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Pharma

Increased confidence in drug metabolite identification through intelligent data acquisition strategies and multiple fragmentation techniques on the Orbitrap Tribrid MS platform

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Keywords

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Overview

- The LC/MS analysis of drug metabolites relies on the accurate and sensitive detection of components in complex matrices. In addition, confident metabolite annotation generally requires fragmentation data for each metabolite to allow structure elucidation.
- Intelligent data acquisition technologies available on the Thermo Scientific™ Orbitrap™ Tribrid™ mass spectrometer platform, such as Thermo Scientific™ AcquireX™ data acquisition software and real-time library search, streamline the process to acquire sample relevant MS^2 and MS^n data for deeper drug metabolite profiling.
- Orthogonal fragmentation methods such as Ultraviolet Photodissociation (UVPD) can provide complementary fragments to allow determination of transformation sites for drug metabolites from structurally informative fragments.

Introduction

Metabolite identification (MetID) is a crucial and integral part of drug discovery and development. In this context, metabolism describes the breakdown and conversion of xenobiotics into molecules that can be excreted from the body, as shown in Figure 1. However, rapid metabolism reduces the duration and potential efficacy of pharmaceuticals, so in early drug discovery stages, metabolic profiling is employed

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to identify metabolic soft spots to inform optimization of lead compounds to attain desired pharmacokinetics. Additionally, metabolic profiling plays a critical role in evaluating the safety of the molecule, as in some cases the intermediates in the drug metabolism can have toxic effects.

Along with NMR, the predominant tool for identification of drug biotransformation products is liquid chromatography coupled with mass spectrometry (LC/MS). The instruments used need to be sensitive enough to detect critical metabolites and collect fragmentation data to provide crucial information for structure elucidation, enabling identification of the site of metabolism.

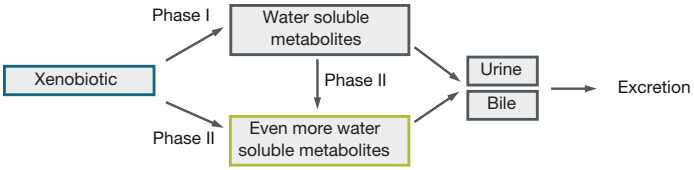

Figure 1. Overview of drug metabolism for a xenobiotic compound with Phase I and II denoting different transformation reactions to 1) oxidize or introduce reactive groups and 2) conjugate polar species to increase hydrophilicity

Increasing relevant data acquisition through adaptive instrument methods

For structural elucidation of drug metabolites and to determine the transformation site, fragmentation data is typically needed. To obtain fragmentation coverage of all metabolites, the fragmentation data are ideally acquired on all relevant metabolites together with full scan mass spectral data in a single injection and then analyzed using software such as Thermo Scientific™ Compound Discoverer™ software and Thermo Scientific™ Mass Frontier™ software. However, one of the main challenges in the profiling of drug metabolites, both with in vitro and in vivo samples, is that complex background signals from matrix components can obscure low level metabolites during data acquisition, making the untargeted fragmentation data acquisition difficult.

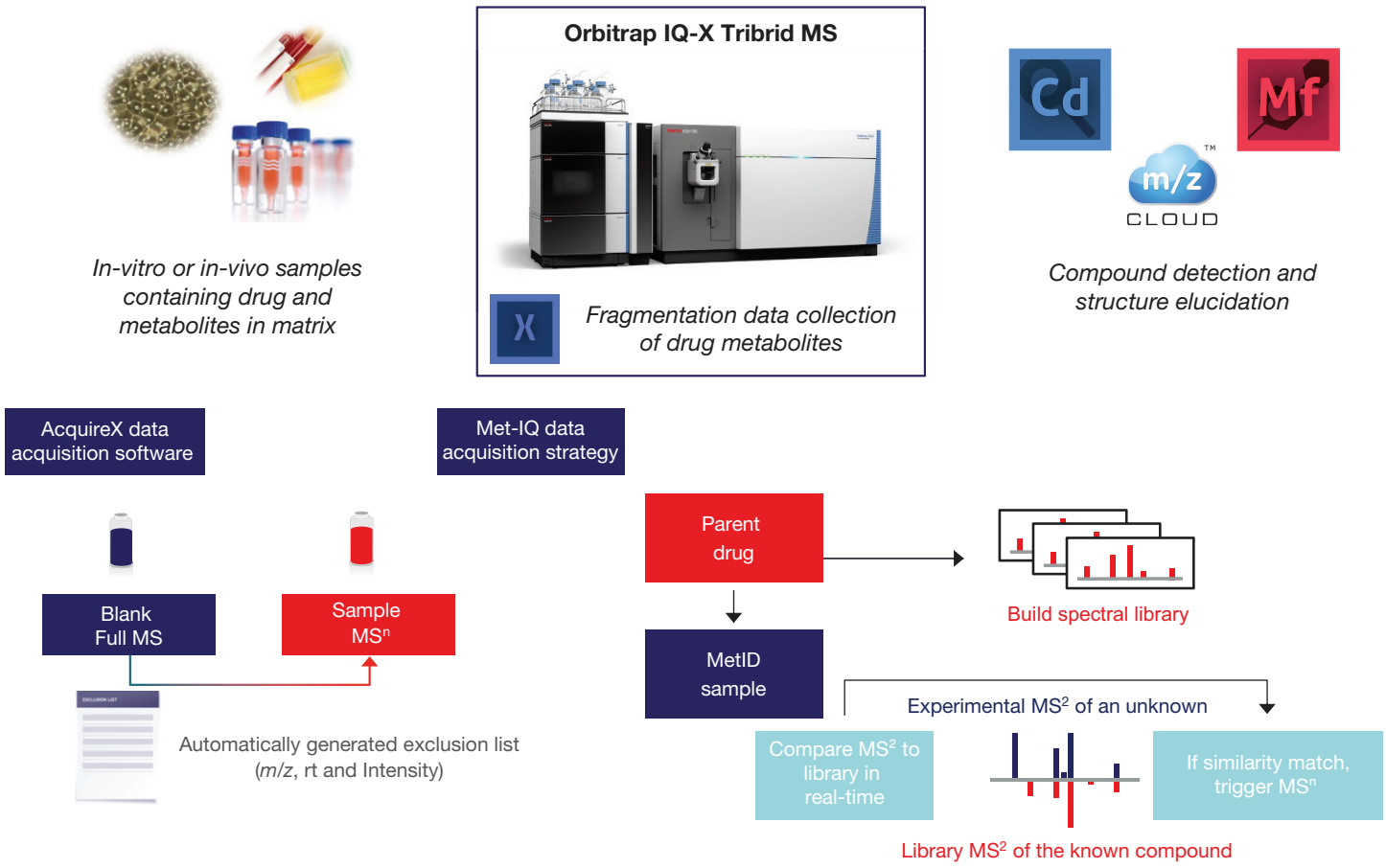

Figure 2. Workflow overview for drug metabolite identification using the Orbitrap IQ-X Tribrid mass spectrometer with AcquireX data acquisition software and the Met-IQ data acquisition strategy coupled to a Thermo Scientific™ Vanquish™ UHPLC system to collect full scan and fragmentation data, allowing software-assisted metabolite detection and structure elucidation

To address these challenges, innovative tools were introduced on Thermo Scientific Orbitrap mass spectrometers to optimize the relevance of the acquired data and provide more insight. In particular, the Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer is designed to reveal complex chemical structures for compound identification and structure elucidation of small molecules using proven tribrid architecture, combining a quadrupole, linear ion trap, and Thermo Scientific Orbitrap HRAM technologies to acquire information-rich MS^n data from every sample. Multiple fragmentation techniques, namely collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), and optional UVPD, are available at any stage of MS^n, with subsequent mass analysis in either the ion trap or ultra-high resolution Orbitrap mass analyzer, providing users the ultimate flexibility and capability to gain comprehensive structural information on a single LC-MS system. Intelligent data acquisition techniques, such as the AcquireX intelligent data acquisition workflow and the Met-IQ data acquisition strategy using real-time library search, allow for automated method modifications and decisions between and during injections.

Focused analysis of pertinent compounds with AcquireX technology

The AcquireX data acquisition technology aims to generate more meaningful fragmentation data through the automated generation of sample-specific inclusion and exclusion lists for data-dependent acquisition experiments. In particular, the AcquireX background exclusion workflow is well suited for the analysis of MetID samples. As outlined in Figure 2, after the injection of a matrix blank sample, such as a control microsomal preparation without the drug, the instrument automatically generated a list of both constant background signals and eluting matrix compounds (recording *m/z* ratios, retention time, and peak intensity). This comprehensive exclusion list was used in subsequent injections to focus acquisition of fragmentation data on "new" features in the incubated samples containing the drug and their metabolites.

The impact of this technology is highlighted in Figure 3, using the example of a minor isomer of nefazodone oxide. In the case of a "conventional" data-dependent fragmentation data acquisition (ddMS/MS), the automatically generated background exclusion list resulted in the high abundant background ions not being triggered for fragmentation, allowing the sampling of lower abundant features including the metabolite of interest at *m/z* 486.2268. This technology, which is also available on the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer, as well as the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer and Thermo Scientific™ Orbitrap Ascend™ Tribrid™ mass spectrometer, has been shown to improve 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.[1,2]

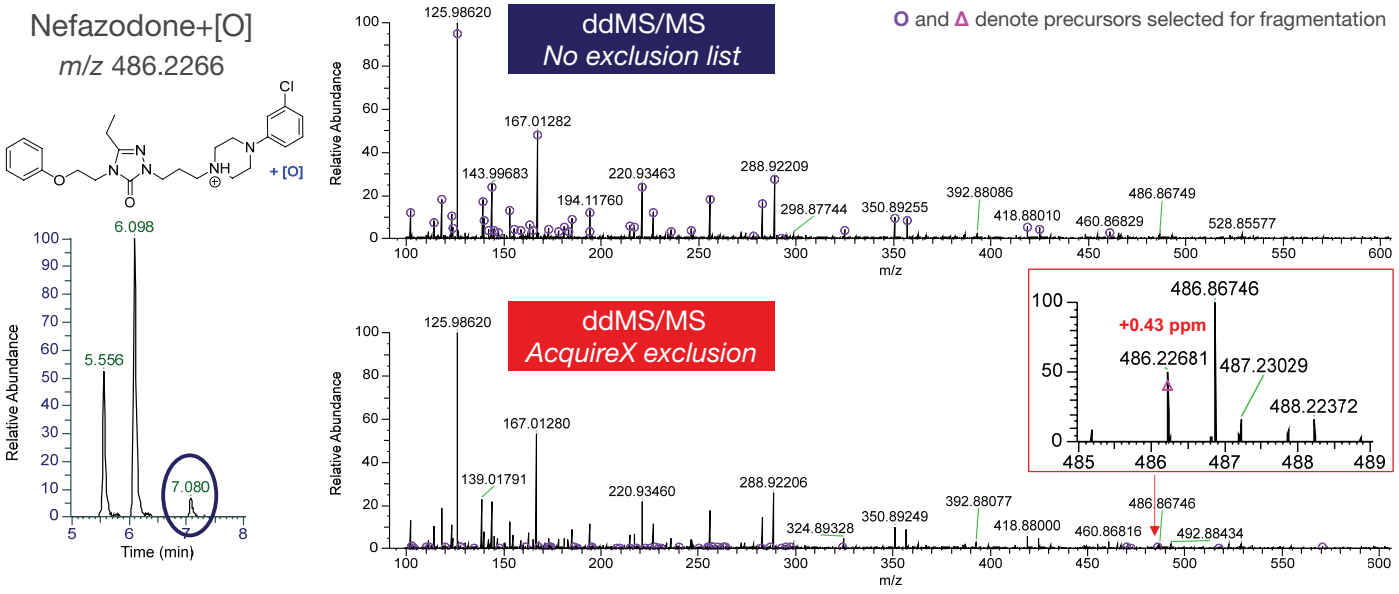

Figure 3. Comparison of the nefazodone + [O] precursor at 7.08 min selected for fragmentation without and with the AcquireX background exclusion workflow, showing the impact of the automatically generated exclusion list to target low abundant metabolites of nefazodone for fragmentation instead of more abundant matrix components and background ions (adapted from reference 3).

Intelligent MS^n data acquisition for in-depth structural characterization

The unique architecture of the Orbitrap IQ-X Tribrid mass spectrometer allows for multi-stage fragmentation in the ion trap. The utility of this is highlighted in the example of the nefazodone oxide metabolite shown in Figure 4. From the MS^2 spectrum alone, the site of oxidation could not be determined, as the main fragment at *m/z* 290 has multiple sites where oxidation is possible. However, the subsequent fragmentation created additional fragments in the MS^3 spectrum, for which structures could be assigned using the fragment ion prediction in the Mass Frontier software to localize the oxidation to the ethyl sidechain based on the predicted structures of the fragments at *m/z* 170 and *m/z* 156.

The setup of MS^n experiments can be challenging, however, often requiring targeted methods to acquire meaningful data. Additionally, the acquisition of MS^n spectra for all precursors lengthens cycle time and reduces the total number of precursors selected for fragmentation. On the Orbitrap IQ-X Tribrid mass spectrometer, a novel data acquisition strategy using real-time library search (RTLS) technology allows the acquisition of these data in a targeted fashion without requiring thorough knowledge of the compounds of interest. This is enabled by on-the-fly spectral searching against a customizable mzVault™ spectral library to allow for decision-based triggering of MS^n scans.

The Met-IQ data acquisition strategy specifically uses RTLS to enable selective detection and characterization of unknown compounds that are structurally related to known compounds, such as metabolites of a parent drug.[4] Compounds that meet the scoring criteria set in the method can trigger additional scan behaviors, including MS^3 or alternate fragmentation. In this way, additional structural information is gained on the most important compounds, and cycle time is freed up for additional MS^2 on other precursors in their absence.

The performance of the Met-IQ workflow was evaluated on several model compounds, including losartan, incubated with human microsomes.[5] During analysis, the RTLS node compared experimental spectra against a library consisting of the parent drug. Only compounds that had a similarity match to the parent drug triggered MS^3 scans, as shown in Figure 5. In the example of the metabolite at *m/z* 615, the similarity threshold was exceeded due to the shared fragment ions with the parent molecule highlighted in yellow, resulting in the acquisition of MS^3 data.

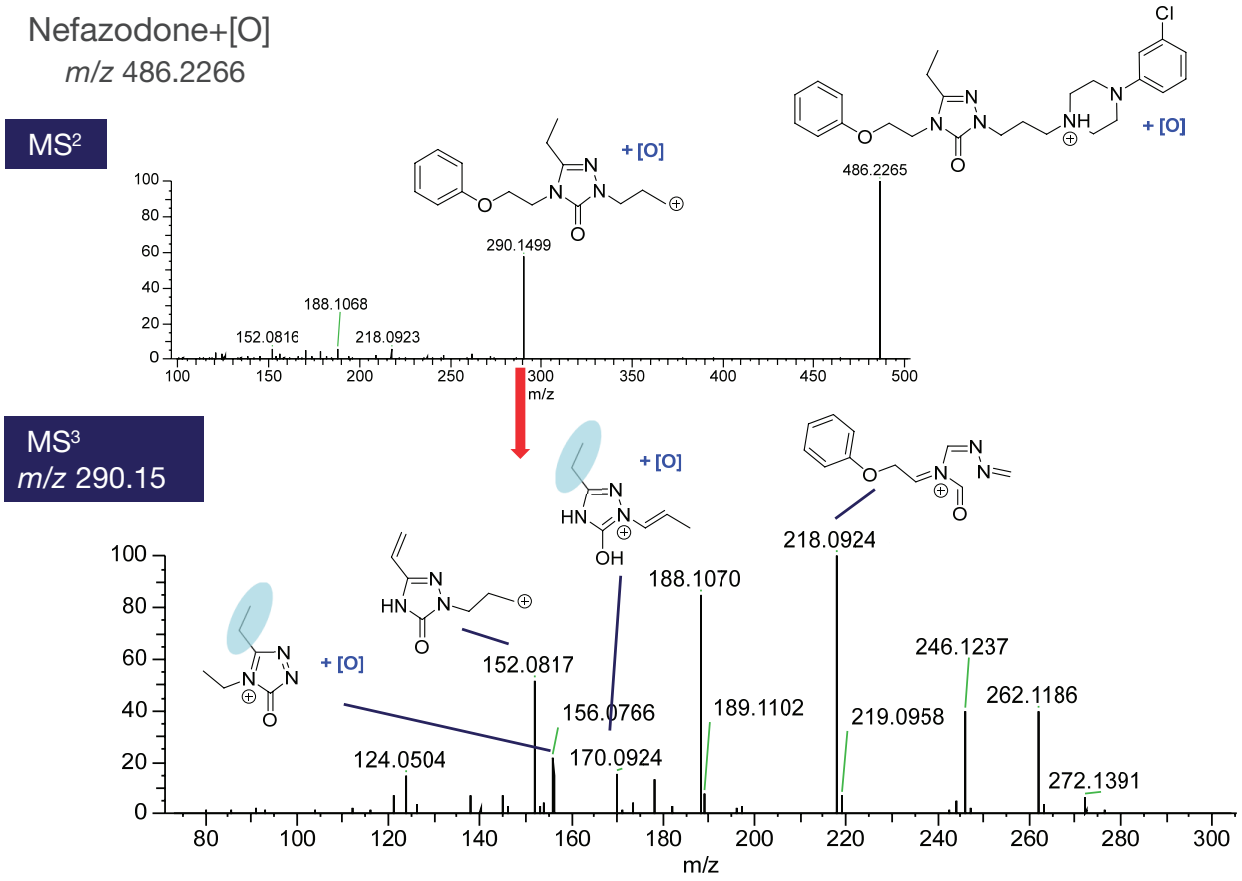

Figure 4. Assignment of fragment ions observed in the MS^3 fragmentation spectrum of the oxidized nefazodone metabolite allows localization of the transformation to the ethyl sidechain.

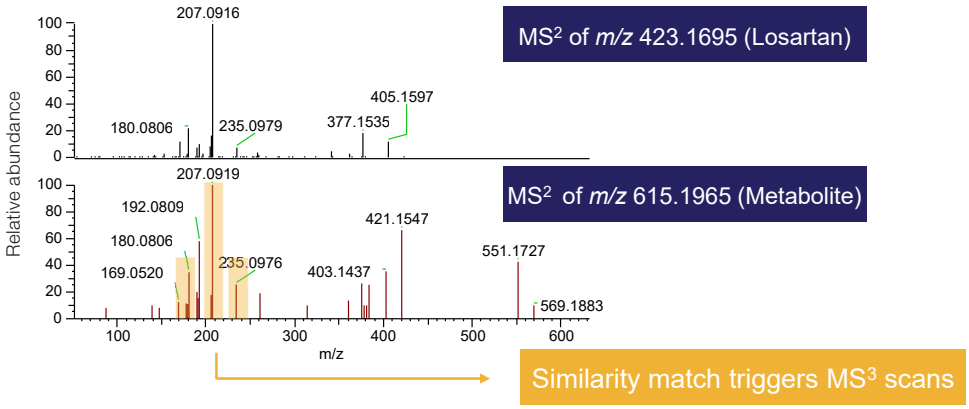

Figure 5. In the Met-IQ workflow, selective MS³ data acquisition was triggered by the similarity match of the acquired fragmentation spectrum of the metabolite at *m/z* 615 to the spectral library generated from the losartan parent.

For losartan, the Met-IQ data acquisition strategy increased the number of losartan metabolites with MS³ data from 10 to 24 compared to a traditional ddMS³ experiment. This is because the instrument spent less time collecting MS³ on non-relevant compounds and was therefore able to interrogate more compounds of interest overall.

Orthogonal fragmentation techniques for increased insight of challenging metabolites

Fragmentation spectra using collision activated dissociation, such as HCD and CID, provide evidence needed to determine transformation sites in most cases, as shown above. However, if only non-specific fragments are generated or the modification is readily cleaved with HCD or CID, the determination of transformation sites can be challenging, and the distinction of isomeric metabolites may not be possible. This can be the case for conjugated phase II metabolites such as glucuronides and glutathione adducts, which are often readily cleaved in HCD experiments. Since formation of acyl glucuronides and GSH adducts is associated with idiosyncratic toxicity in late stages of drug development and post-marketing, it is critical to identify and understand the mechanism for their formation.[6]

The Orbitrap IQ-X Tribrid mass spectrometer offers the optional capability to fragment precursors with UVPD, using a 213 nm laser system with 2.5 kHz repetition rate, generating unique fragments complementary to CID and HCD to obtain detailed structural information. UVPD differs from CID and HCD in that it utilizes photons generated from a laser source to increase the internal energy of a selected precursor ion until there is sufficient internal energy present to overcome the barrier to dissociation. Photons are directly absorbed by target molecules depending on their UV absorption profile. The UVPD laser is embedded in the mass spectrometer chassis and the fragments are generated in the linear ion trap for subsequent detection by either the ion trap or Orbitrap mass analyzer. Figure 6 shows the benefit of UVPD in generating unique fragments from precursors where CID or HCD predominantly cleaved at the same position to produce uninformative fragments.

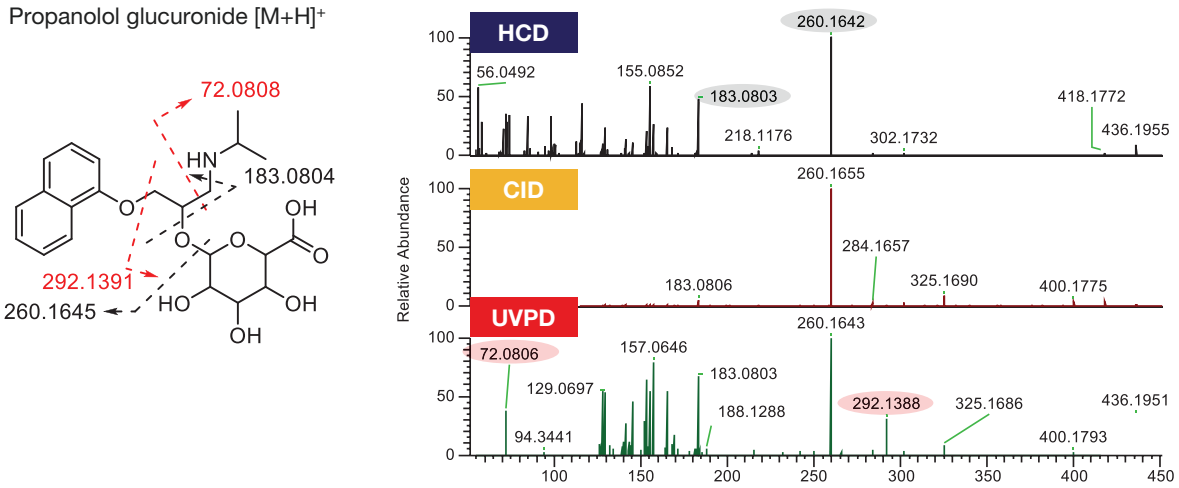

Figure 6. Fragmentation spectra of propranolol glucuronide using HCD, CID, and UVPD fragmentation, with unique informative fragment ions generated by UVPD highlighted (adapted from reference 7).

The utility of this orthogonal dissociation technique for MetID experiments is shown in the analysis of the metabolism of diclofenac.[8] In the case of diclofenac acyl-glucuronide, HCD fragmentation resulted in almost identical spectra for the metabolite and its parent, as shown in Figure 7, as the glucuronide was cleaved preferentially. However, additional fragments could be observed in the UVPD fragmentation spectrum to confirm the addition of the glucuronide at the carboxylic acid of diclofenac.

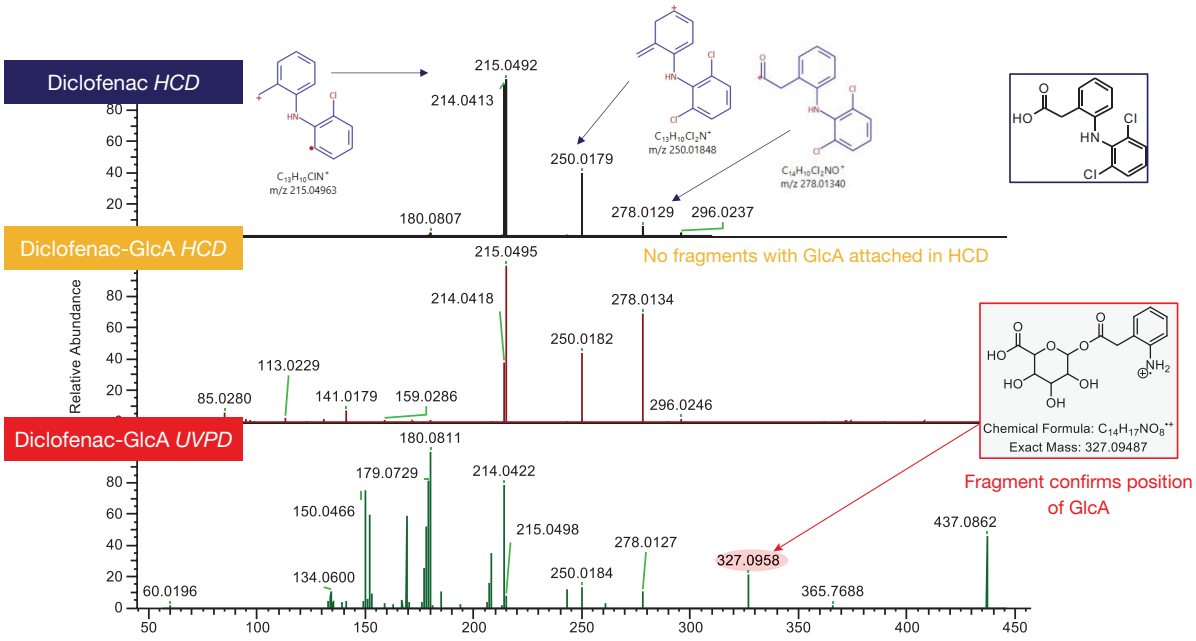

Figure 7. Comparison of the HCD spectrum of diclofenac (top) with the HCD spectrum for its glucuronate metabolite (middle), showing no additional fragments to permit confirmation of the glucuronide position, and the UVPD spectrum (bottom), which allowed the determination of the acyl-glucuronide bond based on the assigned fragment structure for the unique ion at *m/z* 327

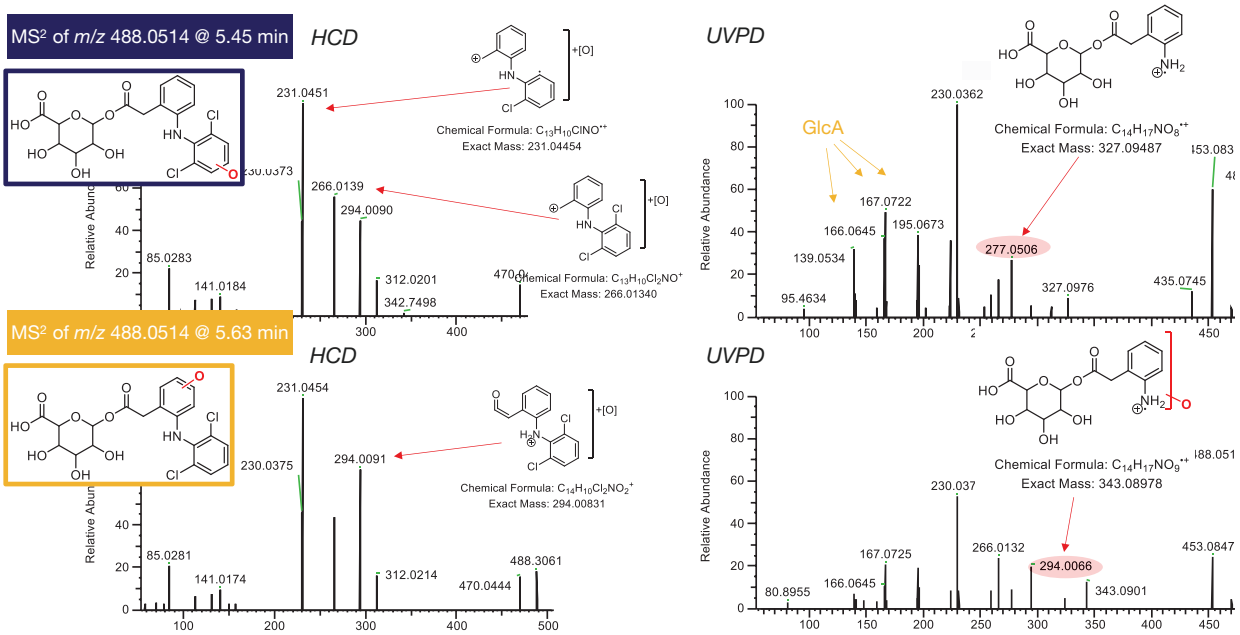

Figure 8. Comparison of the HCD and UVPD spectra for two isomeric oxidized and glucuronidated metabolites of diclofenac (M+[O]+[GlcA]), with assigned fragment structures for the highlighted UVPD fragments, allowing the distinction of the oxidation site and confirmation of the acyl glucuronidation

Moreover, the metabolism of diclofenac also created multiple isomeric metabolites with glucuronide addition in addition to the initial oxidation, which could be separated chromatographically. However, the HCD spectra shown in Figure 8 for the metabolites at 5.45 min and 5.63 min were very similar and did not allow determination of the oxidation position or linkage of the glucuronide.

Conversely, in the UVPD spectra of the two, the presence of a glucuronide containing fragment differing in mass by 16 Da at *m/z* 327 and *m/z* 343 allowed the confirmation of the acyl-glucuronidation and distinguished the two metabolites with respect to the oxidation site.

Summary

As highlighted throughout this white paper, the guided data acquisition of fragmentation data on Thermo Scientific Orbitrap Tribrid mass spectrometers allows for more meaningful data acquisition, improving efficiency for routine metabolite identification.

- The AcquireX background exclusion workflow significantly improves the fragmentation data coverage for relevant metabolites, reducing the need for re-analysis of samples after initial data processing.
- The Met-IQ data acquisition strategy uses real-time library search to guide data acquisition based on structural similarity to provide more meaningful deep fragmentation data, streamlining the structure elucidation of complex metabolites.
- Orthogonal fragmentation techniques such as UVPD can provide complementary information to HCD and CID to aid in elucidating the site of metabolism, especially for labile metabolites.

Notes

The data shown in this technical note were acquired on the Orbitrap IQ-X Tribrid mass spectrometer. The highlighted instrument features (AcquireX, Met-IQ, RTLS, UVPD) are also available on the Orbitrap Eclipse Tribrid mass spectrometer and Orbitrap Ascend Tribrid mass spectrometer.

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