E96 Method Validation for the Detection of 22 Benzodiazepines, Including Clonazolam, Etizolam, and Flubromazolam, Using Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

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Learning Overview: After attending this presentation, attendees will understand the analytical method for the determination of benzodiazepines, including clonazolam, etizolam, and flubromazolam, in human whole blood.

Impact on the Forensic Science Community: This presentation will impact the forensic science community by demonstrating a reliable method for quantifying the target compounds by incorporating the analysis of emerging designer benzodiazepines into a previously accepted method for the analysis of classic benzodiazepines and their metabolites.

Benzodiazepines are a heterocyclic class of drugs used to treat a range of conditions, including anxiety, insomnia, and alcohol withdrawal.[1] In the United States between 1996 and 2013, the number of adults who filled a benzodiazepine prescription increased from 8.1 million to 13.5 million.[2] Benzodiazepine dosage also increased by more than triple during this period.[2] From 2002 to 2015, there was a 4.3-fold increase in the total number of deaths involving benzodiazepines.[2] The adverse effects of benzodiazepine abuse and addiction continue to be overlooked, most likely because 75% of deaths involving benzodiazepines also involve an opioid.[2] Designer benzodiazepines are structural analogs of controlled benzodiazepines that have been synthesized to mimic the pharmacological effects.[3] Clonazolam, etizolam, and flubromazolam are three designer benzodiazepines that have recently been observed in South Carolina casework. These drugs are marketed online as "research chemicals" and "not for human consumption." Given the increase of designer benzodiazepines being shipped to the United States with very similar molecular formulas and structures to already scheduled benzodiazepines, there is a pressing need for confirmatory methods that can differentiate between analogous compounds.

Samples (1.0mL) were prepared for analysis using Solid Phase Extraction (SPE) with an elution solvent of ethyl acetate containing 3% ammonium hydroxide on Clean Screen® 10mL DAU columns. Chromatographic separation was achieved on a Liquid Chromatograph Tandem Mass Spectrometer (LC-MS/MS) system (Agilent® Technologies® 1290 LC coupled with an Agilent® Technologies® 6430 triple quad mass spectrometer) using positive electrospray ionization and dynamic multiple reaction monitoring mode with a UCT Selectra® DA column (50mm x 2.1mm, 3µm). Solvent A was 0.1% formic acid in Dionized (DI) water and solvent B was 0.1% formic acid in methanol. Mobile phase was introduced in a gradient programmed with 35% B, isocratic for one minute that was increased to 70% B over 7.5 minutes, increased again to 95% B for 1.6 minutes before the gradient was returned to the initial conditions and held for the remaining 1.8 minutes. The flow was set to 0.4mL/min with a total run time of 11 minutes.

The method was developed to be a sensitive assay with optimal run time. The following studies were all conducted in concurrence with the Scientific Working Group in Toxicology (SWGTOX) guidelines: bias, precision, calibration, dilution integrity, carryover, limit of detection, limit of quantification, and stability. Interference and ionization suppression/enhancement studies are currently in progress. The designer benzodiazepines had a defined limit of quantitation at 10ng/mL and limit of detection at 5ng/mL. The method was stable up to 72 hours and free from carryover at 7.5 times the highest calibrator. For the three designer benzodiazepines, within-run precision ranged from 3.6% to 8.2% for the Lower Limit Of Quantification (LLOQ) at 10ng/mL and 1.4% to 3.0% for the high control at 600ng/mL. For the low and high controls respectively, the between run precision ranged from 4.8% to 8.2%, and 2.7% to 4.7%, and the accuracy ranged from -5.7% to -0.56% and -2.2% to -0.6%.

Reference(s):

1. National Center for Biotechnology Information. *PubChem Compound Database*; CID=134664. [Internet]. Accessed July 10, 2018. https://pubchem.ncbi.nlm.nih.gov/compound/134664.
2. Bachhuber, Marcus A., Sean Hennessy, Chinazo O. Cunningham. Increasing Benzodiazepine Prescriptions and Overdose Mortality in the United States, 1996–2013. *American Journal of Public Health*, 2016 April; 106(4): 686–688.
3. Moosmann, Bjoern, Leslie A King, and Volker Auwärter. Designer Benzodiazepines: A New Challenge. *World Psychiatry*, 2015 June; 14(2): 248.

Benzodiazepines, Designer, LC/MS/MS