Metabolomics Studies of Drug Containing Urine Samples by Rapid Ultra-high Pressure Chromatography and High Resolution Mass Spectrometry With a LTQ Orbitrap Mass Spectrometer

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Overview

Purpose: This study demonstrates the synergistic value of fast high resolution chromatography, high resolution and high mass accuracy mass spectrometry data analysis using the SIEVE™ (Thermo Electron Corp, Waltham MA) software package for separation and identification of novel and varying concentrations of important metabolites.

Methods: A sample set representative of a typical metabolomics study was generated by spiking multiple levels of three classes of drugs (amphetamines, Opiates and Primidones) into normal human urine. A control urine sample was also prepared. Fast and normal chromatographic separations were evaluated. All compounds were detected using a LTQ Orbitrap™ (Thermo Electron Corp, Waltham MA) hybrid mass spectrometer to acquire high mass accuracy full scan and nominal mass accuracy data dependent product ion spectra. All of the data was analyzed using the SIEVE software to produce novel and differential concentration metabolite profile information.

Results and Conclusions: Results demonstrate the synergistic value of fast chromatography combined with high resolution high mass accuracy mass spectrometry for detecting changes in biological matrices that are not observable in total ion chromatograms. Detection of these differences requires the use of sophisticated software that normalizes minor variations in retention time and then creates AxB type correlations. These results are validated by the use of cluster analysis.

Introduction

Metabolomics represents considerable promise for correlating phenotypic changes with the underlying chemical composition when comparing relative composition and concentration differences between a control and modified sample set. One of the greatest challenges for metabolomics studies involves identifying small changes within a highly complicated matrix. We have studied the effect of very high resolution (and ultra-high pressure) chromatography and high mass accuracy toward addressing issues of sample complexity. This report describes the use of an AxB metabolomics type of comparison to determine the differences in control and treated urine samples. The treated samples consist of urine drug spiked with a cocktail of drugs at a range of concentrations. These samples will be analyzed using a novel high speed HPLC for compound separation, coupled to a very high resolution and high mass accuracy Linear Ion Trap Orbitrap (LTQ OT) mass spectrometer for analyte detection and identification. The SIEVE software package is used to determine statistically significant differences between small changes in control and concentration variant analytes with in the very complicated urine matrix.

Methods

Samples Information: Single stock mixtures of three classes of drugs, opiates, amphetamines and primidones were purchased (Alltech-Applied Science Laboratories) and diluted for use in this study. A single time point urine sample was collected from a healthy non-smoking male. This sample was aliquoted in to 5 mL portions to be used for both control and sample matrices. Each of the sample matrices were spike with a different concentration of the three drug mixtures; ranging from 10pg/uL to 1ng/uL and designated A, B, C, and D as indicated below. The sample and control solutions and internal standard consisting of Reserpine at a final concentration 250 pg/uL was added. The spiked drugs are listed in the following tables:

Amphetamine drugs	*Opiates*	*Primidone drugs*
d-Amphetamine	Codeine	Ethosuximide
Phentermine	Morphine	α-Methyl-α-propylsuccinimide
d-Methamphetamine	Hydromorphone	Methorbital
3,4-MDA	Oxycodone	Barbital
3,4-MDMA	Nalorphine	Methsuximide
3,4-MDE	Diacetylmorphine	Phensuximide
		Normethsuximide
		Mephenytoin
		Ethotoin
		Methobarbital
		PEMA
		Phenobarbital
		Methyl PEMA
		10,11-Dihydrocarbamazepine
		Primidone
		Phenytoin
		Carbamazepine
		4-Methylprimidone

Sample A, B, C, and D are urine samples spiked with different concentration of drugs listed as the following composition:

Sample name	*Opiates*	*Primidone drugs*	*Amphetamine drugs*
Control	0	0	0
Sample A	10 pg/µL	10 pg/µL	10 pg/µL
Sample B	50 pg/µL	100 pg/µL	100 pg/µL
Sample C	100 pg/µL	500 pg/µL	500 pg/µL
Sample D	250 pg/µL	1,000 pg/µL	1,000 pg/µL

Sample Preparation: The mixture were lyophilized and reconstituted with 1 ml of water. Equal volume of methanol was added and centrifuged for 10min. The remaining precipitate were reconstituted with 2 ml of 1:1 methanol : water solution and centrifuged. The collected supernatants were lyophilized again and subsequently reconstituted in 2 mL of water. For comparison, the control urine sample without drug spiking were prepared following same procedure.

Chromatography: The reconstituted samples were analyzed using a Accela™ (Thermo Electron Corp, Waltham MA) hyperbaric liquid chromatography system coupled to an LTQ Orbitrap mass spectrometer via an Ion-Max electrospray interface. The chromatography system consisted of quaternary Pump and a high throughput autosampler. The HPLC columns used in this study were Hypersil-Gold™ (Thermo Electron Corp, Waltham MA) columns and are described in greater detail below. The resulting HPLC-MS and HPLC-MS/MS data was analyzed using the Thermo Electron SIEVE software package.

Three technical replicates of each prepared sample were injected directly on to an HPLC column for normal or fast chromatography with and eluted with a water + 0.1% formic acid (solvent A) / acetonitrile + 0.1% formic acid (solvent B). The fast chromatography was performed using a 1.9 micron particle size column (2.1x50 mm) with a mobile phase flow rate of 0.6 mL/min and the conventional separation used a 5 micron particle size column (2.1x100 mm) and a mobile phase flow rate of 0.25 ml/min. The following mobile phase gradients were used:

1.9 micron particle size column (2.1x50 mm), 10 µL injection.

Time (min.)	A% (0.1% FA in water)	B% (0.1% FA in ACN)	Flow rate
0	95	5	600 uL/min
1	95	5	600 uL/min
11	5	95	600 uL/min
12	5	95	600 uL/min
12.1	95	5	600 uL/min
15	95	5	600 uL/min

5 micron particle size column (2.1x100 mm), 10 µL injection.

Time (min.)	A% (0.1% FA in water)	B% (0.1% FA in ACN)	Flow rate
0	95	5	250 uL/min
1	95	5	250 uL/min
30	5	95	250 uL/min
31	5	95	250 uL/min
31.1	95	5	250 uL/min
36	95	5	250 uL/min

LTQ Orbitrap™, Accela™ ChromAlign™ and SIEVE™ are trademarks of Thermo Electron Corp, Waltham MA.
Spotfire DecisionSite™ is a trademark of Spotfire, Inc., Somerville, MA.

Mass Spectrometry: The ESI interface was operated using a sheath gas flow rate of 40 units for the 0.6 mL/min flow rate and 20 units for the 0.25 ml/min flow rate. The aux gas was set to 10 units for 0.6 ml/min and 5 units for 0.25 mL/min mobile phase flow rate. The spray voltage was set to 5.0 kVolts.

The LTQ Orbitrap was operated in parallel mode so that while the Orbitrap mass analyzer was acquiring the high resolution high mass accuracy full scan mass spectrum, the LTQ was acquiring MS² and MS³ spectra in data dependent mode. The Orbitrap mass resolution was set to 30,000 at m/z 400 with a scan range of 100 to 1000 acquired in approximately 0.3 seconds. No internal mass calibration was used. The LTQ isolation for MS³ studies on the most abundant ions was set to 2 mass units with a normalized collision energy of 35%. Operating in this fashion, three nominal mass data dependent MS³ scans were acquired for each high mass accuracy full scan spectrum.

Data Analysis: SIEVE is designed to support the discovery of both metabolite and protein biomarkers by facilitating the analysis of data from large-scale, label-free, LC-MSn, differential expression experiments in a statistically significant manner. SIEVE was used in this experiment to determine differential levels of opiates, primidone drugs and amphetamines in a series of human urine samples. Spotfire DecisionSite™ Browser (Spotfire, Inc., Somerville, MA) is used for data visualization in SIEVE, allowing the user to review overall semi-quantification results and interactively inspect the reconstructed ion chromatograms for the differentially expressed components. A proprietary algorithm within SIEVE called ChromAlign™ (Thermo Electron Corp, Waltham MA) corrects for any chromatographic variation across the samples being analyzed in order to minimize the effects of shifts in retention time..

Results

Figure 1 and Figure 2 showed the LTQ Orbitrap full scan base peak chromatograms of each urine sample using fast and normal-speed chromatography, respectively. The total separation for the fast chromatography was 15 minutes compared with 36 minutes for the normal-speed method. Close inspection of the chromatograms suggest a slight improvement with the faster chromatography owing to the increased chromatographic resolution and narrowed peak-shape. The fast chromatography provided better retention time reproducibility between the three replicate injections with a standard deviation of between 0 to 0.03. compared with a retention time standard deviation. However, the standard deviation of between 0.01 to 0.25 for the normal-speed chromatographic method. Consequently the fast chromatographic provides clear advantages in separation and speed compared with normal-speed chromatography using conventional column technology.

FIGURE 1. Orbitrap full scan base peak chromatograms of sample control, A, B, C, and D using fast chromatography.

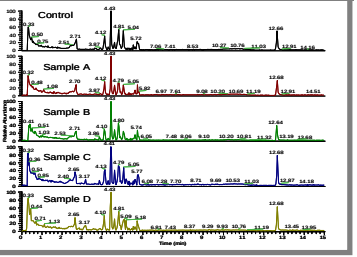
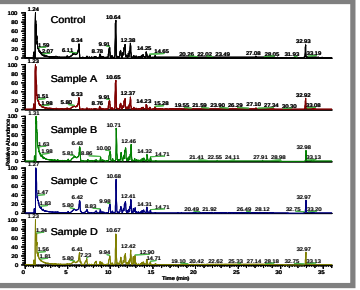


FIGURE 2. Orbitrap full scan Base peak chromatograms of sample control, A, B, C, and D using normal-speed chromatography.



While all the spiked analytes were in urine were well separated using both fast and normal-speed chromatography, the fast chromatographic method produced sharper peak shapes with an average peak width at the base of 5 seconds. This sharper peak shape also resulted in an increase in the analyte related signal-to-noise ratio. This observation is compound related and is most notable for the mass chromatogram for the MH+ of Methsuximide as shown in Figure 3. Consequently, the fast chromatography can actually provide increased detectability for some compounds.

Signals for Individual drugs were extracted from the total ion chromatograms using accurate mass *m/z* values calculated from the monoisotopic mass for the MH+ (positive ion detection) or M-H- (negative ion detection) species of each compound as was appropriate. For these extracted mass chromatograms an accurate mass limit of 5 ppm was used to determine "real" drug signals from the urine background peaks. Thus the high resolution accurate mass capability of the Orbitrap mass analyzer provides an additional dimension of analytical separation necessary for such complicated mixtures. Figure 3, and Figure 4 show examples of these extracted ion chromatograms from the Orbitrap full scan analysis of sample B for fast and normal-speed chromatography, respectively. Using 5 ppm accurate mass filtering also increased the signal-to-noise ratio (data not shown) for the individual compound signals compared with nominal mass accuracy detection by the LTQ alone. Table 1 shows the mass accuracy of the spiked compounds as detected in the urinary matrix. The extreme mass accuracy permitted unambiguous identification of the spiked drugs. These identifications were validated by the co-injection of high level standards containing only the drugs of interest.

FIGURE 3. Fast chromatography extracted ion chromatograms for the MH+ signals for the spiked analytes in sample B. Total separation time was under 15 minutes (5ppm accurate mass filter limit).

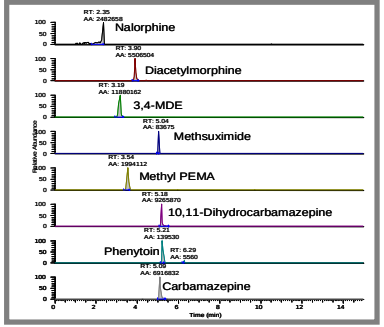


FIGURE 3. Normal-speed chromatography extracted ion chromatograms for the MH+ signals for the spiked analytes in sample B. Total separation time was under 36 minutes (5ppm accurate mass filter limit).

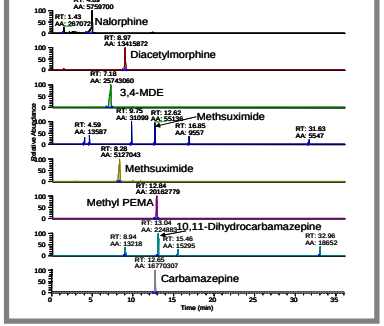


Table 1. Mass accuracy of the detected analytes in sample B using the Orbitrap mass analyzer.

Compound	Theoretical Monoisotopic Mass (Da)	Experimental monoisotopic Mass (Da)	Mass Error (ppm)
Codeine	300.1594	300.1585	-3.00
Morphine	286.1438	286.1430	-2.79
Hydromorphone	286.1438	286.1427	-3.84
Oxycodone	316.1543	316.1537	-1.90
Nalorphine	312.1594	312.1589	-1.60
Diacetylmorphine	370.1649	370.1639	-2.70
d-Amphetamine	136.1121	136.1115	-4.41
Phentermine	150.1277	150.1272	-3.33
d-Methamphetamine	150.1277	150.1270	-4.66
3,4-MDA	180.1019	180.1012	-3.89
3,4-MDMA	194.1176	194.1170	-3.09
3,4-MDE	208.1332	208.1326	-2.88
Methsuximide	204.1019	204.1016	-1.47
Phensuximide	190.0863	190.0856	-3.68
Normethsuximide	190.0863	190.0854	-4.73
Mephenytoin	219.1128	219.1121	-3.19
Ethotoin	205.0972	205.0966	-2.92
PEMA	207.1128	207.1122	-2.89
Methyl PEMA	221.1285	221.1278	-3.16
10,11-Dihydrocarbamazepine	239.1179	239.1174	-2.09
Primidone	219.1128	219.1121	-3.19
Phenytoin	253.0972	253.0964	-3.16
Carbamazepine	237.1022	237.1019	-1.26
4-Methylprimidone	233.1285	233.1280	-2.14
Reserpine	609.2807	609.2789	-1.31

The raw data from each of the technical replicates for samples A-D were analyzed using the SIEVE software package. Figure 5 shows an example of overlapping base-peak chromatograms after alignment of the data using the ChromAlign algorithm for samples B and D in which the difference in spiked analytes was 1-5 for the opiates and 1-10 for the primidones and amphetamines. Differences in analyte concentrations are not evident in base-peak chromatograms as shown in figure 5.

FIGURE 5. An example of the RT alignment for a portion of the base-peak chromatograms for the technical replicates for samples B and D.

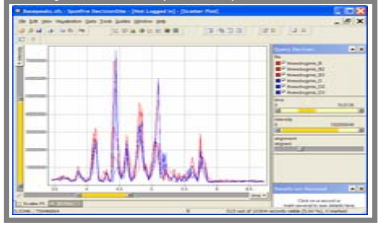


Figure 6 shows a summary of the results from the experiment comparing Sample B to Sample D. In this case, results were sorted by lowest pValue – the lowest indicating the most statistically significant differences. Columns in this table include m/z range for each frame, retention time range for each frame, pValue and ratio of change.

FIGURE 6. A component table showing the components with the most significant differences between sample B and sample D. The relative ratios between the two samples are show as well as a significance score (p-value). Entries highlighted are related to the spiked compounds .

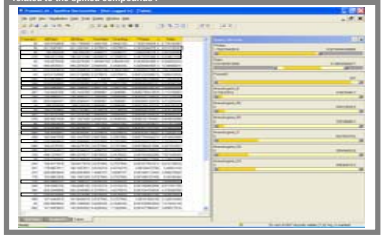


Figure 7 shows a consolidated scatter plot view of all the components for the B vs D experiment (x-axis: m/z, y-axis: ratio of change) before any filters were applied. As expected, most of the components center around a relative ratio of 1, indicating no significant change in relative concentration. Differences were expected and observed at levels of 1:5 (Opiates) and 1:10 (Primidone drugs and Amphetamines). As shown in this figure, SIEVE detected differences in components despite a relatively small number of replicate injections, variations in chromatography, matrix effects inherent in the sample and the low levels of drugs present in the samples. Additionally, the user can readily validate these ratios by clicking through to the underlying reconstructed ion chromatograms to further interrogate the data as needed. When a filters of pValue<0.02 and ratios between 1 and 50 are applied, the most significant differences owing to the spiked compounds are found between 1 and 10 with significant number near 5 or 10 due to the spiked concentrations

FIGURE 7. (A) A consolidated scatter plot of all component ratios for the B vs D comparison. (B) Same data using filters of p< 0.02 and ratio of 1 to 50.

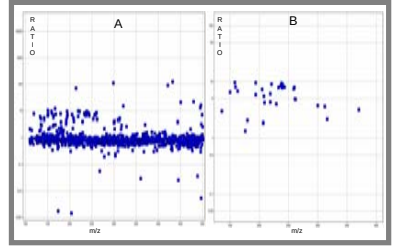
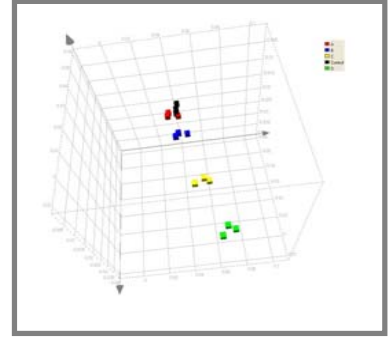


Figure 8 shows a cluster analysis of the data from the four spiked samples and control each with three technical replicates analyzed using the fast chromatographic method. The technical replicates cluster very tightly with significant separation between each sample suggesting that the effects of the spiked compounds were significant even though the total ion chromatograms between samples were not visibly different.

FIGURE 8. A cluster analysis of the four spike samples and the control data using the fast chromatographic method.



Conclusions

- Fast chromatography offers significant gains over normal-speed chromatography by reducing analysis time while still retaining or improving chromatographic resolution and sensitivity with an improvement in signal to noise ratios for some compounds.
- High resolution accurate mass detection of compounds provides an additional degree of separation valuable for complicated metabolomics studies. The high mass accuracy also provides valuable information for reducing or even unambiguously identifying unknown compounds.
- The differences in component concentrations were readily identified by the SIEVE software program. While the spiked compounds were not evident in the total-ion chromatograms of all of the samples when compared to the control, the effect was sufficient to be observed by SIEVE and by cluster analysis.