

Applikationsbericht

## UPLC-MS/MS Method for Quantitation of EtG and EtS in Human Urine

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*For forensic toxicology use only.*

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## Abstract

This application note highlights the development of a rapid, simple dilute and shoot method for the definitive identification and quantitation of ethylglucuronide (EtG) and ethylsulfate (EtS) in human urine using UPLC-MS/MS, for forensic toxicology.

### Benefits

Simple dilute and shoot sample preparation method.

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## Introduction

Ethanol consumption has been linked to significant socio-economic burdens worldwide.<sup>1</sup> As a result, there is a growing need for the detection and identification of ethanol use. Over the years, ethylglucuronide (EtG) and ethylsulfate (EtS) have emerged as reliable biomarkers of recent ethanol use.<sup>2,3</sup> EtG and EtS are minor water soluble phase II metabolites of ethanol and are detectable in urine up to 80 hours following ethanol consumption.<sup>2,4</sup> Definitive confirmation of EtG and EtS as a biomarker of ethanol use is performed for a wide range of testing purposes. The authors report the development of a rapid and simple dilute and shoot method for definitive identification and quantitation of EtG and EtS in human urine using UPLC-MS/MS.

### Materials

#### Urine samples

Human urine samples for the preparation of calibrators and quality controls (QC) were obtained from volunteer donors with no recent (at least a week) use of ethanol. Prior to use, samples were confirmed negative for EtG by immunoassay analysis. Authentic samples were collected as part of routine casework. All samples were stored at -20°C without addition of preservatives.

### Reference standards

Drug reference material for EtG (Ethyl-β-D glucuronide, 1.0 mg/mL), and EtS (Ethylsulfate, 1.0 mg/mL) and deuterated analogues, EtG-D5 (Ethyl-β-D glucuronide D5, 1.0 mg/mL), and EtS-D5 (Ethyl-D5 sulfate, 1.0 mg/mL) were obtained from Cerilliant Corporation, TX, USA. Deuterated analogues were used for the purpose of internal standardization. Stock solutions containing a mixture of non-deuterated reference

material (EtG: 0.1 mg/mL and EtS: 0.05 mg/mL) or a mixture of internal standard (EtG-D5: 0.1 mg/mL and EtS-D5: 0.05 mg/mL) were prepared in methanol and stored at -20 °C. A daily working internal standard solution was prepared by a 400-fold dilution of the stock in distilled water.

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## Experimental

### Sample preparation

Urine samples were initially clarified by centrifugation for three minutes at 7200 rpm (~4227 x g). Following centrifugation, 50 µL aliquots of urine were loaded into a 96-well plate (Waters 96-well Sample Collection Plate, 2 mL square well). Aliquots were diluted by adding 500 µL of the daily working internal standard solution. Following dilution, samples were mixed on a vortex for one minute.

### LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC CSH Phenyl-Hexyl 2.1 x 150 mm, 1.7 µm (P/N: 186005408)
Column temp.:	50 °C
Mobile phase A:	Water containing 0.1% formic acid
Mobile phase B:	Acetonitrile
Wash solvent:	Acetonitrile/isopropanol / dH <sub>2</sub> O (1:1:1) (800 µL)
Purge solvent:	2% methanol in dH <sub>2</sub> O (2400 µL)
Injection volume:	10 µL

### Gradient

Time (min)	Flow rate (mL/min)	%A	%B	Slope
0	0.5	98	2	Initial
0.1	0.5	98	2	6
5	0.5	40	60	6
6.5	0.5	5	95	1
7	0.5	98	2	1

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*Table 1. Gradient conditions, total run time: 7.5 min.*

## MS conditions

MS system:	Xevo TQD Mass Spectrometer
Data acquisition and processing:	MassLynx v4.1 with TargetLynx
Ionization mode:	ESI
Capillary voltage:	2.5 kV
Acquisition mode:	Multiple reaction monitoring (MRM – Table 2)

## MRM conditions

Compound	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Trace type
EtG	221.1	75.0	Quantifier
EtG	221.1	85.0	Qualifier
EtS	125.0	97.0	Quantifier
EtG-D5	226.1	75.0	Quantifier
EtG-D5	226.1	85.0	Qualifier
EtS-D5	130.0	98.0	Quantifier

*Table 2. MRM conditions for EtG, EtS, and corresponding internal standards.*

## Results and Discussion

A series of calibrators and quality control (QC) samples were prepared by diluting the stock solution of non-deuterated EtG/EtS in negative human urine (Table 3). Following the simple sample preparation, multiple reaction monitoring (MRM) was performed using two transitions for EtG and EtG-D5, and one transition for EtS and EtS-D5 (Figure 1). For EtG a target quantifier/qualifier ion ratio was determined, using the threshold calibrator (EtG/EtS: 500/250 ng/mL), and subsequently used to monitor QC's and unknown samples. Acceptability criteria included +/- 20% of target ion ratio.

QC or Calibrator	% Threshold	EtG conc. (ng/mL)	EtS conc. (ng/mL)
S-200/LOD	40	200	100
S-500	100	500	250
S-1000	200	1000	500
S-2500	500	2500	1250
S-5000	1000	5000	2500
S-10000	2000	10000	5000
QCNEG	0	0	0
QC1	40	200	100
QC2	125	625	312.5
QC3	1600	8000	4000

Table 3. Method calibrators and QC's concentrations and corresponding percent of cut-off (EtG: 500 ng/mL, EtS: 250 ng/mL).

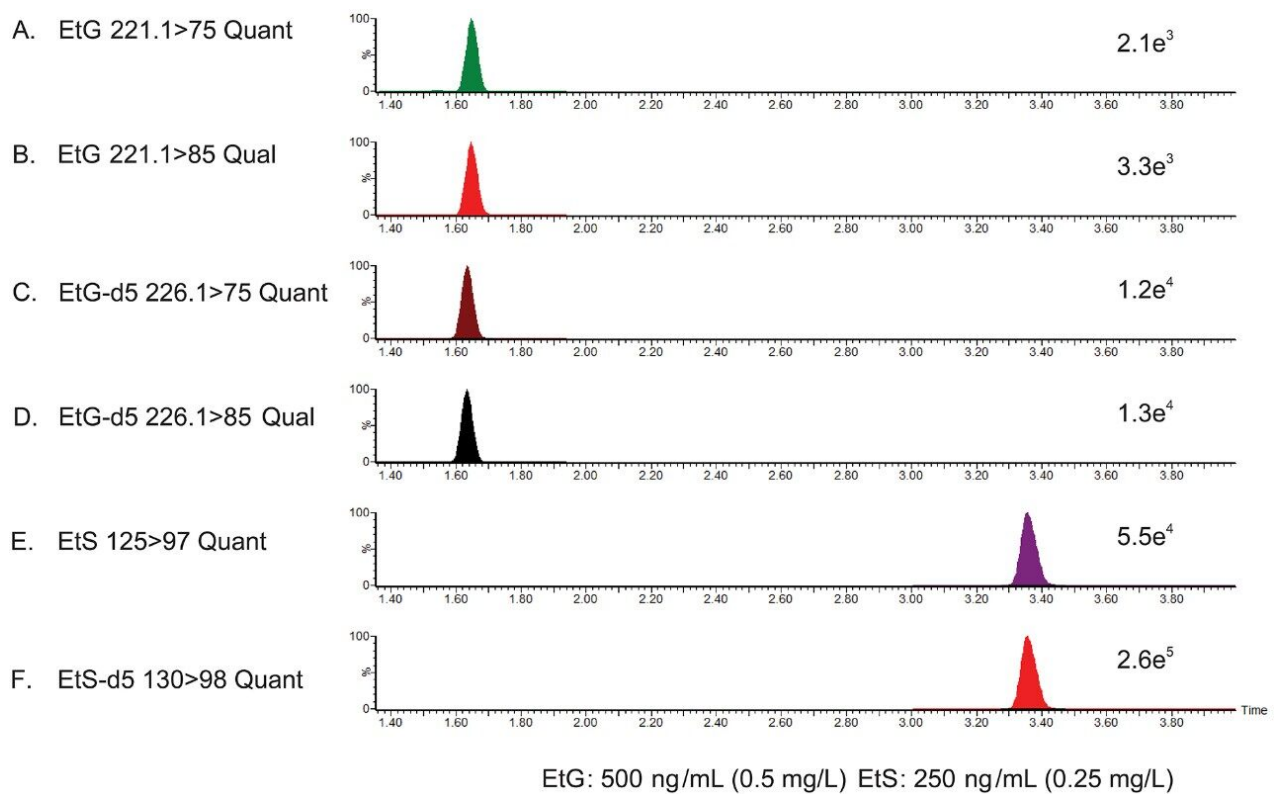
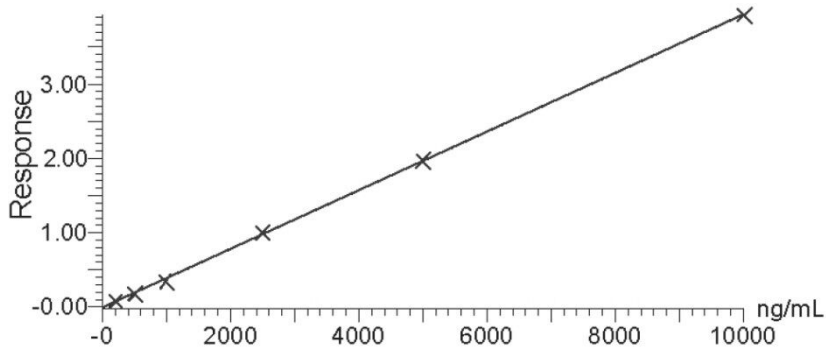


Figure 1. MRM chromatograms from a 10  $\mu$ L injection of a 500/250 ng/mL EtG/EtS urine calibrator. (A) EtG quantifier ion, (B) EtG qualifier ion, (C) EtG-D5 quantifier ion, (D) EtG-D5 qualifier ion, (E) EtS quantifier ion, (F) EtS-D5 quantifier ion.

Calibration curves were generated based on the ratio of the response of the analyte's quantifier ion relative to the response of the quantifier ion for the respective deuterated internal standard. Regression lines were plotted using a 1/x weighting. Calibration curves for EtG ( $r^2$  range: 0.991–0.999) and EtS ( $r^2$  range: 0.997–0.999) were linear over the analytical ranges investigated, and extended from 200 to 10,000 ng/mL and 100 to 5,000 ng/mL for EtG and EtS, respectively (Figure 2). The cut-off for the assay was set at 500 ng/mL for EtG and 250 ng/mL for EtS. The limits of detection (LOD) were determined using the lowest non-zero calibrator approach. LOD's for EtG and EtS were set at 200 ng/mL and EtS 100 ng/mL, respectively.

The precision and accuracy of the method were assessed at three QC concentrations for EtG (200, 625, 8000 ng/mL) and EtS (100, 312.5, 4000 ng/mL). Based on 11 analytical runs, consisting of three or four replicates, the assay precision (%CV) and accuracy for EtG ranged from 8.4 to 19.6, and 98.4% to 103.6%, respectively. The assay precision and accuracy for EtS ranged from 4.7 to 18.2, and 96.4 to 110.8%, respectively. In all, the method showed good precision and accuracy as summarized in Table 4.

- A. Compound name: Ethylglucuronide (EtG)  
Correlation coefficient:  $r = 0.999455$ ,  $r^2 = 0.998910$   
Calibration curve:  $0.000394165 * x + -0.0122341$   
Response type: Internal Std (Ref 2), Area \* (IS Conc./IS Area)  
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



- B. Compound name: Ethylsulfate (EtS)  
Correlation coefficient:  $r = 0.999834$ ,  $r^2 = 0.999669$   
Calibration curve:  $0.000806415 * x + 0.00809353$   
Response type: Internal Std (Ref 4), Area \* (IS Conc./IS Area)  
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

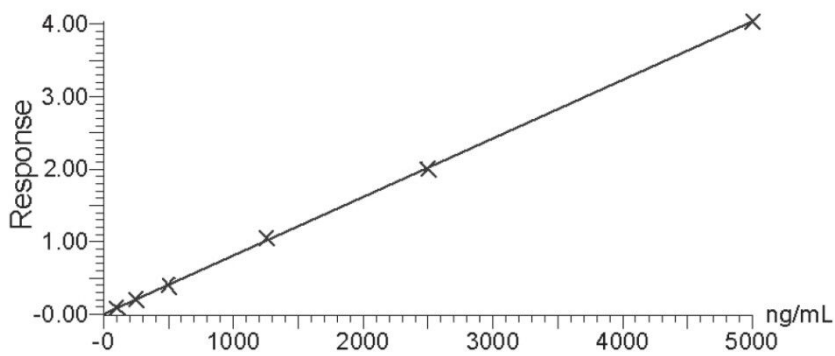


Figure 2. Representative calibration curves for (A) EtG (analytical range: 200 to 10,000 ng/mL) and (B) EtS (analytical range: 100 to 5000 ng/mL).



Compound	QC conc. (ng/mL)	Accuracy (%) (n=35)	Precision (% CV) (n=35)
EtG	8000	103.6	8.4
	625	98.4	12.8
	200	98.4	19.6
EtS	4000	102.9	4.7
	312.5	96.4	10.7
	100	110.8	18.2

*Table 4. Summary of method precision and accuracy data.*

Matrix effects were evaluated using aqueous versus urine based control samples through the analysis of 10 negative urine specimens and aqueous mobile phase spiked with EtG and EtS at 1000 and 500 ng/mL, respectively. Percent matrix effect was calculated using the following formula:  $[(A/B - 1) \times 100\%]$  where A represents the ion response in urine matrix and B represents the ion response without urine matrix present. Ion effects varied from 1% to -58% for EtG and -54% to 94.6% for EtS. Based on dilute and shoot sample injections, ion suppression of greater than 20% was anticipated, however for this reason analyte-matched deuterated internal standards were incorporated into the method to compensate for matrix effects. Normalization of the data using this approach resulted in a robust assay and satisfied the criteria for precision and accuracy. The stability of EtG and EtS were assessed in both primary specimens and prepared samples following a five day storage period at -10 °C and 4 °C, respectively. Results from reanalysis of primary specimens (n=6), calibrator, and QC samples were within 20% of the results obtained on initial analysis.

Identifier	EtG (ng/mL)		EtS (ng/mL)	
	Reference method	Developed method	Reference method	Developed method
case 1	512	710	NA	
case 2	6871	8132	2118	2583
case 3	1431	1840	1411	1847
case 4	2194	1854	872	982
case 5	5892	8087	>5000	
case 6	1542	1506	510	586
case 7	942	1170	332	364
case 8	3174	4340	465	546
case 9	623	316	425	327
case 10	709	389	416	291
case 11	1772	1812	974	991
case 12	7632	7021	1497	1285
case 13	5360	5076	763	842
case 14	2770	2483	1332	1051
case 15	8431	8433	4024	3046
case 16	5220	3941	1056	863
case 17	1838	1503	429	285
case 18	5679	8146	1514	1023
case 19	6512	5063	2224	1695
case 20	2455	1796	332	274
case 21	1710	1581	355	362
case 22	1255	1273	964	842
case 23	2190	1532	744	614
case 24	704	783	250	165
case 25	8448	9354	2793	2761
case 26	1914	1526	1097	768
case 27	1414	1642	333	373
case 28	1759	1391	346	272
case 29	3605	1953	452	284
case 30	1763	1206	1013	1086
case 31	5314	4667	1312	1168
case 32	2166	4603	2341	2018
case 33	1124	927	267	227
case 34	2483	2468	NA	

Table 5. Quantitative EtG and EtS results obtained from reference method (MedTox Laboratories, Inc.) and developed method. Data from the developed method was not tabulated (shaded cells) and indicated when quantitation from the reference method was not available (NA).

Method correlation studies were performed using de-identified casework specimens (n=34) with positive presumptive and confirmatory results for EtG and/or EtS. Initial presumptive results were obtained using a qualitative Microgenics DRI® EtG Enzyme immunoassay analysis with a 500 ng/mL cutoff. Quantitative EtG and EtS results were obtained from MedTox Laboratories, Inc. (Minnesota, USA) using a currently validated

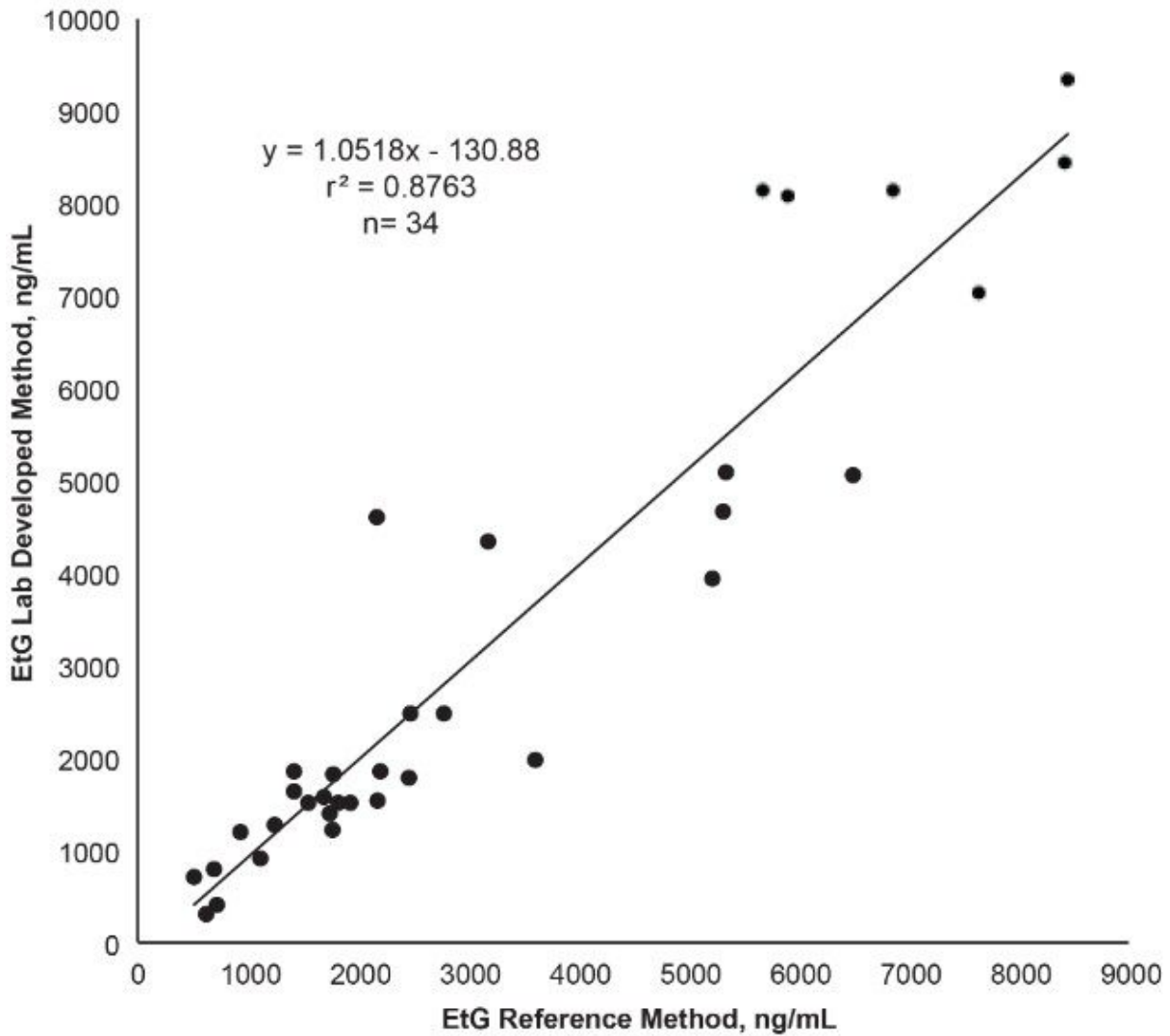
confirmatory LC-MS/MS method (reference method). Following initial analyses, the specimens were stored at -10 °C for a period of six months. Table 5 shows the results from the reference and developed methods. Analysis of the concentrations obtained by both methods shows dispersion in the data as expected between laboratories (Figure 3). However, statistical analysis did not show method bias based upon linear regression analysis using 95% confidence limit for the slope and y-intercept (Table 6).

EtG		
Regression statistics		
Multiple R	0.9361	
R Square	0.8763	
Adjusted R square	0.8725	
Standard error	964.1767	
Observations	34	
Coefficients		
Intercept	-130.8756	
X Variable 1	1.0518	
<i>Standard error</i>	<i>t Stat</i>	
275.7429	-0.4746	
0.0699	15.0581	
<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>
0.6383	-692.5455	430.7942
4.45922E-16	0.9095	1.1941

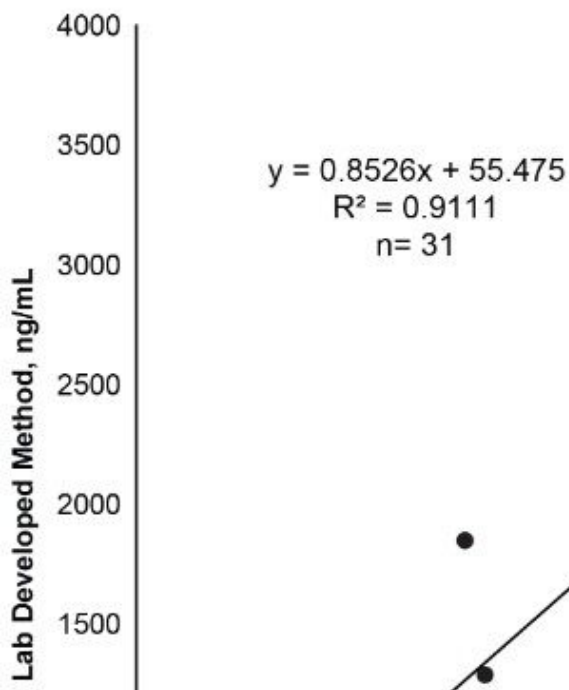
EtS		
Regression statistics		
Multiple R	0.9545	
R Square	0.9111	
Adjusted R square	0.9081	
Standard error	236.7427	
Observations	31	
Coefficients		
Intercept	55.4750	
X Variable 1	0.8526	
<i>Standard error</i>	<i>t Stat</i>	
67.6134	0.8205	
0.0494	17.2436	
<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>
0.4186	-82.8099	193.7599
8.75782E-17	0.7514	0.9537

Table 6. Summary of statistical analysis for correlation study between developed and reference methods.

## EtG Quantification: Lab Developed vs. Reference Method



## EtS Quantification: Lab Developed vs. Reference Method



2. Jaakonmaki PI, Knox KL, Horning EC, Horning MG. *Eur. J. Pharmacol.* (1967) 1:60–70.
  3. Rosano TG, Lin JJ. *Analytical Toxicology.* (2008) 32:594–600.
  4. Wurst FM, Skipper GE, Weinmann W. *Addiction.*(2003) 98 (Suppl 2): 51–61.
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<https://www.waters.com/waters/partDetail.htm?partNumber=186002482>>

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