Ethyl Glucuronide Excretion in Humans Following Oral Administration of and Dermal Exposure to Ethanol

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Abstract

Ethyl glucuronide (EtG) is a direct ethanol biomarker and U.S. Department of Health and Human Services has advised that specificity studies at low EtG levels are needed for distinction of ethanol consumption and incidental exposure. The authors report urinary EtG excretion with ethanol abstinence, dermal exposure and oral consumption. EtG concentration by sensitive liquid chromatography–tandem mass spectrometry measurement in 39 urine specimens from adult alcohol abstainers (< 10–62 μg/L) and in urine from 13 children (< 10–80 μg/L) indicates either unrecognized ethanol exposure or endogenous ethanol metabolism. With repetitive daily dermal exposure to hand sanitizer (60% ethanol) by 9 adults, EtG concentration ranged from < 10 to 114 μg/L in 88 first-morning void specimens. EtG excretion following a 24 g ethanol drink by 4 adults revealed maximum urine EtG concentration (12,200–83,200 μg/L) at 3 to 8 h postdose and an EtG detection window up to 25–39 h, compared to an ethanol window of only 2 to 4 h. Oral ethanol use also showed an increase in the percent (molar equivalent) ethanol excreted as EtG with increasing oral ethanol doses. Human excretion studies show 1. EtG detectable at low concentration (< 100 μg/L) when ethanol use or exposures is not evident, 2. EtG concentration less than 120 μg/L in first morning specimens from adults with repeated dermal exposure to ethanol, 3. EtG levels maximally elevated within 3–8 h and above baseline for up to 39 h after a 24 g ethanol drink, and 4. a dose-dependent increase in the percentage of ethanol excreted as EtG with increasing oral ethanol use.

Introduction

Ethyl glucuronide (EtG, ethyl β-D-6-glucosiduronic acid) is a phase two metabolite of ethanol formed by conjugation reaction with glucuronic acid via UDP-glucurolyosyltransferase catalysis. Urinary excretion of EtG represents a minor pathway of ethanol elimination initially identified in rabbits (1) and later in humans (2). Very early studies in animals indicated an increased percentage of ethanol metabolism to EtG with increasing ethanol dose but confirmation of a dose effect in humans has not been reported. EtG synthesis and initial measurement in both serum and urine in 1995 (3) lead the way

for further development in gas chromatography–mass spectrometry (GC–MS) (4,5), liquid chromatography (LC)–MS (6–8), and LC–MS–MS (9–11) methods of EtG measurement in both research and routine practice. Application of these methods has advanced our knowledge of EtG as a valuable biomarker of recent ethanol use (4,9,12–17). Clinical and forensic monitoring of EtG has increased in routine practice, but the contribution by environmental, hygenic, cosmetic, or other incidental sources of ethanol to urinary excretion of low, but measurable, concentrations of EtG remains in question (18). In addition, EtG generation from endogenous sources of ethanol has been suggested but not yet confirmed. The aim of this study is to evaluate EtG excretion associated with states of ethanol abstinence, dermal exposure, and oral consumption at varying doses.

Experimental

Chemicals

EtG and EtG-d$_5$ (internal standard) were supplied by Medichem in Steinenbronn, Germany. HPLC-grade (0.2-micron filtered) methanol and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ), and ammonium acetate from EDM Chemicals (Gibbstown, NJ). SepPak Vac amino propyl (500 mg) cartridges (3 mL) were purchased from Waters (Milford, MA). Deionized water was obtained from a Siemens Water Technology water purification system (Pittsburgh, PA).

Study design

Pediatric studies were conducted to determine urinary concentration of EtG in children less than 10 years of age where ethanol use is not anticipated. Urine was collected, under an Albany Medical College approved Institutional Review Board protocol, from 13 children who ranged in age from 1 to 10 years. The specimens were stored at 5°C until initial analysis for EtG, ethanol, and creatinine. Urine samples were then re-stored at 5°C and re-analyzed for EtG concentration within 48–72 h to assess reproducibility of initial test results.

Dermal exposure to ethanol from hand-sanitizer use was studied with Albany Medical College Institutional Review

Board approval in nine adult consenting volunteers (six males, three females) who abstained from ethanol consumption and from other known sources of ethanol exposure for at least one week prior to beginning the hand sanitizer protocol. During a five-day exposure period, Avagard D (3M Health Care, St. Paul, MN) hand antiseptic containing 61% (w/w) ethanol was applied 20 times daily during an 8–12-h waking period, with a 20-min minimum time interval between applications. Participants in the study were instructed to apply hand sanitizer (1 full applicator depression of approximately 1 mL), rub hands briskly, and allow the hands to air dry. Subjects were dermally exposed to approximately 10 g of ethanol per day with a cumulative dermal exposure to ethanol of approximately 50 g during the course of the study. A pre-exposure baseline urine was collected on day 1 of the study. Following day 1, first morning void specimens were then collected on days 2–5 of hand wash application with an additional seven days of morning urine collection immediately following the exposure period. The specimens were stored at 5°C until analyzed for EtG, ethanol, and creatinine.

EtG excretion following controlled oral doses of ethanol was studied in four consenting adult male subjects, all social drinkers, with a mean body weight of 75 kg (range 61–83 kg). Participants were required to abstain from both drinking alcohol and from any other known sources of ethanol exposure for at least one week prior to beginning each dosing study. A baseline urine specimen was collected prior to consumption of the ethanol drink for each dosage studied. The ethanol dose was prepared from a calculated volume of a single-source vodka (32% by laboratory analysis) diluted in an approximately 300 mL of water or juice, with complete consumption of the drink in the first 20 min of the study. Ethanol doses of 24, 12, 6, or 3 g were studied, corresponding to a respective mean dose of 0.33, 0.16, 0.08, and 0.05 g ethanol/kg mean body weight. With the 24 g dosing, the total urine void was collect throughout the study with subjects instructed to collect or pool urine for postdose intervals of 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 5 h and then for each subsequent 5-h interval up to 75 h. Subjects recorded the actual time and volume of each collection. For assessment of EtG excretion at progressively lower ethanol doses of 12, 6, and 3 g, total urine void was collected throughout the study in 5-h intervals postdose for up to 75 h. A minimum of one-week washout with abstinence from ethanol use and other known sources of ethanol exposure was maintained between studies. Urine specimens were stored at 5°C until analyzed for EtG, ethanol, and creatinine.

Analytical

EtG was determined by LC–MS–MS in accordance with forensic guidelines (11) and with optimization of a solid-phase extraction method reported by others (5,19). Aminopropyl SepPak cartridges were conditioned by consecutive washing with 3 mL methanol,

3 mL distilled water, and 3 mL acetonitrile prior to sample loading. Aliquots (25 µL) of matrix-matched calibrators, controls, and urine specimens with pre-addition of 50 µL EtG-d_5 (125 µg/L), 33 µL HCl (1 N), and 350 µL acetonitrile were loaded on extraction columns by vacuum control. The columns were washed with 3 mL acetonitrile, 3 mL ethanol, and 2 mL 1% ammonia in methanol. EtG was eluted with 5 mL of an ammonia/water/methanol (1:1:98 by volume) mixture, followed by pH neutralization and dry down with nitrogen at 35–45°C. Dried extracts were reconstitutioned in 200 µL acetonitrile/methanol (90:10). Samples were injected onto an Atlantis HILIC silica analytical column (100 mm × 2.1-mm i.d., 5-micron, Waters, Milford, MA) fitted with a Atlantis HILIC guard column (10 mm × 2.1-mm i.d., 5-micron), using an isocratic mobile phase solution of 7% ammonium acetate (5 mM, pH 5.5) in acetonitrile at a flow rate of 0.4 mL/min and an isocratic temperature of 20°C. LC–MS–MS analysis was performed by an Alliance 2795 LC system interfaced with a Quattro Micro API tandem MS (Waters), employing an electrospray (ESI) probe ionization source in negative mode with desolvation at 400°C, ionization voltage of –4 kV, and a 50 L/h cone gas flow rate. MassLynx version 4.1 software (Waters) was used for instrument data management. Analysis was performed by multireaction monitoring, using precursor ion at m/z 221 and the fragment ions at m/z 75 (quantifier) and 85 (qualifier) for EtG and precursor ion at m/z 226 and fragment ion at m/z 75 for the internal standard EtG-d_5. Representative chromatograms with transition ion mionitoring are shown in Figure 1. Lower limit of detection (LOD) of EtG (4 µg/L) was established with quantification ion detection and qualifying ion ratio criteria in spiked urine pools with baseline EtG concentration of less than 5 µg/L. Lower limit of quantification (LLOQ) was 10 µg/L, with quantitative EtG recovery averaging 92% (range 85–104%) at 10 µg/L and 102% (range 92–112%) at 100 µg/L. The percent coefficient of variation (%CV) profile for control pools with mean EtG concentrations of 13 µg/L (15.7%CV),
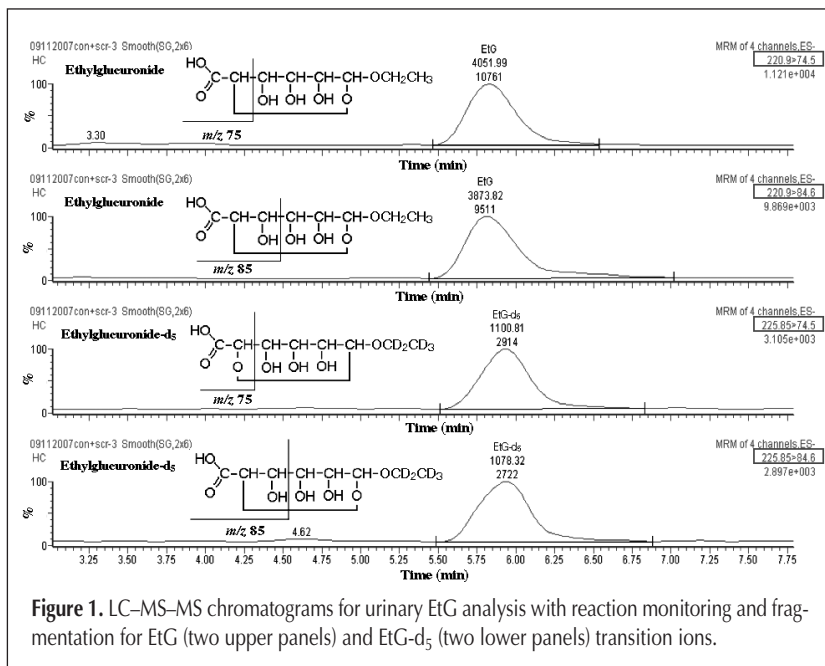

Figure 1. LC–MS–MS chromatograms for urinary EtG analysis with reaction monitoring and fragmentation for EtG (two upper panels) and EtG-d_5 (two lower panels) transition ions.

85 µg/L (7.4%CV), 125 µg/L (5.3%CV), 313 µg/L (2.3%CV), 721 µg/L (2.8%CV), and 9190 µg/L (6.5%CV) was determined. Upper limit of quantification (ULOQ) was 10,000 µg/L based upon analysis of EtG standards (25, 50, 100, 200, 500, 1000, 5000, and 10,000 µg/L) prepared in urine pools with low baseline EtG concentration, using a regression analysis R^2 criteria of > 0.98. Linearity was further assessed on each analytical run by serial dilution of urine pools with an EtG concentration in the range of 10,000 µg/L. Samples with concentrations exceeding the ULOL were diluted and reanalyzed.

The ethanol in urine was measured by a kinetic alcohol dehydrogenase method with ultraviolet monitoring of reduced nicotinamide adenine dinucleotide using a Microgenics DRI assay performed on a Microgenics MGC 240 analyzer (Fremont, CA) with an LLOQ of 10 mg/dL. Urinary creatinine was also measured on a Microgenics MGC 240 analyzer using the instrument manufacturer's alkaline picrate reagent (LLOQ = 20 mg/L).

Results and Discussion

EtG was measurable in a majority of urine samples collected from adults without a known source of ethanol use or exposure. Figure 2 shows the distribution of EtG concentration in 39 samples from adults abstaining from both ethanol consumption and other known sources of oral, dermal, or respiratory ethanol exposure. EtG was detected and quantitated in over 85% of urine samples with a median EtG concentration in adult abstainers of 19 µg/L and a maximum concentration of 62 ug/L. In an additional study with urine from 13 pediatric subjects where alcohol use was not anticipated, urine from 10 of the 13 subjects contained concentrations of EtG (11–80 µg/L) above the LLOQ, and in urine from the 3 remaining subjects EtG exceeded the 4 µg/L LOD (Figure 3). Ethanol at a threshold of 10 mg/dL was not detected in urine from any of the adult abstainers or pediatric subjects.

The source of the low level EtG concentration determined in urine from human subjects without evident ethanol use is not clear. Repeat EtG analysis of pediatric specimens (stored at 5°C) within 48 to 72 h of the initial test, as displayed in Figure 2, did not show any evidence of in vitro EtG generation associated with sample storage, as suggested as a possible occurrence in a recent study (20). Agreement between initial and follow-up testing in pediatric samples was within the analytical precision parameters of the assay, except for one subject where a decline in concentration may have been due to other factors such as β-glucuronidase activity as reported by others (21). Early studies concluded that EtG is exclusively formed after consumption of ethanol and urinary EtG was not measurable by GC–MS and LC–MS (LLOQ range 70–100 µg/L) in ethanol abstainers (5,7,22).

The low levels of EtG in urine of ethanol abstinent individuals would not be measurable with the LLOQ of these earlier methods, and this may explain the discrepancy with the current findings. Although unrecognized ethanol exposure cannot
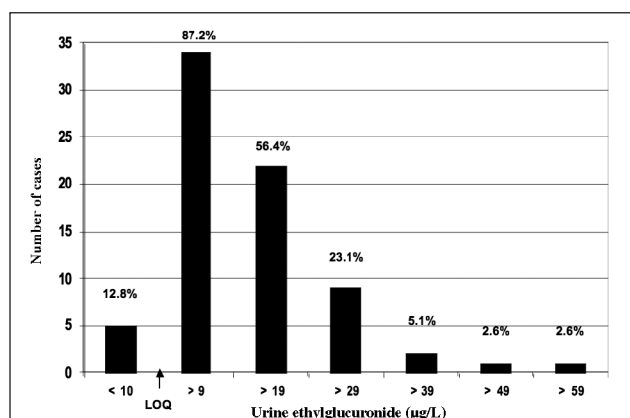
Figure 2. Urinary EtG concentrations in adult subjects abstaining from alcohol use and exposure.
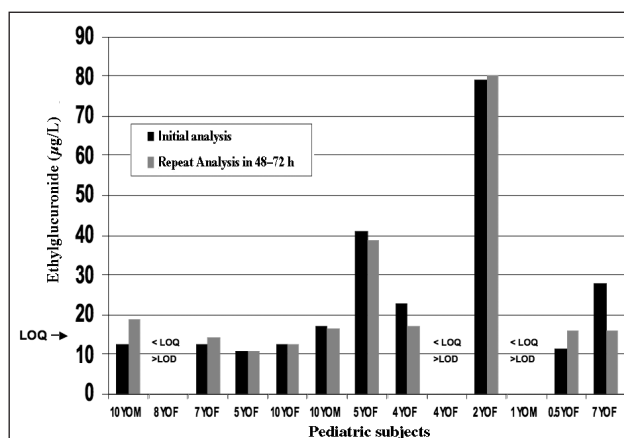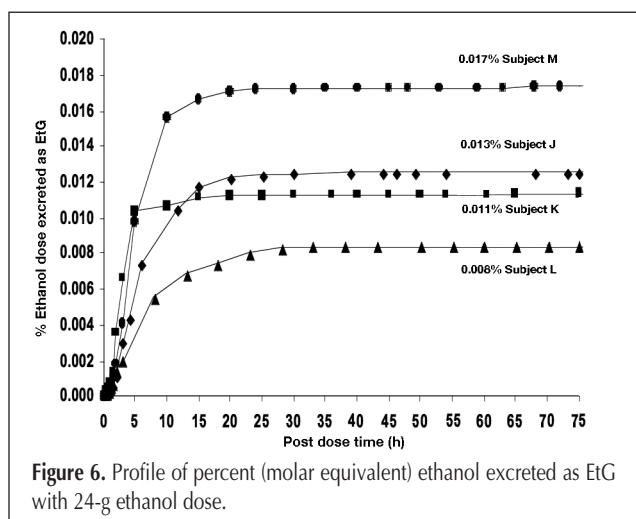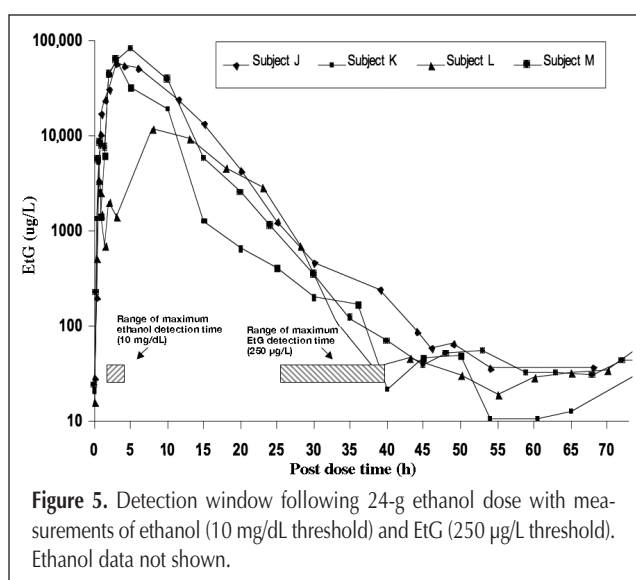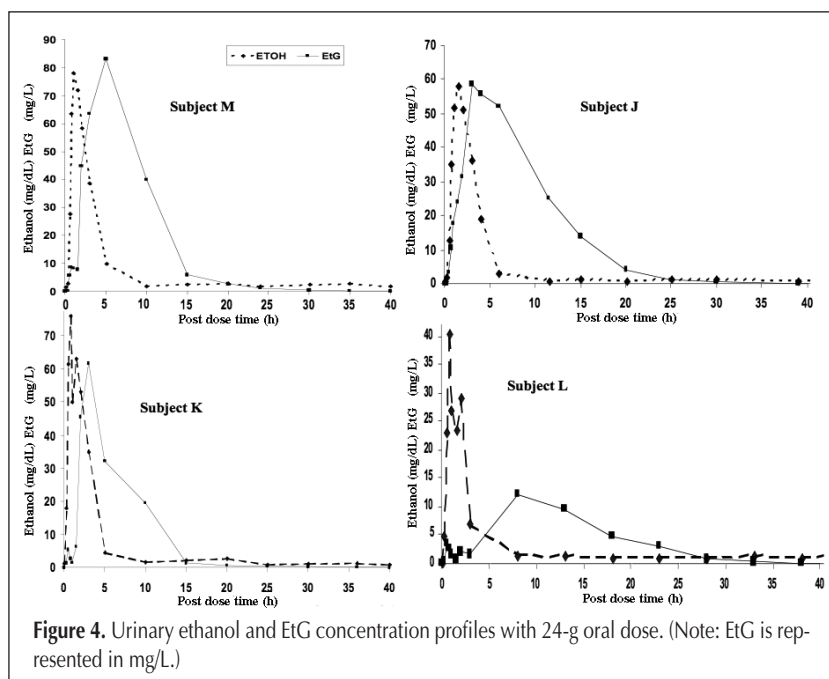
Figure 3. Urinary EtG concentrations in pediatric subjects with repeat analysis.

Table I. Urine EtG Concentrations (µg/L) with Repetitive Dermal Ethanol Exposure

Time of Collection	Subject								
	1	2	3	4	5	6	7	8	9
Baseline	< 10*	12	13	26	30	16	31	20	62
Day 2	41	18	21	93	50	89	43	106	40
Day 3	< 10*	< 10*	21	74	48	36	27	32	27
Day 4	27	< 10	15	28	27	114	18	58	42
Day 5	24	21	55	70	38	105	34	25	28
Day 6	20	10	21	93	46	86	18	90	35
Day 7	< 10*	22	14	27	94	33	19	47	78
Day 8	13	< 10*	11	23	56	29	16	22	19
Day 9	13	16	< 10*	22	21	19	32	28	< 10
Day 10	< 10*	14	16	< 10*	52	43	22	19	11
Day 11	< 10*	10	17	31	27	22	33	48	no data
Day 12	10	14	18	12	16	66	28	32	no data

* > LOD of 4 µg/L.

Figure 4. Urinary ethanol and EtG concentration profiles with 24-g oral dose. (Note: EtG is represented in mg/L.)

Figure 5. Detection window following 24-g ethanol dose with measurements of ethanol (10 mg/dL threshold) and EtG (250 µg/L threshold). Ethanol data not shown.

Figure 6. Profile of percent (molar equivalent) ethanol excreted as EtG with 24-g ethanol dose.

be ruled out as a source of the low concentration of urinary EtG in abstinent subjects, EtG may originate from the very low physiological ethanol concentrations in the blood. Ethanol generation by intestinal bacteria has been demonstrated (23–26) and may result in ethanol absorption and metabolism to EtG. Median plasma ethanol concentration of 0.014 mg/dL has been determined by a sensitive headspace GC method with health volunteer samples (27) and a review of endogenous ethanol studies in the early 1990s shows consensus on the presence of ethanol at maximum concentrations almost always less than 0.1 mg/dL (28). More recently, Logan and Jones (29) also reported endogenous ethanol in peripheral venous blood of healthy individuals, as well as those suffering with metabolic disorders, ranging in concentration up to 0.08 mg/dl. Even though these levels are below the level of ethanol quantification applied in routine clinical and forensic practice and have no known performance impairment effect, absorption and metabolism of ethanol from endogenous fermentation may contribute to low urinary EtG levels in the absence of external ethanol use or exposure.

The effect on urinary EtG concentration from dermal ethanol absorption with repetitive use of ethanol-containing hand sanitizer was studied in nine adult subjects. EtG concentration in first morning void specimens during and following five days of repeated daily use of a commercially available hand sanitizer is displayed in Table I.

Creatinine concentration averaged 1530 mg/L and ethanol was below the limit of quantification (10 mg/dL) in all subject specimens. EtG was quantitated in pre-exposure urine collections from all subjects with measurable concentration ranging from 12 to 62 µg/L in eight of the nine subjects. During the five days of dermal exposure to ethanol, EtG concentration range from less than 10 to 114 µg/L with concentration, exceeding 100 µg/L in only three specimens obtained from two subjects (subject 8 day 2; subject 6 days 4 and 5). In the seven-day postexposure study of first morning specimens EtG concentration ranged up to 94 µg/L. The dermal study shows that EtG concentration may be inconsistently elevated above baseline abstainer levels during exposure, but levels above 120 µg/L were not observed in first morning void specimens during or following exposure. There was also no evidence that EtG accumulates with repetitive daily exposure by the dermal route. Only one other study of EtG excretion with dermal ethanol exposure has been reported to our knowledge. Rohrig and Ross (30) collected urine at approximately 4-h intervals during dermal application of commercial brand of hand sanitizer (62% ethanol) at intervals of 0.25, 0.5, or 1 h. They reported a maximum ETG concentration of 62 µg/L using a LC–MS–MS method with an LLOQ of 50 µg/L. This report is consistent in finding low level EtG elevation above baseline in an occasional sample collected during dermal ethanol exposure, with the majority of exposure-collected specimens having EtG measurements that are not distinguishable

from pre-exposure measurements.

Urinary EtG concentration, following consumption of a drink containing 24 g ethanol, was studied with serial urine collections in four subjects. Panels in Figure 4 show comparative profiles of urinary EtG and ethanol concentration in the first 40 h following a 24-g dose. The time to reach maximum EtG concentration (T_{max}) averaged 4.8 h (range 3–8) compared to an average T_{max} for ethanol of 1.3 h (range 0.8–1.5). The maximum concentration (C_{max}) average for EtG was 53,900 μg/L (range 12,200–83,200) and for ethanol was 58 mg/dL (range 41–76). C_{max} for EtG exceeded an administrative threshold of 250 μg/L by 50–330-fold following a 24-g ethanol dose. During the first 24 h following the ethanol dose 95–99% of EtG was eliminated, with 81–91% of the EtG excreted in the first 10 to 12 h. In order to appreciate post-peak levels and detection time for EtG in urine, a log profile of urinary EtG concentration over the entire study period is displayed in Figure 5. Maximum time for detection of urinary ethanol at an LLOQ of 10 mg/dL (data not shown) averaged only 3 h (range 2–4), compared to a relatively prolonged detection time for EtG averaging 33 h (ranged 25–39) using a 250 mg/L administrative EtG threshold. By 38–46 h postdose, urine EtG concentration declined to levels measured in the ethanol-abstinent state and remained at concentrations of less 68 μg/L throughout the remainder of the study for all subjects. Kinetic parameter of T_{max} and maximum detection time for both ethanol and EtG in the current study compare closely with those reported in earlier studies (31,32).

Total EtG excreted following the 24 g ethanol dose averaged 14.3 mg (range 9.73–20.0) and compared closely with an total EtG excretion averaging 10 mg (SD 5) as reported previously in a 25-g ethanol dose study with three male and four female subjects (10). The relative percent (molar equivalent) of ethanol excreted in the urine as EtG with the 24-g dose ranged from 0.008 to 0.017% for the subjects in our study. Figure 6 profiles the relative percent excretion over the study period and shows that over 95% of renal EtG excretion is complete within first 24 h after ethanol is consumed. Because extra renal routes of EtG excretion are not evident (31), urine excretion of EtG may closely approximate the total body elimination of ethanol via the glucuronidation pathway. An endogenous or unrecognized source of EtG production may have contributed up to one percent to the total EtG output measured but was not considered a significant contributor to the excretion rate calculation.

The effect of varying ethanol dose on the relative percent ethanol excreted as EtG was also studied over an ethanol dosage range of 3 to 25 g. Profile of percent ethanol excreted as EtG for individual subjects is displayed in Figure 7 and shows a dose-related reduction in the molar equivalent percent of ethanol excreted as EtG. The interindividual variability in percent excretion as EtG was evident at each dosage of ethanol and when all subject data was analyzed across dosages, as shown in Figure 8, a direct relationship between dose and percent ethanol excretion as
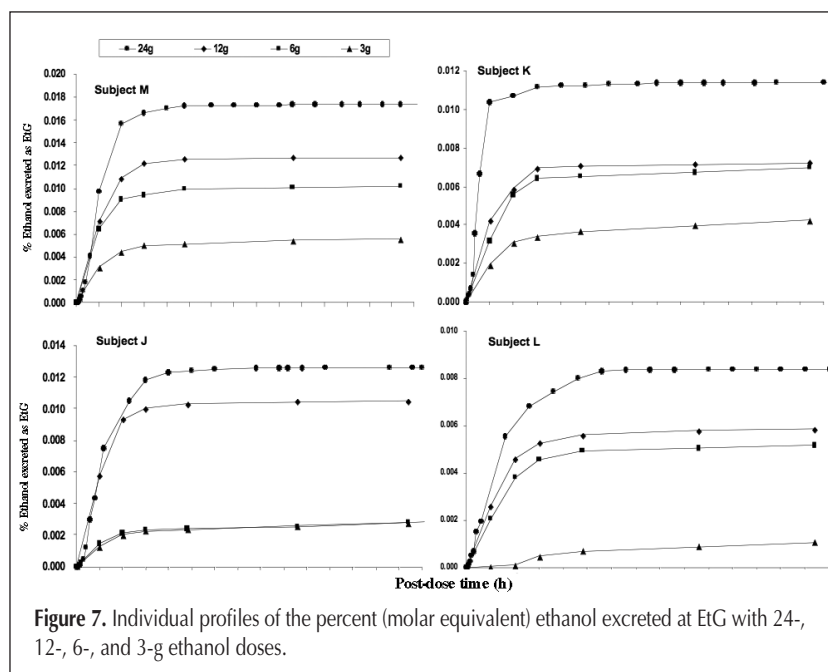

Figure 7. Individual profiles of the percent (molar equivalent) ethanol excreted at EtG with 24-, 12-, 6-, and 3-g ethanol doses.
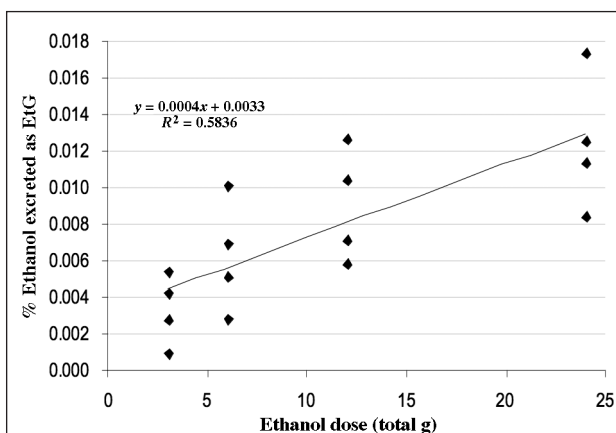

Figure 8. Effect of ethanol dose on percent metabolism to EtG. Mean and range of the percent (molar equivalent) ethanol excreted as EtG for all subjects.
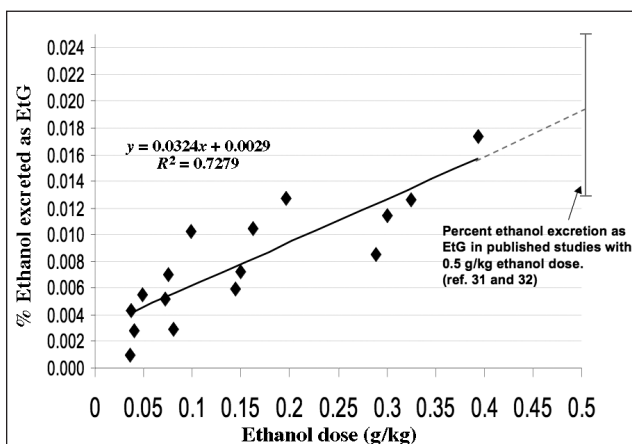

Figure 9. Effect of weight-adjusted ethanol dose on percent (molar equivalent) ethanol excretion as EtG for all subjects with comparison to published data.

EtG was determined by regression analysis with an R^2 of 0.58. Because body weight may contribute to the interindividual variability in percent excretion rate, EtG excretion data was re-evaluated with the ethanol dose normalized for body-weight (g ethanol/kg body weight), with the resultant regressed analysis showing an improved relationship ($R^2 = 0.730$) between dose and percent excretion (Figure 9). Others investigators have used higher doses of ethanol and reported a higher percentage of ethanol excreted as EtG. At a dose of 0.5 g ethanol/kg body weight percent excretion ranges of 0.013–0.022% (31) and 0.013–0.025% (32) have been determined. The range of percent excretion from these studies is plotted on Figure 9 and with an extrapolation of the regression line determine in the current study a 0.019% excretion rate is predicted at the 0.5 g/kg dose. The dose response relationship established in the current study predicts very closely the mean excretion rate found in these earlier investigations. A dose dependent decrease in the metabolism of ethanol to EtG in humans has not been previously reported but is consistent with a very early finding of a qualitatively similar dose relationship in ethanol-treated rabbits (1). A possible explanation for the dose dependency is the development of saturation kinetics in the primary oxidative pathway for ethanol elimination via alcohol dehydrogenase resulting in progressively greater substrate availability for the minor non-oxidative pathway via glucuronidation.

Conclusions

Human studies using a sensitive and validated EtG method show EtG is detectable at low concentration (< 100 µg/L) when ethanol use or other exposures are not evident, indicating either unrecognized exposure or metabolism of endogenously produced ethanol. In dermal exposure to ethanol, EtG levels remain less than 120 µg/L in first morning specimens from subjects with repeated hand washing with an ethanol-containing sanitizer, and EtG accumulation with repeated dermal ethanol exposures was not evident. In comparison, oral consumption of 24 g ethanol resulted in EtG concentrations exceeding 250 µg/L for up to 25 to 39 h, significantly exceeding the maximum detection time of 2 to 4 h for ethanol. Oral ethanol use also results in a dose-dependent effect on EtG excretion with proportionately higher rates of EtG excretion when higher doses of ethanol are taken.

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