Validation pre-face

Benzodiazepine analysis using LC/MS/MS
Waters Acquity UPLC H-Class System/ Xevo TQD System LC/MS/MS
[Serial Numbers: J14CHA622G (column housing unit), K14SDl251G (Sample Manager-FTN), K14QSMS01A (Quaternary Solvent Manager), QCA863 (Xevo TQD)] [DOJ #31401]
Toxicology Unit-Milwaukee
Written by Jonathan T. Tomko
December 8, 2022

Objective: This report briefly describes the work that was conducted as part of the validation of the new benzodiazepine assay. With any new method, method optimization is important and changing specific aspects of an experiment can led to methodology that may not be right for the laboratory or does not meet the desired specifications.

Initially, the primary focus of method optimization was on the instrument. A BEH C18 column and a BEH phenyl column of identical length, I.D. and particle size (100*2.1 mm, 1.7 µm) were evaluated to see which column would give better resolution, peak separation and acceptable chromatography. This was the early stages of development, so it was easy to evaluate this aspect of the method using the benzodiazepine spiking solutions that were used with casework. In general, both columns would have been acceptable for use in this new method, but benzodiazepines seemed to be better retained, and thus better separated from one another on the C18 column compared to the phenyl column.

The next components on the instrument to optimize is the mobile phase composition, gradient and additives. The literature showed that methanol and acetonitrile are both suitable organic solvents for separation and elution of benzodiazepines. Water is the aqueous solvent commonly used when performing reverse phase separations in liquid chromatography. When comparing methanol to acetonitrile for use in the mobile phase, the major difference is that the benzodiazepines eluted faster with acetonitrile. This would reduce run time on the instrument; however, the benzodiazepines do not have time to interact with the column to allow for adequate separation to occur. In addition, the currently validated method for buprenorphine and norbuprenorphine used methanol and water for the gradient so it was hard to justify the use of acetonitrile over methanol. In the future, acetonitrile can still be considered for mobile phase compositions and should not be eliminated for consideration during method development.

Several additives to the mobile phase could be considered too. Additives such as Trifluoroacetic acid (TFA), acetic acid, formic acid, acetate, formate, ammonia, ammonium bicarbonate, ammonium acetate, and ammonium formate could all be used for liquid chromatography with mass spectrometry detection. Based on the literature, it appears that ammonium formate, and formic acid were the most common additives for benzodiazepine analysis, but one article did use aqueous ammonia as their additive. A vital piece of knowledge is that precipitation of the buffer salt may happen with buffer additives without the proper precautions. Routine flushing of the column with LC grade water first, and then acetonitrile will help prevent precipitation of the buffer salt. However, one should assess if and why a buffer would

be needed for their analysis. In the end, the buprenorphine validated method used formic acid as an additive and several literature articles used formic acid as their additive for benzodiazepines analysis. Therefore, the use of formic acid as the additive was justified and effective for the analysis of benzodiazepines.

After selection of the column (BEH C18) and the mobile phases (methanol, water, and formic acid in water), the gradient needed to be optimized. After several injections of unextracted benzodiazepines, it was noticed that amino metabolites of benzodiazepines typically eluted off the column around 50% aqueous as compared to others which seemed to elute around 25% aqueous. With this knowledge, those percentages of aqueous were changed via trail and error to achieve the desired separation. In the end a gradient that runs a total of 9 minutes with benzodiazepines eluting sooner than 4.5 minutes was sufficient for separation.

With the gradient, mobile phases, and column optimized, it was time to assess different extractions. Review of the literature suggests that liquid-liquid extractions (LLE) and solid phase extractions (SPE) were viable options to explore. Benzodiazepines were extracted using both types, and both showed promising results. One difference between the two extractions was that the LLE was faster than SPE. With that in mind, further evaluation of an LLE was performed using different bases (sodium bicarbonate, sodium carbonate, borate buffer) and different organic solvents (ethyl acetate, and n-butyl chloride). No noticeable difference was observed when extracting benzodiazepines with the different bases. This made sense since the addition of base basified the sample matrix which allows benzodiazepines to be extracted using a suitable organic solvent. Next to assess was which organic solvent for the extraction to use. It is noted that the COVID-19 pandemic played a part in availability of chemicals, solvents, and other lab supplies. In addition, n-butyl chloride is an expensive solvent that is chlorinated and considered harmful to the environment. The extractions were repeated with one basifying agent (sodium bicarbonate) with ethyl acetate and n-butyl chloride. Again, both organic solvents showed promising results and effectively extracted the benzodiazepines from the sample matrix. Therefore, a liquid-liquid extraction using sodium bicarbonate and ethyl acetate was to be used for the validation of the new benzodiazepine method since the extraction was faster, and economical.

Validation experiments for calibration, bias and precision were carried out for the liquid-liquid extraction and seemed to be suitable for the needs of the laboratory. However, several discussions took place after the results were presented to others. An important note that was brought up is that the calibration range was smaller with this extraction when several literature articles had calibration ranges with higher upper limits of quantitation. Bias and precision studies showed that the extraction was reliable since bias and precision was below 20%. However, ion suppression and/or enhancement studies were showing that this extraction was not suitable. At the low concentration for this experiment there was considerable suppression (ranged from -3 to -50%) depending on the analyte. At the high concentration for this experiment, ion enhancement was ranging from 75 to 250%. Ion enhancement is the likely cause of the smaller calibration range when using this LLE as compared to other LLE or SPE methods that were presented in the literature. Other important notes that were brought up in discussion was that LLE does not produce a clean sample when comparing to SPE and this in turn would require more maintenance on the

instrument to maintain optimum performance. This ultimately means more instrument down time despite a faster extraction and analysis time. After further discussions with Waters Inc., the Milwaukee County Medical Examiner's office, and other colleagues within the toxicology community, it was best to not validate the liquid-liquid extraction. The data that was collected for this extraction was saved and stored within the unit validation documents. A SPE method for the analysis of benzodiazepines was experimented with and optimized to ensure it met the laboratory and accreditation requirements.