Acetaminophen Protein Adducts in Hospitalized Children Receiving Multiple Doses of Acetaminophen

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Abstract

Previous reports have questioned the safety of multiple doses of acetaminophen administered to ill children. Acetaminophen protein adducts (adducts) are a biomarker of acetaminophen-induced liver injury and reflect the oxidative metabolism of acetaminophen, a known mechanism in acetaminophen toxicity. In this prospective observational study, we analyzed adduct concentrations in 1034 blood samples obtained from 181 hospitalized children (1 to 18 years inclusive) who received 2 or more doses of acetaminophen. Linear regression analysis showed that serum adduct concentrations increased as a function of the cumulative acetaminophen dose, which could be attributed, in part, to a long half-life of adducts (2.17 ± 1.04 days [mean ± standard deviation]) in children. However, few patients (2%) were found to have adduct concentrations higher than 1.0 nmol/mL, a previously identified toxicity cut point for the diagnosis of acetaminophen-induced liver injury in patients with alanine aminotransferase values exceeding 1000 IU/L. A small cohort of patients with suspected infection was noted to show higher adduct concentrations. In addition, adduct concentrations showed a stronger correlation with cumulative acetaminophen doses in adolescents compared with children ($R^2 = 0.41$ vs 0.26). No other covariates (body weight, body mass index z score, sex, race, or surgery) remarkably correlated with adduct elevation. In summary, low levels of adducts can be detected in hospitalized children receiving multiple doses of acetaminophen, and adduct levels correlate with cumulative acetaminophen dose.

Keywords

acetaminophen protein adducts, biomarkers, cumulative doses, hepatotoxicity, pediatrics

Pain and fever are symptoms commonly encountered in childhood, and acetaminophen (APAP) remains a drug of choice for treatment in this population. APAP is generally considered safe when used on an as-needed basis at doses recommended by the manufacturer.[1] Supratherapeutic doses of APAP, including those associated with intentional overdose, may cause severe liver injury in some cases.[2] Notably, some reports have questioned the safety of APAP when administered at the upper end of recommended doses over a multiple-day period.[3,4] A study published by Watkins et al reported that more than 30% of adults administered APAP 4 g/day for 4 days experienced elevations in alanine aminotransferase (ALT) exceeding 3 times the upper limit of normal,[5] whereas a later study found only mild or modest ALT elevations with prolonged APAP administration.[6] In children, liver injury secondary to repeated dosing of APAP is thought to be rare but has been observed in several case reports.[7–10] To date, development of occult toxicity in children receiving APAP over multiday periods has not been extensively evaluated, and it remains to be clarified whether repeated doses of APAP increase the risk of liver injury in children.[11] Some clinical conditions such as glutathione depletion, which may occur with inadequate nutrient intake, may further increase the risk of hepatotoxicity.[12] As safety data on the long-term use of APAP in children are lacking, more research is needed to address this question.

APAP is primarily metabolized by phase II metabolic enzymes through glucuronidation and

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sulfation in the liver.[13] After the administration of a therapeutic dose, phase I enzymes (cytochromes P450 [CYP] 2E1,[14] 2A6,[15] and 2D6[16]) contribute to around 5% of APAP metabolism.[17] Oxidation of APAP forms the toxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is rapidly detoxified to APAP-glutathione and then further converted to other nontoxic metabolites. When high exposure to APAP depletes hepatic glutathione, NAPQI covalently binds to cysteine residues of proteins in hepatocytes to form NAPQI-protein conjugates (APAP protein adducts). APAP protein adducts are released from hepatocytes as these cells rupture and can subsequently be detectable in peripheral blood.[18] Measurement of adducts by high-performance liquid chromatography with electrochemical detection (HPLC-EC) has shown that adducts are both highly sensitive and specific for APAP toxicity of ALT > 1000 IU/L, with a proposed concentration of 1.0 nmol/mL, signaling APAP-induced liver injury.[19–21]

The majority of previous research measuring APAP protein adducts has focused on single acute overdose or chronic APAP administration in adults.[22–24] In this prospective clinical study, we compared adduct measurements with APAP doses and biochemical measures of liver injury in hospitalized children who received multiple doses of APAP according to the standard of care. We also examined the relationships between clinical factors and adduct concentrations.

Methods

Study Design and Analytical Methods

This was a prospective, multicenter observational study designed to evaluate biomarkers of APAP toxicity in pediatric patients. Participants were enrolled with informed permission/assent under a protocol reviewed and approved by the institutional review boards at the participating institutions (see acknowledgments for the institution list). Written informed consent was obtained from the participant's parent/legal guardian before participation in the study. Hospitalized patients aged 1-18 years inclusive who were receiving or expected to receive at least 2 doses of APAP during the hospitalization were eligible for participation. Patients with a history of acute or chronic APAP overdose within 14 days and those with a known history of liver disease or dysfunction were excluded.

Information collected on study participants included demographic data along with APAP dosing (prior to and during hospitalization), formulation, comedications, dosing interval, and the primary reason for the hospitalization (Table 1). Blood samples were collected for serum transaminase levels (aspartate aminotransferase [AST] and ALT) throughout the study and measured in local clinical laboratories of participating institutions. For quantitation of APAP protein adducts, blood samples were collected 8 and 24 hours after the first dose of APAP ("first dose" was defined as the first dose of APAP received once patients were enrolled in the study, designated by signed consent); subsequent samples were collected during predefined study periods for patients continuing to receive APAP as follows: days 2-6, days 7-14, days 15-21, and days 22-28. In addition, a blood sample was obtained 48 hours after discontinuation of APAP. Scavenged blood samples (ie, samples no longer needed for clinical purposes) were also obtained for measurement of APAP protein adducts. Blood samples were collected in nonethylenediaminetetraacetic acid-containing vials and centrifuged within 1 hour of blood draw. The serum portion was removed and stored at -70°C until the time of sample analysis. The samples were shipped to a central, research laboratory and analyzed as

Table 1. Demographics and Clinical Characteristics of Patients

Demographic Characteristics	Patients, n (%)
Total	181
Age (years)	
Infants (1 to <2)	11 (6.1)
Children (2 to <12)	102 (56.4)
Adolescents (12 to <18)	68 (37.6)
Sex	
Male	103 (56.9)
Female	78 (43.1)
Race	
African American	36 (20.0)
White	130 (72.4)
Asian	3 (1.7)
Other[a]	12 (6.6)
Surgery	
Yes	76 (42.0)
No	105 (58.0)
Primary admission diagnosis	
Cardiovascular	51 (28.2)
Fluids/electrolytes/nutrition	4 (2.2)
Gastrointestinal	14 (7.7)
Infection (suspected)[b]	22 (12.2)
Musculoskeletal	16 (8.8)
Neuropsychiatric	26 (14.4)
Oncologic	9 (5)
Respiratory/ENT	23 (12.7)
Trauma/burn	14 (7.7)
Other[c]	2 (1.1)
Physiological characteristics	**Mean ± SD**
Body weight (kg)	38.6 ± 30.3
Height (cm)	126.6 ± 32.3
BMI z score	0.4 ± 1.8

[a]More than 2 or unknown races.
[b]Suspected infection included patients with either suspected or presumed infection.
[c]Missing information on primary admission diagnosis.

Table 2. Summary of APAP Dosing and Sampling

	Mean	Median	5%-95% Percentiles[b]	Min-Max
Number of doses	7.8	5.0	2.0-19.0	2.0-76.0
Interval of dosing (hours)	11.0	6.3	3.1-34.9	2.0-42.0
Duration of treatment (days)	3.7	1.8	1.0-14.0	1.0-26.3
Dose (mg/kg)[a]	12.3	12.1	5.6-17.6	3-35.1
Number of samplings	5.7	4.0	1.0-14.0	1.0-26.0

[a]Because it is a multiple-dose study, dose is defined as the mean dose (the total dose divided by the total number of doses during the entire treatment) for an individual patient, resulting in 181 (mean) doses calculated for 181 patients.
[b]Percentile instead of standard deviation (SD) was presented because the distributions of these variables were highly skewed.

singlets in batches within 6 months of sample collection. APAP protein adduct analysis was conducted using a previously reported HPLC-EC method[19] by a single laboratory technician who was blinded to the clinical histories of the study participants. The lower limit of quantification (LLOQ) for the assay was 0.03 nmol/mL, with a coefficient of variation (CV) of less than 15%. Body mass index (BMI) z scores were calculated using the online calculator[25] based on patients' body weight, height, sex, and age.

Exploratory Data Analysis

Patient demographics, clinical conditions, and APAP dosing and sampling history were summarized (Tables 1 and 2). The clinical data were explored by graphical analysis and Pearson correlation analysis to assess the relationships among APAP doses, adduct concentrations, and transaminase levels. AST and ALT values were linked to adduct measurements obtained within 48 hours of the adduct sample if adduct measurements were not obtained at the same time as the hepatic transaminase tests. The evaluated APAP doses included unnormalized and body-weight-normalized mean and cumulative doses. The cumulative dose was defined as the total dose received before a blood sample was obtained for adduct measurement for an individual patient; the mean dose was calculated as the cumulative dose divided by the number of doses that occurred before a given adduct measurement. Where possible, the calculated APAP doses included any reported doses prior to hospitalization. The adduct concentrations and APAP doses were evaluated both as untransformed and log-transformed values.

Linear Regression Analysis

A standard parametric linear regression model was developed in R (version 3.4) to investigate the relationship between the natural log-transformed cumulative dose (mg/kg) and natural log-transformed adduct concentrations. Given the sparse and unbalanced sampling scheme, for simplicity, only adduct concentrations obtained within 48 hours of an APAP dose were included in the regression analysis. The base bivariate linear model consisted of an intercept term and a slope term multiplied by the cumulative dose as the explanatory variable for adduct concentrations. The goodness of fit was assessed by diagnostic plots and coefficient of determination (R^2). A sensitivity analysis omitting the patients dosed with APAP prior to admission was performed to evaluate the impact of potential data inaccuracy in the screening period. After the linear relationship between the cumulative dose and adduct concentrations was established, multiple regression analysis and analysis of covariance (ANCOVA) were performed to evaluate the correlation between various covariates (age, body weight, BMI z score, sex, race, surgery, and primary admission diagnosis) and adduct concentrations. In addition, regression and correlation analysis were performed on the patient data stratified by categorical covariates (eg, age group, race, and primary admission diagnosis). For any cohort showing a significant covariate effect, the distribution of sampling points and formulations were analyzed to investigate the potential confounding of unbalanced sampling and/or prevailing formulation on the statistical analysis. The age groups were categorized as infants (1 to <2 years), children (2 to <12 years), and adolescents (12 to <18 years).

Individual Patient Analysis

Clinical data were screened for atypical patterns as well as outliers. The patients displaying either a high adduct level (≥1.0 nmol/mL) or a high transaminase level (≥1000 IU/L) were examined on an individual basis to investigate the possible causes for elevated adduct concentrations or abnormal liver function.

Adduct Half-Life Analysis

The apparent adduct half-life was derived from the terminal slope of the natural log-transformed adduct concentrations when sufficient ($n \geq 3$) sampling points after the most recent APAP dose were available. Those sampling points included adduct measurements both within and beyond 48 hours post-APAP administration. The R package PKNCA[26] package was used to compute the terminal slope (λ_z) and the half-life. Only terminal phases showing good linearity (adjusted $R^2 > 0.7$) were used for slope calculation. The estimated half-life was compared with previously reported adduct half-life[20] by the Student t test. The potential covariates affecting the adduct half-life were evaluated by Spearman's rank correlation test for continuous variables (age, body weight, and BMI z score) and 1-way analysis of variance for categorical variables (sex, race, surgery, and primary admission diagnosis).

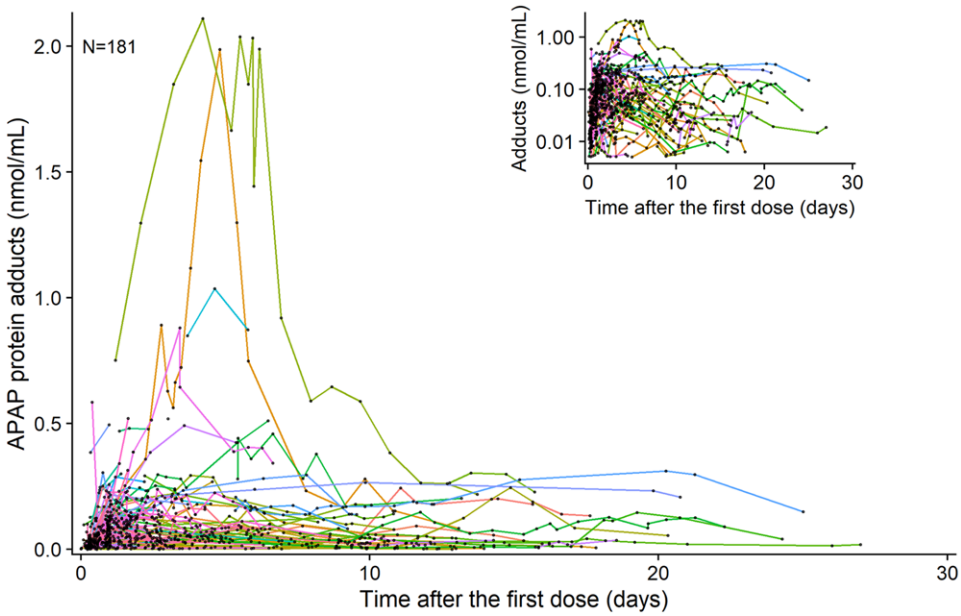

Figure 1. Profile of APAP protein adduct concentrations for all patients (top right: adducts on a semilog scale). Colors in line represent the individual patients.

Results

Exploratory Data Analysis

A total of 1034 adduct measurements were obtained from 181 research patients. Accordingly, a total of 1034 cumulative doses and 1034 mean doses were computed. The demographic characteristics of the pediatric patients are summarized in Table 1. APAP formulations administered consisted of injections (6.9%), suppositories (15.3%), elixirs (16.6%), solutions (28.2%), and tablets (32.3%), with 35.2% of patients having received more than 1 formulation. In addition, 29% of the patients received either opioid (hydrocodone, codeine, or oxycodone)- or diphenhydramine-containing formulations. The number of available blood samples per patient ranged from 1 to 26, and most of the patients (73%) had 2-7 samples obtained (Table 2). Transaminase concentrations were available for 174 of the 181 patients. Among the 174 patients, 112 patients had both AST and ALT measurements, whereas the remaining patients had only ALT measurements. None of the patients had renal insufficiency or reported taking alcohol or isoniazid, which have the potential to interact with APAP hepatic metabolism through CYP2E1 pathway.[27,28]

The adduct concentration varied widely across patients spanning a full order of magnitude (Figure 1). However, 98% of adduct concentrations (1012 of 1034) were below the previously determined toxicity cut point of 1.0 nmol/mL.[20,21] High adduct concentrations were noted in 3 of 181 patients (2%), and these 3 patients are discussed further in the following section of individual patient analysis. For correlation analysis, the highest strength of association ($R^2 = 0.31$) was found between the natural log-transformed cumulative doses (mg/kg) and natural log-transformed adduct concentrations, which was followed by a marginal correlation ($R^2 = 0.04$) between the mean APAP dose (mg/kg) and the adduct concentration. The AST and ALT values were low or modestly elevated (<1000 IU/L) for the majority of the patients (99.5%) with 19-182 IU/L (5%-95% percentiles) and 9-163 IU/L(5%-95% percentiles) for AST and ALT, respectively. Only 1 patient showed AST and ALT levels greater than 1000 IU/L.

Linear Regression Analysis

Figure 2 depicts the regression line describing the positive and linear relationship between the log-transformed cumulative dose and log-transformed adduct concentration. This analysis covered 96% of the available adduct concentrations, as the remaining ones were beyond 48 hours after the most recent dose. The slope of the base model was estimated to be 0.75 ± 0.04 ($P < .001$, mean $\pm$ SE), demonstrating a significant increase in adduct concentrations as the cumulative dose increases. The estimated R^2 value indicated that 31% of the variability in adduct concentration could be explained by cumulative dose. When this analysis was performed on the data (n = 68) excluding patients who received APAP prior to admission, the slope of regression and R^2 showed minor disparity (Supplemental Figure S1) from the model using the entire cohort data, demonstrating that inclusion of the

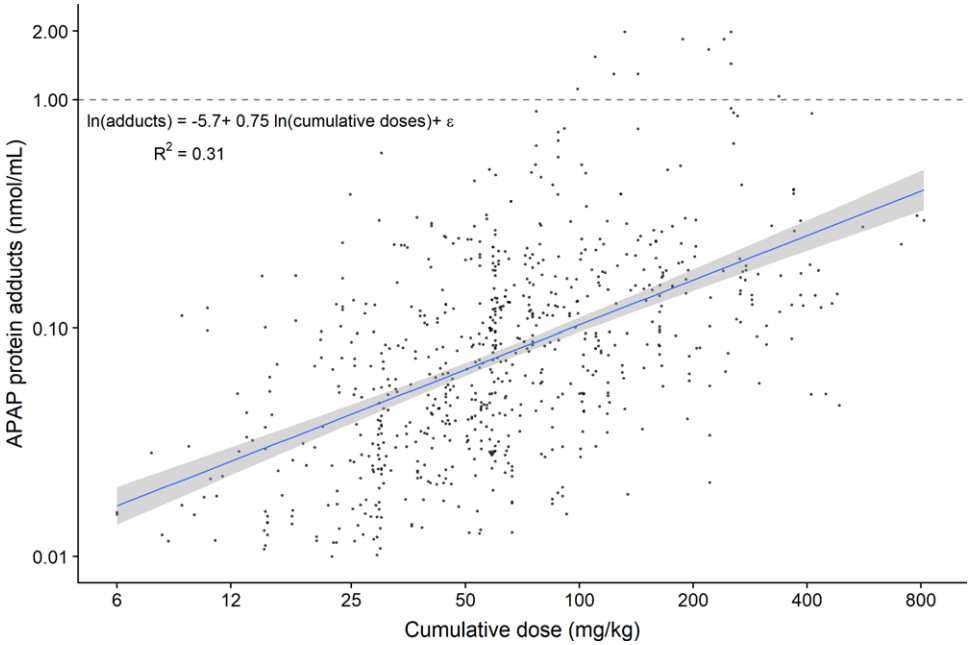

Figure 2. Linear regression of APAP protein adduct concentrations within 48 hours after the most recent dose. Solid line, regression line of adduct concentrations against cumulative doses. Shaded area of regression line, 95% confidence interval for predicted adduct concentrations. Dotted line, cut point of 1.0 nmol/mL for diagnosis of APAP toxicity. ϵ, random error.

preadmission APAP dosing data had minimal impact on the results.

Multiple regression analysis and ANCOVA indicated that the addition of an admission diagnosis improved the model fitting to a relatively large extent, whereas age demonstrated only a mild effect on the linear model (Supplemental Table S1). Variations in slope and/or intercept were also observed as a function of diagnosis and age. Consequently, we further evaluated the effect of diagnosis and age using stratified data analysis (Figure 3). Patients admitted with suspected or presumed infection (n = 22) had remarkably higher adduct concentrations than those observed for patients admitted for noninfectious diseases (mean concentrations, 0.35 vs 0.11 nmol/mL). The number of sampling points (mean, 4.5 samples; median, 3.0 samples) for patients in this cohort did not show a significant deviation from those (mean, 5.7 samples; median, 4.0 samples) of the entire patient population. The infection and noninfection groups had a similar distribution of formulations as well. This observation indicates that neither sampling schemes nor formulation contributed to the relationship between adduct concentrations and infection. The subgroup of infants showed a distinctive slope of 1.35, representing more rapid elevation of adduct concentrations than other age groups, whereas all adduct concentrations were far below 1.0 nmol/mL for this subset. Compared with children, adduct concentrations in adolescents were more concentrated along the regression line, leading to a stronger correlation (R^2 = 0.41 vs 0.26) of adduct levels with the cumulative doses. No sampling bias was found in the stratified data by age group. The most commonly used formulations (frequencies ≥ 25%) stratified by age were suppository (33%) for infants, solution/elixir (48%) for children, and tablet (61%) for adolescents.

Individual Patient Analysis

The clinical histories of 3 patients with adduct concentration ≥ 1.0 nmol/mL were reviewed and are summarized as case reports in Supplemental Table S2. There was no evidence that any comedication was associated with these abnormal adduct levels. When adduct concentrations exceeded 1.0 nmol/mL, all these patients had cumulative doses close to or greater than 100 mg/kg. By comparison, the distribution of hepatic transaminases and adduct levels for children with cumulative doses greater than 100 mg/kg ranged from 14 to 131 IU/L for AST, 7 to 214 IU/L for ALT, and 0.02 to 2.1 nmol/mL for adducts.

A fourth patient received two APAP doses (15 mg/kg per dose) in the 24 hours prior to cardiac surgery. AST and ALT prior to surgery were 66 and 18 IU/L, respectively. However, the patient developed cardiac arrest after surgery, and AST and ALT rose to 3940 and 9741 IU/L, respectively. Because her adduct concentrations were all below 0.10 nmol/mL, the elevation of transaminase was attributed to hepatic ischemia instead of APAP dosing.

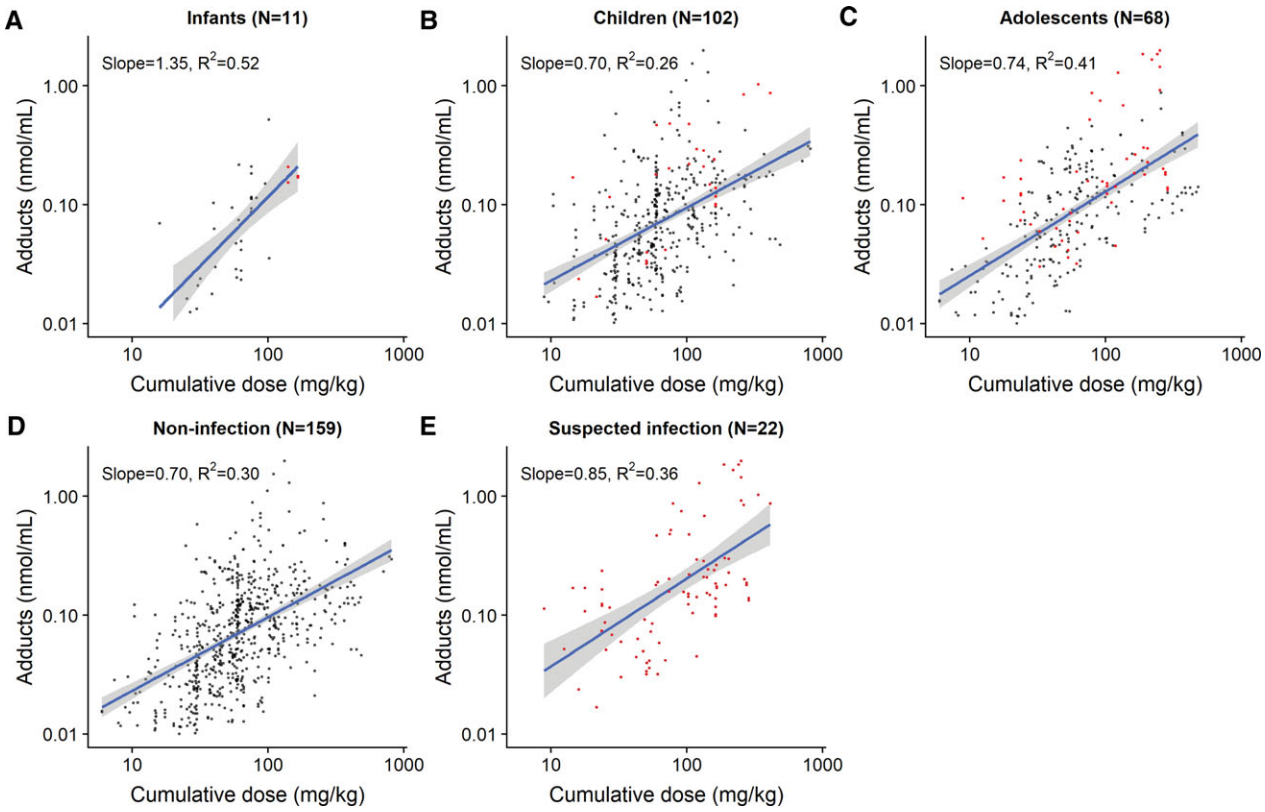

Figure 3. Analysis of adduct concentrations stratified by age (A, B, C) and primary admission diagnosis (D, E). Red dots, adduct concentrations in patients with suspected infection. Solid line, regression line of adduct concentrations against cumulative doses. Shaded area of regression line, 95% confidence interval for predicted adduct concentrations.

Adduct Half-life Analysis

The adduct concentrations of 8 patients were used to determine the adduct half-life because only these patients had sufficient sampling points ($n \geq 3$) for calculation of the terminal slope (Figure 4). The half-life was estimated to be 2.17 ± 1.04 days (mean $\pm$ standard deviation [SD]), ranging from 1.04 to 4.13 days. The large variation in half-life limited the statistical power of comparison; hence, we could not determine whether the adduct half-life we observed after multiple therapeutic doses was longer than the half-life (1.47 ± 0.30 days)[29] reported for children after acute overdose. In addition, no covariates were found to significantly affect the half-life ($P > .05$ for all tested covariates). The individual half-life and associated demographic characteristics are listed in Supplemental Table S3.

Discussion

To date, very few clinical studies have investigated the potential toxicity of repeated therapeutic doses of APAP administered to hospitalized children.[7,30–32] The present study evaluated the relationships between APAP doses, AST and ALT values, and APAP protein adduct levels in 181 hospitalized children. Our data demonstrate that APAP protein adduct levels can be detected at low levels in hospitalized children receiving therapeutic doses of APAP. Moreover, we found that a relatively low percentage of hospitalized children (2%) had adduct levels above the previously identified toxicity cut point for adducts of 1.0 nmol/mL, a value associated with ALT elevation >1000 IU/L in adults following acute APAP overdose. Our findings contribute to a body of literature evaluating the relationships of adducts to transaminase values in patients in different clinical settings.[23,24,32] Although 98% of adduct concentrations were below 1.0 nmol/mL, we observed a dose-dependent relationship for APAP protein adducts, a finding previously noted in experimental models of APAP toxicity.[33–35] To our knowledge, the present study is the first to observe and quantify this relationship in the clinical setting of APAP dosing in hospitalized children. It should be noted that the present study was associated with considerable variability because of varying disease conditions, sampling times, APAP formulations, and dosing frequency. To overcome such wide variability, we applied log-transformation to the adduct data aiming to capture the changes in the magnitude of adduct concentrations given certain APAP doses. A linear and positive relationship was found between

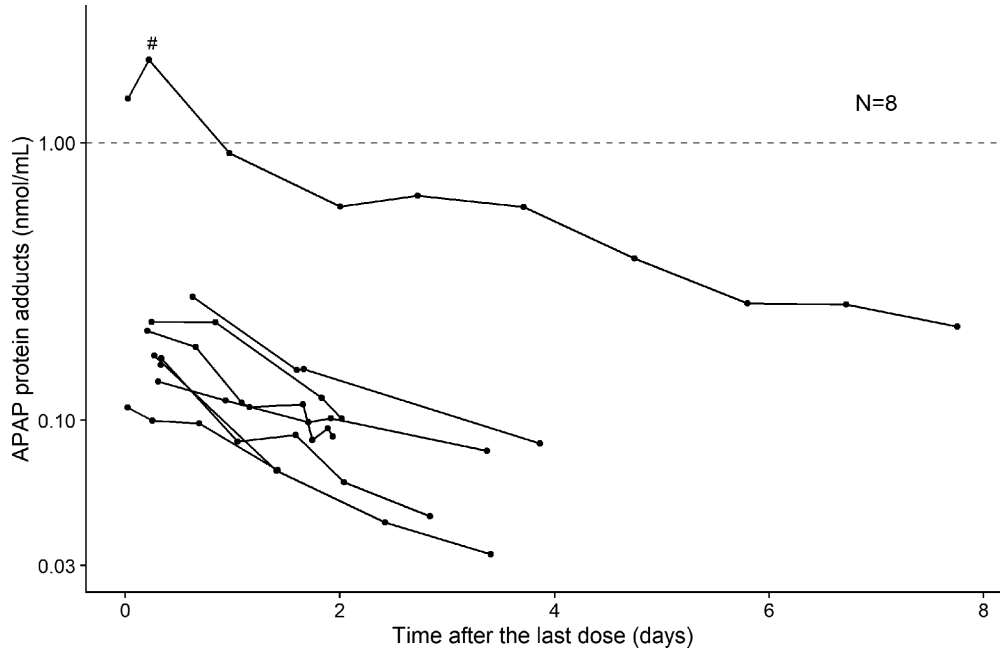

Figure 4. Analysis of adduct half-life. Black dots represent adduct concentrations after the last dose of APAP. **#**, Patient with ≥1.0 nmol/mL adduct concentration. The case report of this patient is included in Supplemental Table S2.

the log-transformed cumulative dose and the log-transformed adduct concentrations, with an R^2 of 0.31. Given the great diversity of the study patients (eg, age, race, and admission diagnosis) and heterogeneity of the dosing scenarios (eg, formulations and dosing interval) in this observational study, this R^2 value demonstrates a fair correlation between the cumulative APAP dose and adduct concentration.

Among clinical covariates, patients with suspected infection had higher adduct concentrations than patients with other admission diagnoses. The reason for this observation is unclear. A previous study, in which mice with active adenoviral infection were treated with APAP, found that mice were protected from APAP liver injury and that the CYP2E1 activity was reduced.[36] Multiple other mechanistic pathways have been implicated in APAP toxicity (eg, oxidative and/or nitrosative stress, mitochondrial injury) and may have contributed to the findings that we observed in this study. Moreover, infants showed a distinctive adduct profile with rapid adduct elevation (Figure 3A), which may be related to their unique physiological characteristics. Because of the limitation of this small cohort (n = 11), we could not make further inference at this stage. The higher correlation between the cumulative APAP dose and adduct concentration in adolescents (Figure 3C) is most likely associated with 2 factors: (1) less diversity in formulations used in adolescents as tablets accounted for more than half of the APAP formulations and (2) the relative maturation of drug metabolism pathways in adolescents compared with children.

Other than the identified covariate effect, the remaining sources of variability can likely be attributed to factors not directly investigated in the present study. For instance, the liver abundance of CYP2E1 carries considerable interindividual variability in humans, although CYP2E1 is considered to lack clinically relevant polymorphisms. Various data sources have reported 4- to 20-fold variation in CYP2E1 level per unit human microsomal protein.[37] In addition to the potential variability of adduct formation mediated by CYP2E1, the observed adduct half-life in the current study was also highly variable (RSD, 48%). This may have, in part, contributed to the elevation of adduct levels following repeated APAP dosing because the dosing intervals (6 to 11 hours) were much shorter than the observed adduct half-life (2.17 days). Another possibility contributing to the adduct elevation is the accumulation of APAP following chronic dosing in children, as reported by Nahata et al.[30] However, other reports failed to observe accumulation of APAP,[38,39] and our study did not include APAP measurements that are needed to further explore this possibility.

The clinical implication of the above findings remains to be seen. The HPLC-EC assay has remarkable sensitivity, with an LLOQ of 0.03 nmol/mL.[19] Previous data using time-course and dose-response designs in experimental models of APAP toxicity have shown that liver adducts and circulating adducts may be detected

prior to ALT elevations.[33–35] The exact mechanism of adduct release from cells is still unknown. A recent study examined whether adducts are released by extracellular vesicles, a known pathway for extracellular cargo transport; it found no adducts in extracellular vesicles.[40] Although adducts have a strong correlation with ALT values in patients with ALT > 1000 IU/L, the correlative relationship was found to be weaker at lower ALT concentrations in an earlier study,[41] which agreed with our findings. This observation may be because of the variations in the mechanisms of adduct and hepatic transaminase release, variation in assay sensitivity between analytical laboratory methods, or other factors.

Conclusions

APAP protein adduct levels obtained from a large cohort of hospitalized children with various admitting diagnoses who were receiving APAP were low and not at levels previously associated with APAP-related acute liver injury. Because patients with higher cumulative doses of APAP had higher adduct levels, careful monitoring of the need for scheduled APAP versus "as-needed" dosing appears prudent.

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Declaration of Conflict of Interest

Dr. Laura James is a part owner of Acetaminophen Toxicity Diagnostics (ATD), LLC. ATD is developing a rapid assay for the measurement of acetaminophen protein adducts in human blood samples.

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Data Sharing

The data supporting the findings of this study may be shared on request from the corresponding author Dr. Laura James at JamesLauraP@uams.edu.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.