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Metabonomics evaluation of urine from rats given acute and chronic doses of acetaminophen using NMR and UPLC/MS☆

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ABSTRACT

Urinary metabolic perturbations associated with acute and chronic acetaminophen-induced hepatotoxicity were investigated using nuclear magnetic resonance (NMR) spectroscopy and ultra performance liquid chromatography/mass spectrometry (UPLC/MS) metabonomics approaches to determine biomarkers of hepatotoxicity. Acute and chronic doses of acetaminophen (APAP) were administered to male Sprague-Dawley rats. NMR and UPLC/MS were able to detect both drug metabolites and endogenous metabolites simultaneously. The principal component analysis (PCA) of NMR or UPLC/MS spectra showed that metabolic changes observed in both acute and chronic dosing of acetaminophen were similar. Histopathology and clinical chemistry studies were performed and correlated well with the PCA analysis and magnitude of metabolite changes. Depletion of antioxidants (e.g. ferulic acid), trigonelline, *S*-adenosyl-L-methionine, and energy-related metabolites indicated that oxidative stress was caused by acute and chronic acetaminophen administration. Similar patterns of metabolic changes in response to acute or chronic dosing suggest similar detoxification and recovery mechanisms following APAP administration.
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1. Introduction

Hepatotoxicity is the number one reason for drug recall and hence it is of major concern to the FDA and to consumers. Drug-induced toxicities account for nearly half the cases of acute liver failure in adults over age 50 in the United States with the leading cause of these drug-induced toxicities attributable to acetaminophen (APAP) [1]. APAP is a widely used over-the-counter analgesic and antipyretic drug. The pharmacological effects of acetaminophen may be associated with binding to the cyclooxygenase isozyme, COX-3, which inhibits the enzyme resulting in a decrease in pain or fever [2]. The toxicity of acetaminophen has been studied extensively [3–5]. While much is known about the metabolism and toxicity of acetaminophen [2,6–9], a complete mechanistic understanding of how acetaminophen causes hepatic necrosis has not been elucidated.

Metabonomics is a quantitative approach to study "the dynamic metabolic response of living systems to pathophysiological stimuli or genetic modification" [10]. The ability to measure global alterations in metabolism in tissues and biofluids that precede conventional biochemical and pathological changes has contributed to the emergence of metabonomics as a promising scientific platform for safety assessment. Metabonomics has the ability to rapidly assess potential toxicity and monitor toxicity during chronic drug administration without the scientific bias associated with predefined clinical measurements [11]. Pattern recognition and expert systems have been developed to allow analysis of complex metabonomics data sets, including use of a training set that consists of samples from specimens exhibiting a specific toxicity or pathology and a control group in order to generate a defined classification scheme. Once a model of the metabonomic data is generated and spectral biomarker(s) are determined, standard analytical techniques are used to identify the biomarker(s). In this manner, spectral biomarkers and the associated metabolites that define differences between groups can be identified. This approach has been pioneered by the group at Imperial College in London [10]. The Consortium on Metabonomic Toxicology (COMET) was formed between Imperial College-London and six pharmaceutical com-

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panies to develop an expert model of toxicity based on proton nuclear magnetic resonance (^{1}H NMR) analysis of urine samples from 147 studies, chiefly of renal and hepatic toxins [12,13]. The urine samples analyzed in this paper were collected as part of the COMET initiative and obtained from Pfizer through a material transfer agreement with the National Center for Toxicological Research (NCTR).

In this paper, ^{1}H NMR and ultra performance liquid chromatography/mass spectrometry (UPLC/MS) were applied to investigate APAP-induced hepatotoxicity resulting from either acute or chronic dosing. Our work indicates that both an acute single-overdose as well as chronic repeated overdosing with acetaminophen causes significant metabolic changes which could lead to hepatotoxicity. Both UPLC/MS and NMR techniques have provided complimentary metabonomics information through the use of multivariate analysis of spectroscopic data. In addition, the combination of UPLC/MS and principal component analysis (PCA) was utilized to detect the drug-related metabolites in urine samples from rats treated with APAP.

2. Experimental

2.1. Sample collection

Thirty 6–8-week-old male Sprague-Dawley rats (CRL:CD(SD)IGS BR) (Charles River Laboratories, Portage, MI) weighing 225–250 g were used for the acute dosing study while 40 rats of the same age, strain, and weight were used for the chronic study. Animal care was performed in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" and was authorized by the Pharmacia Animal Care and Use Committee. The animals were fed Purina Lab Certified Rodent Diet #5002 *ad libitum*, and were allowed to acclimate for a period of 7 days before they were placed into plastic metabolic cages for 2 days before treatment. During the study, room temperatures remained within 19–23 °C, daily relative humidity remained within 45–65%, and fluorescent lighting was provided on a 12 h on, 12 h off cycle (6 a.m. to 6 p.m.), using a cycle of light during the daytime and darkness at night. To reduce contamination, the rats were placed in clean cages each day. Urine was collected into vials containing 1 mL of 0.1% sodium azide that were surrounded by wet ice. The urine samples were centrifuged at 500 × *g* and 4 °C for 10 min, and stored at −70 °C for several years before they were shipped overnight to NCTR on dry ice. Precautions at NCTR were made to limit the amount of freeze thaw cycles and amount of time samples were at room temperature [14–16].

Serum was collected routinely for clinical chemistry analysis. Blood was collected from the caudal vena cava into serum separator tubes, allowed to clot for 30–60 min at room temperature, and centrifuged at 300 × *g*. Serum was removed and frozen at −20 °C until analysis, which was generally performed within 1–3 days of collection. Analytes included creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), glucose, sodium, potassium, calcium, phosphorus, albumin, total protein, and total bilirubin. Sections of liver and kidney were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. Lesions were scored on a 5-point scale (minimal, mild, moderate, marked, and severe).

For the acute study, groups of 10 rats per dose were orally gavaged with a single dose of either 0.2% carboxymethylcellulose (CMC, vehicle control), 400 mg acetaminophen/kg body weight or 1600 mg APAP/kg body weight at a volume of 10 mL/kg. Predose (−16 to 0 h), 0–8 h, 8–24 h, and 24–48 h urine samples were collected for 5 of the 10 animals in each dose group. For the other five animals in each dose group, urine was collected from −16 to 0 h, 0 to 8 h, 8 to 24 h, 24 to 48 h, 48 to 72 h, 72 to 96 h, 96 to 120 h, 120 to 144 h, and 144 to 168 h post-dosing. Half of the animals in each dose group were sacrificed at 48 h while the other half was sacrificed at 168 h post-dosing.

For the chronic study, groups of 10 rats per dose were administered orally by gastric intubation once daily for 7 days either 0.2% CMC, 200 mg APAP/kg body weight, 400 mg APAP/kg body weight, or 800 mg APAP/kg body weight in 0.2% CMC. Predose (−16 to 0 h), 0–8 h, 8–24 h, 24–32 h, 32–48 h, 48–56 h, 56–72 h, 72–80 h, 80–96 h, 96–104 h, 104–120 h, 120–144 h, and 144–168 h urine samples were collected for five of the ten animals in each dose group. For the other five animals in each dose group, urine samples were collected from −16 to 0 h, 0 to 8 h, 8 to 24 h, 24 to 32 h, 32 to 48 h, 48 to 56 h, 56 to 72 h, 72 to 80 h, 80 to 96 h, 96 to 104 h, 104 to 120 h, 120 to 144 h, 144 to 168 h, 168 to 192 h, 192 to 216 h, 216 to 240 h, 240 to 264 h, 264 to 288 h, and 288 to 312 h post-dosing. Half of the animals in each dose group were sacrificed at 168 h while the other half was sacrificed at 312 h post-dosing.

2.2. Conditions

2.2.1. Chemicals

HPLC grade acetonitrile and water were purchased from Burdick & Jackson (Muskegon, MI). Formic acid, leucine-enkephalin, imidazole, and all the MS standards were obtained from Sigma–Aldrich (St. Louis, MO). NMR solvents, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TMSP) and deuterium oxide (D_2O) were obtained from Cambridge Isotope Laboratories (Andover, MA).

2.2.2. ^{1}H NMR spectroscopy

Urine samples were prepared for analysis by the addition of 200 μL of sodium phosphate buffer (pH 7.4) and 60 μL of a mixture of 100 mM imidazole and 10 mM TMSP in D_2O to 400 μL of urine. The samples were centrifuged at 14,800 × *g* for 12 min at 10 °C and 600 μL was transferred to 5 mm o.d. tubes. NMR spectra were acquired on a Bruker Avance 600 MHz spectrometer (Bruker, Billerica, MA) operating at 600.133 MHz for proton and equipped with a triple resonance cryoprobe. The Bruker "noesy1dpr" pulse sequence was utilized, which irradiates the water peak during the mixing time (100 ms) and during a delay time of 2 s. A power level of 58.41 dB was employed for presaturation. For each sample, 32 scans were collected into 65,536 points. The pulse width for proton was 8.10 μs at a power level of 2.60 dB. A spectral width of 9615.385 Hz was employed and the total acquisition time was 3.41 s.

2.2.3. Data analysis for ^{1}H NMR spectroscopy

Spectra were processed using ACD/Labs 1D NMR Manager (ACD/Labs, Toronto, Canada). The raw FIDs were zero filled to 131,072 points, multiplied by a 0.3 Hz exponential function and Fourier transformed. The transformed spectra were then phased using the simple method and baseline corrected using the "SpAveraging" method with a box half width of 61 points and a noise factor of 3. All spectra were autoreferenced to the TMSP peak at 0.0 ppm. The spectra were overlaid in the processing window and grouped. The NMR spectral region between 4.49 and 6.24 ppm containing the resonances for water and urea was removed prior to integration. Also, the NMR spectral regions 2.13–2.19 ppm and 6.83–7.85 that contained resonances for imidazole, acetaminophen, and acetaminophen metabolites were removed prior to integration. Three bins, 3.58, 3.64, and 3.88, associated with acetaminophen glucuronide were also removed prior to PCA analysis. The intel-

ligent bucketing module was employed for integration with the bucket width set to 0.04 ppm and the looseness set to 50% for bin size optimization. The table of integrals was exported as a text file for statistical analysis. The spectra from the 8 h timepoints were not included in the analyses due to the presence of resonances from acetaminophen and acetaminophen metabolites as well as diurnal variation between the 8 h timepoints and the other timepoints. Forty-eight hours after dosing in the acute study, acetaminophen and acetaminophen metabolites in urine were not detectable by NMR methods used in this study.

All statistical analyses were performed using Statistica version 6.0 (Statsoft, Tulsa, OK). PCA based on covariance of the data was applied to the bucketed intensities. Scaled-to-maximum, aligned, and reduced trajectories (SMART) analysis was employed to compare the responses to acute and chronic dosing with acetaminophen [17]. SMART analysis was completed using the first two principal components (PCs) generated from PCA. Metabolite identification within the individual spectra was accomplished using Chenomx NMR Suite (Chenomx, Calgary, Canada), which has a database of >250 metabolites. The metabolite concentrations were normalized to the concentration of creatinine in each individual sample, which is generally applied to take into account the effects of urine dilution.

2.2.4. Ultra performance liquid chromatography

Samples were prepared for UPLC/MS analysis by centrifuging 100 μL of rat urine at 16,000 × *g* for 12 min at room temperature followed by transfer of the supernatant to autosampler vials. Metabolites were separated using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA). A 5 μL aliquot of rat urine was introduced to a Waters bridged ethyl hybrid (BEH) C18 (2.1 mm × 10 cm, 1.7 μm) column held at 40 °C. The UPLC mobile phase consisted of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B). While maintaining a constant flow rate of 0.15 mL/min, the metabolites were eluted using a tri-linear gradient of 0–30% B from 0 to 6 min, 30–50% B from 6 to 9 min, and 50–95% B from 9 to 11 min. The final gradient composition was held constant for 1 min followed by a return to 100% A at 12.1 min. After each injection, a strong/weak wash cycle was employed on the autosampler to eliminate the carryover between analyses. The weak needle wash solvent was 0.1% formic acid in water and the strong needle wash solvent was 50:50 acetonitrile:water.

2.2.5. Time-of-flight mass spectrometry

The mass spectrometric data were collected with a Waters LCT Premier single time-of-flight (TOF) mass spectrometer (Waters, Milford, MA) equipped with an electrospray ion source. The LCT-Premier was operated in W optics mode with a resolution of 11,000 using dynamic range extension (DRE). The source temperature was set to 120 °C with a cone gas flow of 50 L/h, a desolvation temperature of 200 °C and a desolvation gas flow of 550 L/h. The capillary voltage was 3.2 kV for positive ionization mode and 2.6 kV for negative ionization mode while the cone voltage was 40 V in both modes. A scan time of 0.5 s with an inter-scan delay of 0.05 s was used for all analyses. Leucine-enkephalin at a concentration of 250 pg/μL (in 50:50 acetonitrile:0.1% formic acid) was used as the lock-mass in positive mode ($[M+H]^+ = 556.2771$), and at a concentration of 25 ng/μL for negative mode ($[M-H]^- = 554.2615$). The lock spray frequency was 5 s and the lock mass data were averaged over 10 scans during collection. Full scan mode from *m/z* 50 to 850 and from 0 to 12 min was used for data collection in both positive ion and negative ion modes. Compounds detected by MS were confirmed by comparison with authentic standards.

2.2.6. UPLC/MS data analysis

Raw UPLC/MS data were analyzed using Micromass MarkerLynx Application Version 4.0 (Waters, Milford, MA). MarkerLynx was employed for peak selection and peak alignment. The original data were processed using the following parameters: initial retention time 0 min, final retention time 12 min, mass tolerance 0.02 Da, retention time tolerance 0.1 min and 20 masses in a 0.2 min retention window. The raw data were then transformed into a single matrix containing aligned peaks with the same mass/retention time pair along with peak normalized intensities and sample name. The resulting 3D dataset was analyzed by PCA. A conservative approach was used to remove acetaminophen and acetaminophen metabolites including their corresponding isotopic ions from dosed MS spectra. The acetaminophen-related ions were identified based upon PCA analysis of the UPLC/MS data from −16 h urine control and 8 h urine samples from rats dosed with 1600 mg APAP/kg body weight. The ions most responsible for the variance in the PCA loadings plot and the ions that were only observed in the dosed samples were identified as APAP or APAP-related xenobiotic metabolites. These drug-related metabolites were removed from subsequent PCA analyses of the UPLC/MS spectra to investigate changes in the endogenous metabolite profile that results from APAP administration. In total, approximately 95 APAP-related ions in negative mode and 65 APAP-related ions in positive mode were manually excluded from dosed UPLC/MS spectra within the MarkerLynx software. Many of the ions removed from the UPLC/MS spectra were isotopes and salt adducts of APAP metabolites. This method removed only the ions seen in the spectra from dosed animals. It is possible that some endogenous ions could also have been removed that may have been present at high concentrations in samples from dosed animals. The ions in the PCA loadings plots with the greatest contribution to the clustering behavior were manually inspected from the raw data to ensure measured mass accuracy. For ease of analysis due to the large size of UPLC/MS data sets and to clarify separation of the data points, the PCA plots of UPLC/MS data were generated from a limited set of spectra. The set of spectra for PCA was limited to samples collected at the 48 h timepoint and administered 1600 mg APAP/kg in the acute study and 200 mg APAP/kg and 800 mg APAP/kg in the chronic study.

2.2.7. HPLC ESI-MS/MS sample preparation

Urine samples were prepared for HPLC/MS analysis by dilution with H_2O (1:1 by volume) in HPLC/MS sample vials. *S*-Adenosyl-L-methionine (SAMe) standard solutions were prepared in HPLC grade water at concentrations ranging from 12.5 to 200 pg/μL.

2.2.8. HPLC ESI-MS/MS of S-adenosyl-L-methionine

ESI-MS/MS was carried out with a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Manchester, U.K.). The dwell time was set at 0.3 s. The capillary voltage was 3.03 kV, the cone voltage was 60 V, and hexapoles 1 and 2 were both set at 0 V. The source and desolvation temperatures were 120 and 300 °C, respectively. The cone gas flow rate was 65 L/h and the desolvation gas was adjusted to 300 L/h. Argon was used as the collision gas and set at a pressure of 2.13 mTorr. Quantitative analysis was done in positive ionization mode using the MRM *m/z* transition of 399 > 298 and 399 > 250 with a collision energy of 14 V for SAMe. The specific MRM transitions were determined by direct infusion of a standard. The chromatography was conducted with a Waters Acquity HPLC system. An Aquasil C_{18} reversed-phase column (3 μm particle size, 1 mm × 250 mm; Thermo Electron Corp. Bellefonte, PA) was used. Solvent A was a solution of 0.1% formic acid solution in water and solvent B was a solution of 0.1% formic acid, 10% H_2O, and 89.9% CH_3CN. A gradient separation was performed with 100%

Table 1
Acute acetaminophen clinical chemistry, *$p < 0.05$ and †$p < 0.01$

Clinical chemistry parameter	Control	400 mg/kg, 24 h	400 mg/kg, 48 h	400 mg/kg, 168 h	1600 mg/kg, 24 h	1600 mg/kg, 48 h	1600 mg/kg, 168 h
Creatinine (μM)	31.8 ± 6.0	29.2 ± 4.3	38.9 ± 4.8*	26.5 ± 0.0†	40.7 ± 12.6	108 ± 142	24.7 ± 4.0*
BUN (μM)	4338 ± 753	4141 ± 811	5569 ± 649†	5426 ± 924	6605 ± 5130	21349 ± 35967	4927 ± 587
ALT (IU/I)	47.4 ± 14.8	54.0 ± 6.8	38.0 ± 2.3*	40.0 ± 8.8	3144 ± 3903*	1170 ± 1148	40.4 ± 8.0
AST (IU/I)	96.3 ± 25.4	89.9 ± 13.2	80.2 ± 9.0*	91.6 ± 26.2	8860 ± 10574*	3416 ± 3270	81.8 ± 11.2
ALP (IU/I)	415 ± 80	459 ± 65	383 ± 29	365 ± 57	556 ± 110C†	543 ± 104	327 ± 54*
Bilirubin (mM)	3.93 ± 0.98	4.10 ± 1.65	3.76 ± 2.23	3.42 ± 0.00*	12.3 ± 6.7†	8.89 ± 4.49*	2.05 ± 0.76†

A from 0 to 3 min, increased to 50% B from 3 to 6 min, and to 100% B from 6 to 10 min. At 10 min, the concentration was held constant for 4 min and then returned to the starting conditions for 10 min prior to the next injection. The flow rate was set at 50 μL/min and 2 μL injections were used for both standards and samples. A calibration curve was constructed using Microsoft Excel from the range of standards. The concentration of the analyte in the sample was calculated from the standard calibration curve. The bar graph was constructed based upon the percent change with respect to the control group at 0 h. An average of both transitions was used to calculate the percent change in SAMe.

3. Experimental results

3.1. Clinical chemistry results

Doses were chosen to produce hepatic necrosis at the high dose and no overt histological change at the low dose. Table 1 reports the average values for selected clinical chemistry parameters for the acute acetaminophen study that were significantly different ($p < 0.05$) from their time-matched control at either the 400 or 1600 mg APAP/kg dose level or at both levels. As expected, ALT, AST, ALP, and bilirubin, which are markers of liver damage were elevated compared to control values at 24 h for the 1600 mg/kg dose group but not for the 400 mg/kg dose group. Bilirubin remained elevated at 48 h. BUN and creatinine increased in the high-dose group in the acute study at 24 and 48 h, suggesting renal toxicity in addition to hepatotoxicity in some of the animals. There was individual variation in this finding and the group changes were not statistically significant.

In the chronic study, the clinical chemistry profile did not show evidence of hepatic injury in the groups given 200, 400, or 800 mg/kg at 24 or 168 h post-dosing (Table 2). There was no significant increase in ALT, AST, or ALP. Although there was a statistically significant increase in bilirubin in the 800 mg/kg group at 24 h, this was not interpreted as evidence of hepatic injury because the magnitude of the increase was minimal and within the reference range for healthy rats. Similarly, there was no evidence of renal injury at these doses.

3.2. Urine volume and body weight

In the acute study, urine volume increased from 0 to 8 h and body weight decreased at both the 48 and 168 h sacrifice times in the animals given 1600 mg/kg of APAP. In the control animals, mean urine volume from 0 to 8 h was 5.7 ± 1.5 mL; in the animals given 400 mg/kg of APAP, it was 4.9 ± 1.8 mL; and in the animals given 1600 mg/kg of APAP it was 10.2 ± 3.0 mL ($p < 0.05$). In the control animals at 48 h, mean body weight was 236.3 ± 17.2 g; in the animals given 400 mg/kg of APAP, it was 240.3 ± 9.0 g; and in the animals given 1600 mg/kg of APAP, it was 201.2 ± 30.0. At 168 h, mean body weight was 267.0 ± 20.9 g for the control animals, 258.8 ± 15.3 g for the 400 mg/kg group, and 225.3 ± 27.0 ($p < 0.05$) for the 1600 mg/kg group.

In the chronic study, urine volume was increased in the high-dose (800 mg/kg per day) group at various times during the treatment period ($p < 0.05$ at 8, 48, 72, 96, 120, and 168 h). Urine volume increased during the first 24 h in the group that received 400 mg/kg per day ($p < 0.05$), but then returned to normal. The animals given 800 mg/kg per day lost approximately 20 g when weighed 24 h after dosing. After that, rats in this group gained weight at a similar rate as the other groups, but because of the initial weight loss, their average weights remained less.

3.3. Histopathology

Consistent with the goals of the dose selection, the high dose (1600 mg/kg) of APAP caused multifocal, centrilobular hepatic necrosis in all five of the rats sacrificed at 48 h post-dosing. The severity ranged from mild to severe. Additionally, there were regenerative responses by 48 h, including mild to moderate proliferation of hepatic stellate cells in two of five rats and a mild to moderate increase in hepatocellular mitotic figures in three of five rats. In contrast, none of the animals given the low dose (400 mg/kg) of APAP had hepatic necrosis or regenerative changes at 48 h post-dosing. At 168 h post-dosing, there was almost no hepatic necrosis, but regenerative changes were present. In the five rats given the high dose (1600 mg/kg) of APAP, one individual had minimal multifocal, randomly distributed hepatic necrosis at 168 h. Two rats had moderate to marked multifocal fibrosis and four had minimal hepatocellular hyperplasia. In the rats given the low dose (400 mg/kg) of APAP, at 168 h one individual had a single focus of mild necrosis and another individual had multifocal minimal single-cell hepatocellular death. No regenerative changes were seen in this group.

Significant renal lesions were restricted to the rats given the high dose (1600 mg/kg) of APAP. Four of five rats sacrificed at 48 h post-dosing had necrosis of epithelial cells of the proximal convoluted tubules. The lesions were minimal and consisted only of single-cell necrosis in three rats, but in one rat, the necrosis was severe and affected primarily the S3 portion of the proximal convoluted

Table 2
Chronic acetaminophen clinical chemistry, *$p < 0.05$ and †$p < 0.01$

Clinical chemistry parameter	Control	200 mg/kg, 24 h	200 mg/kg, 168 h	400 mg/kg, 24 h	400 mg/kg, 168 h	800 mg/kg, 24 h	800 mg/kg, 168 h
Creatinine (mM)	29.2 ± 4.3	28.3 ± 4.0	33.6 ± 4.0	26.5 ± 0.0	35.4 ± 0.0†	26.5 ± 0.0	35.4 ± 6.3
BUN	5676 ± 569	5641 ± 465	6140 ± 587	5426 ± 639	5855 ± 407	4641 ± 978	6283 ± 997
ALT (IU/I)	37.9 ± 3.4	41.0 ± 4.4	31.4 ± 11.6	39.2 ± 4.1	37.2 ± 2.7	43.2 ± 5.0	39.6 ± 1.5
AST (IU/I)	80.6 ± 4.2	91.4 ± 21.8	69.8 ± 12.4*	94.6 ± 8.8	80.2 ± 7.7	84.0 ± 15.0	75.4 ± 4.3
ALP (IU/I)	396 ± 68	504 ± 96	404 ± 97	369 ± 50	374 ± 97	476 ± 92	432 ± 112
Bilirubin (mM)	2.91 ± 1.41	3.76 ± 0.76	2.74 ± 1.53	2.39 ± 1.53	4.79 ± 1.87	4.79 ± 0.76†	3.08 ± 0.76

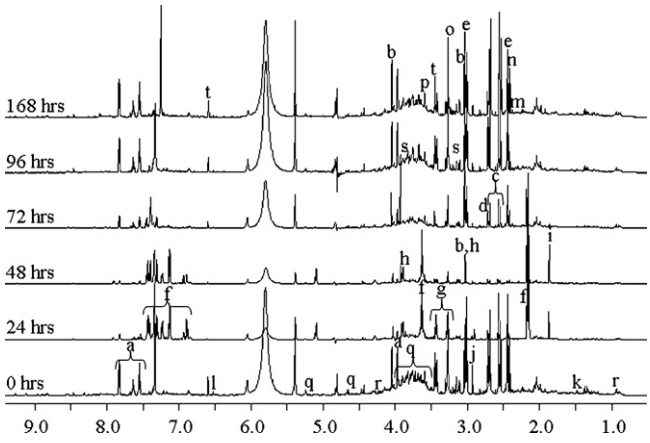

Fig. 1. Representative NMR spectra from the high-dose group (1600 mg APAP/kg) at 0, 24, 48, 72, 96, and 168 h post-dosing. Select resonances have been labeled: a, hippurate; b, creatinine; c, citrate; d, dimethylamine; e, 2-oxoglutarate; f, APAP and APAP metabolites; g, taurine; h, creatine; i, acetate; j, *N*,*N*-dimethylglycine; k, alanine; l, fumarate; m, pyruvate; n, succinate; o, trimethylamine-*N*-oxide; p, glycine; q, glucose; r, *N*-isovalerylglycine; s, betaine; and t, *trans*-aconitate.

tubules. No necrotic changes were seen 168 h post-dosing, but in two of the five rats, there were minimal multifocal regenerative changes consisting of multinucleate cells in the S3 portion.

In contrast, the histopathology data collected for the rats in the chronic study showed no treatment-related lesions in either the liver or the kidney. One of the rats in the 800 mg/kg dose group was noted to be an outlier due to pre-existing renal lesions that were unrelated to treatment. This animal had chronic/active, multifocal severe nephritis and pyelitis.

3.4. Analysis of urine samples by ^{1}H NMR spectroscopy

Fig. 1 shows representative NMR spectra from the high-dose group (1600 mg APAP/kg) at 0, 24, 48, 72, 96, and 168 h post-dosing. There are distinct changes in both the aliphatic, 2.14–2.22 ppm, and aromatic regions from 6.81 to 7.85 ppm of the 24 and 48 h spectra compared with the 0 h spectra. At 72 h, while many resonances are still altered, the spectra are similar to those of the 0 h spectra. By 96 h, the spectra are quite similar to the 0 h spectra by visual inspection alone. Several metabolites were decreased at 48 h relative to the 0 h timepoint including citrate, 2-oxoglutarate (2-OG), hippurate, taurine, *trans*-aconitate, glucose, and *N*,*N*-dimethylglycine (DMG) while concentrations of creatine and acetate increased relative to 0 h. Citrate, 2-oxoglutarate, and *trans*-aconitate are Krebs cycle intermediates that were significantly reduced at 48 h in the 1600 mg/kg dose group. The concentration of trigonelline, also known as 1-methylnicotinate, that is produced during the methylation process of the conversion of SAMe to *S*-adenosylhomocysteine (SAH) was also significantly decreased in both dose groups relative to control at the 48 h timepoint. Table 3 reports the concentrations of select metabolites normalized to creatinine in the acute acetaminophen groups detected by NMR at 0, 48, and 144 h. Concentrations that were significantly different from control ($p < 0.05$) are denoted in the table. Several concentrations were still significantly different from the average control value at 144 h indicating that the animals had not completely recovered following dosing.

The same metabolites that were evaluated for the acute study were also evaluated in the chronic study. Table 4 reports the concentrations of select metabolites normalized to creatinine in the chronic acetaminophen study detected by NMR at 0, 48, 144 h, and 312 h. Concentrations that were significantly different from control ($p < 0.05$) are denoted in the table. Similar changes in the concentrations were noted as described for the acute study. Significant decreases in 2-OG, citrate, glucose, DMG, and hippurate were all noted for the 800 mg/kg dose group 48 h after dosing was initiated. Additionally, the average creatine concentration increased significantly relative to the 0 h controls for the highest dose group 48 h after dosing was initiated. Trigonelline was also significantly decreased relative to the control value at 48 h in the 800 mg/kg dose group. The similar patterns of metabolite changes at the 48 h timepoint in response to acute or chronic dosing indicate that the same detoxification mechanisms are being activated in response to the drug administration.

PCA of the NMR spectra without the regions containing acetaminophen metabolites was used initially to determine whether the high dose, low dose, and control samples could be distinguished from each other in the acute study. The low dose samples at all timepoints clustered closely with the control samples while the samples from the high-dose group clustered apart from the control and low dose samples at all timepoints other than 0 h (data not shown). Fig. 2A and B shows the scores and loadings plots respectively for PCA based on covariances of the NMR data from the control and acute high-dose group samples at all timepoints. The scores

Table 3
Average metabolite concentrations normalized to creatinine from NMR spectra from the acute dosing study of acetaminophen toxicity

Postulated identity	400 mg/kg and 1600 mg/kg, 0 h	400 mg/kg, 48 h	400 mg/kg, 144 h	1600 mg/kg, 48 h	1600 mg/kg, 144 h
2-Oxoglutarate	3.64 ± 0.85	3.54 ± 0.76	3.19 ± 0.71	0.19 ± 0.33†	5.07 ± 1.89
Acetate	0.047 ± 0.020	0.034 ± 0.004†	0.032 ± 0.006†	1.90 ± 0.56†	0.039 ± 0.017
Betaine	0.135 ± 0.105	0.140 ± 0.121	0.113 ± 0.120	0.052 ± 0.017†	0.063 ± 0.018†
Citrate	5.64 ± 0.92	5.65 ± 0.71	5.28 ± 0.26	1.21 ± 0.55†	5.92 ± 1.28
Creatine	0.017 ± 0.005	0.017 ± 0.004	0.014 ± 0.003	1.07 ± 0.24†	0.015 ± 0.007
Dimethylamine	0.182 ± 0.034	0.173 ± 0.013	0.139 ± 0.012†	0.156 ± 0.022*	0.179 ± 0.047
Glucose	0.342 ± 0.087	0.268 ± 0.059*	0.227 ± 0.041†	0.208 ± 0.041†	0.239 ± 0.058*
Glycine	0.078 ± 0.011	0.071 ± 0.013	0.071 ± 0.011	0.054 ± 0.015†	0.057 ± 0.006†
Hippurate	1.46 ± 0.16	1.31 ± 0.17*	1.27 ± 0.12*	0.58 ± 0.13†	1.33 ± 0.49
Lactate	0.048 ± 0.017	0.041 ± 0.010	0.030 ± 0.008†	0.038 ± 0.011	0.032 ± 0.008†
Lysine	0.055 ± 0.009	0.063 ± 0.010*	0.053 ± 0.006	0.030 ± 0.010†	0.046 ± 0.003†
N,*N*-Dimethylglycine	0.121 ± 0.051	0.112 ± 0.051	0.078 ± 0.051	0.035 ± 0.016†	0.073 ± 0.039*
N-Isovalerylglycine	0.029 ± 0.005	0.028 ± 0.005	0.020 ± 0.002†	0.012 ± 0.004†	0.016 ± 0.003†
Pyruvate	0.030 ± 0.033	0.033 ± 0.011	0.030 ± 0.007	0.027 ± 0.013	0.042 ± 0.006†
Succinate	0.391 ± 0.056	0.368 ± 0.054	0.330 ± 0.050*	0.119 ± 0.020†	0.276 ± 0.051†
Taurine	0.725 ± 0.443	0.834 ± 0.327	0.879 ± 0.309	0.442 ± 0.245*	0.437 ± 0.236
Trigonelline	0.30 ± 0.09	0.11 ± 0.01†	0.30 ± 0.06	0.03 ± 0.01†	0.28 ± 0.05
Trimethylamine-*N*-oxide	0.201 ± 0.031	0.187 ± 0.032	0.172 ± 0.031	0.140 ± 0.015†	0.214 ± 0.020
trans-Aconitate	0.758 ± 0.051	0.650 ± 0.055†	0.589 ± 0.044†	0.280 ± 0.071†	0.687 ± 0.061

The average is the average value from all rats at that dose and timepoint. Significant differences are denoted as *$p < 0.05$ and †$p < 0.01$.

Table 4
Metabolite concentrations normalized to creatinine from NMR spectra of rats in the chronic dose study of acetaminophen

Postulated identity	All doses, 0 h	200 mg/kg, 48 h	200 mg/kg, 144 h	200 mg/kg, 312 h	400 mg/kg, 48 h	400 mg/kg, 144 h	400 mg/kg, 312 h	800 mg/kg, 48 h	800 mg/kg, 144 h	800 mg/kg, 312 h
2-Oxoglutarate	3.15 ± 0.47	3.18 ± 0.84	3.31 ± 0.89	1.97 ± 0.51†	2.60 ± 0.90	2.98 ± 0.56	2.05 ± 0.37†	0.86 ± 0.63†	3.36 ± 1.09	1.97 ± 0.39†
Acetate	0.18 ± 0.62	0.04 ± 0.01	0.05 ± 0.05	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.07 ± 0.05	0.26 ± 0.69	0.14 ± 0.16
Betaine	0.18 ± 0.08	0.19 ± 0.16	0.11 ± 0.06†	0.12 ± 0.03†	0.12 ± 0.05†	0.12 ± 0.06*	0.12 ± 0.08	0.09 ± 0.03†	0.11 ± 0.04†	0.09 ± 0.03†
Citrate	5.90 ± 0.67	5.71 ± 1.19	5.33 ± 0.88	3.43 ± 0.47†	4.88 ± 1.16*	5.08 ± 0.81*	3.45 ± 0.35†	1.91 ± 1.02†	5.11 ± 1.46	3.55 ± 0.57†
Creatine	0.04 ± 0.02	0.02 ± 0.01†	0.02 ± 0.01†	0.02 ± 0.01*	0.01 ± 0.00†	0.01 ± 0.01†	0.02 ± 0.01*	0.90 ± 0.63†	0.02 ± 0.01†	0.04 ± 0.02
Dimethylamine	0.23 ± 0.02	0.20 ± 0.02†	0.18 ± 0.02†	0.16 ± 0.01†	0.20 ± 0.02†	0.18 ± 0.03†	0.17 ± 0.01†	0.20 ± 0.02†	0.22 ± 0.03	0.19 ± 0.02†
Glucose	0.74 ± 0.13	0.38 ± 0.08†	0.31 ± 0.05†	0.33 ± 0.07†	0.31 ± 0.09†	0.20 ± 0.04†	0.34 ± 0.06†	0.32 ± 0.10†	0.23 ± 0.19†	0.33 ± 0.05†
Glycine	0.12 ± 0.04	0.09 ± 0.02†	0.05 ± 0.01†	0.06 ± 0.01†	0.06 ± 0.02†	0.02 ± 0.01†	0.06 ± 0.01†	0.03 ± 0.02†	0.04 ± 0.01†	0.04 ± 0.01†
Hippurate	1.74 ± 0.31	1.60 ± 0.22	1.71 ± 0.17	1.10 ± 0.46*	1.59 ± 0.32	1.50 ± 0.50	1.14 ± 0.54	0.95 ± 0.45†	1.92 ± 0.27	1.37 ± 0.42
Lactate	0.16 ± 0.15	0.07 ± 0.01†	0.05 ± 0.01†	0.05 ± 0.01†	0.05 ± 0.01†	0.03 ± 0.01†	0.05 ± 0.01†	0.08 ± 0.09	0.11 ± 0.19	0.19 ± 0.27
Lysine	0.08 ± 0.03	0.05 ± 0.01†	0.06 ± 0.01†	0.09 ± 0.02	0.05 ± 0.02†	0.04 ± 0.02†	0.09 ± 0.02	0.05 ± 0.01†	0.05 ± 0.01†	0.03 ± 0.03*
N,*N*-Dimethylglycine	0.11 ± 0.03	0.12 ± 0.04	0.09 ± 0.04	0.05 ± 0.02†	0.11 ± 0.06	0.09 ± 0.05	0.04 ± 0.01†	0.05 ± 0.02†	0.06 ± 0.02†	0.04 ± 0.03†
N-Isovalerylglycine	0.04 ± 0.01	0.03 ± 0.01†	0.03 ± 0.01†	0.02 ± 0.01†	0.03 ± 0.00†	0.02 ± 0.00†	0.02 ± 0.00†	0.03 ± 0.01†	0.02 ± 0.01†	0.03 ± 0.01
Pyruvate	0.05 ± 0.03	0.03 ± 0.01†	0.03 ± 0.01†	0.02 ± 0.00†	0.03 ± 0.01†	0.02 ± 0.01†	0.02 ± 0.01†	0.02 ± 0.01†	0.02 ± 0.01†	0.02 ± 0.01†
Succinate	0.53 ± 0.10	0.50 ± 0.13	0.42 ± 0.05†	0.34 ± 0.04†	0.50 ± 0.08	0.36 ± 0.04†	0.32 ± 0.04†	0.25 ± 0.13†	0.32 ± 0.17†	0.32 ± 0.17
Taurine	0.71 ± 0.39	0.73 ± 0.36	0.80 ± 0.50	0.88 ± 0.49	0.84 ± 0.32	0.72 ± 0.24	0.93 ± 0.45	0.76 ± 0.36	0.31 ± 0.15†	1.65 ± 0.45†
Trigonelline	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.01	0.10 ± 0.01†	0.12 ± 0.02	0.14 ± 0.01	0.10 ± 0.01†	0.08 ± 0.03†	0.14 ± 0.02	0.11 ± 0.01†
Trimethylamine-*N*-oxide	0.25 ± 0.04	0.24 ± 0.04	0.22 ± 0.03*	0.18 ± 0.03†	0.22 ± 0.05	0.25 ± 0.05	0.19 ± 0.05	0.16 ± 0.05†	0.35 ± 0.12*	0.23 ± 0.03
trans-Aconitate	0.95 ± 0.11	0.88 ± 0.10*	0.75 ± 0.07†	0.63 ± 0.05†	0.77 ± 0.11†	0.75 ± 0.06†	0.67 ± 0.07†	0.53 ± 0.18†	0.77 ± 0.11†	0.76 ± 0.08†

The average is the average value from all rats at that dose and timepoint. Significant differences are denoted as $^{*}p < 0.05$ and $^{\dagger}p < 0.01$.

plot (Fig. 2A) shows that the samples from the 24 and 48 h timepoints separate from control along PC1. After 48 h, all of the animals move back towards the control region. However, three of the rats did not completely return to the control region by 168 h separating from the control region along PC2. Fig. 2B shows the loadings plot from the PCA. The major bins that separated dosed animals from control were related to creatine, taurine, acetate, 2-OG, DMG, and citrate.

PCA analysis of the urine samples from the chronic study showed that the metabolic trajectory of the 800 mg/kg chronic dosed animals moved from the pre-dose control region along PC1 and then returned towards the control region at all time points beyond 168 h following the final dose (Fig. 3A). The scores plot shown in Fig. 3B indicates that the separation from the control region was due to the same bins that separated the control and high-dose group in the acute study (data not shown), again indicating that the same detoxification mechanisms are being employed following drug administration.

SMART analysis was employed to compare the trajectories following acute and chronic dosing with acetaminophen. SMART analysis is useful in that it removes the natural variation that occurs between animals and allows for a comparison of different dose levels and dose regimens [17]. Fig. 4 shows the SMART trajectories for all dose groups including solvent control for both the acute and chronic studies. Fig. 4 shows that the control and low dose trajectories (400 mg/kg acute and 200 mg/kg chronic) are not well defined and do not show much deviation from the starting point. The trajectory that results from the 400 mg/kg dose in the chronic study moves away from the origin along PC1 and starts to come back after 48 h into the dosing regime. A very similar trajectory is noted for the 800 mg/kg dose group in the chronic study although the magnitude of the trajectory is larger. The trajectory following an acute dose of 1600 mg APAP/kg is very similar to the high-dose trajectory in the chronic study in terms of magnitude and path with the greatest deviation from control at 48 h. Since the animals in the acute study received only a single dose of APAP, the trajectory quickly returns to the control region while the trajectories for the animals in the chronic study at the middle and high dose indicate that these rats have altered metabolic states until dosing ceases. The similarities in the trajectories indicate that similar pathways are being perturbed following administration of APAP regardless of whether animals were administered a single acute dose or received multiple doses over a 7-day period.

3.5. Analysis of urine samples by UPLC/MS

Metabolites in urine comprise a wide range of molecules including acidic, basic, amphoteric and neutral components making it impossible to analyze all of the biomolecules with a single ionization mode. In order to analyze as many metabolites as possible, the mass spectrometer was operated in both positive ionization mode and negative ionization mode as described in the experimental section. The LCT was run under dynamic range enhancement (DRE) mode, which expands the linear response range of the MS with slightly decreased mass accuracy to around 6–10 ppm. Typical positive ion total ion chromatograms (TICs) of 8 h urine samples from control and treated rats in the high-dose group for the acute study are shown in Fig. 5A and B, respectively and negative ionization mode TICs for 8 h urine samples from control and treated animals are shown in Fig. 5C and D, respectively. In addition to the identification of several acetaminophen metabolites, numerous changes were observed in the urinary metabolite profiles of acetaminophen-dosed animals compared to control animals using UPLC/MS.

In order to investigate alterations in the endogenous metabolite profile caused by the administration of APAP, it was important

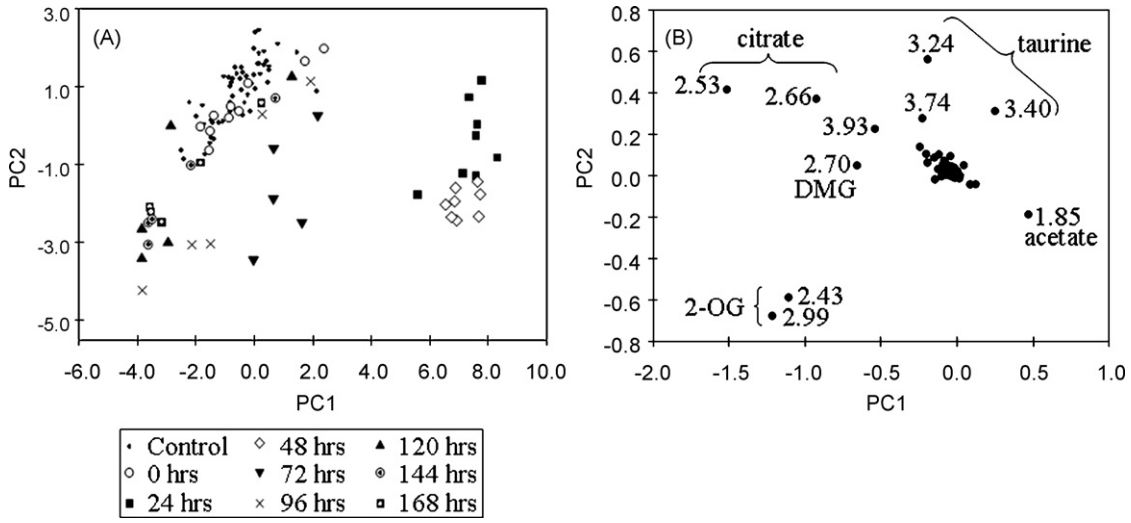

Fig. 2. (A) The scores plot for PCA based on covariances of the NMR data from control and high-dose acute APAP dosing samples at all timepoints. (B) The corresponding PC1 and PC2 loadings.

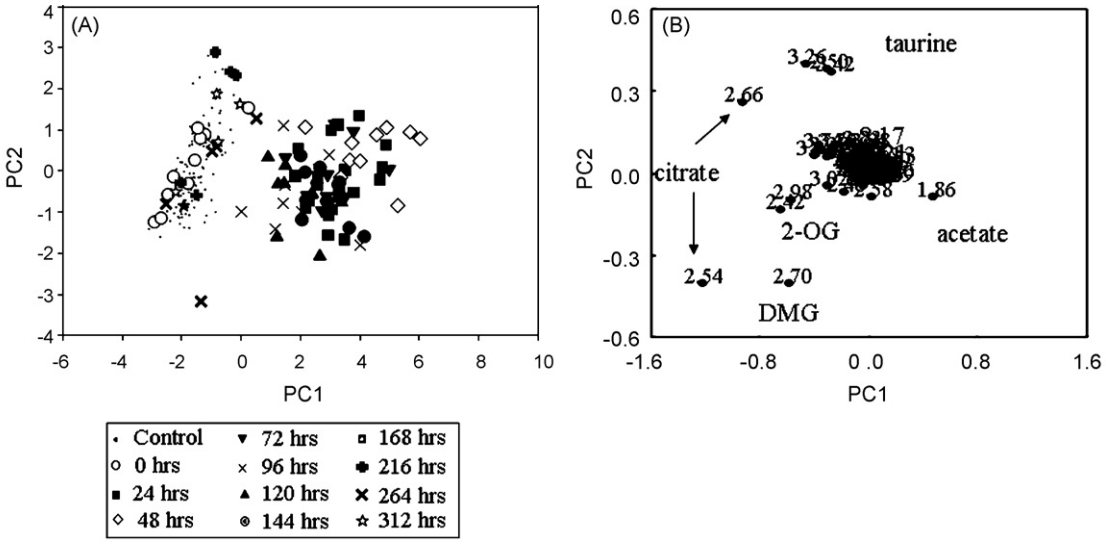

Fig. 3. (A) The scores plot for PCA based on covariances of the NMR data from control and chronic APAP dosing samples at all timepoints. (B) The corresponding PC1 and PC2 loadings.

to eliminate ions from APAP and APAP-related metabolite ions prior to data analysis. These ions were identified by comparing data from the high-dose (1600 mg/kg) and control groups using multivariate statistical analysis. The UPLC/MS spectra at 8 h were evaluated by PCA for markers that were unique in the dosed data sets. To ensure that drug-related components (acetaminophen parent compound and its metabolites) were not included in the analyses, peaks found in the dosed samples that were not also observed in the controls were eliminated. This was based on the conservative assumption that any endogenous compounds would be expected in both control and dosed samples. We acknowledge that endogenous compounds in the dosed animals that were below detection limit or were absent in the control animals may have also been eliminated from analysis. APAP and its metabolites were identified by PC loadings, exact masses, and previously published reports regarding acetaminophen metabolites [6,18,19]. Also, investigation of the ion trend plots facilitated the identification of APAP and known and unknown APAP metabolites. Table 5 (from positive UPLC/MS data) and Table 6 (from negative UPLC/MS data) summarize all of the known and identified ions that correspond to APAP and APAP metabolites in the 8 h post dose samples. Some major APAP metabolites are also labeled in Fig. 5. In total, approximately 95 peaks in negative mode and 65 peaks in positive mode were observed as APAP metabolites and their isotopic

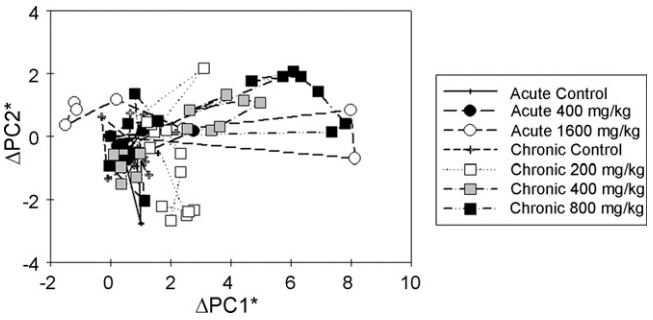

Fig. 4. SMART analysis of NMR data from acute and chronic acetaminophen studies. Each data point stands for the average toxicity response of the group at each different time point.

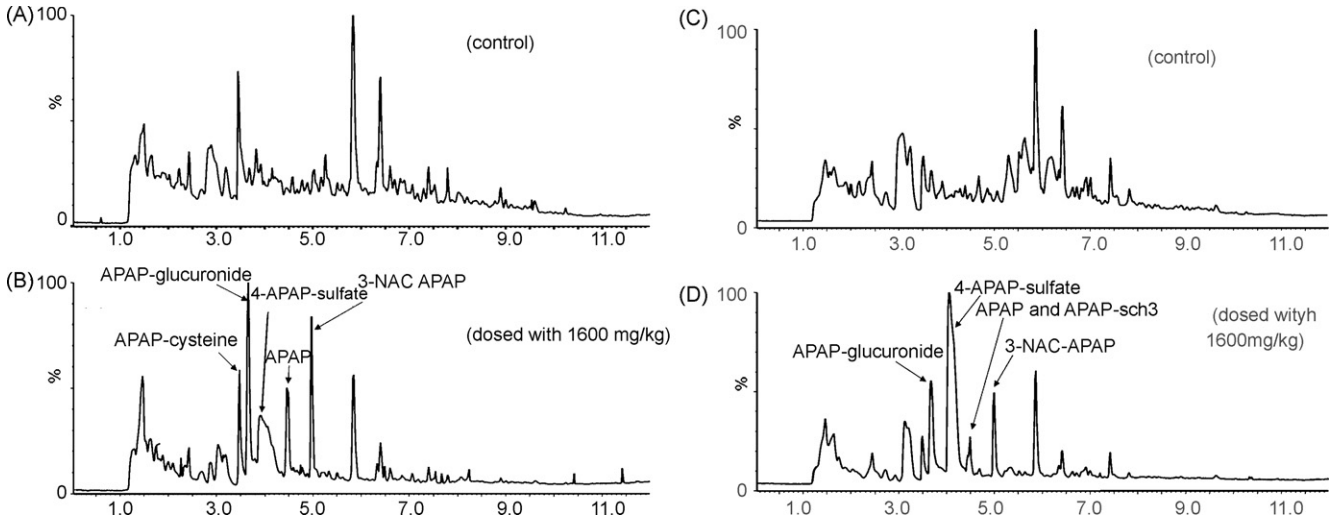

Fig. 5. Typical positive ionization mode total ion chromatograms (TIC) for control (A) and treated rats (B) in the high-dose group, and negative ionization mode TICs for control (C) and treated animals (D) with 1600 mg APAP/kg at 8 h. Labeled peaks correspond to APAP or major APAP-related metabolites.

ions in dosed samples, which were not seen in control urine samples at either −16 or 0 h. The extracted ion chromatograms (EICs) showed that there was no detectable evidence for the presence of the ions listed in Tables 5 and 6 in the control samples (data not shown).

After identification of APAP-related ions, PCA was used to evaluate whether there were differences between control, low dose and high-dose samples for both positive ion and negative ion UPLC/MS data and for analysis of both acute and the chronic studies with the APAP-related peaks excluded. Fig. 6A shows the 2D scores plot for control and rats dosed with 1600 mg/kg in the acute study, 200 mg/kg in the chronic study, and 800 mg/kg in the chronic study 48 h after the initial dose was administered. There is a clear separation of the three dosed groups from the control group. Further, the 200 mg/kg dose group in the chronic study separates from the high-dose groups in both studies along PC2. The high-dose groups from both studies form a tight cluster in the positive mode data. Fig. 6B shows the associated loadings plot for the positive mode LC/MS data. Fig. 7A and B shows the scores and loadings plots for the control and three dose groups based upon the negative mode UPLC/MS data. The dosed and control groups separate along PC1 with the 200 mg/kg dose group from the chronic study intermediate between the two high-dose groups and the control animals. Similar to the positive mode data, the animals from the highest dose groups in the chronic and acute studies form a tight cluster apart from the control group.

The changes in ions detected in positive ion UPLC/MS from PCA analysis of the acute data at 24, 72, 120, and 168 h are summarized in Table 7, which provides compound information including chromatographic retention time, molecular mass detected, molecular mass calculated, postulated identity, elemental composition, timepoints decreased and timepoints increased. Ion trend plots were used to determine whether an ion was increased or decreased at a given timepoint. The measured masses reported in Tables 7 and 8 were reexamined from the raw MS spectra. The principal ions that decreased in positive ion mode following acetaminophen administration were hippurate (*m*/*z* 359.12), phenylalanine (*m*/*z* 166.08), phenylacetylglycine (*m*/*z* 387.15), pantothenate (*m*/*z* 220.11), pipecolinate (*m*/*z* 130.08), and allantoin (*m*/*z* 159.05). The identities of the components were based on the

Table 5
Principal positive APAP-related metabolite ions at 8 h post-dosing with 1600 mg APAP/kg animal

Retention time (min)	Measured mass[a] (Da)	Calculated mass (Da)	Postulated identity	Proposed elemental composition ion
3.66	328.1052 or 655.1985	328.1032	APAP glucuronide	$C_{14}H_{18}NO_8$
3.97	383.0890	383.0913	APAP–sulfate–APAP	$C_{16}H_{19}N_2O_7S$
3.98	232.0258	232.0280	4-APAP sulfate	$C_8H_{10}NO_5S$
4.45	365.0630	365.0630	APAP-*S*-*S*-APAP	$C_{16}H_{17}N_2O_4S_2$
4.47	303.1342 or 152.0711	152.0712	APAP	$C_8H_{10}NO_2$
4.98	313.0848 or 625.1638	313.0858	3-NAC-APAP	$C_{13}H_{17}N_2O_5S$

[a] Measured mass was manually adjusted by inspection of the raw data since the detected mass from the PCA loading plot deviated from accurate mass due to detector saturation.

Table 6
Principal negative APAP-related metabolite ions 8 h after dosing with 1600 mg APAP/kg animal

Retention time (min)	Measured mass[a] (Da)	Calculated mass (Da)	Postulated identity	Proposed elemental composition ion
3.66	326.0876 or 653.1811	326.0876	APAP-glucuronide	$C_{14}H_{16}NO_8$
4.47	301.1189 or 150.0555	150.0555	APAP	$C_8H_8NO_2$
4.98	311.0702 or 623.1465	311.0702	3-NAC-APAP	$C_{13}H_{15}N_2O_5S$
3.98	230.0143 or 461.0314	230.0123	4-APAP sulfate	$C_8H_8NO_5S$
4.49	212.0370	212.0381	3-APAP-$SOCH_3$	$C_9H_{10}NO_3S$

[a] Measured mass was manually adjusted by inspection of the raw data since the detected mass from the PCA loading plot deviated from accurate mass due to detector saturation.

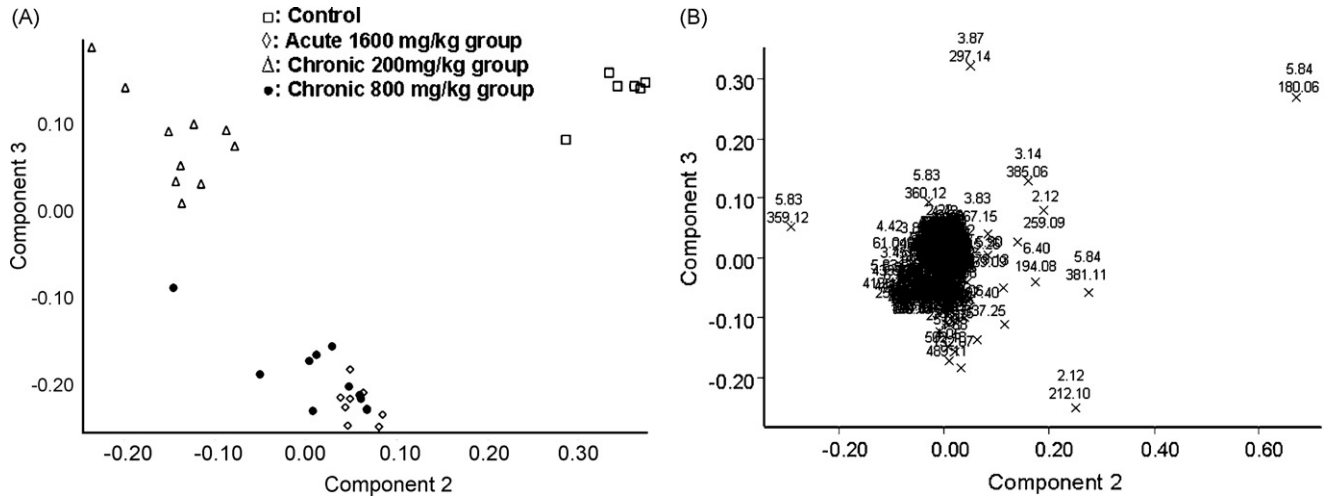

Fig. 6. The scores plot (A) and loadings plot (B) for PCA based on covariances of the UPLC/MS data in positive ionization mode from control, acute high dose (1600 mg/kg), chronic low dose (200 mg/kg) and chronic high-dose (800 mg/kg) group samples at 48 h timepoints. Five animals were included in this analysis.

exact mass measurement and further confirmed with retention times and exact masses of authentic standards or from a similar study by Lenz et al. [20,21]. Other unidentified ions that decreased at all timepoints after dosing with acetaminophen were at retention times (RT) of 1.66, 3.51, 3.87, 3.92, 5.27, 6.40, 6.86, and 7.30 min and *m*/*z* 138.06, 232.13, 303.06, 261.14, 338.08, 235.10, 196.06, and 267.13 respectively. Many of the unidentified ions were decreased at 24 h but started to return to the control region by 96 h. However, the concentrations were lower than the control value over the entire time course of the study. The principal ions that increased in concentration detected in positive ion mode were unidentified ions with retention times of 1.57, 1.63, 5.84, and 5.84 min and *m*/*z* 121.06, 174.07, 240.09, and 560.16, respectively. Other ions at RT of 2.36 and 5.68 *m*/*z* 244.09 and 310.09 increased 24 h after dosing but were back within the control level by 96 h.

The altered ions identified by negative mode UPLC/MS analysis are summarized in Table 8, which provides compound information including chromatographic retention time, detected molecular mass, calculated molecular mass, postulated identity, proposed elemental composition, timepoints decreased and timepoints increased. Most of the ions reported in Table 8 decreased with respect to the 0 h timepoint and include ions for hippurate (*m*/*z* 357.10), indoxysulfuric acid (*m*/*z* 212.00), benzenediol sulfate (*m*/*z* 188.98), ferulic acid sulfate (*m*/*z* 273.00), citrate (*m*/*z* 191.01), 2-oxoglutarate (*m*/*z* 145.01) and pipecolinate (*m*/*z* 128.07). All of these compounds were identified based upon the exact mass measurement and confirmed by authentic standards or published values. Ions that eluted at RT 3.87, 4.26, 4.66, 4.66, and 5.22 min with *m*/*z* 295.12, 239.99, 246.99, 261.00, and 285.06, respectively were also decreased in negative mode. Many ions including hippurate, citrate, 2-oxoglutarate and other unidentified ions were reduced after dosing across all timepoints. Unidentified ions with RT of 1.97, 5.70, and 9.50 min and *m*/*z* 267.03, 308.07, and 269.04, respectively increased in concentration in the negative mode scans. Ions at *m*/*z* 269.04 and 267.03 were greater than the controls at all timepoints while the ion at *m*/*z* 308.07 increased at 24 h and then returned to the control levels by 72 h.

The altered ions in the chronic study show similar patterns of change as those in the acute study. As discussed above, these ions were detected on the basis of PCA analysis from both positive mode and negative mode UPLC/MS data at 24, 48, 72, and 96 h. Data are not shown here but are similar as that in Tables 7 and 8. This comparable pattern of metabolite change between acute and chronic studies again suggests that similar detoxification mechanisms are activated.

Fig. 8A and B shows the percent change of SAMe in dosed urine samples versus controls at 0 h in the acute and chronic APAP studies, respectively. Fig. 8A shows that the SAMe levels in the acute study drop significantly at 24 h for both the low-dose and high-dose groups. By 72 h, the percent change of SAMe for the low- and

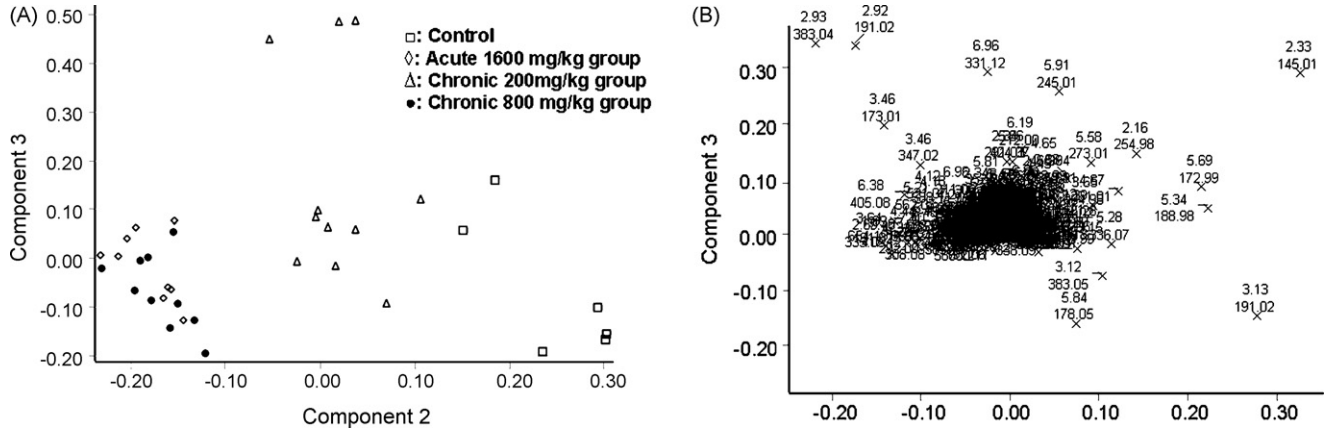

Fig. 7. The scores plot (A) and loadings plot (B) for PCA based on covariances of the UPLC/MS data in negative ionization mode from control, acute high dose (1600 mg/kg), chronic low dose (200 mg/kg) and chronic high-dose (800 mg/kg) group samples at 48 h timepoints. Five animals were included in this analysis.

Table 7
Principal positive ions altered after dosing with 1600 mg APAP/kg at 24, 72, 120, and 168 h

Retention time (min)	Measured mass[c] (Da)	Calculated mass (Da)	Postulated identity	Proposed elemental composition ion	Timepoints decreased	Timepoints increased
1.31	167.0167	167.0178		$C_2H_5N_3O_6$	24, 72	
1.57	121.0619	121.0613		$C_3H_9N_2O_3$		All
1.58	76.0401	76.0399	Glycine[a]	$C_2H_6NO_2$	All	
1.63	174.0765	174.0766		$C_7H_{12}NO_4$		All
1.64	159.0530	159.0518	Allantoin[a]	$C_4H_7N_4O_3$	72, 120, 168	
1.66	138.0632	138.0641		$C_3H_{10}N_2O_4$	24, 72	
1.89	189.1256	189.1239	*N*-Acetyllysine[a]	$C_8H_{17}N_2O_3$	All	
2.14	130.0864	130.0868	Pipecolinate[a]	$C_6H_{12}NO_2$	All	
2.36	244.0910	244.0907		$C_6H_{16}N_2O_8$	72, 120, 168	24
3.51	232.1316	232.1311		$C_{11}H_{20}O_5$	All	
3.83	367.1478	367.1492		$C_{15}H_{21}N_5O_6$	24	72, 120, 168
3.87	303.0697	303.0703		$C_{10}H_{13}N_3O_8$	All	
3.92	261.1448	261.1450		$C_{11}H_{21}N_2O_5$	All	
4.01	166.0850	166.0868	Phenylalanine[a]	$C_9H_{12}NO_2$	All	
4.15	220.1176	220.1185	Pantothenate[a]	$C_9H_{18}NO_5$	24, 120, 168	
5.27	338.0866	338.0876		$C_{15}H_{16}NO_8$	All	
5.68	310.0917	310.0927		$C_{14}H_{16}NO_7$		24
5.83	180.0660	180.0661	Hippurate[a]	$C_9H_{10}NO_3$	All	
5.84	240.0983	240.0984		$C_{10}H_{14}N_3O_4$		All
5.84	560.1699	560.1696		$C_{32}H_{24}N_4O_6$		All
6.07	192.0910	192.0899		$C_{10}H_{12}N_2O_2$	All	
6.40	194.0808	194.0817	Phenylacetylglycine[b]	$C_{10}H_{12}NO_3$	All	
6.40	235.1044	235.1056		$C_8H_{11}N_8O$	All	
6.86	196.0629	196.0637		$C_{12}H_8N_2O$	All	
7.30	267.1309	267.1305		$C_8H_{19}N_4O_6$	All	

[a] Identity from the authentic standard with the corresponding standard deviation of retention time in the range of ±0.01 min.
[b] Identity from the work by Lenz and co-workers [20,21].
[c] Measured mass was manually adjusted by inspection of the raw data since the detected mass from the PCA loading plot deviated from the accurate mass due to detector saturation.

high-dose studies have recovered to predose levels. Fig. 8B shows the percent change of SAMe versus predose controls in the chronic study were significantly lower during the first four days of chronic dosing for both 400 mg/kg and 800 mg APAP/kg doses. This trend is suspected to continue until the dosing was stopped after 7 days. SAMe returned to predose levels at 336 h, which was 1 week after the chronic dosing was stopped.

4. Discussion

The principal effect of the high dose of 1600 mg APAP/kg, given as a single oral dose, was multifocal necrosis of the liver and, to a lesser magnitude, of the renal proximal convoluted tubules. In response to the hepatic necrosis, there was evidence of regenerative changes as early as 48 h. The hepatic necrosis was almost completely resolved by 168 h after dosing, although regenerative changes were still present. In contrast, in the rats given the low dose, 400 mg APAP/kg, none of the animals had hepatic necrosis or regenerative changes at 48 h post-dosing. At 168 h post-dosing, two of five rats given the low dose had a few necrotic changes (a single focus of mild necrosis in one and minimal single-cell death of hepatocytes in another), but these small changes were not accompanied by regeneration as seen in the high-dose group. Consistent with the histopathology results, the markers of liver damage (ALT,

Table 8
Principal negative ions altered after dosing rats with 1600 mg APAP/kg at 24, 72, 120, and 168 h

Retention time (min)	Measured mass[d] (Da)	Calculated mass (Da)	Postulated identity	Proposed elemental composition	Timepoints decreased	Timepoints increased
1.57	195.0515	195.0505	Gluconic acid[a]	$C_6H_{11}O_7$	120, 168	
1.97	267.0354	267.0352		$C_8H_{11}O_{10}$		All
2.14	128.0710	128.0712	Pipecolinate[a]	$C_6H_{10}NO_2$	24, 72	
2.33	145.0127	145.0137	2-Oxoglutarate[a]	$C_5H_5O_5$	All	
3.15	191.0199	191.0192	Citrate[a]	$C_6H_7O_7$	All	
3.87	295.1294	295.1294		$C_{14}H_{19}N_2O_5$	All	
4.26	239.9962	239.9940		$C_6H_8SO_8$	All	
4.66	246.9909	246.9912		$C_9H_9SO_7$	All	
4.66	261.0064	261.0069		$C_8H_7SO_7$	All	
5.22	285.0612	285.0610		$C_{12}H_{13}O_8$	All	
5.30	188.9879	188.9858	Benzenediol sulfate[b]	$C_6H_5SO_5$	24	
5.54	273.0067	273.0069	Ferulic acid sulfate[c]	$C_{10}H_9SO_7$	24	
5.70	308.0745	308.0743		$C_{11}H_{16}O_{10}$		24
5.83	357.1080	357.1087	Hippurate[a]	$C_9H_8NO_3$	All	
6.16	212.0010	212.0018	Indoxysulfuric acid[a]	$C_8H_6NO_4S$	All	
9.50	269.0442	269.0426		$C_8H_{14}PO_8$		All

[a] Identity from the authentic standard with the corresponding standard deviation of retention time in the range of ±0.01 min.
[b] Identity from the work by Lenz and co-workers [17,18].
[c] Identity from the tandem mass spectrum of the metabolite.
[d] Measured mass was manually adjusted by inspection of the raw data since the detected mass from PCA loading plot was deviated from the accurate mass due to detector saturation.

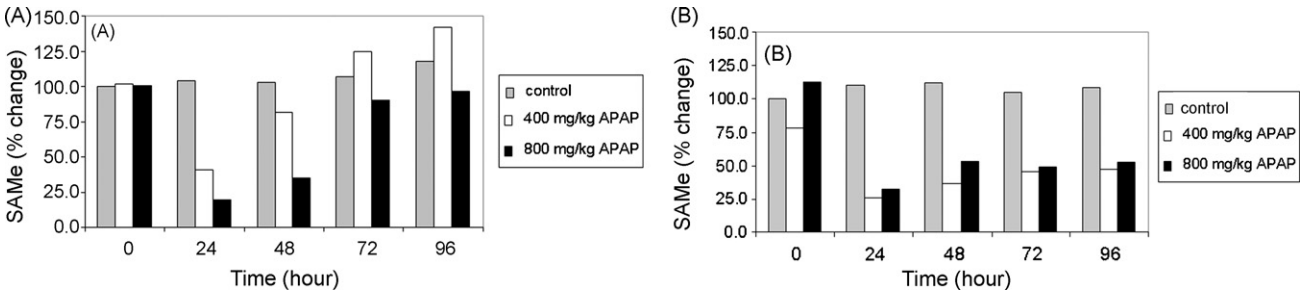

Fig. 8. The bar graph shows the percentage change in SAMe vs. predose controls after 400 mg/kg and 1600 mg APAP/kg acute doses studies (A) at 0, 24, 48, 72, and 96 h. (B) Percent change in SAMe vs. predose controls after 400 mg/kg, and 800 mg/kg daily chronic dosing with APAP studies at 0, 24, 48, 72, and 96 h.

AST, ALP, and bilirubin) were all markedly elevated at 24 h for the 1600 mg/kg dose group but not for the 400 mg/kg dose group. By 48 h, although the mean values of ALT and AST continued to be markedly elevated, the group means were not statistically significant due to substantial individual animal variation. This is likely due to the variable onset and rate of regenerative changes. BUN and creatinine increased in the high-dose group, especially at 48 h post-dosing, suggesting renal damage.

There was one animal in the high-dose group from the acute study that had high levels of creatinine and BUN at both the 24 and 48 h timepoints. This animal was sacrificed at 48 h post-dosing and had severe necrosis of the S3 portion of the renal proximal convoluted tubules and marked centrilobular hepatic necrosis. There was no urine provided for this animal at 24 or 48 h so a direct assessment of metabolite changes could not be made and associated with the clinical findings and histopathology data. It is well documented that high doses of acetaminophen can cause both hepatic and renal toxicity in Sprague-Dawley rats [22,23]. In the chronic study, there was no evidence of toxicity, as evidenced by histopathology or clinical chemistry.

In a metabonomics study, one expects to detect changes related to efficacy, toxicity, and recovery [8,9,12,24,25]. Our metabonomics analyses show the ability to detect altered levels of acetaminophen metabolites and endogenous metabolites associated with *N*-acetyl-*p*-benzo-quinone imine (NAPQI) conjugation that includes gradual glutathione depletion. NMR, UPLC/MS, and HPLC/MS analyses showed decreases in antioxidants and energy-related metabolites following dosing with acetaminophen. Additionally, the NMR and MS analyses showed numerous similarities in the metabolic changes regardless of whether the animals were administered a single acute dose or whether they were administered APAP over several days. The NMR and MS data indicated that the largest changes occurred within 48 h after dosing with APAP in the acute study followed by a gradual return towards the control region by 72–96 h. This observation was consistent with histopathology results, which showed regenerative changes as early as 48 h and the hepatic necrosis almost completely resolved by 168 h after dosing with 1600 mg/kg. For the chronic study, the largest changes from control also occurred within 48 h; however, the metabolic states remained altered until dosing ceased at 168 h. None of the animals in the chronic study showed significant morphologic changes in the liver at the time of sacrifice.

Analysis of the PCA scores plot from the NMR data (Fig. 2A) showed that two of the animals in the acute study returned to the control region 48 h after dosing with 1600 mg APAP/kg indicating that in these two animals, there was not a prolonged toxic effect following dosing with APAP. The remaining three animals had not returned to the control region by 168 h. Histopathological analysis of the liver tissue showed less severe findings for these two animals with one having minimal hepatocellular hyperplasia and the other having no marked pathology. The other three animals had, respectively, (1) minimal hepatocellular hyperplasia plus a single focus of minimal hepatocellular necrosis, (2 and 3) minimal hepatocellular hyperplasia and marked multifocal fibrosis. The pathology findings correlated with the PCA analysis, which showed that two of the animals recovered following dosing while the other three were metabolically different from controls 7 days post-dosing. In the low dose group at 7 days post-dosing, two of the animals showed minimal to mild hepatocellular necrosis although none of the animals showed hepatic necrosis or regenerative changes at 48 h post-dosing with 400 mg APAP/kg.

Slightly lower levels of glucose, acetate, hippurate and *trans*-aconitate were detected in the NMR spectra of the 48 and 144 h low dose urine samples. Krebs cycle intermediates, 2-OG, citrate, pyruvate, and succinate were markedly reduced 48 h after dosing with 1600 mg APAP/kg. Besides the TCA cycle intermediates, NMR detected reductions in glucose, trimethylamine-*N*-oxide (TMAO), dimethylamine, hippurate, glycine, *N*,*N*-dimethylglycine, *N*-isovalerylglycine, taurine, and betaine at 48 h after the 1600 mg/kg dose was given while creatine and acetate were significantly elevated. The NMR analysis of the 1600 APAP mg/kg dose group at 48 h had the lowest levels of the energy-related metabolites 2-OG, citrate, succinate, and *trans*-aconitate observed at any time point in the acute studies or the chronic studies. A significant increase in acetate suggests a switch in energy metabolism from glycolysis to fatty acid beta-oxidation, which could be caused by the increase in free radicals as a result of acetaminophen dosing [26]. Previous studies by Coen et al. [8,9] on the hepatotoxicity of APAP have indicated an increased rate of hepatic glycolysis as well as impairment of fatty acid beta-oxidation in liver mitochondria. In addition, the metabolite *N*-methylnicotinate, also known as trigonelline, was noted to be significantly decreased in both dose groups in the acute study at 48 h post-dosing and in the highest dose group in the chronic study at 48 h after initiation of dosing. This methylated metabolite is a byproduct of the conversion of SAMe to SAH. The decrease in *N*-methylnicotinate may be related to a depletion of SAMe as it is consumed in the transsulfuration pathway in order to regenerate glutathione stores, which are depleted in order to detoxify the reactive metabolites of acetaminophen. SAMe is significantly diminished in the acute and chronic studies at both low and high doses, consistent with the role of glutathione in detoxifying the toxic APAP metabolite, NAPQI, and the gradual glutathione depletion. Additionally, increased excretion of 3-(*N*-acetyl-L-cysteinyl)-4-acetamidophenol (3-NAC-APAP; an APAP metabolite related to glutathione) detected in urine at 24 and 48 h clearly indicated the depletion of glutathione in the high-dose acute APAP study. The NMR spectra of urine from the 1600 mg/kg acetaminophen dose group at 48 h also had the lowest levels of taurine, which is also consistent with the decrease in SAMe and other antioxidant decreases as the sulfur group of taurine is involved with recovery from oxidative stress due to APAP toxicity [27]. Decreases in the levels of metabolites, especially

SAMe and many other antioxidants, may provide further insight into the changes that occur in response to drug-induced hepatotoxicity which should be further evaluated in liver tissue. SAMe has been shown to be protective against several toxins including acetaminophen, cyclosporin A, galactosamine and ethanol [28–33]. SAMe is the primary contributor of the sulfur atom needed for glutathione production via the transsulfuration pathway that is used to detoxify NAPQI [34]. Only when glutathione is sufficiently depleted does NAPQI bind to intracellular proteins to any significant degree. This leads to mitochondrial damage and eventually to cell damage. In our analyses, SAMe was diminished in the 1600 mg/kg acute and all three chronic doses of APAP in each of these studies. However, hepatotoxicity was not observed in terms of pathology or standard clinical chemistry. Therefore, other events besides a reduction in SAMe and antioxidants must occur before hepatotoxicity is noted, such as a critical amount of energy depletion and protein binding [35].

Hippurate, phenylacetylglycine, citrate, and 2-OG were found to decrease in the UPLC/MS spectra soon after acute dosing with 1600 mg APAP/kg and chronic dosing at 800 mg APAP/kg. The urinary levels of hippurate have been shown to correlate with dietary changes [36] and with intestinal microbiota changes [37–39]. UPLC/MS also detected many other compounds including phenylalanine and pantothenate (vitamin B5), which also appeared to decrease following dosing in both acute and chronic dose groups. Since both phenylalanine and pantothenate are primarily derived from diet, one possible explanation is that the rats dosed with acute 1600 mg APAP/kg or chronically with 800 mg APAP/kg may not have been eating or absorbing as many nutrients as the controls and lower dose group animals. However, it is acknowledged that changes in the concentration of phenylalanine in urine can also be due to variations in the intestinal microbiota and its metabolism and recycling of amino acids. Pantothenate (vitamin B5) is needed to form coenzyme A (CoA) and is directly involved with many physiological functions of the cell. Follow-up metabonomics studies in which dietary intake is monitored and the control animals are pair-fed the same amount of feed as the treated animals would be required to address the hypothesis that some of the endogenous metabolites are altered by decreased feed consumption rather than directly altered by APAP toxicity.

UPLC/MS detected reductions in pipecolinate at all timepoints in the 1600 mg/kg dose group and early timepoints in the chronic 800 mg/kg study. Pipecolinate is involved in the lysine metabolism pathway and the decrease may be due to a decrease in free lysine as a result of lysine arylation with APAP [40]. UPLC/MS also detected glycine as being reduced in the high-dose acute samples and this may be in part related to conjugation of the toxic APAP metabolite, NAPQI with glutathione since glycine is required for the synthesis of glutathione [41]. Glycine is also involved with the formation of creatine during drug-induced liver necrosis [42]. Ferulic acid sulfate, a metabolite of ferulic acid, was decreased in the high-dose acute group at all timepoints other than 168 h and all timepoints in the chronic 800 mg/kg study. The observation here indicates that ferulic acid is likely to be chemically oxidized and may play a role in detoxifying acetaminophen metabolites with free radicals.

In addition, the control and high-dosed urine samples at 8 h were evaluated to identify ions related to APAP and its metabolites. These ions were removed prior to statistical analyses of UPLC/MS data to investigate the alterations in the endogenous compounds. The analysis of APAP metabolites using the 8 h samples showed that 3-NAC-APAP was observed in both positive and negative UPLC/MS data of high-dose samples. The acetaminophen mercapturic acid derivative that was observed in 8 h samples has also been seen by LC/MS in a study of human urine samples [43]. Two important, well-known APAP metabolites, acetaminophen sulfate and acetaminophen glucuronide, have also been detected by a combination HPLC-NMR-MS study [18]. APAP-*S*-*S*-APAP (*N*,*N*′-[dithiobis(4-hydroxy-3,1-phenylene)]diacetamide) and 3-APAP-$SOCH_3$ (acetaminophen-3-methyl sulfoxide) were tentatively identified based on the exact mass accuracy and the report by Bessems and Vermeulen [19].

The results from our NMR, UPLC/MS, and HPLC/MS analyses of urine samples from rats dosed with APAP compare favorably with results obtained in previous metabonomics studies on APAP. Previous transcriptomics and metabonomics studies of acetaminophen toxicity in liver tissue showed glucose was down and liver lipid content was increased due to mitochrondrial dysfunction. In addition, metabonomics data for plasma in the same study show increased levels of glucose, pyruvate, acetate, and lactate [9]. Our observations are consistent with liver mitochondrial dysfunction in that the observed urinary levels of Kreb's cycle intermediates, glucose, and lactate were severely diminished at 48 h after an acute high dose of APAP. Although glutathione was not directly detected by LC/MS due to it being easily oxidized, the presence of 3-NAC-APAP and the decrease in SAMe in urine suggests oxidative stress in the mitochondria.

5. Conclusions

Since the current clinical chemistry markers of liver toxicity are not necessarily the most sensitive indicators, it is important to identify new biomarkers of hepatotoxicity that can be used in preclinical and clinical settings. Both NMR and UPLC/MS were able to detect multiple time dependent metabolic changes in acetaminophen drug metabolites, antioxidants, and energy-related metabolites, in the urine of rats following acute or chronic dosing with acetaminophen when compared with control samples. PCA of the UPLC/MS data from urine samples obtained from the 8 h control and 1600 mg APAP/kg dose groups was able to detect many of the known acetaminophen metabolites. These ions were removed from further analyses in order to find endogenous biomarkers of liver toxicity due to acetaminophen overdose. Analysis of NMR and UPLC/MS spectra of urine from high-dose urine samples from the acute acetaminophen studies found patterns of metabolic change that supported clinical chemistry and histopathology results. The concentrations of energy metabolites and antioxidants were altered the most at 48 h after the 1600 mg/kg toxic dose of acetaminophen. Many of the energy-related metabolites and antioxidants detected as significantly altered in the acute study were also altered following chronic dosing, although the chronic study showed no major changes in histopathology or clinical chemistry parameters. Additionally, SMART analysis of acute and chronic spectra showed consistent metabolic changes which indicate that the same metabolic pathways were being perturbed and similar detoxification and recovery mechanisms were being employed.

The UPLC/MS spectra showed that many antioxidants in urine were lowered significantly which gives new insight into antioxidant protective mechanisms in the liver of rodents in addition to the previously published role of the antioxidants glutathione and *N*-acetylcysteine [44–47]. The analyses and spectral changes reported in this manuscript are in agreement with the recent COMET model of 80 toxins that correctly predicted the acute APAP study as hepatotoxic while chronic dosing with APAP was not classified as hepatotoxic [48]. Follow-up studies are needed in order to verify that the reduction in antioxidant levels are due to a protective mechanism against reactive metabolites by the liver and not actually due to dietary changes. As described recently by Coen et al. [49] there is also a need to look at blood and tissue samples to determine

the correlation between urinary metabolic changes as described in this manuscript and those occurring in blood and tissue. This would be similar to the metabonomics study that was done by Waters et al. [50] who evaluated urine, blood, and tissue samples after a toxic dose of α-naphthylisothiocyanate.

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