Introduction

Liquid chromatography triple quadruple 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.

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 (ULTAK 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 ISD solution (200 ng/mL in 0.5% formic acid in H2O).

SPE Procedure:
Combine 100 µL of urine, 50 µL of ISTs (4000 ng/mL in water), and 850 µL of water

1. Condition SPE cartridge (BondElut SAX 200 mg 3 cc, Agilent PN: 12012126) 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.
5. 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: A20211MG2
Injection Volume 20 µL
Needle Wash 1:1:1 MeOH:AQN: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 (min.) (% 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.

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 were observed (figure 2a). However, real samples and controls (figure 2b) show major interferences for the EtS qualifier transition.

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).

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).

Table 1. LC Parameters

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precision</th>
<th>Reproducibility</th>
<th>Dwelling Time</th>
<th>CE (V)</th>
<th>CAV (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtG</td>
<td>* 7.8</td>
<td>9.0</td>
<td>96</td>
<td>85</td>
<td>79.0</td>
</tr>
<tr>
<td>EtS</td>
<td>* 7.8</td>
<td>7.0</td>
<td>96</td>
<td>65</td>
<td>82.0</td>
</tr>
<tr>
<td>ISTD</td>
<td>* 9.4</td>
<td>8.6</td>
<td>96</td>
<td>65</td>
<td>89.0</td>
</tr>
</tbody>
</table>

Table 2: MRM Interference Matrix

Accuracy, reproducibility and sample results
Commercially available quality control (QC) materials (ULTAK) 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 4 and correlation between the two procedures are shown in figures 6 and 7.

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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