Acetaminophen Elimination Half-Life in Humans Is Unaffected by Short-Term Consumption of Sulfur Amino Acid-Free Diet

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ABSTRACT

Sulfation and glutathione (GSH) conjugation are important pathways for elimination of acetaminophen (APAP). Previous studies in rodents show that limitation of dietary sulfur amino acids (SAAs) reduces biosynthesis of 3'-phosphoadenosine-5'-phosphosulfate, the precursor for sulfation, and GSH, the precursor for the mercapturic acid pathway. The amount of SAA needed for the metabolism of two doses of APAP is equivalent to 62% of the recommended dietary allowance (RDA) for SAA in humans. A decrease in the activity of these metabolic pathways could lead to decreased elimination of the reactive metabolite of APAP and possibly affect risk of APAP use. To determine whether intake of a SAA-deficient diet alters APAP metabolism, a pilot clinical study with a double-blind, cross-over

design was performed. Subjects received the RDA for SAA for 3 days for equilibration. After admission to the clinical research unit, subjects were given a chemically defined diet with 100 or 0% of the RDA for SAA for 2 days. On day 3, two doses of APAP (15 mg/kg) or placebo were given successively within a 6-h interval. Plasma samples were collected at baseline and hourly for 12 h, and two 6-h urine aliquots were collected during this time course. The data show that SAA limitation 1) did not change the pattern of APAP metabolites in plasma or urine and 2) did not alter APAP pharmacokinetics. Thus, the results show that 2 days of diet completely devoid of SAA have no effect on APAP metabolism or disposition in healthy humans.

Acetaminophen (APAP) is one of the most widely used antipyretic and analgesic drugs in the world. After therapeutic doses, approximately 3% APAP is excreted in the urine unchanged (Brodie and Axelrod, 1948). Approximately 90% of APAP is metabolized by way of conjugation reactions where two-thirds is metabolized through glucuronidation and one-third through sulfation (Brodie and Axelrod, 1948). The remaining 5 to 9% is converted by human cytochrome P450 2E1 to its reactive intermediate, *N*-acetyl-*p*-benzoquinimine (Dahlin et al., 1984). In the presence of glutathione (GSH), this metabolite is rapidly detoxified and excreted in the urine as GSH, cysteine (Cys), and mercapturic acid conjugates (Mitchell et al., 1974; Andrews et al., 1976).

Sulfur amino acids (SAAs), methionine (Met) and Cys, are required for drug metabolism through the use of Cys for

biosynthesis of sulfate and GSH (Jones et al., 1995; McCarver and Hines, 2002). Previous studies of SAA availability and APAP metabolism showed that prolonged dietary deficiency of Cys and Met significantly lengthened the half-life ($t_{1/2}$) of a low dose of APAP in rodents (Price and Jollow, 1989). The markedly slower elimination of APAP in SAA-deficient animals was caused primarily by a depression of sulfate conjugation. Short-term dietary deficiency of Cys and sulfate slowed the elimination of APAP from the blood and significantly decreased the urinary excretion of the APAP-sulfate conjugate (Gregus et al., 1994). Moreover, rodents with diet-induced SAA deficiency converted more APAP into its toxic metabolite, as suggested by increased excretion of APAP-Cys and APAP-mercapturate conjugates (Gregus et al., 1994). Taken together, these studies suggest that nutritional deficiency of SAA can affect the individual pathways of APAP metabolism by impairment of sulfate conjugation. This impairment could potentially enhance the susceptibility to APAP-induced liver injury.

Although the mean SAA intake in Americans is considerably higher than the recommended daily allowance (RDA) of 1.9 g/day (Food and Nutrition Board, 2005), individual SAA

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ABBREVIATIONS: APAP, acetaminophen; AUC, area under the curve; CL, clearance; E_h , redox potential; RDA, recommended dietary allowance; SAA, sulfur amino acid; $t_{1/2}$, half-life; V_d , volume of distribution; HPLC, high-performance liquid chromatography.

intake is variable and can range from <0.3 to >5 g per day (Flagg et al., 1994). Considering that a molar equivalent of approximately 0.2 g of Cys is used for metabolism of a maximal therapeutic dose of 1 g of APAP, four doses per day would consume 0.8 g, or more than half of the RDA. Because of variation of intake in the population, the total equivalents of SAA needed for metabolism of the maximum daily dose of APAP can exceed individual intake of SAA.

The present study was designed to determine whether short-term SAA insufficiency alters APAP metabolism in healthy individuals. The experiment was designed with two study periods in which each individual was equilibrated to either the RDA or 0% of the RDA for SAA before APAP administration in two successive doses of 15 mg/kg, 6 h apart. Young healthy individuals were studied under conditions where there are no known risks for either APAP use or short-term SAA insufficiency. The specific hypothesis was that 2 days of a SAA-free diet would cause changes in the metabolic pattern and pharmacokinetics of APAP in the plasma and urine.

Materials and Methods

Materials. Authentic standards of APAP metabolites (APAP-glucuronide, APAP-sulfate, APAP-Cys, APAP-GSH, and APAP-mercapturate) were a generous gift from Dr. José Manautou (University of Connecticut, Storrs, CT). Methanol and acetic acid were high-performance liquid chromatography (HPLC) grade.

Human Subjects. This study was reviewed and approved by the Emory Investigational Review Board. The study was designed to determine the effect of 2 days of SAA-deficient diet on APAP metabolism as part of a study that evaluated the effect of APAP on plasma cysteine and glutathione pools. The latter is described along with experimental details in an accompanying article (Mannery et al., 2010). In brief, 12 healthy volunteers (18–40 years old) with a body mass index < 27, no acute and/or chronic illness, and no current smoking history were studied under two different conditions with APAP (two doses; 15 mg/kg) and SAA intake (0 or 100% of RDA) (Table 1). Before each inpatient study period, subjects were given an equilibration diet for 3 days, admitted into the Emory General Clinical Research Center, and given a chemically modified diet containing either 100 or 0% SAA for 2 days. During those 5 days, the subjects consumed no alcohol to avoid induction of cytochrome P450 2E1. On day 6 of each study period, APAP (15 mg/kg) or placebo was administered under fasting conditions at 8:30 AM. Subsequently, a meal was given at 12:30 PM, and a second dose was administered at 2:30 PM. Plasma samples were collected hourly for 12 h after APAP or placebo administration, and two 6-h urine samples were collected during this time. Meals were prepared by the bionutrition unit at the Emory University Hospital General Clinical Research Center, and adequate hydration and vitamin, mineral, and electrolyte requirements were provided to meet or exceed recommended allowances (Raguso et al., 2000). Water intake was ad libitum. Body weight was determined daily, and vital signs were obtained every 8 h.

APAP Analysis. APAP and its metabolites in urine were analyzed by using a HPLC method as described previously (Howie et al., 1977; Chen et al., 2003). Aliquots of urine samples (20 μ l) were

injected into a Zorbax SB 5 μ M C₁₈ reverse-phase column (4.6 mm $\times$ 250 mm; VWR, West Chester, PA). APAP and its metabolites were eluted by using a mobile phase composed of 12.5% HPLC-grade methanol, 1% acetic acid, and 86.5% water, run at a flow rate of 1.2 ml/min. The elution of metabolites was monitored at a wavelength of 254 nm. Retention times of APAP and its metabolites were determined by comparison with authentic standards. Quantification was based on integrated peak areas. Concentration of APAP and its metabolites was calculated by using an APAP standard curve (Howie et al., 1977).

APAP and its metabolites in plasma were analyzed by using the HPLC method of Moldéus (1978). Perchloric acid (3 N) was added 2:1 to plasma samples to precipitate protein. After centrifugation and filtration, the supernatant was used for analysis. A linear gradient was used to separate APAP and its metabolites with a flow rate of 1.7 ml/min. Solvent A consisted of 1% aqueous acetic acid. Solvent B was composed of 1% aqueous acetic acid/methanol/ethyl acetate (90:15:1). The mobile phase was kept at 75% A and 25% B for 7 min. This was followed by a 20-min linear gradient that finished at 99% B. The composition of the mobile phase was restored to initial conditions by using an 8-min linear gradient. All other HPLC conditions were the same as those described above.

Statistics. Results are expressed as means $\pm$ S.E.M. We used SPSS software (version 17; SPSS Inc., Chicago, IL) for all analyses. Area under the curve (AUC) values were calculated by using the trapezoidal rule. Paired *t* tests with the Bonferroni correction were used to determine whether time points for respective groups were significantly different. Results were considered significant at *p* < 0.05.

Results

Subject Characteristics. The study population is described in an accompanying article (Mannery et al., 2010). Fifty percent of the subjects were male, the mean age was 25 ± 4 years, and there was no significant difference between males and females. Half of the subjects (*n* = 6) were African American, four were white, one was Hispanic, and one was Native American. All were healthy, with no history or evidence of acute or chronic illness, and none were taking prescription medications. There were no adverse reactions to SAA insufficiency or APAP dose. Because of uncontrollable scheduling difficulties, only eight subjects completed APAP study periods with both SAA sufficiency and insufficiency.

Plasma APAP Metabolite Concentration Was Not Affected by SAA Insufficiency. The effects of SAA limitation on plasma APAP concentration are shown in Fig. 1A, with corresponding AUC data in Fig. 2A. Measurements of the plasma concentration of APAP showed that the peak concentration occurred at 9:15 AM under SAA-insufficient conditions (Fig. 1A). The peak plasma appearance of APAP was delayed to 10:15 AM under SAA-sufficient conditions. The pattern of APAP concentration in the plasma was similar under both dietary conditions (Fig. 1A). Despite a significant effect at the 3:15 PM time point, AUC data show that an SAA-insufficient diet had no overall effect on plasma APAP concentration (Fig. 2A).

TABLE 1

Experimental design

Plasma samples were collected hourly for 12 h following administration of APAP (15 mg/kg) or placebo at 8:15 AM on day 6. Study periods described above were randomized for order and conducted at least 1 week apart.

Study Period	Days 1–3, Outpatient Equilibration	Days 4–5, Inpatient $\pm$ SAA	Day 6, Inpatient $\pm$ APAP
1	3 Days with 100% RDA for SAA	2 Days in GCRC with 100% RDA for SAA	Two sequential doses of APAP
2	3 Days with 100% RDA for SAA	2 Days in GCRC with SAA-free diet	Two sequential doses of APAP

Limitation of SAA did not alter the pattern of APAP-glucuronide concentration in the plasma (Fig. 1B). Despite a significant increase in APAP-glucuronide at the 7:15 PM time point, AUC data confirm that SAA limitation had no overall effect on plasma APAP-glucuronide concentration (Fig. 2A). Similarly, SAA insufficiency did not alter the pattern of appearance or concentration of plasma APAP-sulfate (Figs. 1C and 2B). The pattern of appearance or concentration of APAP-Cys, APAP-GSH, and APAP-mercapturate were not significantly altered by SAA limitation (Figs. 1D and 2B). However, it should be noted that there was a relatively large variation among individuals for the time course data, so this conclusion is based largely on the AUC data (Fig. 2B).

APAP Pharmacokinetics Were Not Affected by SAA Insufficiency. To determine whether SAA limitation altered APAP pharmacokinetics, the volume of distribution (V_d), $t_{1/2}$, clearance (CL), and rate of elimination were calculated after the first dose of APAP. The results showed that the $t_{1/2}$ for APAP under conditions of SAA insufficiency was not significantly different from the $t_{1/2}$ under control conditions (Table 2). The V_d was also similar to control values, indicating that the total aqueous space available for APAP distribution was not affected by SAA insufficiency (Table 2). There was no significant effect of SAA limitation on CL or AUC for APAP, which indicates that SAA insufficiency had no effect on the elimination of APAP (Table 2).

Urinary Excretion of APAP Metabolites Was Not Affected by SAA Limitation. Excretion of APAP in the urine was not affected by SAA limitation (Fig. 3A). Although the concentration of APAP excreted into the urine was higher during the 2:00 PM to 8:00 PM period, this increase was not significant (Fig. 3A). SAA insufficiency did not cause significant changes in the excretion of the glucuronide conjugate (Fig. 3B) or the sulfate conjugate (Fig. 3C) in the urine. SAA limitation appeared to cause an increase in the concentration of APAP-Cys, APAP-GSH, and APAP-mercapturic acid conjugates excreted in the urine during the 2:00 PM to 8:00 PM period; however, the difference was not significant overall (Fig. 3C). Expression of APAP and metabolites as a percentage of total urinary recovery also showed no effect of SAA insufficiency on APAP metabolism (Fig. 4).

Discussion

The present research was a component of a 2×2 factorial human study to determine whether APAP alters plasma Cys or GSH pools in healthy individuals with adequate or inadequate SAA intake (Mannery et al., 2010). APAP decreased plasma Cys with both 0 and 100% SAA intake, having the greatest effect after the 2nd dose of APAP with 100% SAA. These results suggest that metabolism adapts to insufficient SAA intake by mobilizing body stores, i.e., by protein and/or GSH breakdown. Consistent with this, plasma GSH was

Fig. 1. Plasma concentrations of APAP (A) and APAP metabolites (B–D) after 2 days of an SAA-adequate diet (100% of the RDA) or an SAA-insufficient diet (0% of the RDA). APAP and metabolite concentrations were determined by HPLC. Data are expressed as means $\pm$ S.E. for each time point. *, significant at $p < 0.05$; $n = 8$.

Fig. 2. AUC analysis for plasma APAP (A) and metabolites (B) after 2 days of an SAA-adequate diet (100% of the RDA) or an SAA-insufficient diet (0% of the RDA). AUC was calculated by using the APAP metabolite concentrations measured by HPLC analysis (see Fig. 1). *, significant at $p < 0.05$; $n = 8$.

TABLE 2
Pharmacokinetic constants for plasma APAP after short-term SAA insufficiency

	100% SAA	0% SAA	<i>p</i> Value
Max plasma concentration (nmol/ml)	65.4 ± 7.2	61.6 ± 8.9	
AUC (mg · L ⁻¹ · h)	15.6 ± 1.9	14.0 ± 0.97	0.41
V _D (L)	75.8 ± 9.3	79.4 ± 8.0	0.70
<i>t</i> _{1/2} (h)	2.8 ± 0.3	2.8 ± 0.3	0.98
Rate of elimination (h ⁻¹)	0.3 ± 0.03	0.3 ± 0.03	0.81
CL (L · h ⁻¹)	19.0 ± 1.2	20.67 ± 0.3	0.31

decreased in the SAA-insufficient state after the 2nd dose, indicating that the combination of SAA insufficiency and APAP metabolism decreased hepatic GSH. Rodent studies show that hepatic GSH release is proportional to hepatic GSH concentration (Ookhtens et al., 1985). Moreover, human studies using isotopic tracer methods showed that the fractional turnover of GSH is decreased in humans during SAA insufficiency. Together, the data show that APAP at normal therapeutic doses alter Cys metabolism, but that homeostatic mechanisms largely preserve both Cys and GSH pools.

Accumulating evidence suggests that the redox balance of the plasma Cys and GSH pools can be important in disease mechanisms even without major changes in concentrations of Cys and GSH per se (Moriarty-Craige and Jones, 2004; Jones and Liang, 2009). Cell surface proteins in endothelial cells contain Cys residues that undergo redox changes in response to a more oxidized E_hCySS (Go and Jones, 2005), and platelet activation is responsive to changes in extracellular E_hGSSG (Essex and Li, 2003). The SAA insufficiency caused a more oxidized plasma Cys/CySS redox potential value (E_h CySS) but had no effect on plasma GSH/GSSG redox potential value (E_h GSSG) (Mannery et al., 2010). Whether the effect on E_hCySS has any health consequences is unknown, but it is worthy of study because the magnitude of effect is similar to changes in E_hCySS that have been associated with disease processes (Mannery et al., 2010). It should be noted, however, that APAP administration resulted in the oxidation of plasma E_h CySS values in the SAA-replete state but not in the SAA-insufficient state, suggesting that adaptive responses to SAA insufficiency probably function to protect against adverse effects.

Because the therapeutic, nontoxic doses of APAP significantly decreased plasma GSH concentrations in humans with SAA insufficiency (Mannery et al., 2010), one may expect that SAA insufficiency would also perturb APAP metabolism. However, the present data show that SAA limitation

had no effect on the pattern of APAP metabolite concentrations in the plasma (Fig. 1) or urine (Fig. 3). The results are consistent with the interpretation that short-term SAA insufficiency does not compromise APAP metabolism. Pharmacokinetic parameters including half-life, volume of distribution, elimination rate, and CL rate were unaffected by consumption of a SAA-insufficient diet for 2 days (Table 2). Thus, even though APAP affects plasma GSH under SAA-insufficient conditions, the data provide no evidence that APAP metabolism is altered or that risk of adverse effects would be increased by 2 days of SAA insufficiency.

These results are consistent with previous findings concerning APAP metabolism. Glucuronidation is the major pathway for APAP metabolism and is regulated independently of the other pathways. Glucuronidation can be limited by a relative insufficiency of glucose, hypoxia, and alcohol consumption, but the pathway is not known to be influenced by SAA intake. Sulfation is the second most important pathway and, in the short term, is resistant to SAA insufficiency because of the large sulfate pool in the body. In the present study, no measurements of sulfate were obtained, so it is not known whether the SAA insufficiency caused a significant decrease in sulfate availability. However, the effects on Cys and GSH were modest so that one can anticipate that adaptive changes protecting these pools also protected the sulfate pools. The lack of effect on the mercapturate pathway for APAP metabolism is also consistent with known characteristics that are largely determined by the extent of bioactivation of APAP (Mitchell et al., 1973).

The results of the present study in humans with 2 days of SAA-free food and APAP at 15 mg/kg are different from those obtained in rats given 75 mg/kg APAP after 3 days with Met but lacking inorganic sulfate or Cys (Gregus et al., 1994). The latter showed that APAP metabolism and pharmacokinetic properties were altered because of the reduced capacity of rats to sulfate APAP. The decrease in sulfation was not

Fig. 3. Effect of sulfur amino acid insufficiency on urinary APAP (A) and APAP metabolite (B–D) recovery. Urine was collected in 6-h intervals after administration of APAP and analyzed by HPLC. Results showed no significant differences caused by consumption of SAA-deficient food for 2 days.

Fig. 4. Percentages of APAP and APAP metabolites recovered in urine while consuming a diet insufficient (A) or adequate (B) in SAAs. Data are expressed as percentage of cumulative 12-h urinary excretion of APAP metabolites (APAP, APAP-glucuronide, APAP-sulfate, APAP-Cys, APAP-GSH, and APAP-mercaptopurine) after two doses of APAP (15 mg/kg) given 6 h apart.

compensated by an increase in glucuronidation (Gregus et al., 1994). Instead, the decrease in sulfation resulted in increased conversion of APAP into its toxic metabolite, as suggested by increased excretion of APAP-Cys, APAP-GSH, and APAP-mercaptopuric acid in the urine. These findings indicate that an SAA-deficient diet causes a decrease in detoxification by sulfation and an increase in toxicity by APAP, which could be more severe after repeated doses of APAP. The current study shows that two 15 mg/kg doses of APAP after 2 days of

SAA-free food in humans do not provide evidence for a corresponding shift in metabolism. It must be pointed out, however, that the present study was designed with a modest period of SAA insufficiency and only two doses of APAP to minimize risk of toxicity.

The present study does not completely exclude the effects of SAA insufficiency on APAP toxicity in select subpopulations. The participants in the present study were young, had no known acute or chronic disease, did not smoke, and did not

abuse alcohol. Consequently, extrapolation of these present findings to at-risk populations is inappropriate. Similarly, individuals with malabsorption or prolonged periods of inadequate SAA intake may exhibit different responses.

In conclusion, the present research shows that APAP pharmacokinetics and distribution of metabolites are not affected by SAA deficiency in young, healthy adults. Although there are many unexplained cases of human toxicity after administration of therapeutic doses of APAP, results of the current study indicate that variation in short-term SAA availability is not likely to be a major factor.

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