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

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Running Title Page

Running Title: Acetaminophen metabolism and sulfur amino acid deficiency

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Abbreviations: APAP: acetaminophen; AUC: Area Under the Curve; CL: clearance; Cys: cysteine; $E_h$: redox potential; GSH: glutathione; $k_{elim}$: rate of elimination; Met: methionine; RDA: Recommended Dietary Allowance; SAA: sulfur amino acids; $(t_{1/2})$: half-life; $V_d$: Volume of Distribution

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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 (SAA) reduces biosynthesis of 3´-phosphoadenosine-5´- phosphosulfate (PAPS), the precursor for sulfation, and GSH, the precursor for the mercapturatic acid pathway. The amount of SAA needed for the metabolism of 2 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 sulfur amino acid (SAA) deficient diet alters APAP metabolism, a pilot clinical study with a double-blind, crossover design was performed. Subjects received the RDA for SAA for 3 days for equilibration. Following 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 three, 2 doses of APAP (15 mg/kg) or placebo were given successively with 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 a) did not change the pattern of APAP metabolites in plasma or urine and b) did not alter APAP pharmacokinetics. Thus, the results show that 2 days of diet completely devoid of SAA has no effect on acetaminophen 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, about 3% of 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-benzoquinonime (NAPQI) (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-containing amino acids (SAA), 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 of a low dose of APAP in rodents (Price and Jollow, 1989). The markedly slower elimination of APAP in SAA-deficient animals was due primarily to 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-mercaptopurpur 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.
While the mean SAA intake in Americans is considerably greater than the Recommended Daily Allowance (RDA) of 1.9 g/day (FNB, 2005), individual SAA intake is variable and can range from < 0.3 g to > 5 g per day (Flagg et al., 1994). Considering that a molar equivalent of about 0.2 g of Cys is used for metabolism of a maximal therapeutic dose of 1 g APAP; 4 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 2 study periods in which each individual was equilibrated to either the RDA or 0% of the RDA for SAA prior to APAP administration in 2 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 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 which evaluated effect of APAP on plasma cysteine and glutathione pools. The latter is described along with experimental details in an accompanying paper (Mannery et al, 2010). Briefly, 12 healthy volunteers (18-40 y old) with a body mass index (BMI) < 27, no acute and/or chronic illness, and no current smoking history were studied in under 2 different conditions with acetaminophen (2 doses; 15 mg/kg) and SAA intake (0% or 100% of RDA) (Table 1). Prior to the 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% SAA or 0% SAA for 2 days. During these 5 days, the subjects consumed no alcohol to avoid induction of CYP 2E1. On Day 6 of each study period, APAP (15 mg/kg) or placebo was administered under fasting conditions at 08:15. Subsequently, a meal was given at 12:30 and a second dose was administered at 14:30. Plasma samples were collected hourly for 12 hours following APAP or placebo administration, and two 6-h urine samples were collected during this time. Meals were prepared by the Bionutrition unit, 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 using an HPLC method as previously described (Howie et al., 1977; Chen et al., 2003). Aliquots of urine samples (20 µl) were injected into a Zorbax SB 5µM C18 reverse phase column (4.6 mm × 250 mm). APAP and its metabolites were eluted using a mobile phase composed of 12.5% HPLC-grade methanol, 1% acetic acid, and 86.5% water, run at 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 using an APAP standard curve (Howie et al., 1977).

APAP and its metabolites in plasma were analyzed using the HPLC method of Moldeus (Moldeus, 1978). Perchloric acid (3N) was added 2:1 to plasma samples to precipitate protein. Following 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 minutes. This was followed by a 20 minute linear gradient that finished at 99% B. The composition of the mobile phase was restored to initial conditions using an 8 minute linear gradient. All other HPLC conditions were the same as those described above.

**Statistics.** Results are expressed as means ± S.E.M. We used SPSS software (version 17; SPSS Inc, Chicago, IL) for all analyses. Area under the curve (AUC) values were calculated using the trapezoidal rule. Paired t-tests with the Bonferroni correction were used to determine if 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 manuscript (Mannery et al, 2010). Fifty percent of the subjects were male, the mean age was 25 ± 4 y and there was no significant difference between males and females. Half of the subjects (n = 6) were African American, 4 were Caucasian, 1 was Hispanic, and 1 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 8 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 Figure 1A, with corresponding AUC data in Figure 2A. Measurements of the plasma concentration of APAP showed that the peak concentration occurred at 09:15 under SAA insufficient conditions (Fig 1A). The peak plasma appearance of APAP was delayed to 10:15 under SAA sufficient conditions. The pattern of APAP concentration in the plasma was similar under both dietary conditions (Fig 1A). Despite observing a significant effect at the 15:15 time point, AUC data show that an SAA insufficient diet had no overall effect on plasma APAP concentration (Fig 2A).

Limitation of SAA did not alter the pattern of APAP-glucuronide concentration in the plasma (Fig 1B). Despite observing a significant increase in APAP-glucuronide at the 19:15 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 (Fig 1C; Fig 2B). The pattern of appearance or concentration of APAP-Cys, APAP-GSH, and APAP–mercapturate were not significantly altered by SAA limitation (Fig 1D; Fig 2B). However, it should be noted that there was a relatively large variation among individuals for the time course data, so this conclusion is largely based upon 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 (Vₐ), half-life (t₁/₂), clearance (CL), and rate of elimination (kₑₐₘᵢₑ) were calculated following the first dose of APAP. The results showed that the t₁/₂ for APAP under conditions of SAA insufficiency was not significantly different from the t₁/₂ under control conditions (Table 2). The Vₐ 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). While the concentration of APAP excreted into the urine was higher during the 14:00 to 20:00 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, -GSH, and –mercapturic acid conjugates excreted in the urine during the 14:00 to 20:00 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 4A; Fig 4B).
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 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 which 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 which have been associated with disease processes (Mannery et al.,
2010). It should be noted, however, that APAP administration resulted in the oxidation of plasma $E_{\text{h}}$ CySS values only in the SAA replete state but not in an SAA insufficient state, suggesting that adaptive responses to SAA insufficiency probably function to protect against adverse effects.

Because the therapeutic, non-toxic doses of APAP significantly decreased plasma GSH concentrations in humans with SAA insufficiency (Mannery et al., 2010), one may expect that this would also perturb APAP metabolism. However, the present data show that SAA limitation had no affect 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 clearance 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 which are largely determined by the extent of bioactivation of APAP (Mitchell et al., 1973).

The results of the present study in humans with 2 d of SAA-free food and APAP at 15 mg/kg are different from those obtained in rats given 75 mg/kg APAP following 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 due to a reduced capacity of rats to sulfate APAP. The decrease in sulfation was not 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, -GSH, and – mercapturic 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 insufficient and only 2 doses of APAP to minimize risk of toxicity.

The present study does not completely exclude 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 following 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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REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig 1. Plasma concentrations of APAP and APAP metabolites following 2 days of an SAA adequate diet (100% of the RDA) or an SAA-insufficient (0% of the RDA) diet. APAP and metabolite concentrations were determined by HPLC. Data are expressed as means ± SE for each respective time point. *Significant at p < 0.05, n = 8.

Fig 2. Area Under the Curve (AUC) analysis for plasma acetaminophen and metabolites following 2 days of an SAA adequate diet (100% of the RDA) or an SAA-insufficient (0% of the RDA) diet. Area Under the Curve (AUC) was calculated using the APAP metabolite concentrations measured by HPLC analysis (See Fig 1). *Significant at p < 0.05, n = 8.

Fig 3. Effect of sulfur amino acid insufficiency on urinary APAP and APAP metabolite recovery. Urine was collected in 6-h intervals following administration of APAP and analyzed by HPLC. Results showed no significant differences due to consumption of SAA deficient food for 2 days.

Fig 4. Percentages of APAP and APAP metabolites recovered in urine while consuming diet adequate or insufficient in sulfur amino acids. Data are expressed as percentage of cumulative 12-h urinary excretion of APAP metabolites (APAP, APAP-glucuronide, APAP-sulfate, and APAP-Cys, -GSH, -mercapturate) following 2 doses of APAP (15 mg/kg) given 6 h apart.
Table 1. Experimental Design

<table>
<thead>
<tr>
<th>Study Period</th>
<th>Days 1-3, Outpatient Equilibration</th>
<th>Days 4-5, Inpatient ± SAA</th>
<th>Day 6, Inpatient ± APAP</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>3-days w/ 100% RDA for SAA</td>
<td>2-days in GCRC w/ 100% RDA for SAA</td>
<td>2-sequential doses of APAP</td>
</tr>
<tr>
<td>2</td>
<td>3-days w/ 100% RDA for SAA</td>
<td>2-days in GCRC w/ SAA-free diet</td>
<td>2-sequential doses of APAP</td>
</tr>
</tbody>
</table>

Blood sampling: ↑↑↑↑↑↑↑↑↑↑↑↑

*Study periods described above were randomized for order and conducted at least one week apart*
Table 2: Pharmacokinetic constants for plasma APAP following short term SAA insufficiency

<table>
<thead>
<tr>
<th></th>
<th>100% SAA</th>
<th>0% SAA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max plasma concentration (nmol/ml)</td>
<td>65.4 ± 7.2</td>
<td>61.6 ± 8.9</td>
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</tr>
<tr>
<td>AUC (mg·L⁻¹·h)</td>
<td>15.6 ± 1.9</td>
<td>14.0 ± 0.97</td>
<td>0.41</td>
</tr>
<tr>
<td>V₅ (L)</td>
<td>75.8 ± 9.3</td>
<td>79.4 ± 8.0</td>
<td>0.70</td>
</tr>
<tr>
<td>t½ (h)</td>
<td>2.8 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>0.98</td>
</tr>
<tr>
<td>kₑₙₐₑₙₙ (h⁻¹)</td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.03</td>
<td>0.81</td>
</tr>
<tr>
<td>CL (L·h⁻¹)</td>
<td>19.0 ± 1.2</td>
<td>20.67 ± 0.3</td>
<td>0.31</td>
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</tbody>
</table>
Fig 1
Fig 2
Fig 3
A.

- Glucuronide: 65%
- Sulfate: 24%
- Cys, GSH, NAC: 5%
- APAP: 6%
- 0% SAA

B.

- Glucuronide: 65%
- Sulfate: 25%
- Cys, GSH, NAC: 4%
- APAP: 7%
- 100% SAA

Fig 4