Disposition of Acetaminophen at 4, 6, and 8 g/day for 3 Days in Healthy Young Adults

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The objective of this study was to determine the disposition and tolerability of 1, 1.5, and 2 g acetaminophen every 6 h for 3 days. Group I healthy adults received acetaminophen (4 then 6 g/day) or placebo; Group II received acetaminophen (4 then 8 g/day) or placebo. Acetaminophen and metabolites were measured in plasma and urine. Hepatic aminotransferases were measured daily. At steady state, acetaminophen concentrations were surprisingly *lower* than predicted from single-dose data, although sulfate formation clearance (fCL) was lower as expected, indicating cofactor depletion with possible sulfotransferase saturation. In contrast, glucuronide fCL was unexpectedly *higher*, strongly suggesting glucuronosyltransferase induction. This is the first evidence that acetaminophen induces its own glucuronidation. No dose-dependent differences were detected in fCL of thiol metabolites formed via cytochrome *P*4502E1. Hepatic aminotransferases stayed within reference ranges, and the incidence and frequency of adverse events were similar for acetaminophen and placebo. Although dose-dependence of acetaminophen disposition was reported previously, this study shows a novel finding of time-dependent disposition during repeated dosing. Unexpected increases in glucuronide fCL more than offset decreases in sulfate fCL, thus increasing acetaminophen clearance overall. Thiol metabolite fCL remained constant up to 8 g/day. These findings have important implications in short-term (3 day) tolerability of supratherapeutic acetaminophen doses in healthy adults.

Acetaminophen causes liver toxicity with large acute overdoses. Potential mechanisms have been studied extensively in animals[1,2] and linked to the cytochrome *P*450-catalyzed formation of a reactive electrophile, which was ultimately identified as *N*-acetyl-*p*-benzoquinone imine (NAPQI).[3] Evidence in humans supports a similar etiology. At acute toxic overdoses in humans and experimental animals,[4] acetaminophen disposition is nonlinear with dose owing to depletion of activated sulfate, 3′-phosphoadenosine-5′phosphosulfate, and its precursor, inorganic sulfate. This observation has led to the hypothesis that, following repeated doses at or slightly above those approved for therapy, acetaminophen will accumulate in plasma and give rise to a more than dose-proportional increase in the amount of the toxin, NAPQI, formed. The purpose of this study was to evaluate this hypothesis. Contrary to expectation, it was disproved.

Acetaminophen has a mixed-competitive and sequential biotransformation pattern that is illustrated in **Figure 1**. About 85% is metabolized by conjugation, mainly glucuronidation via UDP-glucuronosyltransferase (UGT) enzymes. The UGT isoform, UGT1A6, mainly catalyzes the glucuronidation of acetaminophen,[5,6] whereas UGT1A1 catalyzes the glucuronidation of bilirubin in liver. Acetaminophen is also substrate for two sulfotransferases, SULT1A1 and SULT1A3.[7,8] Sulfation of acetaminophen is partly governed by availability of inorganic sulfate, which is the rate-limiting factor in the formation of the cofactor of sulfation, 3′-phosphoadenosine-5′phosphosulfate. The other rate-limiting factor is sulfotransferase activity.

About 8–10% of an acetaminophen dose is oxidized by cytochrome *P*4502E1 (CYP2E1), an N–nitrosodimethylamine demethylase that is mainly expressed in the liver. CYP2E1 produces the highly reactive intermediate, NAPQI, from acetaminophen, which is conjugated with glutathione to produce the inert, nontoxic thiol metabolites (cysteine, mercapturate, methylthioacetaminophen, and methanesulfinylacetaminophen). Other human cytochrome *P*450 isoforms, including 1A2, 2A6, 3A4, and recently 2D6, have been reported to form NAPQI using *in vitro* techniques,[9–11] but the contributions produced *in vivo* relative to CYP2E1 are negligible using the selective inhibitor disulfiram.[12]

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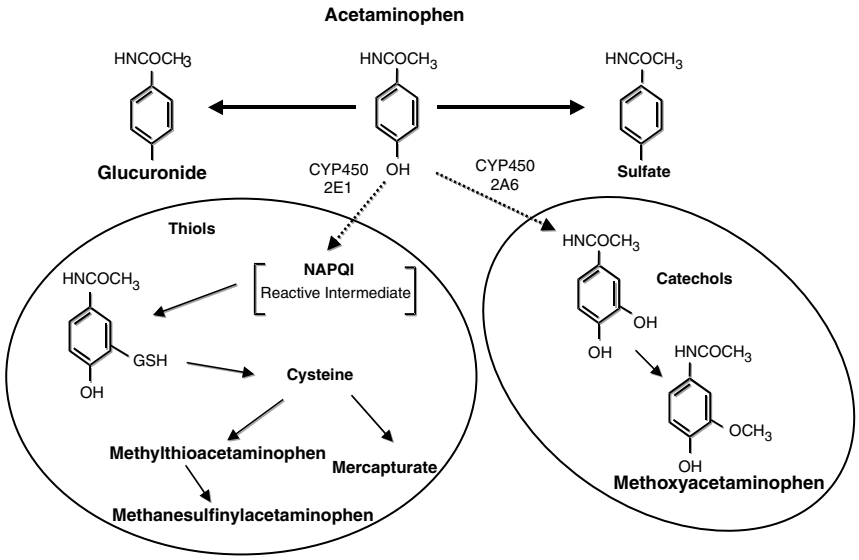

Figure 1 Metabolic pathways of acetaminophen.

Acetaminophen also is oxidized at a low percentage by cytochrome *P*4502A6 to form inert catechols (*e.g.*, methoxyacetaminophen).[9]

The toxicity profile of acetaminophen in acute overdoses, in which very large amounts are consumed within a short time, has been well characterized.[13] Exact mechanisms involved in acetaminophen hepatotoxicity are not fully elucidated; however, higher concentrations of NAPQI may be formed during acute overdoses, which react with other substrates as stores of glutathione are depleted. High concentrations of NAPQI are responsible for centrilobular necrosis of the liver unless a patient is treated with the antidote *N*-acetylcysteine soon after acute ingestion.[13,14]

In contrast, limited information is available on the epidemiology, medical assessment, and clinical course of repeated supratherapeutic doses (Repeated supratherapeutic ingestion is defined as more than one ingestion of acetaminophen during a period exceeding 8 h that results in a cumulative dose greater than 4 g in 24 h.[12]) of acetaminophen, although a recent prospective study was designed to characterize the potential toxicity profile in patients treated for repeated supratherapeutic doses of acetaminophen in poison control centers.[15] Unlike acute acetaminophen overdose, no nomogram exists to guide risk assessment and treatment of these cases. In addition, there are no published data available on the multiple-dose disposition of acetaminophen at the higher-than-recommended dose of 4 g daily. Because toxicity is a function of disposition, which is known to be nonlinear after higher single doses, characterization of the pharmacokinetics and metabolism after repeated supratherapeutic dosing is an important step toward achieving an understanding of the potential risks.

The primary objective of this study was to determine the multiple-dose disposition of acetaminophen at and above 4 g/day to partially address the void of information on acetaminophen at these doses. The secondary objective was to assess tolerability. We examined the controlled use of acetaminophen at 1, 1.5, and 2 g every 6 h (totaling 4, 6, and 8 g daily, respectively) for 3 days. Dosing for this duration was based on the half-lives of acetaminophen and its metabolites and the estimated time to reach steady-state pharmacokinetics. Clinical signs and symptoms and the results of daily aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used to assess tolerability and monitor hepatic function.

In a retrospective review of pooled aminotransferase data collected during placebo treatments of 13 phase I studies,[16] 20% of the subjects ($n = 93$) had at least one aminotransferase value above the upper limit of the reference range (ULRR) (The reference range of a clinical chemistry test is derived from assaying concentrations or activities in serum samples from the general population residing in the geographical area of the Clinical Laboratory.), and 7.5% had at least one value twice the ULRR during those studies. Therefore, a parallel group of subjects was randomized to placebo caplets to avoid potential bias in associating elevations in aminotransferase enzymes with acetaminophen exposure. In addition, an in-house, 3-day, drug-free baseline period was included in this study's design to assess daily variability of aminotransferase activities. Acetaminophen and its metabolites were measured in plasma and urine to characterize the disposition of higher acetaminophen doses over 3 days of therapeutic and supratherapeutic dosing.

RESULTS

Pharmacokinetics of acetaminophen

A schematic of the dosing regimen and staggered periods for both study groups is shown in **Figure 2**. **Table 1** summarizes selected acetaminophen pharmacokinetic parameters and statistical comparisons within each group. Previous studies report that acetaminophen pharmacokinetics are nonlinear[17,18] after single doses at the high end of the dose range

from 0.325 to 3 g. In this study, acetaminophen pharmacokinetics were linear and dose proportional at doses up to 2 g after the first dose and also after reaching steady state.

Plasma concentrations of acetaminophen accumulated less than expected from first-dose half-life and dosing interval after 3 days of multiple dosing with 1, 1.5, or 2 g every 6 h. Mean accumulation ratios ranged from 1.29 to 1.42. Time-dependent changes in acetaminophen pharmacokinetics over the 3 days were observed in the plasma concentration–time profiles. **Figure 3** includes both dosing profiles for Group II, which received multiple doses of 1 and 2 g of acetaminophen. Although not shown, results for Group I present in **Figure 2** were similar. The time-dependent changes in pharmacokinetics were illustrated by fitting a one-compartment exponential model to data from the first four doses and the fitted curve simulated over time for all doses. In general, the fitted curve generated from data for the initial doses of 1, 1.5, or 2 g of acetaminophen did not effectively predict plasma concentrations after the final dose at steady state. This was especially apparent for the 2-g dosing regimen in which predicted concentrations of acetaminophen were markedly higher than actual concentrations. Lower actual concentrations at steady state indicate an increase in oral clearance with acetaminophen exposure over time. Analysis of variance detected significant differences in mean oral clearances ($P<0.0001$) between the first (CL/F,1) and final doses (CL/F,ss) at each dosing level (**Table 1**).

For drugs that exhibit time-independent and linear pharmacokinetics, values of the first-dose area under the concentration–time curve extrapolated to infinity (AUC_∞) and final-dose AUC over the dosing interval ($AUC\tau$) should be equal. Such a linear relationship between these AUC parameters is depicted by a dashed line in **Figure 4**. Consistent with the multiple-dose pharmacokinetic profiles, $AUC\tau$ values for 4- and 6-g/day dosing regimens were slightly below the line, whereas $AUC\tau$ values for 8 g/day were markedly lower.

Table 1 Comparison of acetaminophen pharmacokinetics after the first and final doses

Dose regimen	AUC_∞ (μg·h/ml)	$AUC\tau$ (μg·h/ml)	Ratio of geomeans (90% CI)	CL/F,1 (ml/kg/min)	CL/F,ss (ml/kg/min)	Mean difference
Group I (n=12)	First	Final		First	Final	
4 g/day	55.8 (11.7)	47.1 (13.4)	0.83 (79–88%)	4.30 (0.66)	5.22 (1.07)	0.915 $P<0.0001$
6 g/day	82.0 (18.7)	70.0 (18.5)	0.85 (80–90%)	4.41 (0.74)	5.21 (0.91)	0.797 $P<0.0001$
Group II (n=12)						
4 g/day	61.7 (7.7)	50.2 (9.3)	0.81 (76–85%)	3.88 (0.53)	4.83 (0.83)	0.954 $P<0.0001$
8 g/day	131.2 (16.2)	92.7 (11.0)	0.71 (67–75%)	3.90 (0.47)	5.17 (0.82)	1.268 $P<0.0001$

AUC_∞, area under concentration-time curve extrapolated to infinity after the first dose; $AUC\tau$, area under concentration-time curve over the 6-h interval after the final dose; CI, confidence interval; CL/F,1, oral clearance after the first dose; CL/F,ss, oral clearance at steady state after the final dose. Parameter values are listed as mean (SD). Analysis of variance was used to construct the two-sided 90% confidence limits and test for differences in means.

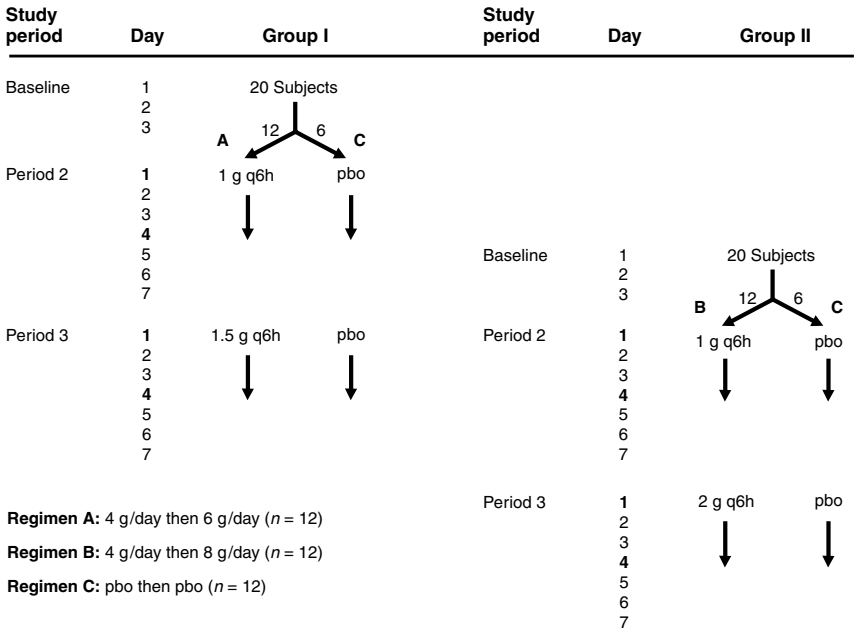

Figure 2 Schematic of the dosing regimen and staggered periods for both study groups. Adapted with permission from *Clinical Toxicology* (Temple *et al*).[19]

Pharmacokinetics of acetaminophen glucuronide and sulfate

Table 2 summarizes AUC parameters for the glucuronide and sulfate conjugates and the statistical comparisons within each study group. Time-dependent changes were apparent in the multiple-dose pharmacokinetic profiles for the glucuronide and sulfate conjugates at each dosage both for Groups I and II. Mean profiles for Group II only at 4 and 8 g/day are shown in **Figure 5**.

A first-order exponential equation was fit to glucuronide data from the first four doses, and the fitted curves were simulated over all doses. Profiles for the initial doses of 1, 1.5, and 2 g of acetaminophen predicted glucuronide plasma concentrations for the final dose that were markedly lower than actual concentrations. The higher actual glucuronide plasma concentrations at steady state indicate an increase in formation clearance (fCL), and they were more pronounced at 8 g of acetaminophen per day. The mean fCL of glucuronide after the final dose of 8 g/day was about 23% higher ($P = 0.0091$, paired t-test) than that after the final dose of 4 g/day.

A first-order exponential equation was fit to sulfate data from the first four doses, and the fitted curves were simulated over all remaining doses. These profiles predicted sulfate plasma concentrations for the final dose at steady state that were markedly higher than actual concentrations. The lower actual sulfate plasma concentrations indicate a decrease in fCL, and they were more pronounced at 8 g of acetaminophen per day. The mean fCL of sulfate after the final dose of 8 g/day was 36% lower ($P < 0.0001$, paired t-test) than that after the final dose of 4 g/day.

Table 2 contains results of statistical equivalence tests between first-dose AUC_{∞} and final-dose $AUC\tau$ for the glucuronide and sulfate metabolites. Ratios of geometric means ($AUC\tau/AUC_{\infty}$) for the glucuronide were >1 at all doses, and no comparisons were shown to be equivalent,

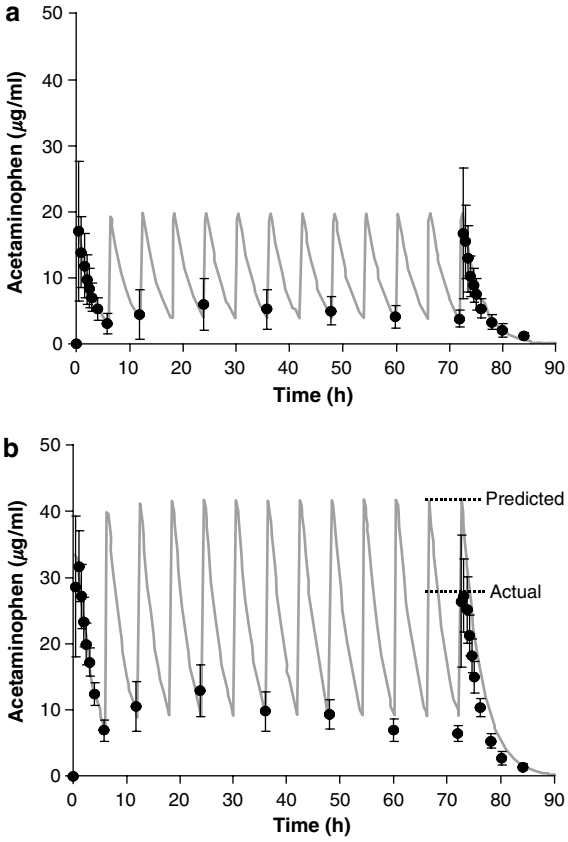

Figure 3 Multiple-dose, simulated pharmacokinetic profiles of acetaminophen for Group II at (**a**) 4 g/day and (**b**) 8 g/day are overlaid on mean (SD) plasma concentrations. The profile at 8 g/day predicted higher concentrations at steady state than those measured.

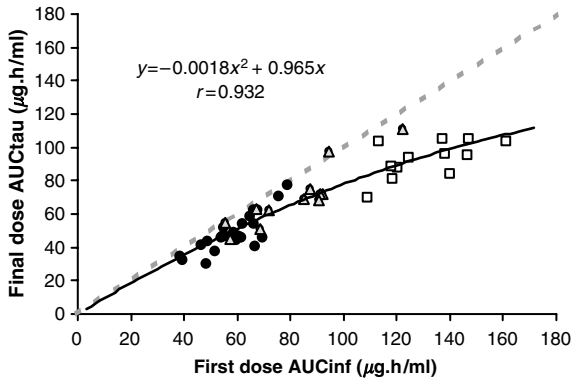

Figure 4 Final dose $AUC\tau$ for individual subjects are plotted as a function of first-dose AUC_{∞}, and they are shown relative to expected values (- - -) for linear kinetics.

Table 2 Comparison of total exposures after the first and final doses for the glucuronide and sulfate metabolites

Dose regimen	Glucuronide AUC (μg·h/ml)		Ratio of geomeans (90% CI)	Sulfate AUC (μg·h/ml)		Ratio of geomeans (90% CI)
Group I (n=12)	AUC_{∞}	$AUC\tau$		AUC_{∞}	$AUC\tau$	
4 g/day	133.7 (28.7)	164.5 (25.0)	1.25 (118–131%)	39.0 (10.7)	22.8 (7.7)	0.62 (54–70%)
6 g/day	219.7 (42.8)	259.0 (41.7)	1.19 (113–125%)	49.6 (13.5)	26.3 (9.6)	0.57 (50–65%)
Group II (n=12)						
4 g/day	129.0 (14.6)	159.8 (17.0)	1.24 (118–130%)	37.0 (8.2)	25.3 (5.7)	0.68 (62–74%)
8 g/day	283.2 (37.4)	374.1 (38.3)	1.32 (126–139%)	67.2 (16.6)	28.1 (7.2)	0.42 (38–45%)

AUC_{∞}, area under concentration-time curve extrapolated to infinity after the first dose; $AUC\tau$, area under concentration-time curve over the 6-h interval after the final dose; CI, confidence interval. Parameter values are listed as mean (SD). Analysis of variance was used to construct the two-sided 90% confidence limits.

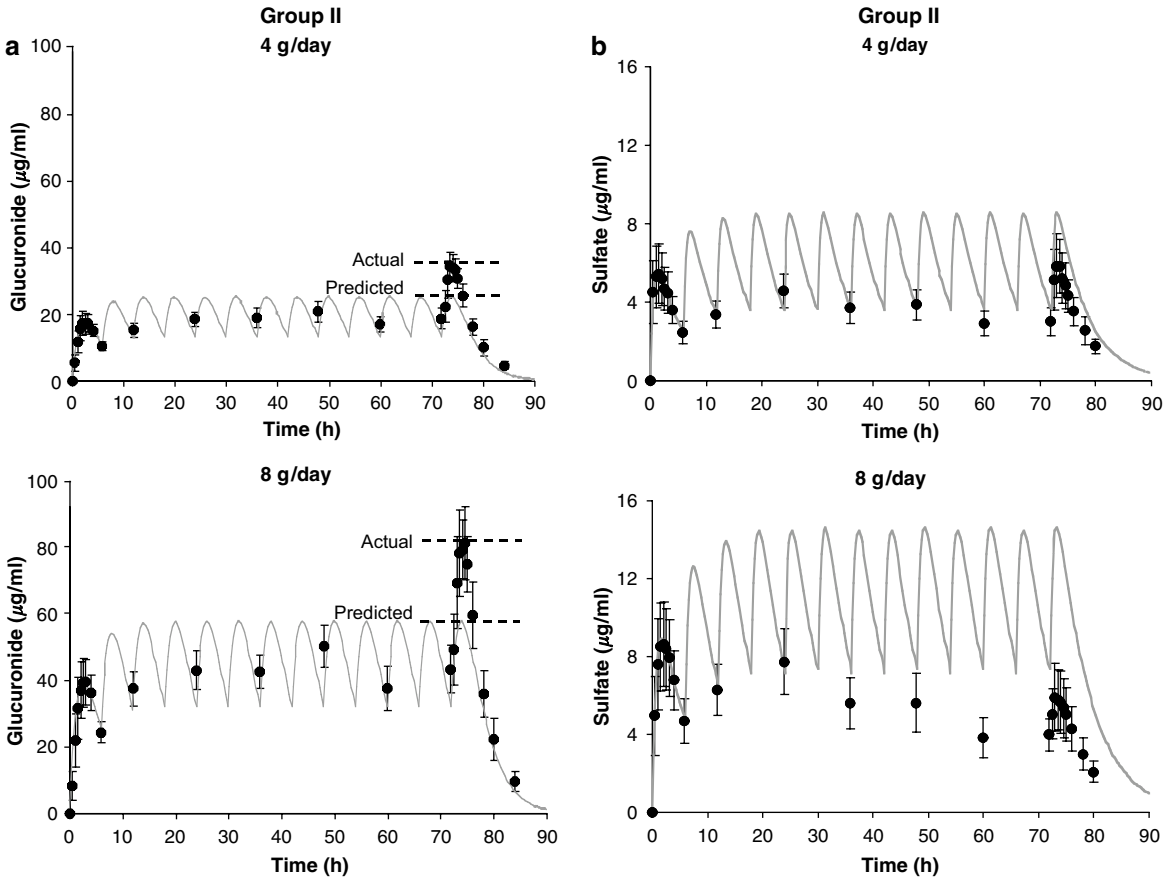

Figure 5 Multiple-dose, simulated pharmacokinetic profiles of (**a**) glucuronide and (**b**) sulfate conjugates at 4 and 8 g/day of acetaminophen for Group II are overlaid on mean (SD) plasma concentrations. Actual steady-state concentrations were higher and lower than predicted by simulation for glucuronide and sulfate, respectively, at both doses.

except for the 1.5-g dose. This is consistent with induction of glucuronosyltransferase enzymes and shunting to this conjugation pathway. Ratios of geometric means ($AUC\tau/AUC_{\infty}$) for the sulfate were <1 at all doses, and no comparisons were shown to be equivalent. This time-dependent change likely reflects depletion of the cofactor, 3′-phosphoadenosine-5′phosphosulfate, with possible saturation of the low-capacity sulfate conjugation pathway.

Excretion of acetaminophen and metabolites in urine

Mean (SD) acetaminophen and metabolites excreted in urine, expressed as a percentage of the total acetaminophen dose, are summarized in **Table 3**. A paired *t*-test was used to compare means for acetaminophen doses of 4 versus 6 g/day for Group I and 4 versus 8 g/day for Group II. Compared with the 4-g/day dosing regimen, there was a statistically significant increase in the percentage excreted as acetaminophen ($P=0.0002$) and methoxyacetaminophen ($P=0.0008$) at 6 g/day and as glucuronide ($P=0.0390$) at 8 g/day. Statistically significant decreases in the percentage excreted as the sulfate were detected at both 6 and 8 g/day compared with 4 g/day ($P=0.0003$ and $P<0.0001$, respectively).

Total thiols are the sum of cysteine, mercapturate, methylthioacetaminophen, and methanesulfinylacetaminophen metabolites that represent the amount of an acetaminophen dose oxidized by CYP2E1 to form NAPQI which is subsequently conjugated with glutathione. Although statistical differences were detected in mean amounts of individual thiols excreted between dosages (4 and 6 or 8 g/day), the percentage of total thiols excreted in the urine was not statistically different (**Table 3**).

Mean (SD) fCLs of acetaminophen metabolites are summarized in **Table 4**. Using a paired *t*-test, there was a statistically significant increase in the fCL of glucuronide ($P=0.0091$) at 8 g/day and as methoxyacetaminophen ($P<0.0001$) at 6 g/day compared with 4 g/day means. Statistically significant decreases in fCL of sulfate ($P<0.0001$) were detected at both 6 and 8 g/day. Mean fCL of total thiols were independent of repeated therapeutic (4 g/day) and supratherapeutic (6 and 8 g/day) acetaminophen doses for 3 days.

Safety and tolerability

Of the 37 enrolled subjects, 36 completed the study per protocol. One subject elected to withdraw after the first 1-g acetaminophen dose because of difficulty in blood collections. Twenty-one of the subjects reported 53 adverse events, of which 96% were rated mild. The incidence and frequency of adverse events were similar across the daily acetaminophen

Table 3 Within-group comparison of mean (SD) percentage[a] acetaminophen and metabolites excreted in urine

	Group I (n=12)		Group II (n=12)	
	4 g/day	6 g/day	4 g/day	8 g/day
Acetaminophen	2.87 (0.53)	3.77 (0.70)[b]	3.20 (0.87)	3.00 (0.97)
Glucuronide	61.3 (8.4)	61.3 (10.5)	58.6 (7.6)	66.6 (14.5)[b]
Sulfate	20.0 (4.72)	14.3 (3.58)[b]	18.8 (4.78)	11.0 (3.70)[c]
Methoxyacetaminophen	4.04 (1.45)	5.39 (2.17)[b]	4.19 (1.47)	3.85 (1.40)
Total thiols[d]	10.24 (3.91)	9.75 (4.83)	8.80 (2.13)	8.32 (1.85)
Cysteine	3.23 (1.01)	2.65 (1.16)[b]	3.00 (0.64)	2.37 (0.56)[b]
Mercapturate	3.99 (0.97)	3.09 (0.94)[b]	3.68 (0.97)	2.80 (0.66)[b]
MT+MS	3.02 (2.52)	4.23 (3.22)[b]	2.13 (1.21)	3.15 (1.36)[b]
Percentage recovered (%)	98.2 (6.5)	97.7 (14.6)	93.0 (12.0)	92.8 (18.6)

MS, methanesulfinylacetaminophen; MT, methylthioacetaminophen. [a]Reported as the percentage of acetaminophen dose. [b]$P<0.05$, paired t-test. [c]$P<0.0001$, paired t-test. [d]The sum of cysteine, mercapturate, MT, and MS.

Table 4 Comparison of acetaminophen clearances and metabolite formation clearances within group by dose

	Clearance	Group I (n=12)			Group II (n=12)		
	(ml/kg/min)	4 g/day	6 g/day	Paired t-test	4 g/day	8 g/day	Paired t-test
Acetaminophen	CL/F,ss	5.22 (1.07)	5.21 (0.91)	ns	4.83 (0.83)	5.17 (0.82)	ns
Acetaminophen	CLr	0.144 (0.022)	0.189 (0.034)	P=0.0001	0.148 (0.033)	0.150 (0.037)	ns
Glucuronide	fCL	3.24 (0.91)	3.24 (0.91)	ns	2.85 (0.68)	3.42 (0.82)	P=0.0091
Sulfate	fCL	1.031 (0.280)	0.746 (0.212)	$P<0.0001$	0.916 (0.281)	0.563 (0.193)	$P<0.0001$
Methoxyacetaminophen	fCL	0.202 (0.059)	0.269 (0.078)	$P<0.0001$	0.196 (0.057)	0.198 (0.070)	ns
Total thiols[a]	fCL	0.534 (0.234)	0.504 (0.247)	ns	0.423 (0.117)	0.428 (0.114)	ns

CL/F,ss, oral clearance at steady state after the final dose; CLr, renal clearance; fCL, metabolite formation clearance; ns, not significant. Parameter values are listed as mean (SD). [a]The sum of cysteine, mercapturate, methylthioacetaminophen, and methanesulfinylacetaminophen.

doses and when compared with placebo. The most frequently reported adverse events were headache (five subjects on placebo and seven on 8 g/day); asthenia (five subjects on placebo and two on 4 g/day); and nausea (two subjects on placebo, two on 4 g/day, and one each on 6 and 8 g/day).

There were no clinically relevant changes in vital signs or clinical laboratory profiles observed during the study. AST and ALT activities measured throughout the study were consistent across daily doses and when compared with placebo. All remained within reference ranges during the study except from one subject who had an AST equal to 43 U/l that just exceeded the upper limit of 42 U/l and was deemed clinically insignificant. More details of the clinical results of this study are reported elsewhere.[19]

DISCUSSION

The major findings in this study were that, at steady state, (i) acetaminophen plasma concentrations (hence, body burden) accumulated *less* than expected from the first-dose half-life over the range of 4–8 g/day; (ii) flux of acetaminophen dose eliminated via oxidation to NAPQI and captured by conjugation with glutathione increased in proportion to dose; (iii) sulfation capacity was exceeded, as expected, as dose and duration of acetaminophen dosing increased; and (iv) glucuronidation capability increased with the duration of repeated administration of therapeutic and supratherapeutic doses of acetaminophen. The last of these findings was completely unexpected and accounts for the less-than-dose-proportional accumulation of acetaminophen in plasma and the absence of a more-than-dose-proportional flux through NAPQI and glutathione conjugation that would otherwise occur from exceeding sulfation capacity. This is a completely novel finding, and is of fundamental importance in considering the potential for toxicologic consequences of repeated ingestion of supratherapeutic doses of acetaminophen.

Based on what is known from single-dose pharmacokinetic studies of acetaminophen,[17] sulfation capacity was expected to be exceeded as dose increased beyond 4 g/day, possibly leading to a greater fraction of dose being eliminated via oxidation to NAPQI and conjugation with glutathione. Although sulfation capacity was exceeded, there was no net

diversion of acetaminophen to NAPQI formation. This result occurred because repeated therapeutic and supratherapeutic dosing of acetaminophen quickly increased the fCL of the glucuronide conjugate, implicating acetaminophen as an inducer of UDPGT, presumably UGT1A6. The net effect of the decrease in sulfation fCL and increase in glucuronide fCL was *increased* acetaminophen oral clearance. The apparent induction of UDPGT balanced the declining capacity of sulfation to the extent that the fraction of acetaminophen dose eliminated by oxidation to the putative toxic metabolite, NAPQI, did not increase (or decrease) with dose at steady state.

Two acetaminophen pharmacokinetics studies[20,21] in healthy adults have shown that a lower amount of sulfate conjugate and a higher amount of glucuronide conjugate is excreted after 4 days of multiple dosing (650 mg every 6 h) compared with the initial single dose. Our results with multiple dosing of 1, 1.5, and 2 g of acetaminophen are consistent with these observations. In another study with the 650-mg acetaminophen multiple-dose regimen,[22] serum sulfate levels were reduced by 28% on the fourth day, but renal clearance (CLr) of inorganic sulfate was also reduced by 33%. Although inorganic sulfate was not measured in plasma or urine in our study, this is the most likely explanation for the decrease in fCL of the sulfate conjugate. Tubular reabsorption of inorganic sulfate in the kidney regulates serum sulfate levels when body stores are decreased, presumably as part of a homeostatic mechanism to conserve sulfate.[23] With prolonged use of acetaminophen at 650 mg per dose,[20] plasma sulfate levels were elevated, suggesting that sulfotransferase activity rather than sulphate depletion may be the major factor limiting the rate of acetaminophen sulfation in these cases.

In this study, the four thiol metabolites were quantified in urine, whereas plasma concentrations of only cysteine and mercapturate were measured. Lower steady-state concentrations of the latter thiols at higher doses were observed, indicating that they are not proportional to acetaminophen dose. However, larger amounts of the two other thiol metabolites (methylthioacetaminophen and methanesulfonyl-acetaminophen) were excreted in urine at the 8-g/day dose compared with the 4-g/day dose (**Table 3**). Increases in formation of these latter metabolites with dose suggest thiolase induction, which would decrease cysteine plasma concentrations directly and channel cysteine away from the competing mercapturate pathway. After acetaminophen is oxidized by CYP2E1 and conjugated with glutathione, dose-dependent shifts in subsequent mixed-competitive and sequential biotransformation are apparent, resulting in this different urinary excretion pattern of individual thiol metabolites. However, the net flux of acetaminophen through glutathione conjugation appears dose proportional, as the mean fCLs of total thiols were nearly identical for repeated dosing of 4 and 8 g/day.

CONCLUSION

Acetaminophen plasma concentrations did not accumulate in plasma during 3 days of repeated therapeutic and supratherapeutic doses up to 8 g/day. Indeed, accumulation was less than expected from single-dose studies. Although acetaminophen clearance increased with time, it became approximately constant at steady state over the range of doses examined. This net result is due to two competing nonlinearities: unexpected increases in glucuronide formation that more than offset decreases in sulfate conjugation over time. These data suggest cofactor depletion with possible saturation of the sulfate pathway. However, increases in glucuronide plasma concentrations were more pronounced, indicating induction of glucuronosyltransferase, presumably UGT1A6, especially at 8 g/day.

No changes were detected in total thiol metabolites formed from the highly reactive, toxic intermediate (NAPQI) with doses up to 8 g/day. Dose-proportional, total glutathione conjugate recovery in the urine demonstrated linearity of thiol fCL, because velocity of formation is equal to the product of acetaminophen plasma concentration and fCL. Moreover, serum AST and ALT activities remained within reference limits during the study, and repeated acetaminophen dosing for 4, 6, and 8 g/day for 3 days was generally well tolerated clinically in this relatively small number of healthy adults. These findings, together with the aforementioned changes in acetaminophen disposition, may have important implications for the short-term (3 day) tolerability of higher-than-recommended daily doses of acetaminophen in healthy adults.

METHODS

Subjects. Healthy men and women between 18 and 45 years of age and 140–200 pounds (63.6–90.9 kg) body weight were eligible for the study. The enrolled population was about 50% male, and all subjects were white with the mean age of 25.3 ± 5.4 years. Subjects were considered healthy based on medical history, a physical examination, vital sign measurements, and an electrocardiogram. They could be social drinkers of alcohol, but were excluded if they had a history of alcohol abuse. Subjects who were tobacco users were excluded as well as female subjects taking oral contraceptives. In addition, subjects had a normal clinical laboratory profile, which included hematology, blood chemistry, and urinalysis at screening and baseline. They tested negative for hepatitis B antigen, hepatitis C antibody, drugs of abuse, and pregnancy (if female). A detailed review of the clinical results from this study are published elsewhere[19] and a summary is presented herein.

Study design. This multiple-dose, pharmacokinetic study had a double-blind, placebo-controlled, parallel-group design with three dosing regimens. It was conducted in accordance with the principles of good clinical practice and relevant articles of the Declaration of Helsinki principles. The protocol and informed consent were approved by the Research Ethics Committee, Royal Group Hospitals (Belfast, Northern Ireland). Subjects were housed for the duration of the study in the clinical research center, Bio-Kinetic Europe, Belfast, Northern Ireland. The three dosing regimens were (a) 1 g of acetaminophen every 6 h for 3 days (4 g/day), washout for 4 days, and 1.5 g of acetaminophen every 6 h for 3 days (6 g/day); (b) 1 g of acetaminophen every 6 h for 3 days (4 g/day), washout for 4 days, and 2 g of acetaminophen every 6 h for 3 days (8 g/day); and (c) placebo for 3 days, washout for 4 days, and placebo for 3 days.

Forty eligible men and women were divided equally into Groups I and II, which were treated via a staggered-start study design shown in **Figure 2**. The first 20 subjects checked into the clinical center for

a 3-day, drug-free baseline period. During this time, AST and ALT activities were measured daily, and the protocol stated that 18 subjects with the least variability in activities would be eligible for the dosing periods. Because the variability was low for all subjects, with no distinction among them, 18 subjects were selected randomly and were further randomized 2:1 to receive either multiple doses of acetaminophen at 4 then 6 g/day or multiple doses of placebo. Subjects in Group II began 7 days after Group I finished the baseline period, and the 18 eligible subjects were randomized 2:1 to receive either multiple doses of acetaminophen at 4 then 8 g/day or multiple doses of placebo. The staggered start allowed for Group I to complete the 6-g/day acetaminophen regimen while Group II completed the 4-g/day regimen, and for predesignated site personnel to review analytical and safety data before Group II proceeded with the 8-g/day acetaminophen regimen. To maintain double-blind status, the latter personnel had no other role in the conduct of the study.

Safety assessment. Vital signs, clinical laboratory test results, and the occurrence and seriousness of any adverse events were considered in the safety assessment. Clinical laboratory profiles were determined at screening and at the end of both dosing periods. To monitor hepatic function during the study, AST and ALT were measured daily. However, they were measured three times daily while subjects were dosed with acetaminophen at 6 and 8 g/day. Subjects were discharged from the clinical center after a physical examination and final blood sample for the final clinical laboratory profile.

Sample collection. Serial blood samples were collected over the first and final acetaminophen doses and before selected morning and evening doses. Specifically, 7-ml samples were collected into tubes containing sodium heparin at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, and 6 h after the first dose and at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 12 h after the final dose. They were centrifuged, and the plasma was separated into tubes that were frozen at −20°C until assayed for acetaminophen and metabolites.

Urine samples were also collected. The total amount of urine excreted over 24 h was collected on the final day of each multiple-dose regimen of acetaminophen and placebo. During this interval, urine samples for each subject were pooled into containers with 3 g of ascorbic acid and stored at 4°C. A 500-ml aliquot was transferred to a storage container and frozen at −20°C until assayed for acetaminophen and metabolites.

Bioanalytical methods. Using validated high-pressure liquid chromatography assays by PPD Development (Middleton, WI), plasma and urine samples were analyzed for acetaminophen and the following metabolites: acetaminophen glucuronide, acetaminophen sulfate, acetaminophen cysteine, and acetaminophen mercapturate. Commercial sources of the standards included US Pharmacopoeia (Rockville, MD) for acetaminophen, Sigma-Aldrich (St Louis, MO) for the glucuronide, and Magellan Laboratories (Research Triangle Park, NC) for the remaining metabolites. Standard curves for acetaminophen were linear from 0.20 to 50.0 μg/ml in plasma and 25.0 to 225 μg/ml in urine. Curves for the metabolites in plasma were linear from 1.25 to 50.0 μg/ml for glucuronide and sulfate, 0.20–10.0 μg/ml for cysteine, and 0.10–5.0 μg/ml for mercapturate. Standard curves in urine were linear from 400 to 3,600 μg/ml for glucuronide, 100–900 μg/ml for sulfate, and 25.0–225 μg/ml for cysteine and mercapturate.

Urine samples were also analyzed for three minor acetaminophen metabolites: methylthioacetaminophen, methanesulfinylacetaminophen, and methoxyacetaminophen. The samples had been collected in containers with 1.82 mM L-ascorbic acid to minimize degradation of metabolites. Standard curves were linear from 1.93 to 171 μg/ml (methylthioacetaminophen + methanesulfinylacetaminophen), 1.00–125 μg/ml (methylthioacetaminophen), 1.00–50.0 μg/ml (methanesulfinylacetaminophen), and 4.00–500 μg/ml (methoxyacetaminophen). The additional metabolite standards were supplied by Custom Synthesis Services (Madison, WI).

For the quantification of acetaminophen in plasma, 500-μl samples were extracted with ethyl acetate, evaporated to dryness, reconstituted with 50:50 methanol:water, and microfuged. For the quantification of metabolites in plasma, 200-μl samples were treated with 36% perchloric acid, vortexed, and centrifuged. The supernatant was then treated with 4.5 M potassium phosphate monobasic solution, vortexed, and centrifuged.

For the quantification of acetaminophen and metabolites in urine, 50-μl samples of urine were diluted with water, glucuronidase, or sulfatase as applicable. After 30 min, an aliquot of 36% perchloric acid was added to each sample and centrifuged. An aliquot of 4.5 M potassium phosphate monobasic solution was added to the supernatant, vortexed, and centrifuged. All final extracts and supernatants from both plasma and urine samples were analyzed using high-pressure liquid chromatography with UV absorbance detection.

Data analysis. WinNonlin Enterprise Version 4.1. (Pharsight, Mountainview, CA) was used in the noncompartmental pharmacokinetic analyses. Standard parameters for the first single dose, including AUC_{∞}, were estimated for acetaminophen, glucuronide, and sulfate. Oral clearance after the first dose (CL/F,1) also was estimated for acetaminophen.

Noncompartmental methods were used to estimate standard multiple-dose pharmacokinetic parameters for acetaminophen, glucuronide, sulfate, cysteine, and mercapturate for the final dose. These include $AUC\tau$ and average plasma concentration at steady state ($C_{AVG,ss}$). In addition, Cl/F at steady state (CL/F,ss) and the accumulation ratio were estimated for acetaminophen.

The amount excreted and percentage of acetaminophen dose in urine were determined for acetaminophen and the glucuronide, sulfate, cysteine, mercapturate, methylthioacetaminophen, methanesulfinylacetaminophen, and methoxyacetaminophen metabolites. The fCL of each metabolite were estimated as the product of the fraction of dose excreted and acetaminophen CL/F,ss. Acetaminophen CLr was estimated as the product of the fraction of dose excreted unchanged and CL/F,ss, whereas CLr for glucuronide, sulfate, cysteine, and mercapturate was estimated by the ratio of the amounts excreted to their $AUC\tau$ values.

Statistical analysis. Single- and multiple-dose pharmacokinetic parameters for acetaminophen and its metabolites were evaluated by descriptive statistics. To assess dose-dependent changes in the formation and/or CLr of metabolites, a paired *t*-test was used to compare metabolite fCL, percentage of each analyte excreted in urine, and CLr between the dosages (4 and 6 or 8 g/day) within each group of subjects.

In addition, estimates for AUC_{∞}, $AUC\tau$, CL/F,1, and CL/F,ss were evaluated by analysis of variance. The general linear models procedure of SAS® was used in the analysis of variance for a one-sequence crossover design that included factors to test for random effects of subject and fixed terms for day, dose, and day × dose. To assess time-dependent changes in pharmacokinetics, the ratios of least-square means for the log-transformed area parameters ($LAUC\tau/LAUC_{\infty}$) were compared within each multiple-dose regimen using the shortest 90% confidence intervals test. The ratio of log-transformed area parameters, which compares the first to last doses, will approach unity for time-independent disposition. For dose-dependent changes, the area terms for acetaminophen were normalized to the 1,000-mg dose.

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Data from this paper were previously presented, in part, at the following meetings: Society of Toxicology, Salt Lake City, UT, March 2003 (Gelotte C.K., Auiler J.F., Lynch J.M., Temple A.R., Bowen D.L. Tolerability and repeat-dose pharmacokinetics (PK) of acetaminophen (APAP) at 4, 6, and 8 g/d in healthy adults. *Toxicol. Sci.* **72**, S-1, 145 (2003)); North American Congress of Clinical Toxicology, Chicago, IL, September 2003 (Gelotte C.K., Auiler J.F., Temple A.R., Lynch J.M., Bowen D.L. Clinical features of a repeat-dose multiple-day pharmacokinetics trial of acetaminophen at 4, 6 and 8 g/day. *J. Toxicol. Clin. Toxicol.* **41**, 726 (2003)); International Congress of Therapeutic Drug Monitoring & Clinical Toxicology, Basel, Switzerland, September 2003 (Gelotte C.K., Auiler J.F., Lynch J.M., Temple A.R., Bowen D.L. Three-day dosing of paracetamol up to 8 g/d in healthy adults: pharmacokinetic (PK) and clinical laboratory outcomes. *Ther. Drug Monit.* **25**, 529 (2003)); American Society for Clinical Pharmacology and Therapeutics, Miami Beach, FL, March 2004 (Gelotte C.K., Auiler J.F., Lynch J.M., Temple A.R., Slattery J.T., Bowen D.L. Metabolite patterns measured during repeated dosing of acetaminophen (APAP) at 4, 6, and 8 g/day in healthy adults. *Clin. Pharmacol. Ther.* **75**, 79 (2004)); and European Association of Poisons Centers and Clinical Toxicologists, June 2004, Strasbourg, France (Gelotte C.K., Auiler J.F., Lynch J.M., Temple A.R., Slattery J.T., Bowen D.L. Time- and dose-dependent changes in the pharmacokinetics and metabolite patterns of paracetamol dosed at 4, 6, and 8 g/day in healthy volunteers. *J. Toxicol. Clin. Toxicol.* **42**, 468 (2004)). Some clinical data from this study are detailed further in: Temple A.R., Lynch J.M., Vena J., Auiler J.A., Gelotte C.K. Aminotransferase activities in healthy subjects receiving three-day dosing of 4, 6, or 8 grams per day of acetaminophen. *Clin. Toxicol.* **45**, 37–45 (2007).

CONFLICT OF INTEREST

Cathy K Gelotte, Joanna F Auiler, and Joseph M Lynch are employed by McNeil Consumer Healthcare. Anthony R Temple was employed at McNeil Consumer Healthcare at the time this research was conducted. Anthony R Temple is now retired and serving as a consultant to McNeil Consumer Healthcare. They each own stock in Johnson & Johnson. John T Slattery serves as a consultant to McNeil Consumer Healthcare.

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