[APPLICATION NOTE]

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Quantitative Analysis of Barbiturates in Urine Using UPLC/MS/MS

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APPLICATION BENEFITS

- Elimination of extraction step
- Elimination of derivatization step prior to analysis
- Improved sample throughput

WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ Mass Spectrometer

ACQUITY® BEH C_{18} Column

Waters® maximum recovery vial

KEY WORDS

Barbiturates, UPLC/MS/MS

INTRODUCTION

Barbiturates act as central nervous system depressants producing effects ranging from mild sedation to general anesthesia. They have largely been replaced by benzodiazepines as prescription medicines, owing to their relatively low therapeutic index and their high potential for dependence. However, it is known that the use of barbiturates is still common in certain regions of Eastern Europe;[1] consequently, their analysis is still of key importance in both forensic analysis and workplace drug testing.

Barbiturates have traditionally been measured by GC.[2,3] The arrival of newer technologies into the modern laboratory, such as UPLC®/MS/MS, often leads to an overall requirement to consolidate analytical methods and transfer existing methodologies to the newer platforms. Furthermore, UPLC/MS/MS permits the development of more sensitive techniques.

We report a quantitative method based on simple dilution and UPLC/MS/MS. The method has been verified, and its performance evaluated using authentic samples. Data were compared to results obtained with a traditional method that used liquid-liquid extraction followed by derivatization and analysis by GC/MS.

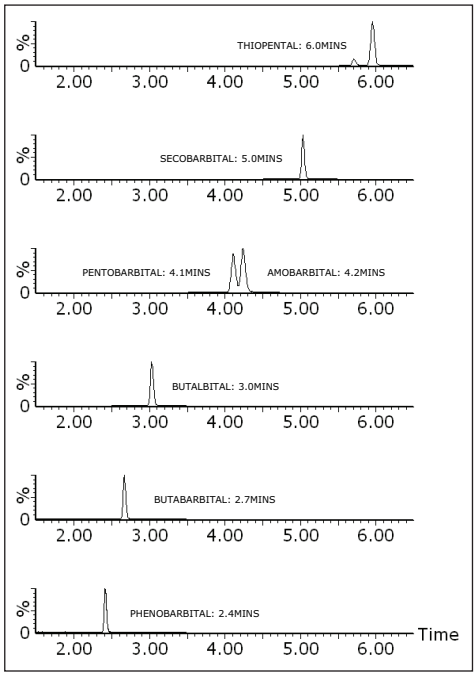

Figure 1. Chromatogram showing barbiturates spiked into urine at 500 ng/mL.

EXPERIMENTAL

Method Conditions

UPLC Conditions

System:	ACQUITY UPLC
Column:	ACQUITY BEH C_{18} 2.1 x 100 mm with BEH C_{18} 2.1 x 5 mm Vanguard pre-column
Column temp.:	50 °C
Sample temp.:	5 °C
Injection volume:	15 µL (PLNO)
Strong wash:	0.001% formic acid in acetonitrile
Weak wash:	0.001% formic acid in water
Flow rate:	400 µL/min
Mobile phase A:	0.001% formic acid in water
Mobile phase B:	0.001% formic acid in acetonitrile
Gradient:	Hold at 5% B for 0.5 min, then switch to 27.5% B, hold until 4 min, then switch to 35% B, hold until 5.25 min, then switch to 90% B, hold until 6.25 min, then switch to 5% B.

MS Conditions

Mass Spectrometer:	Xevo TQ
Ionization mode:	ESI negative
Capillary voltage:	2.75 kV
Cone voltage:	25 V
Collision energy:	12 eV
Desolvation temp.:	500 °C
Desolvation gas:	1000 L/h
Cone gas:	25 L/h
Acquisition mode:	Multiple reaction monitoring (MRM), as shown in Table 1.

Sample description

Phenobarbital and thiopental were purchased from Sigma Aldrich (Dorset, UK) and dissolved in methanol to 1 mg/mL. All other barbiturates (1 mg/mL) and deuterated internal standards (ISTDs) at 0.1 mg/mL were obtained as certified standard solutions from LGC Standards (Teddington, UK). Deuterated internal standards were not available for all of the barbiturates.

Quality control reference urine samples (Bio-Rad Liquichek Urine Toxicology Control: C2, C3, C4, S1, and S2) were obtained from Bio-Rad Laboratories Ltd (Hemel Hempstead, UK).

Urine samples for method development were obtained from donors at Waters Corporation.

Nineteen samples containing pre-analyzed barbiturates were obtained from Concateno, London, UK.

Sample preparation

Urine, either sample or calibrator, was centrifuged at 13,000 rpm for 5 min, then 50 µL was transferred to a Waters maximum recovery vial and diluted with 950 µL water containing 25 ng of each available ISTD.

Barbiturate	Precursor ion (*m/z*)	Product ion (*m/z*)	ISTD
Amobarbital	225.1	182.0	Pentobarbital-d5
Butabarbital	211.1	168.0	Phenobarbital-d5
Butalbital	223.0	180.0	Butalbital-d5
Pentobarbital	225.1	182.0	Pentobarbital-d5
Phenobarbital	231.1	188.0	Phenobarbital-d5
Secobarbital	237.1	194.1	Secobarbital-d5
Thiopental	241.1	57.9	Secobarbital-d5
Butalbital-d5	228.0	185.0	
Pentobarbital-d5	230.1	186.9	
Phenobarbital-d5	236.1	193.0	
Secobarbital-d5	242.1	199.1	

Table 1. MRM transitions for analytes and ISTDs.

Data management

MassLynx™ *v*4.1 incorporating TargetLynx™ Application Manager

RESULTS AND DISCUSSION

Method verification

The MRM transitions for all of the barbiturates and ISTDs are shown in Table 1. All were monitored using a single transition. Figure 1 shows a chromatogram of a 500 ng/mL barbiturate-spiked urine.

To investigate linearity for all barbiturates, spiked urine calibrators were prepared at 0, 25, 50, 100, 250, 500, 1000, 750, 1250, and 1500 ng/mL. Samples were diluted 20-fold with water, containing ISTDs as previously described, and subsequently analyzed by UPLC/MS/MS.

Quantification was performed by integrating the area under the peak for each analyte MRM trace, and referencing to the appropriate ISTD peak area. Data were processed using the TargetLynx Application Manager, and calibration curves plotted with a 1/x weighting. Interday coefficient of determination (assessed over five days) was >0.995.

The limit of detection (LOD) was defined as the lowest concentration, which produced a signal to noise ratio >5:1 in spiked urine. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal to noise ratio >10:1, and demonstrated a mean concentration bias <20% of target, and a %RSD of <20% in spiked urine. The LOD and LLOQ are summarized in Table 2.

Barbiturate matrix effects (from six different sources of blank urines) were investigated in triplicate at the following concentrations: 100 (low), 500 (medium), and 1000 ng/mL (high). Matrix effects were determined by comparing the response in spiked urine sample to the response in water. The results for each barbiturate are shown in Table 2. The %RSD for the six urines at each concentration was <20%.

Interday accuracy and precision were assessed by analyzing three quality control (QC) concentrations (150, 600, 1200 ng/mL) over five different days. The mean achieved values for the quality control replicates over the five-day period at the three concentration levels were within 10% of target and the %RSD was <15%, as shown in Table 3.

	LOD	LLOQ	% Matrix effects		
	ng/mL	ng/mL	100 ng/mL	500 ng/mL	1000 ng/mL
Amobarbital	5	20	102.7 (2.4)	101.8 (2.3)	101.4 (2.3)
Butabarbital	5	20	85.1 (12.9)	83.2 (14.1)	83.6 (14.0)
Butalbital	5	20	104.0 (4.6)	96.0 (4.4)	95.3 (4.7)
Pentobarbital	5	20	97.4 (1.8)	98.4 (3.1)	98.5 (2.5)
Phenobarbital	5	20	84.8 (14.8)	85.6 (12.9)	86.2 (12.1)
Secobarbital	5	20	105.7 (2.8)	102.9 (2.4)	102.7 (1.7)
Thiopental	5	20	93.5 (1.8)	91.4 (2.8)	91.0 (3.3)

Table 2. LOD, LLOQ, and mean % matrix effects (n=6) for barbiturate-spiked urine at low, medium, and high concentrations. The figures in brackets are %RSD.

	Interday accuracy % target			Interday precision %RSD		
	150 ng/mL	600 ng/mL	100 ng/mL	150 ng/mL	600 ng/mL	1200 ng/mL
Amobarbital	104.1	108.0	99.3	5.7	2.4	1.6
Butabarbital	103.3	106.0	101.0	5.6	2.1	2.6
Butalbital	103.4	108.3	99.9	5.8	2.1	3.3
Pentobarbital	102.5	108.4	101.9	4.9	1.7	1.6
Phenobarbital	103.3	106.8	98.4	5.3	2.3	3.2
Secobarbital	105.3	108.7	98.3	4.9	1.2	1.4
Thiopental	103.5	107.0	97.3	7.9	4.2	3.4

Table 3. Interday accuracy and precision (n=20) for barbiturate-spiked urine at three QC levels.

Analysis of authentic urine samples and quality control reference urine samples

A total of nineteen authentic urine samples, and five quality control reference urines were diluted and analyzed using UPLC/MS/MS, and the concentrations of detected barbiturates calculated. For positive identification of barbiturates in the UPLC/MS/MS method, the analyte retention time had to be within 0.2 min of the expected retention time. Phenobarbital was the only barbiturate present in the authentic urine samples and was detected in all nineteen samples of which seventeen results fell within the calibration range. A phenobarbital positive urine sample at 375 ng/mL is shown in Figure 2 with a negative control for comparison.

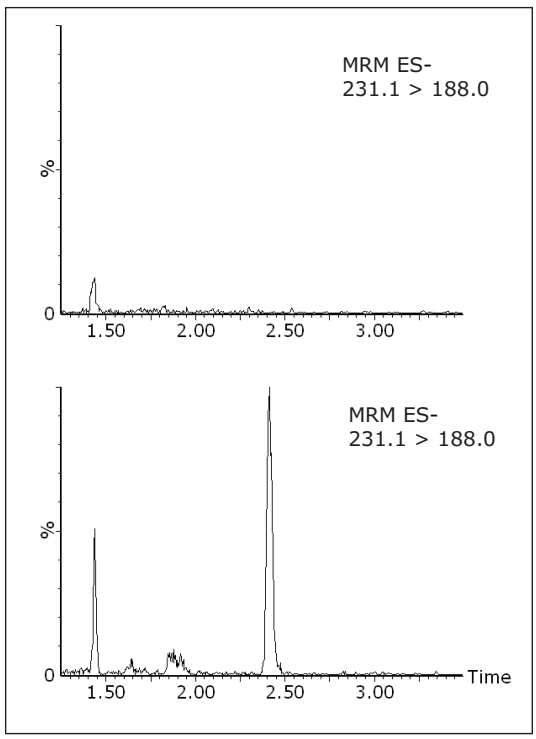

Figure 2. A positive result for phenobarbital at 375 ng/mL. The top trace is a blank urine calibrator and the bottom trace is the authentic sample.

These values were compared to those obtained at a separate laboratory using a liquid-liquid extraction, followed by derivatization and analysis by GC/MS. The correlation (r^2) between the two data sets was excellent, as shown in Figure 3. Amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital were found in commercial reference urines C2, C3, and C4; while secobarbital was the only barbiturate found in reference urines S1 and S2. The correlation between the UPLC/MS/MS data and the vendor's stated concentration by GC for the commercial reference urines was >0.9971.

Utilizing a simple sample dilution rather than a liquid-liquid extraction reduces the sample preparation time and utilizes smaller sample volumes, for example, 50 µL compared to the 1 mL required for the liquid-liquid extraction. Prior to injection, modern GC/MS methods require methylation of the barbiturates using trimethylanilinium hydroxide and ethyl acetate in the hot injection port of the GC. Derivatization of barbiturates is not needed for UPLC/MS/MS analysis, thus this step can be eliminated. The combination of these factors allows for higher sample throughput.

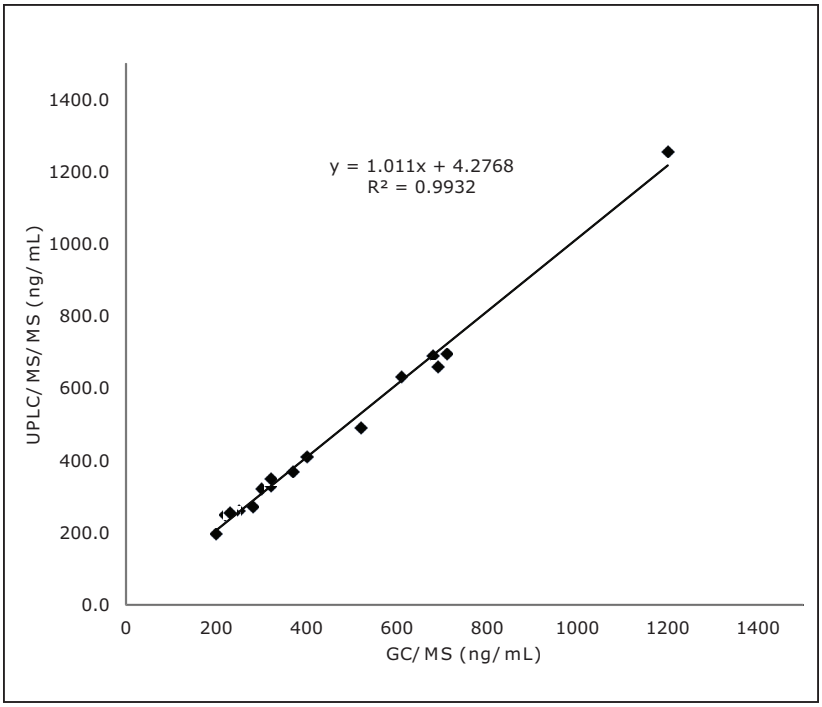

Figure 3. Comparison of GC/MS and UPLC/MS/MS analysis of phenobarbital.

CONCLUSIONS

Barbiturates need to be monitored in both forensic and workplace drug testing laboratories; therefore, an accurate, reliable, and robust method is needed to quantify these compounds in biological samples.

The Xevo TQ MS meets the sensitivity requirement for barbiturates in this particular matrix, without the need for a post-extraction concentration step. When analyzing barbiturates by UPLC/MS/MS, the use of a very simple sample dilution step eliminates both the liquid-liquid extraction and post-extraction derivatization steps that are required for GC/MS analysis. The elimination of the extraction step would reduce the time taken to prepare a typical batch of samples by more than 50%.

The 8.5-min ACQUITY UPLC System separation method run time is similar to the current GC methods for barbiturate analysis; therefore, when coupled with the simple sample dilution, it allows for high sample throughput.

UPLC/MS/MS showed excellent correlation with an alternative GC/MS method for the analysis of phenobarbital in nineteen human urine samples.

A full validation by the user would be necessary prior to adoption in a laboratory.

Acknowledgments

Concateno plc, London, UK for supplying the anonymized authentic urine samples.

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September 2014 720004466EN AG-PDF

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