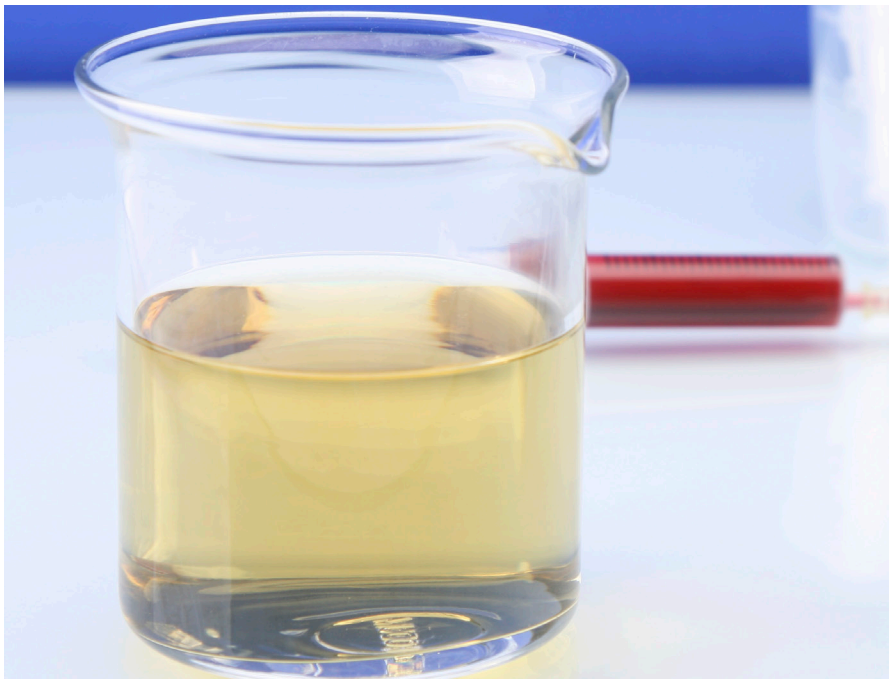

APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Workflow for Quantification of Benzodiazepines in Urine Using UHPLC- TOF

Introduction

There is a great need by forensic toxicologists to develop robust analytical methods to accurately and quickly measure benzodiazepines in biological fluids including urine in cases such as sexual assault and fatalities.

Immunoassays have traditionally been used for testing 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.

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GC/MS offers its own challenges in the analysis of benzodiazepines as most of the compound classes 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 (non-clinical applications) in biological fluids due to its sensitivity and selectivity.

We present an alternative technique to quantitate benzodiazepines in urine utilizing a rapid dilute and shoot LC method in combination with time-of-flight mass spectrometry (TOF MS). The detection limits of benzodiazepines analyzed by the TOF were 100-200 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 spectral information, allowing for screening of non-target compounds.

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

Experimental

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

Calibration Curve(s)

Urine (0.5 mL) was diluted with 0.5 mL of methanol containing varying concentrations of a mixture of benzodiazepines. 5 µL of the sample was injected on column. 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 over four minutes
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.

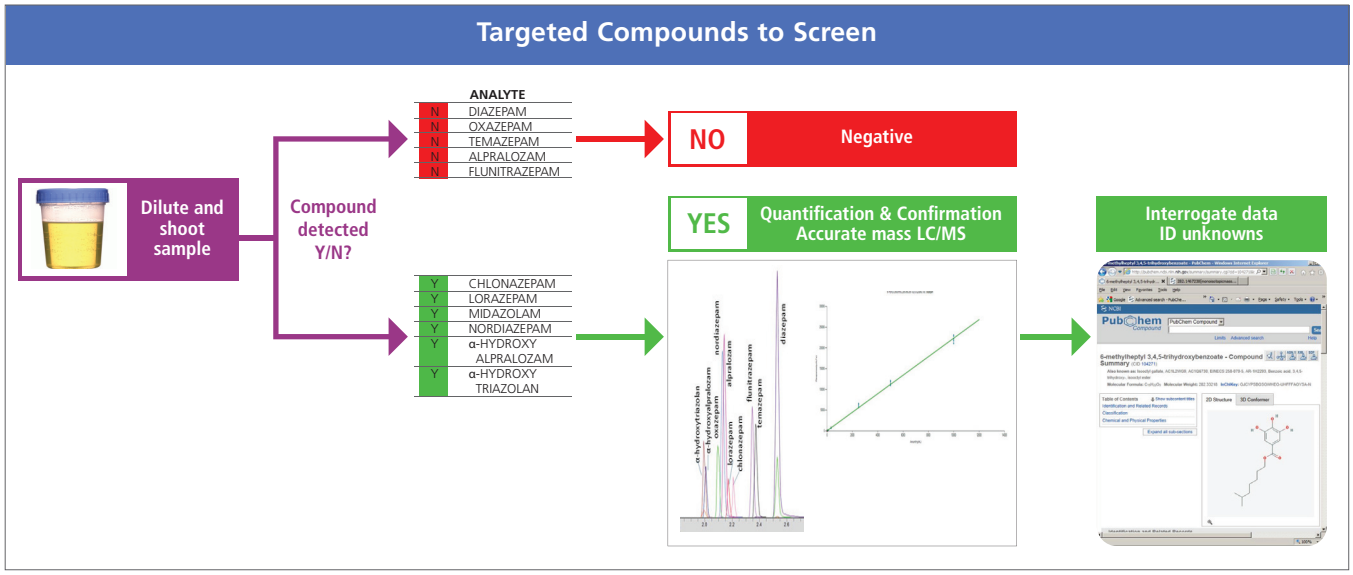

Figure 1. Workflow for identification and quantification of benzodiazepines in urine.

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 an individual compound 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.

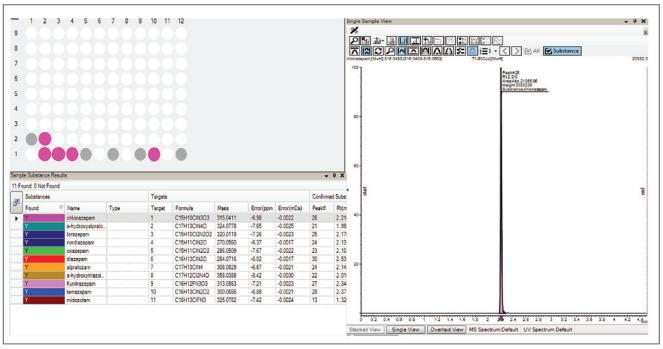

Figure 2. AxION Solo Software: The top left hand corner shows the presence (pink) and the absence (grey) of chlonazepam in different samples (vials). The remaining benzodiazepines detected in the selected vial are displayed in the table (lower left).

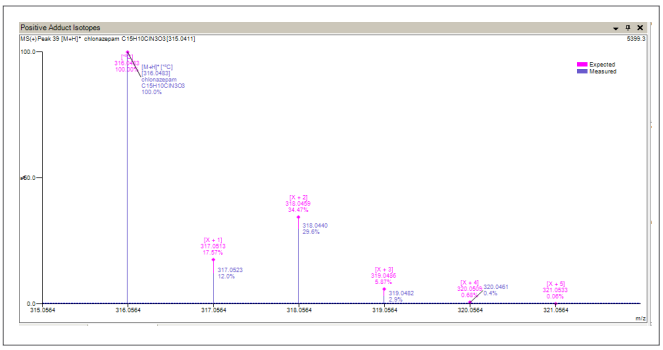

Figure 3. The accurate mass of chlonazepam for A, A+1, are < 2 ppm. The isotope ratios for A+1, A+2 are within 5% of expected ratios.

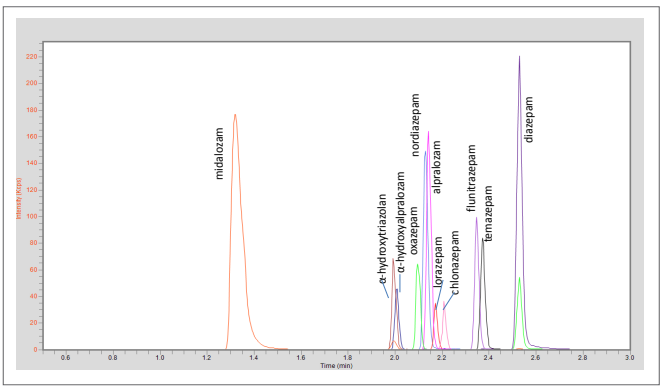

Figure 4. Analysis of benzodiazepines by UHPLC-TOF MS spiked in urine < 3 mins.

Quantification

The overall assay sensitivity was determined to be in the 1- 2 ng/mL range for all of the drugs spiked into urine, (Table 1). The limit of quantification (LOQs) measured by the TOF instrument were 100-200 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 benzodiazepines.

The linearity of a representative drug, diazepam, is shown in Figure 5. The assay showed linearity over four orders with an r^2 value of 0.998. 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 urine 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.

Analyte	LOQ (ng /mL)
Diazepam	1
Oxazepam	2
Temazepam	2
Alpralozam	2
Flunitrazepam	2
Chlonazepam	2
Lorazepam	2
Midazolam	2
Nordiazepam	2
α-Hydroxy Alpralozam	2
α-Hydroxy Triazolan	2

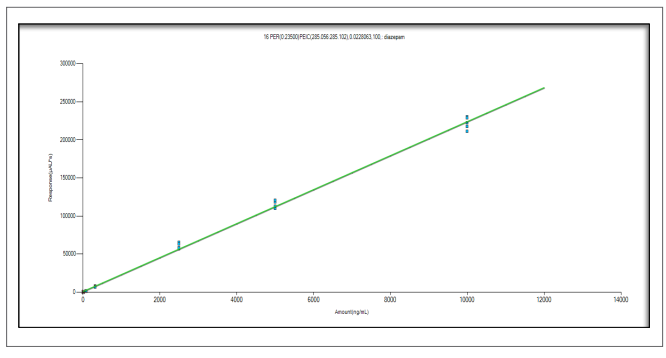

Figure 5. Shows linearity for diazepam spiked in urine over 1-10,000 ng/mL concentration range (r^2 = 0.998).

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

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

Conclusions

The method required little to no 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 1-2 ng/mL concentration of benzodiazepines spiked in urine. The detection limits of these drugs were 100-200 times lower than that required by immunoassays. 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 testing of untargeted compounds. For rapid large scale testing of batches of samples PerkinElmer AxION Solo software provides a quick and easy platform to detect the presence or absence of benzodiazepines.

Table 3. Shows the theoretical 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	359.0470	-2.5 ppm	
α-OH Alpralozam	325.0851	325.0856	-1.5 ppm	
Nordiazepam	271.0633	271.0640	-2.5 ppm	
Midalozam	326.0855	326.0848	2.1 ppm	
Lorazepam	321.0192	321.0185	2.2 ppm	
Chlonazepam	316.0483	316.0481	0.6 ppm	
Flunitrazepam	314.0935	314.0941	-1.9 ppm	
Alpralozam	309.0902	309.0898	1.3 ppm	
Temazepam	301.0738	301.0735	0.9 ppm	
Oxazepam	287.0582	287.0587	-1.7 ppm	
Diazepam	285.0789	285.0783	2.1 ppm	

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