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

Application Note

Forensic Toxicology

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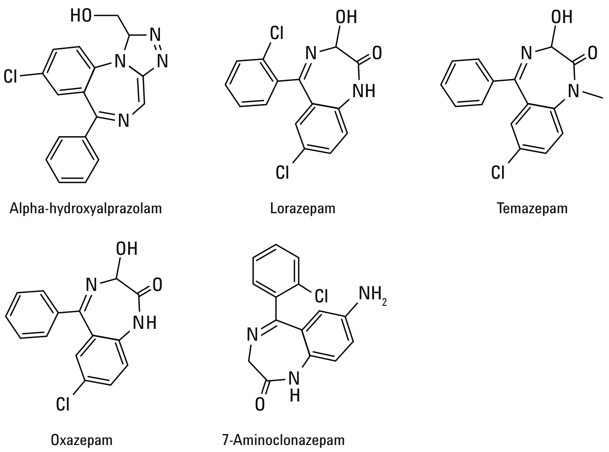
Abstract

The need for greater analytical capacity and throughput for the analysis of benzodiazepines in urine for forensic toxicology has placed demands on traditional analytical technologies. An ultrafast method for quantifying six benzodiazepines as a panel in urine was developed using an Agilent RapidFire High-throughput Mass Spectrometry system. Alpha-hydroxyalprazolam, lorazepam, temazepam, oxazepam, and 7-aminoclonazepam were accurately and precisely measured within a linear range of 50–12,500 ng/mL. All five analytes and a common internal standard, nordiazepam-D5, were simultaneously measured at 14 seconds per sample, providing a throughput of greater than 250 samples per hour.



Alpha-hydroxyalprazolam

Lorazepam

Temazepam

Oxazepam

7-Aminoclonazepam

Figure 1. Chemical structures of the five benzodiazepines in the panel.

Introduction

Traditionally, the quantification of benzodiazepines involves analysis by immunoassays followed by a confirmatory test by GC/MS and more recently by LC/MS assays. The steady increase in sample volume has led to the need for greater analytical capacity within forensic toxicology laboratories, placing a strain on traditional technologies. In the present study, we evaluated the ability of the Agilent RapidFire High-throughput Mass Spectrometry System, an ultrafast SPE/MS/MS system, to quantitate across different drugs of a benzodiazepine class (Figure 1) in urine at low ng/mL concentrations, with sample cycle times of under 15 seconds per sample.

Experimental

The RapidFire/MS/MS system consisted of the following modules: an Agilent RapidFire 365, an Agilent 6490 Triple Quadruple Mass Spectrometer, MassHunter Qualitative Analysis B.05.00, and MassHunter Quantitative Analysis B.05.00. Samples were analyzed at a rate of 14 seconds per sample. Analyte and internal standard ions were monitored simultaneously in all experiments for all five benzodiazepine drugs.

Chemicals and reagents

Alpha-hydroxyalprazolam, lorazepam, temazepam, oxazepam, 7-aminoclonazepam, and nordiazepam-D5 (1.0 mg/mL in methanol) were purchased from Cerilliant, Round Rock, TX. All other solvents and reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Table 1. RapidFire/MS/MS Conditions.

RapidFire conditions	
Buffer A	Water; 1.5 mL/min flow rate
Buffer B	10 % Acetonitrile; 1.25 mL/min flow rate
Buffer C	50:50 Methanol:Isopropanol + 0.1 % Formic acid; 1.25 mL/min flow rate
Back chimney wash	Water (aqueous), Methanol (organic)
Injection volume	10 µL
SPE cartridge	Agilent RapidFire cartridge A2 (reversed-phase C4 chemistry, G9205A)
RF State 1	sip sensor
RF State 2	3,000 ms
RF State 3	3,000 ms
RF State 4	5,000 ms
RF State 5	500 ms
Triple quadrupole conditions	
Gas temperature	200 °C
Gas flow	14 L/min
Nebulizer	45 psi
Sheath gas temperature	250 °C
Sheath gas flow	11 L/min
Nozzle voltage	500 V
Capillary voltage	3,500 V
Time filtering peak width	0.03

Table 2. MRM transitions.

Compound ID	Q1	Q3	Frag	CE	Dwell	CAV
Alpha-Hydroxyalprazolam	325.1	297.1	380	28	20	4
Alpha-Hydroxyalprazolam	325.1	215.9	380	44	20	4
Lorazepam	321	275	380	21	20	4
Lorazepam	321	229	380	35	20	4
Temazepam	301.1	255.1	380	29	20	4
Temazepam	301.1	177	380	45	20	4
Oxazepam	287.1	241.2	380	21	20	4
Oxazepam	287.1	104	380	36	20	4
7-Aminoclonazepam	286.1	222.1	380	25	20	4
7-Aminoclonazepam	286.1	121.1	380	33	20	4
Nordiazepam-D5 (IS)	276.1	165.1	380	31	20	3
Nordiazepam-D5 (IS)	276.1	140.1	380	31	20	3

Sample preparation

Pooled standard calibrators were prepared by spiking 12,500 ng/mL of each of the above mentioned drugs into drug-free human urine. Serial dilutions were used to achieve the remaining standard calibration concentrations. Fifty microliters of 1 M sodium acetate pH 5 containing the internal standard nordiazepam-D5 at 500 ng/mL was added to 200 µL of each sample and vortexed. Next, 10 µL of β-glucuronidase was added to each sample, vortexed and incubated between 55–60 °C for 2 hours. Post incubation, the samples were vortexed again and then centrifuged at 10,000 rpm for 5 minutes. Samples were then diluted 1:50 with water, transferred to 96 well-plates and centrifuged prior to injection on the Agilent RapidFire/MS system.

Data analysis

The peak integration was performed using the Agilent MassHunter Quantitative analysis software. The AUC of each analyte was normalized by the AUC of nordiazepam-D5 internal standard. The entire dataset was subjected to linear regression with 1/x weighting.

Results and Discussion

Samples were prepared by spiking benzodiazepines into drug-free human urine followed by an incubation with glucuronidase and then dilution with water (1:50). Samples were then analyzed by SPE/MS/MS using the RapidFire/MS/MS system and a reversed phase C4 cartridge at 14 seconds per sample. This RapidFire/MS/MS methodology is capable of throughputs greater than 250 samples per hour providing a high-throughput and very efficient analysis.

Pooled benzodiazepine standard curves containing each of the five benzodiazepines were analyzed separately to obtain intra- and interday precision and accuracy values. Intra- and interday accuracies determined for the benzodiazepine analytes were within 15 % and coefficient of variation values were all less than 10 % for concentrations within the measured range (Tables 3–8). The analytes had excellent linearity within the measured ranges with R^2 values greater than 0.99 (Figures 2–7). Signal suppression was not observed for any of the analytes.

Benzodiazepines were quantified between 50–12,500 ng/mL and were determined to have limits of detection (LODs) of less than 25 ng/mL. We recommend using a single blank solvent injection for concentrations higher than 6,250 ng/mL to minimize the potential for carryover. The addition of a blank injection after a highly concentrated sample increases total sample analysis time to approximately 28 seconds for that sample, which is still several fold faster than traditional LC/MS/MS methods.

This method consisting of a standard glucuronidase incubation and dilution for sample preparation followed by quick analysis on RapidFire/MS/MS provides a very efficient mode of quantitatively measuring benzodiazepines in urine compared to traditional LC/MS/MS methods.

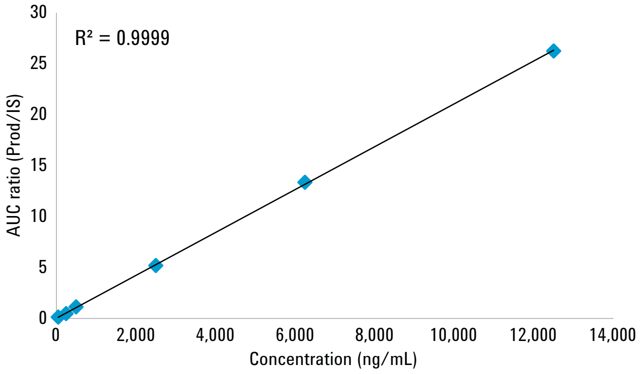


Figure 2. α-Hydroxyalprazolam representative standard curve in urine.

Table 3. Intraday and interday precision and accuracy for α-hydroxyalprazolam.

α-Hydoxyalprazolam ng/mL	Intraday % Accuracy (n=4)	Intraday % Precision (n=4)	Interday % Accuracy (n=4)	Interday % Precision (n=4)
50	101.88	6.84	103.30	8.74
250	95.34	5.68	93.15	4.85
500	101.58	2.45	102.56	5.27
2,500	99.31	5.69	101.50	8.55
6,250	99.24	7.21	100.21	9.14
12,500	103.20	4.40	104.14	5.98

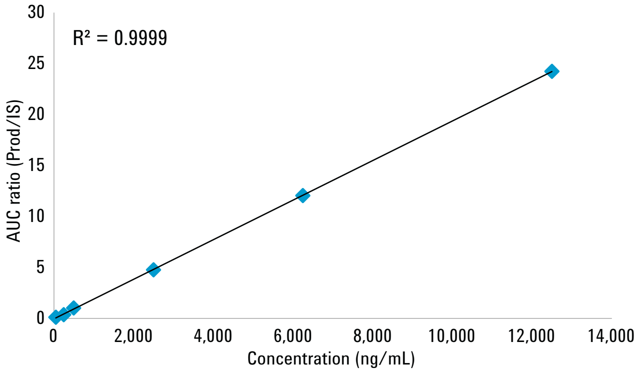


Figure 3. 7-Aminoclonazepam representative standard curve in urine.

Table 4. Intraday and interday precision and accuracy for 7-aminoclonazepam.

7-Aminoclonazepam ng/mL	Intraday % Accuracy (n=4)	Intraday % Precision (n=4)	Interday % Accuracy (n=4)	Interday % Precision (n=4)
50	108.11	8.02	106.00	7.62
250	89.22	5.37	87.66	3.58
500	99.56	6.30	103.02	8.11
2,500	96.18	6.37	98.67	8.92
6,250	104.04	1.17	103.16	7.18
12,500	105.84	3.65	106.39	5.68

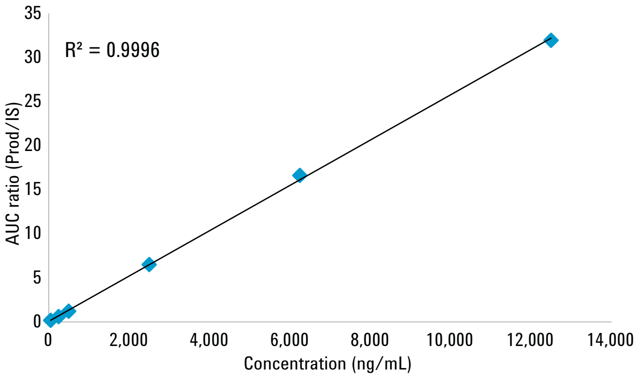


Figure 4. Oxazepam representative standard curve in urine.

Table 5. Intraday and interday precision and accuracy for oxazepam.

Oxazepam ng/mL	Intraday % Accuracy (n=4)	Intraday % Precision (n=4)	Interday % Accuracy (n=4)	Interday % Precision (n=4)
50	103.05	7.90	103.58	9.54
250	90.21	6.18	92.30	5.68
500	102.47	5.96	102.39	5.81
2,500	103.75	8.31	100.66	8.29
6,250	100.58	5.22	101.51	6.56
12,500	100.66	6.79	102.42	5.11

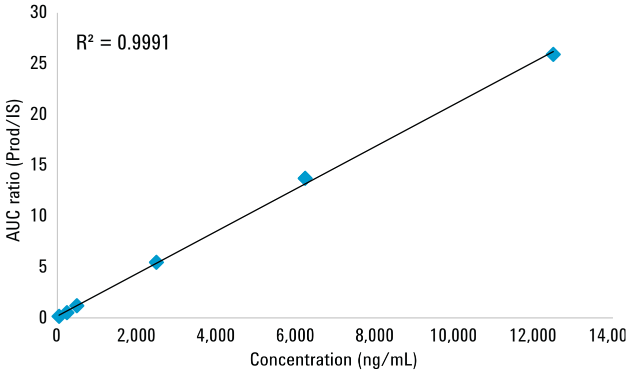


Figure 5. Lorazepam representative standard curve in urine.

Table 6. Intraday and interday precision and accuracy for lorazepam.

Lorazepam ng/mL	Intraday % Accuracy (n=4)	Intraday % Precision (n=4)	Interday % Accuracy (n=4)	Interday % Precision (n=4)
50	101.37	5.45	105.66	7.39
250	97.94	4.29	95.50	4.49
500	99.71	6.89	103.46	6.86
2,500	101.64	3.81	102.46	5.66
6,250	101.54	4.35	98.29	3.92
12,500	98.28	2.73	98.09	3.41

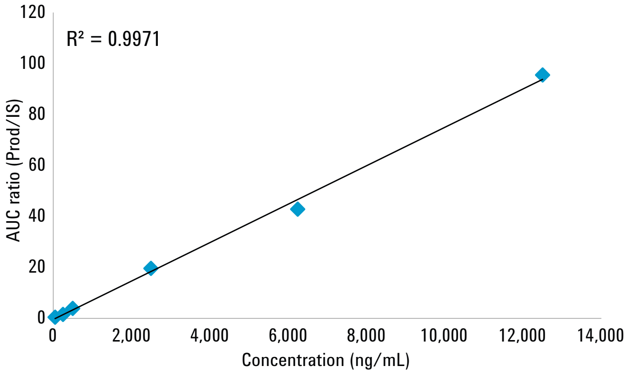


Figure 6. Temazepam representative standard curve in urine.

Table 7. Intraday and interday precision and accuracy for temazepam.

Temazepam ng/mL	Intraday % Accuracy (n=4)	Intraday % Precision (n=4)	Interday % Accuracy (n=4)	Interday % Precision (n=4)
50	104.09	4.55	105.37	3.82
250	96.18	4.44	93.71	4.36
500	96.66	6.35	100.14	6.00
2,500	101.35	4.60	102.81	6.70
6,250	102.75	0.79	99.40	4.24
12,500	100.49	2.52	101.66	3.75

Conclusions

Analytes from the benzodiazepine class were accurately and precisely quantified using an Agilent High-throughput RapidFire Mass Spectrometry System. Samples containing five benzodiazepine analytes were simultaneously analyzed at 14 seconds per sample, using a high-throughput analytical method of quantitation for these analytes that is capable of analyzing more than 250 samples per hour. This SPE/MS/MS methodology provides comparable results to LC/MS/MS, but at >10x the speed and efficiency of typical LC/MS/MS methods.

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