Application Note: 547

Quantitation of 14 Benzodiazepines and Benzodiazepine Metabolites in Urine Using a Triple Stage Quadrupole LC-MS System

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Key Words

- **TSQ Quantum Ultra**
- **Forensic Toxicology**

Introduction

Benzodiazepines have a broad range of therapeutic use and are widely prescribed as safe drugs for the treatment of insomnia, anxiety and seizures and for their amnesic effects prior to medical procedures. They are also abused for their psychoactive effects, in suicide and in drug-facilitated sexual assault. Simple, robust and precise analytical methods are needed to quantitate these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of 14 benzodiazepines and metabolites in urine. These compounds include: 2-hydroxyethylflurazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, α-hydroxyalprazolam, α-hydroxytriazolam, alprazolam, desalkylflurazepam, diazepam, lorazepam, midazolam, nordiazepam, oxazepam and temazepam.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Deuterated analog internal standards were used for all compounds except α-hydroxytriazolam and lorazepam. Isotopic contribution from the di-chlorinated parent interfered with the d4 internal standards. Deuterated α-hydroxyalprazolam and oxazepam, respectively, were used instead. After hydrolysis, methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subject to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 6.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

The timed selected reaction monitoring (T-SRM) was used. T-SRM allows the instrument to scan only for those compounds that are expected to be eluting at a certain time. The data for a particular target compound is acquired only in a short window around the known retention time, not throughout the entire run. Using T-SRM significantly reduces the number of SRM transitions that are monitored in parallel at a certain retention time. At a constant acquisition rate (cycle time) a significantly longer scan time (dwell time) is available for each transition resulting in higher sensitivity and lower quantitation limits, improved RSDs and more data points per chromatographic peak.

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low, middle and high end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve. Matrix effects were investigated by preparing samples in 8 different lots of human urine at twice the limit of quantitation (LOQ) of the method and monitoring peak area recovery compared to samples prepared in water.

Results and Discussion

The method is linear from 25 to 10,000 ng/mL with R^2 values > 0.99 for all 14 compounds (Figure 1). All calibrators back calculate to within 15% of nominal (20% for LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. The %CV was less than 10% for all QC levels.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Table 1 shows the matrix effect results.

Figure 2 shows an SRM chromatogram at LOQ.

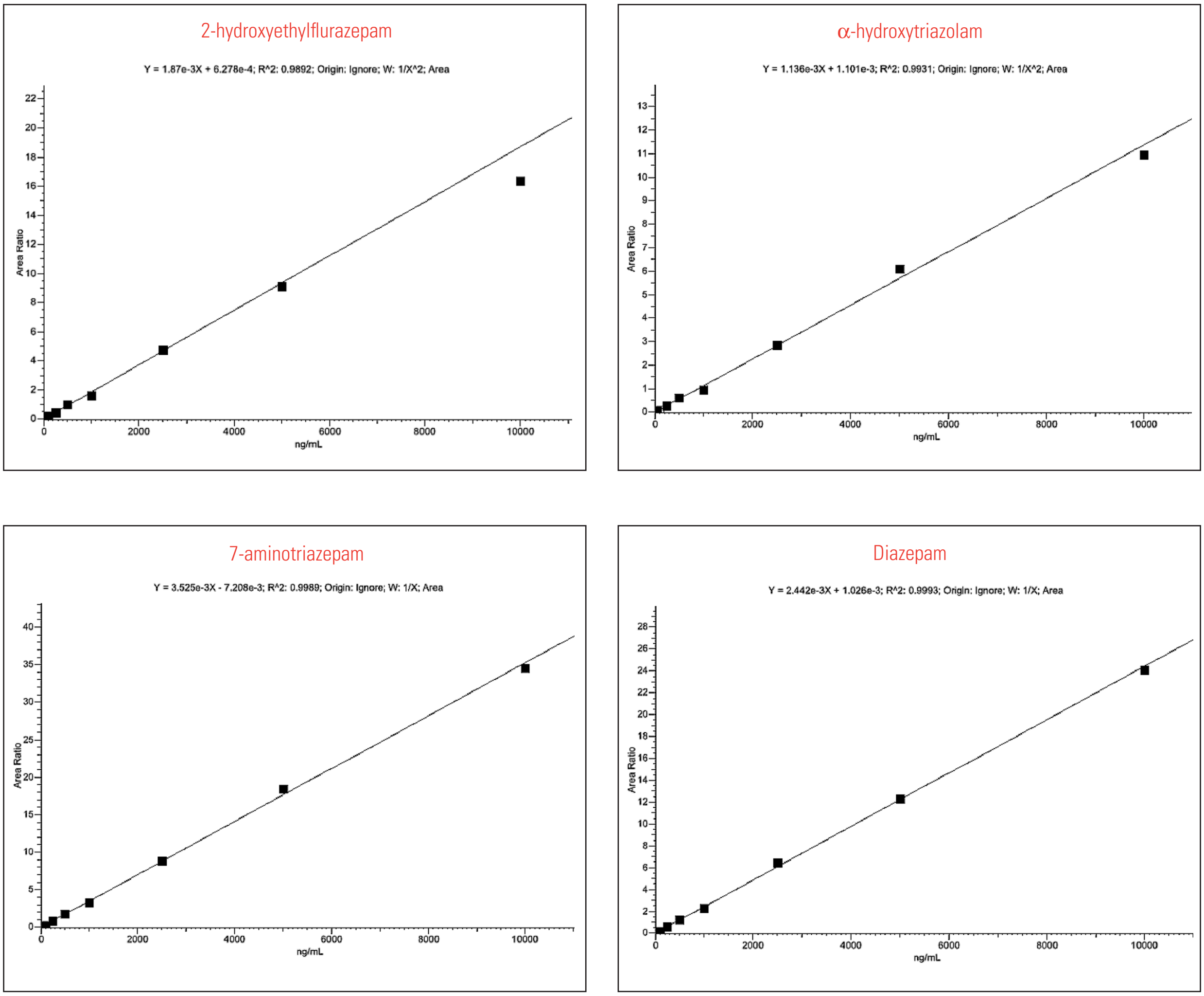

Figure 1. Representative calibration curves for some benzodiazepines showing linearity from 25-10,000 ng/mL in urine

Table 1. Percent recovery of 14 benzodiazepines in eight lots of urine

Compound	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F	Lot G	Lot H
2-hydroxyethyl-flurazepam	83.6	94.7	113	106	131	107	101	102
7-amino-clonazepam	90.9	92.4	93.1	90.0	95.5	98.5	92.0	92.2
7-aminoflunitrzepam	97.1	98.0	100	101	97.6	108	94.9	96.5
7-aminonitrazepam	88.9	99.6	94.9	101	94.0	101	96.5	89.3
α-hydroxyalprazolam	107	104	90.9	112	105	106	113	99.3
α-hydroxytriazolam	95.5	107	101	96.9	87.5	90.7	109	107
alprazolam	108	101	107	110	107	98.9	92.7	95.5
desalkylflurazepam	108	89.3	104	97.6	103	98.9	105	103
diazepam	105	102	113	106	105	111	89.3	103
lorazepam	104	93.1	94.9	95.8	91.1	94.4	108	107
midazolam	113	111	110	101	104	107	105	95.6
nordiazepam	112	99.3	112	109	98.4	109	95.6	102
oxazepam	96.4	91.5	96.7	96.9	92.0	99.3	95.1	96.0
temazepam	105	98.2	99.1	95.5	101	99.1	98.2	101

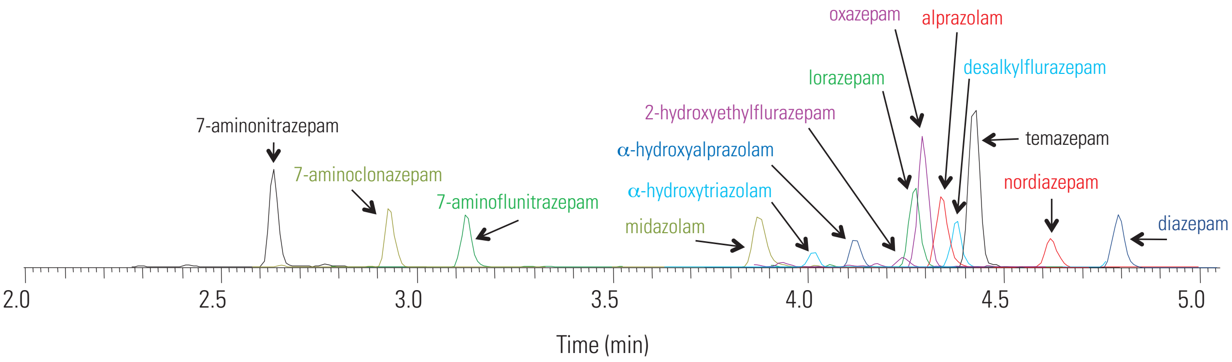

Figure 2. SRM chromatogram of 14 benzodiazepines and metabolites in urine at a concentration of 25 ng/mL

Conclusion

A robust dilute and shoot method with simple and easy sample preparation for the analysis of 14 benzodiazepines in 6.5 minutes was developed for forensic toxicology use. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Aria Transcend system. Multiplexing with the Transcend™ system would result in a run time of 3.5 minutes per sample.

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AN63475_E 09/11S

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