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# Ethyl Sulfate: A Metabolite of Ethanol in Humans and a Potential Biomarker of Acute Alcohol Intake

**Anders Helander[1,*] and Olof Beck[2]**

*[1]Department of Clinical Neuroscience and [2]Department of Medicine, Karolinska Institutet and University Hospital, Stockholm, Sweden*

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## Abstract

**This study identified ethyl sulfate (EtS) in human urine and compared the excretion characteristics of EtS with that of ethanol and ethyl glucuronide (EtG). Urine samples were collected from healthy subjects after a single ethanol dose, and also selected from routine clinical samples. Simultaneous analysis of EtS and EtG was performed by direct electrospray liquid chromatography–mass spectrometry in the negative ion mode, with selected-ion monitoring of the pseudomolecular ions at *m/z* 125 for EtS ($M_w$ 126 g/mol) and *m/z* 221 for EtG ($M_w$ 222 g/mol). The identity of EtS in authentic urine specimens was established by co-chromatography with reference substance, the presence of product ions (*m/z* 97 and 80 from *m/z* 125) with correct relative intensity, and a correct sulfur isotope ratio for $^{34}S$ (*m/z* 127). After healthy subjects drank ethanol, EtS showed a much longer, dose-dependent elimination half-life than the parent compound. No EtS was detected in urines collected after abstention from ethanol for several days prior to sampling. Among 354 consecutive clinical samples, 86 were positive for both EtS and EtG with a mean EtG/EtS molar ratio of 2.3 (median 1.7). Another three urine samples were only positive for EtS and four only for EtG. The present results confirm that sulfate conjugation is a normal but minor metabolic pathway for ethanol in humans, and EtS a common constituent in the urine after alcohol intake. It is also indicated that the concurrent determination of EtS and EtG will improve sensitivity, when being used as biomarkers of recent drinking.**

## Introduction

Following consumption of alcoholic beverages, the bulk of the ethanol dose taken into the human body is metabolized in the liver in a two-stage oxidation process, first to acetaldehyde by alcohol dehydrogenase and then further to acetic acid by aldehyde dehydrogenase. The remainder is excreted unchanged in urine, sweat, and expired air (1). However, another very small fraction (< 0.1%) (2) of the ethanol dose undergoes a phase II conjugation reaction with glucuronic acid, catalyzed by endoplasmic reticulum UDP-glucuronosyltransferase, to produce ethyl glucuronide (ethyl β-glucuronide; EtG) (Figure 1), which is eventually excreted in the urine (3–5). In recent years, the development of improved analytical methods based on mass spectrometric detection (6–8) has renewed the interest in the pharmacology of EtG with primary focus on its use as a biomarker of recent alcohol consumption with clinical and forensic applications (9–11). Being a direct derivative of ethanol, EtG is considered specific for alcohol consumption and it also shows a much longer window of detection than the parent, unconjugated compound, implying a higher sensitivity for detection of recent drinking (2,12).

Previous experiments on laboratory animals (e.g., rat and rabbit) have indicated that ethanol may also undergo sulfate conjugation with 3'-phosphoadenosine 5'-phosphosulfate through the action of cytosolic sulfotransferase to produce ethyl sulfate (EtS) (Figure 1) (13–16). The formation of EtS was demonstrated by incubating lung or liver tissue slices or isolated microsomes with ethanol, but there were large variations between tissues and species. Furthermore, after administration of an oral dose of ethanol and injection of $^{35}S$-labelled sulfate to rats, EtS was also suggested to be excreted in the urine and mainly during the first 24 h (14). A general limitation in

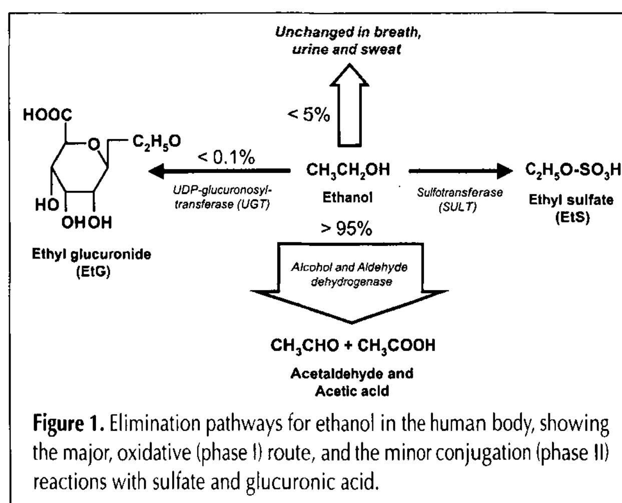


**Figure 1.** Elimination pathways for ethanol in the human body, showing the major, oxidative (phase I) route, and the minor conjugation (phase II) reactions with sulfate and glucuronic acid.

* Author to whom correspondence should be addressed. Dr. Anders Helander, Alcohol Laboratory, L7:03, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden. E-mail: anders.helander@cns.ki.se.



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these studies was the lack of conclusive identification of EtS and precise quantitation by reliable analytical methods, because results were obtained by indirect measurements using labeled compounds or enzymatic hydrolysis followed by measurement of the liberated ethanol. However, quite recently, using a specific and sensitive liquid chromatographic assay with mass spectrometric detection (LC–MS), sulfation of ethanol to produce EtS was established as a minor elimination pathway for ethanol in humans (17).

This study was undertaken to further confirm the identity of EtS in human urine after alcohol intake, to compare the urinary excretion characteristics with that of ethanol and EtG, and evaluate if EtS may also be useful as a biomarker of recent alcohol consumption. Identification and quantitation of EtS was performed by LC–MS and LC–MS–MS methods.

## Experimental

### Chemicals

EtS (ethyl sulfuric acid sodium salt, molecular weight 126.1 g/mol) was purchased from Tokyo Kasei Kogyo (TCI, Tokyo, Japan). EtG (ethyl-β-D-6-glucuronic acid, molecular weight 222 g/mol) and penta-deuterated EtG (EtG-$d_5$, molecular weight 227 g/mol, used as internal standard) were purchased from Medichem Diagnostica (Steinenbronn, Germany). All other chemicals were of analytical grade, and the water was of HPLC grade (> 18 MOhm).

### Urine samples

Urine samples were selected from those sent to the laboratory for routine measurement of the 5-hydroxytryptophol/5-hydroxyindoleacetic acid ratio used as a biomarker of acute alcohol intake (18,19). Urine samples were also collected from 9 healthy individuals (5 men and 4 women, aged 24–42 years) before and at timed intervals after drinking two different doses of ethanol (0.15 or 0.5 g/kg body weight) in 30 min in a fasting state. To study the effect of water-induced diuresis, two male subjects drank 0.15 g/kg ethanol and then 1 L of water immediately after the 6-h urine collection. All participants had abstained from alcoholic beverages for at least 48 h before the experiments began, according to self-report. The urine specimens were stored at –20°C until analysis. The procedures followed were approved by the ethics committee at the Karolinska University Hospital.

### LC–MS measurement of EtS and EtG

The concentration of EtS in urine was determined by a direct, negative ion electrospray LC–MS method used for quantitative analysis of EtG (8), by extending the analysis time and monitoring the pseudomolecular ion for EtS. The method allowed for the simultaneous determination of EtS and EtG (17). Urine was mixed 1:10 (v/v) with the internal standard (EtG-$d_5$ in distilled water; in future work, EtS-$d_5$ at *m/z* 130 is also used) and a 10-μL portion was injected directly into the LC–MS system (Perkin-Elmer 200 LC system and Sciex API 2000 MS). The chromatographic column was a (5-μm 100 × 2.1-mm i.d.) Hypercarb column (ThermoQuest, UK) equipped with a 10 × 2.0-

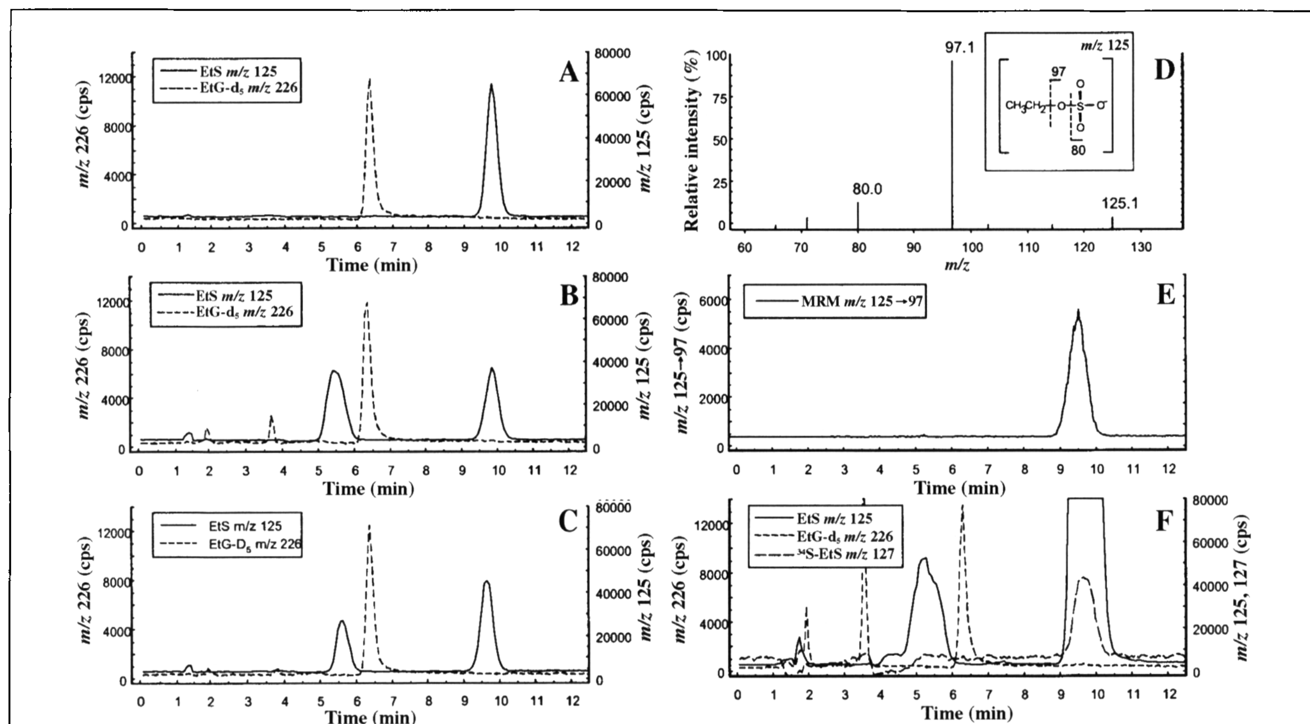


Figure 2. Chromatograms obtained from the analysis of EtS by the LC–MS method (electrospray ionization in negative mode) with the peaks for EtS (*m/z* 125; solid line) and EtG-$d_5$ (*m/z* 226, internal standard; broken line) for a standard sample containing ~2 mg/L EtS (A), an authentic urine sample containing ~1 mg/L EtS (B), and a 1:1 mixture of the standard and authentic urine samples (C). The product ion spectra of the pseudomolecular (*m/z* 125) ion for EtS (D), an LC–MS–MS recording of the major product ion transition (*m/z* 125 → 97) (E), and the presence of the $^{34}S$ isotope of EtS (*m/z* 127) (F), in the analysis of authentic urine samples collected after ethanol ingestion.

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mm Hypercarb guard cartridge. The mobile phase consisted of 25 mmol/L formic acid with 5% acetonitrile and was pumped isocratically at 200 µL/min at ambient temperature. Analysis was performed using selected ion monitoring of the pseudomolecular ions ($[M-H]^-$) at $m/z$ 125 for EtS, 221 for EtG, and 226 for EtG-$d_5$.

A calibration curve covering 0.1–100 mg/L (0.8–800 µmol/L; $r^2 = 0.999$, $p < 0.0001$) EtS was prepared by serial dilution with water and run with every batch of samples. The detection limit for EtS was ~ 0.05 mg/L (~ 0.4 µmol/L; signal-to-noise ratio of 3). The EtS concentration of unknown samples was determined from the peak-area ratio between EtS and internal standard by reference to the calibration curve. Standards were stored at –20°C until use and were found to be stable for at least 12 months.

The ethanol concentration in the urine samples collected during the drinking experiments was determined enzymatically using yeast alcohol dehydrogenase [limit of quantification (LOQ) = 1 mmol/L]. Urinary creatinine was determined by the routine Jaffé reaction on a Hitachi 917 analyzer (LOQ = 0.5 mmol/L).

## Results

### Identification of EtS in human urine

The identity of EtS in authentic urine specimens was established by co-chromatography with EtS reference substance (Figures 2A–C) when monitoring the pseudomolecular ion at $m/z$ 125, and by the presence of the major product ions of EtS ($m/z$ 97 and 80 from $m/z$ 125) by LC–MS–MS (Figures 2D–E). The identity was further supported by the correct relative abundance (peak area) of the $^{34}S$ isotope at $m/z$ 127 (5.3–5.4%; EtS standard 5.2%) in clinical samples containing high EtS concentrations (Figure 2F). No interfering peaks at $m/z$ 125 with a similar retention time as EtS has so far been observed. In 32 urine samples collected from 9 healthy individuals following self-reported abstention from ethanol for several days prior to sampling, no EtS was detected.

### Urinary excretion of EtS after alcohol consumption

The abstention from alcohol for at least 48 h before starting the drinking experiments, according to self-reports, was supported by all urine samples collected immediately prior to alcohol consumption being negative for EtS and EtG. In the first urine sample collected at 1 h after drinking, both EtS and EtG were readily detectable. The urinary excretion profile for EtS was dependent on the dose of ethanol ingested. After healthy subjects drank a single dose of ethanol at 0.15 g/kg (one representative result is shown in Figure 3) or 0.5 g/kg (17) in 30 min in a fasting state, the urinary EtS concentration typically peaked in the 1–2-h and 4-h collections, respectively, and remained detectable for > 12 h and > 24 h. This compares with urinary ethanol being detectable for only about 2 h and 6 h, respectively, after ethanol doses of 0.15 and 0.5 g/kg (data not shown).

When two male subjects drank 1 L of water at 6 h (immediately after the 6-h urine collection) after the intake of 0.15

| | EtS positive | EtS negative |
|---|---|---|
| EtG positive | 86 | 3 |
| EtG negative | 4 | 261 |

**Figure 4.** EtS and EtG were analyzed in 354 consecutive clinical urine samples. In 98% of the cases, the results agreed qualitatively, but in 7 cases only 1 of the conjugated metabolites could be detected.

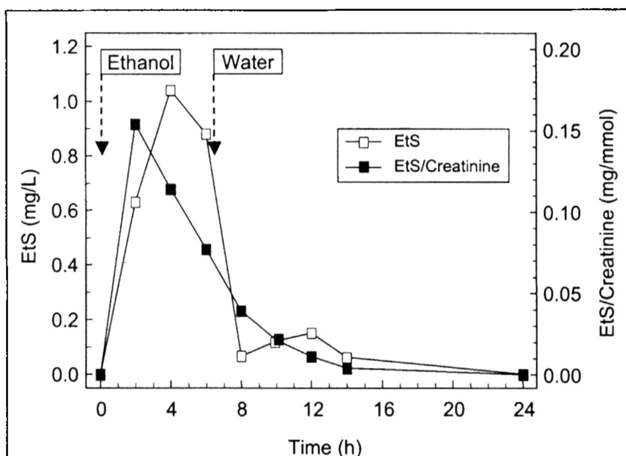


**Figure 3.** Time-course of urinary EtS excretion after oral ingestion of 0.15 g/kg ethanol by one healthy male subject. In this individual, EtS was detected in the 14-h collection but not at 24 h. EtS excretion was also expressed in relation to creatinine, to compensate for internal urine dilution due to the intake of 1 L of water immediately after the 6-h urine collection.

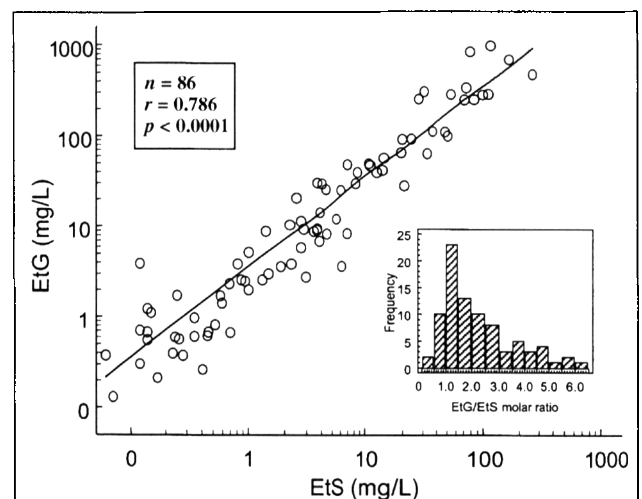


**Figure 5.** Correlation between urinary EtS and EtG concentrations in the 86 clinical urine specimens that were positive for both compounds (Equation: $y$ mg/L = 14.596 + 3.463$x$ mg/L). Inset: Distribution of the corresponding EtG/EtS molar ratios (one outlier with a molar ratio of 18.1 was omitted in this figure) (mean 2.3, median 1.7).



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g/kg ethanol, this caused a marked reduction in the EtS concentration in the next urine sample collected at 8 h (the results for one subject are shown in Figure 3). However, when EtS was instead expressed in relation to urinary creatinine, to compensate for variations in urine dilution, no similar effect of the water-induced diuresis was observed (Figure 3).

### EtS and EtG in routine clinical urine samples

Among 354 consecutive clinical urine samples selected from those sent to the laboratory for routine testing of recent alcohol consumption, 86 (24%) were positive for both EtS (mean 18.5 mg/L, median 2.86, range 0.05–264) and EtG (mean 77 mg/L, median 7.52, range 0.13–997) (Figure 4). Another 3 urine samples were only positive for EtS (range 0.05–0.30 mg/L) and 4 only for EtG (range 0.20–0.78 mg/L). Accordingly, of the 93 positive samples identified, 92.5% were positive for both ethanol conjugates, 3.2% only for EtS, and 4.3% only for EtG.

In the 86 urine specimens that were found positive for both compounds, the EtS and EtG values showed a highly significant, linear correlation over the entire concentration range (Figure 5). The slope for the linear regression curve demonstrated higher concentrations for EtG on the average, the mean value for the EtG/EtS molar ratio being 2.3 (median 1.7). However, there were large interindividual variations in this ratio [range 0.3–6.1 (one outlier showed 18.2), 2.5th–97.5th percentile 0.5–5.8] (Figure 5, inset).

## Discussion

Using mass spectrometric technique, the formation of EtS following ethanol ingestion was recently demonstrated in humans (17). In the present work, the identification of EtS in human urine was further secured and was based on three separate observations: co-chromatography with EtS reference substance, the correct sulfur isotope ratio of the pseudomolecular ion, and the presence of two product ions of the pseudomolecular ion with correct relative intensities. In addition, the identification of EtS has recently been confirmed by independent workers (20,21).

The presented mass spectrometric method for EtS was useful also for quantitative bioanalytical work, when EtS was evaluated as a biomarker of recent alcohol consumption. For this application, sufficient selectivity was obtained by monitoring the pseudomolecular ion at $m/z$ 125 in the negative mode using electrospray ionization. No chromatographic interference was observed, when applying this LC–MS method for the routine measurement of EtS in several hundred clinical samples. The use of single LC–MS rather than LC–MS–MS is advantageous from a clinical laboratory perspective because of the lower costs for instrumentation. If further selectivity is requested, or required, such as in forensic cases (22), this can be achieved by recording of the major product ion transitions (MS–MS or multiple reaction monitoring transitions) for EtS ($m/z$ 97 and $m/z$ 80 from $m/z$ 125), as demonstrated herein and also by others (20,21).

Sulfate conjugation is an important inactivation and detoxification pathway for a multitude of xenobiotics and small endogenous molecules (23). However, compared with glucuronidation, which was demonstrated to account for only < 0.1% of overall ethanol metabolism in humans (2), sulfation is even less important for the elimination of ethanol, because the urinary EtS concentration was lower than the EtG concentration in the majority of the clinical urine specimens (a mean EtG/EtS molar ratio of 2.3). Even though sulfation and glucuronidation are completely different metabolic processes, and no absolute agreement between these ethanol metabolites is to be expected, there was a highly significant, linear correlation between EtS and EtG. This similarity may reflect the fact that both compounds are direct derivatives of ethanol, albeit being formed by different enzyme systems.

One potentially important difference between EtS and EtG was observed for some urine specimens. Out of the total of 93 clinical samples that were positive for these conjugated metabolites, 7 (~ 7.5%) contained only one of them. Because these cases contained rather low concentrations of either compound, it is possible that these urine specimens had been collected at the end of the respective elimination curves, and that the differences in sensitivity observed were due to different time windows for EtS and EtG. Alternatively, genetic polymorphisms are known to occur for both sulfotransferases and UDP-glucuronosyltransferases and this might influence the metabolism and excretion patterns of EtS and EtG (24). This observation means that measuring both compounds will improve the possibility to detect recent intake of alcohol.

## Conclusions

These results demonstrated that sulfate conjugation is a normal but minor (< 0.1%) metabolic pathway for ethanol in humans and EtS a normal constituent in the urine following intake of alcohol. EtS remained detectable in the urine many hours longer than the parent, unconjugated compound, the time depending on the dose of ethanol ingested. As previously shown for EtG, testing urine for the presence of EtS thus provides a very sensitive means to determine if a person has recently consumed any alcohol, even if ethanol itself is not detected. The results further demonstrated that the urinary EtS concentration can be markedly lowered simply by drinking excess amounts of water or fluid prior to testing. In routine use, expressing urinary EtS as a ratio to creatinine therefore may be recommended to compensate for (intentional) urine dilution. Of the clinical samples that were positive for EtS and/or EtG, > 90% were positive for both conjugates, but a few were only positive for either compound. Accordingly, the concurrent determination of EtS and EtG, which is possible with the presented LC–MS method, will improve the overall sensitivity for detection of recent drinking.

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