Introduction

Metabolomics is used within the pharmaceutical industry to investigate biochemical changes resulting from pharmacological responses to potential drug candidates. The ability to identify markers of toxicity/efficacy can significantly accelerate drug discovery and help define the appropriate clinical plan. Data from liquid chromatography-mass spectrometry (LC-MS) metabolomic profiling experiments contains large amounts of chemical background that often confounds biomarker discovery. New mass spectrometer technology and data processing software were utilized here to reduce chemical background in animal experiments investigating the relation of animal age and nutrition to discerning drug-induced changes.

In typical LC-MS metabolomics studies, much of the data is redundant (multiple ions per component) and irrelevant (chemical noise). External factors that influence metabolic profiles (age, nutrition) increase biological variation. Because many of the chemical entities are unknowns, it is especially important to filter false positives before implementing structure elucidation. Ultra-high resolution instruments combined with ultra-high performance LC (UHPLC) separations address the issues of chemical noise and redundancy by providing sufficient resolution to distinguish metabolites from chemical background. Accurate mass data allows sophisticated processing needed to recognize related signals. This leads to significant reduction in data size and providing improved quantitation of targeted metabolites. Biological factors have profound impact on metabolic profiles and even modest metabolic changes can obscure drug-induced metabolic effects. Understanding normal metabolic changes in rats helps to minimize “biological noise” and provides more confidence in assigning specific drug-related metabolic changes.

Goal

To develop a new automated workflow and instrumentation for metabolomics biomarker discovery.

Experimental

Sample Preparation

Blood samples were taken from groups of male rats (fully satiated, acute and chronic fasting, different ages) and analyzed using LC-MS. Protein was removed from serum samples (50 µL) by the addition of 100 µL of cold methanol with 0.1% formic acid. Samples were dried down and reconstituted in 200 µL of H₂O/methanol 90:10. N-benzoyl-D₅-glycine internal standard (tR = 4.27 min, m/z 185.0969) was spiked into every sample.

Liquid Chromatography

Chromatographic separation was achieved using a Thermo Scientific Open Accela 1250 UHPLC system and a Thermo Scientific Hypersil GOLD aQ column (150 x 2.1 mm, 1.9 µm particle size). The injection volume was 3 µL. The chromatographic conditions were as follows:

- **Column temperature**: 50°C
- **Flow rate**: 600 µL/min
- **Solvent A**: 0.1% formic acid in H₂O
- **Solvent B**: 0.1% formic acid in acetonitrile
- **Gradient**:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Mass Spectrometry

High resolution accurate mass (HRAM) data was acquired in both positive and negative ion mode using a Thermo Scientific Q Exactive Orbitrap mass spectrometer (Figure 1) operated at 70,000 resolution (FWHM).

Data Processing

The data was analyzed using Component Elucidator (CE) data processing algorithms in Thermo Scientific SIEVE 2.0 software to determine the metabolic effects of food deprivation on the rats.
Results and Discussion
Figure 2 illustrates the high quality LC-MS data obtained for the N-benzoyl-D$_5$-glycine internal standard. The positive ion data for serum QC replicates was obtained between 25 to 35 hours after mass calibration and demonstrates excellent peak area and mass measurement stability on a UHPLC timescale. The chromatographic peak width was 3.6 s at the base, and 15 scans were acquired across the peak.

Figure 3 illustrates the value of obtaining 70,000 resolution for determining elemental composition of endogenous metabolites. The expanded view around the A+2 isotope (m/z 313) shows a single $^{34}$S is present. This assignment is not possible at 35,000 resolution (simulation) since the $^{13}$C$_2$ isotope is unresolved from $^{34}$S at the lower resolution.
The data processing workflow for component elucidation is shown in Figure 4. The software interprets the data like an analyst does. Instead of treating each data file as a separate entity, the data is processed in a batch and information gained in one run is used to verify information gained in the next run. In this way data gaps are minimized because each component is defined at its maximum concentration in the dataset. In samples where the concentration is much lower, the same component is identified and quantified using a more targeted approach.

A high degree of data reduction was achieved. The processing removed much of the noise from the system, leading to tighter statistical groupings and more confidence in the differential analysis and putative assignments.

Table 1 describes the rat study designed to monitor the effect of fasting on metabolic profiles. Figure 5 shows that the principal component analysis (PCA) nicely clusters the control group of fed rats, the pooled QCs, and 4, 12, and 16 hour fasted serum. There is clearly a difference between fed versus fasted serum and time of fasting. Figure 6 shows metabolites that are increasing (Met, 20:4 FA) and decreasing (Pro, 18:2 LPC) with fasting time. Figure 7 illustrates that the same patterns of uric acid are observed in serum analyzed in both positive and negative ion mode. Biological variability is the primary source of noise in these data since there is excellent reproducibility in pooled serum QC replicates for each metabolite.
Background Subtraction
Based on a series of blank runs

Start with Most Abundant Ion
Extracted ion traces

Automatically Interpret Spectra
Charge state, adducts, isotopes confirm chromatographic co-elution

Elucidate Monoisotopic Adduct Ion
Add to Component List
Quantitative comparison

Chromatographic Alignment
Search for missing components

Peak Area Normalization
Total useful signal or internal std

Build Quantitation Table
All components and samples statistically significant differences

Annotate Components
Local database or ChemSpider search

Figure 4. Data processing workflow in SIEVE 2.0 software with component elucidation algorithms

Figure 5. Principal component analysis (PCA) of rat serum negative ion LC-MS data

Table 1. Rat fasting study design

<table>
<thead>
<tr>
<th>Group</th>
<th>Male, n</th>
<th>Fasting Time</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>1: 1101-1105</td>
<td>5</td>
<td>16hr – Fast</td>
<td>Dark Cycle Control (No Fast)</td>
</tr>
<tr>
<td>2: 2101-2105</td>
<td>5</td>
<td>12hr – Fast</td>
<td>2 hr Fast</td>
</tr>
<tr>
<td>3: 3101-3105</td>
<td>5</td>
<td>4hr – Fast</td>
<td>4 hr Fast</td>
</tr>
<tr>
<td>4: 4101-4105</td>
<td>5</td>
<td>8hr – Fast</td>
<td>8 hr Fast</td>
</tr>
<tr>
<td>5: 5101-5105</td>
<td>5</td>
<td>16hr – Fast</td>
<td>12 hr Fast</td>
</tr>
<tr>
<td>6: 6101-6105</td>
<td>5</td>
<td>16hr – Fast</td>
<td>16 hr Fast</td>
</tr>
</tbody>
</table>

Note: Keep with Figure 5

1 Rats fasted during 6:00 PM to 6:00 AM dark cycle to capture peak feeding time
Figure 6. Examples of metabolite changes upon fasting

Figure 7. Overall method robustness, uric acid positive and negative ion data
Conclusion
The Q Exactive™ Orbitrap LC-MS provides a precise and robust platform for untargeted metabolomics studies. To deal with the numerous sources of noise inherent to these studies, intelligent data reduction tools found in SIEVE™ software can be used to significantly reduce the chemical noise. In addition, the use of systematic studies help to characterize biological noise, while metabolomic pre-screening can help indentify biological outliers to ensure homogeneity within an entire study.

As demonstrated in this study, fasting is a significant variable in model design, and fasting data can help contextualize drug-induced changes in many metabolites. Fasting in rats was found to have a profound impact on metabolomic profiles. Although most metabolic changes were modest in extent, fasting exacerbated or obscured some drug-induced metabolic effects.