APPLICATION NOTE 65953

Confident drug metabolite identification using an intelligent data acquisition and processing workflow

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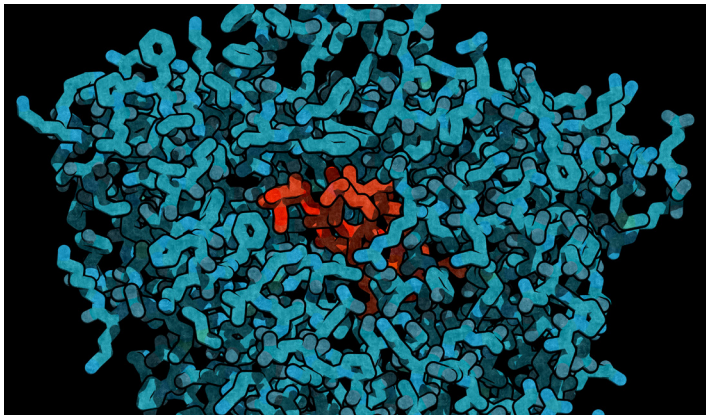
Application benefits

- Achieve comprehensive metabolite profiling through high-quality HRAM MS and MS/MS data acquired with high resolution, high scan speeds, fast polarity switching, and internal calibration.
- Improve metabolite identification and overcome matrix effects by triggering MS/MS analysis of low-level metabolites using an advanced data acquisition workflow.
- Obtain confident results using intelligent data processing software.
- Increase overall productivity through high-quality all-inclusive data acquisition, easy to use interface for instrumental control and method setup, and advanced data processing software.



Introduction

In vitro and *in vivo* drug metabolism studies are an essential part of drug discovery and development. High-resolution accurate mass (HRAM) mass spectrometry (MS) is considered the gold standard for metabolite profiling with its high mass accuracy, selectivity, specificity, and isotope fine structure enabling unequivocal determination of the elemental composition of metabolites, while the MS/MS and MS^n fragments provide crucial information for metabolite structure identification.[1]

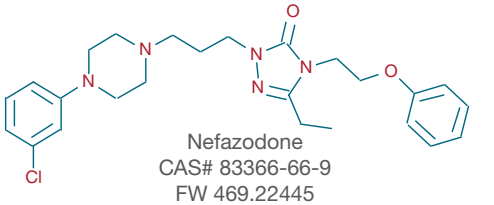
This application note presents a case study for metabolite profiling of model pharmaceutical compounds using a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system. Thermo Scientific™ Compound Discoverer™ small molecule identification software was used for data processing.

The results demonstrate how the high resolution, high mass accuracy, fast scan speed, and rapid polarity switching capabilities of the Orbitrap Exploris 240 mass spectrometer deliver confident structural identification. High-quality full scan and MS/MS data with sub-ppm mass accuracy were generated for confident metabolite detection and structural characterization. Thermo Scientific™ AcquireX™ intelligent data acquisition was used to exclude background ions in a real-time and automated fashion. MS/MS acquisitions of the low-abundant metabolites masked by matrix were triggered, increasing the metabolite identification efficiency circa 30–50%.

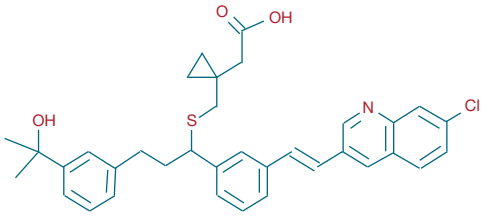
Experimental

Reagents and consumables

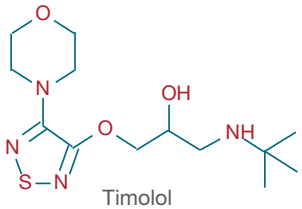
- Water, UHPLC-MS Grade (Thermo Fisher Scientific, P/N W8-1)
- Acetonitrile, UHPLC-MS Grade (Thermo Fisher Scientific, P/N A9561)
- Formic Acid, Optima™ grade LC/MS (Fisher Chemical, P/N: A117-10X1AMP)
- Ammonium formate (Sigma-Aldrich, P/N: 516961-100G)
- Selected model compounds were purchased from Sigma-Aldrich (Figure 1)



Nefazodone
CAS# 83366-66-9
FW 469.22445



Montelukast
CAS# 158966-92-8
FW 585.21044



Timolol
CAS# 26839-75-8
FW 316.15691

Figure 1. Structures of model compounds: nefazodone, montelukast, and timolol.

Sample preparation

Model compounds (10 µM) were incubated with human and rat liver microsomes (1 mg/mL) in the presence of NADPH (1 mM), UDPGA, and GSH (1 mM) as co-factors for 30 minutes. A negative control, used for background subtraction, was prepared with the same incubation conditions in the absence of drug substrates. The enzymatic reaction was stopped by adding acetonitrile to the samples with a volume to volume (v/v) ratio of 3:1 at 0 and 30 minutes, respectively, followed by centrifugation at 13,000 rpm for 15 min for protein precipitation. The supernatants were concentrated to the original volume under a stream of nitrogen, then spiked into human and rat plasma, respectively, in 1:5 (v/v) ratio to mimic *in vivo* study. The spiked solutions were deproteinated with the same procedure described above and used for the subsequent LC-MS analysis.

Liquid chromatography

Chromatographic separations were carried out on the Vanquish Horizon UHPLC system consisting of the following modules:

- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A)
- Thermo Scientific™ Vanquish™ Diode Array Detector FG (VF-D11-A)

1 µL of samples were injected onto a Thermo Scientific™ Hypersil GOLD™ VANQUISH™ C18 UHPLC column (2.1 × 50 mm, 1.9 µm, PN: 25002-052130-V) was used with the gradients specified below at a flow rate of 0.5 mL/min and a column temperature of 50 °C. Mobile phases were: H_2O/5 mM ammonium formate/0.05% formic acid (A) and Acetonitrile:H_2O 9:1/5 mM ammonium formate/0.05% formic acid (B) with the following LC gradients:

Nefazodone		Montelukast		Timolol	
Time (min)	%B	Time (min)	%B	Time (min)	%B
0	5	0	5	0	5
0.5	5	0.5	5	0.5	5
5	40	6	90	6	15
7	95	7	95	7.2	95
8.2	95	8.2	95	9.2	95
8.4	5	8.4	5	8.4	5
10.5	5	10.5	5	10.5	5

Mass spectrometry

The mass spectrometry analysis was carried out on a Orbitrap Exploris 240 mass spectrometer (P/N BRE725535) equipped with a Thermo Scientific™ OptaMax™ NG ion source.

In this study, two data acquisition methods were used:

1. Full MS followed by Top3 ddMS^2 with positive/negative polarity switching
2. Full MS followed by Top5 ddMS^2 with positive mode only

A Thermo Scientific™ EASY-IC™ internal calibration ion source was employed for all data acquisitions to ensure high mass accuracy throughout, Figure 2. All MS data acquisition methods were set up using the drag-and-drop user interface method editor. Figure 2 shows the method of full MS followed by Top3 ddMS^2 with polarity switching using EASY-IC internal calibration.

Data processing

Compound Discoverer 3.2 small molecule identification software (OPTON-31055) was used for data processing and reviewing.

Results and discussion

High scan speed yields high-quality full scan and DDA MS^2 data with polarity switching

To capture metabolites with different ionization preferences, rapid positive/negative polarity switching is commonly used in drug metabolism analysis. In this study, the initial screening was carried out using full MS followed by Top3 DDA MS^2 with polarity switching. The high scan speed of the Orbitrap Exploris 240 instrument enabled a duty cycle of ~1 second for 8 scan events data acquisition: full MS followed by 3 DDA MS^2 with polarity switching at resolution 60,000 (MS) and 15,000 (MS/MS), see Figure 3.

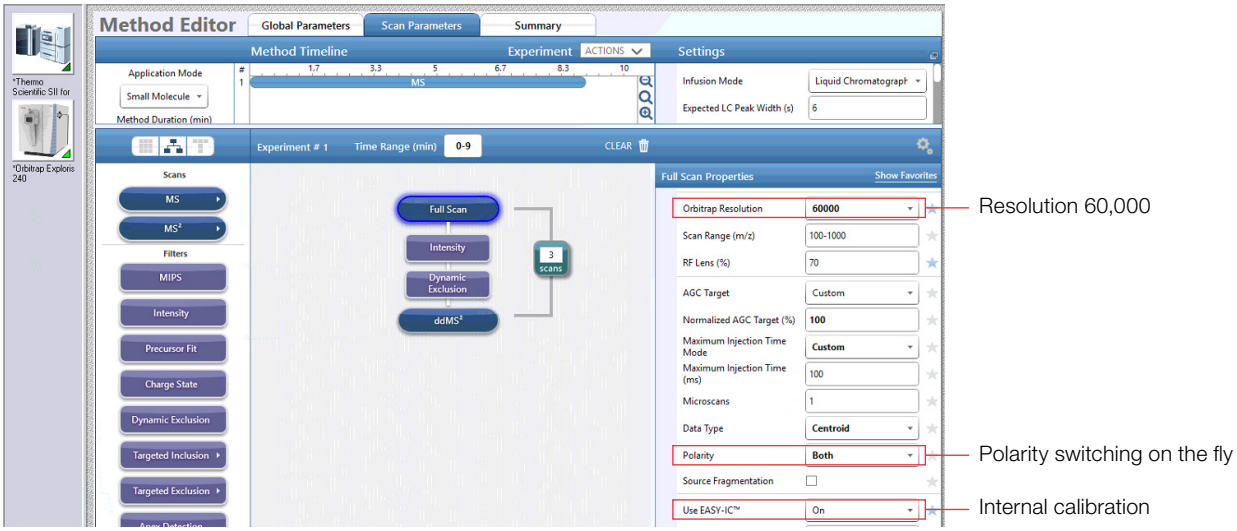


Figure 2. Method editor for data acquisition with polarity switching and internal calibration.

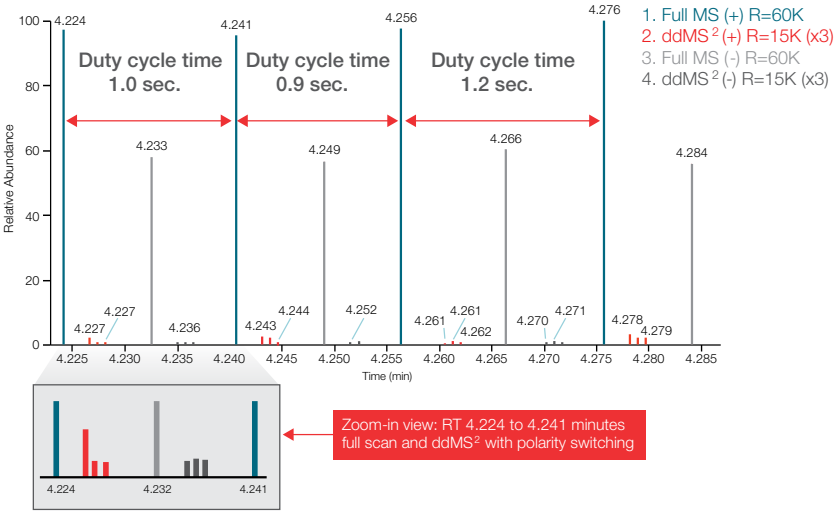


Figure 3. Acquisition cycle showing fast scan speed for high full MS and ddMS^2 with polarity switching acquisition.

As a result, a high-quality HRAM MS dataset with sub-ppm mass accuracy, isotope fine structure, and information rich Higher-energy Collisional Dissociation (HCD) MS/MS was collected; this provided ample information for definitive metabolite identification and structure elucidation.

Figure 4 shows that when using high-resolution polarity switching data acquisition, a timolol metabolite at RT 4.28 minute was detected with an accurate mass in both polarities: *m/z* (+)349.1541 and *m/z* (-)347.1395. The isotope fine structure showed that the Δmass of 1.9950 between 349.1541(A0) to 351.1491(A2 isotope) with a ratio of 1:0.04 indicates the presence of sulfur. Based on the accurate mass and isotope fine structure results, this metabolite was confidently identified, and the elemental composition confidently confirmed as $C_{13}H_{24}O_5N_4S$ with a formula weight of 348.1467 Da.

AcquireX intelligent data acquisition workflow improves metabolite profiling

Finding drug-related components present in complex biological matrices is challenging. To overcome the background interference, one common practice is manually generating background exclusion lists and inclusion lists by repeat injections and data acquisitions.

The Orbitrap Exploris 240 mass spectrometer includes novel AcquireX intelligent data acquisition workflows. AcquireX workflows use an advanced algorithm to generate a background exclusion list from the control and an inclusion list from the sample. The lists are then inserted into the data acquisition method(s), and the updated method conducts DDA MS/MS which triggers MS/MS analysis of only those ions of interest. AcquireX data acquisition conducts list generation and methods update in real-time and automatically, Figure 5.

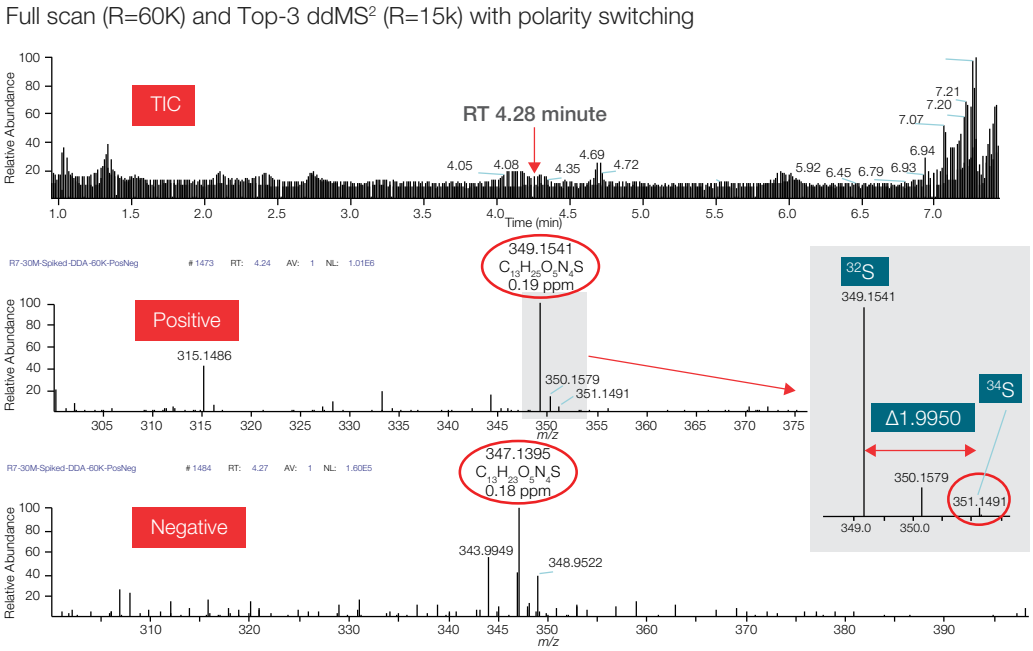


Figure 4. Polarity switching acquisition provided sub-ppm mass accuracy and isotope fine structure for confident metabolite identification.

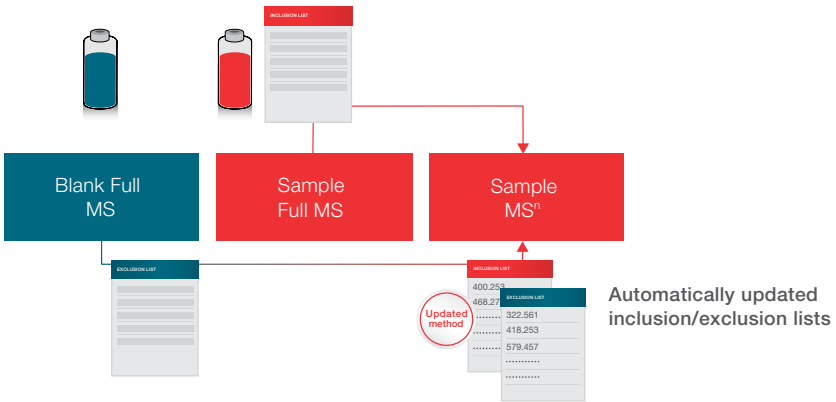


Figure 5. Illustration of the AcquireX intelligent automated production of inclusion & exclusion lists.

There are four AcquireX workflows that are designed to meet the needs of different applications:

- Background Exclusion
- Background Exclusion & Component Inclusion
- Iterative Precursor Exclusion
- Deep scan

Using AcquireX workflows, high-quality full scan and MS/MS data of drug-related compounds are obtained in one run, with no need for repeat injections and building of exclusion or inclusion lists offline.[2] Compared with traditional DDA, the AcquireX data acquisition strategy reduces false positives, while increasing the confidence and efficiency of routine metabolite analysis.

In this study, the AcquireX background exclusion workflow was used for model compounds metabolite analyses. A mass exclusion list was generated using the no-drug control, and the modified data acquisition methods were used for the analysis of rat liver microsome (RLM) incubation samples.

The AcquireX workflow modified method increased MS/MS coverage for potential metabolites by actively excluding matrix background ions and selectively triggering drug-related ions for ddMS^2. As a result, more low-level metabolites were identified. For example, a timolol metabolite *m/z* 349.1541 was identified by triggering MS/MS using the AcquireX background exclusion workflow, Figure 6. The same metabolite was not triggered for MS/MS using the conventional ddMS^2 method due to its low relative intensity <9% and the background interference.

When using the AcquireX Background Exclusion workflow, matrix ions are excluded from triggering DDA. This allows users to lower the MS/MS triggering intensity threshold to trigger more low-level metabolites and increase identification efficiency.

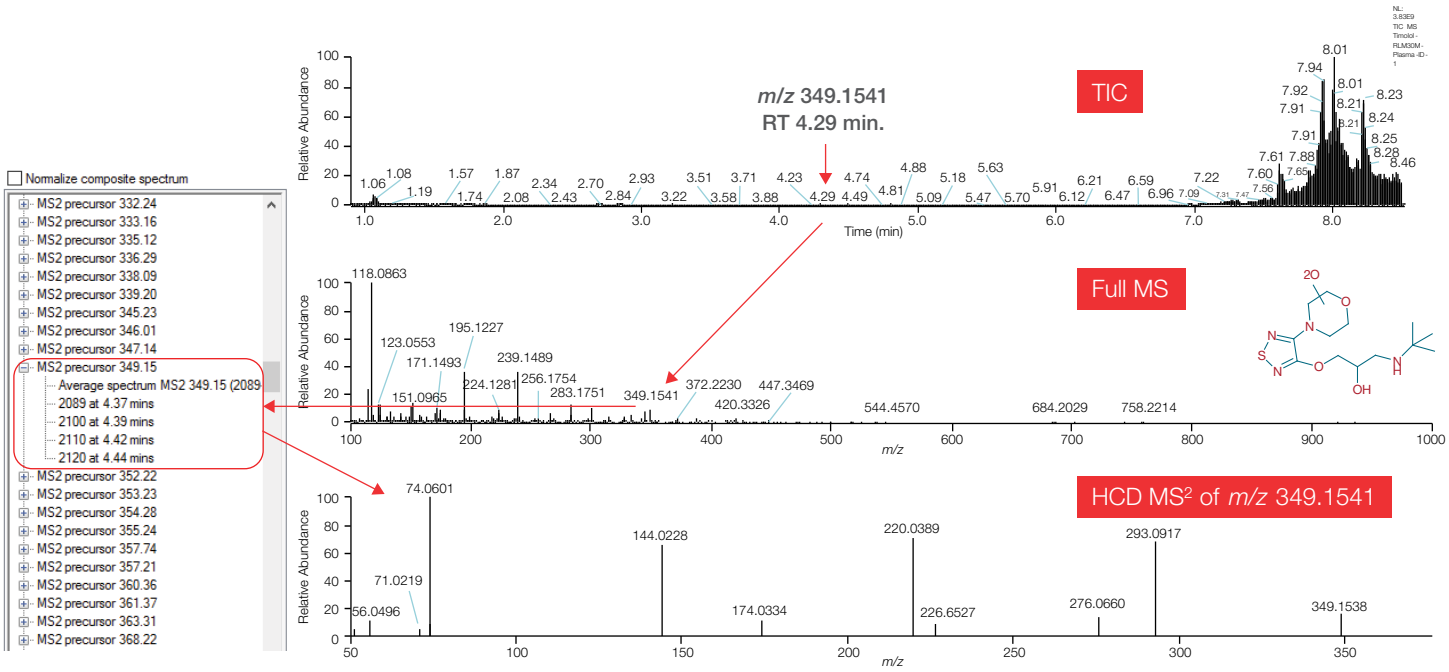


Figure 6. AcquireX background exclusion acquisition triggered low-level timolol metabolite.

Comparison of conventional DDA and AcquireX workflow

Nefazodone RLM 30 minutes incubation sample (10 µM) was spiked into deproteinated rat plasma at a 1:100 ratio (v/v) for a final concentration of 0.1 µM. The spiked samples were analyzed using both conventional DDA and the AcquireX background exclusion workflow. Data processed with Compound Discover software show that traditional DDA triggered 10 metabolites for MS/MS, and AcquireX background exclusion workflow triggered 21 metabolites for MS/MS.

Therefore, using AcquireX background exclusion workflow, more low abundant metabolites were triggered for MS^2, and 50% more nefazodone metabolites were identified (Table 1).

Table 1. Nefazodone metabolites identified using conventional DDA and AcquireX Background Exclusion workflow: 10 versus 21.

RT [min]	Molecular Weight	Formula	Transformations	Composition Change	DDA	AcquireX data acquisition
2.33	391.22195	$C_{19}H_{29}N_5O_4$	Oxidation, Oxidation	-(C_6H_3Cl) +(O_2)		✓
3.18	375.22704	$C_{19}H_{29}N_5O_3$	Oxidation	-(C_6H_3Cl) +(O)		✓
3.65	375.22704	$C_{19}H_{29}N_5O_3$	Oxidation	-(C_6H_3Cl) +(O)	✓	✓
4.05	196.07685	$C_{10}H_{13}ClN_2$	Reduction		✓	✓
4.29	409.18807	$C_{19}H_{28}ClN_5O_3$	Hydration	-(C_6H_4) +(O)		✓
4.41	359.23213	$C_{19}H_{29}N_5O_2$		-(C_6H_3Cl)	✓	✓
4.53	407.17242	$C_{19}H_{26}ClN_5O_3$	Oxidation	-(C_6H_6) +(O)		✓
4.57	373.21139	$C_{19}H_{27}N_5O_3$	Desaturation, Oxidation	-(C_6H_5Cl) +(O)		✓
4.67	393.19315	$C_{19}H_{28}ClN_5O_2$	Reduction	-(C_6H_4)		✓
4.77	307.15321	$C_{15}H_{21}N_3O_4$	Hydration	-($C_{10}H_{11}ClN_2$) +(O_2)		✓
4.79	407.17242	$C_{19}H_{26}ClN_5O_3$	Oxidative Deamination to Alcohol, Glycine Conjugation	-(C_6H_6) +(O)		✓
5.22	501.21428	$C_{25}H_{32}ClN_5O_4$	Desaturation, Oxidation	+(O_2)	✓	✓
5.72	291.15829	$C_{15}H_{21}N_3O_3$	Reduction	-($C_{10}H_{11}ClN_2$) +(O)	✓	✓
5.73	305.13756	$C_{15}H_{19}N_3O_4$	Oxidation	-($C_{10}H_{13}ClN_2$) +(O_2)	✓	✓
5.82	485.21937	$C_{25}H_{32}ClN_5O_3$		+(O)	✓	✓
5.89	305.13756	$C_{15}H19N_3O_4$	Oxidation	-($C_{10}H_{13}ClN_2$) +(O_2)	✓	✓
6.22	457.18782	$C_{23}H_{28}ClN_5O_3$	Oxidation, N-Dealkylation			✓
6.28	485.21937	$C_{25}H_{32}ClN_5O_3$	Oxidation	+(O)	✓	✓
6.44	501.21428	$C_{25}H_{32}ClN_5O_4$	Desaturation, Oxidation	+(O_2)	✓	✓
6.65	469.22445	$C_{25}H_{32}ClN_5O_2$	Parent Compound		✓	✓
6.70	483.20372	$C_{25}H_{30}ClN_5O_3$	Desaturation, Desaturation	-(H_2) +(O)		✓
6.79	485.21937	$C_{25}H_{32}ClN_5O_3$		+(O)		✓

Montelukast and timolol RLM 30 minutes incubation samples, both at 2 µM final concentration, were analyzed using conventional DDA and the AcquireX background exclusion workflow.

The results show that for montelukast, the conventional DDA method triggered MS/MS for 4 metabolites, while AcquireX background exclusion workflow triggered MS/MS for 11 metabolites, see Table 2. Similarly, for timolol; the conventional DDA triggered MS/MS for 5 metabolites, while AcquireX background exclusion workflow triggered MS/MS for 7 metabolites, see Table 3.

Table 2. Montelukast metabolites identified using conventional DDA and AcquireX Background Exclusion workflow: 4 versus 11.

RT [min]	Molecular Weight	Formula	Transformations	Composition Change	DDA	AcquireX data acquisition
4.48	908.28916	$C_{45}H_{53}ClN_4O_{10}S_2$	Oxidation, GSH Conjugation 2	$+(C_{10}H_{17}N_3O_7S)$	✓	✓
4.55	908.28916	$C_{45}H_{53}ClN_4O_{10}S_2$	Oxidation, GSH Conjugation 2	$+(C_{10}H_{17}N_3O_7S)$	✓	✓
5.00	892.29425	$C_{45}H_{53}ClN_4O_9S_2$	GSH Conjugation 2	$+(C_{10}H_{17}N_3O_6S)$		✓
5.48	617.20027	$C_{35}H_{36}ClNO_5S$	Oxidation, Oxidation	$+(O_2)$		✓
5.62	617.20027	$C_{35}H_{36}ClNO_5S$	Oxidation, Oxidation	$+(O_2)$	✓	✓
5.83	601.20536	$C_{35}H_{36}ClNO_4S$	Oxidation	$+(O)$		✓
6.10	601.20536	$C_{35}H_{36}ClNO_4S$	Oxidation	$+(O)$	✓	✓
6.10	583.19479	$C_{35}H_{34}ClNO_3S$	Desaturation	$-(H_2)$		✓
6.41	601.20536	$C_{35}H_{36}ClNO_4S$	Oxidation	$+(O)$		✓
6.54	601.20536	$C_{35}H_{36}ClNO_4S$	Oxidation	$+(O)$		✓
6.60	585.21044	$C_{35}H_{36}ClNO_3S$				✓
6.83	585.21044	$C_{35}H_{36}ClNO_3S$	Parent Compound		✓	✓

Table 3. Timolol metabolites identified using conventional DDA and AcquireX Background Exclusion workflow: 5 versus 7.

RT [min]	Molecular Weight	Formula	Transformations	Composition Change	DDA	AcquireX data acquisition
1.74	246.1151	$C_9H_{18}N_4O_2S$			✓	✓
3.22	290.1413	$C_{11}H_{22}N_4O_3S$			✓	✓
4.22	314.14126	$C_{13}H_{22}N_4O_3S$	Desaturation	$-(H_2)$	✓	✓
4.29	332.15183	$C_{13}H_{24}N_4O_4S$		$+(O)$	✓	✓
4.40	348.14674	$C_{13}H_{24}N_4O_5S$	Oxidation, Oxidation	$+(O_2)$		✓
6.13	316.15691	$C_{13}H_{24}N_4O_3S$	Parent Compound		✓	✓
7.14	314.14126	$C_{13}H_{22}N_4O_3S$	Dehydration	$-(H_2)$	✓	✓
7.12	346.13109	$C_{13}H_{22}N_4O_5S$	Desaturation, Oxidation, Oxidation	$-(H_2)+(O_2)$		✓

Intelligent data processing using Compound Discoverer software

With high-quality HRAM full scan and HCD ddMS^2 data acquisition, an effective data mining tool is essential for confident metabolite identification. In this study, the data processing was carried out using Compound Discoverer small molecule identification software, which uses a fully customizable node-based processing workflow to process HRAM full scan MS, MS/MS data, and fine isotope pattern for structure identification and database searching.[3]

Predefined processing workflow templates are available for quick method setup in Compound Discoverer software. For this study, the workflow template, *MetID with Stats Expected and Unknown with Background Removal*, was used (Figure 8). This workflow uses Targeted and User Defined approaches to capture the expected and the unexpected or unknown metabolites.

A targeted approach to identifying expected metabolites

- Compound Discoverer software detects the metabolites from the expected metabolite list which was generated based on the intelligent dealkylation/dearylation predictions and common metabolic pathways list.
- Subsequently, the FISh Scoring node (FISh = fragment ion search) automatically annotates the MS^2 fragments which match and compare with parent MS^2, and color codes/annotates the fragment ions with mass shift and proposes the type of biotransformation if possible. This facilitates localizing the site of biotransformation.

User-defined approach to identify the unexpected or unknown metabolites

- Pattern Scoring node finds compounds that have user-defined unique isotopic patterns. This is useful for radiolabeled study data processing, and compounds containing sulfur, chlorine, bromine, etc.
- Compound Class Scoring finds compounds that share the same MS^2 fragments with the parent and known metabolites.

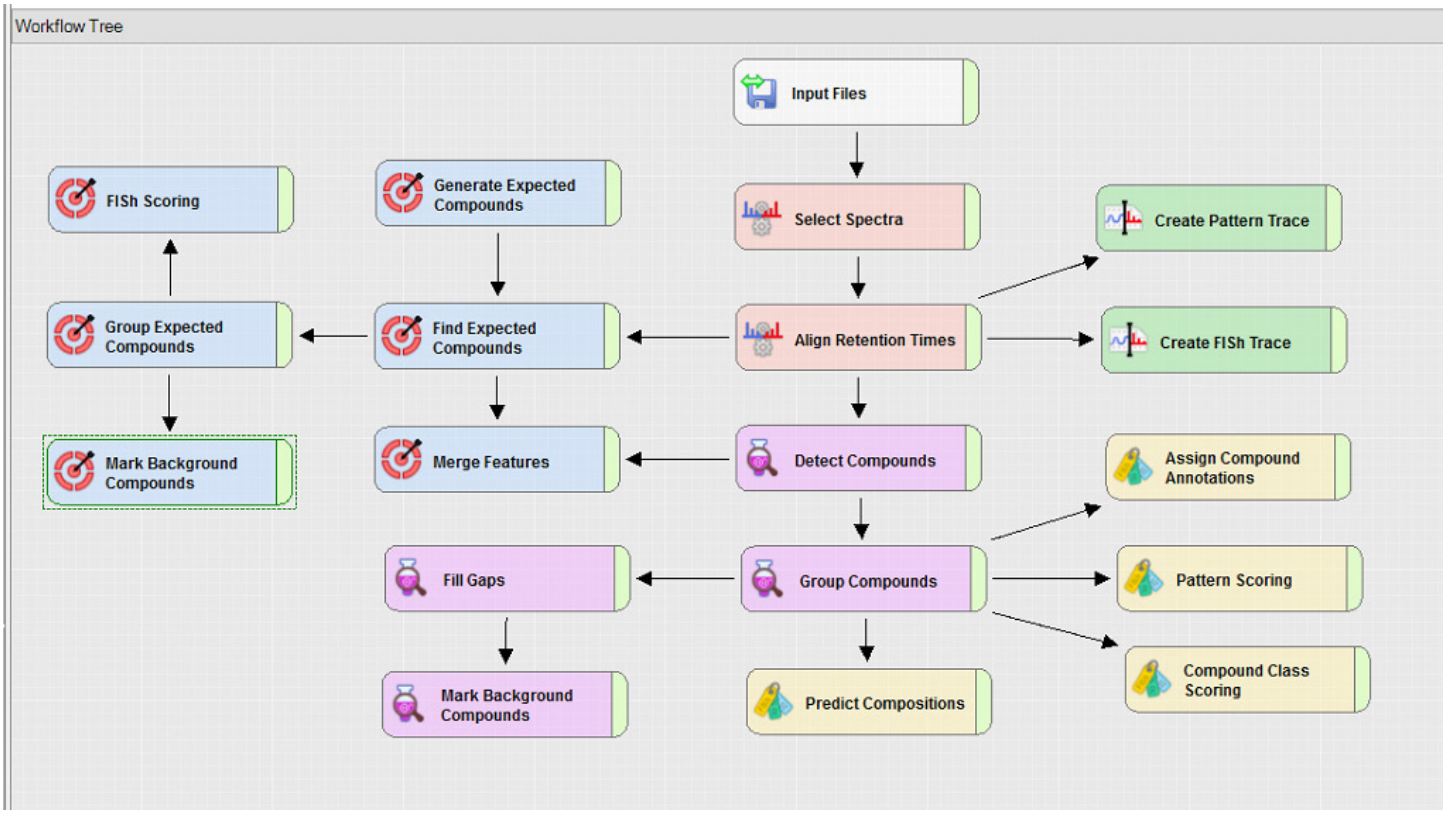


Figure 8. Compound Discoverer software predefined processing workflow: MetID with Stats Expected and Unknown with Background Removal.

The processed results showed that the expected timolol metabolites were readily identified using the Find Expected Compounds node. The auto annotation and mirror plot of MS^2 fragments from parent timolol and its metabolite aided quick determination of the site of modification (Figure 9).

The unexpected N-dealkylated metabolites of timolol were identified through Class Coverage and Pattern Matches nodes and the structures were proposed, see Figure 10.

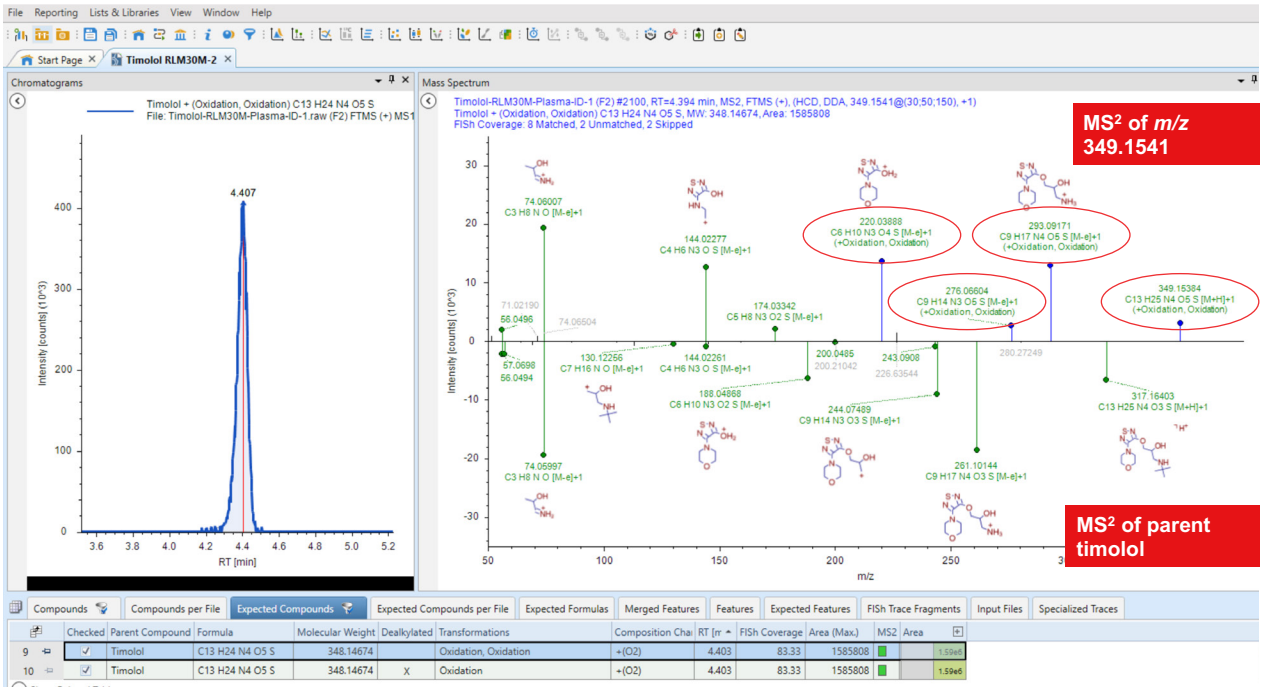


Figure 9. Auto annotation and mirror plot of MS^2 facilitate structure elucidation—green lines represent unchanged fragments matching with the parent compound fragments, and the blue lines represent the modified fragments compared to the parent compound.

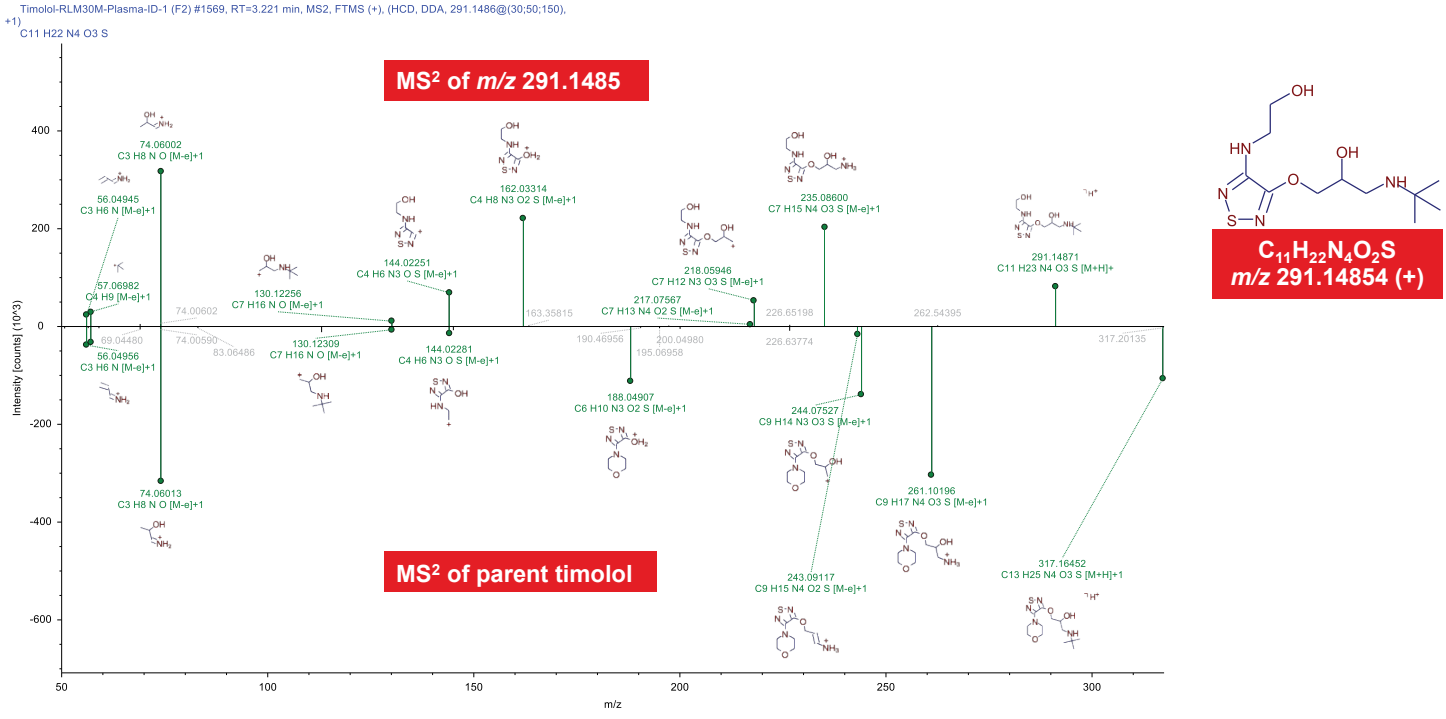


Figure 10. Unexpected N-dealkylated metabolite of timolol identified using Class Coverage and Pattern Matches software features.

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Conclusion

The results demonstrate the high-confidence structural elucidation capabilities enabled by the exceptional spectral clarity and mass accuracy in data generated from the Orbitrap Exploris 240 mass spectrometer.

Coupled with Vanquish Horizon UHPLC system and Compound Discoverer software, the Orbitrap Exploris 240 MS provides a comprehensive workflow for routine metabolite profiling.

The AcquireX background exclusion workflow improves metabolite identification by around 30~50% relative to conventional acquisition methods through the increased triggering of MS^2 of the drug-related metabolites with greater confidence and efficiency.

The system demonstrated that it is well suited for challenging metabolite profiling studies to identify low abundance metabolites present in complex biological matrices.

References

1. Zhu M. et al. Drug Metabolite Profiling and Identification by High-resolution Mass Spectrometry. The Journal of Biological Chemistry 2011, 286 (29), 25419-25425
2. Smart Note: AcquireX Intelligent Data Acquisition Technology for Orbitrap Tribrid Mass Spectrometers – Collect more Meaningful Data, Not Just More Data (SN65392-EN 0720M)
3. White Paper: Compound Discoverer Software – Compounding Insights for Small Molecule Research (WP65210-EN 0518S)

Find out more at **thermofisher.com/AcquireX**

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