APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Workflow for the Testing and Quantification of Benzodiazepines in Plasma Using UHPLC Time-of-flight Mass Spectrometry

Introduction

There is great need by forensic toxicologists and clinical researchers to develop robust analytical methods that accurately and quickly measure benzodiazepines in biological fluids including plasma in cases such as overdoses, sexual assault and fatalities.

Immunoassays have traditionally been used for screening of benzodiazepines but this approach can be challenging giving false positive results – needing confirmation by techniques such as GC/MS. Immunoassays are not always sensitive enough to detect low levels of the drug in matrices such as urine and blood. Also, the results obtained

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from immunoassays correlate to the total concentration of benzodiazepines ingested but cannot identify the specific benzodiazepines consumed by the user. GC/MS offers its own challenges in the analysis of benzodiazepines as most of the compound class are polar and often thermally labile thus requiring derivatization prior to analysis. Unlike GC/MS, LC/MS does not require time consuming derivatization of samples and is ideally suited for the rapid analysis of these compounds. Among the LC techniques, LC/MS/MS is often used to quantitate drugs of abuse in (non-clinical applications) biological fluids due to its sensitivity and selectivity.

We present an alternative technique to quantitate benzodiazepines in plasma utilizing a rapid protein precipitation method with a fast LC separation method in combination with time-of-flight mass spectrometry (TOF MS). The detection limits of benzodiazepines analyzed by the TOF were 20-100 times lower than those required by non-specific immunoassays. In addition to the wide quantitative dynamic range of the AxION® 2 TOF MS, which rivals capabilities of the triple quadrupole instruments, the TOF also provides full spectrum information which allows for detection of non-target compounds. Due to the variety of the illicit and abused drugs available and high incidence of drug abuse, it is vital that labs have an approach that is fast, yet generic in nature and not targeted.

In this application note we present a rapid workflow for the testing and quantification of benzodiazepines in plasma.

Experimental

A workflow for the testing and quantification of benzodiazepines is shown in Figure 1.

Calibration Curve(s)

To 250 µL of plasma, 500 µL of ACN containing 1 % acetic acid and varying concentrations of a mixture of benzodiazepine standards was added. The samples were voretxed, then centrifuged (10,000 RPM for 10 min). The supernatant (500 µL) was mixed with water (500 µL) and injected on column (5 µL). Each calibration level was injected five times.

LC conditions:

Pump:	PerkinElmer Flexar™ FX-15 UHPLC pump
Flow:	0.4 mL/min
Mobile phase A:	Water (0.1% formic acid)
Mobile phase B:	Acetonitrile (0.1% formic acid)
Gradient conditions:	20% B to 90% B in 4 min (linear gradient),
Injection volume:	5 µL in partial fill mode
Column:	PerkinElmer Brownlee™ SPP C-18, 2.1x50 mm, 2.7 µm (part number N9308402), 25 °C

MS conditions:

Mass spectrometer:	PerkinElmer AxION 2 TOF MS
Ionization source:	PerkinElmer Ultraspray™ 2 (Dual ESI source)
Ionization mode:	Positive

Internal calibration was performed using *m/z* 195.0876 and 622.02896 as lock mass ions.

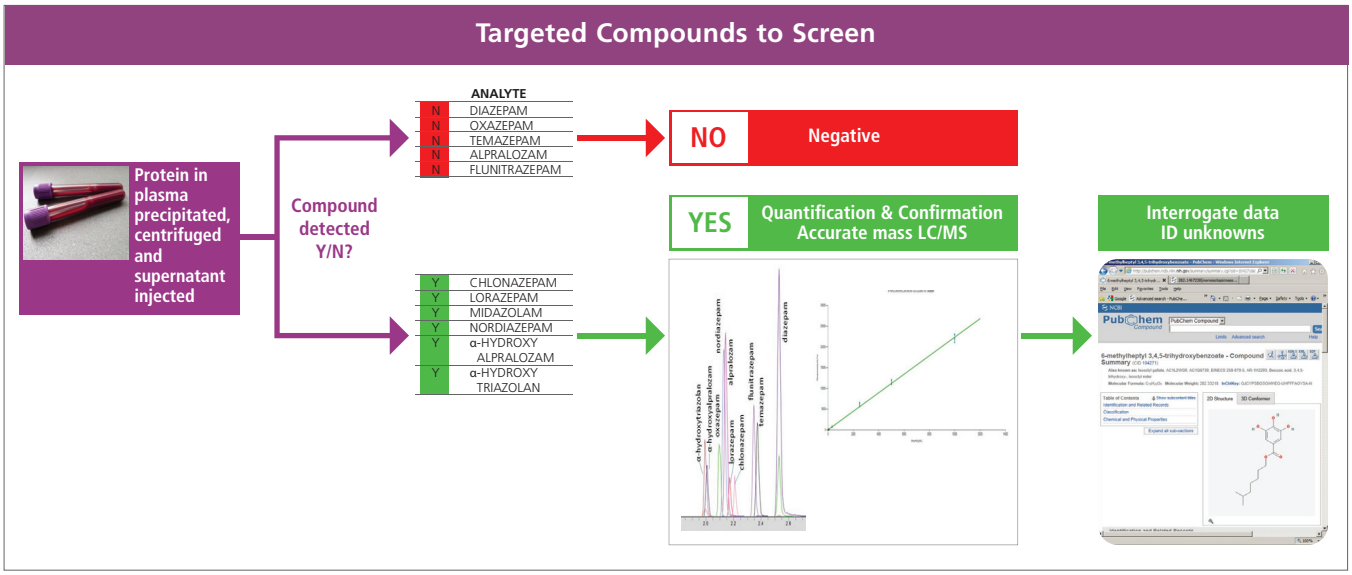

Figure 1. Workflow for testing, identification and quantification of benzodiazepines in plasma.

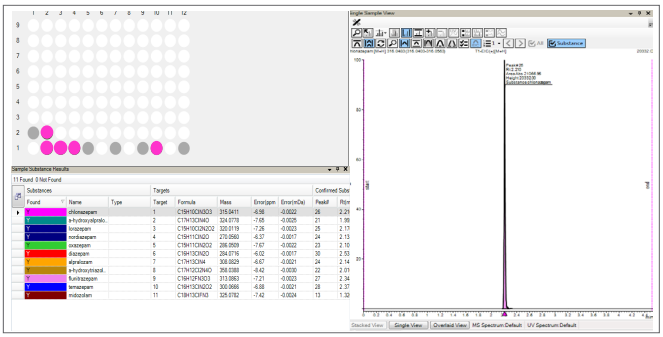

Figure 2. AxION Solo Software: The top left hand corner shows the presence (pink) and the absence (grey) of chlonazepam in different samples (vials). The remaining benzodiazepines detected in the selected vial are displayed in the table (bottom left).

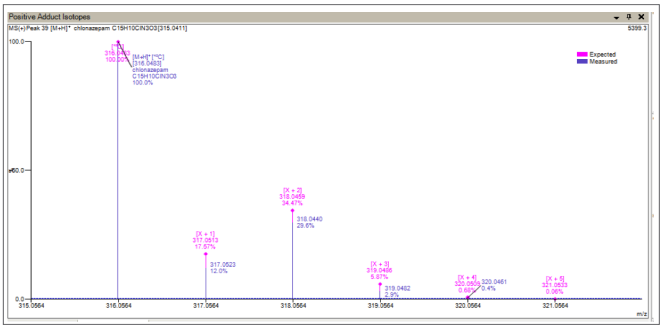

Figure 3. The accurate masses of chlonazepam for A, A+1 are within 2 ppm of expected. The isotope intensity ratios for A+1, A+2 are within 5% of expected.

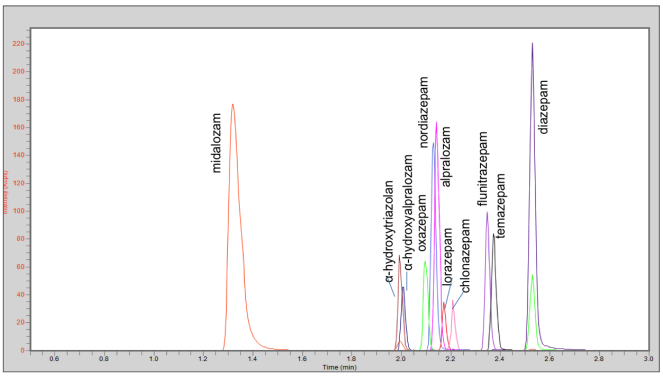

Figure 4. Analysis of benzodiazepines by UHPLC-TOF MS spiked in plasma < 3 mins.

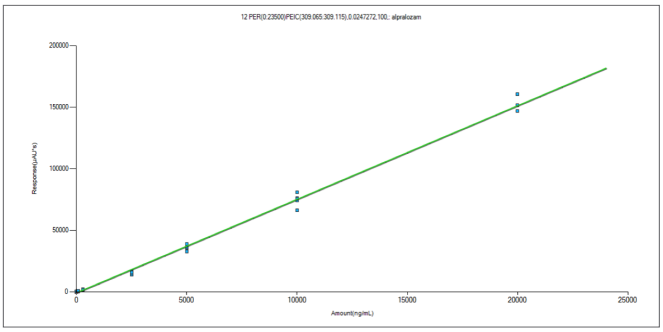

Figure 5. Shows linearity for alpralozam spiked in serum over 2- 20,000 ng/mL concentration range(r^2 = 0.998, n = 5 injections per level).

Results

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 compounds can be coded with a specific color for ease of identification. The software identifies the presence of a drug 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.

Confirmation/Quantification

The overall assay sensitivity was determined to be in the 2-10 ng/mL range for all of the drugs spiked into serum, (Table 1). The limit of quantification (LOQs) measured by the TOF instrument were 20-100 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 a representative drug, 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 drugs. The presence of a given drug in a serum sample can be confirmed by accurate mass and isotope profile provided by TOF MS. As shown in Table 3, the accurate masses of each of the benzodiazepines are < 3 ppm.

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

Analyte	LOQ (ng /mL)
Diazepam	2
Oxazepam	5
Temazepam	5
Alpralozam	2
Flunitrazepam	5
Chlonazepam	5
Lorazepam	10
Midazolam	5
Nordiazepam	5
α-Hydroxy Alpralozam	5
α-Hydroxy Triazolan	5

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

Analyte	Concentration Range (ng/mL)	r^2
Diazepam	2-20,000	0.992
Oxazepam	5-20,000	0.996
Temazepam	5-20,000	0.997
Alpralozam	2-20,000	0.996
Flunitrazepam	5-10,000	0.991
Chlonazepam	5-20,000	0.993
Lorazepam	10-20,000	0.998
Midazolam	5-10,000	0.998
Nordiazepam	5-20,000	0.997
α-Hydroxy Alpralozam	5-20,000	0.998
α-Hydroxy Triazolan	5-20,000	0.998

Conclusions

Even in a challenging matrix such as plasma, the method required minimal sample preparation or method development, saving hours of time and the use of costly reagents and consumables. The AxION 2 TOF was easily able to identify 2-10 ng/mL concentration of benzodiazepines spiked in serum. The detection limits of these drugs were 20-100 times lower than that required by immunoassays. The AxION 2 TOF provides wide dynamic range capabilities similar to that of a triple quadrupole mass spectrometer and also offers the screening of untargeted compounds. For rapid large scale screening of batches of samples, PerkinElmer AxION Solo software provides forensic laboratories with a quick and easy platform to detect the presence or absence of benzodiazepines.

Table 3. Shows the theoritical mass, observed mass and mass error of benzodiazepines.

Analyte	Theoretical Mass of Benzodiazepines	Observed Mass of Benzodiazepines	ppm Error	Structure
α-OH Triazolan	359.0461	359.0456	1.3 ppm	
α-OH Alpralozam	325.0851	325.0841	3.1 ppm	
Nordiazepam	271.0633	271.0636	-1.1 ppm	
Midalozam	326.0855	326.0848	2.1 ppm	
Lorazepam	321.0192	321.0184	2.4 ppm	
Chlonazepam	316.0483	316.0484	-0.3 ppm	
Flunitrazepam	314.0935	314.0928	2.2 ppm	
Alpralozam	309.0902	309.0896	1.9 ppm	
Temazepam	301.0738	301.0731	2.3 ppm	
Oxazepam	287.0582	287.0584	0.3 ppm	
Diazepam	285.0789	285.0789	0.0 ppm	

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