Fast and simultaneous analysis of ethanol metabolites and barbiturates using the QTRAP® 4500 LC-MS/MS system

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Overview

In this technical note, we describe a fast and sensitive method to analyze ethanol metabolites (ethyl glucuronide and ethyl sulfate) and barbiturates (amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital) in human urine using the SCIEX QTRAP®/Triple Quad™ 4500 LC-MS/MS system (Figure 1). Sample preparation is based on a simple “dilute and shoot” methodology. We evaluated both analytical performance and method robustness.

Introduction

Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) is a widely used analytical tool for quantitation of compounds in forensic samples. While most analytes in forensic 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 bariturates such as amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital. Typically, for LC-MS/MS analysis of a comprehensive forensic analytical 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, we have described a method for analysis of a comprehensive forensic drug panel in one injection using polarity switching. 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, we investigated an experimental design to run one injection in positive mode for most analytes after performing a hydrolysis step in the sample preparation. 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.

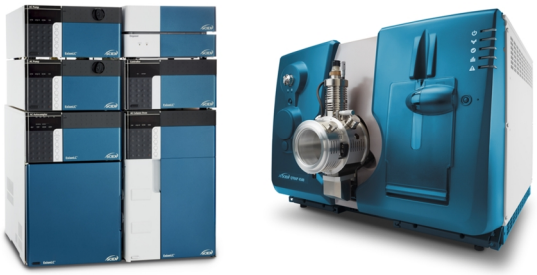


Figure 1: SCIEX ExionLC™ AC HPLC and QTRAP®/Triple Quad™ 4500 LC-MS/MS System

In this study, we describe a fast and sensitive method to analyze ETG, ETS, amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital in human urine in a single injection with SCIEX QTRAP®/Triple Quad™ 4500 LC-MS/MS system. 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 800 continuous injections of urine samples

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. All the standards were procured from Cerilliant.

Table 1: MRM Transitions (Period 1:ETG and ETS, 0-1.8 min; Period 2: barbiturates, after 1.8 min)

Analyte	Q1	Q3	Time (msec)	DP	EP	CE	CXP
ETG 1	*221*	*85*	*50*	*-60*	*-10*	*-20*	*-5*
ETG 2	*221*	*75*	*50*	*-60*	*-10*	*-20*	*-5*
ETS 1	*125*	*97*	*50*	*-52*	*-3*	*-21*	*-7*
ETS 2	*125*	*80*	*50*	*-52*	*-3*	*-40*	*-6*
ETG D5	*226*	*85*	*20*	*-60*	*-10*	*-20*	*-5*
ETS D5	*130*	*98*	*20*	*-52*	*-3*	*-21*	*-7*
Amobarbital 1	*225.2*	*42*	*10*	*-60*	*-10*	*-40*	*-10*
Amobarbital 2	*225.2*	*182*	*10*	*-60*	*-10*	*-18*	*-10*
Butabarbital 1	*211*	*42*	*10*	*-65*	*-10*	*-40*	*-10*
Butabarbital 2	*211*	*168*	*10*	*-65*	*-10*	*-18*	*-10*
Butalbital 1	*223.1*	*42*	*10*	*-65*	*-10*	*-40*	*-10*
Butalbital 2	*223.1*	*180*	*10*	*-65*	*-10*	*-16*	*-10*
Pentobarbital 1	*225.1*	*42*	*10*	*-70*	*-10*	*-40*	*-10*
Pentobarbital 2	*225.1*	*182.1*	*10*	*-70*	*-10*	*-19*	*-10*
Phenobarbital 1	*231.1*	*42.1*	*10*	*-70*	*-10*	*-40*	*-10*
Phenobarbital 2	*231.1*	*188*	*10*	*-70*	*-10*	*-14*	*-10*
Secobarbital 1	*237.1*	*42.1*	*10*	*-70*	*-10*	*-40*	*-10*
Secobarbital 2	*237.1*	*194.1*	*10*	*-70*	*-10*	*-17*	*-10*
Butalbital D5	*228.1*	*42*	*10*	*-65*	*-10*	*-40*	*-10*
Secobarbital D5	*242.1*	*42*	*10*	*-70*	*-10*	*-40*	*-10*

Calibrator Preparation

Blank human urine was used to prepare calibrators. Four levels of calibrators in human urine were prepared (50, 100, 300 and 1000 ng/mL).

Sample Preparation

- 100 µL urine sample was mixed with 10 µL internal standards solution, and then diluted with 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 ExionLC™ AC HPLC system at 30°C. Phenomenex Kinetex Phenyl-hexyl column (50 × 4.6 mm, 2.6 µm, 00B-4495-E0), Phenomenex SecurityGuard ULTRA UHPLC Phenyl (AJ0-8774) and ULTRA holder (AJ0-9000) were 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 min. Injection volume was 10 µL.

For autosampler, the needle rinse solution was methanol:ACN:isopropanol (1:1:3, v/v/v). Rinse sequence was:

1. Rinsing volume: 1 mL
2. Needle stroke: 54 mm
3. Rinsing speed: 35 µL/sec
4. Sampling speed: 15 µL/sec
5. Rinse dip time: 5 sec
6. Rinse mode: before and after aspiration

Table 2: MS Timings and Source Conditions

Source Parameters	Period 1 (0.0-1.8 min)	Period 2 (1.8 to 5.0 min)
Curtain gas	30	*30*
CAD	*10*	*9*
Spray voltage (V)	*-2000*	*-4500*
Temperature (℃)	*600*	*650*
GS 1	*50*	*60*
GS 2	*50*	*50*

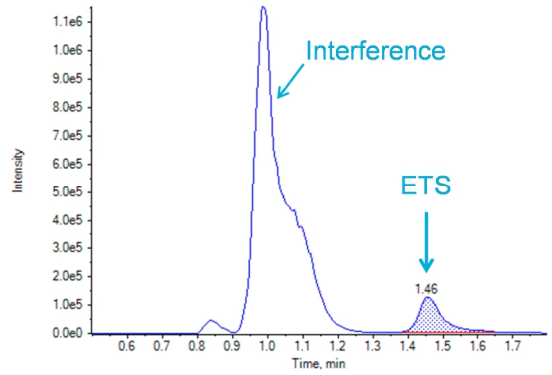
MS/MS Conditions

The SCIEX QTRAP® 4500 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 (Table 1). The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in negative polarity and parameters were optimized for optimum sensitivity (Table 2). Analyst® software version 1.6.3 was used for data acquisition. LC-MS/MS data was processed using the MultiQuant™ software version 3.0.

Results and Discussion

A Phenomenex Kinetex Phenyl-hexyl column (50 × 4.6 mm, 2.6 µm) was used for LC separation and a guard column was used for LC column protection. 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, we were able to achieve baseline separation between a frequently-observed strong interference for one of the monitored MRM transitions for ETS (125/80 m/z) in urine samples (Figure 2).

Figure 2: LC separation of ETS



(ETS; 1.46 min, 125 à 80 *m/z*, 100 ng/mL) and a frequently observed interference in human urine (found at 1 min).

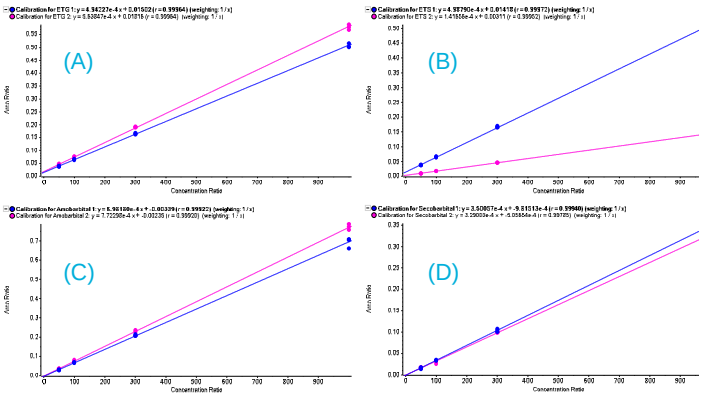
Analytical Sensitivity

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 (50 ng/mL) with ease (Figure 3).

Calibration Curves

Figure 4 shows some typical calibration curves of a few analytes; ETG, ETS, amobarbital and secobarbital (n=3).

Figure 4: Representative Calibration Curves

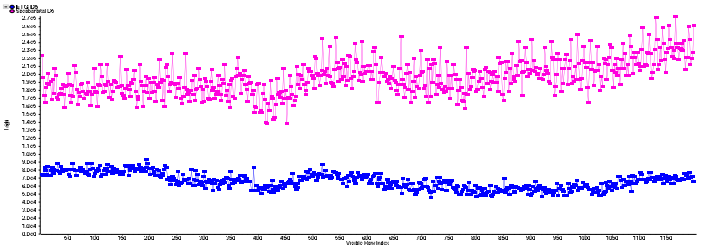


(A) ETG; (B) ETS; (C) amobarbital and (D) secobarbital (n=3)

Robustness

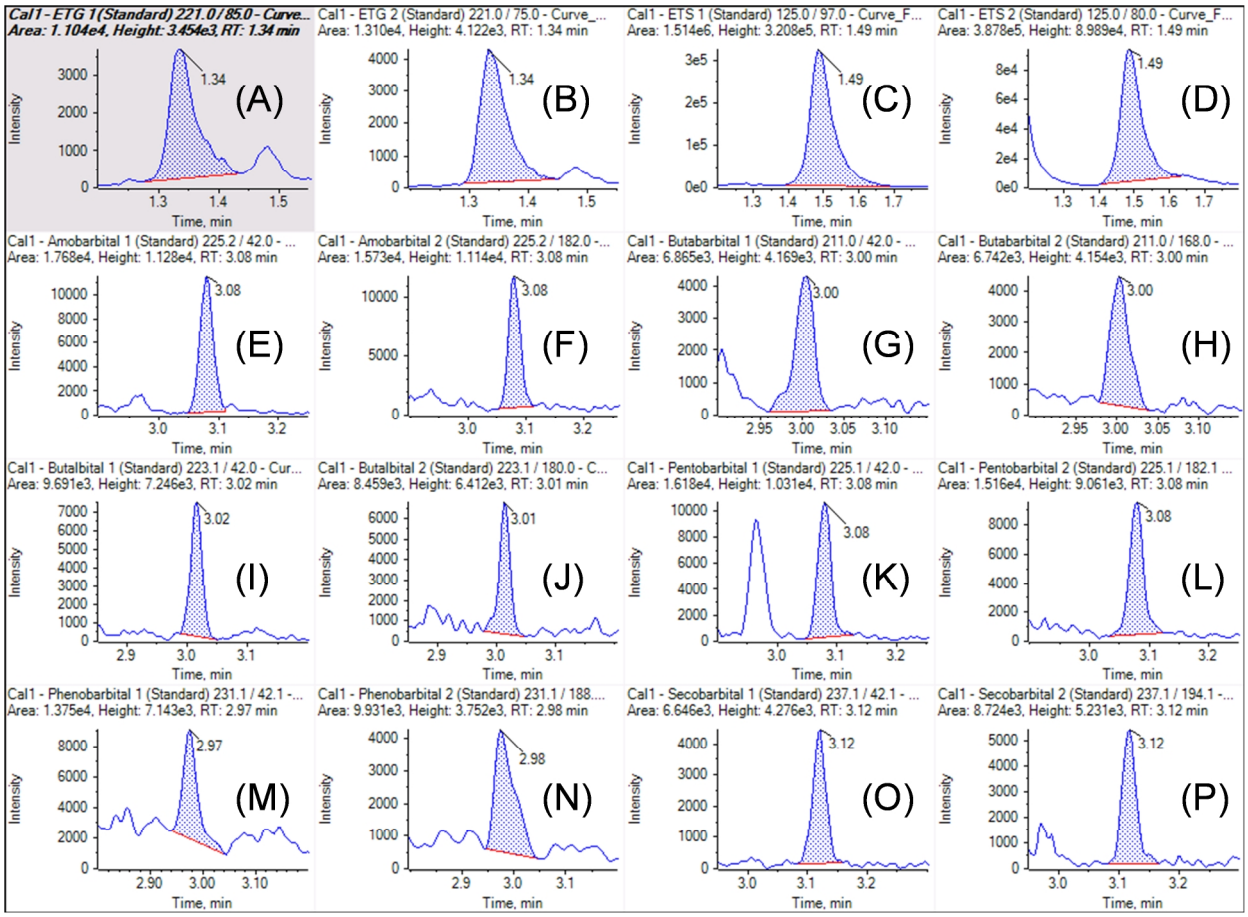
It is critical to prove the method robustness with real human urine samples. Over 840 injections of diluted urine samples spiked with various amount of these analtyes were performed during a >3 day period. No deterioration in either chromatographic separation or sensitivity was observed. Figure 5 shows the signals of ETG-d5 and secobarbital-d5 over 55 hours of 600 continuous injections of urine samples (blank, calibrators and QCs). Figure 6 shows the consistency of the retention times of all the internal standards during this period.

Figure 5. ETG-d5 and Secobarbital-d-5 Signal Stability



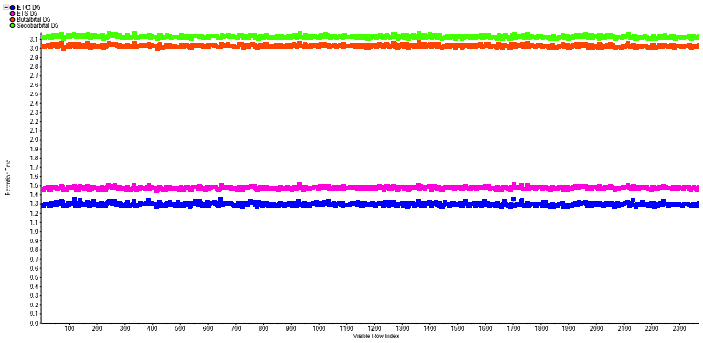
600 Continuous injections of urine (~55 hours)

Figure 3. 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).

Figure 6. Internal Standard Retention Time Stability



600 Continuous injections (from injection #241 to #840) of urine samples in 55 hours

Conclusion

In this technical note, we demonstrated a method to simultaneously analyze ethanol metabolites and barbiturates in human urine using QTRAP®/Triple Quad 4500 LC-MS/MS system. Sample preparation is based on a simple “dilute and shoot” methodology. The method has a total runtime of 5 minutes, shows good sensitivity and is very robust. More than 800 continuous injections of human urine samples were performed on a single LC column with no deterioration in performance evident.

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