Determination of urinary ethyl glucuronide and ethyl sulfate by LC/MS/MS for clinical research

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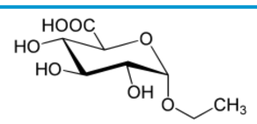
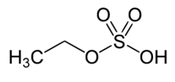
Introduction

Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is ideally suited for the rapid analysis of multiple analytes. A highly sensitive and specific LC/MS/MS analytical method has been developed for the quantitation of ethyl glucuronide and ethyl sulfate. A dilution procedure and a solid phase extraction (SPE) procedure are evaluated and compared based on ease of use, analyte recovery and post-extraction cleanliness.



Ethyl glucuronide (EtG)
C8H14O7
Neutral Mass: 222.07



Ethyl sulfate (EtS)
C2H6O4S
Neutral Mass: 126

Figure 1. Chemical structures of EtG and EtS

Calibrators were created by spiking synthetic urine (Surine-Cerilliant) with various concentrations of EtG and EtS standards (Cerilliant). The chromatographic system consists of a Polaris 3 C18-Ether column coupled with a guard column and a mobile phase comprised of acetonitrile and water containing 0.1% formic acid. Quantifier and qualifier transitions were monitored. EtG-D5 and EtS-D5 internal standards (Cerilliant) were included to ensure accurate and reproducible quantitation. Urine controls (UTAK Laboratories) were used and samples were kindly supplied by collaborators. The separation of EtG and EtS from isobaric interferences is especially critical; without proper separation by retention time, impurities present in both compounds can cause interferences with one another and lead to inaccurate quantitation.

Experimental

Sample Preparation

Simple dilution and solid phase extraction (SPE) were investigated for robustness and sensitivity. Protein precipitation was also evaluated (data not shown), but did not show a significant improvement over either simple dilution or SPE.

Dilution Procedure:

Vortex and centrifuge urine. Transfer 50 µL of supernatant to a clean tube. Add 450 µL of ISTDs solution (200 ng/mL in 0.5% formic acid in H2O).

SPE Procedure:

Combine 100 µL of urine, 50 µL of ISTDs (4000 ng/mL in water), and 850 µL of water

1: Condition SPE cartridge (BondElut SAX 200 mg 3 cc, Agilent PN: 12102126) with 2 mL of MeOH followed by 2 mL of water
2: Add sample
3: Wash with 1 mL of acetonitrile. Dry at full vacuum for 5 minutes
4: Elute with 2 mL of 5% formic acid in methanol (to elute EtG) and 2 mL of 2% HCl in acetonitrile (to elute EtS). Apply vacuum 5" Hg for 60 seconds.

Evaporate with nitrogen at 40°C and reconstitute with 1 mL of 0.5% formic acid in water

LC Method

Agilent 1290 HPLC binary pump, well plate sampler with thermostat, temperature-controlled column compartment

Parameter	Value
Analytical Column	Agilent Polaris 3 C18-Ether, 3x150mm, 3µm, PN: A2021150X030
Guard Column	Agilent Polaris 3 C18-Ether MetaGuard 2 mm, 3µm, PN: A2021MG2
Injection Volume	20 µl
Needle Wash	1:1:1:1 MeOH:ACN:IPA:H2O + 0.1% formic acid in Flush port for 15 seconds
Mobile Phase A	Water + 0.1 % Formic Acid
Mobile Phase B	Acetonitrile + 0.1 % Formic Acid
Pump gradient	Time (min.) %B Flow (mL/min.)
	0.0 0 0.5
	3.5 15 0.5
	4.0 98 0.7
Stop Time	6.0 98 0.7
Post Time	2 min.

Table 1. LC Parameters

MS Method

Agilent 6460 QQQ with JetStream technology

Ion mode:	AJS ESI(-)
Drying gas:	300 °C, 5 L/min
Nebulizer gas pressure:	40 psi
Sheath gas:	400 °C, 12 L/min.
Capillary voltage:	2500V
Nozzle voltage:	1000V
Q1/Q3 Resolution:	0.7 unit
Delta EMV:	500V

Compound	Prec Ion	Prod Ion	Dwell	Frag (V)	CE (V)	CAV (V)
EtG*	221.1	75	20	110	12	5
EtG	221.1	85	20	110	12	5
EtG-D5	226.1	75	20	110	12	5
EtS*	125	96.9	40	90	14	5
EtS	125	80	40	90	34	2
EtS-D5	130	98	40	90	14	5

Table 2: MRM Transitions table (*Quantifier)

Results and Discussion

The primary objective for method development was to achieve chromatographic resolution between EtG, EtS, and various isobaric interferences in order to achieve accurate quantitation at lower analytical sensitivities. When analyzing EtG/EtS in synthetic urine, no major interferences observed (figure 2a). However, real samples and controls (figure 2b) show major interferences for the EtS qualifier transition.

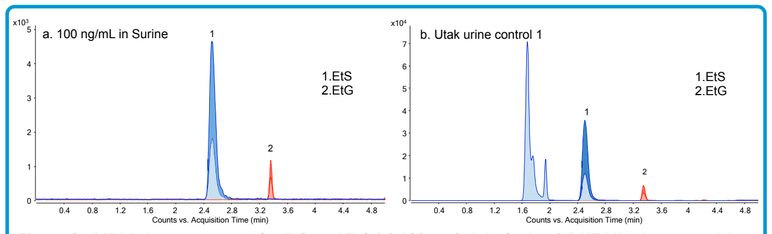


Figure 2. MRM chromatograms for EtG and EtS (a) 100 ng/mL in Surine (b) UTAK urine control 1

The same interference is observed in all samples at various intensities. The SPE procedure removes most of this interference while reducing chemical noise and increasing signal to noise ratio (figure 3a-b).

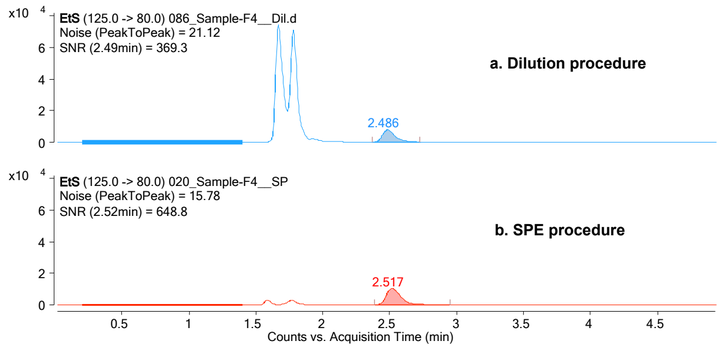


Figure 3. MRM chromatogram for EtS qualifier for sample F4 (a) Dilution procedure (b) SPE procedure

Depending on the sample, several interfering peaks can be observed in any of the EtG/EtS transitions. The proposed LC/MS method is capable of resolving all of these interferences chromatographically (figure 4), producing excellent quantitative results (figure 5, table 3 and table 4).

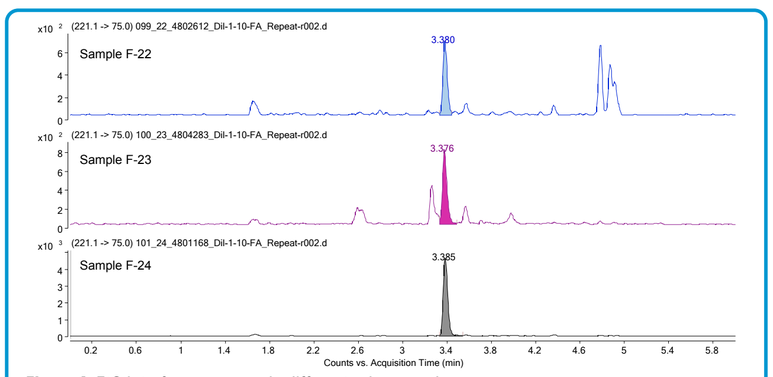


Figure 4. EtG interferences seen in different urine samples.

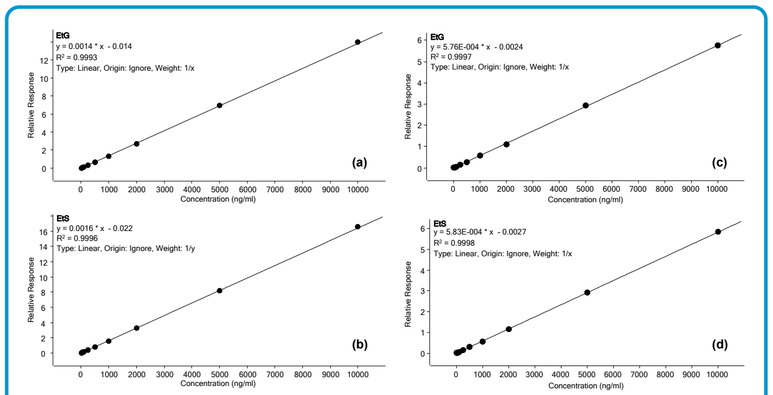


Figure 5. Calibration curves for EtG and EtS using the dilution (a, b) and SPE (c, d) procedures

Dilution procedure			
Compound	R²	Conc. (ng/mL)	Accuracy (%)
EtG	0.9993	25	119.8
		500	92.6
		10000	101.1
EtS	0.9996	25	119.5
		500	95.3
		10000	101.0

Table 3. Accuracy of the dilution procedure

SPE procedure			
Compound	R²	Conc. (ng/mL)	Accuracy (%)
EtG	0.9998	25	109.2
		500	95.7
		10000	100.2
EtS	0.9997	25	112.5
		500	99.1
		10000	100.5

Table 4. Accuracy of the SPE procedure

Results and Discussion

Matrix effects and SPE recovery

Absolute ion suppression and matrix effects were determined for the dilution procedure (table 5). Matrix effects, recovery efficiency and process efficiency were determined for the SPE procedure (table 6). All effects were compensated for by the internal standards.

Compound	Matrix effects %* (n = 9)		Accuracies % With ISTDs corrections** (n = 9)		
	Average	SD	Range	Average	SD
EtG	101.8	6.4	91.7-119.8	100.0	9.1
EtS	72.3	2.5	91.5-119.5	99.4	8.3

Table 5. Matrix effects for dilution procedure

Measurements done at 9 different concentrations ranging from 25 to 10000 ng/mL
* Peak areas from urine spiked compared with H2O spiked solutions
** Calculated concentrations of urine spiked with ISTD corrections versus theoretical concentrations

Compound	Matrix effects % (n = 9)		Recovery efficiency % (n = 9)		Process efficiency % (n = 9)	
	Average	SD	Average	SD	Average	SD
EtG	91.6	8.3	92.6	3.6	84.7	7.7
EtS	98.2	3.4	77.5	3.7	76.1	4.2

Table 6. Matrix effects, recovery efficiency and process efficiency for SPE procedure

Matrix effect % = B/A *100
Recovery efficiency % = C/B *100
Process efficiency % = C/A *100
A: neat standard solutions
B: surine extracted then spiked (post-ext)
C: surine then extracted (pre-ext)

Accuracy, reproducibility and sample results

Commercially available quality control (QC) materials (UTAK) were used to measure the precision of this method. Results (table 7) show excellent precision at both levels and for both sample preparation procedures. Forty urine samples were processed in parallel by the dilution and SPE procedures. Raw data is shown in table 8 and correlation between the two procedures are shown in figures 6 and 7.

Compound	Level 1				Level 2			
	Dilution Measured (ng/mL) n=3	CV (%)	SPE Measured (ng/mL) n=6	CV (%)	Dilution Measured (ng/mL) n=3	CV (%)	SPE Measured (ng/mL) n=6	CV (%)
EtG	475.8	4.0	460	5.3	1737	1.6	1772	3.1
EtS	236.9	2.5	234.4	3.4	898.1	1.1	896.4	2.8

Table 7. Results of UTAK controls by LC/MS/MS

	EtS (ng/mL)			EtG (ng/mL)		
Name	SPE	Dilution	% Diff.	SPE	Dilution	% Diff.
Sample-F1	352.4	352.2	0.1	1288.1	1127.0	13.3
Sample-F2	750.7	728.9	3.0	1148.0	1156.4	-0.7
Sample-F3	379.2	391.4	-3.2	347.7	374.2	-7.4
Sample-F4	395.4	401.8	-1.6	526.3	443.7	17.0
Sample-F5	501.0	487.8	2.7	1231.4	1364.8	-10.3
Sample-F6	553.2	548.0	1.0	1169.5	968.2	18.8
Sample-F7	666.2	689.3	-3.4	932.9	785.0	17.2
Sample-F8	306.8	331.4	-7.7	1278.1	1310.1	-2.5
Sample-F9	559.8	570.0	-1.8	512.2	431.6	17.1
Sample-F10	203.8	223.3	-9.1	986.9	997.5	-1.1
Sample-F11	684.0	705.8	-3.1	778.9	685.2	12.8
Sample-F12	905.8	871.7	3.8	718.2	693.2	3.5
Sample-F13	262.4	267.3	-1.9	372.7	413.6	-10.4
Sample-F14	261.0	286.5	-9.3	417.5	395.6	5.4
Sample-F15	181.5	200.5	-9.9	177.1	183.8	-3.7
Sample-F16	130.7	140.6	-7.3	229.7	222.1	3.4
Sample-F17	646.9	621.5	4.0	1695.0	1417.4	17.8
Sample-F18	328.0	350.2	-6.5	550.1	644.0	-15.7
Sample-F19	368.0	403.1	-9.1	303.0	287.4	5.3
Sample-F20	382.6	415.9	-8.3	361.3	383.1	-5.9
Sample-F21	239.8	259.2	-7.8	759.8	741.2	2.5
Sample-F22	200.2	202.7	-1.2	292.2	322.9	-10.0
Sample-F23	608.2	648.9	-6.5	206.6	201.4	2.6
Sample-F24	277.5	291.1	-4.8	983.2	1103.1	-11.5
Sample-S1	14.0	17.9	-24.1	43.0	62.2	-36.5
Sample-S2	9.8	12.5	-23.8	10.4	13.7	-27.3
Sample-S3	94.7	100.4	-5.8	52.3	52.9	-1.2
Sample-S4	229.5	251.9	-9.3	189.4	217.3	-13.7
Sample-S5	499.6	528.8	-5.7	65.6	85.2	-26.0
Sample-S6	148.5	161.2	-8.2	284.3	287.7	-1.2
Sample-S7	280.4	301.7	-7.3	689.4	682.2	1.1
Sample-S8	169.5	183.8	-8.1	581.6	575.6	1.0
Sample-S9	330.6	299.5	9.9	619.1	602.2	2.8
Sample-S10	374.5	404.8	-7.8	569.5	639.9	-11.6
Sample-S11	1112.2	1218.1	-9.1	268.7	272.1	-1.3
Sample-S12	640.2	682.0	-6.3	1850.9	1700.4	8.5
Sample-S13	621.4	642.9	-3.4	1678.7	1349.8	21.7
Sample-S14	633.3	646.5	-2.1	3191.3	3440.4	-7.5
Sample-S15	1737.5	1789.5	-2.9	6586.6	5678.8	14.8
Sample-S16	1852.1	2021.4	-8.7	6986.7	5957.8	15.9

Table 8. Results of urine samples

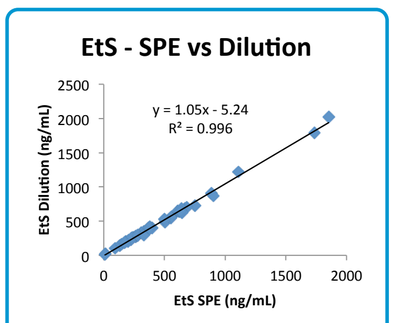


Figure 6. Correlation for EtS results

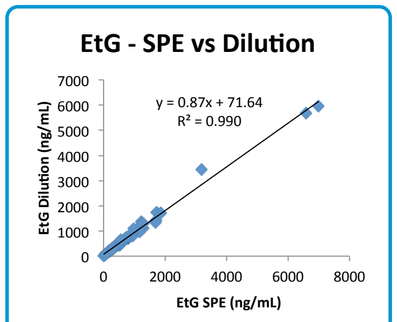


Figure 7. Correlation for EtG results

Conclusions

A method has been developed for quantifying ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine for clinical research. Two sample preparation procedures consisting of a simple dilution from urine and SPE are shown. Chromatographic separation of all analytes and interferences with conditions compatible with LC/MS/MS have been developed. Typical analytical method performance results are well within acceptable criteria.

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