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Note d'application

Metabonomics Analysis of Rat Urine by UPLC-MS, following the Oral Administration of Pravastatin

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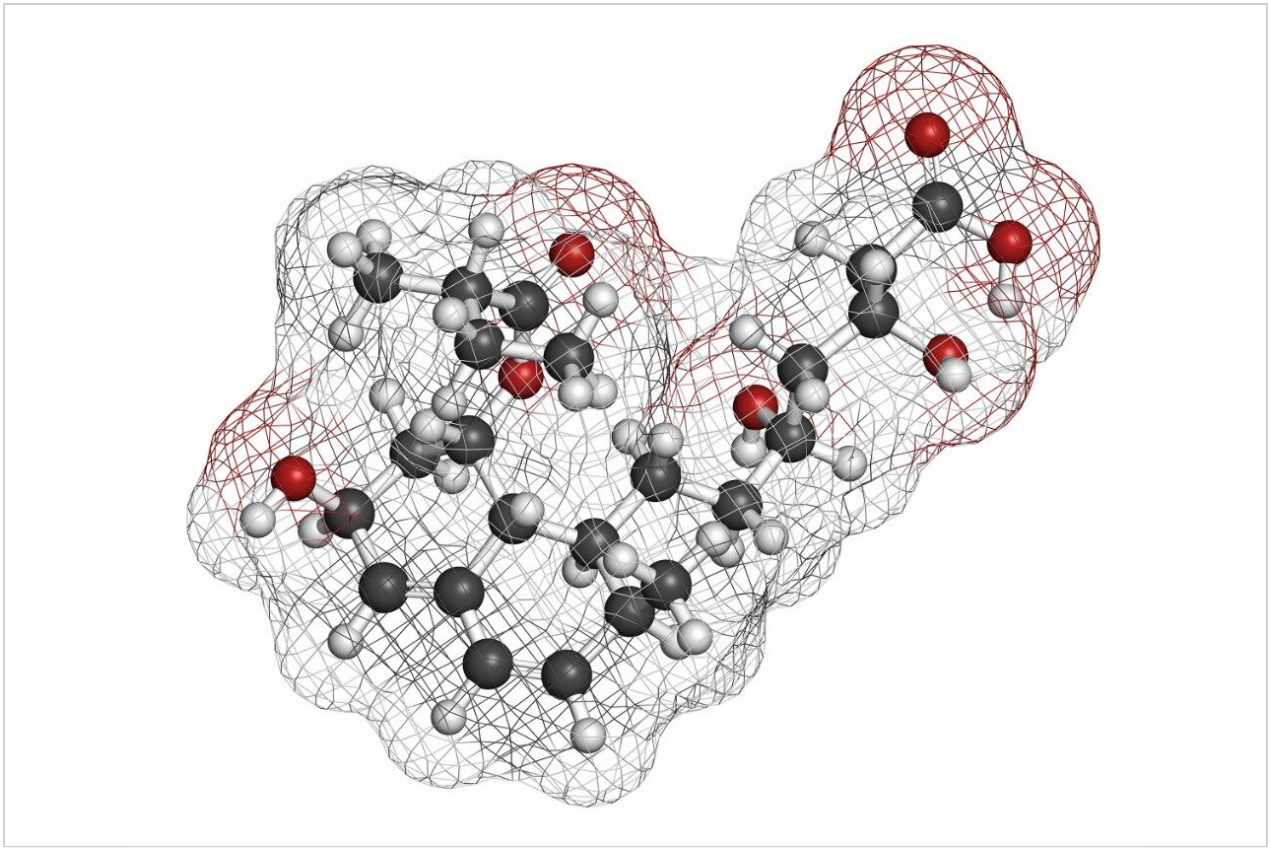

Abstract

Metabonomics is focused on predicting drug toxicity, and disease progression by monitoring the changes in endogenous metabolism. In this application note, Ultra Performance LC oa-ToF MS employing 1 mm i.d. columns and metabonomics-based multivariate statistics were effective in determining the effects of the oral administration of Pravastatin (one of a new class of lipid-lowering compounds) on the endogenous metabolism of the rat.

Rat urine from control and dosed animals on days –1 (pre-dose) through 4 were analyzed by UPLC-MS employing reversed phase gradient elution at the 1mm scale. The MS detection was performed on a Waters Micromass Q-Tof micro Mass Spectrometer using positive ion ESI and LockSpray.

The 1 mm UPLC columns gave produced excellent chromatographic performance with an increase in signal to noise increase of 18%. From the UPLC-MS data its was possible to separate the dosed and control animals into distinct groups, and identify the potential biomarker ions glychenodeoxycholate using exact mass MS.

Introduction

Pravastatin Sodium (Figure 1), marketed as Pravacholas a prescription drug, is one of a new class of lipid-lowering compounds, the HMG-CoA reductaseinhibitors, which reduce cholesterol biosynthesis. These agents are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme catalyzing the early rate-limiting step in cholesterol biosynthesis, conversion of HMG-CoA to mevalonate. It is particularly effective at lowering the concentration of low-density lipoprotein cholesterol.

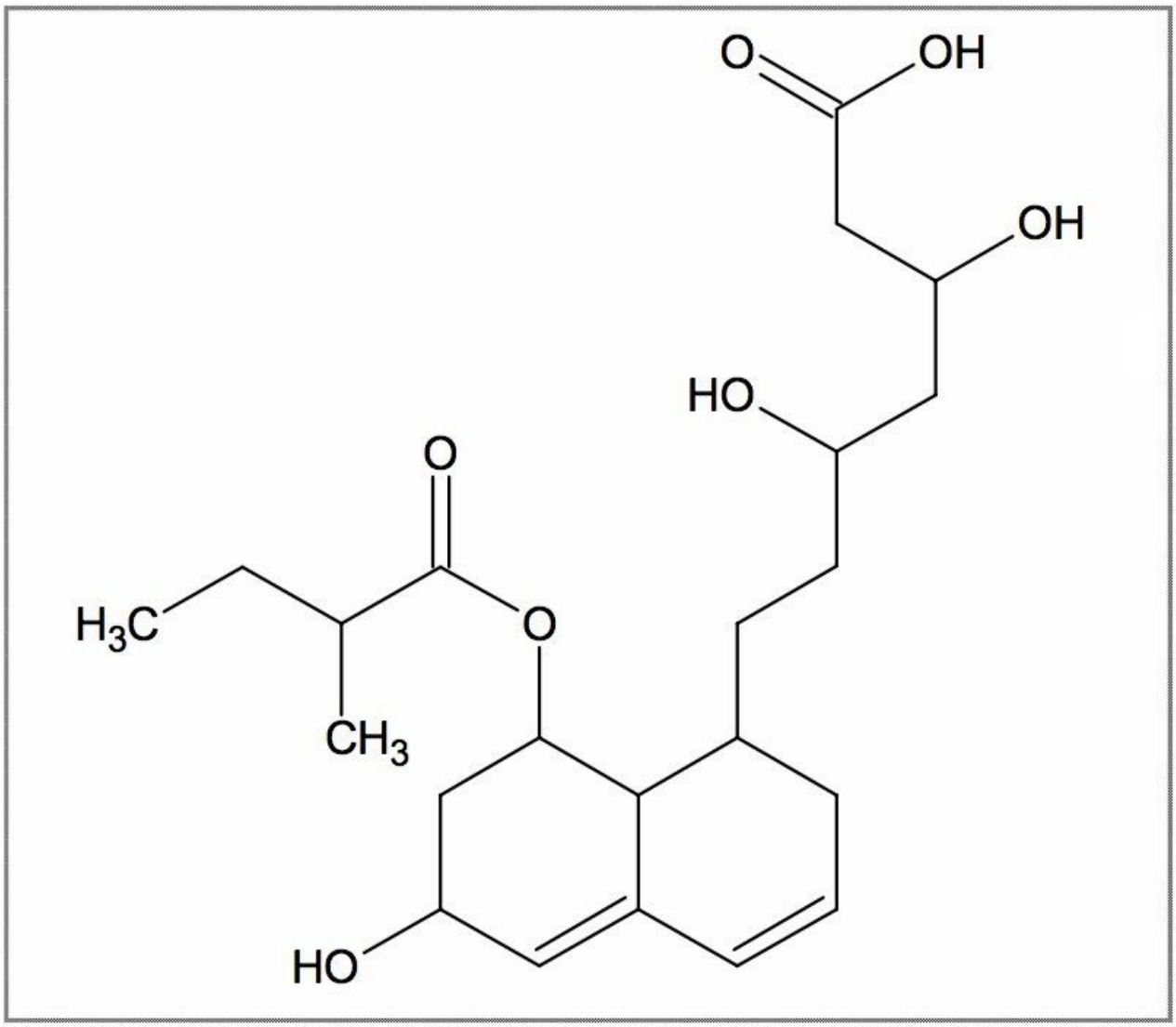

Figure 1. Structure of Pravastatin Sodium.

Pravastatin is administered orally, and unlike other statins, it is not susceptible to metabolism by cytochrome p450 enzymes and eliminated mainly in the bile. Like other statins, however, Pravastatin is generally well tolerated and serious adverse events, including muscle toxicity leading to rhabdomyolysis, are rare.

Waters ACQUITY UPLC System.

Metabonomics is the third of the disciplines of “omics”-based investigations, focused on predicting drug toxicity and disease progression by monitoring the changes in endogenous metabolism. LC-MS, and more recently, UPLC combined with orthogonal acceleration time-of-flight (oa-ToF) mass spectrometry, have been applied to the science of metabonomics to provide exact mass and hence structural identification information to help identify new, novel biomarkers of toxicity or efficacy. Central to the applicability of any analytical technique to metabonomics is the ability to reproducibly detect all the components in the sample in a semi-quantitative manner.

UltraPerformance LC has displayed great potential for complex mixture analysis due to its increased chromatographic performance and sensitivity. The extra performance of provided by UPLC is due to the ability of system to exploit the chromatographic potential of sub-2 µm particle stationary phase. This extra

performance manifests itself as increased chromatography efficiency and resolution, resulting in sharper peaks. These sharper peaks are responsible for the observed increases in sensitivity. As the optimal linear velocity of these 1.7 µm stationary phases is twice that of a 3.5 µm material, we also benefit from faster analysis times with UPLC.

Biological samples from early discovery or small rodent studies are very precious and often only a few microliters are available. As pharmaceutical compounds become increasingly more potent and dosing levels are subsequently reduced, there is a need for increased assay sensitivity to detect all of the components of interest. This can be achieved by either improving the mass spectrometry performance or reducing the chromatography scale. One of the major advantages of the ACQUITY UPLC System is its ability to use 1 mm internal diameter columns, which offer the benefits of both reduced sample consumption and increased sensitivity. In this application note, we show how these 1 mm ACQUITY UPLC Columns have been used to analyze the urine from male rats following the oral administration of Pravastatin.

Experimental

LC Conditions

LC system:	Waters ACQUITY UPLC
Column:	ACQUITY UPLC BEH 1 mm x 100 mm, 1.7 µm
Flow rate:	136 µL/min
Injection volume:	2 µL
Gradient:	0–95% B over 30 min, where: A = 0.1% Aqueous Formic Acid B = 95% MeCN
Column temp:	40 °C

MS Conditions

MS system:	Waters Micromass Q-Tof micro
Ionization mode:	Positive ion ESI
Range:	80–800 *m/z*
Scan rate	0.1 sec, inter-scan 0.05 sec
Capillary voltage:	3 KV
Cone voltage:	30 V
Cone gas:	0 L/hr
Source temp:	120 °C
Desolvation gas:	200 L/hr
Desolvation temp:	200 °C
Collision energy:	5 eV

Rat urine samples were obtained on days –1, 1, 2, 3, and 4 after oral dosing with either vehicle alone or Pravastatin at 20 mg/kg.

Results and Discussion

The chromatographic performance was compared using 1 mm and 2 mm i.d. columns of similar length, operating at the same mobile phase linear velocities. The data in Figure 2 shows the extracted ion chromatogram of *m/z* = 255 for both column types. They performed similarly, with peaks of an average width of 6 seconds at the base, with a peak capacity of 300. The volume of sample injected onto the 1 mm column was scaled to maintain the mass load of the column. The signal-to-noise ratio (P/P) of the 1 and 2 mm columns was determined to be 195:1 and 165:1, respectively –an 18% increase using the 1 mm.

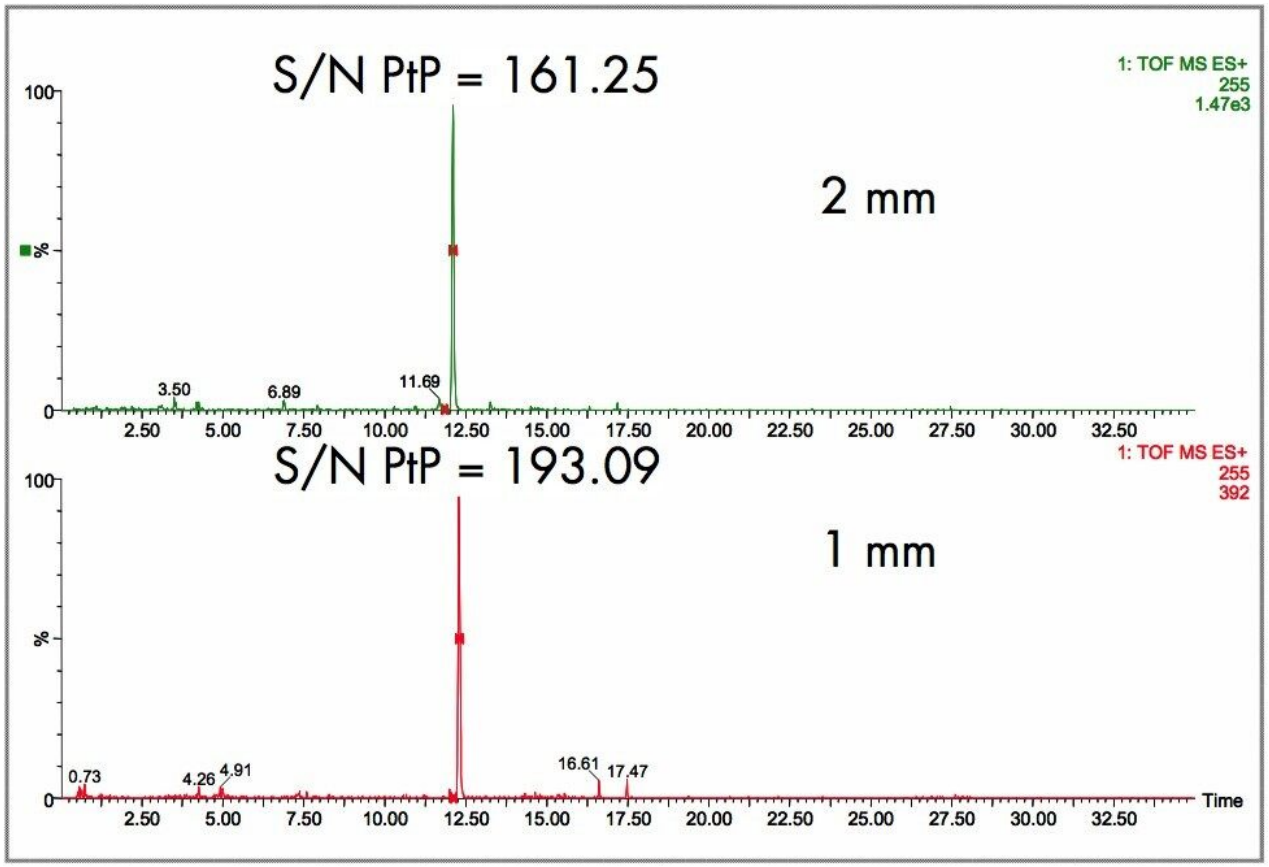

Figure 2. Comparison of the XIC m/z = 255 for 1 mm and 2 mm ACQUITY UPLC BEH 1.7 µm Columns.

A total of 20 samples were processed by positive ion UPLC-ESI MS on the ACQUITY UPLC System coupled with the Q-Tof micro Mass Spectrometer. A representative chromatogram from dose and control samples on day 2 are shown in Figure 3. The first obvious feature of the chromatography is that the data is information-rich, with literally hundreds of peaks. We can also see that there are some obvious differences in the chromatographic separation, with a marked increase in the intensities of the peaks eluting between ~12.5 and 18 minutes in the dosed animals. The resulting UPLC-MS data was subjected to 3-D peak integration and data processing by Principal Components Analysis using the MarkerLynx Application Manager for MassLynx Software.

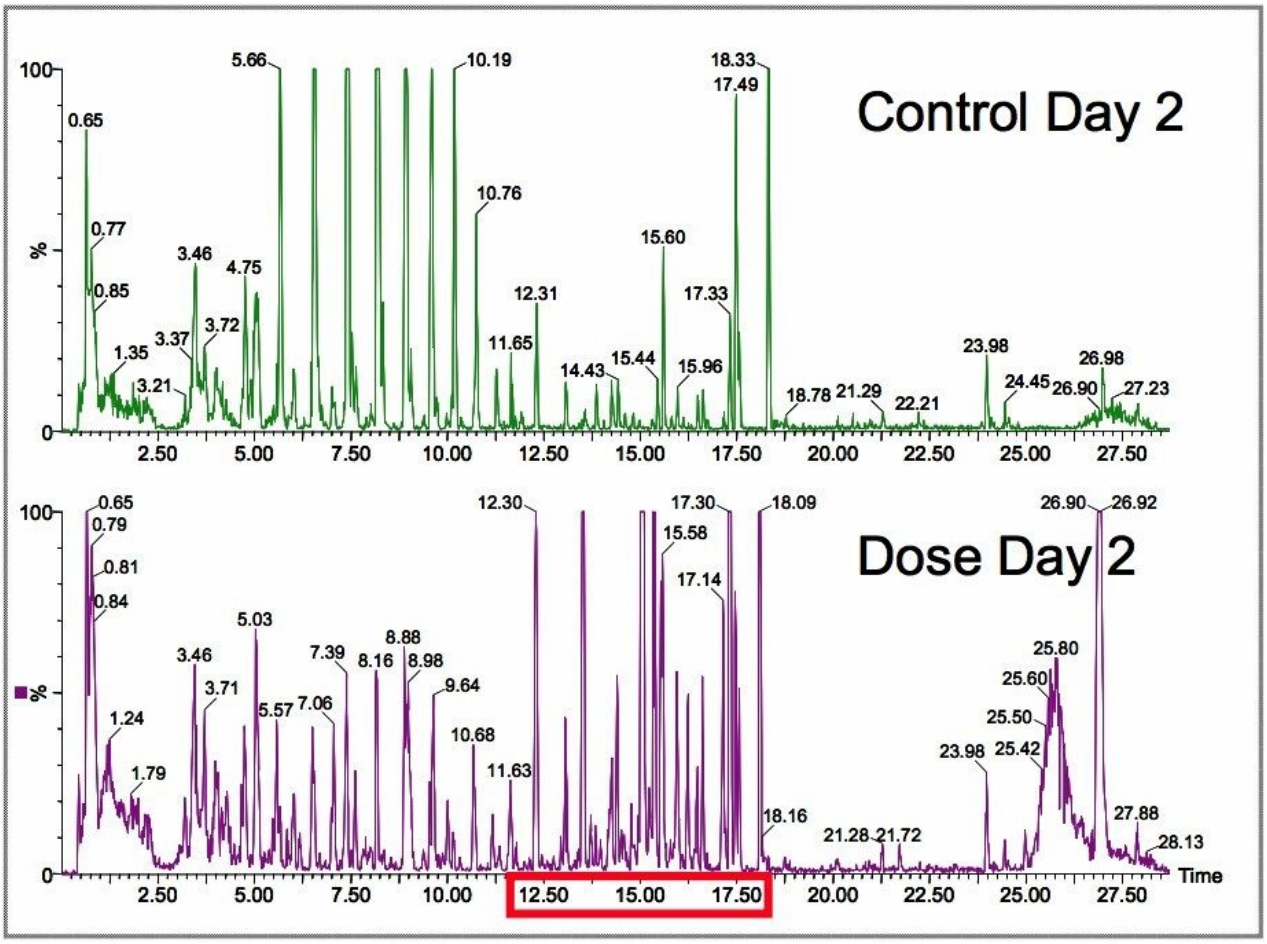

Figure 3. Comparison of rate urine samples from dosed and control animals on day 2.

The Principal Components Analysis revealed that the control samples and those dosed with the Pravastatin were easily separated into two distinct groups (Figure 4), using principal components 1 vs. 2. The pre-dose (day –1) samples from the dose group clustered with the control samples, which add confidence in the validity of the data generated. The day –1 M17 animal appears to be an outlier, mapping to a different position in metabolic space, with respect to the other day –1 animals.

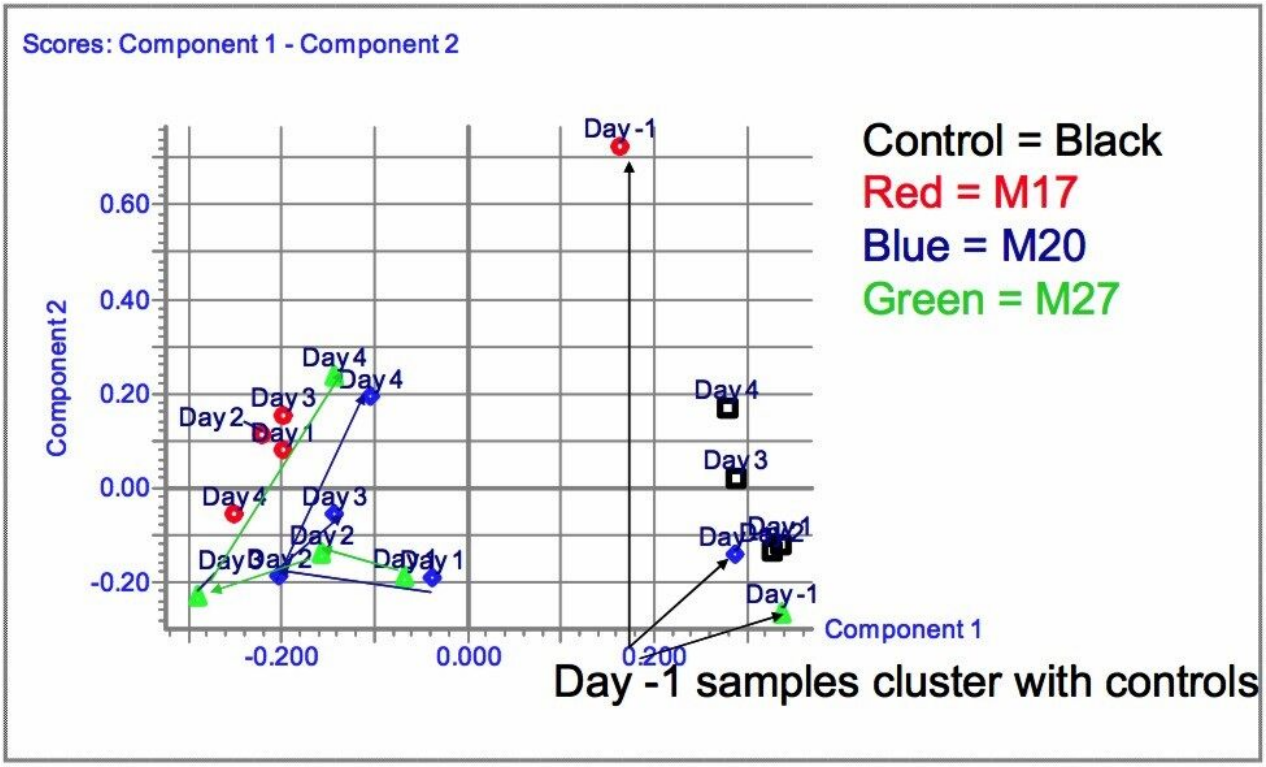

Figure 4. Scores plot of principal components 1 vs. 2.

The corresponding loadings plot from the statistical analysis of the LC-MS data reveals the ions that contribute most significantly to the observed data clustering. The loadings plot from the Pravastatin rat urine analysis is displayed in Figure 5.

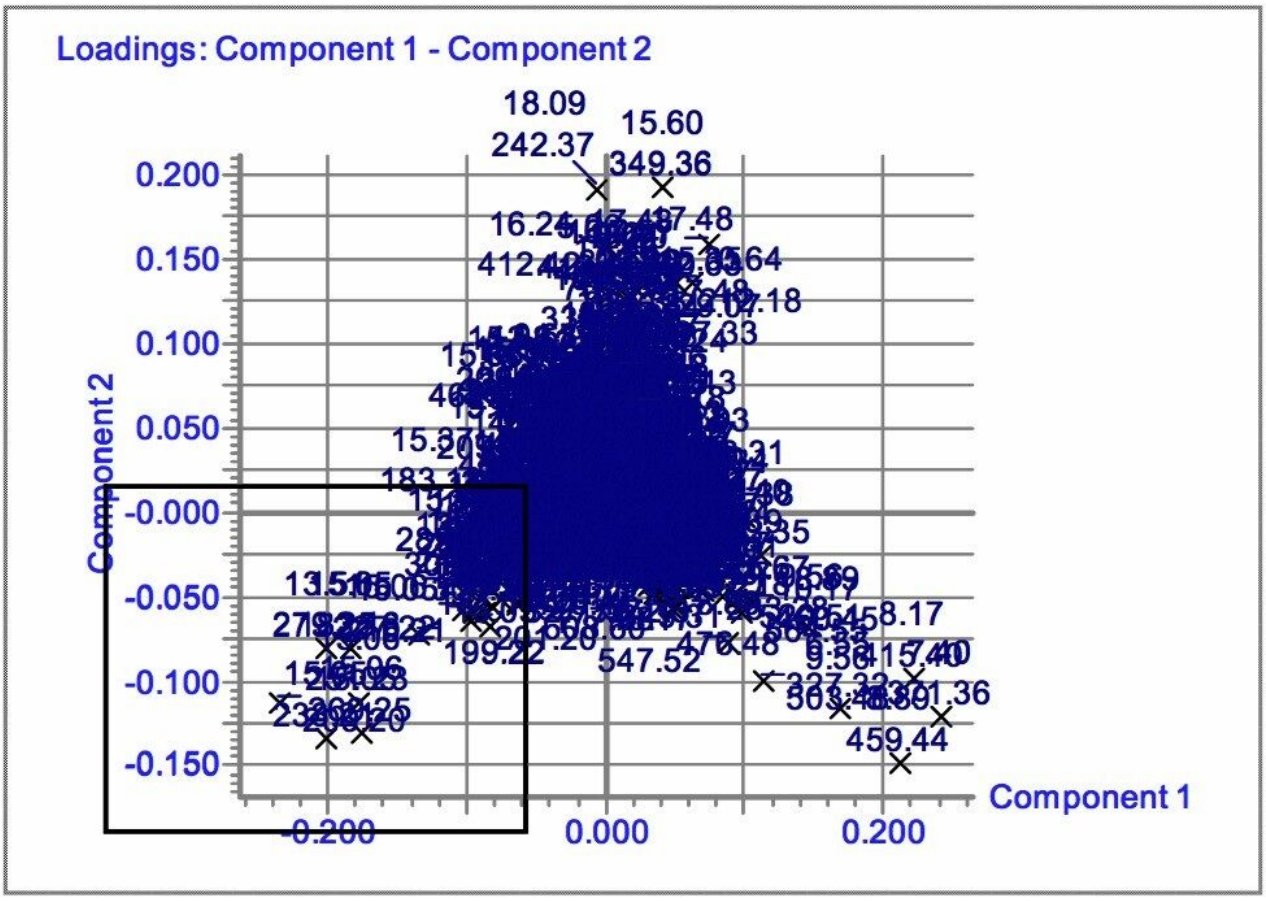

Figure 5. Loadings plot of principal components 1 vs. 2.

From this data, the ions contributing most significantly to the observed variation in the data were determined to be *m/z* = 209 at 15.05 minutes, *m/z* = 233.2 at 15.05 minutes, *m/z*= 279.5 at 13.50 minutes, and *m/z* = 450.32 at 15.50 minutes. All of these ions showed a markedly stronger response in the dosed animal than in the control animals. The extracted ion chromatogram for control and dosed animals on day 2 of *m/z* = 450.32 is given in Figure 6. Upon further exact mass and MS/MS investigation, *m/z* = 450.3219 was determined to be glychenodeoxycholate. The MS spectra of this peak gave a mass of 450.3212, a mass error of 1.6 ppm (Figure 7). Ions that were found to be more abundantin the control animals were *m/z* = 437.39 at 8.17 minutes, 415.6 at 8.70 minutes, and *m/z* = 459.44 at 8.88 minutes.

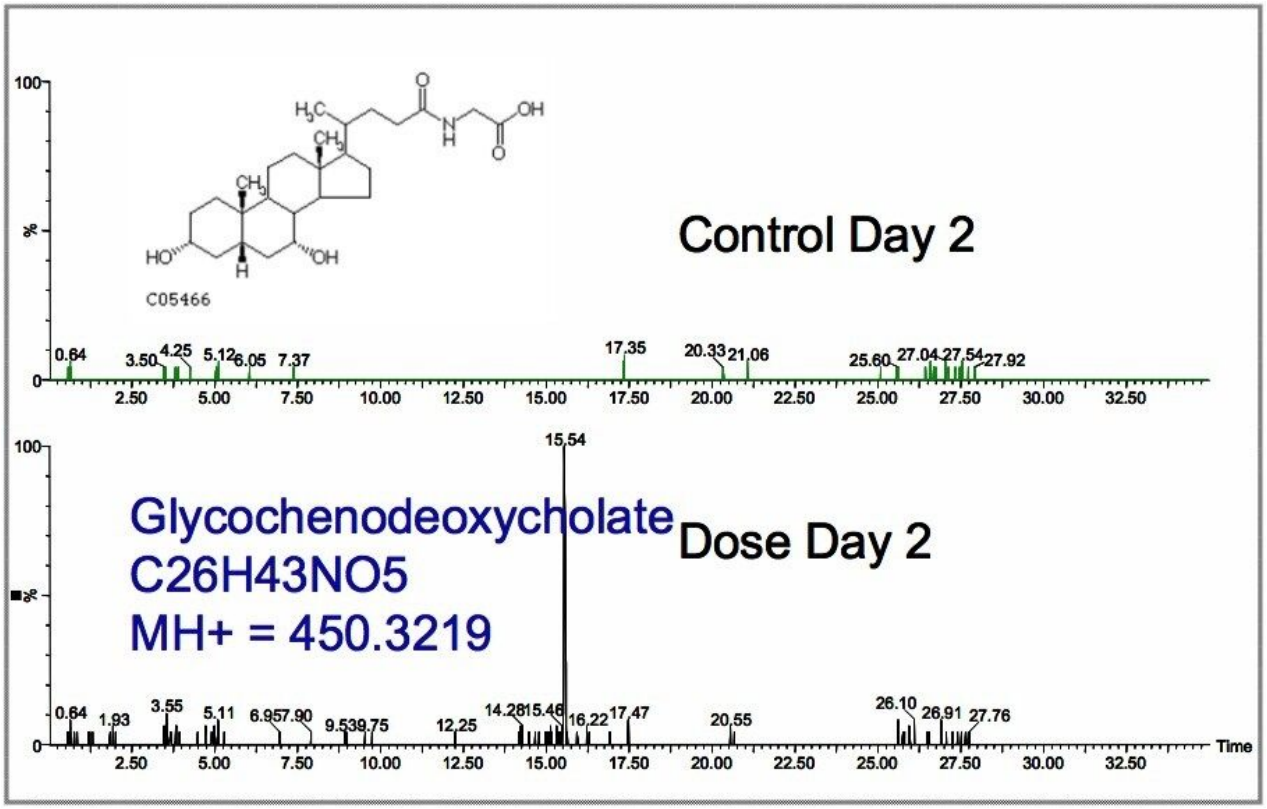

Figure 6. Comparison of the peak intensities for the m/z = 450.32 ion, control and dosed M17, day 2.

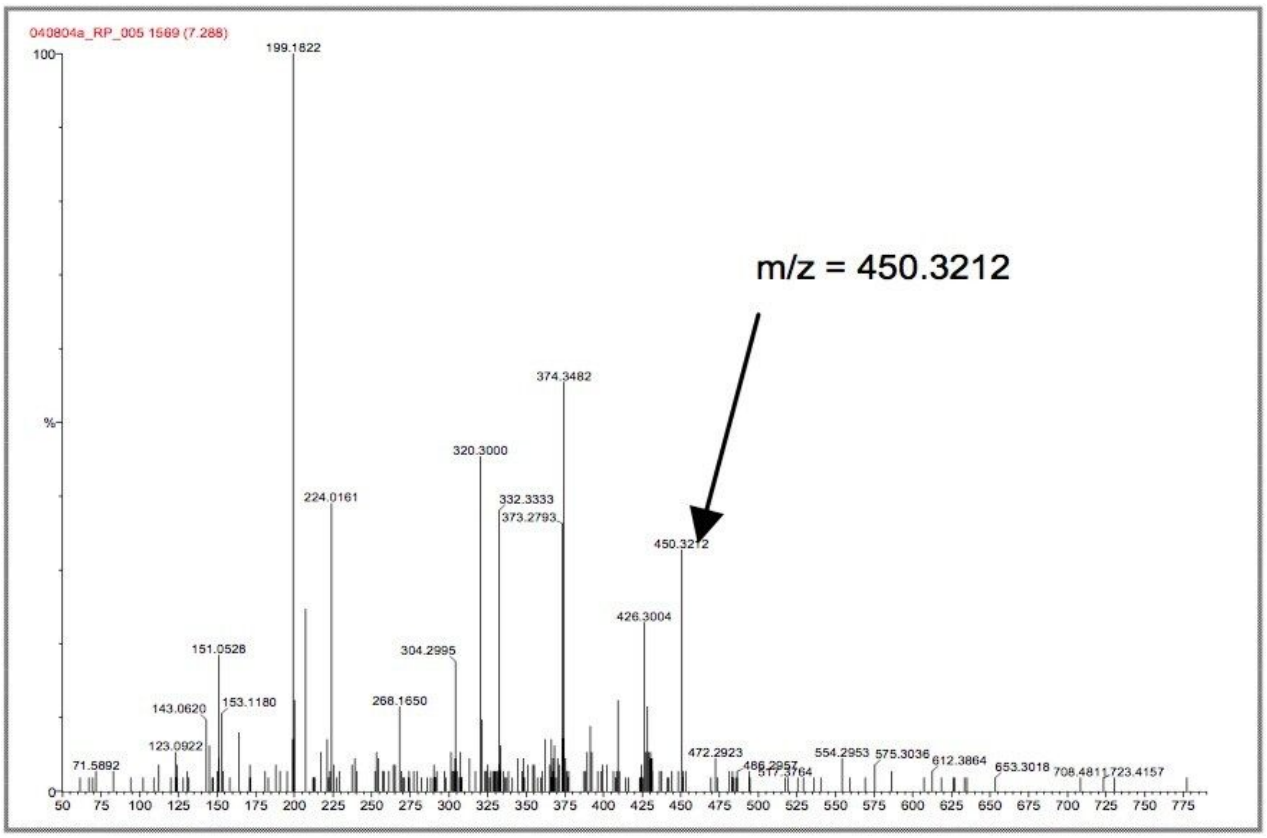

Figure 7. Mass spectrum of the peak eluting at 15.50 minutes.

Conclusion

The use of 1 mm i.d. UPLC Columns has been successfully implemented for the analysis of rat urine from a metabonomics study. These columns showed no reduction in chromatographic performance vs. 2 mm columns, and gave an increase in signal-to-noise increase of 18% for the same column loading. The resulting LC-MS data was processed by MarkerLynx, and from this statistical analysis, it was possible to clearly separate the dosed and control animals, and identify those components contributing to the group clustering.

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