TURNING METABONOMICS DATA INTO KNOWLEDGE

Waters

Hilary Major [1] and Ian Wilson[2]
[1]Waters Corporation, Atlas Park, Simonsway Manchester M22 5PP, UK
[2]Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

INTRODUCTION

Metabonomics/metabolomics along with genomics and proteomics are increasingly being used as part of the "systems biology" approach to understanding the complex interplay of molecules that make up living systems. Metabonomics has been defined as "the quantitative measurement of the dynamic multiparametric responses of living systems to pathophysiological stimuli or genetic modification" [1, 2]. This approach involves comparing the endogenous metabolic profiles of mammalian systems following exposure to an external stimulus e.g. a candidate pharmaceutical, onset of disease or environmental stress, using chemometric data analysis strategies.

Metabonomics has already been widely used to study toxicity [3, 4], as it has the potential to improve the drug discovery process where an early indication of toxicity is of paramount importance in preventing the late attrition of a potential drug candidate. It is also increasingly being applied to the study of disease, and disease models, as a means of discovering novel biomarkers for assessing disease progression and drug efficacy [5].

Originally the most commonly used platform for the analysis of metabonomic samples (e.g. urine and plasma) was ^{1}H NMR [3, 4] which required little or no sample pre-treatment and acted as an unbiased detector for proton containing molecules. Increasingly MS based technologies are being used due to the enhanced sensitivity over ^{1}H NMR for many of the compound classes under investigation. GC/MS is a powerful technique for the analysis of complex mixtures but requires the use of derivatisation reagents to make endogenous metabolites such as sugars and amino acids volatile [6]. UPLC/MS analysis by contrast, like ^{1}H NMR, needs minimal sample preparation (dilution of urine or protein precipitation of plasma) and due to the sub 2µm particle size and high pressure (up to 15,000psi) results in improved chromtographic resolution, increased sensitivity and reduced analysis times.

A major difference between the MS and NMR approaches however is the extra dimension and complexity added by the use of chromatographic separation techniques. The introduction of the MarkerLynx™ Application Manager with its advanced peak deconvolution capabilities and principal components analysis (PCA) allowed the user to visualise their data and extract information about changes to the endogenous metabolite profiles from complex data sets. Any potential biomarkers highlighted by the PCA need to be identified to check their position in the metabolic pathways and asses their biological significance. The ability to search EI data against libraries such as NIST simplify the task immensely and the use of exact mass CI can confirm the molecular masses where no molecular ion is seen by EI. The task of identifying any metabolites highlighted by UPLC/MS has now been simplified by the introduction of MarkerLynx db Database which allows searching of exact mass and/or retention time against a database of metabolites.

This poster describes the use of exact mass EI and CI GC/MS in the identification of potential biomarkers as part of the metabonomic investigations of animal models of type II diabetes.
The database searching capability of MarkerLynx db has been used in the identification of potential markers of nephrotoxicity in the urine from Wistar rats dosed with the model nephrotoxin mercuric chloride as part of a UPLC/MS study of nephrotoxins [7].

METHODS

GC/MS Analysis

Blood samples were collected from 20 week old male Alderley Park Wistar-derived rats and 20 week old male Zucker fa/fa rats, used as models for obesity and insulin resistance. The samples were centrifuged at 3000g for 10 min and the resulting plasma samples were protein precipitated using acetonitrile.100µL of each supernatant was evaporated to dryness under vacuum prior to derivatisation with 20µl methoxylamine hydrochloride (40mg/mL in pyridine) at 28°C for 90min followed by 180 µl MSTFA at 37 °C for 30min.

System: Waters Micromass GCT™ fitted with a HP 6890 GC
Column: J&W Scientific DB5-MS 20m x 180µm x 0.18µm
Carrier gas: helium, 0.8mL/min
Ramp: 85°C for 2 min,15°C/min to 320°C, hold for 5min
CI reagent gas: ammonia
Lock reference: chloropentafluorobenzene (201.9609) for CI; heptacosa (218.9856) for EI
Acquisition: m/z 85 to 700; 0.15sec/spectrum; 0.05sec inter acquisition delay (0.2sec cycle time)

UPLC/MS Analysis

Male Wistar rats were administered either mercuric chloride, a model nephrotoxin, at 2.0mg/kg (dosed) or 0.9%(w/v) saline (controls). Urine samples were collected daily for 9 days pre and post dose.

System: Waters ACQUITY™/ LCT Premier™
Column: ACQUITY UPLC™ BEH C18, 2.1 x100mm, 1.7µm
Temperature: 40°C
Flow rate: 600µL/min
Phase A: 0.1% formic acid in water
Phase B: 0.1% formic acid in acetonitrile
Gradient: 0-20%B in 4mins, 20-95%B over 4-9mins.
Injection volume: 5µL
Ion mode: Electrospray positive
Capillary voltage: 3000V positive
Cone voltage: 35V
Source temp.: 120°C
Desolvation temp: 350°C
Acquisition time: 0.15spectra/sec, inter-scan delay 0.05sec
Lock reference: Leucine enkephalin

RESULTS AND DISCUSSION

All the data were processed using principal components analysis (PCA) via the MarkerLynx Application Manager. Figure 1 shows the PCA scores plot for the EI analysis of the plasma samples of the Zucker fa/fa rats and the Wistar rats. It can be seen that the samples cluster in different areas of the plot. The m/z and retention time pairs responsible for this separation can be obtained from the associated loadings plot (not shown). Library searching (NIST2005) of the EI spectra of these components often reveal a match but as this is generally based on low mass fragment ions the molecular mass can not be confirmed.

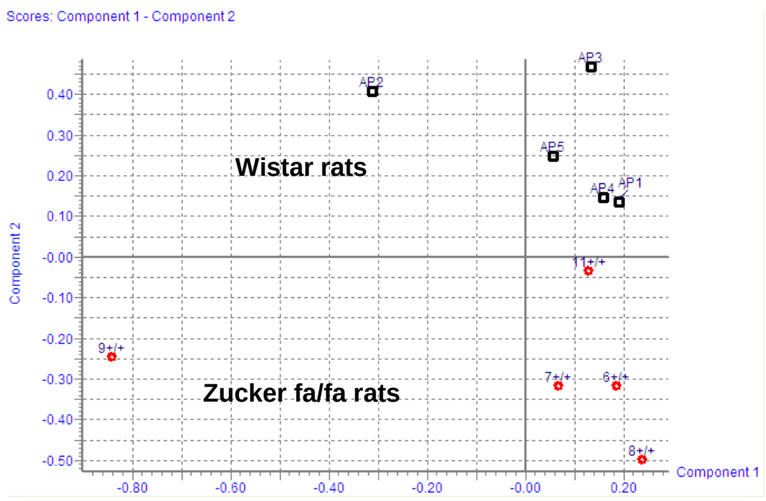

Figure 1. MarkerLynx PCA scores plot from EI GC/MS analysis of rat plasma from Zucker fa/fa and Wistar rats.

The top three library hits for the component eluting at 10.8 min, lower in the Zucker rats, is shown in figure 2. These all show matches for sugar derivatives with molecular mass 540.

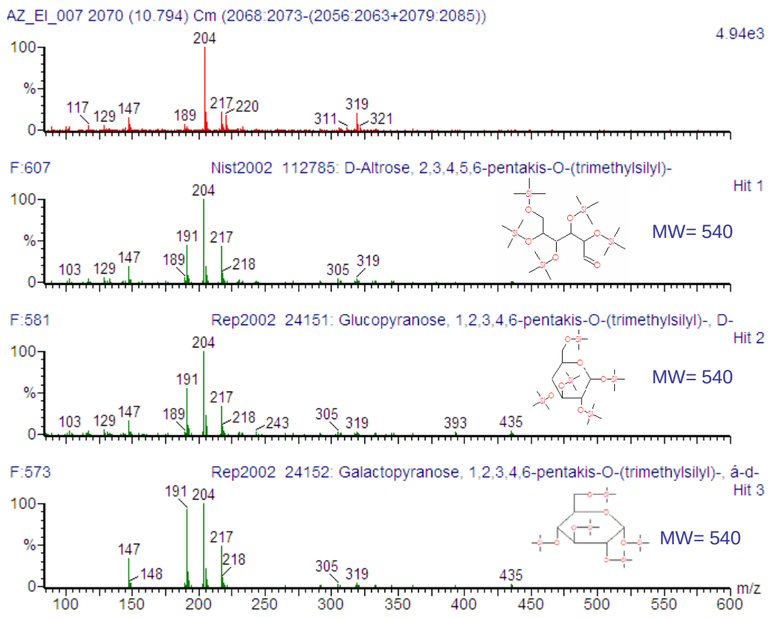

Figure 2. EI library matches for component eluting at 10.8min

Exact mass CI analysis (figure 3) however clearly shows a $(M+H)^+$ at m/z 570. The NIST library has 8 entries which match the formula $C_{22}H_{55}NO_6Si_5$, all sugar derivatives, but none of the EI spectra match. Thus whilst the availability of extensive library data for searching for EI spectra is valuable for metabonomics studies it is still subject to the limitation that many biologically relevant compounds are not yet to be found in the commercially available libraries.

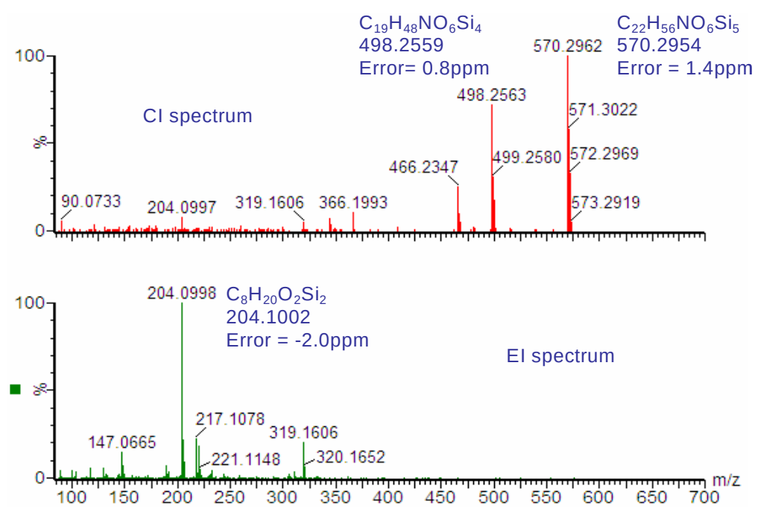

Figure 3. Exact mass EI and CI spectra from component at 10.8min found to be lower in Zucker fa/fa plasma samples.

The scores plot for the UPLC/MS analysis of the urine samples from the control rats and the rats dosed with the nephrotoxin mercuric chloride are shown in figure 4.

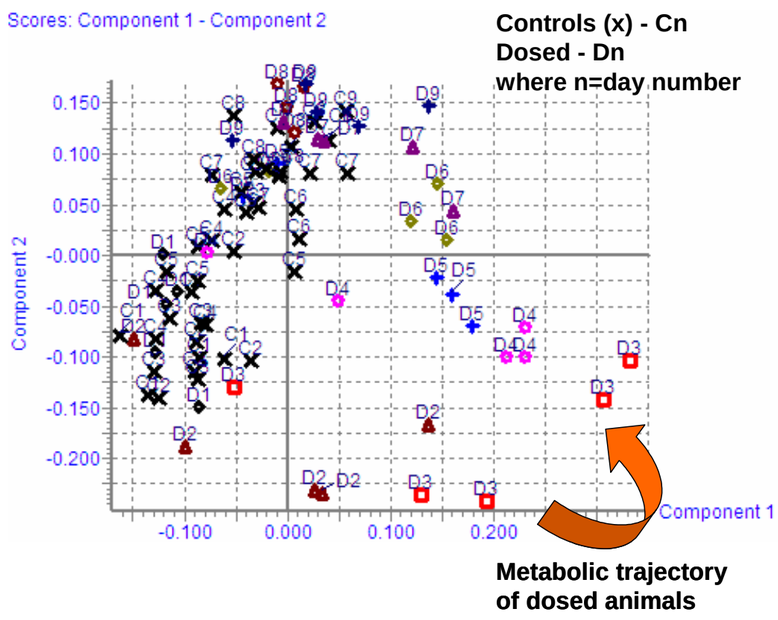

Figure 4. MarkerLynx scores plot for UPLC/MS analysis of urine samples from control and mercuric chloride dosed rats.

The dosed animals show a metabolic trajectory away from the controls with maximum disturbance at day 3 and a return to normal by day 9. Again the m/z and retention time pairs of the ions responsible can be determined and searched against a database within MarkerLynx. Part of the MarkerLynx browser is shown in figure 5 with the database client window showing the results of the exact mass search for m/z 190.0502 at 2.51min.
The search can be based on mass and/or retention time and the user can review the search results which include structures, CAS and KEGG numbers, as well as information about the metabolic pathway, and can append to the database if the metabolite of interest is not present.
In this example kynurenic acid (calculated mass 190.0504), part of the tryptophan metabolism cycle, was found to decrease after dosing with mercuric chloride. The exact mass measurement with an error of <1ppm and the retention time confirmed by analysis of a urine sample spiked with standard gave confidence in this identification.

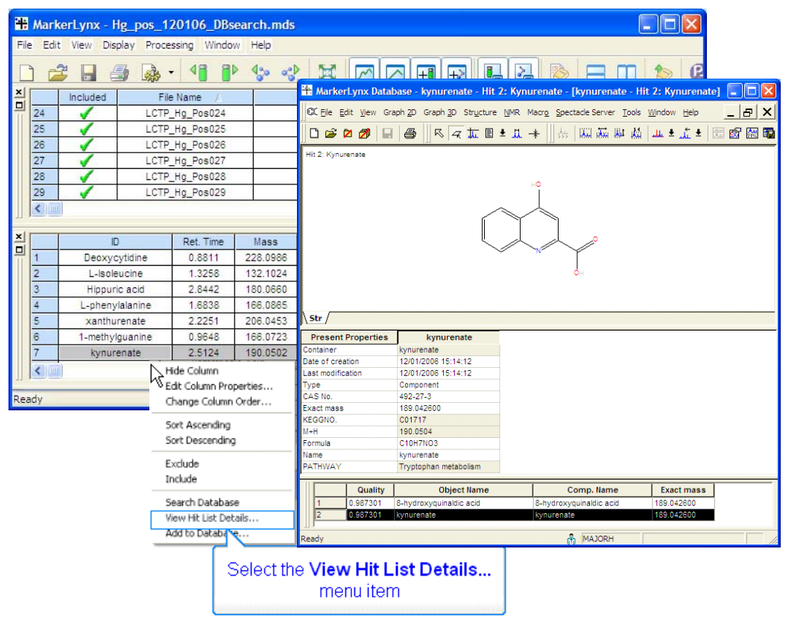

Figure 5. MarkerLynx browser showing the Hit List Details for kynurenate found to decrease in rats after dosing with mercuric chloride.

CONCLUSIONS

- The ability to extract useful information from metabonomic data depends on being able to identify any potential markers highlighted in the multivariate data analysis of the complex data sets.
- The use of EI libraries is well established and in conjunction with exact mass measurements and CI the identification of markers of disease or toxicity is relatively routine, assuming that the metabolite is present in the library.
- The introduction of a searchable exact mass database within the MarkerLynx Application Manager for UPLC/MS adds another tool to help the analyst turn data into knowledge.

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