Ultrafast Analysis of Barbiturates in Urine by the Agilent RapidFire High-Throughput Triple Quadrupole Mass Spectrometry System

Application Note

Forensic Toxicology

Authors

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Abstract

An ultrafast RapidFire/MS/MS method was developed for simultaneous qualitative analysis of common barbiturates in human urine. The need for greater analytical capacity and throughput for the analysis of barbiturates in forensic toxicology laboratories has placed demands on the traditional analytical technologies of HPLC and LC/MS/MS. We developed a method utilizing the Agilent RapidFire/MS/MS system to analyze butalbital, phenobarbital, secobarbital, amobarbital, and pentobarbital in urine with much faster sample cycle times and similar analytical results compared to LC/MS/MS assays. A simple dilute and shoot methodology followed by analysis by RapidFire/MS/MS allows for the accurate and precise measurement of these analytes in urine over a linear range of 50 to 10,000 ng/mL. Samples were analyzed at 16 seconds per sample providing a much higher throughput analysis compared to traditional LC/MS/MS protocols. This new ultrafast method has the speed and accuracy necessary for an efficient forensic screen or qualitative analysis workflow.

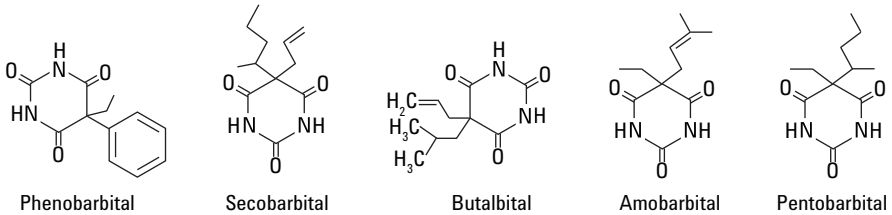

Figure 1. Chemical structures of barbiturates: phenobarbital, secobarbital, butalbital, amobarbital, and pentobarbital.

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Introduction

Barbiturates represent a class of drugs that were originally introduced as sleep inducers and that are often abused. The risks for addiction and abuse have put pressure on forensic toxicology laboratories to measure these drugs reliably and accurately. Steady increases in the need for greater analytical capacity and throughput have placed demands on traditional analytical technologies. We strived to develop a streamlined screening process to provide a fast, cost effective, and reproducible method from start to finish for forensic labs performing high-throughput processing of barbiturate screens.

The Agilent RapidFire High-throughput Mass Spectrometry System is an ultrafast SPE/MS/MS system capable of analyzing samples with cycle times of less than 16 seconds. In the present study, we developed a method to analyze barbiturates in urine using a simple dilute and shoot methodology and the RapidFire/MS/MS system with much faster sample cycle times and similar analytical results compared to HPLC or LC/MS/MS methods. This new method allows for the rapid, accurate and precise detection of barbiturates in urine over a linear range of 50 to 10,000 ng/mL. The chemical structures for the barbiturates used in this study can be found in Figure 1. This methodology provides comparable results to LC/MS/MS,[1] but at >15x the speed and efficiency of traditional HPLC or LC/MS/MS methods.

Experimental

RapidFire triple quadrupole conditions

The Agilent RapidFire/MS/MS system consisted of the following modules: Agilent RapidFire 360, Agilent 6460 Triple Quadrupole Mass Spectrometer using Agilent MassHunter Triple Quadrupole Acquisition Software (B.04.01) with Qualitative Analysis (B.04.00), Quantitative Analysis (B.04.00), and RapidFire Acquisition Software. Samples were analyzed at a rate of 16 seconds per sample. Quantitative and qualitative ions for phenobarbital, secobarbital, butalbital, amobarbital, and pentobarbital and the internal standard butalbital-d5 were monitored simultaneously in all experiments (Table 1).

Table 1. RapidFire/MS/MS Conditions.

RapidFire conditions						
Buffer A (pump 1)	5 mM Ammonium acetate in LC/MS grade water; 1.5 mL/min flow rate					
Buffer B (pump 2)	5 % LC/MS grade acetonitrile in water; 1.25 mL/min flow rate					
Buffer C (pump 3)	50 % LC/MS grade methanol: 50 % HPLC grade isopropanol; 0.6 mL/min flow rate					
Injection volume	10 µL					
SPE cartridge	Agilent RapidFire cartridge F (reversed-phase phenyl, G9208A)					
RF State 1	sip sensor					
RF State 2	3,000 ms					
RF State 3	2,000 ms					
RF State 4	7,000 ms					
RF State 5	1,000 ms					
Triple quadrupole conditions						
Gas temperature	325 °C					
Gas flow	13 L/min					
Nebulizer	25 psi					
Sheath gas flow	11 L/min					
Sheath gas temperature	400 °C					
Nozzle voltage	2,000 V					
Capillary voltage	2,500 V					
ESI mode	Negative					
Analyte	**Q1**	**Q3**	**Dwell**	**Fragmentor**	**CE**	**CAV**
Butalbital-d5	228.1	42.1	50	95	5	2
Phenobarbital	231.1	42.1	50	95	16	2
Amobarbital and pentobarbital (isobars)	225.1	42.1	50	91	16	2
Butalbital	223.1	42.1	50	95	12	2
Secobarbital	237.1	42.1	50	100	20	2

Chemicals and reagents

The analytes Phenobarbital, Secobarbital, Butalbital, Amobarbital, Pentobarbital, and the stable-labeled isotope internal standard Butalbital-d5 were purchased from Cerilliant, Round Rock, TX. All other solvents and reagents were purchased from VWR or Fisher Scientific.

Sample preparation

Calibrators were prepared by spiking drug-free urine with all analytes at 50, 250, 1,000, 5,000, and 10,000 ng/mL concentrations. Sample preparation consisted of a simple dilute-and-shoot procedure. Ten microliters of each urine sample or calibrator were combined with 490 µL of 100 % ultrapure water containing internal standard (butalbital-d5 at 10 ng/mL) in a 2.2 mL, 96 deep well plate. The plate was then sealed with an Agilent PlateLoc Thermal Microplate Sealer, mixed for 20 seconds and centrifuged prior to RapidFire/MS/MS analysis.

Data analysis

System control and data acquisition were performed by Agilent MassHunter triple quadrupole data acquisition software. Calibration curves were constructed using linear least squares regression with $1/X^2$ weighting for the multiple reactions monitoring (MRM). The quantitation, using Agilent MassHunter Quantitative Analysis software, was performed by spectral peak area ratio to a known concentration of the internal standards.

Results and Discussion

Samples were prepared by spiking barbiturates into drug-free human urine and then diluting samples 50-fold with water containing the internal standard. Samples were then analyzed via SPE/MS/MS using the RapidFire/MS/MS system and a phenyl cartridge at 16 seconds per sample (Figure 2). This RapidFire/MS/MS methodology is capable of throughputs greater than 225 samples per hour providing a high-throughput and very efficient mode of analysis.

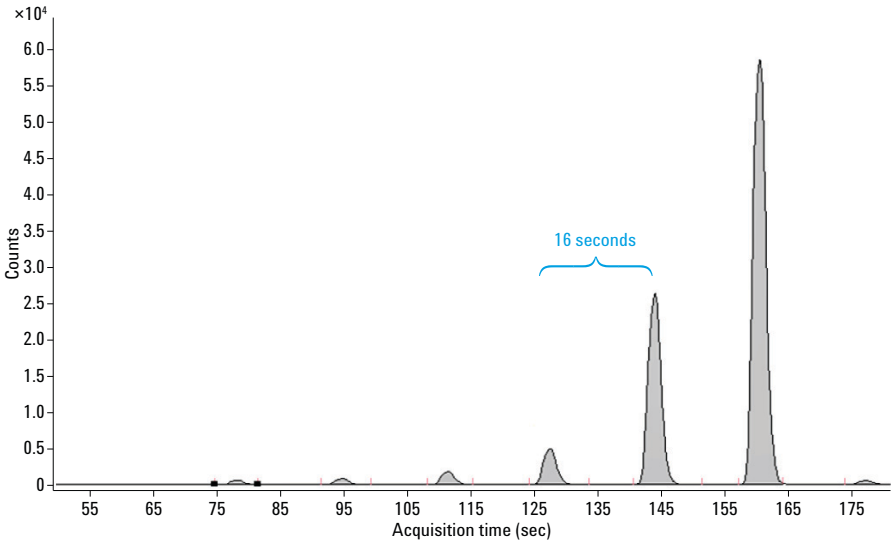

Figure 2. Representative standard curve showing 16 second injection to injection interval.

Phenobarbital, Secobarbital, Butalbital, and Amo/Pentobarbital standard curves in urine had excellent linearity within the measured range (50–10,000 ng/mL) (Figure 3) with an R^2 value greater than 0.995. Amobarbital and Pentobarbital are isobaric and this qualitative method cannot distinguish between these two analytes. The standards were analyzed to obtain intra- and interday precision and accuracy values. Accuracies determined were within 10 % and coefficient of variation values were all less than 10 % for concentrations within the measured range (Table 2).

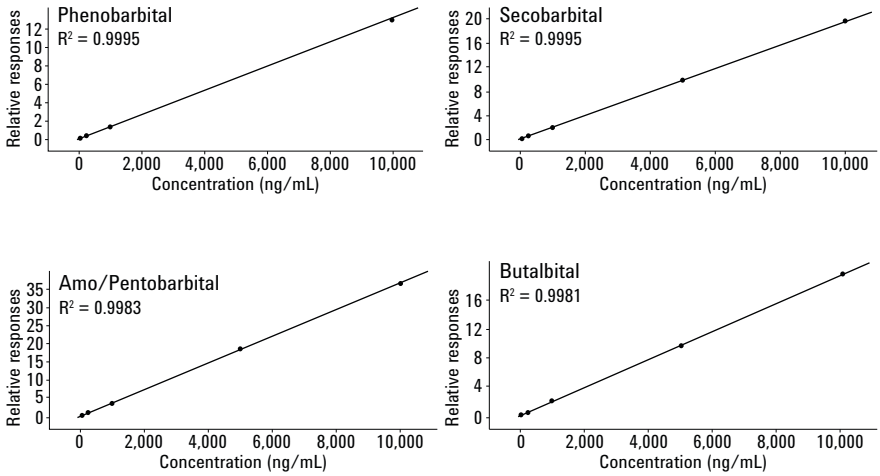

Figure 3. Representative calibration curves for each barbiturate analyte.

Table 2. Interday and Intraday accuracy and precision (n=6) for RapidFire/MS/MS analysis of barbiturates in urine.

Phenobarbital (ng/mL)	Interday % Accuracy	Interday % Precision	Intraday % Accuracy	Intraday % Precision
50	100.4	5.0	101.6	2.3
250	99.0	5.0	97.9	4.5
1,000	99.4	2.6	101.8	3.1
5,000	99.9	5.8	101.1	5.1
10,000	101.4	2.3	102.6	5.7
Secobarbital (ng/mL)	**Interday % Accuracy**	**Interday % Precision**	**Intraday % Accuracy**	**Intraday % Precision**
50	100.0	6.3	101.1	1.1
250	99.1	4.8	97.7	6.4
1,000	96.9	2.7	99.4	3.9
5,000	101.1	4.4	99.9	4.0
10,000	102.6	2.8	101.6	1.5
Butalbital (ng/mL)	**Interday % Accuracy**	**Interday % Precision**	**Intraday % Accuracy**	**Intraday % Precision**
50	99.6	6.0	101.0	1.1
250	102.1	4.5	96.9	6.1
1,000	96.7	4.4	102.0	2.2
5,000	99.3	4.5	100.2	3.0
10,000	102.3	4.4	101.9	1.4
Amo/ Pentobarbital (ng/mL)	**Interday % Accuracy**	**Interday % Precision**	**Intraday % Accuracy**	**Intraday % Precision**
50	100.0	6.2	101.6	1.5
250	99.4	5.9	98.9	7.6
1,000	95.7	4.1	99.0	4.6
5,000	101.2	4.2	101.6	1.3
10,000	103.4	2.3	101.9	1.5

Carryover was assessed by analyzing the AUC of the blank calculated as % of the mean peak area of the 50 ng/mL samples. No significant carryover (0 %) was determined for all barbiturates (Figure 4). When measuring higher concentrations of barbiturates (>10,000 ng/mL), we recommend using one blank injection between wells by injecting a strong organic solution. Matrix effects were also investigated for all analytes by comparing the AUCs of standard curves prepared in 100 % water to those in drug free human urine. No significant matrix effect was observed (<10 %).

This method consisting of dilute and shoot sample preparation followed by quick analysis on RapidFire/MS/MS provides a very efficient mode of qualitatively measuring barbiturates in urine compared to traditional HPLC or LC/MS/MS methods.

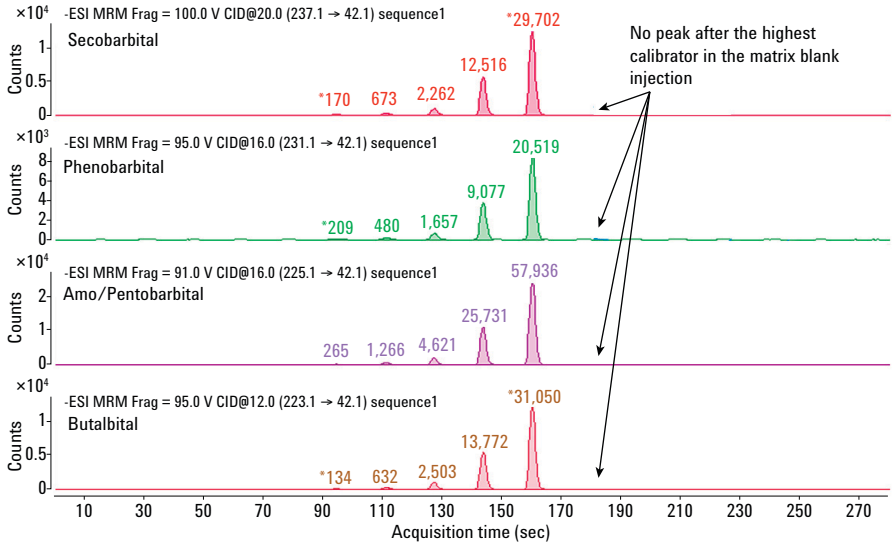

Figure 4. Carryover assessment using a blank injection after the highest calibrator shows that no significant carryover was observed for any analyte.

Conclusion

An efficient, sensitive, and accurate RapidFire/MS/MS method was developed for the qualitative analysis of five common barbiturates in human urine using a dilute and shoot methodology. There is also the potential to add more analytes to this panel through simple modifications to the current method. Samples were analyzed with injection to injection cycle times of 16 seconds, providing a high-throughput method of analysis for these analytes over a wide calibration range. This methodology is capable of throughputs greater than 225 samples per hour. The analytical results were comparable to LC/MS/MS, but at >15x the speed of LC/MS/MS methods. This method provides a very efficient mode for qualitative analysis of Butalbital, Phenobarbital, Secobarbital, Amobarbital, and Pentobarbital in urine compared to traditional analytical methods.

Reference

1. June Feng, Lanqing Wang, Ingrid Dai, Tia Harmon, and John T. Bernert. Simultaneous Determination of Multiple Drugs of Abuse and Relevant Metabolites in Urine by LC/MS/MS. *Journal of Analytical Toxicology*, 31:September **2007**, 359-368.

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