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A NOVEL MS-BASED METABONOMIC APPROACH TO THE DETERMINATION OF BIOMARKERS OF DRUG TOXICITY

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OVERVIEW

- The aim of this study was to determine whether LC/MS could be a viable alternative, or complementary to NMR spectroscopy for toxicology studies in drug discovery and development.
- Analysis of a large batch of samples from a toxicology study.
- An integrated system consisting of the Waters® Micromass® Q-Tof micro™ Mass Spectrometer with LockSpray™, the Alliance® 2795 XC Separations Module and the 2487 Dual Wavelength Absorbance Detector using MassLynx™ Software's MarkerLynx™ Application Manager.
- Multivariate statistical analysis for identification of the potential biomarkers of toxicity within a simple Application Manager (Figure 2).
- Increased levels of creatine in the urine after dosing is indicative of reduced liver function.
- The results show complementary information to ^{1}H NMR studies.

INTRODUCTION

Metabonomics is a rapidly growing area of scientific research primarily utilizing proton NMR spectroscopy as the analytical method of choice. It involves the study of time-related metabolic profile changes that can be the result of exposure to a toxin or drug, to environmental effects or the onset of disease.

These studies are concerned with the complement of endogenous compounds rather than with xenobiotic compounds or metabolites. The primary goal is to identify and quantitate small molecules in a biological system that are affected as the direct result of an external stimulus. Knowledge of these compounds, or biomarkers, can then be used for diagnosis, screening, or to direct further research.

The metabonomics approach is now being widely investigated by large pharmaceutical companies in the area of drug discovery and development where an early indication of toxicity is of paramount importance in preventing the late attrition of a potential drug candidate.

Figure 1. Waters® Metabonomics MS System combines the Waters Micromass® Q-Tof micro™ Mass Spectrometer with LockSpray™, the Alliance® 2795 XC Separations Module and the 2487 Dual Wavelength Absorbance Detector using MassLynx™ Software's MarkerLynx™ Application Manager.

Hydrazine is a commonly used model hepatotoxin in animal studies and here we present data from the analysis of rat urine samples from a toxicological study.

METHODS

Animal Studies

- Male rats (8 per group) were given a single oral dose of hydrazine (30 or 90 mg/kg) formulated in saline.
- The animals were housed in metabolism cages, and urine collected into refrigerated urine racks.
- Urine samples were collected from all animals over the periods -24 to -12h and -12 to 0h (pre-dose samples), and 0-12h, 12-24h, 24-48h, and 48-72h.
- At 72h four animals from each group were sacrificed for histopathological examination.
- Urine samples were collected from the remaining animals for the periods 72-96h, 96-120h, 120-144h and 144-168h after dosing.
- Each animal was sacrificed after completion of the final collection and all urine samples were frozen at -20 °C until analysis.

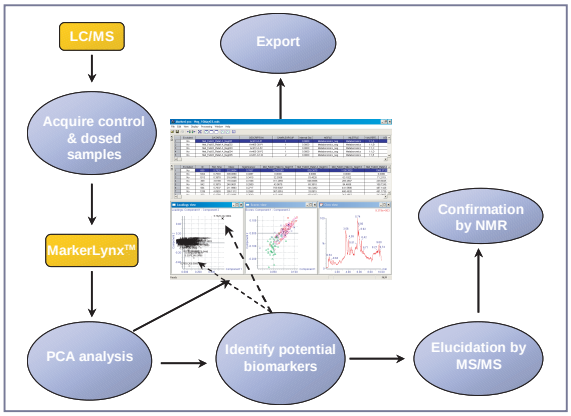

Figure 2. MarkerLynx Strategy.

HPLC Conditions

HPLC	Waters 2795 XC Separations Module
Column	Waters Symmetry® C_{18}, 2.1 x 100 mm, 3.5 µm
Column temperature	40 °C
Mobile phase A	H_2O + 0.1% (v/v) formic acid
Mobile phase B	acetonitrile + 0.1(v/v) formic acid
Gradient	0-0.5 min 0% B
	0.5-4 min linear gradient 0% B to 20% B,
	4-8 min linear gradient 20% B to 95% B
	8-9 min hold at 95% B then return to 0% B
Flow	0.6 mL/min split to ~120 µL/min to MS
Injection volume	10 µL

MS Conditions

MS	Q-Tof micro with LockSpray interface
Ionization mode	Electrospray positive and negative ion
Sample cone	30V
Source heater	120 °C
Desolvation heater	250 °C
Cone gas (nitrogen)	50 l/h
Desolvation gas	500 l/h
Scan	m/z 50-1500, 0.4 sec integration, 0.1 sec interscan delay, centroid mode
Lock frequency	5 secs
Reference	leucine enkephalin, $[M+H]^+$ 556.2771, $[M-H]^-$ 554.2615
Run time	10 min

DATA PROCESSING

- The analysis of large numbers of complex biological samples such as these can generate a wealth of data which require multivariate statistical analysis and pattern recognition methods.
- The MarkerLynx Application Manager has been developed to detect peaks in the data set, create a list of the detected ions with associated intensities and finally perform principal component analysis (PCA).
- The facility to export the detected masses and ion intensities to third party multivariate software packages for further analysis has also been incorporated.
- In this analysis the data was exported and analyzed by PLS-DA using SIMCA multivariate software (Umetrics, Sweden).

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RESULTS

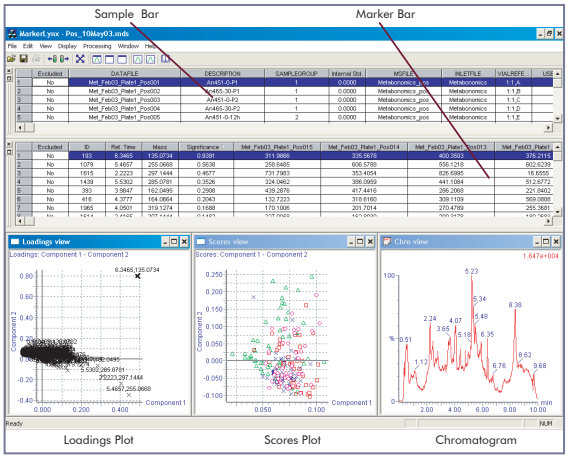

Figure 3. MarkerLynx Positive Ion Results.

Positive Ion Results

- The positive ion data was exported from MarkerLynx (Figure 3) into SIMCA (Umetrics, Sweden) and the PLS-DA scores plot for the positive ion data is shown in Figure 4.
- The scores plot of the principal components provides information about the separation of the dose groups in two dimensions. Only the high dose samples show separation from the predose and controls samples.
- The loadings (weights) plot indicates which ions contribute to the formation of the scores and allows for the identification of those ions of greatest influence to the separation/clustering and potentially the deduction of biomarkers of toxicity or disease state.
- Examination of the weights plot, Figure 5, indicates that the principal ions responsible for the separation in positive ion mode are: m/z 135, 311, 217 and 132 increasing after dosing and m/z 297,162, 260, 319 and 285 decreasing after dosing.
- Exact mass MS and MS/MS identified the ion at m/z 132.0773 as being from creatine (Figure 6).

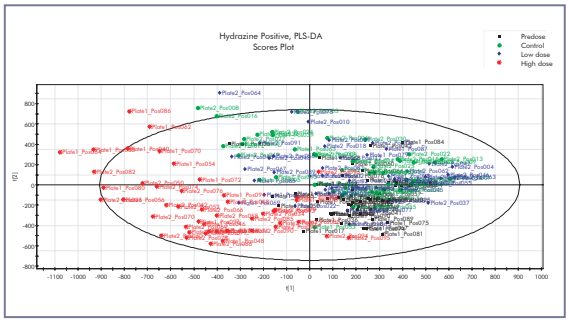

Figure 4. SIMCA PLS-DA Positive Scores Plot.

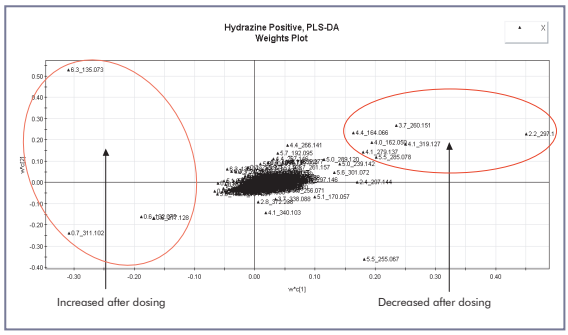

Figure 5. SIMCA PLS-DA Positive Weights Plot.

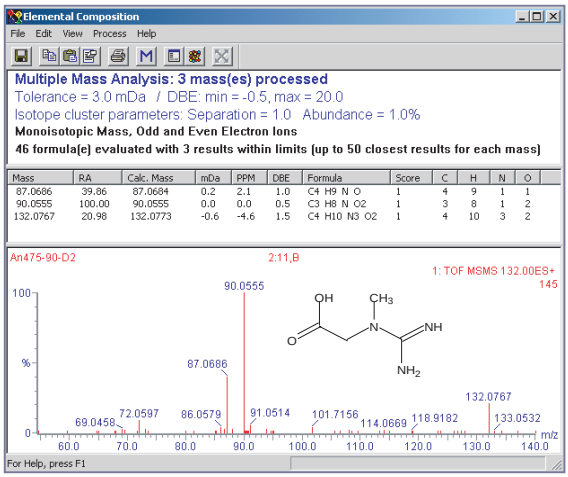

Figure 6. Exact mass MS/MS product ion spectrum for m/z 132 from creatine.

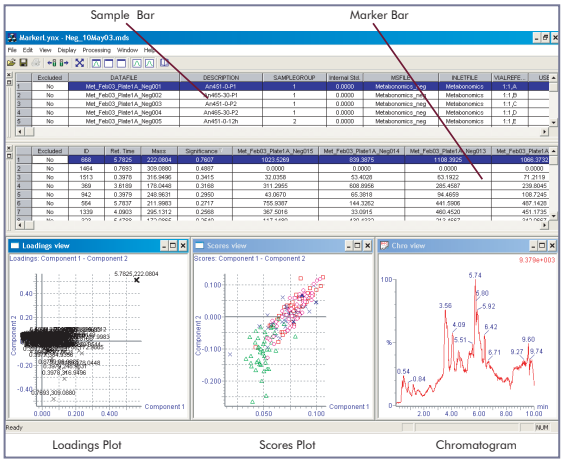

Figure 7. MarkerLynx Negative Ion Results.

Negative Ion Results

- The PCA scores plot (Figure 7) showed the separation between the high dose and the controls and low dose samples was better in negative ion.
- The PLS-DA weights plot (Figure 8) shows the m/z values responsible for the separation are m/z 309, 317 and 192 increasing after dosing and m/z 222, 295 and 178 decreasing after dosing.
- Exact mass MS/MS product ion analysis on m/z 178 confirmed it as being from hippuric acid (Figure 9).
- Hippuric acid was also identified as decreasing after dosing in the ^{1}H NMR study on the same sample set.

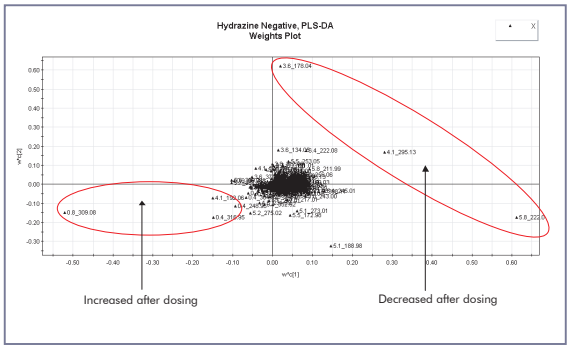

Figure 8. SIMCA Negative ion PLS-DA weights lot.

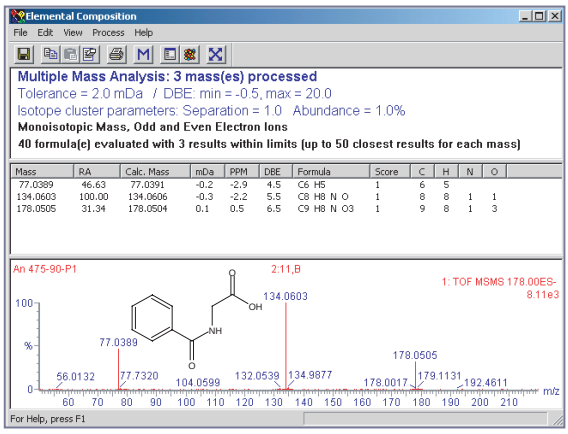

Figure 9. Exact mass MS/MS product ion spectrum for m/z 178 from hippuric acid.

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CONCLUSIONS

- A rapid gradient on a Waters Alliance 2795 XC Separations Module with a Symmetry column was employed to maximize throughput while maintaining chromatographic resolution.
- A Waters Micromass Q-Tof micro fitted with a LockSpray source ensured exact mass MS and MS/MS measurements to aid identification of any potential biomarkers highlighted.
- Data analysis was facilitated by implementation of the MarkerLynx Application Manager.
- PCA separation achieved by MS was similar to that seen by NMR.
- Bacterial contamination of the samples shows a greater variability than the effect of the low dose of hydrazine (also observed by NMR).
- PCA shows that MS can differentiate high dose samples from the controls. The ions responsible for the separation can be identified.
- Further structural elucidation of these potential biomarkers is required by exact mass MS/MS before their biological significance can be determined.
- Creatine was identified by MS as increasing after dosing. Increased urinary levels of creatine and taurine observed in the NMR study were associated with reduced liver function.
- The results presented here clearly show that LC/MS can provide complementary information to ^{1}H NMR in metabonomic applications and may be a viable alternative in the field of drug discovery and development.

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