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Metabonomics: Electrospray Mass Spectrometry Coupled to HPLC Shows Potential for the Screening of Rat Urine in Drug Development

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Introduction

Metabonomics is a rapidly growing area of scientific research [1]. It is a systems approach for studying *in vivo* metabolic profiles and can provide information on disease state, toxicity and gene function [2-4]. In metabonomics the effect of a pharmaceutical candidate on a whole animal or organism is investigated by measuring the changes in endogenous metabolites over a time course following compound administration. The analytical data generated in these studies is analyzed by mathematical techniques such as principal component analysis (PCA) to highlight both subtle and gross differences in the samples [5-7]. This metabonomics approach is now being investigated by large pharmaceutical companies to screen compounds for toxicity, lead compound selection and human disease profiles to name but a few.

To date the vast majority of work in this field has utilized ^{1}H-NMR as the analytical method of choice [1]. While being very effective, NMR has two significant disadvantages namely; poor sensitivity and lack of analyte resolution leading to the masking of low abundance analytes by high concentration components.

Electrospray LC/MS has become the technique of choice for bioanalysis, both quantitative [8] and qualitative [9-10]. Here we describe how electrospray LC/MS can be successfully employed in the metabonomic analysis of rat urine

Experimental

- 20 rat samples were used for this controlled study.
- The rats were orally dosed as per Table 1, where time point 1 is 0-8 hours and time point 2 is 8—24 hours.
- The analysis was performed on a Waters Alliance® HT system connected to a Micromass Quattro Micro™ tandem mass spectrometer.
- The MS data were divided into 10 bins of 100 scans.
- A combined spectrum was created, for each 100 scan bin.
- The spectrum list was transferred to MATLAB where PCA was performed.
- Accurate mass information was determined on a Micromass Q-TOF Ultima™ utilizing a Waters Alliance® HT system.

Column:	Waters Symmetry® C_{18} column (2.1mm x 10 cm , 3.5 µm)
Mobile Phase:	0-30% B gradient over 7 minutes
Flow rate:	600 µL/min where A = 0.1% formic acid (aq) and B = acetonitrile + 0.1% formic acid.
Sample Prep:	Urine diluted1:4 with water
Injection Vol:	20 µL
MS conditions:	Negative ion electrospray, scan mode 100-800 m/z
Scan time :	0.2 seconds, dwell time of 0.05 seconds.
Capillary voltage:	3.5 kV
Cone voltage:	30 V

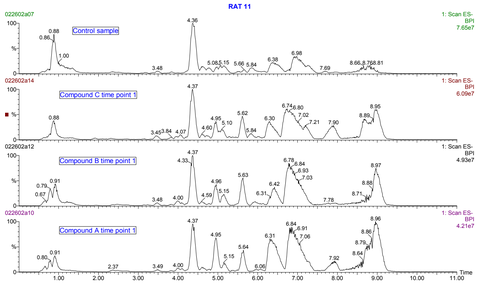
LC/MS

The chromatograms from the control and dosed urine samples at time point 1 reveals a qualitative difference, Figure 1.

LC/MS/MS analysis of the peaks responsible for the qualitative differences confirmed that none of these were dose related metabolites.

Therefore these observed differences are probably due to a change in the metabolic state of the animals.

Figure 1. Comparison of BPI chromatograms of dosed and control rats samples.



Principle Component Analysis (PCA)

The PCA interrogation of the whole data set of retention time and m/z values is presented in Figure 2. Each number represents an individual rat. In this figure, the data relating to the control samples are contained within the circled area.

This data clearly shows that LC/MS can be used to differentiate between the dosed and control animal samples.

Table 2 lists the principle ions found to be responsible for the separation in the PCA interrogation.

Figure 3 illustrates the changes in analyte abundances of selected ions from Table 2, following the administration of pharmaceutical candidates.

Dividing the data into 100 scan bins simplified the PCA data yielding more subtle information. An example is shown for minute 5 (Figure 4).

Table 1. Sample Information

Dose	Time point	Sample ID
Control	1	1-3
A	1	4,6
B	1	7-8
C	1	11-12
Control	2	13-15
A	2	16-18
B	2	19-21
C	2	23-24

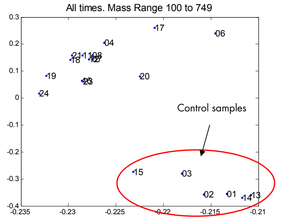


Table 2. The analytes responsible for the PCA separation and the change in relative abundance.

Compound dosed	Analyte m/z value	Change
A	283	10 fold increase
A	461	5 fold increase
A	187	10 fold increase
B	338	2 fold reduction
B	283	10 fold increase
B	461	10 fold increase
B	187	10 fold increase
C	283	20 fold increase
C	187	30 fold increase
A,B,C	192	3 fold increase

Figure 3. Ion intensity comparison of PCA identified ions (m/z 187, 192, 283, 338) in all 20 rat urine samples.

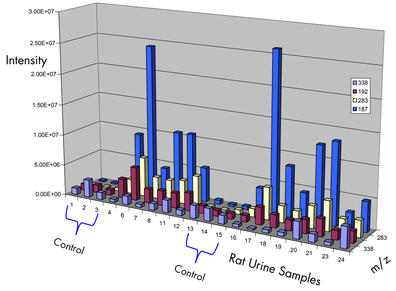


Figure 4. 20 overlaid LC/MS chromatograms that show the ions responsible for the separation in the PCA plot.

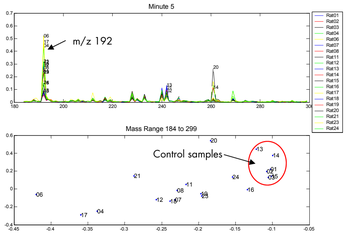
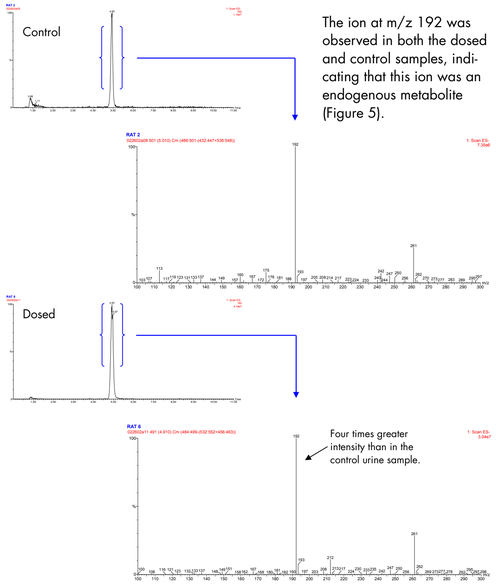
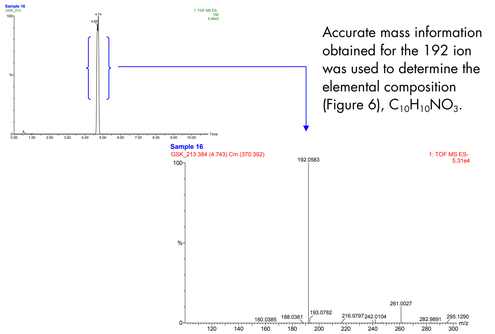


Figure 5. Extracted ion chromatogram (m/z =192) of both control and dosed rat urine samples.



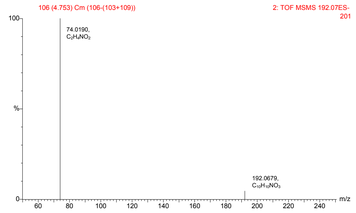
The ion at m/z 192 was observed in both the dosed and control samples, indicating that this ion was an endogenous metabolite (Figure 5).

Figure 6. Accurate mass information from Extracted ion chromatogram (m/z =192).

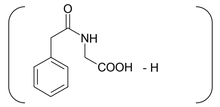


Accurate mass information obtained for the 192 ion was used to determine the elemental composition (Figure 6), $C_{10}H_{10}NO_3$.

Figure 7. Collision induced dissociation mass spectrum of the ion at m/z 192.



The structure of the negative ion at m/z 192 was elucidated by using a combination of accurate mass and MS/MS fragment information (Figure 7). The ion at m/z 192 was determined to be phenyl acetyl glycine (PAG) , Figure 8. This structure was confirmed by comparison of the ^{1}H-NMR of the purified analyte with the authentic compound.



m/z 192

Conclusion

- LC/MS in combination with PCA has been successfully applied to the screening of rat urine.
- With this methodology it was possible to differentiate the control samples from the dosed samples.
- The m/z values of components, *i.e.*, metabolites, responsible for the PCA separation were identified.
- One of the components (m/z 192) responsible for the PCA result was determined to be phenyl acetyl glycine (PAG).
- LC/MS is complementary or even an alternative to proton NMR for metabonomics.

References

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