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

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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 (Figure 1) consisting of a Waters® Micromass® Q-Tof micro™ + LockSpray™ + Waters Alliance® HT + MarkerLynx™ Application Manager
- Multivariate statistical analysis for identification of the potential biomarkers of toxicity within a simple Applications Manager (Figure 2)
- 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 xenobiotics. 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.

Cyclosporin A ($C_{62}H_{111}N_{11}O_{12}$, M.Wt. 1201.8414) is an immuno-suppressive drug widely used following transplant surgery but it is also a known nephrotoxin. As well as resulting in kidney damage other side effects include high blood pressure, headaches, seizures, excessive hair growth, gout and coma.
The aim of this study is to show that LC/MS is complementary to NMR spectroscopy for high throughput toxicity screening for lead candidate selection. The exact masses of key components in a complicated matrix are determined and the chemical structures of the potential biomarkers elucidated by MS/MS.

Experimental

Animal Study

- Male Wistar-derived rats (n=5 per group), approx. 140g in weight acclimatized in metabolism cages for 3 days prior to treatment
- Food and water provided *ad libitum*
- One group dosed with cyclosporin A at 45 mg/kg/day orally
- Urine samples collected daily for 9 days pre- and post-dose from control and dosed animals and stored at -20 °C prior to analysis
- The excretion pattern of small organic molecules in the urine was studied using HPLC/MS

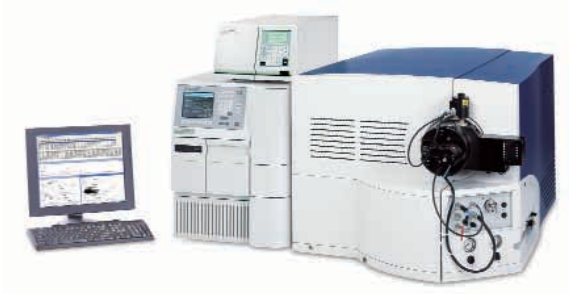

Figure 1. Waters Metabonomics MS System

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HPLC Conditions:

HPLC:	Waters 2795 XC Separations Module
Column:	Waters Symmetry® C_{18}, 2.1x100 mm, 3.5 µm
Mobile Phase:	A: water + 0.1% formic acid B: acetonitrile + 0.1% formic acid
Flow Rate:	600 µL/min split to 120 µL/min to MS
Column Temp:	40 °C
Injection Volume:	10 µL

Gradient:

Time (min)	%A	%B
0	100	0
0.5	100	0
4.0	80	20
8.0	5	95
9.0	5	95
9.1	100	0

MS Conditions:

MS	Q-Tof micro
Ionization mode	Positive Ion Electrospray
Capillary	3200 V
Sample Cone	30 V
Source Temperature	120 °C
Desolvation Temperature	250 °C
Cone Gas Flow	50 L/hr
Desolvation Gas Flow	500 L/hr

MS Acquisition Parameters - LockSpray Enabled

Acquisition Range	m/z 50-1500
Acquisition Rate	0.4 sec
Inter-scan Time	0.1 sec
Mode	centroid
Lock mass frequency	5.0 sec
Lock scans averaged	10
Lock Reference	0.5 ng/m L leucine enkephalin in 1:1 acetonitrile: water + 0.1% formic acid
Lock mass	556.2771

Data Processing

- The analysis of large batches of complex biological samples such as these can generate a wealth of data that require multivariate statistical analysis and pattern recognition methods
- The MarkerLynx Application Manager has been developed to detect peaks in the data set, collect the data and create a list of the detected ions with associated intensities for all samples and finally perform principal component analysis (PCA)
- The ability to exclude xenobiotics automatically has also been incorporated
- Option to export the detected masses and ion intensities to third party multivariate software packages e.g. SIMCA (Umetrics , Sweden) and Pirouette (Infometrix, USA) for further analysis such as PLS-DA (partial least squares discriminant analysis)

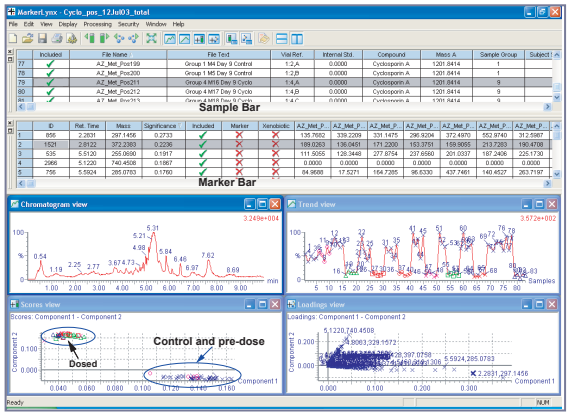

Figure 2. MarkerLynx results

Results

The MarkerLynx results are shown in Figure 2 and consists of

- a sample bar which lists the samples analyzed
- a marker bar which lists the masses and retention times with associated intensities of all the components detected in each of the samples
- a TIC chromatogram view of the selected sample
- a trend view of the selected component (m/z 372.2 at 2.81 min) across all samples
- a scores plot showing the separation and/or clustering between samples (annotation turned off for clearer visualization of the results)
- a loadings plot indicating the m/z and RT values of the ions responsible for the clustering/separation

The PCA scores plot shows clear separation between the pre-dose and control and dosed samples.

The loadings plot indicates the ions responsible for the separations are:

- increased after dosing: m/z 740, 784, 652, 696, 828, 608, 657, 872 etc. These ions all correspond to PEG which was found to be part of the dosing vehicle used to administer the cyclosporin A.

The chromatograms from a day 9 control and dosed urine are shown in Figure 3.

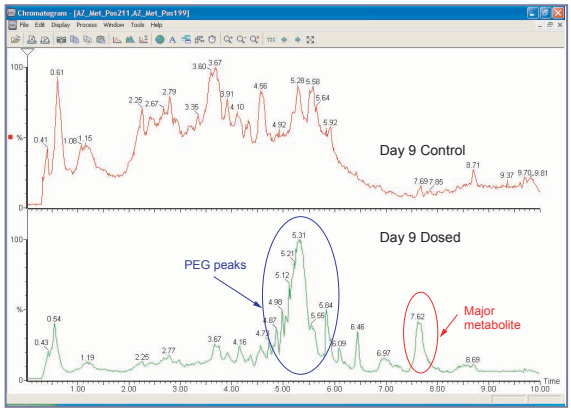

Figure 3. Day 9 control and dosed chromatograms

Exclusion of the significant PEG peaks then showed that among the principal ions responsible for the separation were metabolites of the cyclosporin A. The most significant of these was the hydroxlated metabolite which was present as both the protonated and sodiated species.

The exact mass spectrum and elemental composition report for the major metabolite is shown in Figure 4 showing errors of <1 ppm.

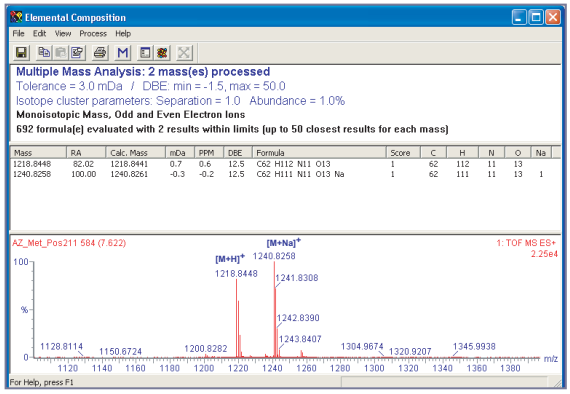

Figure 4. Elemental composition report for major metabolite of cyclosporin A

The MarkerLynx method editor can be used to automatically exclude expected metabolites within user defined limits (see Figure 5).

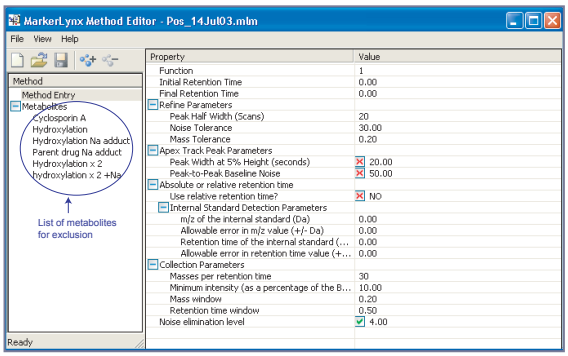

Figure 5a. MarkerLynx method editor

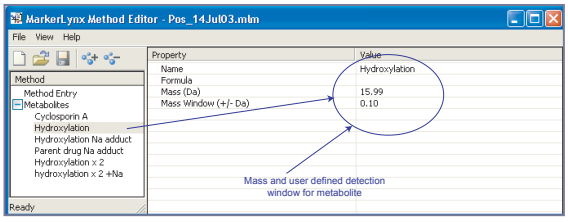

Figure 5b. Metabolite editor

The PEG peaks and metabolites of cyclosporin were excluded from the potential marker table and the PCA plots recalculated. The results are shown in Figure 6.

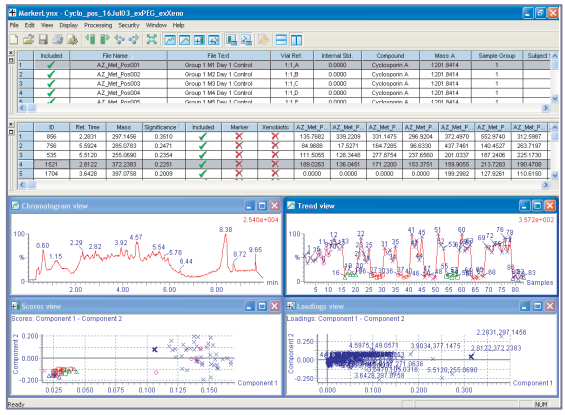

Figure 6. MarkerLynx report after exclusion of xenobiotics and PEG peaks

The PCA plot scores still shows excellent separation between the pre-dose/controls and the dosed samples. The significant ions responsible for the separation all showed a decrease in intensity after dosing and are listed in Table 1.

Principal ions showing a decrease after dosing						
Retention Time (min)	Measured Mass (Da)	Calculated Mass (Da)	Proposed Elemental Composition $(M+H)^+$	Error mDa	Error ppm	Comments
2.28	297.1456	297.1450	$C_{14}H_{21}N_2O_5$	0.6	1.9	
5.59	285.0783	285.0763	$C_{14}H_{13}O_5$	2.0	7.0	
5.51	255.0690	255.0657	$C_{13}H_{11}O_4$	3.3	12.8	
2.81	372.2383	372.2386	$C_{19}H_{34}NO_6$	-0.3	-0.8	
3.64	397.0758					
3.65	105.0316	105.0340	C_7H_5O	-2.4	-23.2	Hippuric acid fragment
3.46	350.0905	350.0836 350.0876	$C_{14}H_{18}N_2O_{10}$ $C_{18}H_{12}NO_6$	6.9 2.9	19.8 8.3	A glucuronide from MS/MS
3.90	377.1475	377.1461	$C_{17}H_{21}N_2O_6$	1.4	3.7	
5.91	271.0636	271.0606	$C_{13}H_{11}O_5$	3.0	10.9	
4.60	149.0571	149.0603	$C_9H_9O_2$	-3.6	-23.8	Cinnamic acid?
2.73	311.1611	311.1607	$C_{13}H_{23}N_2O_5$	0.4	1.3	
1.01	267.1351	267.1345	$C_{13}H_{18}N_2O_4$	0.6	2.3	
3.91	190.0433	190.0504	$C_{10}H_8NO_3$	-7.1	-37.5	Kynurenic acid

Table 1. Principal ions showing a decrease after dosing

The proposed elemental compositions are based on the exact masses reported in the MarkerLynx marker list where data has been collected within user-defined mass and retention time windows for all samples. The elemental compositions have been confirmed by exact mass MS/MS measurements. The results of the exact mass product ion measurements on m/z 149 and m/z 190 with proposed structures are shown in Figures 7 and 8.

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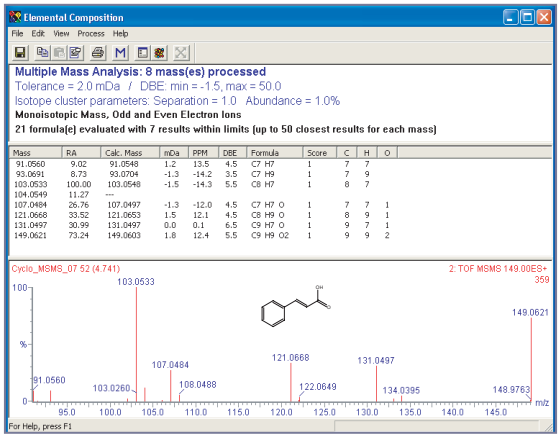

Figure 7. Elemental composition report and proposed structure for m/z 149

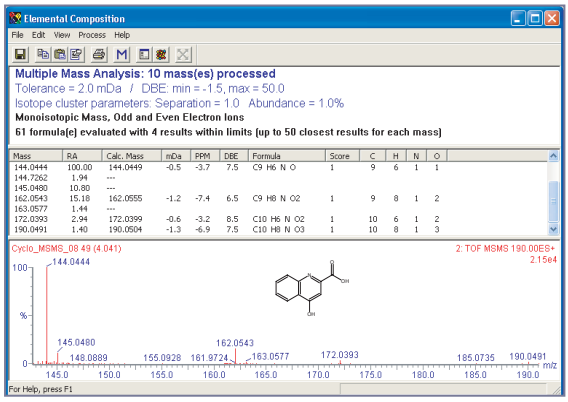

Figure 8. Elemental composition report and proposed structure for m/z 190

Conclusions

- LC/MS in conjunction with PCA analysis has been successfully used to screen rat urine after dosing with cyclosporin
- The control and pre-dose samples could easily be differentiated from the dosed samples
- The m/z values of the ions responsible for the PCA separation were identified
- MS/MS exact mass was used for structural elucidation of the potential biomarkers
- LC/MS data complementary to NMR which had identified glucose, succinate and acetate as increased after dosing with glucose being the main biomarker of nephrotoxicity. These species would not have been retained on the HPLC column under the conditions employed.
- Further structural elucidation required to determine the toxicological significance of the potential biomarkers

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