Protein-Derived Acetaminophen-Cysteine Can Be Detected After Repeated Supratherapeutic Ingestion of Acetaminophen in the Absence of Hepatotoxicity

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Abstract Generation of protein-derived acetaminophen-cysteine (APAP-CYS) is reported after ingestion of large and therapeutic dosages of acetaminophen in healthy and in liver-damaged patients. The incidence of protein-derived APAP-CYS adducts in repeated supratherapeutic dosages of APAP is not known. Methods: for 12 months, a standardized and comprehensive questionnaire was used to interview every consecutive patient at a pain management clinic. Patients found to ingest more than 4 g of APAP per day for a minimum of 14 consecutive days at the time of the encounter were invited to have blood drawn for hepatic transaminases and APAP-CYS adduct levels. Twelve subjects out of 990 interviewees met inclusion criteria. Ten of the 12 had measurable protein-derived APAP-CYS, none had evidence of liver injury. Patients that ingest repeated supratherapeutic amounts of APAP over several weeks may generate APAP-CYS protein adducts in the absence of hepatic injury.

Keywords Acetaminophen · Protein-derived APAP-CYS · Repeated supratherapeutic ingestion · Hepatotoxicity

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Introduction

The biochemistry and relative contribution of different molecules in acetaminophen (APAP) metabolism and toxicity are not completely understood. APAP-induced hepatotoxicity is believed to be mediated by covalent binding of the reactive metabolite N-acetyl-p-benzoquinone imine to essential proteins in the liver [1]. The primary reaction of this metabolite with hepatic proteins results in the formation of 3-(cysteine-S-yl)-APAP adducts, specifically 3-para-cysteinyl acetaminophen. The presence of acetaminophen-cysteine (APAP-CYS) protein adducts in the serum may or may not indicate APAP-induced hepatic injury.

Protein-derived APAP-CYS has been isolated in the sera of patients following recommended (therapeutic) doses of APAP and following APAP overdose with and without accompanying liver injury [2].

There is a paucity of data regarding the relevance or importance of protein-derived APAP-CYS in humans following repeated supratherapeutic ingestion (RSTI) of APAP. Patients with chronic pain commonly take APAP-containing medicines and have been identified as a population at risk for APAP overdose.

We performed a structured survey of patients presenting to a pain management clinic in order to identify individuals that self-report chronic supratherapeutic dosages of APAP (>4000 mg/day) in an effort to better characterize protein-derived APAP-CYS concentrations and hepatic transaminase activity. The purpose of our study was to determine if protein-derived acetaminophen-cysteine can be detected after repeated supratherapeutic ingestion in a population of patients in a pain management clinic.

Methods

The setting for this investigation is an urban chronic pain management clinic run by the anesthesia department of Albert Einstein Medical Center, Philadelphia, PA. The average monthly census of the pain clinic is 90–100 patient visits of which less than five are new referrals. This is an IRB-approved prospective, descriptive study of a consecutive cohort of patients from the pain management clinic. All patient interviews and phlebotomy were performed by research assistants and certified phlebotomists within the clinical spaces of the pain management clinic.

For 12 months, from July 2009 to June 2010, we stationed a research assistant to stay in the pain management clinic for 8 h Monday–Friday (8 a.m.–4 p.m.) and review the medical records of every consecutive patient scheduled for counseling and treatment. The research assistant was a single physician trained in administering structured questionnaires. Past medical history including history of alcohol and drug use was collected on every pain clinic patient. Comprehensive medication reconciliation was performed by the research assistant, and every APAP-containing prescription and OTC medication and their dosages that appeared on the medication list was confirmed via direct structured interviews with the patient.

During the structured interview, patients were asked specifically about the use of additional APAP-containing medications and were shown photographs of commonly prescribed and OTC medications containing APAP (photographs were provided by the authors or copied from manufacturers' websites and other internet sources). The research assistants added up the total APAP dosage for the combined APAP-containing medications. Patients that were confirmed via the structured interviews to have ingested greater than 4 g of APAP/day for at least 14 consecutive days prior to presentation were considered eligible for enrollment in the study. Enrolled patients were specifically questioned about signs and symptoms of hepatic injury including nausea, right upper quadrant abdominal pain, and unexplained or easy bruising.

Patients were excluded if their daily APAP ingestion was calculated to be less than 4 g/day or if they refused participation. Preexisting hepatic disease or alcohol use were not exclusion criteria.

Patients provided informed consent and agreed to phlebotomy for complete blood count, complete metabolic panel, coagulation profile, APAP concentration, and protein-derived APAP-CYS concentrations.

Sample Analysis

Assays for CBC, complete metabolic panel, coagulation profile, and APAP concentration were performed locally at the hospital lab according to standard laboratory practice for each

respective assay. Samples for protein-derived APAP-CYS measurement were collected in a standard non-heparinized tube and immediately centrifuged by the research assistants to separate serum, which was transferred to standard plastic serum tubes and frozen at -80°C centigrade. The samples were batched and sent frozen overnight via UPS to the Center for Human Toxicology in Salt Lake City for measurement of protein-derived APAP-CYS concentration.

Protein-Derived APAP-CYS Measurement

The APAP-CYS protein adducts were measured by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS-MS). The sample of 150 μL of separated serum was passed over a gel filtration column twice. One hundred microliters of eluate from the gel filtration was added to 100 μL of protease (8 U/mL) and allowed to incubate for 24 h at 37°C . After 24 h, 0.6 mL acetonitrile was added and the sample was centrifuged to separation. The supernatant was removed and evaporated at 50°C and reconstituted in 200 μL of 0.1 % formic acid in water. The sample was filtered through a 0.22- μm Ultrafree-MC centrifugal filter, and 20 μL of the filtrate was analyzed via HPLC/MS-MS.

Sample extracts were injected onto an HPLC column (Poroshell 120 SB-C18 50 mm $\text{L} \times 2.0$ mm I.D.; 3.0 μm particle size) coupled to a triple quadrupole tandem mass spectrometer (Agilent 6460 TripleQuad LC/MS-MS) operating in electrospray ionization (ESI) and selected reaction mode (SRM). The limit of quantification for protein-derived APAP-CYS was 0.01 μM .

The research team educated patients that were discovered to be ingesting excessive amounts of acetaminophen as to the FDA-approved correct therapeutic dosage and notified the treating pain physician.

Results

A total of 990 subjects were screened for possible RSTI of APAP over the course of 12 months. Twelve subjects met criteria for enrollment. Results of the serum analysis on these 12 subjects are reproduced in Table 1. None of the subjects complained of symptoms consistent with hepatotoxicity (nausea, right upper quadrant abdominal pain, unexplained or easy bruising) and aside from their chronic pain issues were living their day-to-day lives with little or no interruption referable to hepatic dysfunction.

Twelve of the 990 subjects were noted to have used supratherapeutic APAP dosages over the previous 2 weeks, according to the answers provided via the standardized questionnaire, all agreed to the free blood testing; none of the subjects with historically elevated APAP ingestion refused to

Table 1 Summary of demographic data, APAP dose, and serum biomarkers

Patient	Age	Gender	Ethnicity	APAP dose (grams/day) for 14 consecutive days	ALT (IU/mL)	AST (IU/mL)	APAP (μg/mL)	Protein-derived APAP-CYS (μM)
1	77	Male	AA	4.225	43	23	<1	0.218
2	53	Male	Caucasian	5–7	39	20	4.9	0.230
3	37	Female	Hispanic	8.65	34	19	<1	0.030
4	42	Female	AA	8	34	13	<1	0.047
5	33	Male	AA	5.95–7.95	31	17	19.1	0.062
6	41	Female	AA	5.5–9.1	51	15	<1	<LOQ
7	72	Female	AA	5.85	31	15	26.5	0.401
8	43	Female	Caucasian	4–4.5	50	39	30.2	0.818
9	45	Female	AA	4.5	35	17	N/A	0.025
10	59	Female	AA	4	49	27	<1	<LOQ
11	59	Male	AA	5.4	21	43	<1	0.044
12	66	Female	AA	4	29	14	1.3	0.256

APAP acetaminophen, AA African American, ALT alanine aminotransferase, AST aspartate aminotransferase, LOQ limit of quantitation, CYS cysteine

participate with the experimental protocol. None of the enrolled subjects reported habitual (daily) use of alcohol. Ten of the subjects had measurable protein-derived APAP-CYS concentrations. None of the subjects had any evidence of chemical hepatitis or liver injury as evidenced by serum transaminase levels or coagulation proteins.

All patients were serologically evaluated for viral hepatitis and HIV, but only one, patient #3, was positive for hepatitis C (this was not unknown to the subject and was included as part of her medical history in the clinic medical record). All other subjects were seronegative for hepatitis, and none were positive for HIV. One subject (patient #1) underwent regular hemodialysis. Chronic renal insufficiency may result in accumulation of APAP-CYS [3].

Discussion

In normal acetaminophen (APAP) metabolism, most APAP is linked to either sulfide or glucuronide prior to (primarily) urinary excretion. A percentage (5–15 %) of the APAP dose is oxidized to an intermediate ketone (N-acetyl para-benzo quinone imine or NAPQI) then reduced to form a hydroxide at the para location and a sulfhydryl link to glutathione at the 3 (meta) position on the phenol ring. A small amount (3–5 %) of the parent compound is excreted unchanged [4]. With therapeutic dosing, NAPQI is attached to the sulfhydryl group on the cysteine portion of glutathione, without release from the enzyme and prior to elimination.

During overdose of APAP or when glutathione stores are reduced, NAPQI is released within the cell and forms covalent links with many macromolecules, particularly the sulfhydryl group on cysteine components of proteins (APAP-CYS protein adducts) [4, 5]. There is data to indicate that APAP-

CYS protein adducts form as a byproduct of simple hepatocellular exposure (not overdose) to APAP in the form of NAPQI [2, 6].

3-para-cysteinyl APAP has been demonstrated in human patients with fulminant hepatic failure thought to be related to APAP, as well as following acute overdose of APAP [2] and after ingestion of therapeutic doses of APAP in 4 g/day doses [4,6,7,8].

Our results corroborate the results of previous investigations; protein-derived APAP-CYS concentrations less than 1.1 ng/mL are consistent with APAP exposure, but not necessarily APAP toxicity or liver injury. Ten of the enrolled subjects in our experiment had measurable protein-derived APAP-CYS serum concentrations, although none demonstrated APAP-CYS protein adducts considered clinically alarming (>1 ng/mL) [2, 7].

A number of studies over the past decade [2, 6] describe a dose-dependent generation of APAP-CYS protein adducts; their diagnostic significance, however, is unclear. The presence of APAP-CYS protein adducts indicates recent ingestion of or exposure to acetaminophen. Even low doses (80 mg/kg) of acetaminophen result in the formation of APAP-CYS adducts [6]. Serum levels of APAP-CYS adducts greater than 1.1 nmoles/mL are associated with elevated transaminases; however, there is no observed correlation between peak recorded APAP-CYS concentrations and peak recorded transaminase levels [2].

Several early studies explored the possibility of using APAP-CYS protein adducts to identify and confirm APAP as the etiology behind unexplained hepatic failure [7, 8], but specificity and accuracy for this purpose are unproven.

We interviewed nearly 1000 patients over 12 months and discovered only 12 patients that regularly ingested supratherapeutic dosages of APAP; several of these

individuals were presenting for their initial visit to the pain management clinic when enrolled in this study. This suggests that the pain management specialists in our local clinic are doing an effective job at reducing the habitual use of APAP among the patients referred to them.

Limitations

This study relied primarily on patient history, as reported by the patient themselves, along with data extracted from the written medical record to identify potential subjects; memory can be an inaccurate data source, and medication histories can be inaccurate. We were exceedingly discriminating to err on the side of including only subjects who we believed, based on direct, comprehensive, standardized interviews, pill and prescription counts, and medical record review had a high probability of exceeding 4 g of APAP/day for the preceding 14 days. It is possible that not all of those who the history suggested had ingested >4 g of APAP/day for the preceding 14 days actually did so. It is also possible that the history failed to detect patients taking >4 g of APAP/day. All patients were derived from a select cohort of chronic pain patients, our results are not generalizable to other populations.

Protein-derived APAP-CYS samples may degrade over time if not frozen and prepared correctly. Samples are not expected to degrade if handled properly and processed within 7 months of collection [9]. The longest time between collection and processing for any of our samples was slightly longer than 16 weeks and well within the time expected to prevent any significant degradation.

Control samples were not included as part of the experimental protocol so the possibility of false positive (and false negative) results exist. We do not believe this is likely because false-positive acetaminophen is not typically reported except in the presence of liver injury, which none of the subjects in our population demonstrated [10].

Conclusion

This study adds to the growing literature that demonstrates the dose-dependent generation of APAP-CYS protein adducts in

the absence of liver injury in patients that ingest acetaminophen. Non-acute ingestion of supratherapeutic dosages of APAP, and the consequent generation of APAP-CYS protein adducts is a unique element of this study.

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