USE OF UPLC COUPLED WITH TOF MASS SPECTROMETRY FOR METABONOMIC INVESTIGATIONS OF PREDNISONE DOSING

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ABSTRACT

Fast UPLC separations coupled with high resolution TOF mass spectrometry are used to profile urine samples obtained after dosing with prednisone. Amino acid concentrations are monitored during therapy. PCA is used to identify components leading group separation.

INTRODUCTION

Glucocorticoid therapy is widely used for cancer chemotherapy, immunosupression, and anti-inflammatory activity. Prednisone is one of the most common drugs used for the therapy, however there is a general activation of catabolic processes, including glycolosis and protein turnover. Several studies have attempted to describe the general mechanism for the protein turnover, finding an increase in urea production and excretion.[1-3]

In addition to the classical *in vivo* drug metabolism experiments, metabonomic studies play an important role in assessing drug efficacy, drug safety, and mechanism of activity. Along with the quantification of the drug and its metabolites, profiling of other physiological fluid constituents can illuminate other metabolic processes that are affected by drug therapy.[4,5] Metabolic profiling of physiological fluids such as plasma or urine is complicated by the number of endogenous components (sample complexity) and the number of analyses needed to gather meaningful statistical data.

Ultra performance liquid chromatography (UPLC) is characterized by the high chromatographic efficiency and high speed analysis accorded by the use of sub 2 µm particles.[6] In addition to high efficiency and high speed chromatographic analysis, time of flight mass spectrometry is utilized to obtain high sensitivity full scan data that can be used for identification purposes. Accurate mass measurements generated from this data are used to deduce elemental compositions. In addition, the novel MS^E techniques are used to generate MS/MS like data for the whole dataset, reducing the necessity for dedicated MS/MS or DDA techniques.[7]

Because many hundreds to thousands of chromatographic components can be isolated during a short UPLC run, data reduction techniques must be used to detect, align, and filter out confounding variables so that biological components that are changing with respect to drug therapy can be identified. Principal component analysis and other statistical data treatments were used to characterize the time-course behavior of endogenous compounds found in urine during the drug therapy.[8]

METHODS

Prednisone Dosing and Sample Collection

Morning urine was collected from an adult male during treatment with Prednisone (60 mg/day for two days, with subsequent doses at 50 mg/day for two days, then 40 mg/day for two days). Aliquots were frozen at –40 ° C until analysis. Samples were thawed, centrifuged at 14,000x g for 10 minutes, and then diluted with 10 volumes of water prior to analysis.

Liquid Chromatography/Mass Spectrometry

Waters ACQUITY UPLC

Mobile Phases
- 0.1% Aqueous Formic Acid
- Acetonitrile

Column ACQUITY BEH C8 2.1x100 mm 1.7 µm d_p
- Flow Rate 400 µL/min
- Temperature 50 ° C
- 5 µl Injections

Gradient:
- Ramp from 5 % to 50% B in 5 minutes
- Ramp to 95% B in 0.1 minutes
- Hold 1 minute

Waters Q-Tof Premier TOF MS Analysis
- ESI Positive or Negative Mode
- Capillary= 3.5 kV
- Cone Voltage= 25 V
- Collision Energy = 4 eV
- LockSpray using Leucine Enkephalin as a reference mass
- W Mode resolution (fwhm) = 15,000 @ *m/z* 554

MS^E Experiments
- ESI Positive or Negative Mode
- Capillary= 3.5 kV
- Cone Voltage= 25 V
- Collision Cell 4 eV Low Energy; 15 eV High Energy

LockSpray using Leucine Enkephalin as a reference mass
W Mode resolution (fwhm) = 15,000 @ *m/z* 554

Data Analysis

Quantitative data was processed using QuanLynx. Data was exported to Microsoft Excel for further analysis. Positive and negative ion LC/MS data sets were processed using MarkerLynx to detect and quantify chromatographic components, time-align the chromatographic components, and normalize data from each sample. The data was processed using Principal Component Analysis to identify putative markers. The dataset was exported to SIMCA-P (Umetrics AB, Umea, Sweden) for further evaluation.

RESULTS

	Included	File Name	File Text	Vial Ref.	Internal Std.	Symbol	Sample Group	Spectral Noise	Chromatogram Noise
1	✓	Prednisone_0_01_Neg	D-1 05/04	1:18	0.0000	X	0	1.01	14.41
2	✓	Prednisone_0_02_Neg	D-1 05/04	1:18	0.0000	X	0	1.01	15.32
3	✓	Prednisone_0_03_Neg	D-1 05/04	1:18	0.0000	X	0	1.01	16.36
4	✓	Prednisone_1_01_Neg	D1 05/05	1:19	0.0000	O	1	1.01	14.38
5	✓	Prednisone_1_02_Neg	D1 05/05	1:19	0.0000	O	1	1.01	15.53

	Ret. Time	Mass	Significance	Included	Marker	Prednisone_0_01_Neg	Prednisone_0_02_Neg	Prednisone_0_03_Neg	Prednisone_1_01_Neg	Prednisone_1_02_Neg	Prednisone_1_03_Neg
749	3.9777	369.1739	0.0918	✓	X	108.1746	88.0005	86.6759	45.2684	47.5625	44.8012
377	1.3326	245.0121	0.0911	✓	X	52.1055	55.3329	62.0290	53.8609	50.4118	51.8205
797	2.0121	383.0981	0.0871	✓	X	0.0000	0.0000	0.0000	68.7748	64.7941	69.0481
720	1.7930	369.0821	0.0845	✓	X	0.0000	0.0000	0.0000	66.6124	60.9550	63.7794
357	2.6554	243.0327	0.0825	✓	X	20.9379	20.1160	18.3028	77.7716	70.6752	75.4653
1109	3.3176	481.2443	0.0823	✓	X	64.8481	65.8478	61.7578	40.8444	36.5087	35.7526
1205	2.8526	541.2650	0.0804	✓	X	69.1630	71.6894	67.9621	23.9751	35.7982	35.4118
351	0.7974	233.0075	0.0799	✓	X	21.5446	19.3588	19.6623	8.5094	7.6393	7.5050
146	0.9532	188.9859	0.0780	✓	X	84.4207	83.0368	83.6250	132.8762	162.7309	167.8668

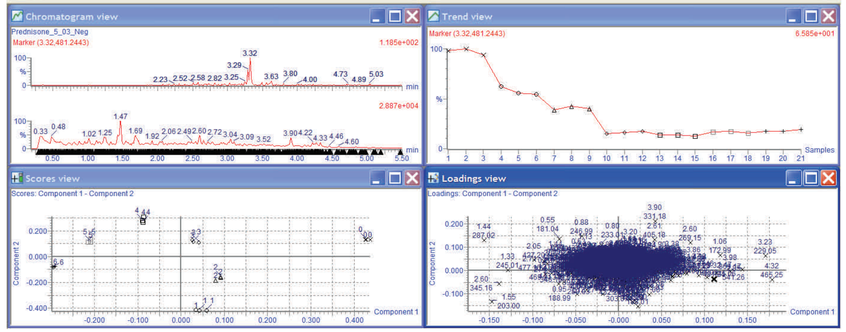

Figure 1. MarkerLynx Browser Report for Negative Ion UPLC/TOF MS for time course study of prednisone dosing. Top Panel Sample Report; Middle Panel Table of identified components; Bottom Panes Chromatogram with identified components and extracted ion current plot for ion at 3.32 RT and *m/z* 481.2443, Marker Trend for ion at 3.32 RT and *m/z* 481.2443, PCA Scores Plot showing group separation, and PCA Loadings Plot of components leading to group separation. Scores Plot labels correspond to Day 0 (predose) through Day 6.

PLS-DA Scores and Loadings Plots

Positive Ion Dataset

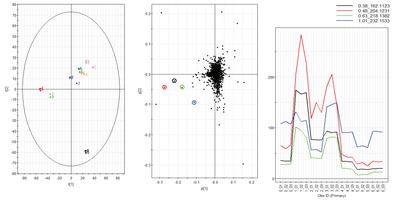

Figure 2. Partial Least Squares Discriminate Analysis plots for positive ion dataset for Day 0 vs. Days 1-6. Left Panel Scores Plot. Center Panel Loadings Plot. Right Plot Extracted Markers showing elevated response during the first three days of drug administration.

O-PLS Scores and Loadings *S*-Plots

Negative Ion Dataset

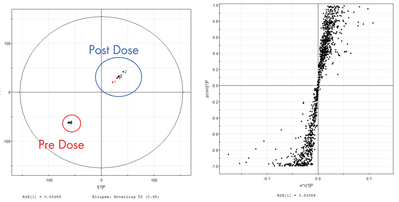

Figure 3. Orthogonal Partial Least Squares plots for negative ion dataset for Day 0 vs. Days 1 and 2. Left Panel Scores Plot. Right Panel Loadings *S*-Plot. Markers with high or low p(corr) values are responsible for the class separation between the pre-dose and post dose samples.

Identification of Markers

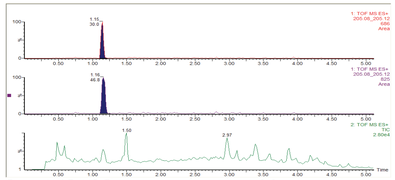

Figure 4. LC/MS Chromatograms for marker at 1.1 minutes and *m/z* 205.098. Top Trace XIC for Pre Dose Middle Trace XIC for Dose Day 2, Bottom Trace MS^E Trace.

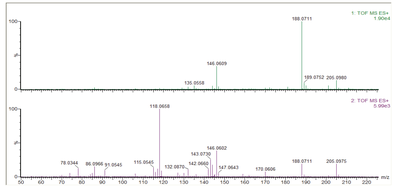

Figure 5. Background subtracted spectra for component at 1.1 minutes. Top Trace Spectrum for low energy acquisition (4 eV), Bottom Trace Spectrum from MS^E high energy acquisition (18 eV).

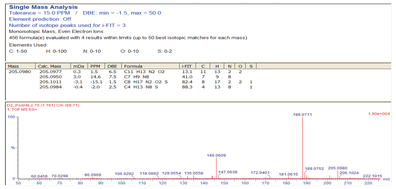

Figure 6. Elemental composition report for low energy spectrum. Database searching of the elemental composition suggests tryptophan as a possible match.

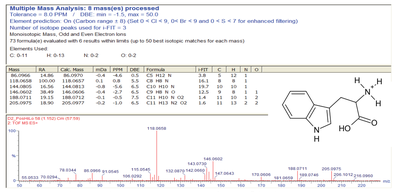

Figure 7. Elemental composition report for MS^E high energy spectrum at 1.1 minutes.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Carnitine	798.00	447.48	334.38	177.25	81.36	78.77
Tryptophan	112.58	148.85	116.56	137.62	121.28	112.01
Valine	86.21	n.d.	110.60	114.83	64.64	71.20
Leucine	n.d.	n.d.	47.11	n.d.	48.66	n.d.
Histidine	335.66	271.26	226.00	239.61	202.09	n.d.
Phenylalanine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glutamine	n.d.	119.93	74.75	113.18	105.60	89.68
Arginine	64.08	133.04	n.d.	164.11	62.82	54.34
Tyrosine	n.d.	99.42	n.d.	n.d.	n.d.	80.06
Glutamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methionine	128.34	233.54	56.29	n.d.	145.28	n.d.
Taurine	n.d.	55.35	n.d.	22.60	39.84	18.53
Threonine	109.86	143.71	81.61	124.51	141.03	113.56

Table 1. Statistically significant changes (97.5% confidence limits) in amounts of amino acid excretion. Data is expressed as percent change from the D0 level. n.d= no statistically significant change between dosed level and pre-dose level

CONCLUSIONS

- **UPLC has high resolution and peak capacity necessary for metabonomic profiling of complex biological separations.**
- **Use of supervised and unsupervised methods for determination of components responsible for group separation.**
- **Use of Q-Tof Premier MSE methodology gives comprehensive MS and MS/MS-like datasets to aid in small molecule biomarker characterization.**
- **Elevation of amino acids and carnitine in urine supports protein breakdown models for prednisone-induced muscle wasting.**

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