Using HRAM Survey Analysis Combined with Rapid MS^2 Data to Develop a Fragmentation Based Detection Workflow for Structure ID Acquisition

Tim Stratton[1], Caroline Ding[1], Christoph Henrich[2], Hans Grensemann[2], Hans Pfaff[2], Martin Strohalm[2], Sebastian Kusch[2]
[1]Thermo Fisher Scientific, San Jose, CA [2]Thermo Fisher Scientific, Bremen Germany

Overview

Purpose: Demonstrate a workflow for metabolite detection and structure elucidation combining HRAM (high-resolution, accurate-mass) HCD MS^2 with a new customizable processing workflow using orthogonal metabolite detection tools to direct multiple fragmentation mode MS^n acquisition.

Methods: Samples of human urine, post-kidney operation, were analyzed by rapid HRAM HCD MS2 for peak detection. Potential metabolite peaks were acquired by multiple-fragment-mechanism MS^n for structure identification where an HCD MS^2 scan was followed by multiple CID MS^3 fragmentation scans.

Results: Multiple metabolites for each of four co- and serially administered drugs commonly used during surgery were detected and identified.

Introduction

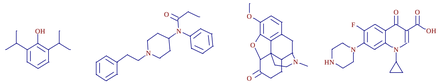
The complexity of biological matrices makes the task of metabolite detection difficult. Identification of which components in a sample or series of samples are of interest is the first step in the final determination of the metabolites' structure. Here we demonstrate the use of fast scanning HCD MS2 combined with a novel data processing workflow to determine peaks of interest in samples of human urine following kidney surgery where multiple drugs were administered (anesthetics, analgesics, antibiotics).

Subsequent to the detection of components of interest, we have demonstrated the use of HRAM MS^n acquisition combining different fragmentation techniques to improve metabolite structure identification. Acquisition combined an MS^2 scan using HCD to generate multiple fragments from a high energy event followed by multiple MS^3 scans using CID fragmentation to determine fragment heritage and structure.

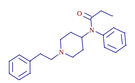
Methods

Sample Preparation

Samples of human urine were acquired post kidney surgery (five samples over the first 12 hours after surgery). Samples (10 mL aliquots) were prepared for analysis by solid phase extraction using Thermo Scientific™ Hypersep C18 SPE columns, (50mg), by washing with 2 volumes of water followed by elution with one volume each of acetonitrile and methanol. Eluates were evaporated to dryness under a stream of nitrogen gas and reconstituted in 1 mL of 90:10 water:acetonitrile for injection.

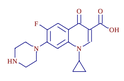


Propofol
$C_{12}H_{18}O$
MW. 178.13577



Fentanyl
$C_{22}H_{28}N_2O$
MW. 336.22016

Hydrocodone
$C_{18}H_{21}NO_3$
MW. 299.15214



Ciprofloxacin
$C_{17}H_{18}N_3O_3F$
MW. 331.13322

Liquid Chromatography

Column: Thermo Scientific™ Accucore™ C18 100 x 2.1 particle (µ)
Injection volume: 2 µL Flow rate: 300 µl/min.
LC Gradient: MP A: H_2O/0.1% formic acid. MP B: ACN/0.1% formic acid

Time (min)	MP A (%)	MP B (%)
0.0	98	2
0.5	98	2
13.0	5	95
13.8	5	95
14.0	98	2
15.0	98	2

Mass Spectrometer

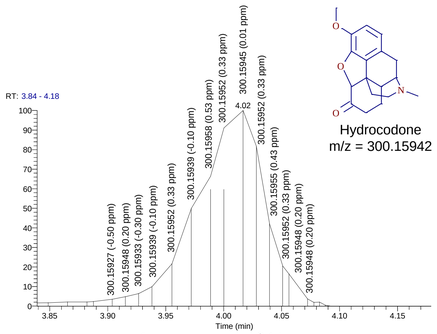
Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mass Spectrometer
Ion source: Easy-IC
Ionization mode: ESI positive
Sheath gas flow rate: 45 units N_2
Auxiliary gas flow rate: 15 units N_2
Spray voltage (KV): +3.5
Capillary temp (°C): 320
S-lens RF level: 60.0
Heater temp (°C): 275

Results

Identifying Peaks of Interest in Survey Analysis

Samples were initially acquired using a high-resolution full scan (60,000 FWHM @ *m/z* 200) and multiple data-dependent HCD MS^2 fragmentation scans. The instrument was operated with a cycle time of 600 msec which achieved an average of 4-5 MS^2 events per full scan. This cycle time, combined with dynamic exclusion, allowed for acquisition on large numbers of co-eluting components in the matrix. Internal calibration was also used during acquisition to assure that mass accuracy was less than 1 ppm. Internal calibration was achieved using a novel source calibration system which requires no calibration solution to be infused.

FIGURE 1. Mass Accuracy Scan-to-Scan: Hydrocodone in Urine #2



Hydrocodone
m/z = 300.15942

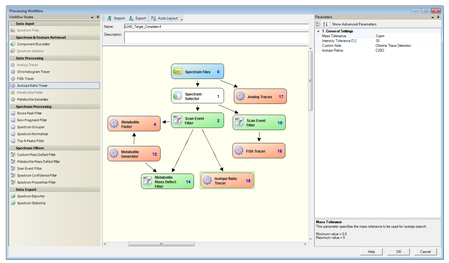
Average accuracy: 0.14 ppm

Processing Workflow for Metabolite Detection

The high-resolution, accurate-mass survey injections were processed using the novel workflow-based Thermo Scientific™ Compound Discoverer™ software allowing for a custom processing approach. The workflow editor (Figure 2) allows a custom workflow to be created to process any data. In this example, metabolites of each of four different drugs present were detected with a workflow that combined multiple orthogonal detection approaches including a combinatorial metabolite search with standard multiple mass defect filter and fragment searching.

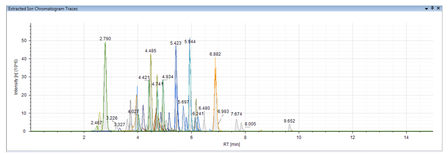
The combinatorial approach uses a list of known single-step metabolic transformations along with simple user inputs to assemble a list of potential metabolite masses. This process also includes the generation of dealkylation products (optional) with subsequent metabolism of these products. The list of biotransformations used is also adjusted to the elemental composition of the parent such that dehalogenation is not applied to structures that do not contain a halogen.

FIGURE 2. Processing Workflow Editor.



To process the survey injections, a series of phase I and phase II modifications were included in the metabolite generator (a total of 32) and the node was allowed to predict transformations of up to 3 sequential steps including 1 or 2 steps of dealkylation. Redundant predictions were removed but the remaining list contained 513 possible metabolites for ciprofloxacin, 622 for fentanyl, 348 for hydrocodone, and 105 for propofol. Peaks detected were confirmed by comparison with fragmentation and mass defect filters before inclusion for MS^n acquisition. Multiple metabolites were detected (Figure 3) based on this approach.

FIGURE 3. Top 50 Potential Metabolites of Propofol, Fentanyl, Hydrocodone, and Ciprofloxacin in Human Urine Sample #2



Acquisition of Combined Fragmentation Mechanism MS^n Data

With the list of potential metabolites from the processing of the survey injections, samples were injected using a HRAM MS^n acquisition method (Figure 4) which combined HCD MS^2 with CID MS^3. The ability to combine fragmentation mechanisms was unique and useful for metabolite structure identification. We made use of the wide fragment mass range of the higher energy HCD mechanism for MS^2 to generate a wide range of fragments to select for subsequent CID MS^3. The lower energy of the collisional dissociation MS^3 event allowed for higher sensitivity and the ability to determine fragment heritage (which MS^2 fragment ion comes from which), this is useful in determining the site of modification as it allows for a more detailed analysis of the structure of the metabolite.

FIGURE 4. Orbitrap Fusion Mass Spectrometer Method Editor for Combined HCD → CID MS^3 Fragmentation Acquisition.

Structure Identification Using HRAM MS^n

The accurate-mass fragmentation spectral trees acquired were interpreted using theoretical fragmentation of the parent drug from which the metabolite was believed to be derived. For peaks conforming to an expected set of single-step enzymatic biotransformations, fragmentation was interpreted using the parent theoretical fragments and only the subset of single-step biotransformations. In many cases, more than one set of biotransformations could explain an observed peak and the interpretation of the peak using each set allowed for easier determination of the correct biotransformation steps involved and subsequently the correct location(s) for the modification(s).

One example of this interpretation is shown in Figure 5 for a reduction / bisoxidative metabolite of ciprofloxacin.

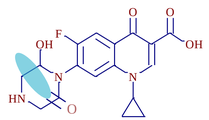
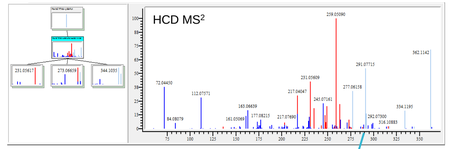
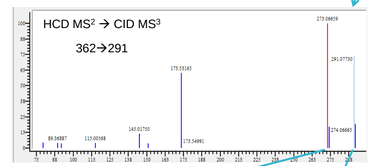
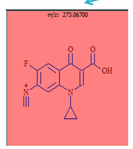
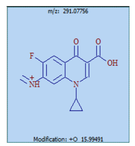


FIGURE 5. Fragmentation of Reduction + Bisoxidation of Ciprofloxacin.









Conclusion

Utilizing a rapid HCD MS^2 survey injection enabled by dynamic scan management along with the mixed-fragmentation mechanism MS^n approach possible on the Orbitrap Fusion mass spectrometer we have demonstrated the ability to detect and identify metabolites from multiple parents in complex biological samples.

Combining these acquisition techniques with a customizable workflow for data processing in Compound Discoverer software we have demonstrated:

- The usefulness of high-scan-rate accurate-mass HCD MS^2 acquisition combined with orthogonal metabolite detection techniques for peak finding.
- Combined fragmentation mechanisms in MS^3 (combining HCD and CID) to access more structure determination information.

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