

# Acetaminophen protein adduct formation following low-dose acetaminophen exposure: comparison of immediate-release vs extended-release formulations

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## Abstract

**Purpose** Acetaminophen (APAP) protein adducts are a biomarker of APAP metabolism, reflecting oxidation of APAP and generation of the reactive metabolite N-acetyl-p-benzoquinone imine. High levels of adducts correspond to liver toxicity in patients with APAP-related acute liver failure. Adduct formation following low-dose exposure to APAP has not been well studied. APAP protein adducts were measured in blood samples collected from fasted individuals who participated in a crossover study of APAP (80 mg/kg)

comparing extended release (ER) and immediate release (IR) formulations.

**Methods** Adducts were quantified in all postdose blood samples using a validated high-performance liquid chromatography electrochemical detection (HPLC-EC) assay.

**Results** Comparison of pharmacokinetic parameters for adducts did not reveal significant differences between ER and IR formulations, with one exception. Formation rates for adducts were faster for IR than the ER formulation ( $0.420 \pm 0.157$  vs.  $0.203 \pm 0.080$  1/h), respectively. Maximum plasma concentrations ( $C_{\max}$ ) of adducts for IR and ER were  $0.108 (\pm 0.020)$  and  $0.100 (\pm 0.028)$  nmol/ml serum, respectively, and were two orders of magnitude lower than adduct levels previously reported in adults with acute liver failure secondary to APAP.

**Conclusions** APAP protein adducts are rapidly formed following nontoxic ingestion of APAP at levels significantly lower than those associated with acute liver failure.

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## Introduction

Acetaminophen (APAP) is the most widely used drug for treating pain and fever around the world. Whereas the drug is generally regarded to be safe when used in doses recommended by the manufacturer, large doses of APAP lead to fulminant hepatotoxicity [1]. In the United States and many European countries, APAP is recognized as a major cause of acute liver failure [1]. The drug is widely available in hundreds of over-the-counter cough and cold remedies and as single-ingredient preparations. In addition, APAP–opioid combination products are widely used to treat moderate pain.

Following therapeutic exposure, APAP is primarily metabolized by glucuronidation and sulfation reactions in the liver, and a small proportion of the drug undergoes oxidation. With supratherapeutic APAP doses, metabolism shifts, and an increased proportion of the parent drug undergoes oxidation via the cytochrome P450 system. Greater amounts of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) are formed, and NAPQI binds to cysteine groups on proteins, forming APAP protein adducts. In previous work, we have shown that high levels of APAP protein adducts are present in the blood of patients who consume large doses of APAP leading to acute liver failure (ALF) [2]. In addition, we found that APAP protein adducts have a long elimination half-life that markedly exceeds that of the parent compound, APAP, permitting detection of APAP toxicity long after the parent compound has cleared the blood [3]. For example, mean elimination half-life of APAP protein adducts for adults with APAP-induced ALF was  $41.3 \pm 8.3$  h. By comparison, the mean elimination half-life for APAP in adults with APAP-induced ALF varied from 5.4 to 18.4 h, depending on the severity of the liver injury and the presence of encephalopathy [4]. In milder forms of APAP toxicity, levels of adducts are inversely related to the lag time of starting treatment with the antidote, N-acetylcysteine [5].

Following proteolytic digestion of human blood samples, adducts can be detected through a highly specific and sensitive assay using high performance liquid chromatography with electrochemical detection (HPLC-EC) [2, 6]. Previous studies have shown that high levels of adducts are present in the blood samples of patients with APAP-induced ALF and are absent in patients with other causes of ALF [2].

The issue of whether or not adducts are formed following lower-dose exposure to APAP is also of interest. Previously, it was generally accepted that APAP protein adducts were only formed during severe APAP toxicity and then only after depletion of hepatic glutathione [7]. The sensitivity and precision of the HPLC-EC method of determination of adducts has extended our capability to specifically detect adducts after

low doses of APAP. In a small study, we previously reported low levels of adducts in patients following therapeutic exposure to multiple doses of APAP [8]. To date, the rate of adduct formation and magnitude of adduct levels have not been examined following single-dose exposure to APAP. Thus, this study examined adduct profiles in blood samples of healthy adults who participated in a study of single-dose APAP in a crossover design comparing immediate-release (IR) and extended-release (ER) formulations.

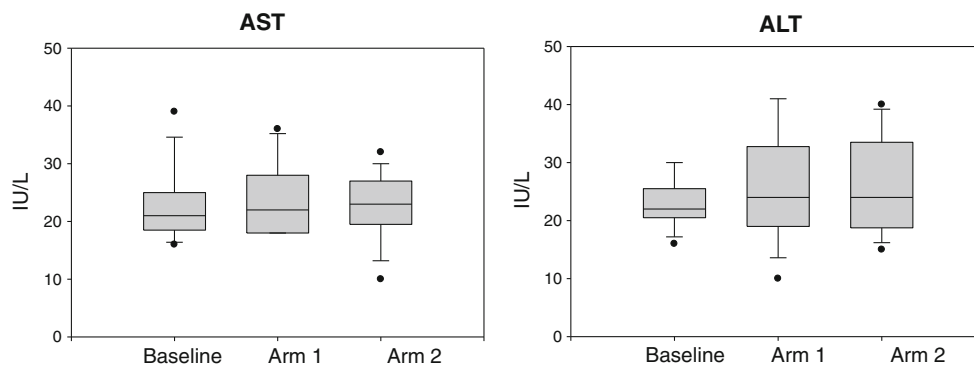
## Methods

### Study population

Banked serum samples obtained from nine adults participating in a recently published study to examine APAP pharmacokinetics were used to analyze APAP protein adducts [9]. The previously published study received institutional review board approval and was an open-label, nonblinded, crossover design in nine healthy adults ages 18–65 years. Exclusion criteria for enrollment were pregnancy, pre-existing liver disease, chronic alcohol consumption of  $>20$  g per day, chronic illness, regular use of any medication, body weight  $>100$  kg, use of an APAP-containing product in the previous 7 days, and abnormal liver function tests. Analysis of adducts in banked samples was approved by the institutional review boards of the participating institutions.

### Study design

Baseline serum bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) tests were performed 1 week prior to the study and 3 days following the completion of each arm of the study. Participants underwent an 8-h fast the evening prior to APAP administration. Using a simulated overdose design, APAP ( $\sim 80$  mg/kg) was administered by mouth either as Panadol Extend<sup>®</sup> [665-mg



**Fig. 1** Summary data for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) for baseline and postdosing of acetaminophen (APAP). Data are presented as median (line within box), 25th to

75th percentile (boundary of box), and 10th and 90th percentile (whiskers). No differences were present between ALT and AST at baseline compared with APAP postdosing

**Table 1** Summary of pharmacokinetic parameters [mean  $\pm$  standard deviation (SD)] for acetaminophen (APAP) protein adducts following administration of immediate- and extended-release formulations

	Immediate release	Extended release
$T_{max}$ (h)	8.4 $\pm$ 1.9	8.6 $\pm$ 3.4
$C_{max}$ ( $\mu$ M)	0.108 $\pm$ 0.020	0.100 $\pm$ 0.028
AUC <sub>0–12</sub> ( $\mu$ M $\cdot$ h)	0.95 $\pm$ 0.17	0.85 $\pm$ 0.21
Half life (h)	23.4 $\pm$ 21.7 <sup>a</sup>	21.0 $\pm$ 14.0 <sup>b</sup>
MRT (h)	36.8 $\pm$ 30.9 <sup>a</sup>	33.3 $\pm$ 20.1 <sup>b</sup>
Formation rate (1/h) <sup>c</sup>	0.420 $\pm$ 0.157 <sup>b</sup>	0.203 $\pm$ 0.080 <sup>b</sup>

$T_{max}$  time to maximum plasma concentration,  $C_{max}$  maximum plasma concentration

<sup>a</sup>  $n=7$  participants

<sup>b</sup>  $n=6$  participants

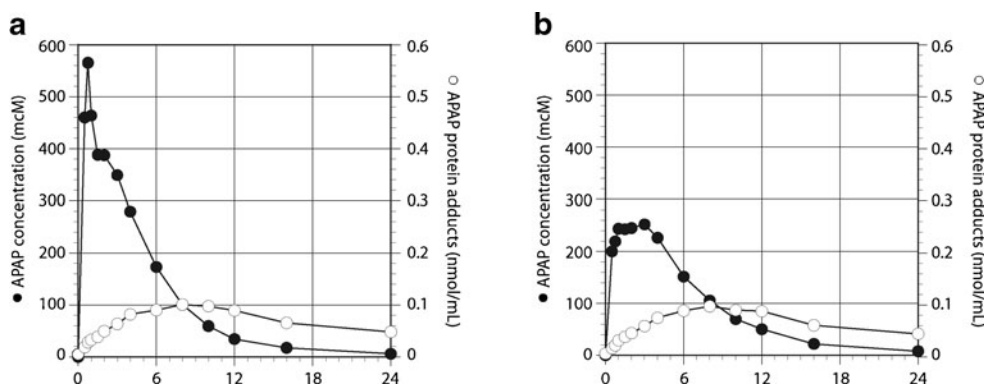
<sup>c</sup>  $p=0.035$

tablets: 69 % slow release (SR) and 31 % extended release, hereafter referred to as EX) or Panadol IR APAP (500 mg tablets, hereafter referred to as IR) [9]. Blood samples (5 ml) were obtained immediately prior to APAP and at 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h postingestion. All participants completed the study protocol, and blood samples were also obtained at 16 and 24 h postingestion in six of the nine participants. One week after completion of the first arm of the study, participants completed the second arm.

#### Analytical method

Serum samples were frozen at  $-80^{\circ}$  for batched analysis of APAP protein adducts using a modification of the previously reported HPLC-EC assay for APAP adducts [3, 6]. Assay modifications included centrifugal gel filtration, high-efficiency proteolytic digestion, and increased sample injection volumes, resulting in improved sensitivity and efficiency of the assay. The lower limit of quantitation for the assay was defined as 0.03  $\mu$ M. The laboratory technician was blinded to the sample group.

**Fig. 2** Summary profile for acetaminophen (APAP) (●) and APAP protein adducts (○) for **a** immediate- and **b** extended-release APAP

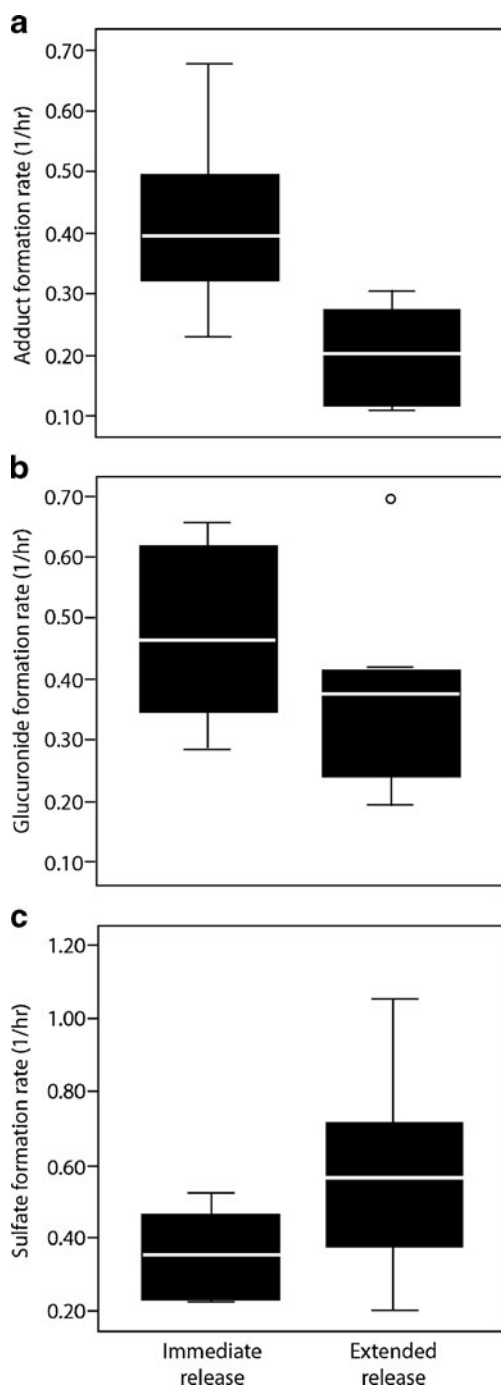


#### Clinical data

Patient data included age (years), gender, weight (kg), APAP dose (mg/kg), and baseline and post-APAP administration AST and ALT levels.

#### Pharmacokinetic analysis

Model-independent pharmacokinetic analyses were used to assess the disposition profiles of APAP, APAP metabolites, and APAP protein adducts. Serum concentration vs. time data were curve fit using a peeling algorithm to generate initial polyexponential parameter estimates. Final estimates of the terminal elimination rate constant ( $\lambda_z$ ) were determined from an iterative, nonlinear, least squares regression algorithm. Individual maximum plasma concentration ( $C_{max}$ ) and time to  $C_{max}$  ( $T_{max}$ ) were obtained by direct examination of the serum concentration versus time profiles. The area under the plasma concentration versus time curve during the sampling period (AUC<sub>0–n</sub>) was calculated using the mixed log-linear method (i.e., trapezoidal between values where  $C_n < C_{n+1}$  and logarithmic when  $C_n > C_{n+1}$ ). Extrapolation of the AUC to infinity (AUC<sub>0–∞</sub>) was achieved by the summation of AUC<sub>0–n</sub> +  $C_n/\lambda_z$ , where  $C_n$  is the predicted plasma concentration calculated from the curve fit and  $\lambda_z$  is the apparent terminal elimination rate constant. For participants in whom a sufficient number of postpeak serum concentrations were available, model-dependent pharmacokinetic analyses were used to arrive at a formation rate constant for each of the APAP metabolites and the adduct. Model-dependent rate constants were calculated from final polyexponential parameter estimates after application of the Akaike Information and Schwartz criteria and examination of the coefficients of variation for the polyexponential parameters estimated from a given model. All pharmacokinetic analyses were performed with Kinetica version 5.0 (ThermoElectron, Philadelphia, PA).



**Fig. 3** Formation rates for **a** acetaminophen (APAP) protein adducts, **b** APAP glucuronide, and **c** APAP sulfate for immediate- and extended-release APAP. The **solid bold lines** indicate data median; **boxes** indicate the 25th to 75th percentiles. **Whisker lines** indicated minimum and maximum data values. **o** denotes outliers

#### Statistical analysis

Pharmacokinetic data were examined using standard descriptive statistics. Relationships between pharmacokinetics of APAP, APAP conjugates, and APAP protein adducts were

examined using both linear and nonlinear least-squares regression. Evaluation of the data for possible formulation dependence in adduct formation was undertaken using univariate analysis of variance (ANOVA). Comparison of pharmacokinetic parameters between formulations was performed using a paired *t* test. Statistical analyses were performed using SPSS version 12 (SPSS, Inc., Chicago, IL, USA), and the significance limit accepted for all analyses was  $\alpha=0.05$ .

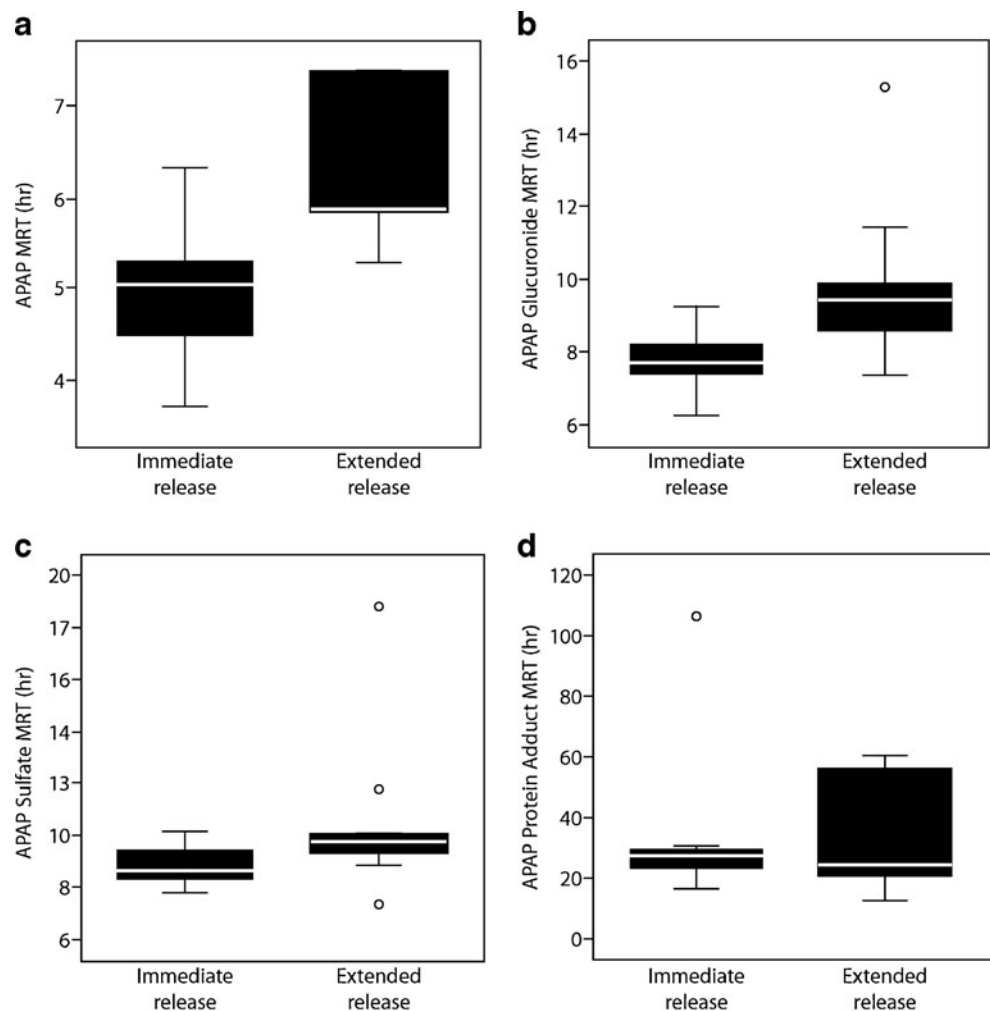
#### Results

Nine participants (eight men; one woman) completed the research protocol. Participant mean weight and age were 73.1 ( $\pm 7.7$ ) kg and 34 ( $\pm 7.1$ ) years, respectively. Mean administered dose of IR and ER APAP was comparable ( $5,778 \pm 565$  mg vs.  $5,763 \pm 665$  mg, respectively;  $p$ =not significant). Weight-normalized doses were also comparable ( $79.1 \pm 2.2$  mg/kg vs.  $78.8 \pm 2.6$  mg/kg), respectively. No differences were detected in AST and ALT from baseline and post-APAP dosing (Fig. 1).

Summary data for adduct pharmacokinetic parameters is presented in Table 1 and of composite plots for APAP and APAP protein adducts in Fig. 2. As reported in the original study [9], APAP concentrations were higher and occurred earlier with the IR formulation compared with the ER formulation. Serum concentration profiles for APAP protein adducts were more protracted than those of APAP, peaked much later, and did not return to baseline over the time course of the study (Fig. 2). Despite significant differences in the release of APAP into blood between the two formulations ( $p < 0.05$ ), no significant differences were detected in  $T_{max}$ ,  $C_{max}$ , or AUC values of adducts (Table 1). No correlation was observed between APAP exposure and the level of adduct formation, as would be expected in a study of very narrow dose range (i.e., 74–84 mg/kg). Formation rates for APAP protein adducts were faster for the IR than the ER formulation (Table 1; Fig. 3a). Although formation rates for glucuronide and sulfate metabolites appeared to be influenced to some extent by formulation, these differences were not statistically significant (Fig. 3b, c).

Analysis of total body exposure for APAP adducts (i.e., AUC) was somewhat limited because of the prolonged elimination half-life (Table 1; Fig. 2) and the limited number of postpeak samples obtained from some of the participants (i.e., only 6/9 had 16- and 24-h samples taken). Thus, determination of AUC<sub>0–12</sub> for the two formulations was performed. No relationship between AUC<sub>0–12</sub> for either APAP or adducts was noted as a function of dose (data not shown). Comparison of mean residence time (MRT) between formulations indicated that the MRT of APAP and glucuronide were shorter following administration of the IR

**Fig. 4** Mean residence time (MRT) for **a** acetaminophen (APAP), **b** APAP glucuronide, **c** APAP sulfate, and **d** APAP protein adducts for immediate- and extended-release APAP. The *solid bold lines* indicate data median, and *boxes* indicated the 25th to 75th percentiles. *Whisker lines* indicated minimum and maximum data values. *○* denotes outliers



formulation compared with the ER formulation ( $5.0 \pm 0.8$  vs.  $6.3 \pm 0.8$  h,  $p=0.004$ ;  $7.8 \pm 0.9$  vs.  $9.9 \pm 2.3$  h,  $p=0.01$ , respectively) (Fig. 4). No differences in MRT for APAP sulfate or APAP protein adducts were observed between formulations.

## Discussion

APAP toxicity is responsible for approximately half of all cases of ALF in the United States [1] and is a major cause of ALF in Great Britain and other countries in Europe [10]. We previously found that measurement of APAP protein adducts by HPLC-EC accurately distinguished between well-characterized cases of APAP-related and other known causes of ALF [2]. It is noteworthy that the elimination half-life of APAP protein adducts is long (Table 1) and exceeds that of the parent compound [3]. Patients with APAP-related ALF typically present for medical evaluation in the later stages of toxicity, and diagnosis of ALF etiology may be challenging due to difficulties in obtaining accurate histories

and the relatively rapid clearance of APAP in the systemic circulation. Thus, APAP protein adduct measurement in patients with ALF of unknown or questionable etiology may be of diagnostic value. In two previous studies, we detected high levels of APAP protein adducts in 18 and 19 % of patients with ALF of unknown etiology [2, 11]. The levels of adducts in patients with APAP-related ALF were  $>1.0$  nmol/ml. Criteria for the diagnosis of APAP-related ALF were ALT values  $>1,000$  IU/L, encephalopathy, and coagulopathy [2, 11].

An important component of biomarker validation is characterizing the dynamic range of the biomarker within various patient populations. The HPLC-EC assay used in this study provides a measurement of the total concentration of protease-cleaved APAP protein adducts in peripheral blood. We previously reported that adducts can be detected at low levels in patients receiving multiple therapeutic doses of APAP in the clinical setting [8]. The availability of samples from a single-dose study comparing two formulations of APAP allowed us to continue this line of research and to compare formation and decay characteristics of APAP and

APAP protein adducts within a single study. Data demonstrate that adducts are formed following single-dose (lower-dose) exposure to APAP, albeit at levels approximately two orders of magnitude lower than observed in APAP-induced ALF. The dose of APAP administered to study participants was approximately 20 % greater than the standard daily dose of APAP previously recommended by the manufacturer (4 g) and approximately half the dose known to be toxic. Due to safety concerns associated with frequent use of APAP, the manufacturers of APAP recently announced the lowering of the daily dose from 4 g to 3 g.

Mean  $C_{\max}$  for adducts (0.10 nmol/ml adducts) in this study was comparable between the two formulations (Fig. 2), suggesting that total APAP dose rather than the APAP absorption profile influences the extent of adduct formation. Importantly, the value for  $C_{\max}$  observed in this study is considerably lower than peak levels of adducts previously observed in patients with APAP-induced ALF. In the ALF study, patient samples were collected at a mean time of 73 h after large overdoses of APAP, and the mean peak levels were 10.85 ( $\pm$ 9.26) nmol/ml adducts [3]. Thus, levels of adducts following low-dose APAP administration in healthy adults are approximately two orders of magnitude below those measured in the acute stages of APAP-related ALF. Finally, differences were noted between ER and IR formulations for the formation rate of APAP protein adducts. Whereas the formation rate for adducts may have been secondary to differences in absorption profiles between the two formulations, this finding also speaks to the precision and sensitivity of the HPLC-EC assay.

The significance of low levels of APAP protein adducts in the blood samples of participants receiving doses of APAP considered to be nontoxic is unclear. The traditional understanding of covalent modification in APAP toxicity is based on time-course studies conducted in the mouse model of APAP toxicity. Early studies showed that APAP protein adducts were formed following depletion of hepatic glutathione and saturation of conjugation pathways [12, 13]. However, these early studies were based on methods for detection of APAP protein adducts that were less sensitive and precise than the HPLC-EC methodology used in this study. The finding of low levels of adducts in peripheral blood in humans receiving low doses of APAP is consistent with the concept of localized depletion of hepatic glutathione and subsequent adduct formation in centrilobular hepatocytes that metabolize APAP as it enters the liver through the portal vein. Measurement of liver glutathione, the main source of glutathione in humans, is not possible due to the invasive nature of the required methodology. Also, peripheral measurements of glutathione do not accurately reflect liver levels of this intracellular defense mechanism for oxidative stress. In contrast to earlier assays used in previously conducted studies [7, 14], the HPLC-EC assay is capable of

detecting adducts at a lower magnitude of APAP exposure prior to the onset of overt hepatocyte injury and below the sensitivity threshold of hepatic transaminase assays available in clinical laboratories. Thus, data presented herein demonstrate the capability, precision, and sensitivity of the HPLC-EC assay for determining APAP exposure, and they complement previous reports on APAP protein adducts [2, 3, 5, 8] by providing new data on anticipated levels of adducts in volunteers following APAP doses considered to be subtoxic.

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