Optimized Metabolite Extraction from Fecal Material for Biomarker Analysis

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Overview
Autism Spectrum Disorder (ASD) is a general term for a group of complex neurodevelopmental disorders that lacks a clinical biomarker. Emerging evidence shows that the intestinal microbiome in ASD subjects can be distinguished from controls, suggesting metabolite differences may be observable due to the action of intestinal microbes. Our lab has previously studied the metabolites sterobilin and sterobilinogen as putative biomarkers for ASD wherein a 45% depletion of sterobilin was observed with a p < 0.001. In this study, we investigate the utilization of extraction techniques for the analysis of metabolites within the feces of a murine model of ASD. Through the utilization of HRMS, we examine the use of syringe filters, solid phase and Bligh-Dyer extractions, as well as in-line columns to further identify metabolites of interest within fecal material as potential biomarkers.1,2

Introduction

What is autism?
- Autism spectrum disorder (ASD) and autism are both general terms for a group of complex disorders of brain development.3
- ASD can be associated with intellectual disability, difficulties in motor coordination and attention, and physical health issues such as sleep and gastrointestinal disturbances.4

What Mouse Model is Used?
- Timothy Syndrome (TS) mice have been shown to be accurate behavioral animal models for autism.4
- TS is a rare autosomal dominant disorder in which 80% of patients tend to develop ASD.4
- There are 3 strains of TS in the colony: TS1-neo (inverted neomycin resistance cassette), TS2-neo, and TS1-no-neo.

What is Sterobilin?
- Sterobilin (C24H14N2O3, MW: 594.34 g/mol) is a member of the group of mammalian metabolites known as bilin tetrapyrroles.5
- Sterobilin is a product of heme metabolism formed from the degradation of bilirubin.5

Methods
Timothy Syndrome Mice
- Fecal samples were collected from mice and stored at -20°C until use.
- Murine fecal samples from 12 TS mice and controls are chosen at random to create a pooled fecal slurry to determine the validity and reproducibility of each extraction technique.

Extraction of Bilins from Excrement
- Bilins need to be extracted from fecal material to be properly analyzed by mass spectrometry.
- 25 mg of fecal slurry were used for each extraction procedure.
- Each extraction and sample were analyzed in triplicate.
- Polypropylene syringe filters, Oasis MAX SPE Cartridges, Bligh-Dyer Extractions, C18 zip tips, and a Polar Advantage II (PA2) liquid chromatography column were all utilized.
- Prior to extraction isotope labelled sterobilin was added to allow for the examination of percent recovery of the compounds of interest. After extraction, a standard of quinapril is added to determine percent recovery of both the standard and labelled sterobilin.

Instrumentation
All analysis was done by a Bruker Daltonics 12T Solarix FT-ICR MS or a Thermo Scientific Orbitrap Q Exactive Focus using electrospray ionization in positive ion mode.

Results

Separation by LC-MS on Orbitrap
- Through the use of a decreased flow rate (0.180 mL/min) coupled to the addition of 0.1% formic acid as a charging agent, unlabeled and labeled sterobilin are able to be resolved from one another. Quinapril also showed as a separate elution for use in quantitation.
- The utilization of intensity affected the calculated average percent loss negatively. The utilization of quanbrowser in the future with a calibration curve of quinapril could more accurately determine the efficiency of the technique.

Summary of Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Percent Loss</th>
<th>Standard Deviation</th>
<th>Coefficient of Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe Filter</td>
<td>47.1 %</td>
<td>5.1 %</td>
<td>4 - 9 %</td>
</tr>
<tr>
<td>Solid Phase Extraction</td>
<td>30.8 %</td>
<td>4.1 %</td>
<td>1 – 8 %</td>
</tr>
<tr>
<td>Bligh-Dyer Top Layer</td>
<td>76.5 %</td>
<td>10.2 %</td>
<td>0.6 – 4 %</td>
</tr>
<tr>
<td>Bligh Dyer Bottom Layer</td>
<td>99.9 %</td>
<td>0.1 %</td>
<td>0.01 – 0.2 %</td>
</tr>
<tr>
<td>Bligh-Dyer Top Layer with Zip Tip</td>
<td>86.2 %</td>
<td>5.6 %</td>
<td>3 – 8 %</td>
</tr>
<tr>
<td>PA-2 Column</td>
<td>90.6 %</td>
<td>1.1 %</td>
<td>1 %</td>
</tr>
</tbody>
</table>

Figure 6: Analysis of different extraction techniques.

Conclusions
- Although the Bligh-Dyer extraction proved to be promising to lessen matrix suppression, the appearance of labelled sterobilin in the bottom layer would alter the calculations of concentrations as both labelled and unlabelled compounds were not effected equally.
- The use of a PA-2 column did separate bilins and other components to enhance signal and lessen suppression from other compounds but requires further evaluation as the intensity provided is not a proper evaluation technique for percent loss.
- Due to the ability of solid phase extraction to be tailored specifically for the extraction of bilins meanwhile lessening matrix suppression, it has been chosen for further analysis.
- Solid phase extraction also proved to have acceptable reproducibility with the least percent loss reported.

Future Work
- Further evaluation of percent loss utilizing PA-2 Column.
- Viability of chosen extraction technique with urine samples.
- Determination of the validity of depletion sterobilin and sterobilinogen across multiple mouse models of autism.
- Further testing of samples for sterobilinogen depletion.
- Testing our putative biomarker in human urine samples.

References

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Figure 1: Structure of Sterobilin
Figure 2: Control and TS2 mice.
Figure 3: Outline of Extraction Protocol.
Figure 4: TIC of a syringe filter sample on the PA2 Column with representative mass spectra of the peaks with unlabeled bilins and quinapril.
Figure 5: Comparison of representative mass spectra by multiple extraction techniques.