A Simple Method to Analyze Barbiturates in Urine Using a Triple Quadrupole Mass Spectrometer

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Application Note 640

Key Words
Barbiturates, forensic toxicology, TSQ Endura MS, urine

Goal
To develop a simple, fast, and robust quantitative method for analysis of five barbiturates in urine that meets forensic toxicology laboratory requirements.

Application Benefits
- Easy dilute-and-shoot sample preparation
- Chromatographic separation of isobaric analytes
- Limited matrix effects
- Method performance meets toxicology lab requirements

Introduction
Sophisticated sample preparation methods such as solid phase extraction (SPE) or disposable pipette extraction (DPX) have been used to extract barbiturates from urine prior to LC-MS/MS analysis in order to improve analytical method sensitivity. However, these methods are not only expensive but also tedious. In this application note, we present a fast and simple dilute-and-shoot LC-MS/MS method to quantify five barbiturates (amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital) in urine using a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer. Although amobarbital and pentobarbital differ only in the position of a methyl group and are, hence, difficult to separate, the resolution achieved in this method using a Thermo Scientific™ Accucore™ C18 column is sufficient for routine analysis.

Methods
Sample Preparation
To prepare samples, 50 µL of urine 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 50, 250, and 2000 ng/mL (LQC, MQC, HQC) were prepared in synthetic urine and processed as described in the previous section.

Liquid Chromatography
A six-minute gradient elution was performed using a Thermo Scientific™ UltiMate™ 3000 UHPLC system with a LPG-3400XRS pump and OAS-3300TXRS autosampler. Mobile phases were 5 mM ammonium acetate in water and 100% acetonitrile (Fisher Chemical Optima™ grade) for phase A and B, respectively. The analytical column was a Thermo Scientific™ Accucore™ C18, 2.6 µm, 50 × 2.1 mm column (P/N 17126-052130).

Mass Spectrometry
Compounds were detected on a TSQ Endura triple quadrupole mass spectrometer equipped with a heated electrospray ionization source (HESI II). Data were acquired in selected-reaction monitoring (SRM) mode. Two SRM transitions for each analyte were monitored and ion ratios were calculated for confirmation (Table 1).

Data Analysis
Data were acquired and processed using Thermo Scientific™ TraceFinder™ software version 3.2.

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 quintuplicate QCs on three different days. Matrix effects were evaluated by spiking urine from seven different donors at a concentration of 100 ng/mL and calculating recovery against the same concentration 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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Table 1. SRM transitions monitored for analytes and internal standards.

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor *(m/z)*	Product *(m/z)*	Collision Energy (V)	Comment
Amobarbital	2.30	1.6	Negative	225.1	182.2	14	quantifying ion
Amobarbital	2.30	1.6	Negative	225.1	42.2	18	confirming ion
Amobarbital-d5	2.30	1.6	Negative	230.2	187.2	14	quantifying ion
Amobarbital-d5	2.30	1.6	Negative	230.2	42.2	18	confirming ion
Butalbital	1.30	1.0	Negative	223.1	180.2	12	quantifying ion
Butalbital	1.30	1.0	Negative	223.1	42.2	18	confirming ion
Butalbital-d5	1.30	1.0	Negative	228.1	185.1	12	quantifying ion
Butalbital-d5	1.30	1.0	Negative	228.1	42.2	18	confirming ion
Pentobarbital	2.30	1.6	Negative	225.1	182.2	14	quantifying ion
Pentobarbital	2.30	1.6	Negative	225.1	42.2	18	confirming ion
Pentobarbital-d5	2.30	1.6	Negative	230.2	187.2	14	quantifying ion
Pentobarbital-d5	2.30	1.6	Negative	230.2	42.2	18	confirming ion
Phenobarbital	0.85	0.8	Negative	231.1	188.1	12	quantifying ion
Phenobarbital	0.85	0.8	Negative	231.1	42.2	18	confirming ion
Phenobarbital-d5	0.85	0.8	Negative	236.1	193.2	12	quantifying ion
Phenobarbital-d5	0.85	0.8	Negative	236.1	42.2	18	confirming ion
Secobarbital	3.20	1.4	Negative	237.2	194.2	12	quantifying ion
Secobarbital	3.20	1.4	Negative	237.2	42.2	18	confirming ion
Secobarbital-d5	3.20	1.4	Negative	242.2	199.2	12	quantifying ion
Secobarbital-d5	3.20	1.4	Negative	242.2	42.2	18	confirming ion

Results and Discussion

Limits of quantitation (LOQs) were defined as the lowest concentrations that had back-calculated values within 20%, RSD for three calibration replicates within 20%, and an ion ratio within the specified range. Using these criteria, the limits of quantitation were 5 ng/mL for butalbital and secobarbital, 10 ng/mL for amobarbital and pentobarbital, and 25 ng/mL for phenobarbital.

The upper calibration range for all analytes was 2000 ng/mL. Figure 1 shows representative calibration curves for all five analytes, collected in triplicate, along with chromatograms for the lowest calibration standard. Calibration standards' precision was better than 15%, and accuracy was within 15%.

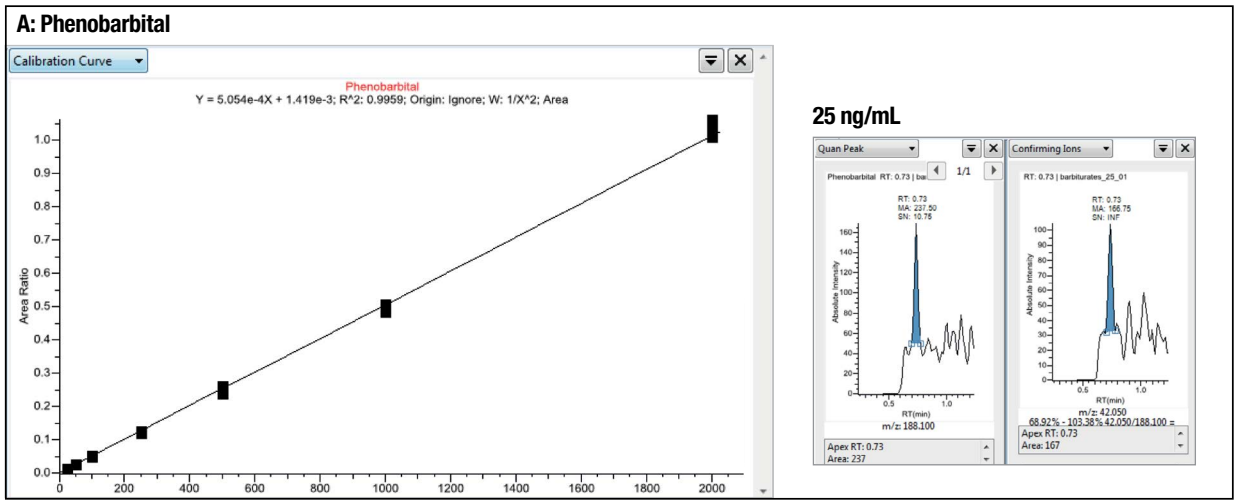
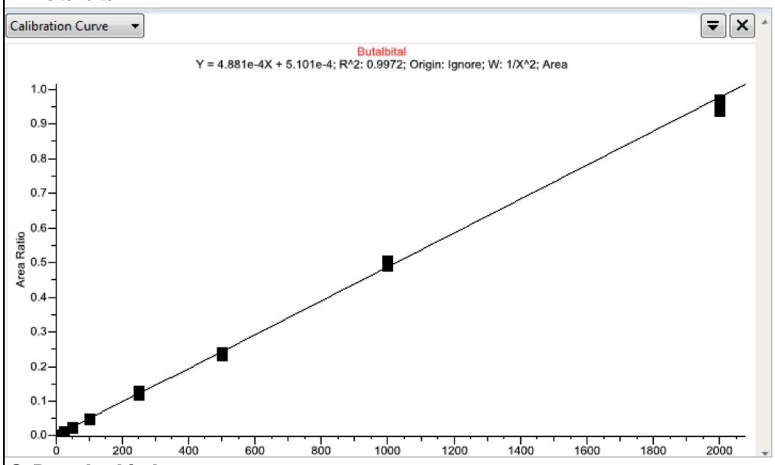
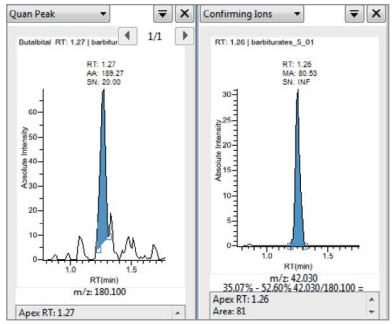


Figure 1. Representative calibration curves for all five analytes, collected in triplicate, along with chromatograms for the lowest calibration standard.

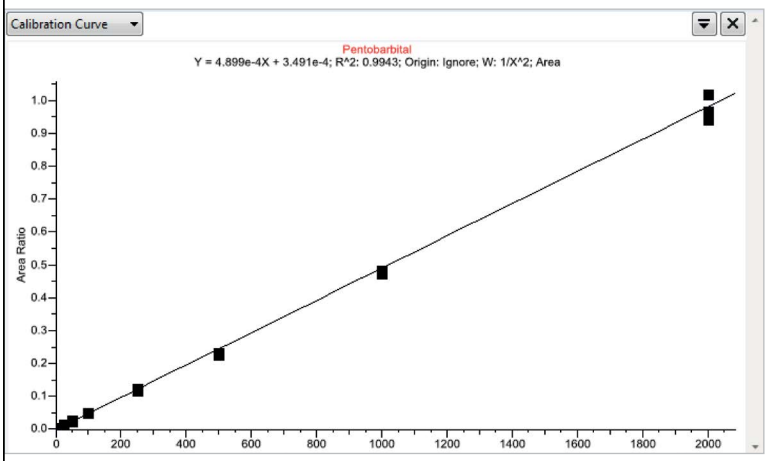
B: Butalbital



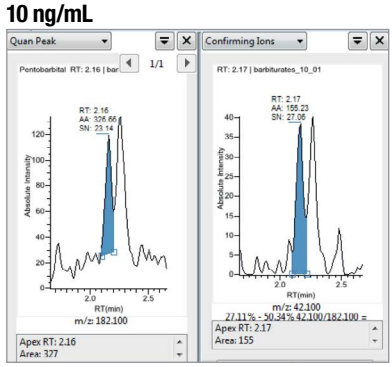
5 ng/mL



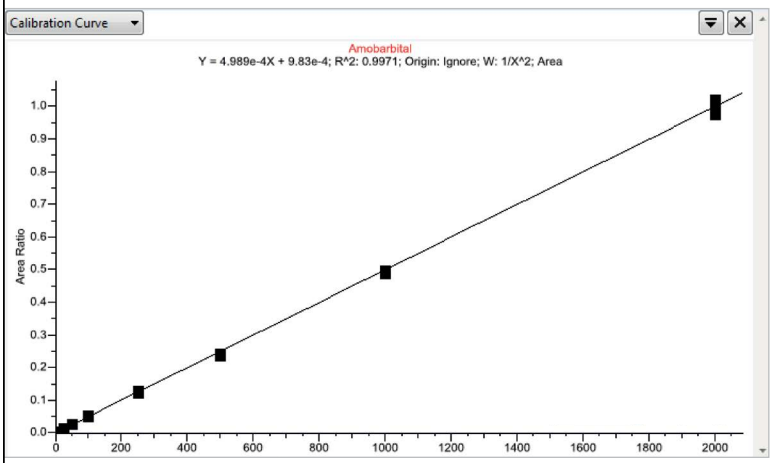
C: Pentobarbital



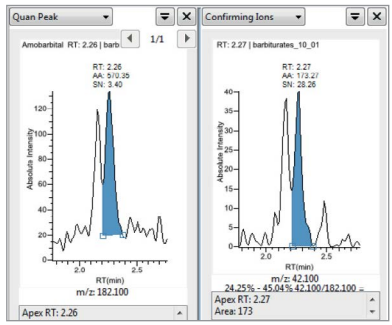
10 ng/mL



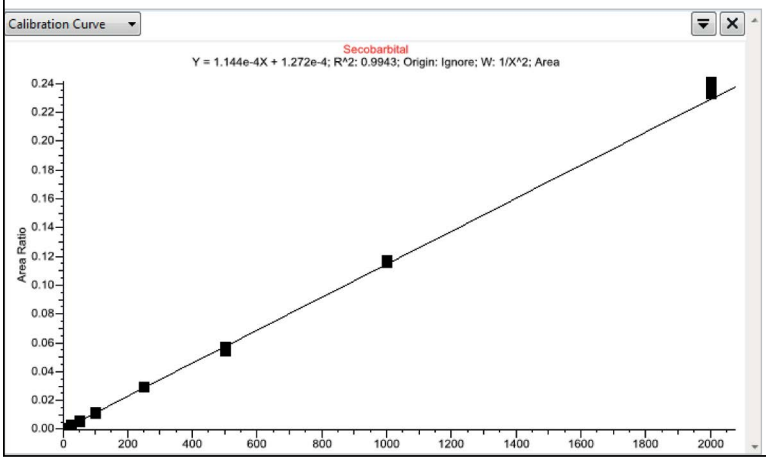
D: Amobarbital



10 ng/mL



E: Secobarbital



5 ng/mL

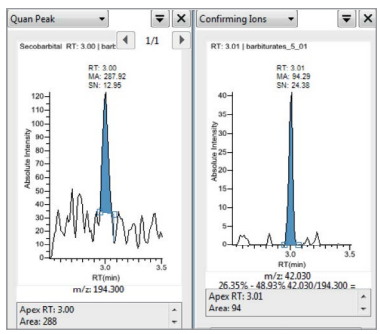


Figure 1 (continued). Representative calibration curves for all five analytes, collected in triplicate, along with chromatograms for the lowest calibration standard.

Intra-assay precision (% RSD) was better than 10% (Table 2), and inter-assay precision (% RSD) was better than 10% (Table 3) for all analytes.

Table 2. Intra-assay precision (n=5).

Analyte	%RSD		
	LQC	MQC	HQC
Phenobarbital	8.0–9.5	5.1–5.7	1.2–2.5
Butalbital	2.7–5.0	2.2–2.6	1.2–2.3
Pentobarbital	3.7–6.8	0.7–2.3	0.9–3.1
Amobarbital	3.0–6.8	1.3–2.6	0.7–1.4
Secobarbital	3.6–6.5	3.0–4.1	0.6–1.1

Table 3. Inter-assay precision (n=15).

Analyte	%RSD		
	LQC	MQC	HQC
Phenobarbital	8.4	5.0	2.1
Butalbital	4.5	2.4	1.9
Pentobarbital	5.4	1.8	1.9
Amobarbital	4.6	1.8	1.4
Secobarbital	6.3	3.8	0.9

Limited matrix effects were observed. Absolute recovery in seven donor samples, calculated as the ratio between analyte peak area in urine matrix and analyte peak area in solvent, ranged from 67.5% to 138.0% (Table 4). However, relative recovery, which was calculated as the ratio between analyte peak area ratio against internal standard in urine matrix and analyte peak area ratio in solvent, ranged from 85.4% to 115.0%. Hence, the matrix effect was corrected by addition of internal standards. Figure 2 presents chromatograms of donor urine spiked with all analytes at a concentration of 25 ng/mL.

Table 4. Absolute and relative recovery rate.

Analyte	100 ng/mL	
	Absolute % Recovery	Relative % Recovery
Phenobarbital	67.5–97.6	85.4–109
Butalbital	89.2–122	90.6–107
Pentobarbital	86.6–129	97.9–115
Amobarbital	90.4–138	96.7–111
Secobarbital	83.8–105	88.0–96.0

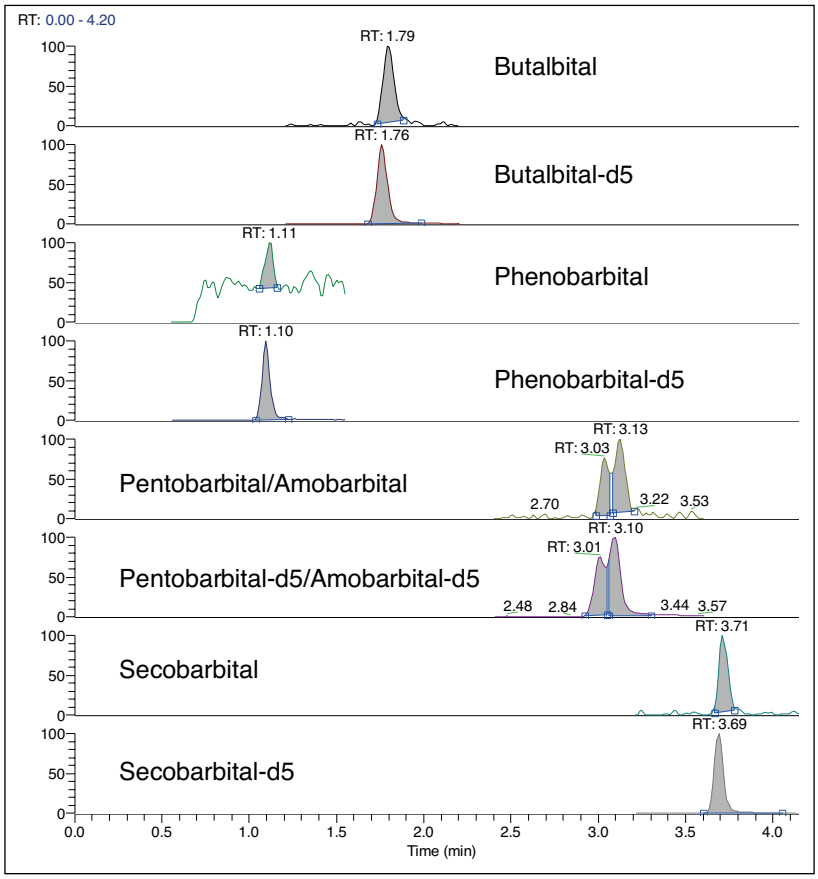


Figure 2. Chromatogram of donor urine spiked with all analytes at a concentration of 25 ng/mL.

Internal standards recovery in 48 donor urine samples ranged from 74.2% to 111.9% (Figure 3).

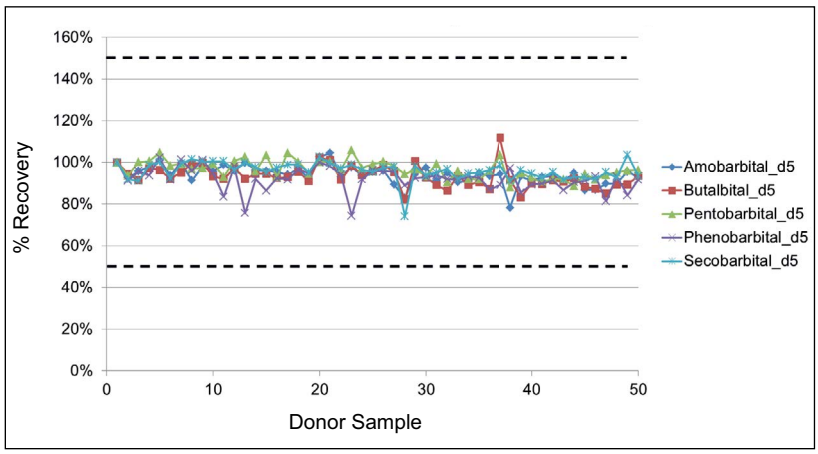


Figure 3. Internal standards recovery in 48 donor urine samples.

Conclusion

In this study, we demonstrated a simple dilute-and-shoot method for the quantification of five barbiturates in urine with a TSQ Endura triple quadrupole mass spectrometer. In addition to offering sensitive, robust, and reproducible data, this method allowed us to chromatographically separate the isobaric compounds, amobarbital and pentobarbital. All results reported in this study met forensic toxicology laboratory requirements.

For forensic use only.