Improving Experimental Efficiency for Drug Metabolite Profiling and Identification

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Overview

Purpose: Drug metabolite profiling and identification are a key step to promote or stop drug compound in early drug discovery stage. It has always been a challenge on how to maximize the amount of metabolite information gathered in a single analysis of drugs that were dosed in mice or incubated with mouse and human liver microsomes. In this study, several features in mass spectrometry were investigated to improve the experimental efficiency.

Methods: Several approaches from using new features of the Thermo Scientific Q Exactive Orbitrap mass spectrometry are presented to improve the efficiency of using HR/AM LC/MS to characterize drug metabolites in complex samples.

Results: Ultra high resolution from the Q Exactive™ Orbitrap mass spectrometry reduces interference peaks. This often improves the quantitation results. Fast polarity switching can cover positive and negative ions from a single injection. Apex-triggering of the MS/MS scans ensures that the highest quality tandem MS data are gathered on metabolites of interest.

Introduction

LC/MS is used routinely for drug metabolite identification and profiling of *in vivo* and *in vitro* assays. The information these assays provide on metabolic soft-spots guides the medicinal chemist in determining what modifications to make to a potential drug structural template to improve its properties. As this is a key piece of information in the development of a drug there is a continuous drive to improve efficiencies in this process. Often the goal is to reduce expensive instrument utilization and conserve precious biological samples. This poster describes several approaches to maximize the amount of metabolite information gathered in a single analysis of drugs that were dosed in mice or incubated with mouse and human liver microsomes.

Methods

Sample Preparation

All samples were collected and prepared in Scripps Research Institute, Jupiter, Florida before shipping to Thermo Fisher Scientific demo lab, San Jose, California.

Microsomal incubations were 200 µl and contained 1 mg/ml hepatic microsomes and 1 µM compound in 100 mM potassium phosphate buffer, pH 7.4. The incubations were brought to 37C with shaking and started with the addition of 1 mM NADPH. 25 µl was removed and quenched with 100 µl acetonitrile at 0, 5, 10, 20, 40 ,and 60 minutes. The samples were centrifuged to precipitate proteins.

The mouse studies were IP dosed at 20 mg/kg in C57Bl6 mice. Compounds were formulated at 2 mg/ml in 10%DMSO/10%tween80/80%water. Blood was collected in sodium EDTA tubes and plasma generated through standard centrifugation techniques. Plasma protein was removed by centrifugation after the addition of 5-times v:v acetonitrile. Brains were collected and processed by the addition of 5-times v:w acetonitrile. The tissue was disrupted with a probe tip sonicator and centrifuged to remove suspended solids and precipitated proteins.

Liquid Chromatography

UHPLC equipped with Thermo Scientific Accela Open AS and 1250 pump. Optimized chromatography conditions were used for each compound.

Column: Thermo Scientific Hypersil Gold PFP, 100 x2 .1 mm, 3µm
Column Temp: 35 ˚C
Injection Vol : 5 µL
LC Pump: Accela 1250 Pump
Mobile Phase A: Water, 0.1% Formic Acid
Mobile Phase B: Acetonitrile, 0.1% Formic Acid
Gradient:

Time / min	% MP-A	%MP-B	Flow Rate / µL/min
0.00	95	5	600
1.00	95	5	600
8.00	5	95	600
10.00	5	95	800
10.01	95	5	800
12.00	95	5	600

Mass Spectrometry

A bench-top Q Exactive Orbitrap mass spectrometry from Thermo Fisher Scientific was used with external calibration. Various MS scan modes, data dependant settings and method strategies were investigated to determine the approaches that provided maximal, high quality, metabolite information while minimizing sample consumption.

=== HESI II Tune Data: ===

Spray Voltage	3800
Capillary Temperature	320
Sheath Gas	55
Aux Gas	15
Sweep Gas	2
Heater Temperature	500
S-lens	40
Lock Mass:	off

Automatic Gain Control (AGC)
1E6 (full-MS scan), 2E5 (SIM), 1E5 (MS/MS)

Data Analysis

Thermo Scientific Xcalibur 2.2 software and MetWorks™ 1.3 software.

Results

Ultra High Resolution to Improve Ion Selectivity

In the profiling and characterization of drug metabolites both quantitative and qualitative information is needed. Full scan, single ion monitoring (SIM) and MS/MS were evaluated for quantitation in terms of both ease of instrument set up and sensitivity of measurement. This included an evaluation of the appropriate resolution to run for each method. Full scan was the most convenient in terms of instrument set up, while high resolution SIM was generally observed as the most sensitive. In very complex matrices high resolution MS/MS was sometimes needed to avoid chemical interferences. As shown in Figure 1, in lower resolution instruments, such as triple quad and ion trap, MS/MS is employed to gain selectivity by lowering baseline noise. The decrease of signal in the process of MS/MS in many cases can be compensated for by the improved selectivity. On a instrument with capable of ultra high resolution (>70,000), selectivity can be obtained at the MS level without the need to develop MS/MS methods in most cases, which conserves absolute signal and makes instrument set up less time consuming. Targeted analysis can be performed by extracted ion chromatogram (EIC) using a 5ppm window around the theoretical *m/z* of the compound of interest to eliminate the majority of chemical interferences (Figure 2 and 3). This makes data review more efficient.

FIGURE 1. MRM of 50 ng/mL peptide HYLNLVTR, 508.4 → 751.4, acquired from typical triple quadrupole mass spectrometry.

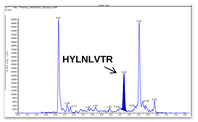


FIGURE 2. EIC of 50 ng/mL peptide HYLNLVTR, m/z 508.2878 with 5 ppm window, acquired from Q Exactive Orbitrap mass spectrometry.

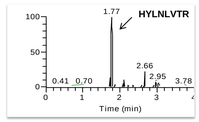
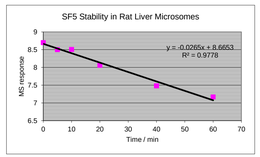
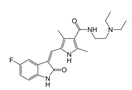


FIGURE 3. T ½ calculation of sunitinib (SF5) in mouse liver microsomes, based on the peak area of SF5 with 5 ppm mass accuracy window.



T1/2	11.4 min
R^2	0.98



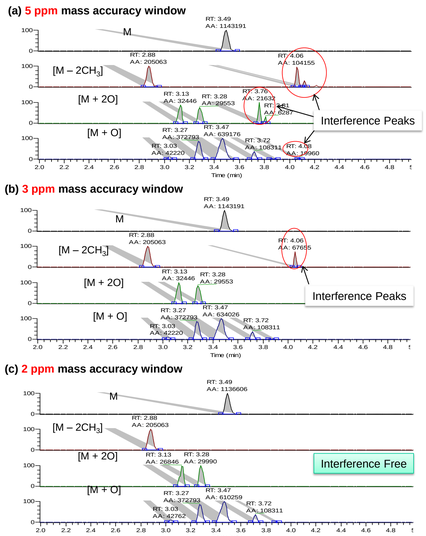
SF5: C22H27FN4O2

High Mass Accuracy to Eliminate Interference

Many drugs has aromatic structure, which leads to produce multiple isomers in phase-I metabolites, such as multiple oxidation sites on an aromatic ring. It's very critical to get the right number of isomers and estimate the most active functional group. Triple quadrupole mass spec user has to acquire MS/MS data to study the fragmentation pattern of each isomers and needs to find the linkage from drug compound to metabolites with respect to their MS/MS data similarity. It is time consuming to understand fragmentation pattern and also highly possible to miss isomers due to low quality MS/MS data.

Being benefited from the ultra high resolution of Orbitrap technique [Ref 1], ions of interests can be separated from background matrix. Additionally, the excellent mass accuracy within 2 ppm can be achieved from Q Exactive Orbitrap mass spectrometry across mass range of *m/z* 50- 2000 without using lock mass. As shown in Figure 4, the number of interference peaks can be reduced significantly by narrowing mass window from 5 ppm to 2 ppm, but peak areas of the metabolites remained the same. Lots of efforts were saved to identify and confirm metabolite isomers.

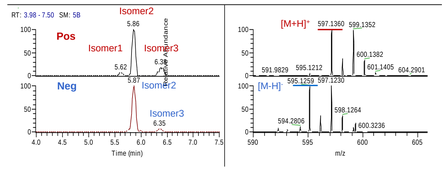
FIGURE 4. EIC of compound 4 and its metabolites with (a) 5 ppm, (b) 3 ppm, (c) 2 ppm mass accuracy window.



Polarity Switching (Pos/Neg) to Monitor Positive and Negative Metabolite Ions

Due to the difference in chemical property between the drug compound and its metabolites, they perform differently in positive ionization and negative ionization sometimes. For instance, oxidative metabolism can also form metabolites that are more readily detected by negative ionization. For traditional high resolution accurate mass (HR/AM) instruments this would require running the sample twice. Here we show the utility of HR/AM polarity switching (pos/neg) in a single LCMS run (Figure 5). This proved to be especially useful for the mouse study where the sample was limited.

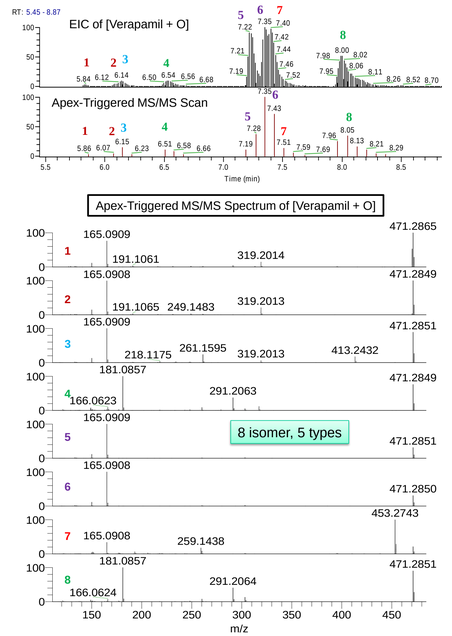
FIGURE 5. Positive, Negative EIC and MS spectrum of [3-1824 + O] metabolite from 30 min mouse liver microsomal incubation.



Apex-Triggered MS/MS Scan to Obtain Qualitative Data

For the qualitative information, data dependant approaches like apex triggered MS/MS were evaluated. The instrument estimates the chromatographic peak apex and triggers an MS/MS scan (Figure 6). This ensured the highest quality data were gathered on metabolites of interest.

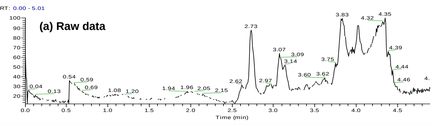
FIGURE 6. EIC and Apex-Triggered MS/MS scan of [Verapamil + O] metabolite from 60 min human liver microsomal incubation [Ref 2].

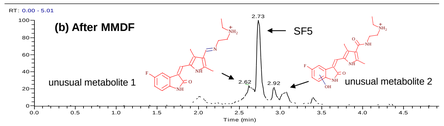


Multiple Mass Defect Filter (MMDF) to Discovery Unusual Metabolites

MMDF is a powerful feature in the latest version of Thermo Scientific MetWorks 1.3 Software that combines the results from up to six different MDFs. MMDFs were able to filter out the vast majority of the background ions in the full scan spectra while preserving those related to the parent drug. As shown in Figure 7, TIC chromatograph was simplified by MMDF and it was more feasible to discover the drug-related compounds, especially, unusual metabolites generated from the dissociated drug compound.

FIGURE 7. Total ion chromatogram (TIC) of SF5 incubated in mouse liver microsoms for 60 min, (a) raw data and (b) data processed by MMDF.





Conclusion

The routine study of drug metabolites in *in vivo* and *in vitro assays* can be more efficient by using new features in the Q Exactive Orbitrap mass spectrometry.

- Ultra high resolution and accurate mass improve ion selectivity and significantly reduce the inference from biological matrix.
- Polarity switching (Pos/Neg scan) allows both negative and positive ions be monitored simultaneously, a great approach for metabolites mapping with a single injection.
- Apex-Triggered MS/MS scan allows a MS/MS spectrum be acquired at the apex of the chromatogram peak along with acquiring the quantitation data, a very efficient way for Quan/Qual analysis.
- Multiple Mass Defect Filter (MMDF) simplifies the full-MS spectrum and make it easier to find unusual metabolites from biological matrix.
- The scan speed on Q Exactive Orbitrap mass spectrometry (4Hz at R=70,000, 12.5Hz at R=17,500) provides superior sampling rate allowing excellent compatibility with UHPLC.

References

1. Michaela Scigelova and Alexander Makarov, "Advances in bioanalytical LC–MS using the Orbitrap™ mass analyzer", *Bioanalysis*, 2009, Vol. 1, No. 4, 741-754.
2. M Walles, T Thum, K Levsen, J Borlak," Metabolism of verapamil: 24 new phase I and phase II metabolites identified in cell cultures of rat hepatocytes by liquid chromatography–tandem mass spectrometry" *Journal of Chromatography B*, 2003, Vol. 798, No. 2, 265–274.

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