Application Note: 527

Screening of 20 Benzodiazepines and Four Metabolites in Whole Blood using UHPLC-MS/MS

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Key Words

- Psychoactive drugs
- TSQ Quantum Ultra
- T-SRM method
- Accela UHPLC System
- Forensic Toxicology

Introduction

Benzodiazepines have a broad range of therapeutic use and are widely prescribed as safe drugs with relatively few side effects for the treatment of insomnia, anxiety and epilepsy. However, they are also abused in cases of crime, suicide, and drug-facilitated sexual assault. These molecules are active at very low concentrations and some of them have very short half lives. For this reason, the analytical methods must show extensive specificity and sensitivity for forensic purposes. We have developed and validated a method for 20 benzodiazepines and four metabolites in whole blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with ultrahigh pressure liquid chromatography (UHPLC) pumps.

Goal

To present a rapid and quantitative forensic screening approach for the analysis of benzodiazepines in blood matrix using UHPLC conditions.

Experimental

Sample Preparation

Extraction was performed using a liquid-liquid extraction (LLE) procedure. After the extraction, the sample was evaporated to dryness and reconstituted with 100 µL of a mixture containing acetonitrile/5 mM ammonium formate pH3 (30/70).

HPLC Conditions

Chromatographic analyses were performed using the Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD 1.9 µm, 50 x 2.1 mm
Flow rate:	0.6 mL/min
Mobile phase A:	Water containing 5 mM ammonium formate, pH3
Mobile phase B:	Acetonitrile containing 0.1% formic acid

A gradient was performed starting from 95% of A to 95% of B in 6 minutes. The injection volume was 10 µL.

MS Conditions

Mass Spectrometer:	Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer
Source:	Heated electrospray ionization (HESI) mode
Ion Polarity:	Positive mode
Spray Voltage:	3000 V
Sheath/Auxiliary gas:	Nitrogen
Sheath gas pressure:	50 (arbitrary units)
Auxiliary gas pressure:	40 (arbitrary units)
Capillary temperature:	300 °C
Scan Type:	Selected reaction monitoring (SRM)
Q1, Q3 resolution:	Unit (0.7 Da FWHM)

Two SRM transitions were monitored for each component to provide ion ratio confirmations (IRC).

Results and Discussion

We validated a timed SRM (T-SRM) method for screening and quantifying 20 benzodiazepines and four metabolites. The run time was less than eight minutes, although most compounds eluted before four minutes. The T-SRM method allows the acquisition of an SRM transition only during a specified time window, not the entire run time. T-SRM divides the task into smaller batches by programming the instrument to look for each SRM only when it is expected to enter the instrument from an upstream LC system. Each time period is then optimized for the retention time of each compound. More time per transition results in better signal-to-noise (S/N) ratios or more scans per peak, allowing better quantitative data.

Standard spiking solutions of the analytes in porcine whole blood at concentrations of 5, 10, 50, 100, 300 and 500 ng/mL were prepared. All benzodiazepine calibration curves were evaluated using linear regression. Excellent linearity with a correlation coefficient of $R^2 > 0.99$ was obtained for each molecule. Seventeen were linear on the entire concentration range from 5 to 500 ng/mL. Six were linear from 10 to 500 ng/mL, and 3 were validated under linear conditions from 5 to 300 ng/mL. In all cases, the concentration range covered the therapeutic ranges.

Intra-method variability was calculated by processing five replicates of four calibration levels: the LOQ (limit of quantitation), two intermediate concentrations, and the maximum concentration. (%CV = coefficient of variance).

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Inter-method variability was determined by processing five replicates of four calibration levels in four different batches run on four different days. All values were below 15% and therefore within the guidelines set for a validated LC-MS/MS method.

Extraction efficiency also was evaluated and calculated at three concentration levels: 10 ng/mL, 100 ng/mL and 300 ng/mL. Values were between 50% and 100%, except for 7 amino-clonazepam which was around 30%.

The lower limit of quantitation (LLOQ) and the limit of detection (LOD) of the compounds were determined based on the calibration curve of S/N ratio versus concentration and the definitions of LOQ and LOD using S/N = 10 and 3. LLOQs were between 0.1 and 3 ng/mL for all molecules. Figure 1 shows the chromatogram obtained from a real sample acquired using the developed UHPLC-MS/MS method.

Conclusion

A rapid UHPLC-MS/MS method for quantifying benzodiazepines in whole blood samples was developed for forensic toxicology. The precision of the analysis meets current consensus guidelines. A T-SRM method was used to increase the acquisition time per compound and achieve better signal-to-noise ratios for the analytes.

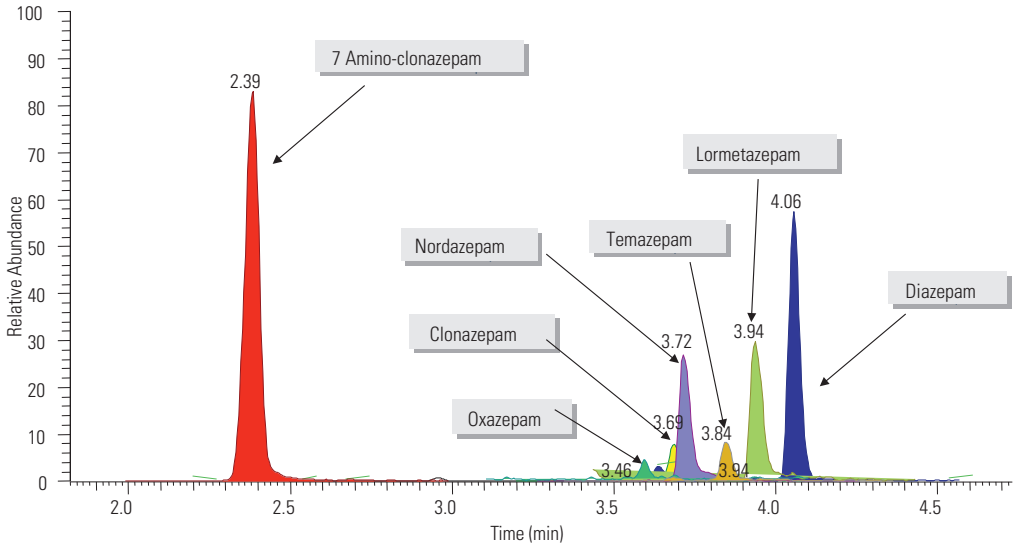

Figure 1. Chromatogram obtained from a real sample acquired using the T-SRM UHPLC-MS/MS method