Rapid and Confident Metabolite Profiling and Identification using Bench-Top Orbitrap Q Exactive and Compound Discoverer

Kate Comstock,[1] Caroline Ding,[1] Tim Stratton,[1] Kelly Wang,[2] Gene Eiserberg[2]
[1]Thermo Fisher Scientific, San Jose, CA; [2]Gilead Sciences, Foster City, CA

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Overview

Purpose: Single software solution for HRAM LC/MS data processing for confident and rapid metabolite identification and structure elucidation.

Methods: High-resolution LC/MS and novel software for metabolite profiling and structure identification.

Results: Compound Discoverer enables fast, efficient, and confident metabolite profiling in an all-in-one UHPLC/HR-MS/MS platform.

Introduction

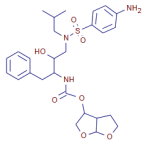
In vitro drug metabolite identification by LC coupled with HRAM MS is an essential component of drug discovery to select and prioritize compounds with the best chance of success. Data processing and data collation are the current rate-limiting steps of the MetID process.

This study demonstrates how using Thermo Scientific™ Compound Discoverer™ Software improves the MetID process. Compound Discoverer software is pipeline-based where each node performs a discrete function. The user has the flexibility to arrange appropriate nodes into a workflow that produces the desired information. A branched pipeline can be used to process a data set in multiple ways to extract maximal information and to bring all the data together for review and reporting.

Methods

Sample Preparation

Darunavir samples (50 uM) were incubated in human liver microsomes (1 mg/mL) fortified with NADPH (1mM) for 0 and 2 hours.



Darunavir
C27 H37 N3 O7 S
MW 547.23522

Liquid Chromatography

Thermo Scientific™ Ultimate™ 3000 RS UHPLC system

Column: Thermo Scientific™ Accucore™ C18 (100 x 2.1 mm), 2.6 μm

Column Temperature: 35 ºC

Gradient Solvents: A: H_2O/0.1% Formic Acid;
B: ACN/0.1% Formic Acid

Flow rate: 500 μl/min

Injection Volume: 5 μl

LC gradient:

Time (min.)	0.0	14.0	18.0	20.0	22.0	22.1	26.0
A%	95	60	60	20	20	95	95
B%	5	40	40	80	80	5	5

Mass Spectrometry

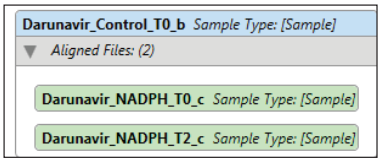
The MS analyses were carried out on a Thermo Scientific Q Exactive™ mass spectrometer using the electrospray technique in the positive ion mode. High-resolution accurate mass (HRAM) full-scan MS and top 3 MS/MS spectra were collected in a data-dependent fashion at a resolving power of 70,000 and 17,500 at FWHM *m/z* 200, respectively. The Stepped NCE (Normalized Collision Energy) setting was 15, 20, 25.

Data Analysis

A study was created in Compound Discoverer for Darunavir. The study files include raw files from t_{0h}, t_{2h} with and without NADPH and a Darunavir standard injection. The standard injection was used to ensure there were no major impurities in the Darunavir drug.

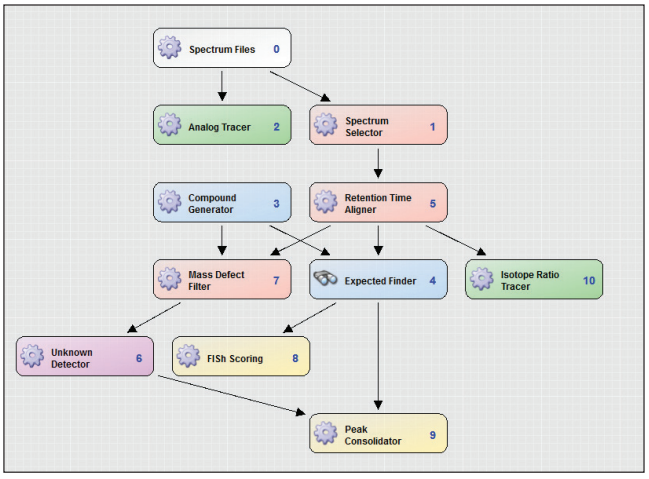
Compound Discoverer software employs a drag-and-drop user interface to facilitate quick and flexible file relationship assignments for downstream control comparison and retention time alignment (Figure 1).

FIGURE 1. Easy grouping of files by dragging and dropping the files to the control. The box represents file relationships between the three files: Control_t0h, NADPH_t0h, and NADPH_t2h for RT alignment and comparison.



The three files, Control_t0h, NADPH_t0h, and NADPH_t2h, were processed in one analysis using Compound Discoverer software. The node-based processing workflow (Figure 2) included both expected metabolite detection and mass-defect-filtered unknown component detection. The Mass Defect Filter node is a spectra filtering tool and was used before the Unknown Detector node. The mass defect filters were created on the basis of parent de-alkylation predictions and expected transformations with a mass tolerance of +/- 50 Da and a mass defect tolerance of +/- 50 mmu. The Expected Finder node includes de-alkylation and de-arylation predictions based on the structure of Darunavir. Common phase I bio-transformations were automatically combined on the basis of the number of maximum occurrences for each transformation and the maximum number of combinatorial steps. The Fragment Ion Search (FISh) Scoring node searches MS/MS spectra for fragment ions in common with the parent fragment ions or those that are shifted by a predicted biotransformation. A positive match confirms the presence of a compound that is structurally related to the parent and provides the location of the modification on the basis of the shifted fragments. Since Darunavir contains one sulfur (S) atom and the full-scan MS data was run at a resolving power of 70,000, the Isotope Ratio Tracer node was used in the workflow to create a 1S fine isotope trace as an additional trace for parent-related compound detection, and UV traces were also included as orthogonal traces. The Compare with Control feature was enabled in the workflow to compare compounds in the three files.

FIGURE 2. Node based processing workflow in Compound Discoverer Software



Results

Compound Discoverer software uses both metabolite prediction and advanced component detection to discover both predicted and unexpected metabolites in the data. Advanced, automated tools that use isotope ratios, isotope fine structure, and correlation of fragment peaks provide confidence in the identification of drug related components and site of modification. Results are consolidated into one peak table. Furthermore, the same peaks found in the sample and control files are collated and consolidated. Overlay chromatograms of the same compound found in NADPH_t2h, NADPH_t0h, and Control_t0h are displayed in the chromatogram window for visual inspection (Figure 3). Adducts are automatically detected and grouped for each compound (Figure 4). Adduct grouping greatly reduces false positives from compound hits.

FIGURE 3. Overlaid XIC provides visualization of how the same compound changes in three files.

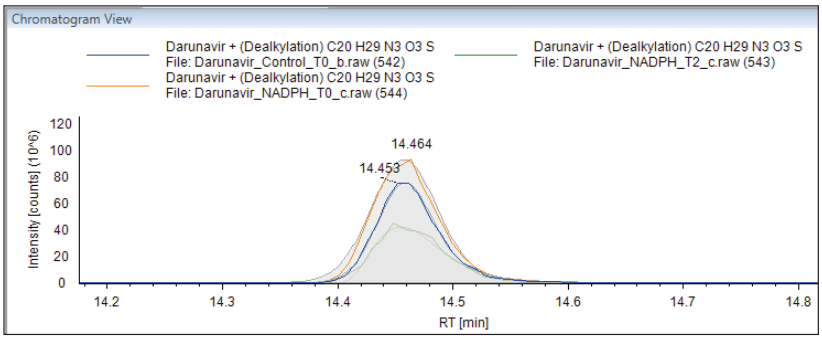
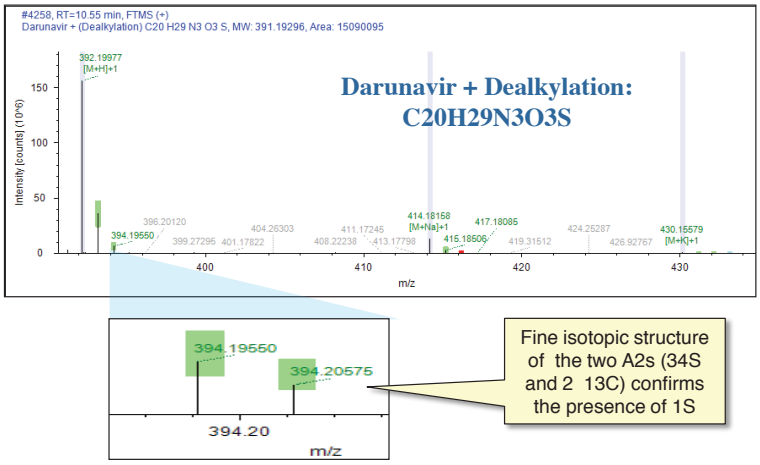
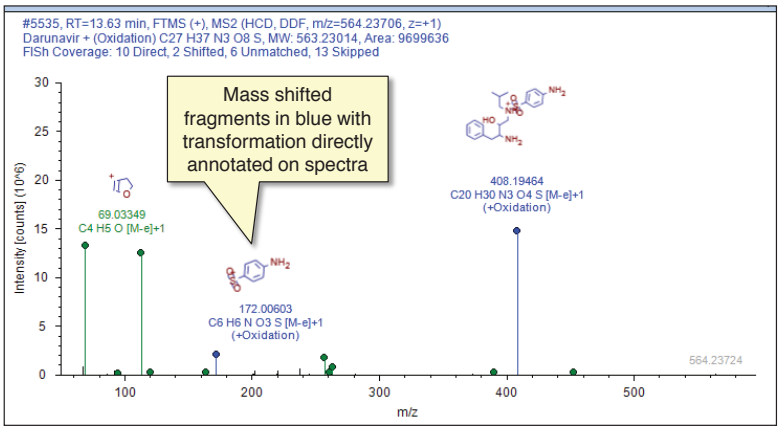


FIGURE 4. Automatic adduct grouping reduces false positive assignments. Isotope patterns are automatically considered with color coding to represent the fidelity of isotope pattern fit for assigned elemental composition.



For each expected metabolite, FISh Scoring automatically compares the MS/MS fragmentation spectra to that of the parent compound and annotates the spectra with matching fragment structures (color-coded in green) and biotransformation shifted fragments (color-coded in blue). This provides a quick visual indication of which part of the molecule has been modified and which part remains unchanged for a rapid determination of the modification location (Figure 5).

FIGURE 5. MSMS fragments are compared to the parent and structures automatically annotated to help determine the location of the modification.



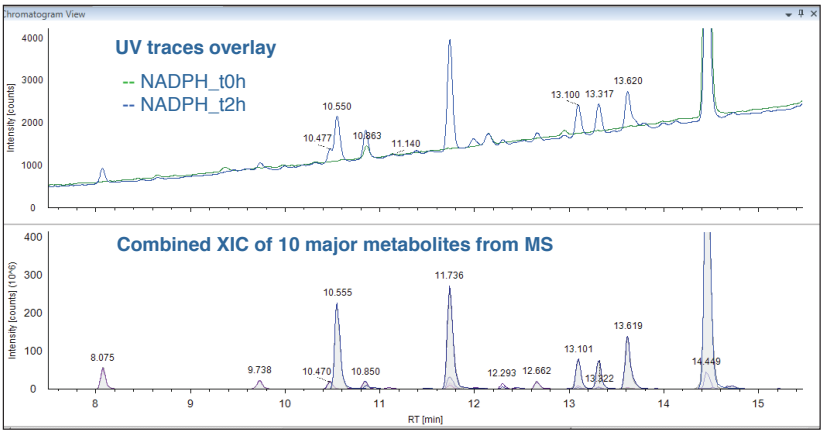
Compound Discoverer software supports multiple monitors and includes a data review window that is easily customized for the MetID review process. The Consolidated Peaks table shows consolidated peaks detected from the Expected Finder and/or Unknown Detector nodes and from all three files in the same table simultaneously. The tables in Compound Discoverer support multiple-column sorting and multiple-property filtering. An area threshold filter of 1% of the parent compound in NADPH_t0h and a RT range filter of 2-17mins were applied to reduce the number of peaks for data review. Furthermore, the In Control status was used as a filtering condition to filter out peaks that were found in both of the t0h samples (NADPH_ t0h and Control_t0h) and that did not change significantly in the t2h sample. The following ten putative metabolites were identified in the NADPH_ t2h sample in addition to the parent compound (Table 1).

TABLE 1. Darunavir in vitro metabolites (area threshold above 1% of parent in NADPH_t0h) detected by HRAM and Compound Discoverer.

Name	RT [min]	Apex m/z	MW	Formula	Formula change	Comment	NADPH_t2h	NADPH_t0h	Control_t0h
Parent	14.46	548.24195	547.23522	C27H37N3O7S		Darunavir	43961061	91409870	69281472
M1	8.08	408.19473	407.18788	C20H29N3O4S	-(C7H8O3)	Dealkylation +oxidation	3192335	0	0
M2	9.74	408.19501	407.18788	C20H29N3O4S	-(C7H8O3)	Dealkylation +oxidation	1604308	0	0
M3	10.48	580.23216	579.22505	C27H37N3O9S	+O2	oxidation + oxidation	899572	0	0
M4	10.55	392.19975	391.19296	C20H29N3O3S	-(C7H8O4)	dealkylation	13789370	58349	12381
M5	10.85	580.23224	579.22505	C27H37N3O9S	+O2	oxidation + oxidation	952287	0	0
M6	11.74	564.23718	563.23014	C27H37N3O8S	+O	oxidation	13099948	33394	0
M7	12.66	580.23218	579.22505	C27H37N3O9S	+O2	oxidation + oxidation	1268479	0	0
M8	13.10	564.23712	563.23014	C27H37N3O8S	+O	oxidation	3934473	16074	0
M9	13.32	564.23724	563.23014	C27H37N3O8S	+O	oxidation	3884732	16909	0
M10	13.62	564.23712	563.23014	C27H37N3O8S	+O	oxidation	9237332	51973	10302

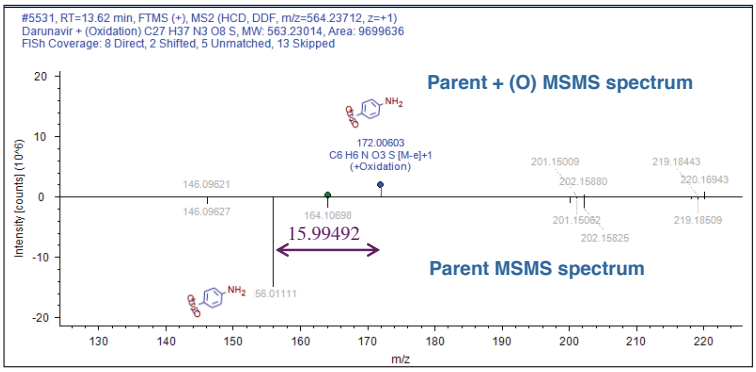
UV traces were used in addition to the various filtered mass chromatograms to help correlate back to non-LC/MS-based studies. Figure 6 shows overlaid UV traces of NADPH_t0h and NADPH_t2h that are added and stacked on top of the combined MS trace of the 10 putative metabolites and parent.

FIGURE 6. Stacked view of combined ms trace from the 10 major metabolites and overlay of UV traces from NADPH_t0h and NADPH_t2h.



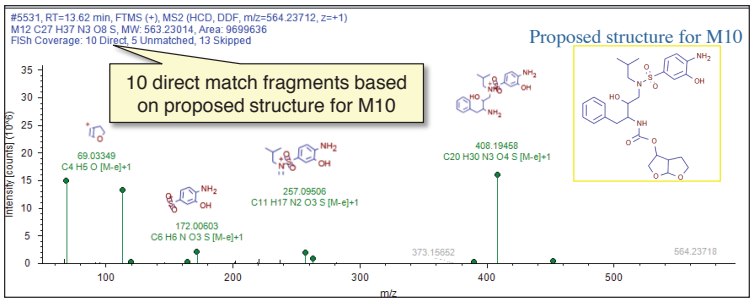
Metabolite structure assignments were facilitated by FISh annotations. An example is given below (Figure 7) showing how a comparison of the FISh-annotated MS/MS spectrum of the oxidated Darunavir metabolite to the parent HCD MS2 spectrum was used to help determine the site of transformation.

FIGURE 7. Mirrored plot of MS/MS spectra for M10 and parent compound with FISh fragment annotations.



Putative metabolite structure assignment was confirmed by re-running the FISh matching and annotations algorithm for the proposed structure in Compound Discoverer. In the example shown below for M10, the FISh annotation based on the proposed structure found ten exact match fragments (Figure 8). Final determination of the structure will require MS3 or higher fragmentation.

FIGURE 8. FISh re-annotated MS/MS spectrum based on proposed putative structure for M10.



Conclusion

Metabolite ID is a complex and time-consuming process. By combining the HRAM full-scan MS and MS/MS data from the bench top Q Exactive instrument, and the advanced processing algorithms in the Compound Discoverer software, a previously tedious process that could take an experienced MetID scientist a few days can be reduced to one day.

• A node-based processing workflow provides flexibility in data processing.

• The ability to extract and support orthogonal pieces of data including analog trace data (from UV, PDA, or other analog detectors including radioisotope detectors) ensures no compound of interest is missed.

• Resolution aware and accurate-mass empowered algorithms that include mass defect filtering, an isotope ratio tracer, a targeted search for parent compounds and their transformation products based on expected modifications and de-alkylation predictions, and the ability to detect untargeted components increases the confidence of compound identification.

•Easy to use software interface for method development and sample comparison increases productivity.

• Flexible data review and multiple monitor support provides simultaneous viewing of chromatographic traces, mass spectra, and data result tables.

• Fully integrated fragmentation prediction from Mass Frontier™ and automatic fragment ion matching with annotations help elucidate the localization of transformations. This leads to more confident structure determinations of the expected transformation products and unknown detected sample components.

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