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Detection of Acetaminophen-Protein Adducts in Decedents with Suspected Opioid-Acetaminophen Combination Product Overdose

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Abstract

Acetaminophen overdose is a leading cause of drug induced liver failure in the United States (US). Acetaminophen-protein adducts have been suggested as a biomarker of hepatotoxicity. The purpose of this study was to determine if protein-derived acetaminophen-protein adducts are quantifiable in post-mortem samples. Heart blood, femoral blood and liver tissue were collected at autopsy from 22 decedents suspected of opioid-acetaminophen overdose. Samples were assayed for protein-derived acetaminophen-protein adducts, acetaminophen, and selected opioids found in combination products containing acetaminophen. Protein-derived APAP-CYS was detected in 17 of 22 decedents and was measurable in blood that was not degraded or hemolyzed. Heart blood concentrations ranged from 11 ng/mL (0.1 μ M) to 7817 ng/mL (28.9 μ M). Protein-derived acetaminophen-protein adducts were detectable in liver tissue for 20 of 22 decedents. Liver histology was also performed for all decedents and no evidence of centrilobular hepatic necrosis was observed.

Keywords

forensic science; acetaminophen; clinical laboratory techniques; protein-derived APAP-CYS; post-mortem redistribution; acetaminophen-protein adducts

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More than 28 billion doses of acetaminophen (APAP) were purchased in the United States in 2008 (1). APAP is used for pain relief, and is available alone or in combination with other analgesics including opioids such as oxycodone, hydrocodone, tramadol, and codeine. APAP toxicity is a leading cause of drug induced liver failure in the United States (2, 3). In 2011, poison control centers received more than 90,000 voluntary reports of accidental and intentional exposures to acetaminophen or acetaminophen containing products (4). Liver injury after APAP overdose is attributed to the formation of a toxic APAP metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) formed by cytochrome P450 2E1 metabolism in the liver (5, 6). This metabolite is highly reactive and short-lived, and binds to macromolecules in hepatocytes causing hepatocellular damage and death (5, 7, 8). Binding of NAPQI to proteins results in a measurable protein-derived APAP-CYS adduct that is distinct from non-protein derived APAP-CYS formed as a cysteine or glutathione metabolite (9–11). (Figure 1, APAP metabolism) Previous studies have suggested that most protein-derived APAP-CYS adducts are formed in hepatocytes and are released into circulation when cells lyse (12). The biomarker protein-derived APAP-CYS is measurable in serum from patients taking APAP even at therapeutic doses (13, 14). Protein-derived APAP-CYS concentrations increase in a dose dependent manner and serum protein-derived APAP-CYS concentrations > 1.1 μ M have been correlated with alanine aminotransferase (ALT) concentrations > 1000 IU/L in patients known to have APAP-induced liver injury (12, 13). APAP itself is often not detectable more than 24–48h after ingestion even in large overdoses. Protein-derived APAP-CYS has a half-life of about 1.5 days after therapeutic dosing and is measurable long after serum APAP concentrations are undetectable (14). Protein-derived APAP-CYS is not found in samples from patients who have not been exposed to APAP (15). Protein-derived APAP-CYS has been proposed as a diagnostic tool in cases of acute liver failure or encephalopathy without an identified cause (15).

In addition to the clinical utility of protein-derived APAP-CYS determination in living patients as a means to determine the cause of liver injury and guide treatment, protein-derived APAP-CYS determination in deceased persons may elucidate causes of liver injury or liver failure and provide additional information to aid in determination of cause of death. APAP is detectable in samples from deceased patients, however concentrations may be difficult to interpret due to lack of a history of ingestion timing or chronicity. The dose-dependence and longer detection time of protein-derived APAP-CYS adducts compared to APAP suggests this biomarker may be suitable for use in postmortem investigation.

For this study, a protein-derived APAP-CYS biomarker was measured in serum (heart and femoral) and liver samples from persons who died of possible opioid-APAP combination product overdose. Liver histology was also performed to detect histologic evidence of centrilobular hepatic necrosis characteristic of APAP toxicity. The objective of this study was to determine if the measurement of APAP-CYS adducts was possible in postmortem samples.

Methods

Sample Selection and Storage

Initial toxicology testing of blood and urine was performed by the state Medical Examiner's office as part of the routine protocols for death investigation. Cases were identified based on history alone prior to autopsy or laboratory analysis. Twenty-two decedents suspected of possibly taking combination APAP/opioid product prior to death were included in this study. Liver tissue, heart and femoral blood were collected at autopsy.

Femoral blood was obtained by percutaneous aspiration. Heart blood was obtained by direct cardiac puncture. Both heart and femoral blood were collected into a 10 mL red topped vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Two liver specimens were also obtained; one specimen was frozen for later protein-derived APAP-CYS analysis and the other specimen was placed into a formalin-containing vial for histopathology examination. Blood was centrifuged for 15 minutes to collect serum. Serum and liver sections were stored at -80°C until analysis. Liver sections in formalin for histology were stored at room temperature.

Ethics

The Institutional Review Board at the University of Utah reviewed this study and awarded exempt status as analyses were carried out on de-identified samples from decedents as part of the death investigation. No names or identifying sample numbers were provided to the study team.

Materials

APAP-CYS was obtained from Toronto Research Chemicals as trifluoroacetic acid salt (TRC; North Rock, CA). APAP was obtained from Sigma (St. Louis, MO). Hydrocodone, oxycodone, hydrocodone- D_3 , oxycodone- D_6 , tramadol- ^{13}C , D_3 were obtained from Cerilliant (Round Rock, TX). Tramadol HCl was obtained from the R.W. Johnson Pharmaceutical Research Institute. LC-MS grade methanol, dichloromethane, and isopropanol were obtained from Honeywell-Burdick & Jackson (Muskegon, MI). ACS grade ammonium hydroxide, potassium phosphate dibasic, potassium hydroxide, trichloroacetic acid, and formic acid were obtained from Fisher Scientific (Fairlawn, NJ). HPLC grade acetic acid was obtained from J.T. Baker (Phillipsburg, NJ). The 0.1 M acetate pH 4 buffer was prepared from acetic acid and potassium hydroxide.

Analyses for APAP, protein-derived APAP-CYS, and selected opioids commonly found in combination with APAP were conducted at the Center for Human Toxicology. All other data (Table 1) were obtained as part of the routine investigation conducted by the Office of the Medical Examiner.

APAP-CYS Analysis

The protein-derived APAP-CYS biomarker was quantified using previously validated high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) procedures in serum and liver samples (limit of quantification for protein-derived APAP-CYS

biomarker: 2.7 ng/mL (0.01 μ M)) (16–17). For the purposes of this study, protein-derived APAP-CYS concentrations are expressed both as ng/mL and μ M to facilitate comparison to other published literature.

Quantification of 3-cysteiny APAP (APAP-CYS) protein adducts in serum and plasma was performed using HPLC-MS/MS (17). Serum (500 μ L) or liver homogenate (150 μ L) are first dialyzed (Spectrum Labs, Santa Domingo, CA; MWCO 3500), and the dialysate then passed through gel filtration columns $\times$ 2 (Bio-Spin $\text{\textcircled{R}}$ 6 Tris columns, 6 kDa molecular weight cut-off, Bio-Rad Laboratories, Hercules, CA) to remove APAP and APAP-CYS not covalently bound to proteins. The eluates are then subjected to enzymatic protease digestion (Protease type XIV from *Streptomyces griseus*, 40 U/mL) for 24 hours at 37°C to liberate protein-bound APAP-CYS. Norbuprenorphine-d3 (NBUP-d3) (200 ng/mL) is added as an internal standard (ISTD). The liberated protein-derived APAP-CYS and ISTD are recovered from the digested matrix by protein precipitation using cold acetonitrile (600 μ L). Sample extracts are injected on to an HPLC column (Poroshell 120SB-C18 50 mm L $\times$ 2.0 mm I.D.; 3.0 μ m particle size) coupled to a triple quadrupole tandem mass spectrometer operating in electrospray ionization mode (Agilent 6460 TripleQuad LC-ESI-MS/MS). The mobile phase was a gradient that used 0.1 % formic acid in water transitioning to 0.1% formic acid in methanol and back again at a flow rate of 0.2 mL/minute. The injection volume was 10 μ L. Positive ion electrospray and nitrogen sheath gas (60 psi) were used for ionization. Argon was used for collision-induced dissociation. Selected reaction monitoring was used in the analysis: APAP-CYS: 271 $\rightarrow$ 140, and 271 $\rightarrow$ 96, and norbuprenorphine-D₃: 417 $\rightarrow$ 417. Separate sets of 0.01 ng/ μ L, 0.1 ng/ μ L, 1 ng/ μ L, and 10 ng/ μ L APAP-CYS working solutions were prepared with water:methanol 50:50 (v/v) and were used to make the calibration standards and controls. Calibration standards ranging from 2.7 ng/mL to 2700 ng/mL were prepared in blank gel filtered matrix of the same type as the study samples. Control samples were also prepared in blank gel filtered matrix at 8 ng/mL, 80 ng/mL, and 2100 ng/mL. The calibration standards and controls were analyzed in the same manner as the study samples and concurrently with the study samples. The dynamic range over which protein-derived APAP-CYS can be accurately quantified is from 2.7 to 2700 ng/mL (0.01 to 10.0 μ M). Samples with protein-derived APAP-CYS concentrations greater than 2700 ng/mL were diluted for repeat analysis. The LOQ for protein-derived APAP-CYS was 2.7 ng/mL (0.01 μ M) in serum and liver homogenate. Intra- and inter-assay imprecision and accuracy was <5.8% as determined by matrix-matched quality control specimens. Protein-derived APAP-CYS was found to be stable in human serum for three freeze-thaw cycles and 24 hours at ambient temperature.

Histopathology

A pathologist with extensive experience in identifying liver injury after APAP exposure completed blinded histopathological analysis. Representative liver samples were prepared and stained according to standard practice. Samples were evaluated for evidence of centrilobular hepatic necrosis.

Opioid Analysis

The quantitative analysis for oxycodone, hydrocodone and tramadol employed solid phase extraction (SPE) and tandem liquid chromatography –mass spectrometry (LC-MS). Opioid analysis in this study was to confirm the analytical work done by the Medical Examiner's office. For the quantitative analysis, a 1 mL volume from each sample was transferred to clean 16 × 100 mm glass culture tubes. Fifty nanograms of oxycodone-D₆, hydrocodone-D₃, and tramadol-¹³C₁D₃ (50µL of 1 ng/µL) were added to each tube to act as the internal standards. A 4 mL volume of Milli Q water and a 1 mL volume of 10 % trichloroacetic acid were added to each tube. The tubes were vortexed and centrifuged at 2000 rpm for 10 minutes on a IEC FLx 40 centrifuge. Following centrifugation, the supernatants were collected and transferred to separate, clean 16 X 100 mm culture tubes. The pH of the supernatants was adjusted to pH 8.5–9.0 by adding 1 mL of 50 % K₂HPO₄ and several drops of 1 N NaOH to each tube. The tubes were centrifuged as described above. The re-centrifuged supernatants were transferred to clean, separate glass culture tubes and set aside for SPE. The SPE procedure was a modification of a method described in an application note (18). Separate Clean Screen® ZSDAU020 SPE columns (United Chemical Technologies, Bristol, PA) were used for each sample. The SPE was performed using a glass manifold that was attached to an in-house vacuum source. The SPE columns were conditioned by addition of 3 mL of methanol and 3 mL of water. The samples supernatants were added to the separate SPE columns. After the sample supernatants passed through, the SPE columns were washed with 3 mL of water, 2 mL 0.1 M acetate, pH 4.0 buffer, and 3 mL of methanol. After the methanol passed through, the SPE columns were dried by applying vacuum for 15 minutes. The SPE columns were eluted by adding 3 mL of dichloromethane:isopropanol:ammonia (80:20:2). The elution liquid was collected into clean, separate, 13 X 100 mm culture tubes. A Turbo Vap® (Caliper Life Sciences, Hopkinton, MA) evaporator set at 40° C was used to dry the extracts under a stream of air. The extracts were reconstituted with 100 µL of 0.1 % formic acid : methanol (97:3). The extracts were transferred to separate, clean conical, 11 mm polypropylene auto sampler vials.

Modification of an LC-MS method used to test for morphine and metabolites was the basis for the LC-MS analysis used in this study (19). The LC-MS system consisted of an Agilent 1100 liquid chromatograph and a Thermo Finnigan (San Jose, CA) TSQ 7000 tandem mass spectrometer. The LC-MS system was operated by Xcalibur (Thermo-Finnigan) software. A YMC ODS-AQ 2 X 50 mm (Waters, Milford MA) column was used for the chromatographic separation. The mobile phase was a gradient that used 0.1 % formic acid (85→60): acetonitrile (15→40) at a flow rate of 0.2 mL/minute. The injection volume was 6 µL. Positive ion electrospray and nitrogen sheath gas (60 psi) were used for ionization. Argon was used for collision-induced dissociation. Selected reaction monitoring was used in the analysis: oxycodone: 316.01→298; oxycodone-D₆: 322.10→304; hydrocodone: 300.01→199; hydrocodone-D₃: 303.01→202; tramadol: 264.07→264 (survivor ion); tramadol-D₄: 268.10→268 (survivor ion).

Separate sets of 0.1 ng/µL 1 ng/µL, and 10 ng/µL oxycodone/hydrocodone/tramadol were prepared with methanol and were used to make the calibration standards and controls.

Calibration standards ranging from 10 -1000 ng/mL were prepared in blank bovine blood. Blank bovine blood was analyzed in the check run and the analytical run. Hydrocodone and Oxycodone were not detected in the blank bovine blood. For tramadol, there was a chromatographic signal near the retention time of tramadol in the blank bovine extract. This signal quantitated tramadol well below the limit of quantitation (10 ng/mL). The blank bovine blood was considered negative for tramadol. Control samples were also prepared in bovine blood at 30 ng/mL, 100 ng/mL, and 650 ng/mL. The calibration standards and controls were analyzed in the same manner as the study samples and concurrently with the study samples. Calibration curves used a quadratic fit and a 1/x weighting.

APAP Analysis

APAP concentration was assayed in heart serum samples due to availability of sample using an APAP Direct ELISA kit appropriate for forensic analysis and according to manufacturer instructions. (Immunalysis Corporation, Pomona, CA catalog # 227-0096). Briefly, serum aliquots and standard curve solutions were incubated with enzyme labeled APAP derivative in a microplate coated with polyclonal antibody. After incubation, the wells were washed, a chromogenic substrate was added for 30 minutes, treated with a stop solution and then read within 60 minutes of color development at a wavelength of 450 nm on a Tecan Sunrise microplate absorbance reader (Tecan, Mannedorf, Switzerland) with Magellan Software (Tecan). The positive reference standard included in the acetaminophen assay kit contained 100 ug/mL of APAP dissolved in synthetic urine with non-azide preservatives. The negative reference standard included in the acetaminophen assay kit was drug free synthetic urine with azide-free preservatives. The laboratory working control was a 20 ug/mL APAP solution prepared in blank human serum. Calibration controls and standards were prepared in blank human serum. The linear range of the acetaminophen assay was 5 ug/mL to 1000 ug/mL.

Results

Analyses performed at the Center for Human Toxicology were performed on serum and liver tissue. Table 1 shows the demographic data of the 22 subjects included in this study. Of the 22 subjects, 12 were male and 10 were female, ranging in age from 26 to 66 years of age. Determination of cause of death, manner of death, and the substances detected on autopsy are established during routine investigation by the office of the medical examiner.

Table 2 shows opioid, APAP and protein-derived APAP-CYS concentrations in serum and liver tissue. Heart serum from 7 subjects had measurable oxycodone, 12 subjects had measurable hydrocodone and one subject had measurable tramadol. Subjects 1 and 15 were excluded from opioid analysis based on review of autopsy findings and negative drug screens from the medical examiner's office.

Heart serum from 8 subjects had measurable APAP concentrations. Seven subjects had measurable APAP in addition to measurable opioid concentrations in heart serum. Measurable concentrations of protein-derived APAP-CYS biomarker ranged from 11 ng/mL (0.1 μ M) to 7817 ng/mL (28.9 μ M) in heart serum, and from 8 ng/mL (0.03 μ M) to 2477 ng/mL (9.2 μ M) in femoral serum. Liver tissue concentrations of protein-derived APAP-

CYS biomarker ranged from 0.1 to 4.8 ng/mg liver. For all subjects, liver histology did not show significant hepatic injury or necrosis, even in decedents with high serum protein-derived APAP-CYS concentrations.

Discussion

Protein-derived APAP-CYS is a promising biomarker for APAP-induced hepatotoxicity in living persons (20). In patients with known APAP-induced liver injury serum protein-derived APAP-CYS concentrations exceed about 1.1 μM in the presence of ALT activity $> 1,000$ IU/L, with progressive increases of adduct concentrations as ALT activity rises. Serum protein-derived APAP-CYS concentrations have been reported to reach levels as high as 41 μM in patients with known APAP-induced liver failure, though are more commonly lower. Serum protein-derived APAP-CYS is also measureable in living patients consuming therapeutic doses of APAP in the absence of hepatotoxicity, with levels ~ 1.1 μM . Thus the finding of dramatic elevation of serum protein-derived APAP-CYS concentrations in patients with acute liver failure of unknown cause may provide strong support for APAP overdose being an etiology.

This is the first study to report the measurement of protein-derived APAP-CYS in postmortem serum and tissue samples. In 22 subjects who possibly ingested an APAP/opioid product, serum satisfactory for analysis for protein-derived APAP-CYS was available from heart blood in 17, and from femoral blood in 18 decedents. In this study, in the absence of histological evidence of APAP-induced liver injury, postmortem serum protein-derived APAP-CYS concentrations ranged as high as 28.9 μM . For decedents with both heart and femoral samples available, excluding decedents with chromatographic interference, measurable protein-derived APAP-CYS concentrations were higher in heart samples than in femoral samples for 10 of 12 sample pairs. Both of these facts support redistribution of protein-derived APAP adducts from intracellular sites of formation into blood after death. This is in keeping with subject 14, for example, who died a natural death without detection of APAP in cardiac blood, but with heart blood that contained protein-derived APAP-CYS at a concentration of 7.4 μM . While hepatic CYP2E1 is mainly responsible for antemortem metabolism of APAP, CYP2E1 has been reported to reside in various organs and tissues, including kidney, colon, duodenum, ileum, esophagus, lung, nasal epithelium, and skin. The relative contribution of these and other organs towards postmortem redistribution of protein-derived APAP-CYS remains unknown.

Centrilobular hepatic necrosis, when present, is not specific for APAP, and is seen, for example, in ischemic hepatitis (shock liver) and septic shock. Given the extremely common use of APAP in and outside the hospital, our data indicate that postmortem serum protein-derived APAP-CYS concentrations in the range seen in living persons with APAP-induced liver injury and failure would not, in and of themselves, provide strong evidence that APAP was responsible for hepatic necrosis.

In living patients who ingest hepatotoxic doses of APAP, serum ALT activity and serum protein-derived APAP-CYS concentrations may not begin to rise into ranges well above normal or above those associated with therapeutic APAP dosing for 18 hours or longer, with

subsequent liver failure not evident until 36 to 72 hours after ingestion. In contrast, following overdoses of opioid/APAP combination products, coma and respiratory depression from the opioid component or co-ingestants with subsequent consequences (e.g., aspiration) can cause death well before the development of APAP induced hepatic necrosis.

In this study, APAP was not detectable in all decedents with measureable protein-derived APAP-CYS. Detection of protein-derived APAP-CYS in serum from decedents without detectable APAP concentrations is consistent with the difference in serum half-life of APAP (about 4 h) and protein-derived APAP-CYS (about 1.5 days) as well as with postmortem redistribution of the adduct.

Limitations to this study include the absence of subjects with known APAP-induced liver necrosis. While we contrasted protein-derived APAP-CYS in postmortem blood in the absence of hepatic necrosis with those in living patients, we have not compared them to postmortem levels in decedents with known APAP-induced liver failure. As is commonly the case in deaths suspicious for drug overdose, the exact times of drug ingestion, dose, or death, are not known. This precludes an attempt at seeking a relationship between time after death and progressive rises in serum adduct levels. Finally, blood obtained by blind femoral sticks is not necessarily venous blood, but can represent femoral arterial blood, which would be expected to be most similar to cardiac blood. Nevertheless, blind femoral needle sticks are commonly used, which increases the external validity of our finding that femoral adduct levels commonly are lower than those in cardiac blood, supporting postmortem redistribution.

Conclusions

Protein-derived APAP-CYS was measurable in 18 of 22 decedents in which ingestion of an opioid/acetaminophen combination product was suspected. Liver histology showed no evidence of centrilobular hepatic necrosis in any decedent. Postmortem protein-derived APAP-CYS concentrations tended to be higher in heart samples than in femoral samples. There was no association between post mortem protein-derived APAP-CYS concentrations and hepatotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
APAP Metabolism

TABLE 1

Demographics.

Subject (No.)	Age, years	Gender	Cause of Death	Manner of Death	Substances Detected on Autopsy
1	40	Male	Positional asphyxia due to mixed drug intoxication	Accident	ethanol, fentanyl
2	40	Male	Drug intoxication	Accident	morphine, hydrocodone
3	31	Male	Drug toxicity	Undetermined	alprazolam, diazepam, diphenhydramine, temazepam, tramadol
4	52	Female	Pneumonia and obesity	Natural	none
5	66	Female	Drug Toxicity	Suicide	acetaminophen, diphenhydramine, zolpidem
6	59	Male	Mixed drug intoxication and pneumonia	Accident	fentanyl, hydrocodone
7	43	Female	Mixed drug intoxication	Undetermined	morphine, alprazolam, carisoprodol, hydrocodone
8	51	Male	Complications of mixed drug overdose	Suicide	alprazolam, diphenhydramine, hydrocodone, zolpidem, acetaminophen
9	22	Male	Drug toxicity	Accident	ethanol, hydrocodone
10	62	Female	Mixed drug intoxication	Suicide	fentanyl, methadone, oxycodone, zolpidem, diazepam
11	40	Female	Mixed drug and alcohol intoxication	Undetermined	ethanol, alprazolam, oxycodone, quetiapine
12	29	Female	Mixed drug intoxication	Undetermined	alprazolam, diphenhydramine, hydrocodone, oxycodone, promethazine
13	26	Female	Mixed drug intoxication	Accident	hydrocodone, diazepam, fentanyl
14	48	Male	Complications of schizophrenia and seizure disorder	Natural	oxycodone, hydrocodone
15	40	Male	Undetermined	Undetermined	none
16	50	Male	Probable cardiac arrhythmia	Natural	hydrocodone
17	27	Male	Blunt force injury to the torso	Accident	ethanol, hydrocodone
18	45	Male	Anoxic encephalopathy and pneumonia due to mixed drug intoxication	Accident	alprazolam, oxycodone, carisoprodol
19	34	Female	Drug toxicity	Accident	clonazepam, cyclobenzaprine, diphenhydramine, meperidine, oxycodone, promethazine, trazodone
20	29	Male	Mixed drug intoxication	Accident	ethanol, alprazolam, chlorpiazepoxide, hydrocodone

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Subject (No.)	Age, years	Gender	Cause of Death	Manner of Death	Substances Detected on Autopsy
21	49	Female	Drug toxicity	Accident	fentanyl and hydrocodone
22	26	Female	Mixed drug toxicity and morbid obesity	Accident	oxycodone and amphetamines

TABLE 2

Opioid, acetaminophen and protein-derived APAP-CYS quantification.

Subject (No.)	Oxycodone, Heart, ng/mL	Hydrocodone, Heart, ng/mL	Acetaminophen, Heart, µg/mL	Protein-Derived APAP-CYS, Heart, ng/mL (µM)	Protein-Derived APAP-CYS, Femoral, ng/mL (µM)	Protein-Derived APAP-CYS, Liver, ng/mg liver
1	Not Tested	Not Tested	ND	CI	20.4 (0.1)	0.4
2	ND	20.5	ND	1302.7 (4.8)	CI	1.7
3	ND	ND	ND	864.1 (3.2)	ND	4.8
4	77.2	ND	ND	CI	CI	1.3
5	ND	ND	497.5	CI	CI	0.1
6	ND	41.7	ND	2084.9 (7.7)	CI	0.4
7	ND	40.6	ND	CI	20.4 (0.1)	2.0
8	ND	353.4	344.1	CI	141.1 (0.5)	0.9
9	ND	163	78.6	432.5 (1.6)	ND	0.1
10	1385.4	ND	103.2	1080.2 (4.0)	2477.3 (9.2)	0.1
11	218.5	ND	ND	11.7 (0.1)	ND	0.3
12	87.5	547.6	260.3	7817.2 (28.9)	1314.7 (4.9)	4.8
13	ND	45.3	ND	1271.5 (4.7)	627.6 (2.3)	0.6
14	55.3	24.2	ND	2001.2 (7.4)	33.9 (0.1)	0.3
15	Not Tested	Not Tested	ND	ND	ND	ND
16	ND	61.7	133.4	ND	ND	2.1
17	ND	109.1	ND	1704.5 (6.3)	8.7 (0.03)	0.1
18	ND	ND	ND	1107.6 (4.1)	276.7 (1.0)	0.6
19	336	ND	ND	320.2 (1.2)	ND	2.2
20	ND	145.9	139.6	816.9 (3.0)	44.3 (0.2)	0.4
21	ND	62.8	ND	ND	ND	ND
22	341.8	ND	86.9	17.2 (0.1)	17.2 (0.1)	0.9

Footnotes: ND is not detected; CI is chromatographic interference. Tramadol was assayed for in all samples but was only detected in sample 3 at 25.6 ng/mL; APAP was detected in heart serum using ELISA methods.