Pharma and biopharma

# Accelerating metabolite identification with mass spectrometry

thermo scientific

# Contents



# 1. Why is metabolite identification critical for drug discovery?

Why do drugs that show high efficacy *in vitro* or in animal models often fail in clinical trials? The answer lies in drug metabolism (Figure 1), which is critical to ensuring drug safety, efficacy and optimal pharmacokinetics. In the liver, cytochrome P450 enzymes convert drugs by oxidation, reduction, hydrolysis and conjugation. Identification and characterization of these metabolites is essential for understanding the metabolic fate of a drug.

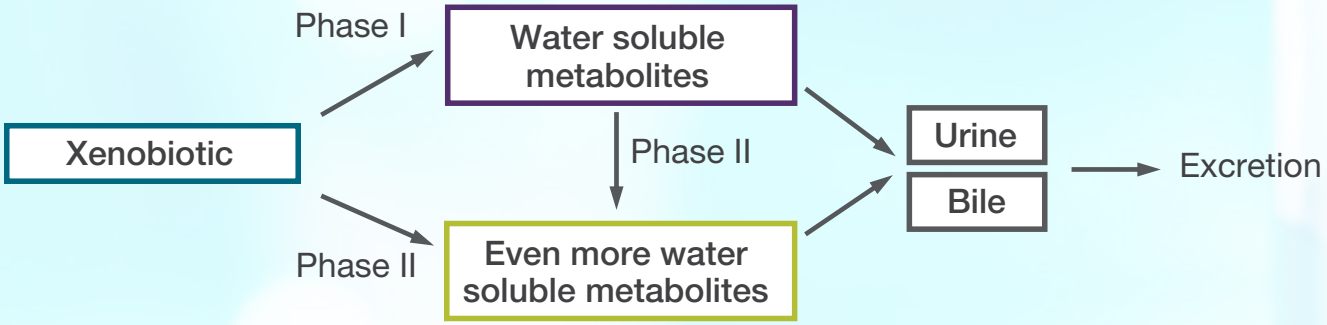



**Figure 1. Overview of drug metabolism for a xenobiotic compound with Phase I and II denoting different transformation reactions to 1) oxidize or introduce reactive groups and 2) conjugate polar species to increase hydrophilicity (Figure taken from white paper).**

Metabolite identification (MetID) provides critical insight into the behavior of a drug in the body and is essential for optimizing lead compounds and refining drug development strategies. Understanding metabolic pathways helps researchers predict and evaluate metabolites that may enhance therapeutic effects or pose toxicity risks. The MetID process involves several steps, including sample preparation, chromatographic separation, mass spectrometry analysis and data processing.

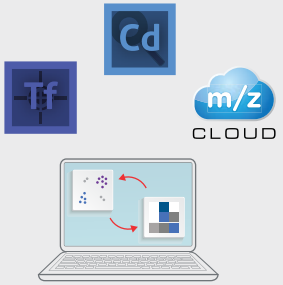
Human metabolites accounting for more than 10% of total drug-related exposure raise safety concerns and require structural characterization. Therefore, the primary focus is to determine the structure and relative concentrations of key metabolites in biological matrices, followed by the synthesis of reference standards and assessment of their safety and efficacy.

**MetID aims to analyze the structure of metabolites in complex biological matrices and determine their *in vitro*/*in vivo* biotransformation and clearance. By integrating this knowledge with drug metabolism studies, scientists can anticipate metabolic variations, identify potential risks early, and develop mitigation strategies that enhance patient safety and support regulatory approval.**

Chromatographic separations → Mass spectrometry data acquisition → Data processing

Thermo Scientific™ Vanquish™ Horizon UHPLC system, Thermo Scientific™ Accucore™ C30 HPLC column, Thermo Scientific™ Hypersil GOLD™ C18 Selectivity HPLC column


Thermo Scientific™ Orbitrap™ Exploris™ 240 mass spectrometer and Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer




Thermo Scientific™ Compound Discoverer™ software, Thermo Scientific™ TraceFinder™ Software, Thermo Scientific™ mzCloud™ mass spectral library

**Figure 2. Workflow overview for drug metabolite identification.**

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## 1.1. How to perform metabolite identification in drug discovery?

MetID relies on liquid chromatography (LC) to separate structurally distinct compounds, followed by mass spectrometry (MS) for detailed structural analysis. However, traditional low-resolution MS methods often fail to identify complex metabolites with confidence due to limited precision and resolving power.

## 1.2. Why is HRAM MS advantageous in metabolite identification?

High-resolution accurate-mass (HRAM) mass spectrometry has become the industry standard, addressing analytical challenges with unmatched mass accuracy, resolving power, and sensitivity. Its high-throughput capabilities make liquid chromatography-high-resolution mass spectrometry (LC-HRMS) the preferred instrumentation for metabolite identification in the pharmaceutical industry.

Among HRMS, Thermo Scientific™ Orbitrap™ HRAM MS stands out for its exceptional accuracy and precision. Leveraging high-resolution, accurate mass (HRAM) capabilities, these advanced systems ensure reliable metabolite identification. Integrating with LC, Orbitrap HRAM MS enhances the separation of both known and unknown compounds, optimizing high-throughput workflows for more efficient and comprehensive analysis (Figure 3).

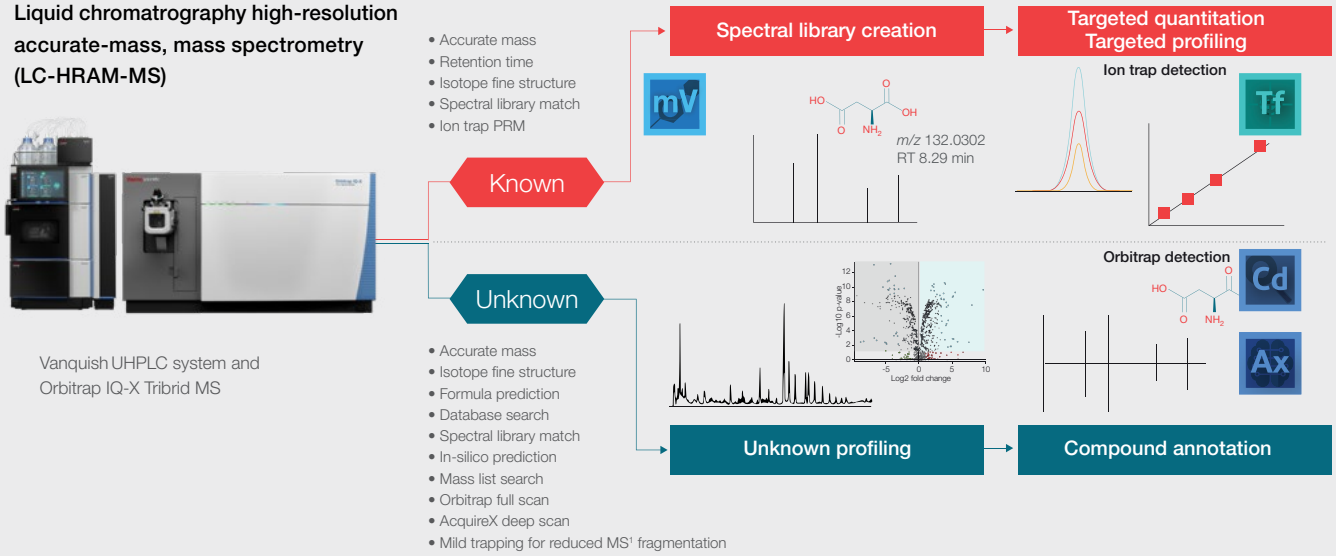


**Figure 3. Orbitrap HRMS enables simultaneous quantification of known metabolites and discovery of unknown compounds within a single integrated workflow.** Using reference standards and isotope labeling, known metabolites are quantified with high precision via spectral matching and $MS^2$ confirmation. Parallel untargeted profiling leverages AcquireX-driven acquisition, statistical analysis, and in-silico tools, including formula prediction, database searching, and spectral libraries, to identify novel metabolite signatures and enhance annotation confidence.

Orbitrap HRAM MS can measure known metabolites and discover unknown compounds simultaneously in a single workflow. Individual reference standards, with or without stable isotope labels, can be used to establish retention time, mass measurement, isotopic fine structure and $MS^2$ spectral confirmation against an in-house library to identify and quantify target metabolites. When stable isotope labels are used, absolute quantification can be achieved. As part of the same workflow, statistical analysis focuses on relevant biological changes in unknown compounds. Intelligence-driven data acquisition strategies, such as Thermo Scientific™ AcquireX software, enable scientists to gain a deeper understanding of the sample while providing an overview of known metabolites. Annotation tools, including formula prediction, database searches, spectral library searches and in-silico prediction, increase annotation confidence.

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# 2. What are the challenges in drug metabolite identification?

## 2.1. Inefficient and time-consuming data processing in metabolite identification

Metabolite identification generates large datasets that require extensive processing and interpretation. Traditional workflows often rely on manual data review, which is time-consuming, error-prone, and inefficient—particularly when handling unknown or unexpected metabolites. Distinguishing true metabolites from background noise and confidently assigning structures to detected compounds remains a significant challenge. Without automated, systematic processing tools, researchers encounter major bottlenecks in workflow efficiency and data reliability. These challenges can result in delays in metabolite characterization, extended drug development timelines, and an increased risk of overlooking potentially toxic or pharmacologically relevant metabolites.

## 2.2. Limited sensitivity and selectivity in metabolite detection

The detection and characterization of low-abundance and reactive metabolites is a critical step in drug metabolism studies. Some drugs form highly reactive metabolites *in vivo*, which typically bind to glutathione (GSH) for elimination. At higher doses, however, these reactive species can contribute to toxicity, making their early and reliable identification essential. Traditional MS techniques often struggle to detect and characterize low abundance or reactive metabolites, resulting in incomplete metabolite profiling. Sensitivity and selectivity are critical to confidently identify these metabolites, particularly in GSH trapping assays where reactive species must be accurately captured to assess potential drug toxicity. If these metabolites are not identified early in the development process, it can lead to unexpected toxicological findings at a later stage of the study. This could result in delays or even termination of the program.

## 2.3. Interference from complex biological matrices

Endogenous compounds present in biological samples can generate background signals that can interfere with the detection of metabolites, particularly for low abundance species. This matrix effect challenge can obscure critical metabolites during data acquisition, reducing the clarity and accuracy of identification. Such interference makes it difficult to collect non-targeted fragmentation data, ultimately compromising comprehensive drug metabolism characterization.

Failure to address this issue can result in metabolites being missed or misidentified, leading to gaps in metabolic profiling. These gaps may impact safety assessments, structure–activity relationships or regulatory submissions.

## 2.4. Complex metabolic pathways and structural elucidation

The metabolism of heterobifunctional PROTAC (Proteolysis Targeting Chimera) drugs presents unique challenges due to their large, complex structures and multi-step biotransformations. Conventional LC-MS workflows can struggle to identify transformation sites with confidence, making it difficult to determine the metabolic fate of these compounds. This uncertainty can hinder the prediction of pharmacokinetic behavior, obscure potential safety risks and complicate regulatory evaluations requiring detailed metabolite characterization.

# 3. How can Thermo Fisher Scientific help you meet these challenges?

## 3.1. Automated data processing with Compound Discoverer software

MetID data processing is often complex and time-consuming. To address these challenges, Compound Discoverer software offers a fully automated and customizable node-based workflow that streamlines metabolite identification, even in complex datasets. This approach significantly reduces manual effort while increasing confidence in results.

In this technical note it is shown that Orbitrap Exploris 240 mass spectrometer, coupled with Vanquish Horizon UHPLC and Compound Discoverer software, provides a high-throughput and automated workflow to efficiently process complex metabolite data and confidently identify isotope-labelled GSH-trapped reactive metabolites in biological matrices.

**How was this performed?**

In the study, the antipsychotic drug clozapine was chosen as a reference compound to demonstrate the data acquisition and processing workflow. GSH conjugates of clozapine were generated in rat liver microsomal incubations containing a 1:1 mixture of unlabeled GSH and stable isotope-labeled GSH ([13C2, 15N]). Samples were analyzed by reversed-phase chromatography on a Thermo Scientific™ Vanquish™ Flex UHPLC system coupled with an Orbitrap Exploris 240 mass spectrometer (Figure 4).

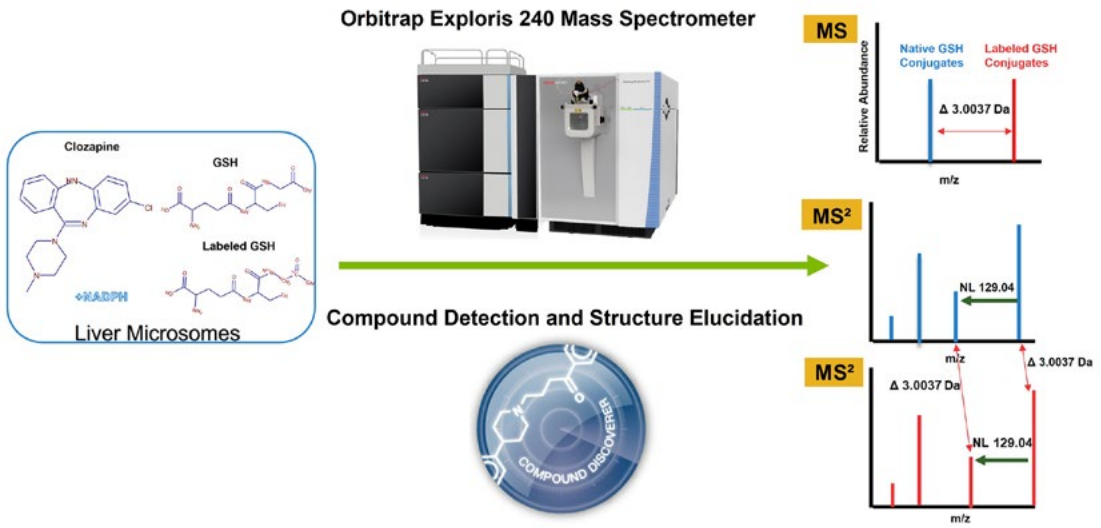



**Figure 4. Workflow overview for the detection and identification of labeled glutathione-trapped reactive metabolites using the Orbitrap Exploris 240 MS and Compound Discoverer software.**

Click on the thumbnails for more information

## What did the study show?

The Orbitrap Exploris 240 mass spectrometer provided HRAM Full Scan and HCD-MS$^2$ data with sub-ppm mass accuracy, enabling confident metabolite detection and structural characterization.

MetID was streamlined using Compound Discoverer software, which employed a single processing workflow (figure 5) for both expected and unknown compounds. To enhance identification confidence:

**Figure 5. Compound Discoverer software workflow tree for the study.** This workflow template was customized based on a combination of nodes from the Compound Discoverer software MetID w Stats Expected and the Unknown w Background Removal Workflow trees to create a combined workflow.

- The Pattern Scoring node flagged compounds based on their distinct isotopic pattern from isotope-labeled GSH incorporation (Figure 6).

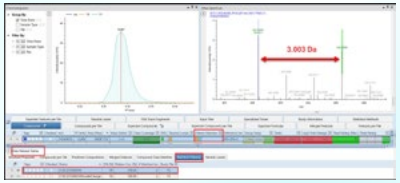


**Figure 6. Pattern Scoring node flags unknown compounds matching user specified artificial isotopic pattern(s) and it adds the related Matched Patterns table to the result file (highlighted in red).**

- The Search Neutral Losses node identified compounds with characteristic GSH neutral loss fragments (Figure 7).

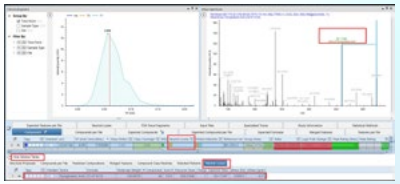


**Figure 7. Search Neutral Losses node reports unknown compounds with specified neutral loss fragment.** Related Neutral Losses table provides more detailed information.

- The FISh Scoring node facilitated in silico fragmentation prediction, automatically annotating spectra with predicted fragment structures for structural elucidation (Figure 8).

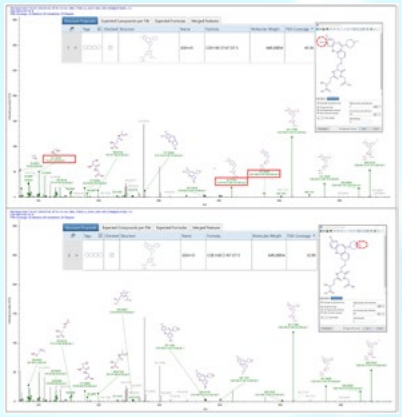


**Figure 8. Fragmentation prediction with user proposed structure.** Applying FISh Scoring to the proposed structure (inset box) of the clozapine-GSH conjugate for fragment ion matching and structure annotation. Due to the difference in oxidation position, MS$^2$ spectral interpretation confirmed that the fragment ions m/z 475.08356, m/z 421.07401, and m/z 101.10738 matched the structure proposal, suggesting hydroxylation on the chlorobenzene ring and not the N-oxide metabolite.

This work demonstrates the integration of Orbitrap Exploris 240 MS, Vanquish Horizon UHPLC and Compound Discoverer software for efficient metabolite data processing. This approach reduces manual effort, increases identification confidence and accelerates the detection of low-abundance reactive metabolites. Using automated data filtering and structural annotation, this workflow successfully identified nine GSH-bound reactive metabolites of clozapine (Table 1).

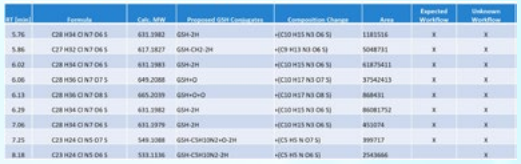


**Table 1. Summary of GSH-capped reactive metabolites of clozapine identified by Compound Discoverer software.**

## 3.2. Confident detection of low-abundance and reactive metabolite

To overcome the challenges of low sensitivity and selectivity, Orbitrap HRAM MS provides high-resolution and accurate mass detection, enabling precise identification of reactive metabolites. Using sub-ppm mass accuracy and automated data processing, it streamlines GSH trapping assays and ensures reliable detection of bioactivation pathways.

The detection of low abundance reactive metabolites remains a significant challenge in metabolite identification. The case study shows how the integration of the Orbitrap Exploris 240 mass spectrometer with Vanquish Horizon UHPLC and Compound Discoverer software increases sensitivity and selectivity, enabling accurate detection of trace metabolites. The high-resolution capabilities of this system enabled the successful identification and characterization of nine GSH-capped reactive metabolites of clozapine (Table 1).

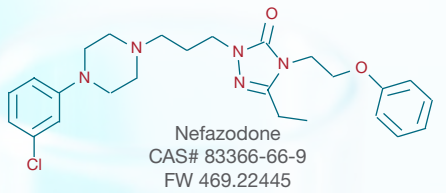
# 3.3. Reducing background interference from complex biological matrices

Accurate metabolite identification in drug metabolism studies is often hampered by background interference from complex biological matrices, which can obscure low-abundance metabolites and reduce identification confidence. To address this, Orbitrap HRAM MS, combined with advanced data acquisition strategies, provides a targeted solution to remove background noise and improve metabolite detection.

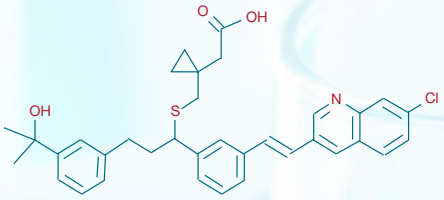
This study demonstrates how the Orbitrap Exploris 240 mass spectrometer, paired with the Vanquish Horizon UHPLC system and Compound Discoverer software, overcomes the challenges of matrix interference using Thermo Scientific™ AcquireX™ intelligent data acquisition. This automated background exclusion workflow enhances metabolite detection efficiency, enabling confident structural characterization of low-abundance metabolites in complex samples.

**How was it done?**

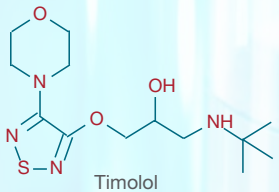
Metabolite formation with model compounds (nefazodone, montelukast, and timolol) were studied using human and rat liver microsomal incubations with relevant co-factors to mimic metabolic reactions (Figure 9). A negative control was included for background subtraction. After incubation, the enzymatic reaction was quenched, and protein precipitation was performed. The processed samples were then spiked into plasma to simulate *in vivo* conditions, followed by LC-MS analysis for metabolite identification.




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 9. Structures of model compounds: nefazodone, montelukast, and timolol.**

Click on the thumbnails for more information

## What was observed?

**Improved detection of low abundance metabolites:** The Orbitrap Exploris 240 MS provided high-resolution, full-scan MS/MS data with sub-ppm mass accuracy, ensuring confident metabolite identification. Polarity switching data acquisition enabled the detection of a timolol metabolite with a retention time (RT) of 4.28 min in both positive (m/z 349.1541) and negative (m/z 347.1395) ion modes. Isotopic fine structure analysis confirmed the presence of sulphur, and the accurate mass data allowed confident identification of the metabolite with an elemental composition of $C_{13}H_{24}O_5N_4S$ (348.1467 Da) (Figure 10).

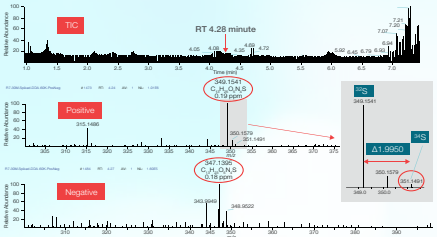



**Figure 10. Polarity-switched acquisition provided sub-ppm mass accuracy and isotopic fine structure for confident metabolite identification.**

**Improved removal of background interference:** The AcquireX intelligent data acquisition workflow (Figure 11) automatically excluded background ions in real time, improving metabolite detection efficiency (Figure 12).

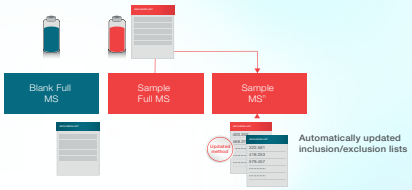



**Figure 11. Illustration of AcquireX intelligent automated creation of inclusion and exclusion lists.**

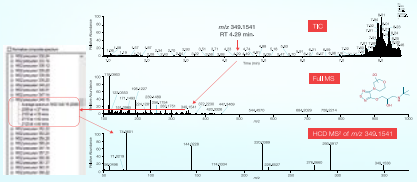



**Figure 12. AcquireX background exclusion acquisition triggered by low level timolol metabolite.**

**Higher metabolite identification rate:** Compared to Data-Dependent Acquisition (DDA) , AcquireX increased metabolite detection efficiency by **30–50%**, enabling the identification of more low-abundance metabolites (Table 2-4).

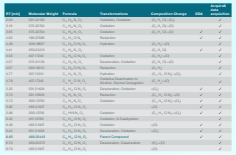


**Table 2. Nefazodone metabolites identified by conventional DDA and AcquireX Background Exclusion workflow: 10 versus 21.**

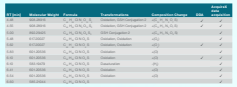


**Table 3. Montelukast metabolites identified by conventional DDA and AcquireX Background Exclusion workflow: 4 versus 11.**

**Table 4. Timolol metabolites identified by conventional DDA and AcquireX Background Exclusion workflow: 5 versus 7.**

**Faster and more reliable structure elucidation:** The fast scan speed and rapid polarity switching of the Orbitrap Exploris 240 MS allowed comprehensive metabolite characterization in a single run (Figure 13).

**Figure 13. Automatic annotation and mirror plot of MS² 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.**

The case study demonstrated that the AcquireX background exclusion workflow improves metabolite identification by **30-50%** compared to conventional acquisition methods (DDA). By increasing MS/MS triggering of drug-related metabolites, it improves detection confidence and efficiency. This approach is highly effective for challenging metabolite profiling studies, enabling the identification of low-abundance metabolites in complex biological matrices with greater accuracy.

## 3.4. Reliable identification of PROTAC metabolites with multi-step fragmentation

PROTACs are bifunctional small molecules designed to induce catalytic protein degradation via the ubiquitin-proteasomal pathway. Their ability to selectively degrade disease-causing proteins has led to rapid progress in drug discovery, with the first phase III clinical trial (ARV-471) starting in 2022. As PROTACs enter clinical development, understanding their metabolic fate is critical to optimizing their efficacy and safety.

Metabolite characterization of PROTACs requires advanced fragmentation strategies to accurately pinpoint transformation sites and elucidate metabolic pathways. This case study demonstrates how multi-stage fragmentation ($MS^n$) on the ThermoScientific™ Orbitrap™ Ascend BioPharma Tribrid™ mass spectrometer, combined with AcquireX intelligent data acquisition and Compound Discoverer software, provides a powerful and automated workflow for identifying and profiling PROTAC metabolites. By utilizing $MS^n$ analysis, this workflow enables confident identification of transformation sites, providing deeper insight into PROTAC metabolism (Figure 14).

**How was it done?**

Model PROTAC compounds (MZ1, dBET1) were incubated with human liver S9 fraction in phosphate buffer at 37°C. Metabolism was initiated with NADPH and stopped at 0 and 4 hours with ice-cold acetonitrile. Samples were centrifuged and the supernatants analyzed by LC-MS. A matrix blank control was included for comparison.

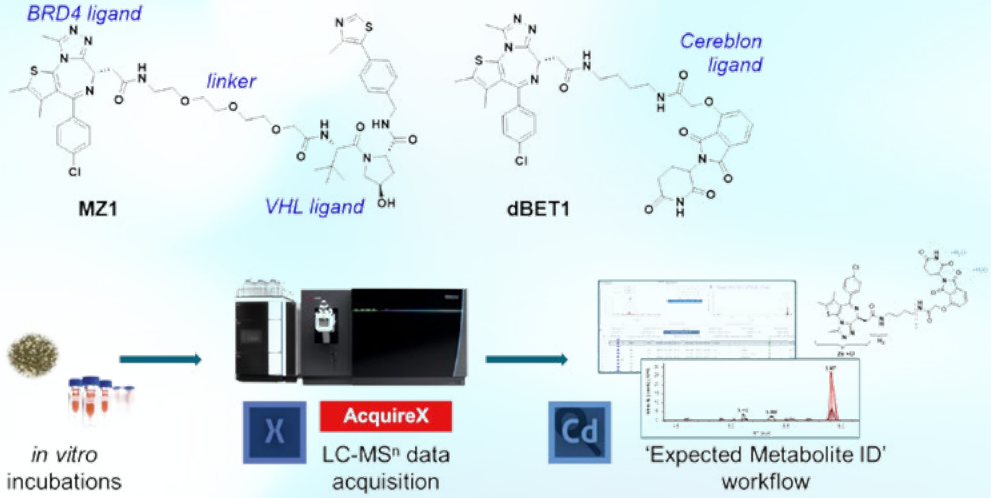



**Figure 14. (A) Structures of the PROTAC model compounds MZ1 and dBET1 with shared BRD4 ligand substructure; (B) Overview of the Metabolite ID application steps from *in vitro* incubation of test compounds, LC-MS data acquisition using Intelligent MSn Data Acquisition to data processing facilitated by the "Expected Metabolite ID" workflow in the Compound Discoverer software.**

Click on the thumbnails for more information

## What was observed?

**Comprehensive metabolite profiling:** A total of 24 metabolites for MZ1 and 12 for dBET1 were detected and structurally characterized using Compound Discoverer software (Figure 15,16). The software's customizable, node-based workflow processed HRAM full-scan MS, $MS^n$ data and fine isotope patterns to enable confident identification of small molecules through structural analysis and database searching.

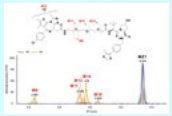


**Figure 15. XICs of MZ1 and its five most abundant metabolites, with their transformation sites marked on the structure above.**

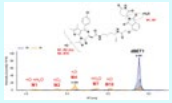


**Figure 16. XICs of dBET1 and its five most abundant metabolites, with their transformation sites marked on the structure above.**

**Identification of fragment ion assignments for metabolites:** Major metabolite transformation including oxidation, linker cleavage and hydrolysis, were confirmed by $MS^n$ fragmentation spectra (Figure 17-20).

**Figure 17. Fragmentation spectra of the [M+Na]+ ion for the oxidized metabolite M18 of MZ1, highlighting the fragment ion assignments allowing the localization of the oxidation site to the BRD4 ligand from the oxidation-shifted $MS^3$ fragment at m/z 395.**

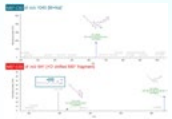


**Figure 18. Fragmentation spectra of the [M+Na]+ ion for the oxidized metabolite M23 of MZ1, highlighting the fragment ion assignments allowing the localization of the oxidation site to the linker portion from the oxidation-shifted $MS^3$ fragments.**

**Figure 19. Comparison of the $MS^2$-CID spectra for the "dBET1+H2O" metabolites M7 and M8 providing evidence for their different ring opening hydrolysis locations.**

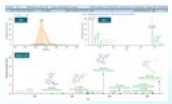


**Figure 20. Overview of the $MS^1$ and $MS^n$ data supporting the metabolite assignment for M14, resulting from a "dealkylation" linker cleavage, with fragment ions matching the in silico predicted fragments of the metabolite automatically highlighted in the Compound Discoverer software.**

**Improved metabolite identification using AcquireX data acquisition:** The Thermo Scientific™ AcquireX™ background exclusion workflow minimized matrix interference and ensured that $MS^n$ acquisition focused only on relevant compounds (Figure 21). This approach expanded fragmentation coverage, reducing the need for additional targeted $MS^n$ experiments.

**Figure 21. Experiment decision tree used to acquire MSn data in versatile fashion, with $MS^3$ data acquired only on precursors exceeding 500 Da to increase efficiency.**

**Improved structural characterization with $MS^n$:** the dual-pressure linear ion-trap, afforded by Orbitrap Ascend, enables stepwise fragmentation, allowing precise localization of transformation sites (Figure 17-20). $MS^3$ fragmentation spectra helped resolve isomeric metabolites and distinguish structural modifications.

Integrating Orbitrap $MS^n$ fragmentation, AcquireX intelligent data acquisition and Compound Discoverer automated data processing, this workflow streamlines metabolite characterization with PROTAC. It provides confident metabolic site localization, even for low abundance and structurally complex metabolites, making it an ideal solution for drug metabolism studies.

# 4. Conclusion

The successful identification and characterization of drug metabolites are critical for optimizing drug efficacy, safety, and pharmacokinetics. Traditional metabolite identification methods often face challenges such as low sensitivity, complex biological matrices, data processing, and structural elucidation of complex compounds such as PROTACs.

Orbitrap HRAM MS solutions, combined with AcquireX Intelligent Data Acquisition and Compounded Software tools, offer a powerful and streamlined approach to overcoming these challenges. By integrating high-resolution mass spectrometry, automated data processing, and advanced analytical workflows, researchers can achieve more confident MetID, faster data analysis, and deeper biological insights—ultimately accelerating the drug discovery and development process.

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**Resources**

**Literature**

- White paper: Increased confidence in drug metabolite identification through intelligent data acquisition strategies and multiple fragmentation techniques on the Orbitrap Tribrid MS platform
- Case study: A streamlined solution for confident detection and identification of isotope-labeled glutathione-trapped reactive drug metabolites using the Orbitrap Exploris 240 MS and the Compound Discoverer software

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