SIMULTANEOUS QUANTITATIVE DETERMINATION OF ETHYL GLUCURONIDE AND ETHYL SULPHATE IN HUMAN URINE USING UPLC® /MS/MS

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OBJECTIVE

To develop and validate a simple and rapid UPLC/MS/MS method for the simultaneous determination of ethyl glucuronide (EtG) and ethyl sulphate (EtS) in human urine

INTRODUCTION

- **EtG and EtS are non-volatile, water-soluble, direct metabolites of ethanol**
- **Both EtG and EtS are formed shortly after ethanol consumption and can be detected much longer than ethanol itself (up to 80 hours for EtG, 30 hours for EtS)[1,2]**
- **EtG has been shown to be susceptible to post sampling synthesis[3] and also bacterial degradation[4,5] which may lead to false positive or negative results respectively. Whilst the possibility of bacterial degradation of EtS can not be excluded[6], it appears to be less susceptible than EtG, making it a more reliable marker**
- **EtG and EtS are formed by different metabolic pathways and therefore simultaneous determination has been found to increase sensitivity and reliability in detecting recent ethanol consumption[7]**
- **EtG testing is now widely used in alcohol withdrawal programmes (to monitor abstinence), within workplace settings and for forensic cases such as post-mortems and drug-facilitated crimes**

Figure 1. System configuration - Waters ACQUITY® TQD

MATERIALS

Specimens

Validation was performed using human urine samples obtained from the St George's Analytical Unit and Wythenshawe Hospital (Manchester, UK). All samples (sodium fluoride preserved) were stored at -20°C until analysis. Synthetic blank urine (Surine®, DYNA-TEK industries, USA) was used as the control material to prepare all the calibrators.

Internal standards

Deuterated analogues EtG-D5 and EtS-D5 (Lipomed, Switzerland) were used as the internal standards (IS). Mixed stock solutions were prepared in water at 20 and 5mg/L respectively.

EXPERIMENTAL

Sample preparation

A simple urine dilution (1:20) was undertaken after centrifugation at 12000rpm (~11000xg) for 10 mins. This dilution also incorporated the addition of the IS. Briefly, IS (10µL) and 0.1% formic acid (940µL) were added to the human urine samples (50µL) before finally vortex mixing for 30 secs.

LC conditions

LC System:	Waters® ACQUITY UPLC®
Column:	ACQUITY UPLC HSS C18 Column (2.1 x 150 mm, 1.8 µm)
Column Temp:	50 °C
Flow Rate:	400 µL/min.
Mobile Phase A:	Water containing 0.05% formic acid
Mobile Phase B:	Acetonitrile
Gradient:	1-100% B over 2.5 min.
Injection Vol:	10µL

MS conditions

MS System:	Waters® TQ Detector mass spectrometer
Ionization Mode:	ESI Negative
Capillary Voltage:	2.5 kV
Acquisition Mode:	Multiple reaction monitoring (MRM)
Data Processing:	MassLynx® v4.1 with TargetLynx™

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
EtG	**221**	**85**
	221	75
EtS	**125**	**97**
	125	125
EtG-D5	226	85
EtS-D5	130	98

*Table 1. MRM conditions used for EtG, EtS and internal standards. **Bold** transitions used as the quantifier ions*

RESULTS

The MRM conditions used for the measurement of EtG, EtS and their respective IS are summarised in Table 1.

A calibration curve (0.25–100mg/L for EtG, 0.05-20mg/L for EtS) was prepared by adding EtG and EtS to synthetic blank urine. Calibrators and quality controls (QC) were diluted by the same procedure as previously described for the samples.

Figure 2 shows the MRM chromatograms obtained from a 10µL injection of a 0.5mg/L urine calibrator. The quantifier/qualifier ion ratios for both compounds were monitored for all calibrators, QC's and samples and were found to be within ±20% of the target ion ratios.

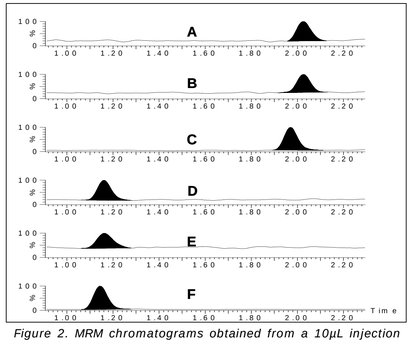

Figure 2. MRM chromatograms obtained from a 10µL injection of a urine calibrator at the cut-off level (0.5 and 0.1mg/L EtG and EtS respectively) for EtG quantifier ion (A), qualifier ion (B), EtG-D5 (C) and EtS quantifier ion (D), qualifier ion (E), EtS-D5 (F)

Figure 3 shows a typical standard curve for EtG and EtS in urine. Calibrators were plotted using 1/x weighting and found to be linear for both compounds, over the investigated range (coefficient of determination r^2 = >0.996).

Limits of detection were 0.2 and 0.04mg/L for EtG and EtS respectively, which is below the cut-offs applied for this analysis i.e, 0.5 and 0.1mg/L respectively.

Intra-assay precision and accuracy were assessed by adding the EtG and EtS to blank patient urine (*n*=5) at four QC concentrations (0.75, 2.5, 7.5 and 50mg/L for EtG and 0.15, 0.5, 1.5 and 10mg/L for EtS). Inter-day precision was assessed by analysing the QC samples in duplicate on five different days. Results are shown in Table 2.

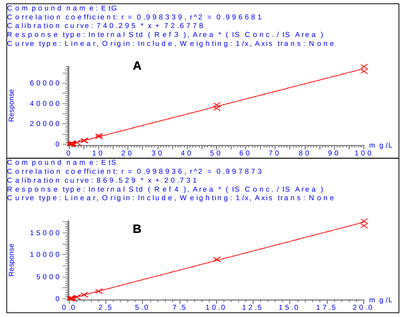

Figure 3. Typical calibration curves obtained for EtG (A) and EtS (B)

The stability of prepared samples and standards was assessed over 24 hours. A prepared calibrator (2.5/0.5mg/L, EtG/EtS) was stored at 5°C in the dark in the ACQUITY sample manager with an injection performed every hour. No significant changes in absolute peak area were found for either compound over the investigated time period.

	QC Level (mg/L)	Intra-day accuracy (%)	Intra-day precision (% RSD)	Inter-day accuracy (%)	Inter-day precision (% RSD)
EtG	0.75	104.6	3.8	107.0	5.5
	2.5	103.3	5.8	100.0	8.0
	7.5	102.0	3.8	98.4	6.2
	50	111.8	8.3	103.3	9.3
EtS	0.15	103.2	1.7	104.3	5.7
	0.5	98.7	2.4	97.5	3.6
	1.5	97.3	2.0	100.5	4.1
	10	97.8	5.6	99.6	6.4

Table 2. Accuracy and precision data for EtG and EtS at 4 QC levels

Matrix effects were assessed in 2 ways, firstly by spiking blank prepared patient samples (*n* = 6) with both compounds and comparing the absolute peak areas against the equivalent concentration of calibrator solution in solvent. The average matrix effects were found to be acceptable (-16% for EtG and -7% for EtS). Secondly, a post-column infusion of both compounds was performed during the injection of a solvent blank and prepared urine samples (*n* = 6). Minimal matrix effects were observed with a simple urine dilution.

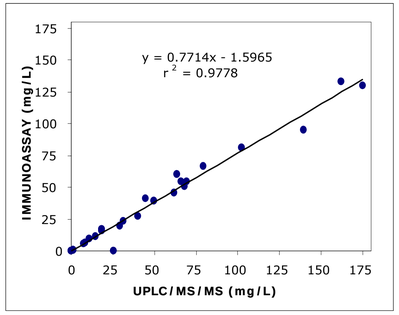

Figure 4. Analysis of EtG concentrations in forensic samples by immunoassay and UPLC/MS/MS. Results which were below the cut-off or >2000mg/L are not plotted.

Forensic samples (*n* = 39) collected from alleged drug-facilitated sexual assault (DFSA) cases which had been previously analysed for EtG using the Microgenics DRI® EtG Enzyme Immunoassay[8], were subsequently analysed using the newly developed UPLC/MS/MS method. EtG and EtS levels are highly influenced by water intake[9] therefore normalisation of EtG and EtS values to the creatinine concentration is recommended, but for the purpose of this comparison results were not normalised.

Preliminary results showed that many samples contained EtG and EtS concentrations which were above the calibration range used. Therefore, all samples were re-prepared by dilution (1:100) with synthetic blank urine, as previously described and re-analysed. The EtG results showed a good correlation (r^2=0.978) but also showed an analytical bias, as shown in Figure 4. Investigations into this bias are still ongoing. EtG and EtS were detectable in samples collected up to approximately 40 hours after the alleged DFSA.

CONCLUSION

- **EtG and EtS testing is becoming more widely used across the world within different settings such as alcohol withdrawal programs, clinical situations, forensic cases and the workplace to identify recent ethanol consumption or to verify abstinence**
- **The developed methodology has been shown to be accurate, precise and sensitive for the simultaneous quantitation of EtG and EtS and can provide rapid results in a single 4 minute chromatographic run**
- **The method has been successfully applied to the analysis of EtG in forensic samples with good correlation when compared to an established immunoassay. There is currently no immunoassay test available for EtS**
- **The speed and simplicity of the developed method make it the ideal solution for reliable, rapid, high-throughput EtG and EtS analysis**

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