Fast and Simultaneous Analysis of Ethanol Metabolites and Barbiturates in Human Urine

Using SCIEX Triple Quad™ and QTRAP® LC-MS/MS Systems

Liquid Chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a widely used analytical tool for quantification of compounds in forensic samples. While most analytes in drug screening applications analyze well with positive ionization, there are analytes that show better ionization efficiency with negative ionization, for example acidic compounds. These analytes include ethanol metabolites such as ethyl glucuronide (ETG), ethyl sulfate (ETS), and the barbiturates such as amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital.

Typically, for LC-MS/MS analysis of a comprehensive drug panel, detection of urinary barbiturates is done in negative ionization mode, and majority of other compound classes are detected in positive ionization mode. In a previous technical note, a method for analysis of a comprehensive forensic drug panel in one injection using polarity switching was described. The sample preparation of that method has a hydrolysis step because many analytes in the panel formed phase II conjugates that need to be de-conjugated with hydrolysis back to the parent drug that typically gives better analytical performance. However, if ETG and ETS are included in the panel, then a separate injection for ETG and ETS detection is required because they cannot undergo hydrolysis. As a two-sample-preparation/two-injection approach is inevitable, an experimental design to run one injection in positive mode for most analytes after performing a hydrolysis step in the sample preparation was investigated. Then perform a second sample preparation, that doesn't include the hydrolysis, on a separate aliquot of the sample. A second injection in negative mode for ETG/ETS and barbiturates was therefore performed.

In this study, a fast and sensitive method to analyze ETG, ETS, amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital in human urine in a single injection with a SCIEX Triple Quad / QTRAP LC-MS/MS system is described. Sample preparation is based on a simple "dilute and shoot" methodology without hydrolysis. Analytical performance was evaluated. In addition, a robustness test for the method was done with over 600 continuous injections of urine samples.

Key Features of Fast and Simultaneous Analysis of Ethanol Metabolite and Barbiturate Quantification in Urine

- Simplified extraction procedure made possible by selective and sensitive LC-MS/MS analysis.
- Fast LC technology allows rapid chromatography and separation of known interferences in a 5-minute LC runtime.
- Advanced LC-MS/MS technology provides enhanced robustness and extended method performance when using diluted samples prepared as proposed.
- Additional options to exploit QTRAP technology to further enhance confidence in results.

Experimental

***Materials*:** Compounds of interest include ETG, ETS, amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital. Internal standards are ETG-D5 and ETS-D5 for ETG and ETS, and butalbital-D5 and secobarbital-D5 for the barbiturates.

***Calibrator Preparation*:** Blank human urine was used to prepare calibrators. Four levels of calibrators across the required concentration range in human urine were prepared.

Sample Preparation:

- 100 µL urine sample was mixed with 10 µL internal standard solution and 890 µL water
- The mixture was then centrifuged at 21,000 rcf for 10 min
- The supernatant was transferred to a glass vial with insert for LC-MS/MS analysis

***Liquid Chromatography*:** HPLC separation was performed using a SCIEX HPLC system. A Phenomenex Kinetex Phenyl-hexyl column fitted with a Phenomenex SecurityGuard ULTRA UHPLC Phenyl was used. Mobile phase A (MPA) and mobile phase B (MPB) were water and methanol with modifier. The LC flowrate was 0.75 mL/min and the total LC runtime was 5 minutes.

***MS/MS Conditions*:** The SCIEX Triple Quad / QTRAP LC-MS/MS system was operated in multiple reaction monitoring

(MRM) mode. Two selective MRM transitions were monitored for each target analyte and one MRM transition for each internal standard. The Turbo V™ Ion Source was used with an electrospray ionization (ESI) probe in negative polarity and parameters were optimized for optimum sensitivity. Analyst® MD Software version 1.6.3 was used for data acquisition. LC-MS/MS data was processed using the MultiQuant™ MD Software version 3.0.

Results and Discussion

As previously stated, a fast LC gradient with a 5-min runtime was used in this method. Overall, both ETG and ETS had good retention using the developed LC conditions. In addition, baseline separation was achieved between a frequently observed strong interference for one of the monitored MRM transitions for ETS in urine samples (Figure 2).

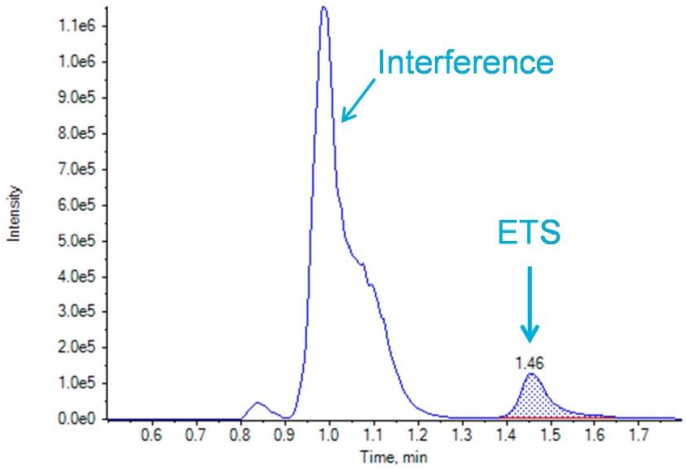

Figure 1: LC Separation of ETS and a Frequently Observed Interference in Human Urine.

Analytical Sensitivity: The processed urine sample had a final dilution factor of 10. With 10 µL injection volume (equivalent of 1 µL unprocessed urine), we were able to detect all the analytes at the lowest concentration required (Figure 2).

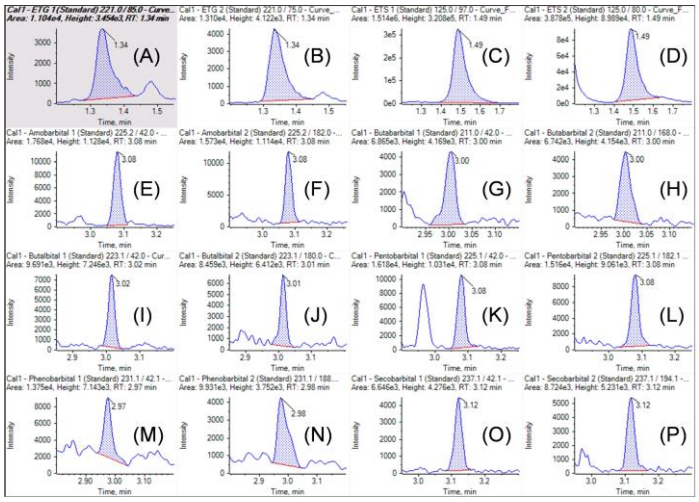

Figure 2. Extracted Ion Chromatograms (XICs) of Both Quantifying and Qualifying Analyte MRM Transitions at 50 ng/mL in Urine. ETG (A, B), ETS (C, D), amobarbital (E, F), butabarbital (G, H), butalbital (I, J), pentobarbital (K, L), phenobarbital (M, N) and secobarbital (O, P).

Calibration Curves: Figure 3 shows example calibration curves of the analytes; ETG, ETS, amobarbital and secobarbital (n=3, quantifier and qualifier transition calibration curves shown).

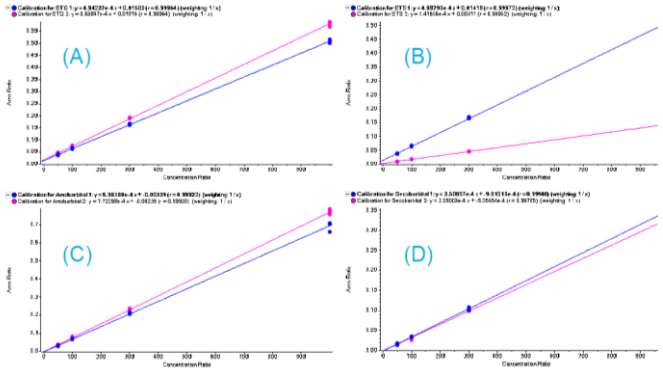

Figure 3: Example Calibration Curves of ETG (A), ETS (B), Amobarbital (C) and Secobarbital (D) Generated Using the Method Proposed.

Robustness: It is critical to prove the method robustness with real human urine samples. Over 840 injections of diluted urine samples spiked with various amount of these analytes were performed during a >3-day period. No deterioration in either chromatographic separation or sensitivity was observed. Figure 4 shows the signals of ETG-d5 and secobarbital-d5 over 55 hours continuous operation incorporating 600 injections of urine samples (blank, calibrators and QCs). Figure 5 shows the consistency of the retention times of all the internal standards during this period.

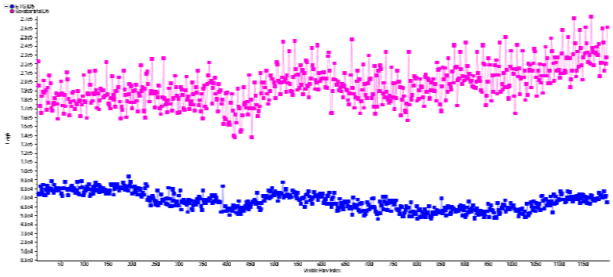

Figure 4: Peak Area Response of ETG-d5 and Secobarbital-d5 Over 600 Injections for 55 Hours Continuous Instrument Operation.

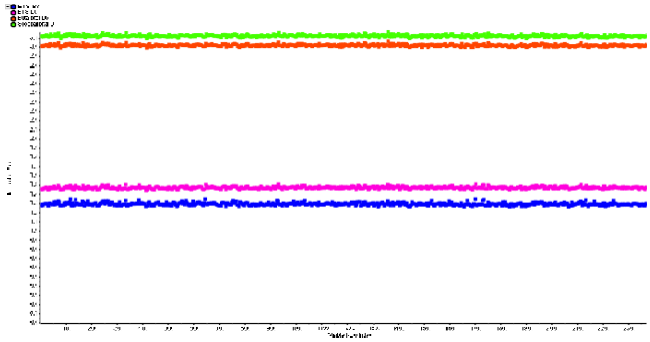

Figure 5. Internal Standard Retention Time Stability over 600 Injections of Urine Samples for 55 Hours Continuous Operation.

Conclusions

In this technical note, a method to simultaneously analyze ethanol metabolites and barbiturates in human urine using a SCIEX QTRAP/Triple Quad LC-MS/MS system is described. Sample preparation is based on a simple “dilute and shoot” methodology. The method has a total runtime of 5 minutes, shows good sensitivity and high robustness. More than 800 continuous injections of diluted human urine samples were performed on in a single uninterrupted analytical run with no deterioration in performance evident.

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