Testing and Quantitation of Benzodiazepines in Urine and Serum by LC-TOF

Authors: Sharanya Reddy, Leslie Sullivan, George Perkins
PerkinElmer Inc, Shelton, CT

1 Introduction

The study presented here details the use of UHPLC-TOF mass spectrometry to detect and quantify a class of compounds known as benzodiazepams.

Screening of these compounds is done using immunoassays, which suffer from non-specificity and poor sensitivity requiring, additional confirmation with GC-MS assays. The GC-MS assays require derivatization of analytes which can be time consuming. However, LC/MS based techniques do not require derivatization. Among the LC techniques, LC/MS/MS is often used to quantitate benzodiazepams in biological fluids however, these assays are only suitable for targeted analysis.

2 Experimental conditions

A PerkinElmer Flexar™ FX-15 LC pump with a PerkinElmer AxION® 2 TOF was used for UHPLC separation and detection of the compounds. The separation was achieved on a PerkinElmer Brownlee SPP C-18, 2X50 mm, 2.7 µm column using a mobile phase gradient of water and acetonitrile containing 0.1% formic acid. The TOF was operated in positive mode.

Benzodiazepam standards were purchased from Cerilliant (Round Rock, TX, US). Calibration curves were set up for urine by spiking urine with varying concentrations of analyte compounds and diluted with methanol (1:1) and injected on column (5 µL). For serum samples, after spiking with varying concentrations of compound mixture, protein was precipitated with acetonitrile containing 1% acetic acid, centrifuged, supernatant diluted 1 to 2 with water and 10 µL injected on column.

3 Results

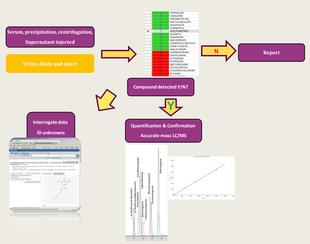

Fig. 1. Workflow for testing, identification and quantification of benzodiazepines in urine and serum

Testing

To rapidly identify the presence or absence of compounds in large batches of samples, AxION Solo™ software was used. AxION Solo provides quick visualization of the presence or absence of analytes in the samples (Figure 2). The presence of individual analytes can be coded with a specific color for ease of identification. The software identifies the presence of a compound based on accurate mass and isotope profile ratio as shown in Figure 3. In addition to searching against spectral information, the software can also search for target analytes based on user defined retention time windows which further improves the specificity of detection. The list of target analytes can be quickly and easily added to as previously unknown analytes are detected in samples. The analysis of benzodiazepines was completed in < 3 min (Figure 4) with all peaks eluting before 2.7 minutes.

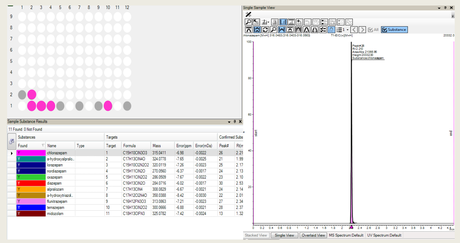

Fig. 2. Axion Solo Software: The top left hand corner shows the presence (pink) and the absence (grey) of chlonazepam in different samples (vials). The remaining benzodiazepines detected in the selected vial are displayed in the table (bottom left).

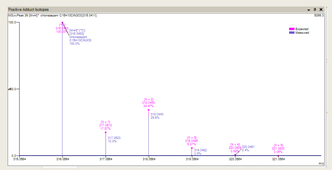

Fig 3. The accurate mass of chlonazepam for A, A+1, are < 2ppm. The isotope ratios for A+1, A+2 are within 5% of expected ratio

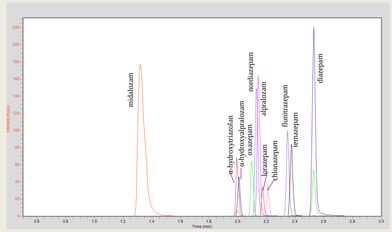

Fig. 4. Analysis of benzodiazepines by UHPLC-TOF MS spiked in urine < 3 mins.

Confirmation/quantification

The overall assay sensitivity was determined to be in the 1-10 ng/mL range for all of the compounds spiked into serum or urine, (Table 1). The limit of quantification (LOQs) measured by the TOF instrument were 20-200 times more sensitive than what is required by the non-specific EMIT immunoassays. When analyzing such low levels of compound carryover must be assessed to ensure that the assay is suitable for use. In spite of the low LOQs provided by the TOF MS, 0% carryover was observed after an injection of the upper limit of quantification (ULOQ) mixture of the benzodiazepine.

The linearity of an example compound, alprazolam is shown in Figure 5. The assay showed linearity over four orders with an r^2 value of 0.996. The majority of the benzodiazepines analyzed showed linearity between 3-4 orders of dynamic range with r^2 values of 0.99 (Table 2). Multiple injections (n=5) of each calibration level showed excellent reproducibility (RSDs< 15%) for each of the analytes. The presence of a given compound in a serum sample can be confirmed by accurate mass and isotope profile provided by TOF MS. The accurate masses of each of the benzodiazepines was < 3 ppm.

Analyte	Urine LOQ (ng/mL)	Serum LOQ (ng/mL)
diazepam	1	2
oxazepam	2	5
temazepam	2	5
alpralozam	2	2
flunitrazepam	2	5
chlonazepam	2	5
lorazepam	2	10
midazolam	2	5
nordiazepam	2	5
α-hydroxy alpralozam	2	5
α-hydroxy triazolan	2	

Table 1. Shows the LOQs of the benzodiazepines in urine and serum

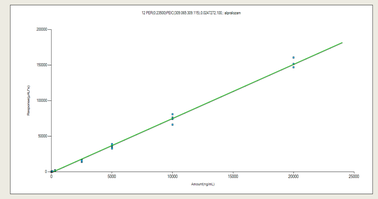

Fig. 5. Shows linearity for alpralozam spiked in serum over 2- 20,000 ng/mL concentration range(r^2 = 0.996).

Analyte	Concentration range (ng/mL)	r^2
diazepam	1-10,000	0.998
oxazepam	2-10,000	0.995
temazepam	2-10,000	0.996
alpralozam	2-10,000	0.995
flunitrazepam	2-10,000	0.997
chlonazepam	2-5,000	0.997
lorazepam	2-5,000	0.998
midazolam	2-2,500	0.999
nordiazepam	2-10,000	0.997
α-hydroxy alpralozam	2-5,000	0.997
α-hydroxy triazolan	2-10,000	0.991

Table 2. Shows the linear dynamic range and regression for each of the diazepams spiked in urine as matrix

4 Conclusions

1. Even in a challenging matrix such as urine or serum, the method required little to no sample preparation or method development, saving hours of time and the use of costly reagents and consumables.
2. The AxION 2 TOF was easily able to identify 1-10 ng/mL concentration of benzodiazepines spiked in urine or serum.
3. The detection limits of these compounds were 20-200 times lower than that required by immunoassays.
4. The AxION 2 TOF with the ADC detector technology provides wide dynamic range capabilities similar to that of a triple quadrupole mass spectrometer and also offers the screening of untargeted compounds.
5. For rapid large scale screening of batches of samples PerkinElmer AxION Solo software provides a quick and easy platform to detect the presence or absence of benzodiazepines.

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