

# Quantification of ethyl glucuronide and ethyl sulfate in human urine by LC-HRAM(MS) for clinical research

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## Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass<sup>®</sup> kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive hardware enables increased confidence in data
- Simple dilute-and-shoot offline sample preparation

## Goal

Implementation of an analytical method for the quantification of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in human urine on a high-resolution accurate mass (HRAM) Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

## Introduction

EtG and EtS are direct metabolites of ethanol and can be used as markers for recent alcohol use. Laboratory-based urine tests can indicate the presence of alcohol and quantify the amounts of EtG and EtS in urine up to 80 hours after consumption. Liquid chromatography coupled to mass spectrometry (LC-MS) enables laboratories to confidently conduct high quality tests, while ensuring accurate results due to the selectivity and specificity of the technology and method. Ease of use further facilitates the convenience of complete EtG and EtS testing kits, which feature fast and simple sample preparation with reliable analyte determination.

An analytical method for clinical research for the quantification of EtG and EtS in human urine using both a full scan (FullMS) mode and a parallel reaction monitoring (PRM) mode is reported in this study. The use of high resolution allows for selectivity and sensitivity even in FullMS mode. The additional use of fragmentation in PRM mode provides enhanced specificity to the analytical method.

Urine samples were simply diluted and injected onto a Thermo Scientific™ Vanquish™ Duo UHPLC system for LC separation. Detection was performed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization (HESI), either by FullMS scan or by PRM using d5-EtG and d5-EtS as internal standards. Method performance was evaluated using the ClinMass LC-MS/MS Complete Kit for Ethylglucuronide and Ethylsulfate in Urine from [RECIPE Chemicals + Instruments GmbH](#) (Munich, Germany) in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurements, and intra- and inter-assay precision for both analytes.

## Experimental

### Target analytes

The concentration ranges covered by the calibrators (MS8713 batch #1437) used are reported in Table 1.

**Table 1. Concentration ranges covered by calibrators**

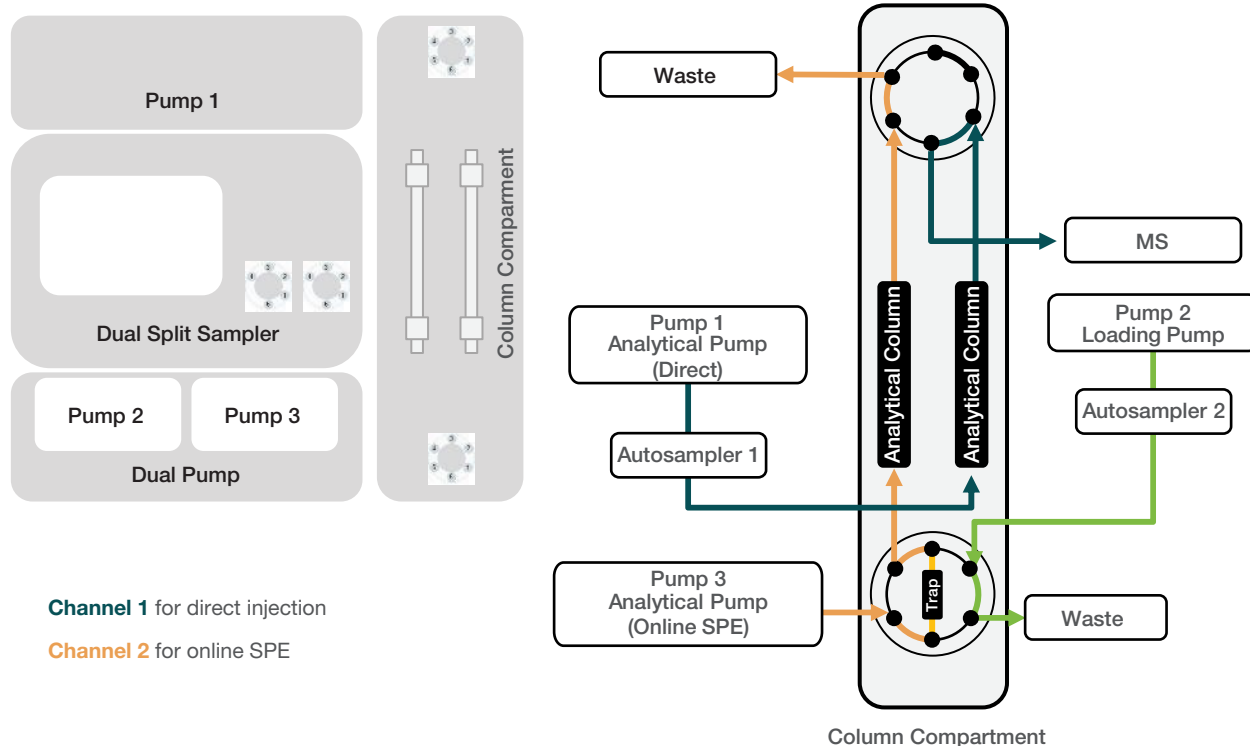
Analyte	Concentration range (ng/mL)
Ethyl glucuronide	81.7–9,368
Ethyl sulfate	29.1–4,953

### Sample preparation

Reagents included seven calibrators (including blank) and three controls (MS8083 batch #1478) from RECIPE, as well as d5-EtG and d5-EtS as internal standards for the quantification. Samples of 50 µL of urine were simply diluted using 1,000 µL of dilution solution provided with the kit, containing the internal standards, and injected onto the LC system.

### Liquid chromatography

A Vanquish Duo UHPLC system configured as a dual channel instrument for both LC-only and online SPE applications (Figure 1), was used for chromatographic separation. The LC-only channel was used in this case, utilizing the mobile phase and the analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. Total runtime was 6.0 minutes.



**Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup**

**Table 2. LC method description**

Gradient profile		
Time (min)	Flow rate (mL/min)	A (%)
0.00	0.0	100
0.01	0.2	100
2.00	0.2	100
2.10	0.7	100
5.90	0.7	100
5.91	0.2	100
6.00	0.0	100
Other parameters		
Injection volume		15 $\mu$ L
Column temperature		40 $^{\circ}$ C

### Mass spectrometry

Analytes and internal standards were detected in both FullMS and PRM modes on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization operated in negative ion mode. A summary of the MS conditions is reported in Table 3.

**Table 3. MS settings**

Parameter	Value
Source type	Heated electrospray ionization (H-ESI)
Vaporizer temperature	460 $^{\circ}$ C
Capillary temperature	350 $^{\circ}$ C
Spray voltage (negative mode)	2,500 V
Sheath gas	60 AU
Sweep gas	1 AU
Auxiliary gas	15 AU
S-lens RF level	60
Data acquisition mode	FullMS-ddMS <sup>2</sup> and PRM

### Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, LLOQ, carryover, accuracy, trueness of measurement, and intra- and inter-assay precision for all the analytes.

To determine the LLOQ, the lowest calibrator was diluted down to 20-fold with blank matrix; a full set of calibrators (six levels), diluted calibrators (four levels), and controls

(three levels) were extracted in replicates of five (n=5), injected in a single batch, and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a percentage coefficient of variation (%CV) < 20% across the entire batch of samples.

Carryover was calculated in terms of percentage ratio between the peak area of the highest calibrator and a blank sample injected just after it.

Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using the quality control samples at three different levels provided by RECIPE, prepared and analyzed in replicates of five on three different days.

Trueness of measurement was also evaluated as percentage bias using certified external quality controls (GTFCh ETG 1/18 B, 1/19 B, 1/20 B, 3/18 B, 3/19 B from Arvecon GmbH, Germany), prepared and analyzed in replicates of five on a single day.

Intra-assay precision for each day was evaluated in terms of %CV using the controls at three different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at three levels in replicates of five prepared and analyzed on three different days).

### Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

### Results and discussion

A linear response with 1/x weighting was obtained for all the analytes in both FullMS and PRM mode, not only in the calibration range covered by the calibrators but also down to lower limits of quantification. A summary of the LLOQs obtained using the two different acquisition modes is reported in Table 4.

**Table 4. Concentrations of the lowest calibrators and the LLOQs**

Analyte	Concentration (ng/mL)		
	Lowest calibrator	LLOQ FullMS mode	LLOQ PRM mode
Ethyl glucuronide	81.7	4.08	4.08
Ethyl sulfate	29.1	14.6	14.6

The percentage bias between nominal and back-calculated concentration was always within  $\pm 15\%$  for all the calibrators ( $\pm 20\%$  for the lowest calibrator) in all the runs. Representative chromatograms for the LLOQ for the analytes and their internal standard using both approaches are depicted in Figure 2. Representative calibration curves in the concentration range covered by the kit (six calibrators) are shown in Figure 3.

No carryover was registered; no peak was detected in the blank sample following the highest calibrator for both analytes.

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control

samples ranging between  $-4.7\%$  and  $6.8\%$  in FullIMS mode and between  $-8.3\%$  and  $4.8\%$  in PRM mode (Table 5).

Good results were obtained also from the evaluation of trueness of measurement, with a percentage bias between  $-4.0\%$  and  $11.2\%$  in FullIMS mode and between  $-5.3\%$  and  $11.3\%$  in PRM mode (Table 6).

The %CV for intra-assay precision was always below  $6.3\%$  in FullIMS mode and  $3.3\%$  in PRM mode. The maximum %CV for inter-assay precision was  $10.0\%$  and  $5.1\%$  in FullIMS and PRM modes, respectively. Results for intra- and inter-assay precision are reported in Table 7 and Table 8 for FullIMS and PRM modes, respectively.

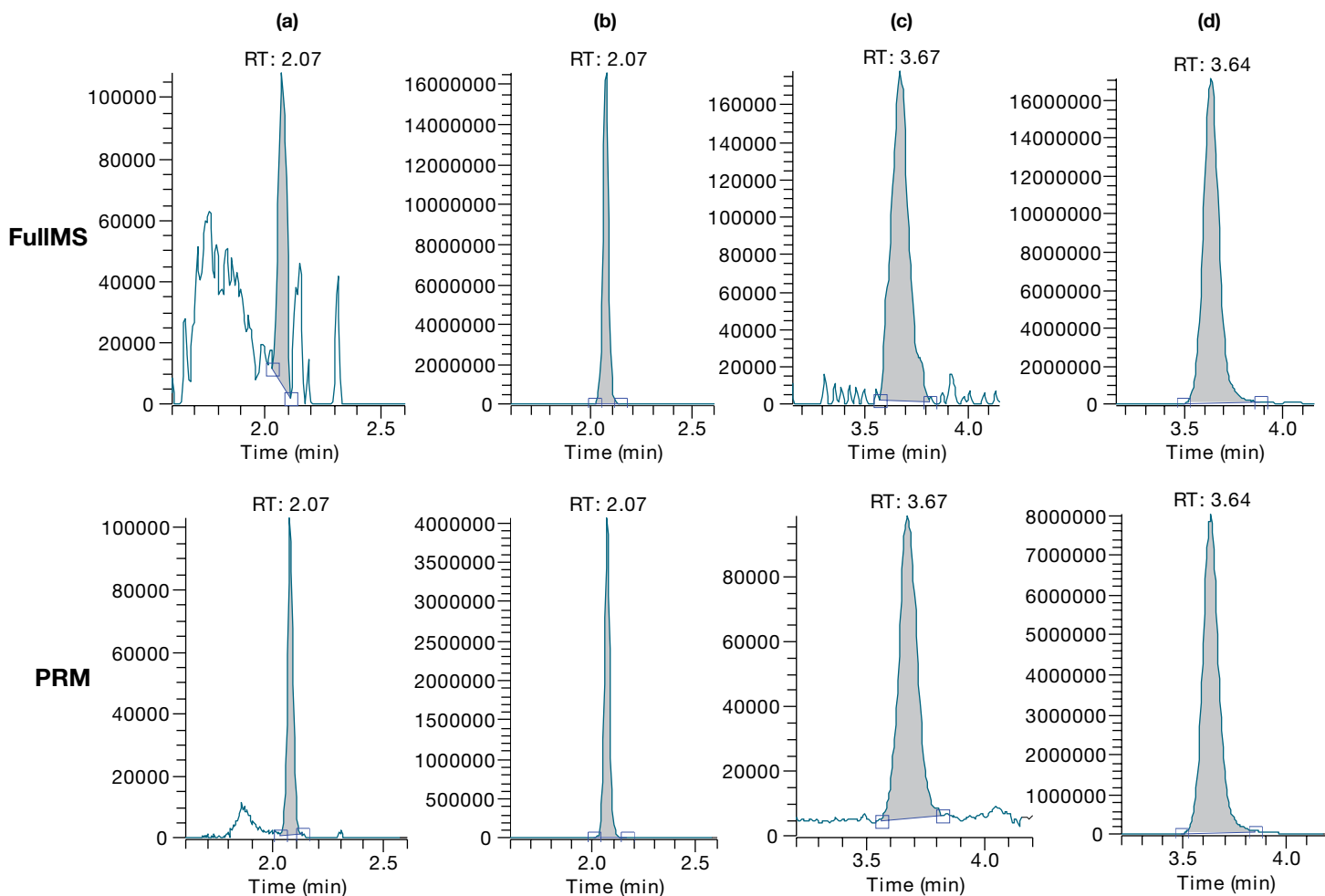


Figure 2. Representative chromatograms of the LLOQ for (a) ethyl glucuronide, (b) d<sub>5</sub>-ethyl glucuronide, (c) ethyl sulfate, and (d) d<sub>5</sub>-ethyl sulfate using FullIMS and PRM acquisition modes

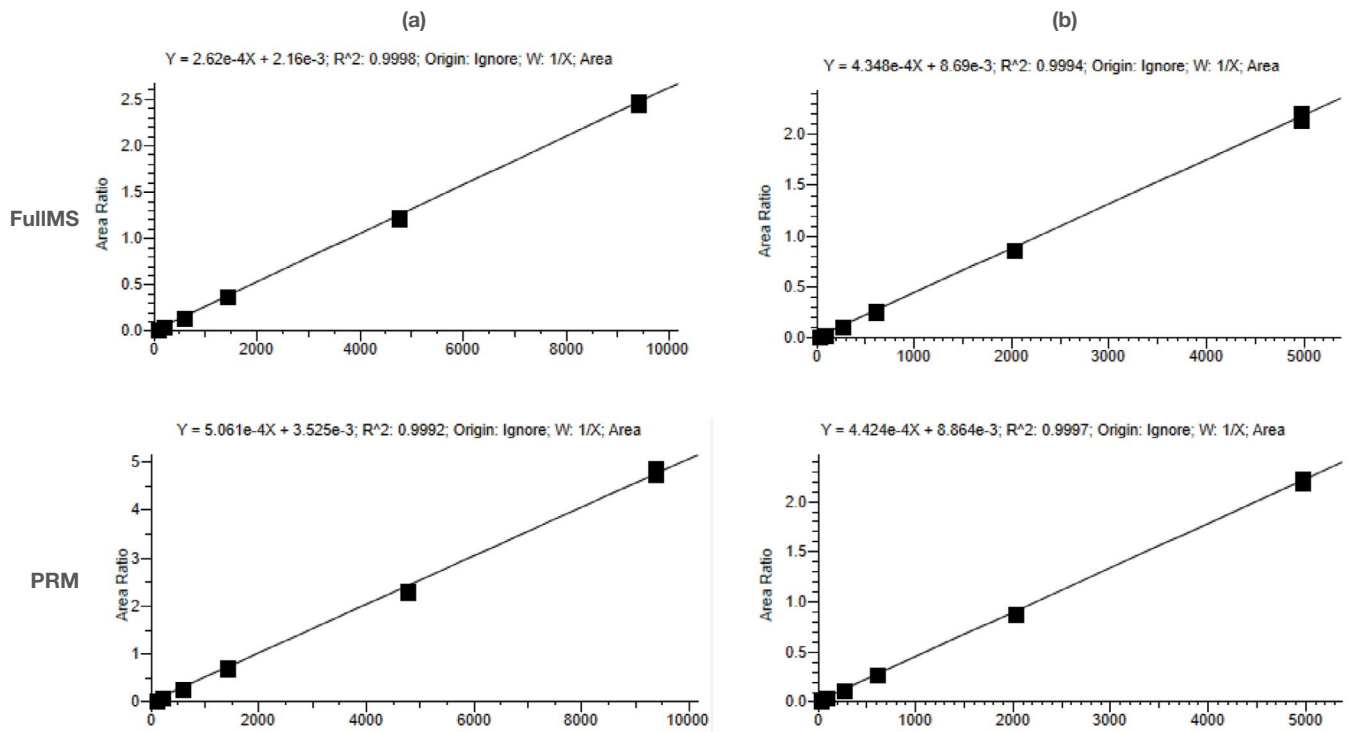


Figure 3. Representative calibration curves for (a) ethyl glucuronide and (b) ethyl sulfate, collected in duplicate, using FullIMS and PRM acquisition modes

Table 5. Analytical accuracy results for control MS8083 batch #1478

Analyte	Control	Nominal concentration (ng/mL)	FullIMS mode		PRM mode	
			Average calculated concentration (ng/mL)	Bias (%)	Average calculated concentration (ng/mL)	Bias (%)
Ethyl glucuronide	Level I	125	134	6.8	126	0.5
	Level II	505	520	3.0	515	1.9
	Level III	1961	1957	-0.2	1961	0.0
Ethyl sulfate	Level I	38.0	36.2	-4.7	34.9	-8.3
	Level II	185	189	2.3	194	4.8
	Level III	745	757	1.6	760	2.0

Table 6. Analytical accuracy results for control MS8083 batch #1478

Analyte	Control	Nominal concentration (ng/mL)	FullIMS mode		PRM mode	
			Average calculated concentration (ng/mL)	Bias (%)	Average calculated concentration (ng/mL)	Bias (%)
Ethyl glucuronide	GTFCh ETG 1/18 B	1030	1055	2.4	1030	0.0
	GTFCh ETG 1/19 B	441	467	5.8	458	3.7
	GTFCh ETG 1/20 B	624	599	-4.0	618	-1.0
	GTFCh ETG 3/18 B	1730	1690	-2.3	1638	-5.3
	GTFCh ETG 3/19 B	2620	2609	-0.4	2571	-1.9
Ethyl sulfate	GTFCh ETG 1/18 B	1060	1156	9.1	1129	6.5
	GTFCh ETG 1/19 B	610	678	11.2	679	11.3
	GTFCh ETG 1/20 B	736	795	8.0	788	7.1
	GTFCh ETG 3/18 B	1550	1676	8.1	1652	6.6
	GTFCh ETG 3/19 B	1850	2030	9.7	2001	8.2

Table 7. Analytical intra- and inter-assay precision results for control MS8083 batch #1478 – FullIMS mode

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3		Average calculated concentration (ng/mL)	CV (%)
		Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)		
Ethyl glucuronide	Level I	131	1.2	140	2.2	130	3.7	134	4.1
	Level II	523	2.2	518	0.5	519	1.3	520	1.5
	Level III	1949	2.2	1942	0.5	1979	1.6	1957	1.7
Ethyl sulfate	Level I	32.9	3.2	40.4	6.3	35.3	3.3	36.2	10.0
	Level II	181	1.9	196	2.2	191	1.8	189	3.9
	Level III	746	2.4	764	2.1	761	1.4	757	2.1

Table 8. Analytical intra- and inter-assay precision results for control MS8083 batch #1478 – PRM mode

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3		Average calculated concentration (ng/mL)	CV (%)
		Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)		
Ethyl glucuronide	Level I	131	1.7	123	2.0	123	1.3	126	3.4
	Level II	522	1.7	505	1.3	517	2.0	515	2.1
	Level III	1955	1.6	1926	2.6	2001	2.3	1961	2.6
Ethyl sulfate	Level I	32.8	3.3	36.3	2.5	35.5	1.6	34.9	5.1
	Level II	192	0.7	197	0.7	193	1.5	194	1.7
	Level III	760	1.2	759	1.4	761	1.0	760	1.1

## Conclusions

An HRAM mass spectrometry-based method utilizing a Vanquish Duo UHPLC system coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap MS is reported here, demonstrating the power of Orbitrap technology in performing accurate qualitative analyses and routine quantitation with high efficiency. A liquid chromatography-HRAM mass spectrometry method for clinical research was developed and implemented for the quantification

of EtG and EtS in human urine. The ClinMass LC-MS/MS Complete Kit for Ethylglucuronide and Ethylsulfate in Urine from RECIPE was used. The method incorporates a quick and simple dilute-and-shoot sample preparation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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