INTRODUCTION

Metabonomics 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 biological systems 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. 1H NMR has been the primary method of choice for analysis of changes to the profiles of complex biofluids, such as urine, as a result of toxic lesions induced by xenobiotics. We have investigated the use of exact mass LC/MS for the determination of potential biomarkers of nephrotoxicity. The gentamicin complex used in this study is an aminoglycoside antibiotic complex and a nephrotoxin.

EXPERIMENTAL

Animal Study

- Male Wistar-derived rats (n=5 per group), approx. 140 g in weight acclimatized in metabolism cages for 3 days prior to treatment
- Food and water provided ad libitum
- One group dosed with gentamicin at 60 mg/kg twice daily, subcutaneous
- 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 LC/MS

HPLC Conditions

HPLC: Waters 2795XC Separations Module
Column: Waters Symmetry® C18,
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:
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
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<td>95</td>
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<tr>
<td>9.1</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
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MS Conditions

MS: Waters Micromass®
Q-Tof micro™
Ionization Mode: Positive Ion Electrospray
Capillary: 3200 V positive
Sample Cone: 30 V
Source Temperature: 120 °C
Desolvation Temperature 250 °C
Cone Gas Flow 50 L/hr
Desolvation Gas Flow 500 L/hr
Argon Collision Gas
MS Acquisition Parameters - LockSpray™ Enabled
Acquisition Range m/z 50-850
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 Leucine enkephalin
0.5ng/µL 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 incorporates a peak deconvolution package which allows detection and retention time alignment of the peaks eluting in each chromatogram.
• Data is collected into a single matrix by aligning peaks with the same mass/retention time along with their associated normalized intensities.
• Finally principal component analysis (PCA) is performed.
• 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 AB, Sweden) and Pirouette (Infometrix, USA) for further analysis such as PLS-DA (partial least squares discriminant analysis).

RESULTS
The MarkerLynx results for the positive ion data 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 across all samples (m/z 255.0868, RT 2.95 min.)
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 i.e. potential biomarkers of nephrotoxicity

Gentamicin is an antibiotic complex produced by fermentation of *Micromonospora purpurea* or *M. echinospora* and consists of 3 closely related components, gentamicin C1, C2 and C1a as shown in Figure 3.

Gentamicin C1 R1=R2=CH₃ C₂₁H₄₃N₅O₇  
C2 R1=CH₃;R2=H C₂₀H₄₁N₅O₇  
C1a R1=R2=H C₁₉H₃₉N₅O₇

Figure 3. Structure of gentamicin complex

Under the HPLC conditions employed the gentamicin complex and any metabolites formed were not retained on the column and eluted with the solvent front. A spectrum and elemental composition report for the gentamicin complex, eluting at 0.3 min, from a day 9 dosed rat urine sample are shown in Figure 4.

The gentamicin and any metabolites are readily removed from the PCA plot leaving only endogenous metabolites.

Figure 4. Spectrum and elemental composition report for gentamicin complex

Figure 5. PCA Scores Plot

The scores plot shows separation of the day 5-9 dosed samples from the controls and predose samples and the associated loadings plot (Figure 6) shows the ions responsible.
The major species responsible for the separation of the day 5 - 9 dosed samples from the predose and controls are tabulated in Tables 1 and 2.

For a few concentrated components where the ions are particularly intense the mass measurements are lower than expected. This is due to detector saturation, otherwise they are typically within 5 ppm of the calculated mass for the postulated elemental composition.

MS/MS exact mass analysis has been used for structural elucidation of the potential biomarkers. The MS/MS product ion spectrum from the ion at m/z 190, which was observed to decrease in intensity after dosing, is shown in Figure 6. This was shown to be kynurenic acid (4-hydroxyquinoline-2-carboxylic acid), a metabolic product of tryptophan.

Xanthurenic acid, m/z 206, also identified as decreasing after dosing is also part of the tryptophan catabolism pathway (Figure 7). The tryptophan derived isoquinolines, kynurenic acid and xanthurenic acid, are not degraded further but are excreted in urine and are partly responsible for the yellow color of urine.

Pantothenic acid, also seen to decrease after dosing, is a building block of coenzyme A. Work is ongoing to elucidate the structures of the other ions determined as being significant in the loadings plot and to evaluate their toxicological significance.
CONCLUSIONS

- LC/MS in conjunction with PCA analysis has been successfully used to screen rat urine after dosing with gentamicin.
- The MarkerLynx Application Manager simplifies the processing by incorporating peak deconvolution and data alignment with PCA in one software package.
- The control and pre-dose samples could easily be differentiated from the day 5 - 9 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.
- Xanthurenic acid and kynurenic acid, part of the tryptophan catabolism pathway, were identified as decreasing after dosing.
- LC/MS data complementary to NMR which had identified glucose, which increased after dosing, as the main biomarker of nephrotoxicity.
- Further MS/MS structural elucidation is required for identification of the other potential biomarkers and their toxicological significance needs to be determined.
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