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A COMPARISON OF ^{1}H-NMR AND LC/MS(TOF) FOR A METABONOMICS EVALUATION OF RAT URINE FROM A TOXICOLOGICAL STUDY

Chris L. Stumpf[1], Maria Anthony[2], Robert S. Plumb[1], John N. Haselden[2], Jennifer H. Granger[1], Brian Sweatman[2], Jose Castro-Perez[3], and Hilary Major[3]
[1]Waters Corporation, Milford, MA; [2]GlaxoSmithKline, Ware, UK; [3]Waters Corporation, Manchester UK

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Introduction

The science of "Metabonomics" focuses on elucidation of markers of disease state, toxicological insult, etc. in biological fluids. These markers are important in drug discovery as they can streamline compound selection. Most drug candidates fail in Phase I or II clinical trials due to poor efficacy or toxicity. By utilizing a Metabonomics approach, poor drug candidates can be identified and eliminated much earlier in the drug discovery process. Metabonomics as practiced typically utilizes rapid screening analytical techniques such as ^{1}H-NMR or more recently LC/MS in combination with multivariate statistical analysis methodologies. In this study, rat urine from a toxicological study was analyzed by ^{1}H-NMR and LC/MS.

Methods

Samples:

Male and female rats were dosed with either vehicle alone, or a novel candidate pharmaceutical compound at 2 or 18 mg/kg. Urine samples were collected 0-8 and 8-24 hours post dose over the course of 90 days. Table 1 illustrates the dosing schedule, where day 33 represents the 0-8 hour collection point and day 34 represents the 8-24 hour collection. Samples were analyzed following a 1:4 dilution with water.

Table 1. Rat Dosing Schedule.

Rat Number	Dose Level	Gender	Time Points
1-8	Control	M	D33,D34,D84,D85
9-16	Low Dose	M	D33,D34,D84,D85
17-24	High Dose	M	D33,D34,D84,D85
25-32	Control	F	D33,D34,D84,D85
33-40	Low Dose	F	D33,D34,D84,D85
41-48	High Dose	F	D33,D34,D84,D85

LC Conditions:

LC System	Waters 2795 Separations Module
Column	Waters Symmetry® C_{18} 3.5 µm 2.1 x 100 mm
Flow Rate	600 µL/min. split to 150 µL/min. into MS
Injection Volume	10 µL
Gradient	Linear, 0-20% B over 0.5-4 min., 20-95% B over 4-8 min., hold at 95% B for 1 min. then return to 0% B at 9 min.

Where A = 0.1% Formic Acid in Water, and B = 0.1% Formic Acid in Acetonitrile

MS Conditions:

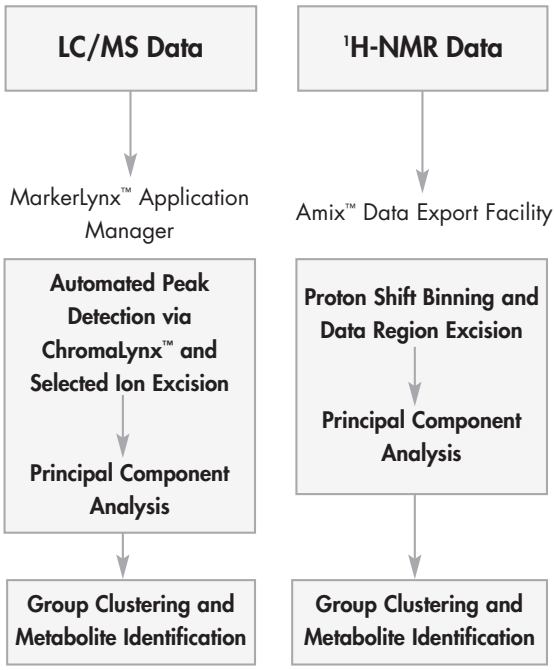
MS System	Waters Micromass® Q-Tof micro™ with a LockSpray™ interface
Ion Mode	Positive or negative electrospray
Cone Voltage	30 V
Capillary Voltage	3.2 kV
Desolvation Temp.	250 °C
Source Temp.	120 °C
Collision Energy	10 eV
Detection Mode	Full scan 50-1400 m/z, centroid mode
Dwell	0.1 sec.
Collision Gas	Argon, 5 x 10^{-3} mbar
Lock Mass	Leucine Enkephalin at a concentration of 50 fmol/µL, a flow rate of 20 µL/min. was employed with a frequency of 10 seconds

^{1}H-NMR:

Single pulse ^{1}H-NMR spectra of all urine samples were obtained at 700 MHz , TSP in D_2O was added to the samples for referencing purposes.

Data Analysis:

Two independent multivariate statistical analyses were conducted on the respective spectroscopic data.



Results

Typical LC/MS and ¹H-NMR data that were used in the Metabonomics approach is shown in Figures 1 and 2. A 12-minute reversed phase total ion chromatogram from a control rat urine sample is shown in Figure 1, while Figure 2 shows a typical rat urine ¹H-NMR spectrum.

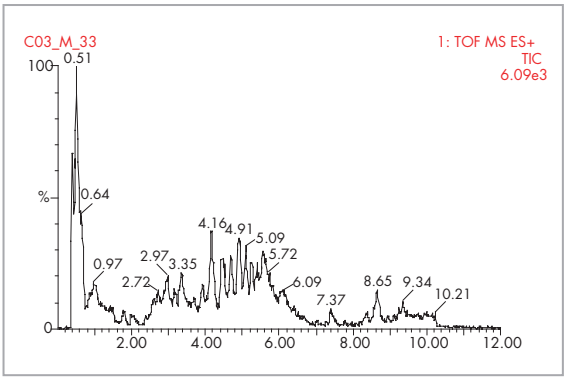


Figure 1. LC/MS total ion chromatogram of control rat urine.

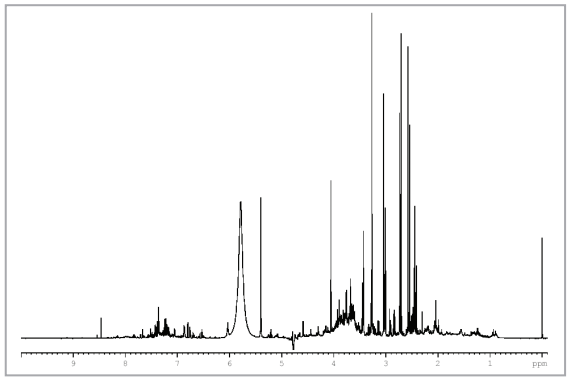


Figure 2. ¹H-NMR spectrum of control rat urine.

Detecting Dose Group Variability:

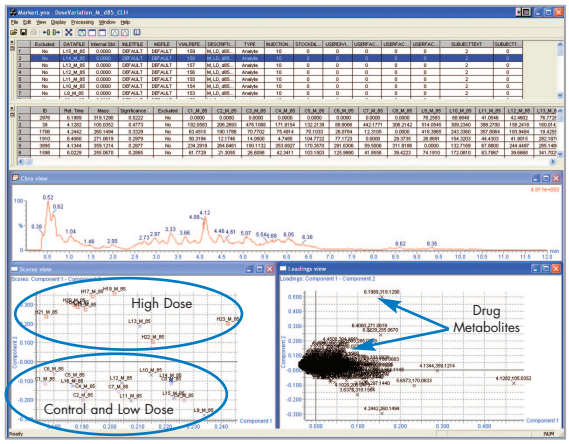


Figure 3. LC/MS data from male rats on day 85 from control, low dose, and high dose. Metabolites of GSK Compound A were located in the loadings plot at m/z 304 and m/z 319; these were independently confirmed by dosing of the radio-labeled compound and LC/MS/MS.

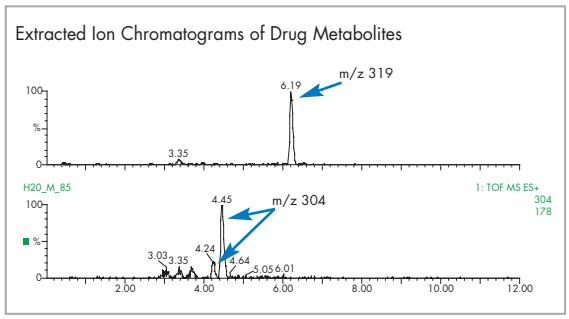


Figure 4. Extracted ion chromatograms of drug metabolites of m/z 304 and 319 from male rat urine where the rat was dosed with 18 mg/kg of GSK compound A and the urine was collected on day 85.

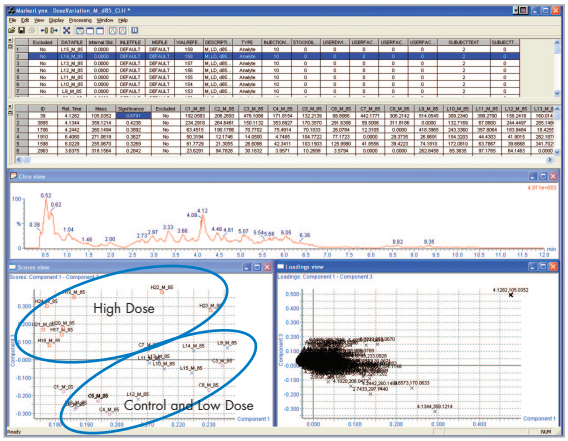


Figure 5. The detected drug metabolites of m/z 304 and 319 were excluded from the analysis and PCA was re-run. The control and low dosage groups still cluster together away from the high dose group indicating that the GSK compound A is producing an effect on the endogenous metabolite profile of the rats in the high dosage group.

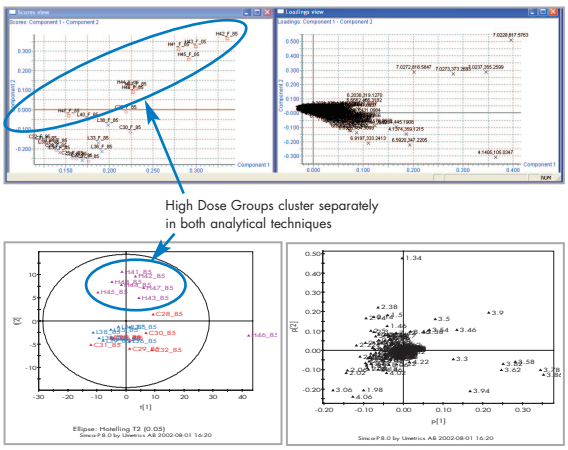


Figure 6. A comparison of the LC/MS (top) and ^{1}H-NMR (bottom) Scores and Loadings plots for the female rats on day 85, 8-24 hours for control, low dose, and high dose. The scores plot of both analytical techniques show that the control and low dose samples cluster together away from the high dose group indicating that the endogenous metabolite profile of the high dose group has changed.

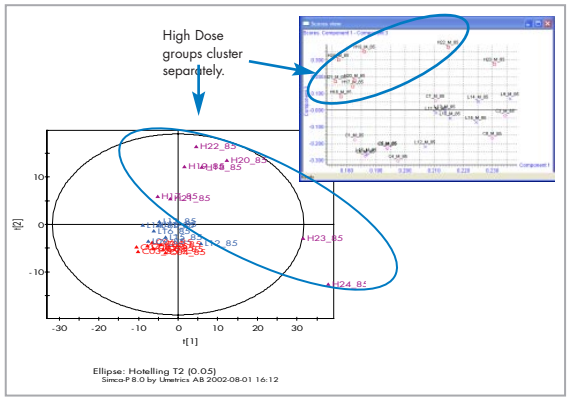


Figure 7. LC/MS and ^{1}H-NMR Scores plots of males rats from control, low dose, and high dose on day 85. Again, high dose group clusters away from control and low dose groups in the scores plot obtained from both analytical techniques.

Detecting Gender and Diurnal Effects:

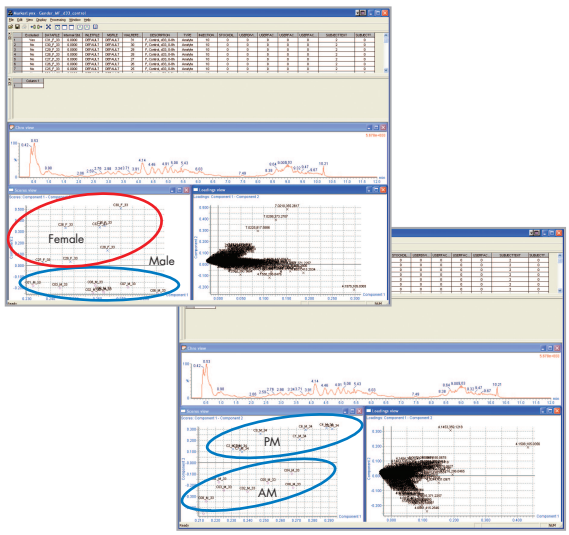


Figure 8. The metabolic profile of male and female control rats are sufficiently different to allow for differentiation by LC/MS and PCA (left). Subtle metabolic differences are discerned in the analysis of male rat urine collected in the morning and evening of day 33 (right). Similar group clustering is observed using ^{1}H-NMR.

Discussion

The LC/MS and ^{1}H-NMR data shown here illustrate that GSK compound A induces a change in the endogenous metabolism of the rats when dosing at 18 mg/kg while the low dose group (2 mg/kg) remains similar to the control group. Because LC/MS and ^{1}H-NMR have different sensitivities with regard to analyte detection, it is likely that the group clusterings found in the PCA scores plots are not always the result of detecting the same chemical moieties. An advantage of the 3D nature of the LC/MS (TOF) data over ^{1}H-NMR is that after metabolite removal (Figures 3-5), more spectral data is retained for subsequent multivariate analysis. A further advantage of LC/MS (TOF) over ^{1}H-NMR is the ability to provide structurally relevant information that can be readily searched in a database. The elemental composition and MS/MS fragment ion information provided by LC/MS is amenable to providing a short list of potential marker compounds. Hence, to conclude this work, an example of metabolite detection and elucidation is illustrated using negative ion ESI LC/MS (TOF) data from the dose group variability study from day 85, 8-24 hours.

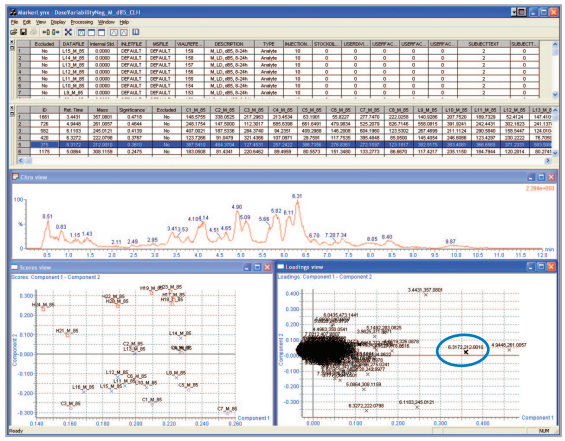


Figure 9. Scores and Loadings Plots of Negative ESI data from dose variability study on day 85, 8-24 hours. The structure of the identified marker at m/z 212 was elucidated.

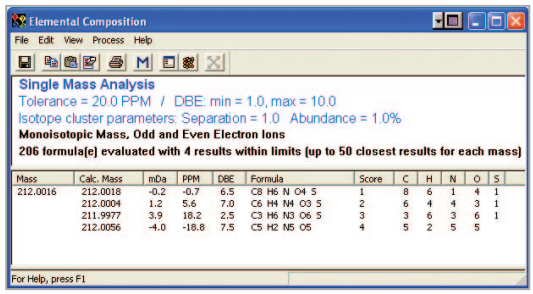
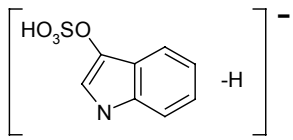


Figure 10. Elemental composition ($C_8H_6NO_4S$) of indoxyl sulphate determined from accurate mass LC/MS (TOF). LC/MS/MS and ^{1}H-NMR confirms structure.



Indoxyl sulphate, m/z 212.0018

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Conclusions

Drug metabolites of GSK Compound A could be detected from the LC/MS and PCA loadings plot (from the dose group variability study).

The LC/MS and ^{1}H-NMR data produced similar group clustering in the dose group variability study.

Endogenous metabolite differences resulting from gender and diurnal effects could be discerned by using LC/MS and multivariate analysis (similar results have been reported with ^{1}H-NMR).

Identification of potentially important biomarkers has been streamlined by using LC/MS-TOF exact mass and an integrated software browser. Hence, the structural elucidation stage of metabolite marker identification can be reached rapidly, allowing more effort to be focused on this challenging area.

WATERS CORPORATION
34 Maple St.
Milford, MA 01757 U.S.A.
T: 508 478 2000
F: 508 872 1990
www.waters.com

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