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Systems based LC-MS metabolite profiling of mice treated with ethanol enriched liquid diets

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Introduction

Changes in endogenous metabolites through ethanol administration has enabled a greater understanding of metabolic stress responses. Alcohol can be delivered via intragastric cannulas or through liquid diets in which animals can be given alcohol for several months and high blood alcohol levels can be maintained. However, in this study a less invasive, four week ethanol enriched liquid diet model was evaluated as an alternative vehicle. To assess the impact of reduced level ethanol exposure, a systems based approach was taken measuring urine, plasma and liver tissue to characterize both ethanol metabolites and endogenous lipid changes.

Samples were measured by liquid chromatography ion trap mass spectrometry (LCMS-IT-TOF), separated by Nexera UHPLC. Metabolite identity was confirmed via the combination of accurate mass formula prediction, authentic standard analysis and database searching (LipidMaps, Metlin, HMDB and KEGG).

Materials and Methods

Animal liquid diets (Dyets, Inc., Bethlehem, PA) were administered to control mice (Dyets #710079) and ethanol treated mice (Dyets #710301). The percentage alcohol derived calorie in the alcohol diet was for week 1 - 10%, week 2 - 20%, weeks 3 and 4 - 35%. When 35% of the calorie was derived from alcohol, the alcohol concentration was ~6.5% (v/v). Urine samples were prepared taking 50uL, diluted with 150uL water. Plasma samples were prepared taking 50 uL plus addition of 150 uL MeOH ultra-centrifuged and 160 uL supernatant removed to which 80uL water was added. Liver samples (ca. 50 mg) were extracted into 1 mL of MeOH/ACN/water 40:40:20 (V/V), for 10 min in an ultrasonic bath followed by ultracentrifugation and supernatant extracted. The pellets were further extracted in 1.5 mL $CHCl_3$:MeOH 3:1 as previously described (1). Urine and plasma was separated by Nexera UHPLC (Shimadzu) using a Phenomenex Kinetex column (XB C18 1.7 um 2.1×100 mm) over 12 minutes (A - 0.1% formic acid solution + 20 mM ammonium formate and B – methanol + 0.1% formic acid + 20 mM ammonium formate) at a flow rate of 0.7 mL/min. Liver tissue was separated over 32.5 min starting at 50% B using a stepwise binary gradient previously described (1). LCMS analysis was performed using an LCMS-IT-TOF (Shimadzu Corporation, Kyoto, Japan) using the mass range *m/z* 70-1250 with positive/ negative switching (100 msec). MS^n data was acquired on selected QA/QC samples.

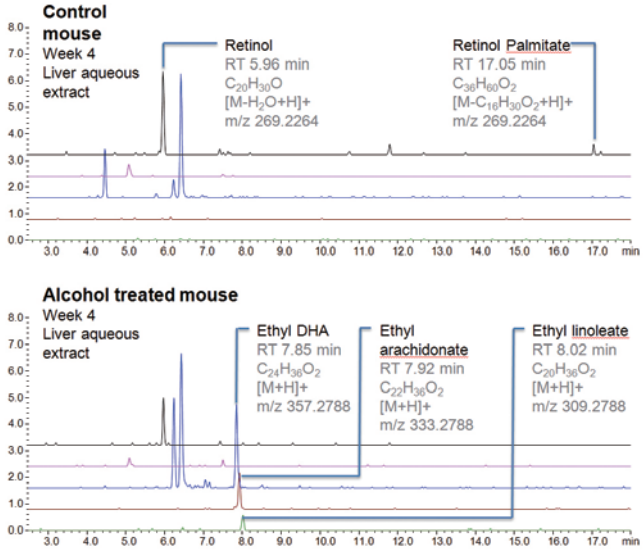

Fig. 1 Chromatograms showing significant differences detected between control and alcohol treated mice from liver tissue aqueous extract (equivalent scaling). 7-keto-cholesterol (pink, *m/z* 401.3414, RT 7.50 min) was also detected at elevated levels in alcohol treated animals

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Results

In this current study the experimental model was changed from previous work to include animal liquid diets as opposed to intra-gastric feeding cannulas. Aqueous liver sample measurements from this study continued to detect ethylated lipid marker compounds, consistent to previous work published from intragastric feeding models (1). Interestingly the profound lipid differences observed from the intragastric feeding model were markedly reduced in the current liquid diet study with the emergence of ethylated lipid markers at week 4 but not at week 2. These included ethyl arachidonate, ethyl linolate and ethyl DHA which were not detected in control or 2 week alcohol treated samples. In broad agreement to previously published work, retinol palmitate and retinol were also significantly reduced in ethanol treated animals (Fig. 1). Two commonly observed phosphatidylcholine compounds (*m/z* 496.3398 & 524.3711) in addition to 7-keto-cholesterol were measured at elevated levels in ethanol treated mice compared to control

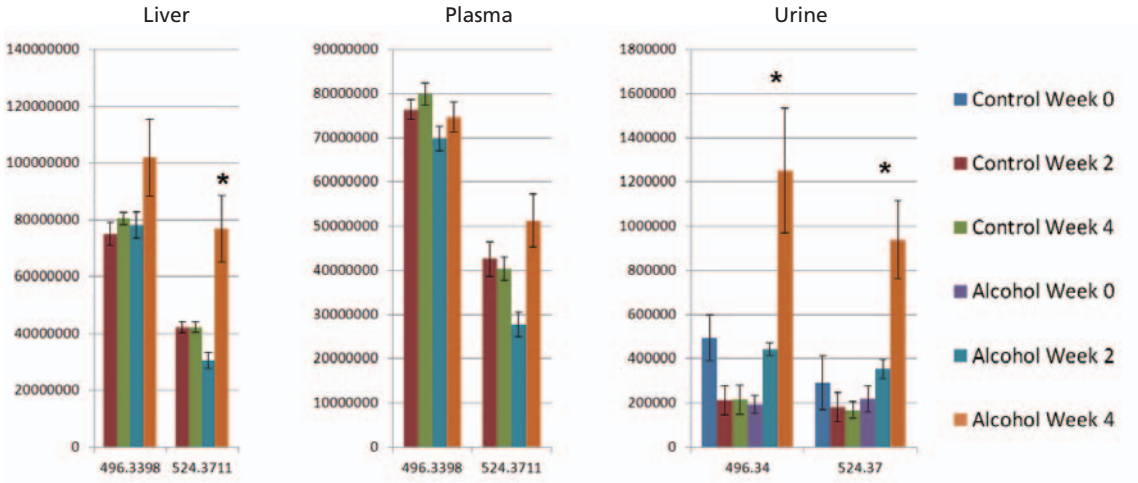

Fig. 2 Two commonly observed phosphatidylcholine compounds (*m/z* 496.3398 & 524.3711). Normalized intensities from liver, plasma and urine, demonstrating significantly increased levels detected in 4 week alcohol treated animals in urine and liver tissue compared to plasma, that was not significantly different (*P<0.05 t-test; alcohol treated week 4 tested against remaining groups).

In contrast to previous work, in this study urine, plasma and liver tissue were analyzed. This systems based approach enabled a targeted metabolomics analysis to monitor known endogenous metabolites and ethanol metabolites. Urine samples were also found to contain increased levels of ethanol metabolites including ethyl glucuronoide and ethyl sulphate, metabolites commonly used to determine recent ethanol consumption (Fig. 3).

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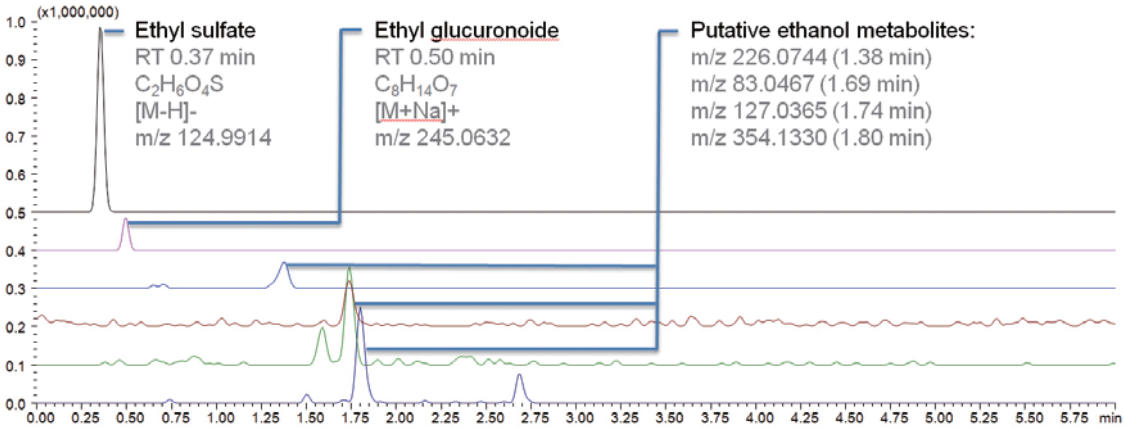

Fig. 3 Ethanol metabolites detected in urine samples from alcohol treated week 4 animals using MetID Solution software (Shimadzu Corporation, Kyoto, Japan). Additional putative ethanol metabolites only detected in 4 week ethanol treated samples were based on accurate mass measurement (LCMS-IT-TOF typically better than 5 ppm mass accuracy).

Using an untargeted profiling approach, ions of significance could be visualized using Profiling Solution software (Fig. 4). Database searching via the human metabolite database (HMDB) found *m/z* 144.1022 corresponded to the osmoprotective compound, proline betaine commonly detected in urine. This was detected in all mouse urine at week 0 but markedly reduced in weeks 2 & 4, possibly indicating the beginning of liquid diet for both control and alcohol treated animals. Ions *m/z* 143.1159, 144.0651 & 146.0812 were detected at elevated levels in week 4 alcohol treated animals and corresponded to fatty acid compounds commonly detected in urine. In particular vinylacetylglycine and isobutyrylglycine have been associated with disrupted metabolism.

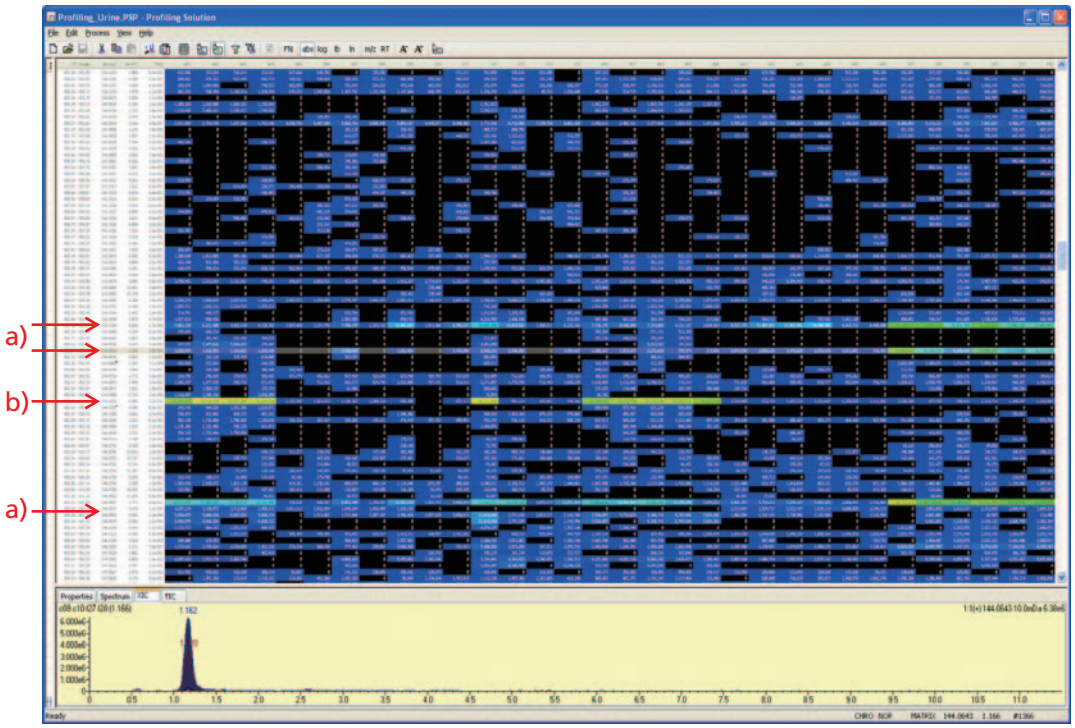

Fig. 4 Profiling Solution unique spectral binning data alignment of mouse urine samples. Visual representation of peak area enables identification of potentially significant ions. Ions detected at elevated levels in week 4 alcohol treated mice: *m/z* 143.1159 (RT 0.67 min), *m/z* 144.0651(RT 1.17 min), *m/z* 146.0807 (RT 1.371). *m/z* 144.1022 (RT 0.40 min) elevated in control and alcohol treated mice at week 0 but lower during weeks 2 and 4.

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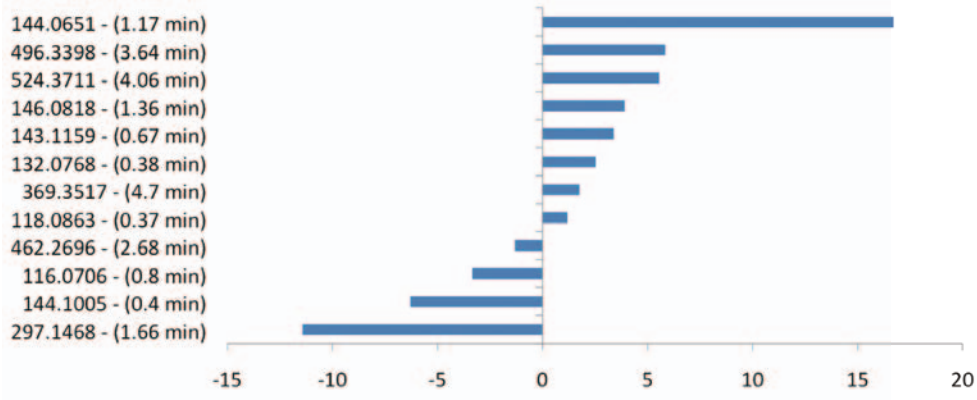

Fig. 5 Fold difference of elevated and decreased ions detected in week 4 alcohol treated mouse urine compared to week 4 control mouse urine.

Data handling of complex matrices such as urine, plasma and liver requires both confidence in LC reproducibility and stability in mass accuracy during several days analysis time. To take advantage of Profiling Solution ability to align data sets and subsequent group data statistical analyses, the same LC gradient was used for urine and plasma. Aqueous and organic extracts of liver tissue also shared the same separation gradient with exception that organic sample separation contained an added 10 minutes 100% isocratic B to elute non polar lipids absent from aqueous samples. Principal components analysis (PCA) was performed on data arrays generated by Profiling Solution. Sample types (urine, plasma, liver aqueous, liver organic) could be distinguished from one another however experimental groups could not be distinguished, indicating that only a small number of ions differed between experimental groups.

Conclution

- The use of targeted and un-targeted data mining techniques successfully identified significant differences between alcohol treated and control mouse bio-fluids.
- Subtle differences in endogenous metabolites following alcohol treatment may indicate that in this study the level of alcohol consumption by liquid diet was lower than in previous experiments using intragastric feeling cannulas.
- Differences detected in this study may indicate areas of metabolism that first become affected through prolonged alcohol consumption.

Reference

(1) Journal of Proteome Research (2011) 10, 705-713

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