Analysis of Five Barbiturates in Urine Using an Affordable High-Resolution Mass Spectrometer

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Application Note 633

Key Words
Q Exactive Focus, barbiturates, forensic toxicology

Goal
To develop a quantitative method for analysis of barbiturates in urine that meets forensic toxicology laboratory requirements.

Application Benefits
- Dilute-and-shoot method
- High selectivity
- Chromatographic separation of isobaric analyte
- Limited matrix effects
- Accurate chromatographic peak integration due to very low background
- Method performance meets toxicology lab requirements

Introduction
High-resolution mass spectrometers are widely accepted in forensic toxicology laboratories for use in screening applications. Conventionally, triple quadrupole instruments have been used for quantitative methods. The high-resolution Thermo Scientific™ Orbitrap™ instruments can be utilized for both screening and quantitative confirmatory methods, as well as structural elucidation experiments. Their ability to perform over a wide range of applications makes them a highly versatile platform for use in toxicology labs. In this note, we show their ability to quantitate five barbiturates (amobarbital, butalbital, pentobarbital, phenobarbital and secobarbital) in human urine.

Methods
Sample Preparation
Sample preparation was a dilute-and-shoot technique. A 50 µL urine sample was diluted with 950 µL of water that contained internal standards at a concentration of 100 ng/mL.

Calibrators and Quality Controls
Calibration standards in the range of 5 to 2000 ng/mL and quality control (QC) samples at concentrations of 25, 100, and 1000 ng/mL (LQC, MQC, HQC) were prepared in synthetic urine.

Liquid Chromatography
A six-minute gradient elution was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000RS liquid chromatography pump with OAS autosampler. Mobile phases consisted of 5 mM ammonium acetate in water and acetonitrile (Fisher Chemical™ Optima™ grade) for mobile phases A and B, respectively. The column used was a Thermo Scientific™ Accucore™ C18, 2.6 µm, 50 x 2.1 mm column (P/N 17126-052130).

Mass Spectrometry
Compounds were detected on a Thermo Scientific™ Q Exactive™ Focus quadrupole-Orbitrap mass spectrometer equipped with a Thermo Scientific™ Ion Max™ source and a heated electrospray (HESI-II) source. Data were acquired in parallel-reaction monitoring (PRM) mode. In this mode, a single precursor ion was selected in the quadrupole with an isolation width of 2.0 *m/z* and fragmented in the HCD cell using an optimized, compound-specific collision energy. The resulting MS/MS product ion spectrum was detected in the Orbitrap detector at a resolution of 35,000 (FWHM at *m/z* of 200).

Method Performance Evaluation
The limits of quantitation (LOQ) and linearity ranges were evaluated by collecting calibration curve data in triplicate in three different batches. Method precision and accuracy were evaluated by running a triplicate calibration curve and quintuplicate replicates of QCs on three different days. Matrix effects were evaluated by spiking urine from seven different donors at concentrations of 10, 25, and 100 ng/mL and calculating recovery against the same concentrations prepared in water instead of urine. Matrix effects were also evaluated by analyzing 48 donor urine samples and calculating internal standards' recovery against a sample prepared in water instead of urine.

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Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software. For each analyte, the precursor exact ion mass in the MS/MS spectrum was used for quantification and the most abundant fragment was used for confirmation. The chromatograms were reconstructed with a mass accuracy of 5 ppm for quantification. Figure 1 shows representative MS/MS spectra for selected analytes with quantifying and confirming ions specified.

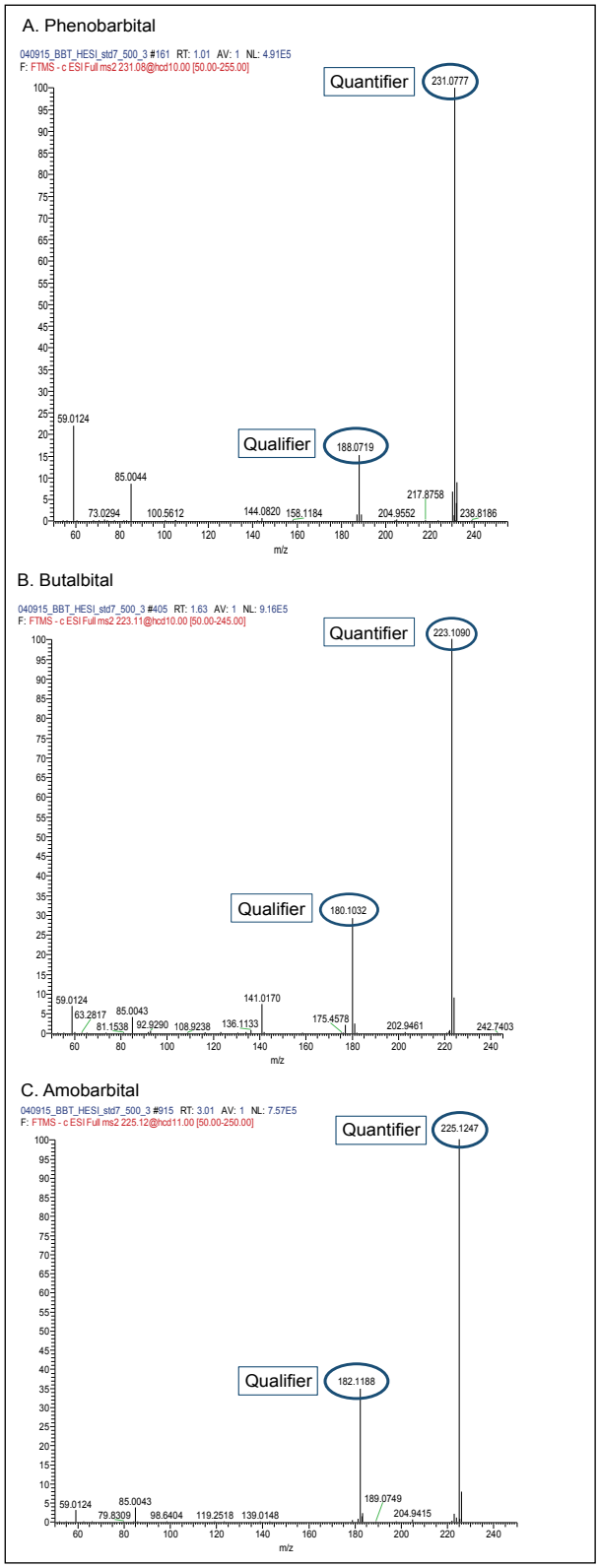

Figure 1. Representative MS/MS spectra for selected analytes with quantifying and qualifying ions specified.

Results

LOQs were defined as the lowest concentrations that had back-calculated values within 20%, RSD for five QC replicates within 20%, and ion ratio within specified range. Using these criteria, the limit of quantitation for butalbital, pentobarbital, amobarbital, and secobarbital was 5 ng/mL, and for phenobarbital was 25 ng/mL.

The upper calibration range for all analytes was 2000 ng/mL. Figure 2 shows representative calibration curves for all five analytes, collected in triplicate, along with chromatograms for the lowest calibration standard. Calibration standards' precision and accuracy were better than 15%.

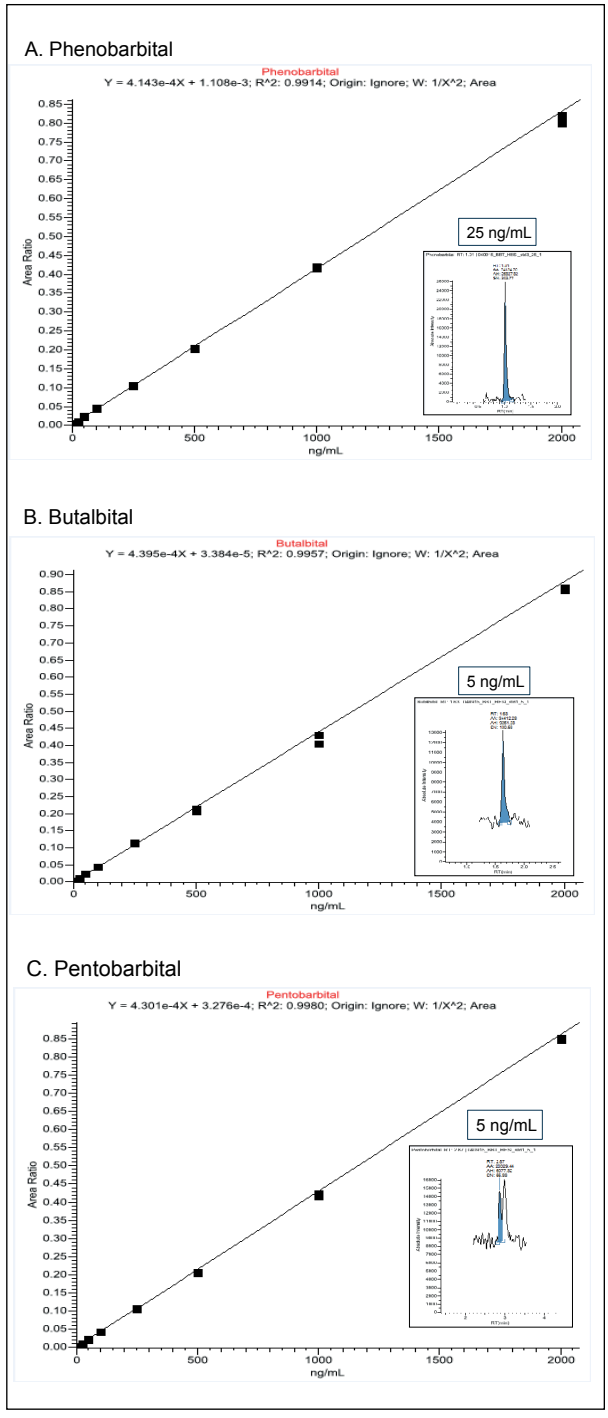

Figure 2. Representative calibration curves in triplicate and chromatograms for the lowest calibration standard. Accuracy of all calibration standards is within 15%.

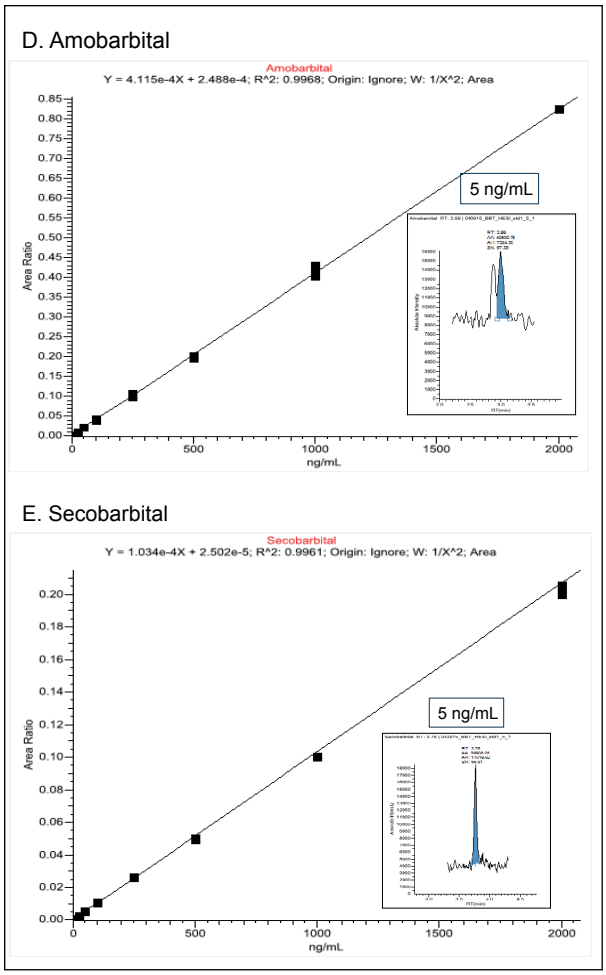

Figure 2 (continued). Representative calibration curves in triplicate and chromatograms for the lowest calibration standard. Accuracy of all calibration standards is within 15%.

Intra-assay precision was better than 8% (Table 1), and inter-assay precision was better than 10% (Table 2) for all analytes.

Table 1. Intra-assay precision.

Analyte	LQC	MQC	HQC
	%RSD		
Phenobarbital	3.5–7.2	2.6–4.6	3.0–3.5
Butalbital	3.0–5.2	2.0–2.8	1.9–3.8
Pentobarbital	2.5–8.0	0.74–2.6	2.0–4.0
Amobarbital	3.6–6.8	2.6–4.3	1.6–2.8
Secobarbital	2.9–4.8	2.2–2.8	1.7–3.3

Table 2. Inter-assay precision.

Analyte	LQC	MQC	HQC
	%RSD		
Phenobarbital	5.5	5.6	4.1
Butalbital	5.7	6.1	5.4
Pentobarbital	6.5	6.2	6.1
Amobarbital	9.0	9.7	7.0
Secobarbital	5.1	5.7	5.1

Limited matrix effects were observed. Recovery in seven donor samples, calculated as ratio between analyte peak area in urine matrix and analyte peak area in solvent matrix, ranged from 85.8% to 115% (Table 3). Figure 3 presents a quantifying ion chromatogram of donor urine spiked with all analytes at a concentration of 25 ng/mL.

Internal standards recovery in 48 donor urine samples ranged from 76% to 108% (Figure 4).

Table 3. Matrix effects in spiked donor urine samples.

Analyte	10 ng/mL	25 ng/mL	100 ng/mL
	%recovery		
Phenobarbital	92.3–104	85.8–105	99.3–108
Butalbital	99.7–110	93.0–105	99.1–109
Pentobarbital	99.1–112	89.2–102	96.3–108
Amobarbital	93.8–113	97.8–112	102–111
Secobarbital	92.3–102	85.3–104	99.7–110

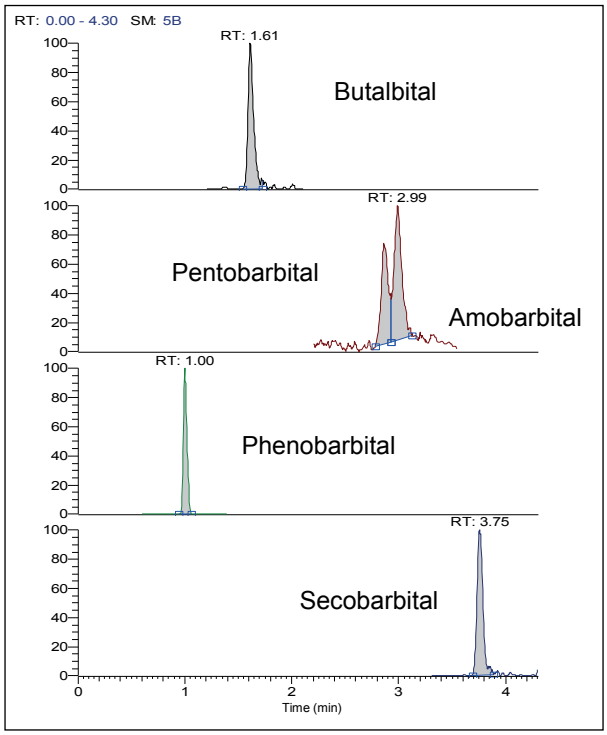

Figure 3. Chromatogram of donor urine spiked with all analytes to a concentration of 25 ng/mL. Chromatogram is reconstructed with a mass accuracy of 5 ppm.

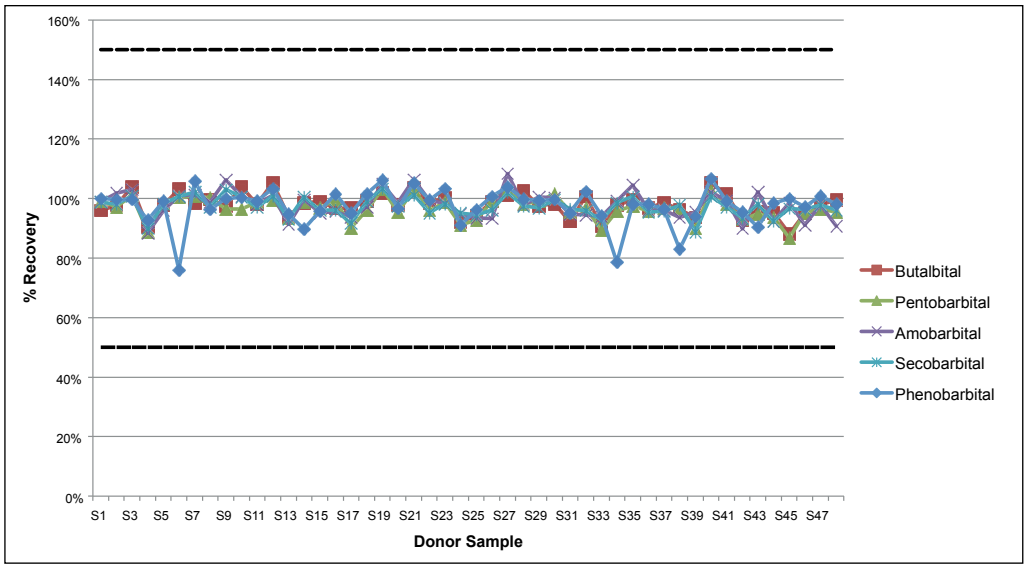

Figure 4. Internal standards % Recovery in donor urine samples.

Conclusion

We demonstrated a simple dilute-and-shoot method for the analysis of five barbiturates in urine implemented on a Q Exactive Focus high-resolution mass spectrometer. The method performance evaluation data indicate that the method can be implemented in forensic toxicology laboratories and show the versatility of Orbitrap technology for these laboratories.

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