Development of a Fast Extraction and LC/MS/MS Analysis Method for Benzodiazepines and Metabolites in Blood and Urine

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Benzodiazepines are widely used for anxiety and sleep disorders such as restless leg syndrome. These drugs are also often prescribed in conjunction with pain management medications, due to their ability to relieve pain caused by stress and anxiety. In collaboration with Northern Tier Research, Restek has developed a fast chromatographic method for the analysis of either extracted or diluted benzodiazepines. Both the chromatographic method and the extraction method have been developed to allow for fast analysis of a variety of benzodiazepines while at the same time maximizing column lifetime.
The compounds analyzed included in the chromatographic method were clonazepam, diazepam, lorazepam, flunitrazepam, triazolam, and 7-amino, desmethyl, and desalkyl metabolites. Analytes were spiked into blank blood and urine, the urine samples were enzymatically hydrolyzed, then all samples were and either diluted or extracted. The diluted samples were diluted 20:1 with mobile phase. The extracted samples were extracted using 130mg Clean Screen® Xcel I cartridges on a positive pressure manifold. The LOD for this method ranged from 0.1 ng/mL for high responding compounds such as flurazepam to 5 ng/mL for poor responding compounds such as clonazepam. Linearity was evaluated from 0.1 ng/mL to 1000 ng/mL. For extracted samples, recoveries ranged from 73% to 101%, with recovery of most compounds around 95%. Column longevity data was collected for both hydrolyzed urine that was extracted on the Clean Screen® Xcel I cartridges and for hydrolyzed urine prepared with a dilute-and-shoot method.

Sample Preparation and Analysis Method:

Sample Extraction (for extracted samples):

1. Hydrolysis Step
To 1-5 mL urine sample add 1-2 mL of 0.1M acetate buffer (pH= 5.0) containing 5,000 units/mL ß-glucuronidase.
Add appropriate volume and concentration internal standards.
Vortex and heat for 1-2 hours at 65 °C.
Allow sample to cool.

2. APPLYING SAMPLE TO COLUMN
Load sample directly to column without any preconditioning.
Pull sample through at a rate of 1-2 mL/ minute.
Dry column thoroughly under vacuum (10 mm Hg) or positive pressure (~ 80-100 psi) for 1 minute.

3. WASH
Wash sample with 1 mL of methylene chloride.
Dry column thoroughly under vacuum (10 mm Hg) or positive pressure (~ 80-100 psi) for a minimum of 5-10 minutes.

4. ELUTION
Elute samples with 1 mL ethyl acetate/ ammonium hydroxide (98/2) Evaporate fraction to complete dryness under stream of dry air or nitrogen at ~ 35 °C. Reconstitute samples with 100µL of mobile phase.

Sample Dilution (for dilute-and-shoot samples:
Prepare a 20:1 dilution of sample using mobile phase containing 30ng/mL internal standard. After dilution centrifuge samples for 5 min @ 10,000x g or 15 min. @ 3,000x g.

Chromatographic Method:
Column: 5µm Ultra Biphenyl 50mm x 2.1mm
Mobile Phase A: H_2O + 2mM Ammonium Formate + 0.2% Formic Acid
Mobile Phase B: ACN + 10% H_2O + 2mM Ammonium Formate + 0.2% Formic Acid
Flow Rate: 0.6mL/min
Injection Volume: 10µL for extracts and 30µL for dilute and shoot samples
Oven Temperature: 40°C
Gradient:

Time	%B
0.00	30
3.00	65
3.10	95
3.50	95
3.60	30
4.50	stop

Note: The gradient profile for this method was developed to optimize elution of the analytes of interest while keeping a short analysis time. All of the analystes will elute at 65%B, therefore after that time, the gradient is ramped quickly to rinse matrix off of the analytical column.

Results and Discussion: Validation Experiments

While linearity, precision, and accuracy were acceptable for both the extracted and diluted samples, the data from the extracted samples showed greater precision and accuracy, and the potential for lower LOQs (Table 1). Validation parameters were evaluated at a range of 0.1 – 1000ng/mL for diluted samples and from 10 – 1000ng/mL for extracted samples. The range for the extracted samples is representative of the range for routine forensic testing. The LOQs for the extracted samples were listed as 10ng/mL, however, analyte response indicates that the true LOQ of the method is considerably lower. A representative chromatogram is shown in Figure 1 and a response comparison between extracted and diluted samples is shown in the inlay below:

Figure 1. 50ng/mL Dilute-and-Shoot Hydrolyzed Urine Sample

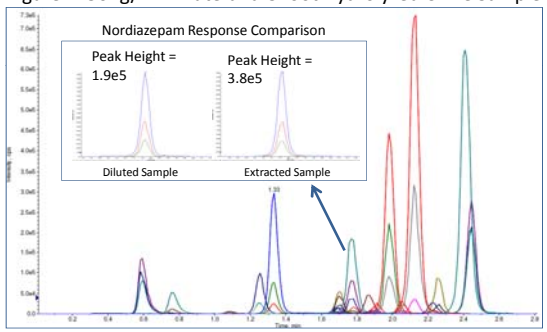


Table 1. Summary of Validation Data

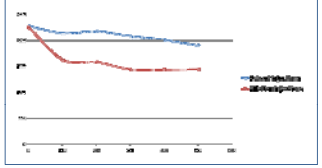
Sample Preparation Method	R^2 Range	LOD Range	Precision (All Compounds)	Accuracy (All Compounds)
Extraction	0.9977 - 0.9997	10*	0.4 - 16.4	80.4 - 114.0
Dilution (Blood)	0.9992 - 0.9997	0.1 - 5	0.2 - 29.7	80.8 - 119.7
Dilution (Hydrolyzed Urine)	0.9977 - 0.9995	0.1 - 5	1.1 - 27.3	87.4 - 174.0

Validation data was generated with triplicate injections of calibration curves and blanks. No LOD range is listed for the extracted samples because response for all compounds was more than acceptable at the 10ng/mL level. Full validation data, including transitions and ion ratios is available as a handout.

Results and Discussion: Lifetime and Reproducibility

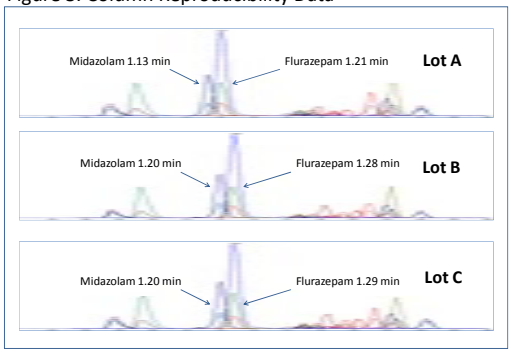
While a robust chromatographic method is necessary for routine drug analysis, column lifetime can be shortened when analyzing 'dirty' samples such as hydrolyzed urine. Guard columns can mitigate column degradation, but overall column lifetime can still be shortened by harsh matrices. An accelerated column lifetime comparison between extracted and diluted samples was conducted. One new column was subjected to repeated injections of a 20:1 dilution of hydrolyzed urine, and separate new column was subjected to repeated injections of extracted hydrolyzed urine. Injections were performed every 30 seconds, effectively exposing the analytical column to matrix at all times during testing. Column efficiency was measured every 100 injections. Over the course of 500 injections, both columns degraded significantly, however the column subjected to injections of hydrolyzed urine degraded approximately twice as fast as the column that was subjected to extract injections (Figure 2).

Figure 2. Column Lifetime Comparison



Column – to – column reproducibility was also evaluated during this project. A benzodiazepine standard was analyzed on three separate lots of Ultra Biphenyl columns. Maximum retention time drift between lots was 0.08 min. (Figure 3).

Figure 3. Column Reproducibility Data



In conclusion, a robust chromatographic method for benzodiazepines, coupled with a fast extraction method can serve to increase laboratory throughput and extend column lifetime.