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Detection and Identification of Acetaminophen (Tylenol) Metabolites using Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS/MS) Analysis

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Abstract:
Acetaminophen (APAP) is a commonly used analgesic and antipyretic drug that can cause liver injury, liver necrosis, and liver failure. APAP-induced liver failure (AILF) is associated with depletion of glutathione and the increased formation of APAP protein adducts from the toxic NAPQI metabolite. There is a hypothesized detoxifying pathway where vitamin C ascorbylates and conjugates the hepatotoxic metabolite *N*-acetyl-p-benzoquinone imine (NAPQI). If the hypothesized metabolite is found, the protocol for treating APAP overdose could be changed from giving large amounts of the drug acetylcysteine to giving intravenous vitamin C. To find the hypothesized metabolites, we collected urine from 10 male subjects' after ingestion of 1000 mg of APAP. The urine was prepared for analysis through saturation with sodium chloride, liquid-liquid extraction and analysis of the extract for metabolites by LC-HRMS/MS. The major metabolites found in the urine collected for eight hours after APAP ingestion (T8) include APAP, APAP-sulfate, APAP- glucuronide, APAP-mercapturate, and 2-hydroxyacetaminophen sulfate. The hypothesized ascorbylated NAPQI was not found in the urine. LC-HRMS/MS can simultaneously quantify APAP, its major metabolites (APAP-sulfate and APAP-glucuronide) in urine samples. Follow-up studies are needed to determine if the secondary pathway through ascorbate conjugation is possible with APAP.

Significance Statement:

Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS/MS) can simultaneously quantify APAP, its major metabolites (APAP-sulfate and APAP-glucuronide) in urine samples. Follow-up studies are needed to determine if the secondary pathway through ascorbate conjugation is possible with APAP to change the way we treat both intentional and unintentional acetaminophen overdose, lower healthcare costs, and to prevent liver damage.

Introduction:

Acetaminophen (APAP, $C_8H_9NO_2$), also known as paracetamol and *N*-acetyl-p-aminophenol, is commonly used for pain relief and reducing fevers. It is considered a safe and effective analgesic and antipyretic when used as directed. While much is known about the metabolism and toxicity of APAP, the binding of vitamin C to detoxify APAP was not reported in a metabolomics analysis of urine from rats given APAP (1). Vitamin C (AscH) acts as a cofactor for a number of 2-ketoglutarate dependent dioxygenases, can also act as a biological antioxidant and act as a pro-oxidant (2, 3, 4). AscH also has a lesser known ability to participate in nucleophilic substitution and in Michael addition reactions (1,2). AscH is essentially an enolate and is therefore capable of forming C-C bonds with electrophiles, which is termed 'ascorbylation' (6).

Exceeding the maximum daily dose of 4,000 mg of APAP is an overdose and can lead to severe liver damage, necrosis, and death (5, 7). APAP overdose is the leading cause of acute liver failure (ALF) in the United States (AAC, 2019; 8). ALF or APAP-induced acute liver failure (AIALF) often requires immediate hospitalization and large, frequent intravenous doses of the drug *N*-acetylcysteine, NAC, (8). While the majority of the overdose-related liver failure causes are intentional (suicide attempt), nearly half are associated with unintentional overdoses by not reading or following dosing instructions and taking multiple APAP-containing medicines at the same time, taking too much at one

time, and/or redosing too soon (10). A number of mechanisms have been linked to the development of liver injury, including glutathione depletion, oxidative stress, formation of reactive oxygen species (ROS), formation of reactive nitrogen species (RNS), mitochondrial dysfunction and disruption of energy metabolism (7). For this reason the metabolism of APAP and its resulting metabolites are being studied.

APAP, like many drugs, is metabolized in the liver (5). Figure 1 shows the metabolism of APAP with both known and hypothesized pathways. Most APAP undergoes Phase II metabolism in the liver and is converted into harmless metabolites through sulfation or glucuronidation creating APAP-sulfate or APAP-glucuronide respectively, which are excreted by the kidneys into the urine (7, 11). A small percentage is converted in the liver by the cytochrome P450 enzymes, primarily CYP2E1, to the reactive and toxic APAP intermediate *N*-acetyl-p-benzoquinone imine (NAPQI) (5; 7,10). NAPQI is electrophilic and can be detoxified through conjugation with glutathione and excreted in the glutathione and *N*-acetyl-cysteine forms (8,9). The portion of NAPQI that is not detoxified by conjugation to glutathione and excreted can covalently bind to proteins and DNA (7, 8). Overdoses of APAP result in the generation of APAP-protein adducts, which are produced when NAPQI binds to cysteine groups on proteins as 3-(cystein-*S*-yl)-APAP adducts (7, 8). The APAP protein adducts have been observed in biofluids (8).

This project seeks to confirm the presence of a newly discovered metabolic pathway involving ascorbic acid (2,6). In previous research from Dr. Stevens' lab, vitamin C has been shown to detoxify other compounds, such as acrolein, via ascorbylation (6). It is hypothesized that there is a neutralized compound created by the ascorbylation of NAPQI through the "Kesinger pathway" (2, 6). An in vitro experiment was conducted by other members in the Stevens lab that appeared to demonstrate the formation of the ascorbylated APAP metabolite. The finding may provide evidence to support a revised hospital treatment protocol to include intravenous vitamin C administration as a protective treatment for ALF and/or lead to reformulation strategies to prevent ALF. Intravenous vitamin C may be more economical than NAC. If intravenous vitamin C is more effective or as effective in treating AIALF as NAC, then the discovery may contribute to lowering healthcare costs for hospitals and patients.

To evaluate whether vitamin C plays a role in detoxifying acetaminophen's toxic metabolite, NAPQI, we gathered urine from 10 male individuals after ingestion of acetaminophen and used LC-HRMS/MS for untargeted metabonomic analysis to try to identify hypothetical ascorbylated metabolites along with known APAP metabolites. Metabolomics is an emerging technology that comprehensively analyzes metabolites in a biological specimen to inform the precision of medicine and to discover specific biomarkers for drug pharmacodynamics (12, 13). Metabolomics has been used to diagnose complex metabolic diseases and is capable of precise analysis of hundreds to thousands of metabolites (1, 12). With the coverage of many metabolites, this technique provides characterization of metabolites that may cause underlying diseases like ALF, discovery of biomarkers that could be used to help diagnose a disease, and the discovery of new therapeutic target organs (12, 14). In this study, we tested the effectiveness of untargeted liquid chromatography high resolution mass spectrometry using previously identified APAP metabolites (1,8,9,10,11**).** Before any research was conducted in the lab a set of hypothesized metabolites were identified and their exact masses were found. To filter through the thousands of compounds in the urine sample,

a mass defect filter (MD) was used to help screen and identify the reactive metabolites by taking the difference between the mass of the isotope and its mass number (15).

Materials and Methods:

Study Demographics: Urine samples were gathered from 10 male subjects who were recruited via Craigslist, OSU Today advertisements, and flyer advertisements placed on OSU campus and the surrounding Corvallis area. The subjects were between 18-50 years of age and who were willing to 1) ingest 1000mg of acetaminophen (Two 500 mg Extra Strength Tylenol Tablets), 2) not consume alcohol 24 hours prior to the study day through the end of the study, and 3) not use acetaminophen-containing products for 24 hours prior to the study through the end of the study. The 500 mg Extra Strength Tylenol was manufactured in Fort Washington, PA and purchased at Bi-Mart. The study was limited to male subjects, and exclusion criteria for subjects included history of smoking, prior history of acetaminophen poisoning or hypersensitivity reactions, having significant acute or chronic illnesses such as kidney or liver disease, and regular consumption or more than two alcoholic drinks per day. The reasons for the exclusion criteria were as follows: when the study was proposed acetaminophen was a FDA Pregnancy Category C medication; excessive alcohol consumption can increase risk of liver disease, older individuals may have reduced liver function, which may reduce their capacity or efficiency of detoxifying enzymes. The study activities took place in the Clinical Research Center (CRC room 407) at the Linus Pauling Science Center (LSPC), Oregon State University, Corvallis, OR. The subjects participated in telephone screenings, where they provided verbal consent to restrict the use of alcohol and acetaminophen containing products for 24-hours prior to their appointment. All participants provided informed, written consent. The Institutional Review Board (IRB) at OSU approved all study protocols (OSU IRB #7579).

Consent and Study Visit 1: The study Nurse, Sandra Uesugi, reviewed the informed consent document, answered questions, and provided additional information if asked. After written consent was obtained the nurse collected a brief health history, heart rate, blood pressure, weight and height, and a 10 mL venous blood sample. Subjects were instructed to empty their bladder into a container before acetaminophen ingestion (T0). The weight of the urine was collected. The acetaminophen dose (1000 mg) was ingested with 8 ounces of water. Subjects were advised of symptoms of acetaminophen toxicity and hypersensitivity, and liver damage. The subjects were given a large plastic container to collect all urine for 8-10 hours following acetaminophen ingestion, along with a discrete tote bag, and were instructed to maintain their usual eating and activity habits throughout the day while following the drug restrictions.

Study Visit 2: Subjects returned to the CRC 8-10 hours after acetaminophen ingestion (T8) and were instructed to empty their bladder into the large plastic container for the last time. The container was weighed again to determine the amount of urine received. Another 10 mL venous blood sample was collected. Subjects were questioned about any adverse effects such as toxicity or hypersensitivity (flu-like symptoms followed by widespread skin pain, rash or redness, blistering, itching, hives). Subjects received $25 for their complete participation in the study. Drug restrictions ended at the completion of this visit. For storage the urine from each subject was inverted in the large container four times and was transferred into two 1 L bottles filled ¾ full to ensure space for freezing.

Bottles were labelled with appropriate subject code (AVC###), date, and T8 and placed into -80ºC freezer on the day of the visit.

Urine Preparation: The urine samples only left the -80ºC freezer when thawing for extraction and during the extraction process. Samples were thawed in a walk-in refrigerator and extracted in a fume hood. A 100 mL aliquot of urine was transferred into 250 mL beaker. Eight scoops of sodium chloride were added to the urine with a spatula and the mixture was stirred with a magnetic stir bar on a magnetic stirrer. The salt-saturated urine was then poured into a 250 mL separatory funnel along with 100 mL of ethyl acetate. The separatory funnel was capped and hand shaken for 2 minutes to ensure mixing and separation of the aqueous and organic phase. After each shake, the stopcock was turned to relieve the vapor pressure in the separatory funnel. After being shaken the separatory funnel, with the stopcock closed, was placed in a stand and the cap removed. The cap was removed to allow for the organic (clear) and aqueous phase (urine) to separate. Once the layers were separated, the stopcock was opened allowing for the urine to flow out of the separatory funnel back into the 250 mL beaker until the top clear organic layer was left in the separatory funnel. Once the clear layer was isolated, it was poured from the top of the separatory funnel into a 500 mL flask. The urine collected in the 250 mL beaker underwent two more extractions with ethyl acetate. Urine samples collected before (T0) and after (T8) acetaminophen ingestion were extracted three times with a total volume of 300 mL of ethyl acetate. After the final extraction, the urine's pH was recorded for each sample. The organic layer was then poured into a 500 mL round bottom flask over a funnel with paper filter that contained sodium sulfate anhydrous. The 500 mL round bottom flask was then connected to a rotary evaporator. Once the organic layer was dry, it was removed from the evaporator and the sides of the flask were rinsed with < 2 mL of ethanol and placed into a test tube with a Pasteur pipette. A different Pasteur pipette was used for each sample transfer. Each test tube was labeled according to the subject and time of collection (T8 or T0) and placed into the refrigerator until all samples had been extracted. A preliminary urine sample was used to determine if the extraction method was suitable for finding APAP metabolites. Once it was deemed viable, the 20 urine samples were extracted using the same method. For the analysis of the 20 urine samples after the extraction, the volume of a 10% aliquot was measured and recorded (Table 2). The weight was recorded for the 2 mL test tube before and after the 10% aliquot was placed into the tube to determine the amount of sample that would be analyzed. The before weight was subtracted from the after weight to determine the weight of the sample before it was resuspended with the 50:50 water:acetonitrile mixture.

Liquid Chromatography High Resolution Mass Spectrometry: Preparation of samples took place in the OSU Mass Spectrometry Center in the Agricultural & Life Sciences building. For preparation of the samples for the HPLC System the volume of each sample was measured using a 1 mL pipette and was recorded. After each sample, the tip of the pipette was discarded. Twenty test tubes were labeled 1-20 and their weights were recorded. 10% aliquot of each sample was calculated and transferred into one of the labeled test tubes. Test tubes 1-10 corresponded to T8 samples and 11-20 test tubes corresponded to T0 samples. After the samples were transferred into the corresponding test tube they were placed into a Speed-Vac with the lid open. Once test tubes were dry, their weights were recorded and each sample was dissolved in 200 µL of methanol 50%

(v/v) aqueous solution. Samples were vortexed and placed in centrifuged for 10 minutes at 4ºC at 15,000 ppm. After centrifugation, the liquid was transferred into HPLC vials. A “QC" was made by pooling 15 µL from each sample. Methanol was placed into a HPLC tube and used as a blank for calibration. A Shimadzu Prominence HPLC System (Shimadzu, Columbia, MD, USA) containing two LC-20AD pumps, a DQU-20A degasser, and an SIL-HTC autosampler was used to analyze the urine samples. The samples were placed in the autosampler. The autosampler and column oven temperatures were set at 10 and 40 °C, respectively. Chromatographic separation of metabolites was achieved on an Inertsil Phenyl-3 column (4.6 mm × 150 mm, 100 Å, 5 µm; GL Sciences, Torrance, CA, USA) eluted with solvent A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) using the settings described in detail by Kirkwood, 2012 (16). Injection volume was 5 µL and the mobile phase flow was 400 µL/min (17, 18,). Sample injections were randomized but the QC was injected after every five samples. The first and final samples injected were methanol.

Metabolite Identification: For qualitative (untargeted) analysis, LC-MS/MS data were imported into PeakView® software (AB Sciex Triple TOF 5600 system) to identify metabolic features using an established in-house MS database. In order to discover possible metabolites, MS acquisition was performed in both positive (ESI+) and negative (ESI-) ionization mode. The database was created by analyzing purchased authentic metabolite standards (IROA Technologies, Boston, MA, USA) using identical analytical protocols and instrumentation used to analyze urine samples in the present study (17, 18). Progenesis QI software (NonLinear Dynamics, Newcastle upon Tyne, UK) was used to process both ionization modes (ESI+ TOF and ESI- TOF) raw data for tentative metabolite identifications. Progenesis QI Software is a bioinformatics tool used for the discovery and analysis of small molecules. Metabolites were tentatively assigned by extensive querying and comparison of molecular features, namely accurate mass (m/z; error < 10 ppm), MS/MS fragmentation pattern (score > 50), and isotope similarity (Score > 80), against Metlin™, IROA, and HMDB databases to reduce unintentional biases (17,18). In Progenesis QI software a confidence score greater than 50 was used for tentative assignment of metabolites (17, 18).

Statistical Analysis: Raw HPLC-MS/MS data was imported into MetaboAnalyst 3.0 to perform normalization (e.g., feature detection, peak alignment, peak integration) using software-designed algorithms (18). Principal Component Analysis (PCA) was performed in MetaboAnalyst using log transformation (19). PCA determined degree of separation based on features detected in both positive and negative ion modes. To identify significantly altered features, paired *t* tests were performed in MetaboAnalyst comparing peak areas at each time point with no normalization performed. To adjust for multiple comparisons given the large number of features, compounds with a p-value below 0.05 were considered. Data for both ESI+ and ESI- TOF were exported as both csv and excel files. The csv files were altered so the data would be in a column format for MetaboAnalyst Statistical and Biomarker Analysis. Statistical Analysis through Heat Map, Fold Change (T8/T0), partial least squares - discriminant analysis (PLS-DA), Volcano Plot, and Biomarker Analysis through Classical univariant ROC curve analysis were collected for both ESI+ and ESI- TOF.

Results:

The major compounds relating to APAP found in the urine collected for eight hours after APAP ingestion (T8) include APAP, APAP-sulfate, APAP- glucuronide, APAP-mercapturate, and 2-hydroxyacetaminophen sulfate. This pilot study collected clinical data from the 10 male subjects who ingested 1000 mg (two 500 mg Extra Strength Tylenol) and is reported in Table 1. To keep patient information confidential, only the study nurse had access to the locked cabinet where the files were located.

An APAP metabolite list with exact mass and structures was compiled to be referred to when using PeakView to sort through the raw data from ESI+ and ESI- TOF. The compound name, its chemical formula, and its exact mass can be found in Table 3. ChemDraw Software was used to create the chemical structure and to calculate the exact mass of the hypothetical ascorbylated compounds.

We were fortunate to obtain two urine samples (T0 and T8) from a male volunteer outside of the study. The two urine samples are referred to as “preliminary urine”. In PeakView, the most prominent peaks found in the raw data from a male subject T0 and T8 urine samples were APAP, APAP sulfate, and APAP mercapturate. The peaks are shown with the accompanying compounds chemical structure in Figure 3.

Using the mass defect filter of 0.01 to 0.1 from APAP metabolites led to the identification of APAP mercapturate, APAP sulfate, and 3-methoxyacetaminophen. The mass defect range (difference between the mass of the isotope and its exact mass) of 0.01 to 0.1 was determined through finding the smaller exact mass and the highest exact mass metabolite from Table 3. Each compound can be found in Table 4 with accompanying retention times. The *m/z* or exact mass is listed for each compound to further aid in the identification.

We focused on the identification of compounds changing after treatment using their p-values. The annotations were prioritized for compounds which p-value was less than 0.05. The compounds identified were APAP, APAP sulfate, 2-hydroxyacetaminophen sulfate, and APAP glucuronide and can be found in Table 5 with their accompanying retention times, exact mass, and mode (ESI+ or ESI-).

Urine is a complex matrix and contains thousands of compounds. Under this approach, we were able to annotate 343 compounds in total. Figure 3 and Figure 5 show the top 20 compounds from ESI+ TOF and ESI- TOF respectively with corresponding selected importance in Tylenol or control urine. The compounds with a higher concentration in the Tylenol urine include APAP, 2-hydroxyacetaminophen, and APAP sulfate. Fifteen compounds in the figure are shown to have higher concentrations in the Tylenol urine; whereas, only five compounds are shown to have higher concentrations in the control (CTRL) urine in ESI+. The top 20 compounds for ESI- were found using Variable Importance in Projection (VIP) score which estimates the importance of metabolites in our study in MetaboAnalyst Software. For ESI- TOF there are eight compounds with higher concentrations in the Tylenol urine and 12 compounds more abundant in the control group.

ROC curves were utilized for further visualization of the identifications. Box and Whisker plots accompany the ROC curves showing each subject’s relative abundance for the compound. Each black dot on the box and whisker plot is a patient and the red dot

is the mean abundance value for the data. The ROC curves for APAP, APAP sulfate, 2-hydroxyacetaminophen, and APAP glucuronide can be seen in Figure 4 and Figure 6.

Table 1:
Summary clinical data for 10 male adults who ingested 1000mg of APAP

Subject	Ht (cm)	Wt (kg)	BMI	Age	T0 urine (g)	T8 urine (g)
AVC001	177	69.2	22.1	26	28.49	1052.33
AVC002	176	76.8	24.88	27	88.39	2180.89
AVC003	191	80.5	22.1	24	387.35	2061.61
AVC004	187	82.3	23.5	33	412.94	1543.39
AVC005	183	121.6	36.3	42	48.81	1313.24
AVC006	179	71.7	22.4	30	172.9	1943.73
AVC007	174	83.8	27.7	35	96.32	1117.97
AVC008	189.5	91.5	25.5	41	101.92	695.85
AVC009	184	102	30.2	32	440.95	759.59
AVC010	183	76	22.7	24	75.3	1407.24
AVERAGE	182.35	85.54	25.738	31.4		
SD	5.783357	15.88725	4.569925	6.501281		

Subject data: Summary clinical data for 10 male subjects labeled “AVC001-AVC010” are presented in Table 1 including their height, weight, BMI, age, and weight for T0 and T8 urine. APAP metabolites were found in all the study samples that had ingested the APAP tablet (T8 Urine). Each metabolite peak obtained from the HPLC was used for each patient to determine differences in concentration levels.

Table 2:
Summary of a 10% aliquot of total 20 samples obtained from 10 subjects

Tube	Name	Before 10% Weight (g)	After 10% Weight (g)	Sample (g)
1	KMS001 T8	1.0566	1.0588	0.0022
2	KMS002 T8	1.0599	1.0581	-0.0018
3	KMS003 T8	1.056	1.0574	0.0014
4	KMS 004 T8	1.0588	1.0611	0.0023
5	KMS 005 T8	1.0574	1.0609	0.0035
6	KMS 006 T8	1.057	1.0592	0.0022
7	KMS 007 T8	1.0222	1.0258	0.0036
8	KMS 008 T8	1.0594	1.0656	0.0062
9	KMS 009 T8	1.066	1.0732	0.0072
10	KMS 010 T8	1.0472	1.0514	0.0042
11	KMS 001 T0	1.0497	1.0541	0.0044
12	KMS 002 T0	1.0444	1.0515	0.0071
13	KMS 003 T0	1.0475	1.0516	0.0041
14	KMS 004 T0	1.0493	1.0589	0.0096
15	KMS 005 T0	1.0498	1.0524	0.0026
16	KMS 006 T0	1.0537	1.0578	0.0041
17	KMS 007 T0	1.0505	1.0562	0.0057
18	KMS 008 T0	1.0527	1.06	0.0073

19	KMS 009 T0	1.0498	1.0634	0.0136
20	KMS 010 T0	1.0448	1.0516	0.0068

Urine sample data: 10% aliquot for 10 male subjects labeled AVC001-AVC010 are presented in Table 2. T8 represents the urine collected for eight hours after the ingestion of the APAP. The table describes test tube weight before 10% aliquot was inserted in grams and after the 10% aliquot was inserted and dried. The weights were recorded with the test tube lids closed.

Figure 1:
Metabolism of APAP in the liver with hypothesized ascorbate conjugation

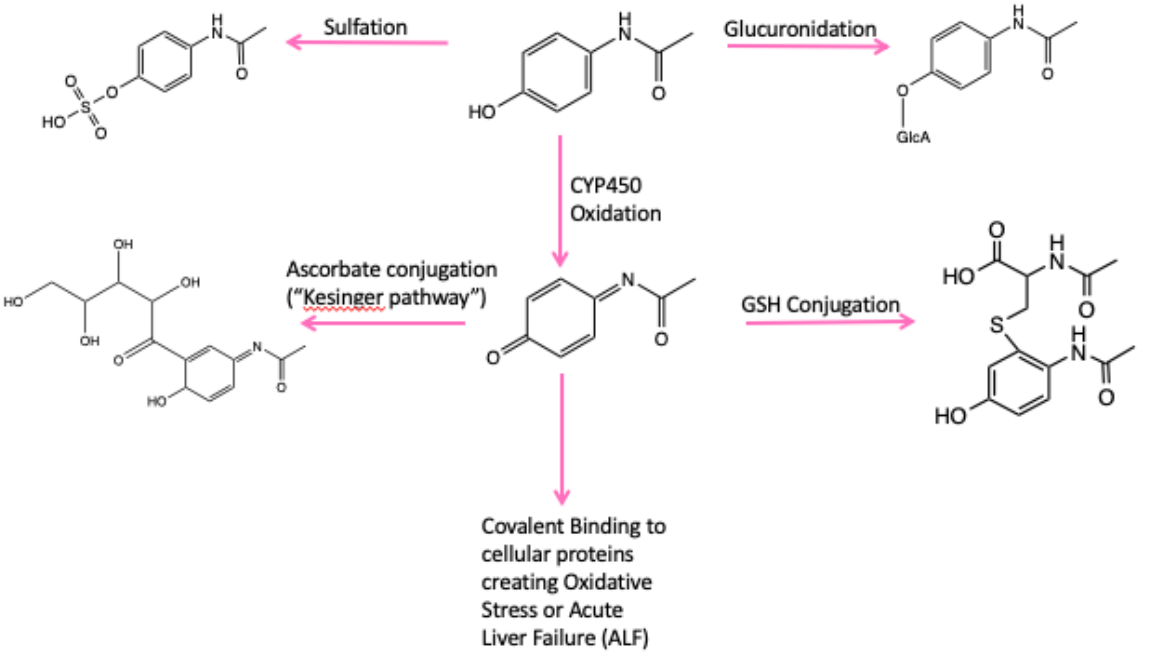


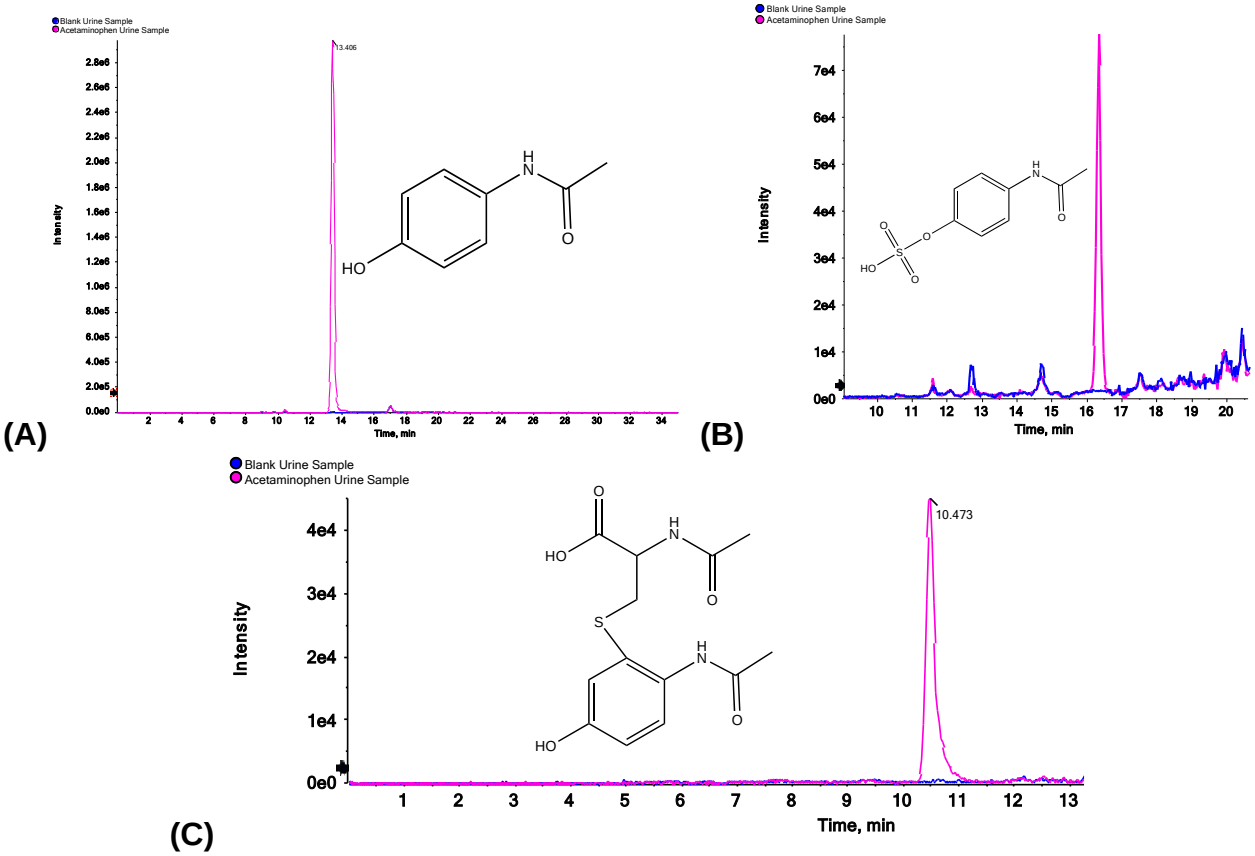
Table 3:
APAP and its potential metabolites. Chemical formulas were used for compound identification (exact mass).

Name	Chemical Formula	Exact Mass	H+ (ESI+)	H- (ESI-)
APAP	$C_8H_9NO_2$	151.0630	152.0710	150.0550
NAPQI	$C_8H_7NO_2$	149.0480	150.0560	148.0400
APAP glucuronide	$C_{14}H_{17}NO_8$	327.0950	328.1030	326.0870
APAP sulfate	$C_8H_9NO_5S$	231.0200	232.0280	230.0120
APAP glutathione	$C_{18}H_{24}N_4O_8S$	456.1310	457.1390	455.1230
APAP cysteine	$C_{11}H_{14}N_2O_3S$	254.0730	255.0810	253.0650
Compound 1*	$C_{14}H_{15}NO_8$	325.0798	326.0878	324.0718
Compound 2*	$C_{14}H_{17}NO_9$	343.0903	344.0983	342.0823
Compound 3*	$C_{13}H_{17}NO_7$	299.1005	300.1085	298.0925
APAP mercapturate	$C_{13}H_{16}N_2O_5S$	312.0780	313.0858	311.0702
Compound 4*	$C_{13}H_{19}NO_7$	301.1162	302.1240	300.1084

Compound 5*	$C_{10}H_{11}NO_4$	209.0688	210.0766	208.0610
Compound 6*	$C_{10}H_9NO_3$	191.0582	192.0660	190.0504
Compound 7*	$C_{16}H_{17}NO_9$	267.0903	268.0981	266.0825
Compound 8*	$C_{10}H_9NO_6S$	271.0151	272.0229	270.0073
Compound 9*	$C_{16}H_{17}NO_9$	367.0903	368.0981	366.0825

Each compound exact mass was calculated through the ChemDraw software. To find the compounds in ESI+ and ESI-, a proton with the exact mass of 1.0078 was added to the exact mass or subtracted from the compounds exact mass respectfully.
*Hypothetical compounds between APAP and ascorbic acid.

Figure 2:
PeakView extracted ion chromatograms (XIC) of (A) acetaminophen, (B) acetaminophen sulfate, and (C) acetaminophen mercapturate from preliminary urine



Preliminary urine sample: A male subject outside the study followed the same protocols as the study subjects for urine collection before and after APAP ingestion. The chromatograms shown are APAP metabolites that were found in the raw data using PeakView. Finding the metabolites in the raw data led to the tentative analysis of acetaminophen, acetanilide, acetaminophen-sulfate, acetaminophen mercapturate, and 3-methoxyacetaminophen in the urine after APAP administration with Progenesis Software.

Table 4:

Compound identifications using mass defect filter of 0.01 to 0.1 from male subject outside the study. This table was elaborated by using Progenesis QI Software.

Name	Retention time (min)	Adducts	m/z	Formula	Mass Error (ppm)	Mode
p-Acetamidophenol (Acetaminophen, Tylenol)	13.35	M+H-H_2O, M+H	152.0706	$C_8H_9NO_2$	-3.6	ESI+
p-Acetamidophenol (Acetaminophen, Tylenol)	13.41	M-H	150.0568	$C_8H_9NO_2$	5.2	ESI-
APAP mercapturate	16.34	M+H-H_2O, M+H, M+Na	313.0859	$C_{13}H_{16}N_2O_5S$	1.9	ESI+
APAP sulfate	10.46	M+H, M+Na	232.0280	$C_8H_9NO_5S$	2.5	ESI+
3-Methoxyacetaminophen	7.82	M+H	182.0803	$C_9H_{11}NO_3$	-4.8	ESI+
Acetanilide	20.36	M-H	134.0617	C_8H_9NO	4.2	ESI-

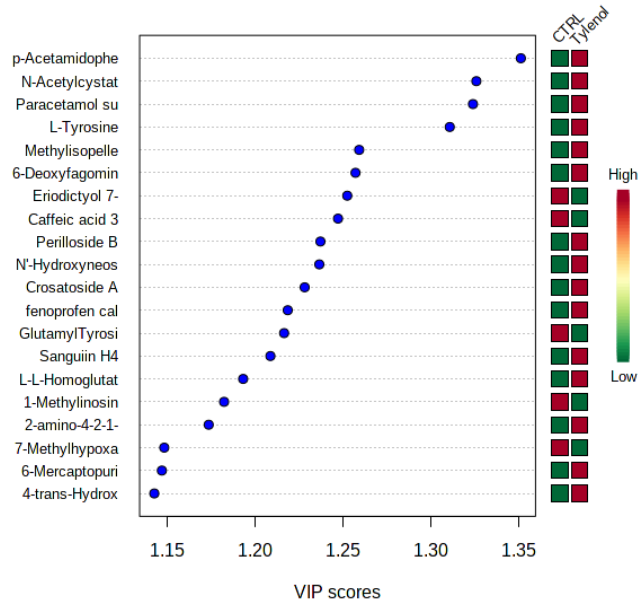
The mass defect filter of 0.01 through 0.1 identified five compounds relating to APAP in the urine. For the ESI+ TOF, there was a total of 172 identified compounds. There were 61 total compounds identified for ESI- TOF. m/z is referring to the compounds exact mass.

Table 5:
Compound identification from the 10 subjects' urine samples. This table was elaborated by using Progenesis QI Software.

Name	Retention time (min)	Adducts	*m/z*	Formula	Mass Error
p-Acetamidophenol (Acetaminophen, Tylenol)	13.19	M+H-H_2O, M+H, M+Na	152.0700	$C_8H_9NO_2$	-3.73
p-Acetamidophenol (Acetaminophen, Tylenol)	13.18	M-H	150.0556	$C_8H_9NO_2$	-2.26
Paracetamol sulfate	10.24	M+H, M+Na	232.0273	$C_8H_9NO_5S$	-0.63
Paracetamol sulfate	10.24	M-H, M+Na-2H	230.0130	$C_8H_9NO_5S$	0.76
2-hydroxyacetaminophen sulfate	9.32	M+H, M+Na	248.0223	$C_8H_9NO_6S$	-0.08
2-hydroxyacetaminophen sulfate	9.29	M-H, M+Na-2H	246.0078	$C_8H_9NO_6S$	0.15
APAP-glucuronide	11.27	M-H, M+Na-2H	326.0883	$C_{14}H_{17}NO_8$	0.49

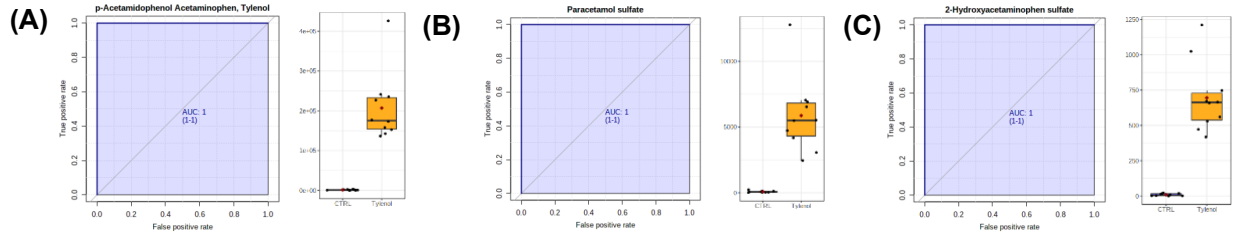
Finding the metabolites in the raw data led to the tentative annotation of acetaminophen, acetaminophen-sulfate, 2-hydroxyacetaminophen sulfate, and APAP glucuronide in the urine collected after APAP administration with Progenesis Software. Annotations were included if the p-value was below 0.05. For ESI+, 94 compounds were tentatively assigned. There were a total of 171 compounds that were identified for ESI-, with a p-value below 0.05.

Figure 3:
Top 20 compounds from ESI+ TOF urine with corresponding relative abundance in the Variable Importance in Projection (VIP) analysis.



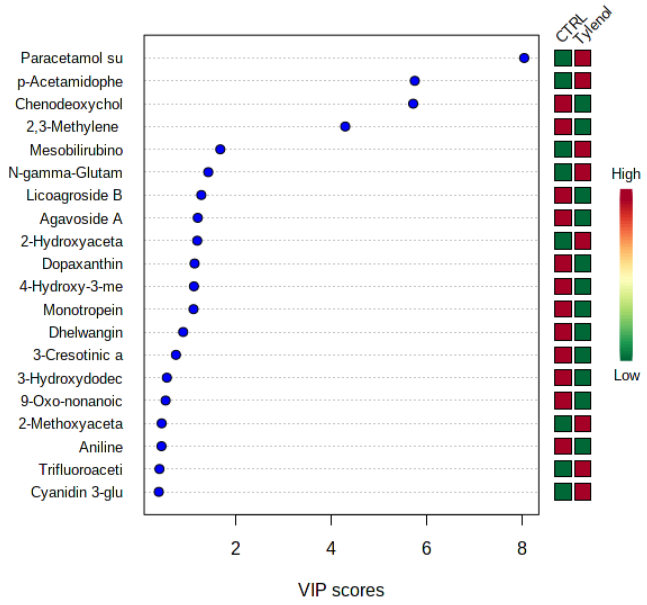
MetaboAnalyst's partial least squares - discriminant analysis (PLS-DA) shows the VIP score (x-axis) which estimates the importance of metabolites in our study. Two compounds out of the top twenty are related to APAP. Which includes, APAP, and paracetamol sulfate. The figure also shows the right side of the y-axis the relative concentrations of that specific compound and whether it is high in the Control (T0) urine or the Tylenol urine (T8). For example, p-Acetamidophenol (Acetaminophen, Tylenol) occurred at high abundance in the Tylenol urine and low abundance in the control urine.

Figure 4
Area Under the Curve ROC curve analysis for individual biomarkers "true positive" identification of APAP structures with accompanying box-and-whisker plot showing concentration.



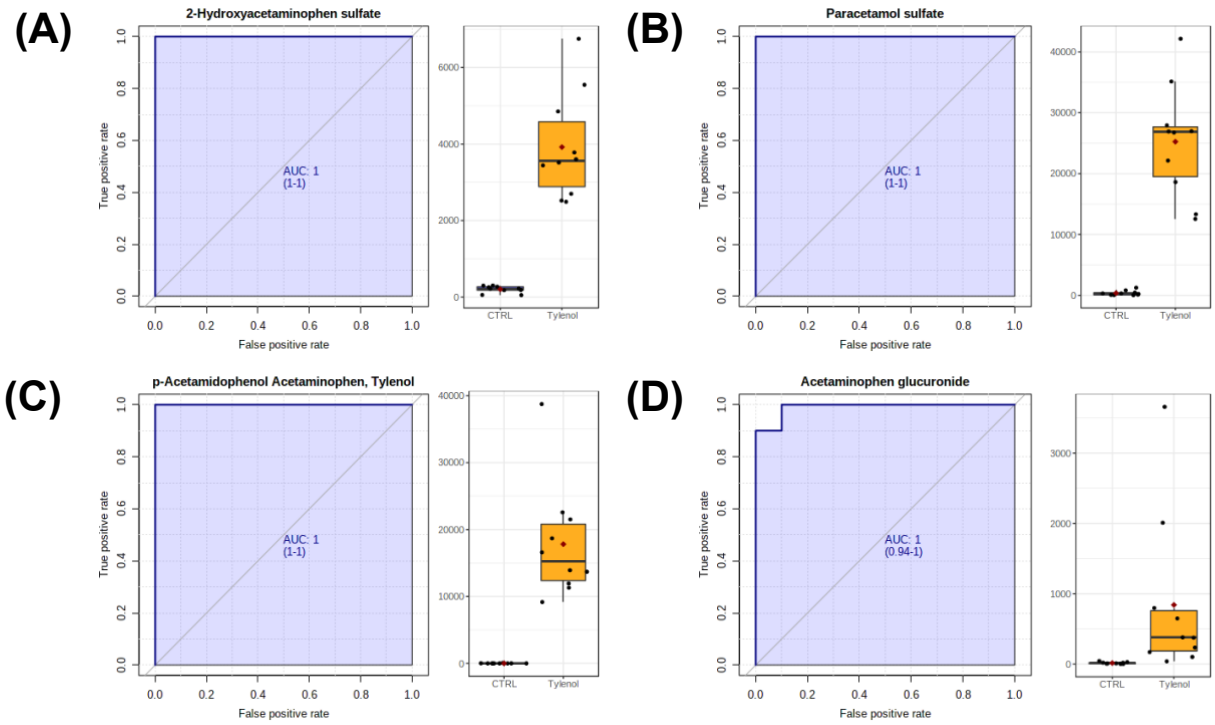
A-C are shown from highest concentration to lowest concentration in the urine samples. The APAP compounds were found in high concentrations in the T8 urine. The box and whisker plots accompany the ROC curve show the compound abundance concentrations for ESI+ TOF compounds. APAP had the highest concentration in the T8 urine. APAP sulfate also known as paracetamol sulfate was found to be the second highest compound followed by 2-hydroxyacetaminophen sulfate.

Figure 5:
Top 20 compounds from ESI- TOF urine with corresponding relative abundance in the Variable Importance in Projection (VIP) analysis.



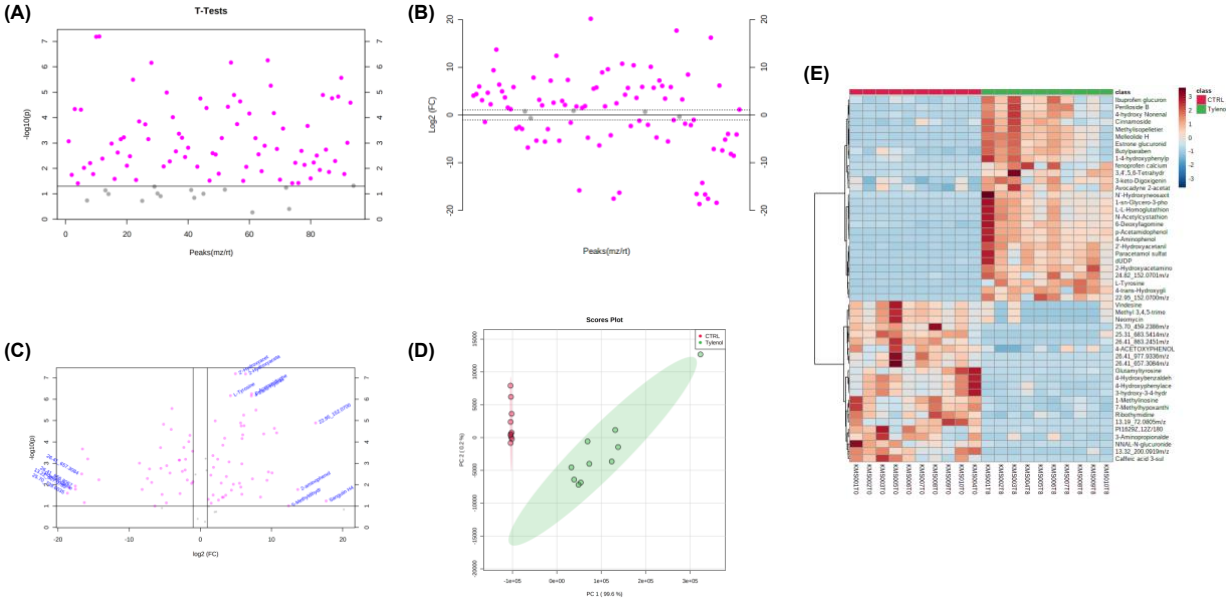
MetaboAnalyst's partial least squares - discriminant analysis (PLS-DA) shows the VIP score (x-axis) which estimates the importance of metabolites in our study. Three compounds out of the top twenty are related to APAP. Which includes, APAP, 2-hydroxyacetaminophen sulfate and paracetamol sulfate. The figure also shows the right side of the y-axis the relative abundance of that specific compound and whether it is high in the Control (T0) urine or the Tylenol urine (T8). For example, paracetamol sulfate occurred at high abundance in the Tylenol urine and low abundance in the control urine.

Figure 6:
Area Under the Curve (ROC curve) analysis for individual biomarkers "true positive" identification of APAP structures with accompanying box-and-whisker plot showing abundance



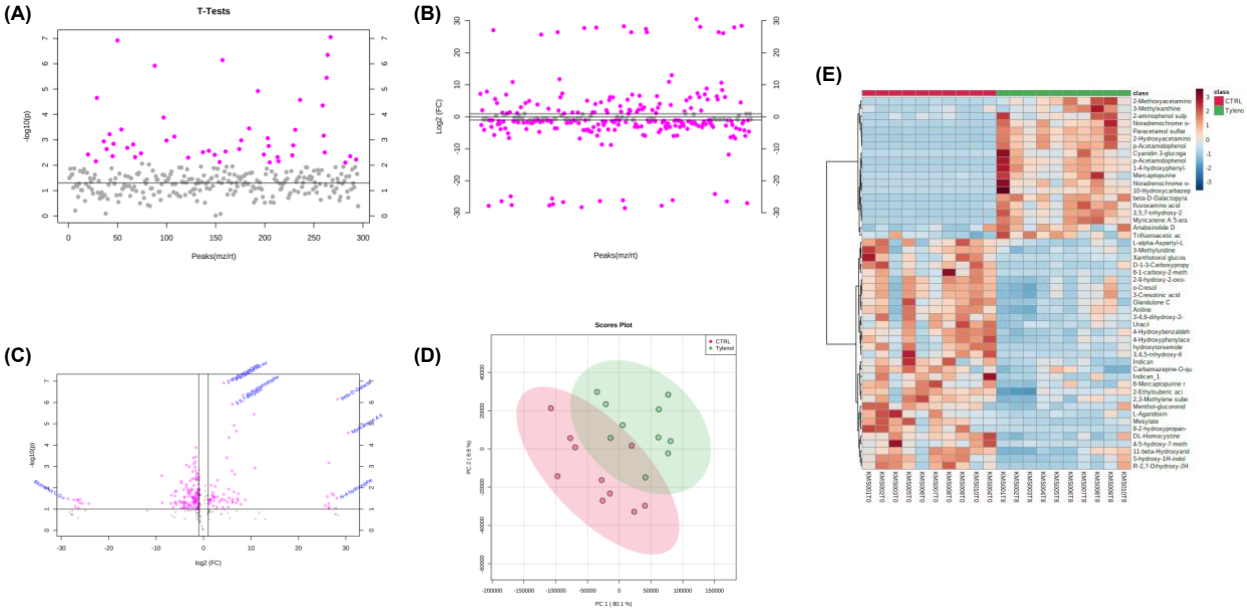
A-D are shown from highest concentration to lowest concentration in the urine samples. The APAP compounds were found in high concentrations in the T8 urine. The box and whisker plots accompany the ROC curve show the compounds abundance for ESI- TOF compounds. 2-hydroxyacetaminophen had the highest concentration in the T8 urine, followed by paracetamol sulfate, APAP, and APAP glucuronide.

Figure 7:
ESI+ TOF tentative compound MetaboAnalyst statistical analysis (t-test, fold change Tylenol/control, volcano, PCA plot, and heat map)



Analysis of similarities and differences of compounds in control and Tylenol urine samples with p values below 0.05 (ESI+). (A) t-test, each dot represents a compound found in the urine. (B) fold change Tylenol/control, the dots above the x axis correspond to compounds more abundant in the Tylenol urine. Those below the x axis correspond to compounds more abundant in the control urine based on 172 potentially identified compounds (C) volcano plot, compares the fold change of the compounds in the Tylenol urine and control urine to the statistical significance level. (D) PCA plot, compares the Tylenol urine to the control urine. The green dots correspond to compounds that are found in all 10 Tylenol samples. The red dots correspond to compounds found in all 10 control samples. (E) heatmap visualizing area under the curve for chromatographic peaks of compounds found in the urine samples.

Figure 8:



Analysis of similarities and differences of compounds in control and Tylenol urine samples (ESI-). (A) t-test, each dot represents a compound found in the urine. (B) fold change Tylenol/control, the dots above the x axis (peaks mz/rt) correspond to compounds more abundant in the Tylenol urine. Those below the x axis correspond to compounds more abundant in the control urine based on 171 identified compounds (C) volcano plot, compares the fold change of the compounds in the Tylenol urine and control urine to the statistical significance level. (D) PCA plot, compares the Tylenol urine to the control urine. The green dots correspond to compounds that are found in all 10 Tylenol samples. The red dots correspond to compounds found in all 10 control samples. (E) heatmap visualizing area under the curve for chromatographic peaks of compounds found in the urine samples.

Discussion:

It has been shown that vitamin C (ascorbic acid) has the ability to ascorbylate 33 natural products but its ability to ascorbylate APAP has not been shown (2, 6, 21, 22, 23, 24, 25, 26). Ascorbic acid has also been shown to detoxify other compounds such as acrolein, via ascorbylation (6). We hypothesize a secondary pathway involving a similar

reaction between vitamin C (ascorbate) and NAPQI. We expected to determine the protective effects of ascorbic acid against damage from NAPQI through the ascorbic acid conjugate of the NAPQI being present in subjects' urine after the ingestion of 1000mg of APAP. The finding of the new pathway and metabolite may provide evidence to support a revised hospital treatment protocol to include intravenous vitamin C administration as a protective treatment for AIALF and/or lead to reformulation strategies to prevent AIALF. This discovery may contribute to lowering healthcare costs for hospitals and patients as intravenous vitamin C may be more economical than NAC.

The preliminary study urine samples confirmed the liquid-liquid extraction method for the urine was sufficient for the identification of APAP and its metabolites (Figure 1, Table 4). By first gathering metabolite exact masses shown in Figure 2, the PeakView® software enabled us to sort through the raw data using the extracted ion chromatogram XIC. Using both ion modes (ESI+ and ESI-) helped characterize the compound we were looking to identify. If the selected compound had a different retention time than its retention time of the corresponding standard in the library then it was likely not the correct peak for the compound. Once the compounds were found in the raw data, Progenesis QI software was able to tentatively assign identifications to the compounds in the urine. The 20 urine samples from the 10 human subjects underwent the same methods as the preliminary study urine along with biomarker analysis that confirmed the presence of APAP, APAP-sulfate, 2-hydroxyacetaminophen sulfate, and APAP-glucuronide in the T8 Urine shown in Figure 2, Table 4 and Table 5. Comparing the exact masses found in Figure 2 to those found in Tables 4 and 5 only in the T8 urine samples further confirms APAP metabolites. Other studies have reported the analysis of APAP in plasma or urine, either alone or together with other compounds (11, 27, 28 29, 30, 31, 32). Using different solvents for high-performance liquid chromatography such as formic acid and ammonium acetate or glacial acetic acid yields the sulfate, glucuronide, cysteine, and mercapturic acid conjugates of acetaminophen (11, 27, 28, 29, 30). These reports are consistent with our findings of the major APAP metabolites, APAP sulfate, and APAP glucuronide.

If the compound is present in both T0 and T8 urine, it is most likely not related to APAP as the subjects were not to consume APAP-containing products before their study date. If the compound was only present in the T8 urine and had the exact masses listed for the metabolites it is likely it is related to APAP. The biomarker analysis ROC Curve from Figure 4 and Figure 6 aided in further validating the identifications found in both PeakView® software and Progenesis QI software because a biomarker is a biological indicator showing the absence, presence or condition of the disease or patient (20). ROC Curves were found for each compound and selecting those with the highest Area Under the Curve (AUC) of 0.8 or 80% corrects "false positive" compounds and above to 1.0 (34). True positive rate (also called sensitivity) measures the proportion of actual positives which are correctly identified for the test (20, 33, 34). False positive rate (specificity) measures the proportion of negatives which were correctly identified as such for the test (20, 33 34). The AUC shown in Figure 4 and Figure 6 is a measure of how well a parameter can distinguish between two diagnostic groups (T8 and T0), where a value of 1.0 shows perfect performance for the compound. Each point on the ROC Curve represents a true positive/false positive pair corresponding to a particular decision threshold. The accompanying box and whisker plot with each compound shows each subjects relative intensity of each ion.

APAP-mercapturate was found in the raw data in PeakView® software using the exact mass (m/z) of 312.08 in both negative and positive mode at 311.07 m/z and 313.08 m/z respectively shown in Figure 2 and Table 4. Although it was found in PeakView® it was not identified in Progenesis or MetaboAnalyst. Another study has shown APAP-mercapturate was confirmed by mass spectrometry by a molecular ion at 312 (33). The hypothesized ascorbic acid conjugate of NAPQI was not found in the raw data in PeakView® software using the exact masses of 325.07 m/z and 299.10 m/z based off the hypothetical chemical formulas shown in Table 3

Statistical analysis comparing the Tylenol urine to the control urine identifies which compounds are more abundant in one sample or the other. In Figure 7 and Figure 8 the heat map (D) shows red and blue color differentiations. The colors in the heatmap indicate the z-score which was calculated for each compound by subtracting the mean for the peak areas for the compound across different samples and dividing it by the standard deviation across all compounds. The red color indicated positive z-score; whereas, a blue color indicates negative z-score. The darker the color the higher the intensity for the compound. The x axis shows in which sample the compound is present or absent in. APAP has very different abundance concentrations between subjects T8 urine. APAP, APAP sulfate, 2-hydroxyacetaminophen sulfate, and APAP glucuronide are relatively similar between ESI+ and ESI- TOF heat maps from Figures 7 and 8 which corresponds the similar retention times captured in Table 2 and Table 3 between ESI+ and ESI-.

Some limitations in the study include the small sample size of only 10 subjects, the exclusion of women, and the targeted metabolite could be in the urine sample but at low levels which would be indistinguishable from the background noise or masked by another compound thus it not be picked up by the detector in the LC-MS, and there could also be further metabolism of the ascorbylated metabolites through either beta oxidation or some other pathway we may be unaware of causing us not to find it in the samples. Although the hypothesized ascorbic acid conjugate was not detected in this study, it does not eliminate its possible existence. The liquid-liquid extraction method used on the urine provided sufficient separation for the quantification of APAP metabolites. The described LC-HRMS/MS can simultaneously quantify APAP, its major metabolites (APAP-sulfate and APAP-glucuronide) in urine samples. Follow-up studies are needed to verify either the existence or non-existence of the hypothesized vitamin C conjugate in both urine and plasma samples and to determine whether the secondary pathway through ascorbate conjugation (“Kesinger Pathway”) is possible with APAP.

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Citations:

1. Sun J, Schnackenberg LK, Holland RD, Schmitt TC, Cantor GH, Dragan YP, and Beger RD (2008) Metabonomics evaluation of urine from rats given acute and chronic doses of acetaminophen using NMR and UPLC/MS. *Journal of Chromatography B* **871**:328–340.
2. Kesinger, NG. (2009) PhD Thesis: Ascorbylation-the biological relevance of covalently bound ascorbic acid. Accessed at: https://ir.library.oregonstate.edu/xmLui/handle/1957/13008
3. Miranda CL, Reed RL, Kuiper HC, Alber S, and Stevens JF (2009) Ascorbic acid promotes detoxification and elimination of 4-hydroxy-2(E)-nonenal in human monocytic THP-1 cells. *Chem Res Toxicol* **22**:863–874.
4. Traber, M.G. and J.F. Stevens, (2011) Vitamins C and E: beneficial effects from a mechanistic perspective. *Free Radic Biol Med* 51(5): p. 1000-13.
5. The Acetaminophen Awareness Coalition (AAC)., "Know Your Dose." 2019. PDF File.
6. Kesinger NG, Langsdorf BL, Yokochi AF, Miranda CL, and Stevens JF (2010) Formation of a Vitamin C Conjugate of Acrolein and its Paraoxonase-mediated Conversion into 5,6,7,8-Tetrahydroxy-4-oxooctanal. *Chem Res Toxicol* **23:**836–844.
7. McJunkin B, Barwick KW, Little WC, and Winfield JB (1976) Fatal massive hepatic necrosis following acetaminophen overdose. *JAMA* **236**:1874–1875.
8. James LP, Letzig L, Simpson PM, Capparelli E, Roberts DW, Hinson JA, Davern TJ, and Lee WM (2009) Pharmacokinetics of acetaminophen-protein adducts in adults with acetaminophen overdose and acute liver failure. *Drug Metab Dispos* **37**:1779–1784.
9. Beger RD, Bhattacharyya S, Yang X, Gill PS, Schnackenberg LK, Sun J, and James LP (2015) Translational biomarkers of acetaminophen-induced acute liver injury. *Arch Toxicol* **89**:1497–1522.
10. Koen YM, Liu K, Shinogle H, Williams TD, and Hanzlik RP (2016) Comparative Toxicity and Metabolism of N-Acyl Homologues of Acetaminophen and Its Isomer 3′-Hydroxyacetanilide. *Chem Res Toxicol* **29**:1857–1864, American Chemical Society
11. Tan Q, Zhu R, Li H, Wang F, Yan M, and Dai L (2012) Simultaneous quantitative determination of paracetamol and its glucuronide conjugate in human plasma and urine by liquid chromatography coupled to electrospray tandem mass spectrometry: Application to a clinical pharmacokinetic study. *Journal of Chromatography B* **893–894**:162–167.
12. Clish CB (2015) Metabolomics: an emerging but powerful tool for precision medicine. *Cold Spring Harb Mol Case Stud* **1**.
13. Schnackenberg LK, Sun J, Bhattacharyya S, Gill P, James LP, and Beger RD (2017) Metabolomics Analysis of Urine Samples from Children after Acetaminophen Overdose. *Metabolites* **7**:46.
14. Nicholson JK, Lindon JC, and Holmes E (1999) "Metabonomics": understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **29**:1181–1189.

15. Zhu M, Ma L, Zhang H, and Humphreys WG (2007) Detection and Structural Characterization of Glutathione-Trapped Reactive Metabolites Using Liquid Chromatography−High-Resolution Mass Spectrometry and Mass Defect Filtering. *Analytical Chemistry* **79**:8333–8341.
16. Kirkwood, J.S., (2012) Vitamin C deficiency activates the purine nucleotide cycle in zebrafish. *J Biol Chem* **287**(6): p. 3833-41.
17. Bading-Taika B, Akinyeke T, Magana AA, Choi J, Ouanesisouk M, Torres ERS, Lione LA, Maier CS, Bobe G, Raber J, Miranda CL, and Stevens JF (2018) Phytochemical characterization of Tabernanthe iboga root bark and its effects on dysfunctional metabolism and cognitive performance in high-fat-fed C57BL/6J mice. *J Food Bioact* **3**:111–123.
18. Housley L, Magana AA, Hsu A, Beaver LM, Wong CP, Stevens JF, Choi J, Jiang Y, Bella D, Williams DE, Maier CS, Shannon J, Dashwood RH, and Ho E (2018) Untargeted metabolomic screen reveals changes in human plasma metabolite profiles following consumption of fresh broccoli sprouts. *Mol Nutr Food Res* **62**:e1700665.
19. Xia, J. Wishart, D.S. (2016) Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Curr. Protoc. Bioinform.* **55**.
20. Xia J, Broadhurst DI, Wilson M, and Wishart DS (2013) Translational biomarker discovery in clinical metabolomics: an introductory tutorial. *Metabolomics* **9**:280–299.
21. Wagner AE, Rimbach G. (2009) Ascorbigen: chemistry, occurrence, and biologic properties. *Clin. Dermatol* **27**:217–224.
22. Fathi AR, Krautheim A, Kaap S, Eger K, Steinfelder HJ. (2000) Michael adducts of ascorbic acid as inhibitors of protein phosphatase 2A and inducers of apoptosis. *Bioorg. Med. Chem. Lett* **10**:1605–1608.
23. Sussangkarn K, Fodor G, Strope D, George C. (1989) New furo[3,2-b]furans derived from L-ascorbic acid. Heterocycles **28**:467–475.
24. Veltri RW, Fodor G, Liu CM, Woolverton CJ, Baseler MW. (1986) A new class of synthetic biological response modifiers: the methylfurylbutyrolactones (Nafocare B). *J. Biol. Response Mod* **5**:444–461.
25. Fodor G, Sussangkarn K, Mathelier H, Fang K, Arnold R, Flippen-Anderson J, Karle I. (1986) Stereospecificity of a new reaction of L-ascorbic acid with cis and trans olefinic 1,4-dicarbonyl compounds. *J. Org. Chem* **51**:3148–3150.
26. Fodor G, Arnold R, Mohacsi T. (1983) A new role for L-ascorbic acid: Michael donor to alpha, beta- unsaturated carbonyl compounds. *Tetrahedron* **39**:2137–2145.
27. Wilson JM, Slattery JT, Forte AJ, and Nelson SD (1982) Analysis of acetaminophen metabolites in urine by high-performance liquid chromatography with UV and amperometric detection. *Journal of Chromatography B: Biomedical Sciences and Applications* **227**:453–462.
28. Cook SF, King AD, van den Anker JN, and Wilkins DG (2015) Simultaneous quantification of acetaminophen and five acetaminophen metabolites in human plasma and urine by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry: Method validation and application to a neonatal pharmacokinetic study. *Journal of Chromatography B* **1007**:30–42.12.

29. Johnson KA, and Plumb R (2005) Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. *Journal of Pharmaceutical and Biomedical Analysis* **39**:805–810.
30. Mrochek JE, Katz S, Christie WH, and Dinsmore SR (1974) Acetaminophen Metabolism in Man, as Determined by High-Resolution Liquid Chromatography. *Clin Chem* **20**:1086–1096, Oxford Academic.
31. Miksa IR, Cummings MR, and Poppenga RH (2005) Multi-residue determination of anti-inflammatory analgesics in sera by liquid chromatography--mass spectrometry. *J Anal Toxicol* **29**:95–104.
32. La S, Yoo HH, and Kim D-H (2005) Liquid chromatography-mass spectrometric analysis of urinary metabolites and their pattern recognition for the prediction of drug-induced hepatotoxicity. *Chem Res Toxicol* **18**:1887–1896.
33. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, and Brodie BB (1973) Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* **187**:185–194.
34. English PA, Williams JA, Martini J-F, Motzer RJ, Valota O, and Buller RE (2016) A case for the use of receiver operating characteristic analysis of potential clinical efficacy biomarkers in advanced renal cell carcinoma. *Future Oncol* **12**:175–182.