metabolite, although not fully resolved, sufficient characteristic fine isotope structure provided further confirmatory evidence (see Figure 5), where the $^{23}Na_1{}^{12}C_{17}{}^{13}C_2{}^{1}H_{20}{}^{16}O_9$ and $^{23}Na_1{}^{12}C_{19}{}^{1}H_{20}{}^{16}O_8{}^{18}O_1$ species are evident. In the case of naproxen, the 10 Hz duty cycle has enabled chromatographic peak shape characterization, although not fully resolved four isomeric species (1-β-O and 2, 3, 4-O acyl glucuronide) were observed at t_r= 5.59, 5.69, 5.71, and 5.86 min. [3,5] The routine and reproducible attainment of ppb mass accuracy is illustrated for ES+ precursor and fragment ion spectra obtained for [naproxen glucuronide +Na]$^+$ (see Figure 6).

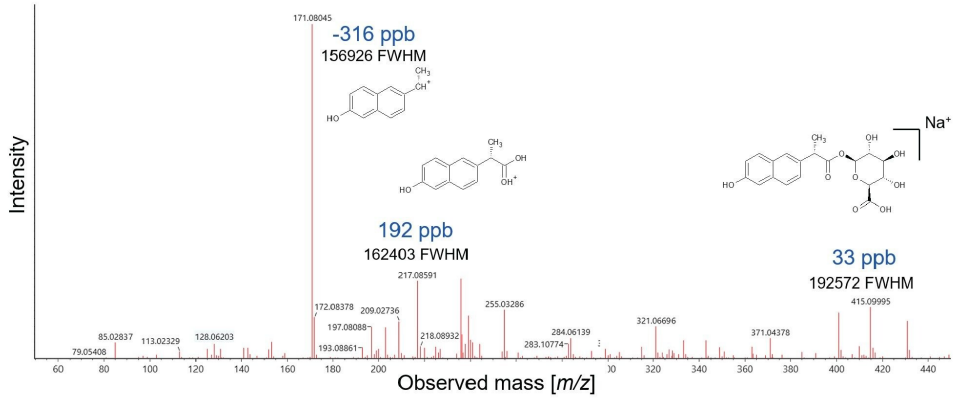

Figure 4. 10 Hz acquisition of UPLC MRT ES+ precursor and fragment ion spectra obtained for [desmethyl naproxen glucuronide +Na]$^+$.

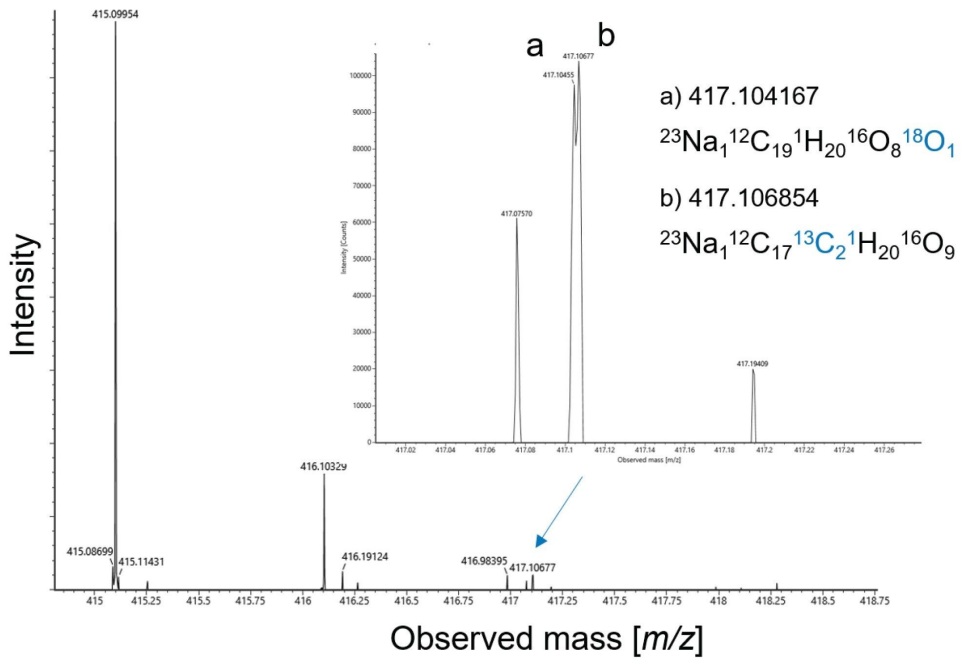

Figure 5. 10 Hz acquisition of UPLC MRT ES+ fine isotope structure for desmethyl naproxen glucuronide [naproxen glucuronide +Na]$^+$.

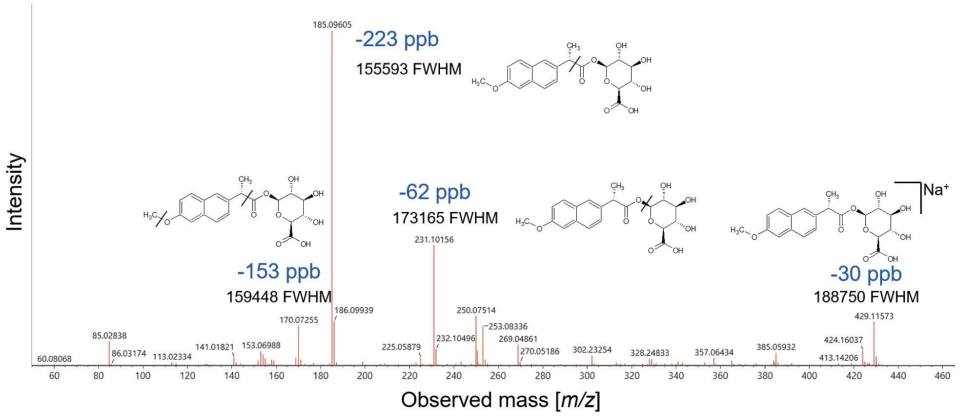

Figure 6. 10 Hz acquisition of UPLC MRT ES+ precursor and fragment ion spectra obtained for [naproxen glucuronide +Na]$^+$.

Metabolites of carbamazepine were also identified; it is an anticonvulsant medication used primarily in the treatment of epilepsy and neuropathic pain. Carbamazepine is metabolized to its active and potentially toxic metabolite, carbamazepine-10,11- epoxide. Aromatic, and benzylic hydroxylation, with ensuing *O*-glucuronidation are also observed. To assess assay robustness and mass accuracy, the experiments performed comprised replicate injections of solvent blank (N=3), post-dose time course points 0, 2, 4, and 6 hours (N=6), with repeat sample set analyses carried out continuously over a 24-hour period.

In Figure 7 we illustrate response reproducibility over the metabolism time course for carbamazepine, acetaminophen, and naproxen. Corresponding response trend plots for the acetaminophen-S-cysteine conjugate, carbamazepine epoxide, and desmethyl naproxen glucuronide conjugate are also presented.

In the case of carbamazepine, it has been observed that the parent drug and metabolites have been observed at T0 hours. Upon an acute dose, the expected initial elimination half-life of carbamazepine is 25—65 hours, which decreases to 4—17 hours with repeated doses.[7,8] RMS mass measurement errors, carbamazepine (558 ppb), carbamazepine epoxide (556 ppb), carbamazepine-O-glucuronide (606 ppb) provide a degree of confidence in the T0 hours sample identifications. The human urine sample analyzed is from a healthy volunteer where repeat carbamazepine doses were administered, and therefore present in the T0 sample. For carbamazepine, several isomeric species have been observed, where ppb mass accuracy provides confidence that isomeric analytes have been identified rather than isobaric. In the case of 9-hydroxymethyl-10-carbamoyl acridan O-glucuronide (fragment ion $C_{14}H_{12}NO_2$) and 10,11-Dihydro-10,11-dihydroxy carbamazepine glucuronide (fragment ion $C_{15}H_{10}NO_2$), characteristic MS^E fragment ions with 347 ppb and 582 ppb were respectively determined. This highlights DIA MS^E fragment ions can provide identification certainty in the presence of a complex matrix.

For carbamazepine, disposition data for sulphonated metabolites is not commonly reported.[8-12] It is proposed three phase II hydroxy sulphonated carbamazepine metabolites (*m/z* 333.05397) were identified. At t_r 4.75 mins for the most intense isomer, fragment ion *m/z* 253.09715 (196 ppb) is observed, corresponding to a SO_3 loss, as well as the characteristic carbamazepine fragment ions, base peak *m/z* 210.09133 (-31 ppb), and *m/z* 208.07568 (-32 ppb). Additionally observed fine isotope structure adds certainty to detection of a carbamazepine sulfonated biotransformation species.

UPLC-MRT-ES+ facilitates unsurpassed mass accuracy at an acquisition rate of 10 Hz, where chromatographic integrity is retained. RMS mass measurement obtained for the major metabolites identified are presented in Table 1. Overall, an RMS error of 549 ppb has been obtained for the detections discussed (N=2651), which are comprised of small molecules between *m/z* 152 and *m/z* 429.

Utilizing an unbiased data acquisition strategy such as DIA MS^E where all precursor and fragment ions are acquired, facilitates a representative profile of whole sample composition to be obtained. Although DIA MS^E acquisition is not as selective as MS/MS or DDA strategies, the MS^E specificity is enhanced using the MRT system resolving power >200,000 FWHM. This enables fragment ions to be mass resolved from coeluting isobaric biological matrix components. As a result, retention time aligned precursor/fragment ions with high mass accuracy are observed. The number of possible elemental compositions generated with ppb high mass accuracy data is reduced and consequently there is a high degree of confidence in analyte identification.[13,14,15]

Applying low intensity processing peak detection thresholds (e.g., 20 counts), enables unbiased data processing to be performed alongside unbiased data acquisition. The impact of mass accuracy can be harnessed using post processing filters and is illustrated in Table 2 for the carbamazepine four hour time point. Post processing mass accuracy tolerance thresholds applied to

precursor and fragment ions, simultaneously enhance identification confidence, and reduce the false detection rate. Additional post processing parameters such as a ” reporting threshold” may be applied, along with metabolite time course point profile assessment to facilitate confirmation of detected biotransformations.

Therapeutic xenobiotic/metabolite	Adduct	*m/z*	RMS error ppb	Retention time (t_r)
Acetaminophen	$[M+H]^+$	152.07061	444	2.92
Acetaminophen sulphate	$[M+H]^+$	232.02742	527	2.72
Acetaminophen glucuronide	$[M+H]^+$	328.10269	538	2.4
Acetaminophen S cysteine conjugate	$[M+H]^+$	271.0747	543	2.6
Acetaminophen acetyl cysteine	$[M+H]^+$	313.08527	565	3.53
Carbamazepine	$[M+H]^+$	237.10224	558	5.89
10 11-dihydroxy carbamazepine glucuronide	$[M+H]^+$	445.12416	611	4.32
Carbamazepine-N-glucuronide	$[M+H]^+$	413.13433	567	5.16
Carbamazepine-10,11-epoxide-N-glucuronide	$[M+H]^+$	429.12924	494	4.73
Hydroxy 9-hydroxymethyl-10-carbamoyl acridan-O-glucuronide	$[M+H]^+$	447.13981	562	4.02
10,11 - Dihydro-10,11-dihydroxy carbamazepine glucuronide	$[M+H]^+$	447.13981	481	4.42
9-(Hydroxymethyl)-10-carbamoylacridan-O-glucuronide	$[M+H]^+$	431.14489	551	5.03
Carbamazepine-O-glucuronide 1	$[M+H]^+$	429.12924	606	4.18
Carbamazepine-O-glucuronide 2	$[M+H]^+$	429.12924	577	4.24
Carbamazepine-O-glucuronide 3	$[M+H]^+$	429.12924	526	4.47
Carbamazepine-O-glucuronide 4	$[M+H]^+$	429.12924	569	4.88
Carbamazepine-10,11-epoxide	$[M+H]^+$	253.09715	556	4.66
Carbamazepine hydroxy sulphate 1	$[M+H]^+$	333.05397	501	4.62
Carbamazepine hydroxy sulphate 2	$[M+H]^+$	333.05397	543	4.75
Carbamazepine hydroxy sulphate 3	$[M+H]^+$	333.05397	634	5.27
Hydroxy carbamazepine 1	$[M+H]^+$	253.09715	509	3.88
Hydroxy carbamazepine 2	$[M+H]^+$	253.09715	565	3.8
Hydroxy carbamazepine 3	$[M+H]^+$	253.09715	496	3.95
Naproxen	$[M+H]^+$	231.10157	651	6.69
Naproxen glucuronide 1	$[M+Na]^+$	429.1156	502	5.59
Naproxen glucuronide 2/3	$[M+Na]^+$	429.1156	529	5.68/5.71
Naproxen glucuronide 4	$[M+Na]^+$	429.1156	604	5.86
Desmethyl naproxen glucuronide	$[M+Na]^+$	415.09995	503	4.83

Table 1. RMS accurate mass measurement obtained for detection of therapeutic xenobiotics and metabolites for analyses performed over a period of 24-hours.

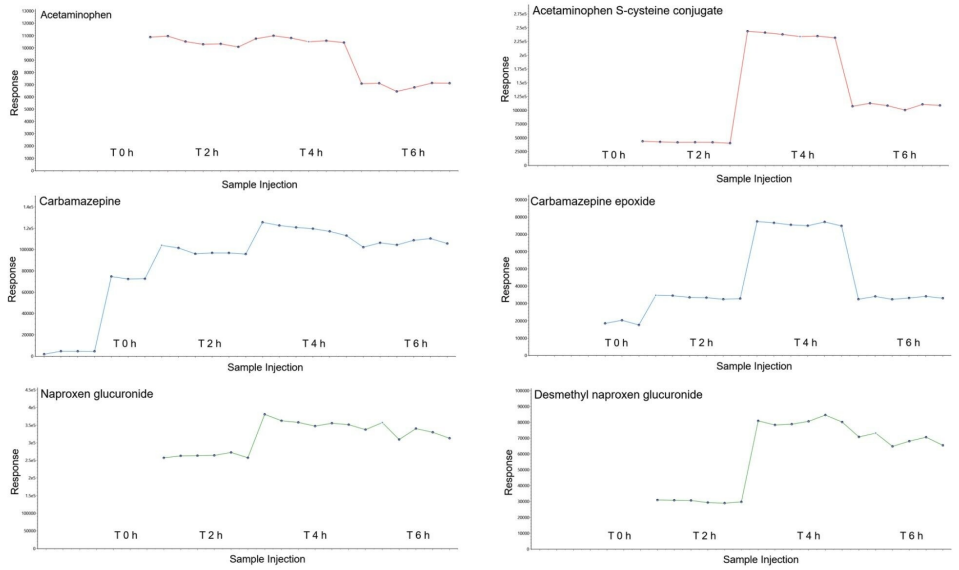

Figure 7. Metabolism response trend plots for therapeutic xenobiotics and metabolites (post dose time course points 0, 2, 4, and 6 hours) identified in a human urine sample using a metabolite identification workflow.

	Applied processing mass accuracy tolerance					
	Precursor 5 ppm	Precursor 5 ppm	Precursor 5 ppm	Precursor 1 ppm	Precursor 1 ppm	Precursor 1 ppm
	Fragment 2 mDa	Fragment 1 mDa	Fragment 0.2 mDa	Fragment 0.2 mDa	Fragment 0.1 mDa	Fragment 0.05 mDa
	t_r 0.1 parent	t_r 0.1 parent	t_r 0.1 parent	t_r 0.1 parent	t_r 0.1 parent	t_r 0.1 parent
Detections	200	200	200	142	142	142
Detections with expected fragments	140	140	133	112	98	88
Detections with theoretical fragments	196	191	181	144	138	119

Table 2. Impact of accurate mass on biotransformations false detection rate determined using a metabolite identification workflow at system resolving power > 200,000 FWHM for carbamazepine four hour time course point.

Conclusion

The complex analysis performed has illustrated LC-MS ES+ at an acquisition rate of 10 Hz encompassing a routine resolving power of >200,000 FHWM. Achieving ppb level mass accuracy for precursor and fragment ions has facilitated a non-targeted urinary screening assay to be performed with a new level of confidence, enabling routine identification of a combination of therapeutic drugs, including major and minor metabolites for the urinary screen of a volunteer patient.

A symbiotic relationship exists between data quality and the power of informatics solutions, where ppb mass measurement for DIA precursor ion and fragment ions provides a high degree of specificity for non-targeted analytical acquisition strategies. Transformative mass measurement affords the opportunity to improve informatics output and analysis efficiency, in the drug discovery and development process. High resolution mass spectrometry facilitates the implementation of more stringent informatics data processing filters, enhancing confidence in the identification of parent drug, metabolite precursor, and fragment ions. Such identification credence applies to both parent drug, expected fragment ions and in-silico fragment ions. Subsequently ppb mass accuracy reduces false detection rate of in-silico biotransformation products, decreasing data interrogation time, providing improved analysis efficiency. Fine isotope structure, which provides an additional identification criterion has also enhanced analyte identification specificity. Overall, the high- resolving power ppb mass measurement performance illustrated reduces the number of potential elemental compositions determined for small molecule identification, which is beneficial when dealing with knowns and unknowns.

The UPLC-MRT-MS performance at an acquisition rate of 10 Hz has been shown in the context of a metabolite identification workflow, where duty cycle facilitating chromatographic fidelity and routine ppb mass accuracy affords the opportunity to enhance identification confidence in many small

molecule application areas, including lipidomic, metabolomic, and natural products analysis.

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