K74 Validation of the Neogen® Enzyme-Linked Immuno-Sorbent Assay (ELISA) Benzodiazepine Kit Using Clonazepam as the Target Molecule for Blood and Urine

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Learning Overview: After attending this presentation, attendees will understand: (1) the effectiveness of the Neogen® ELISA Benzodiazepine kit for screening whole blood and urine specimens using clonazepam as the target molecule, and (2) the challenges with following the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology Laboratories.

Impact on the Forensic Science Community: This presentation will impact the forensic science community by demonstrating the validation of an ELISA method for the screening of a broad range of benzodiazepines.

Objective: The validation of a semi-quantitative method for the rapid screening of whole blood and urine specimens by a Dynex DSX® Automated ELISA System using the Neogen® Benzodiazepine kit and clonazepam, rather than oxazepam, as the target molecule.

Method: Neogen® Benzodiazepine kit assay instructions for incubation times and reagent volumes were followed. The amount of sample added was increased from the recommended 10µL to 20µL. Whole blood samples were diluted 1:5 with Neogen® EIA buffer offline. Urine samples were diluted 1:10 with Neogen® EIA buffer online by the instrument. Performance of the assay was evaluated at one decision point for each matrix: 10ng/mL in whole blood and 25ng/mL in urine. An in-house validation protocol based on the SWGTOX standard practices was followed for the validation of the assay, which included the evaluation of sensitivity, precision, specificity, carryover, drift, ruggedness/robustness, and a case sample comparison.

Results: The theoretical Limit Of Detection (LOD) for clonazepam was calculated to be 2ng/mL in blood and 8ng/mL in urine. The experimental LOD for clonazepam was determined to be at least 5ng/mL in blood and 10ng/mL in urine. Precision was evaluated using the mean of three replicates from five separate runs (n=15) at the decision point and at concentrations +/-50% and +/-100% of the decision point. Although the method was optimized, and precision was demonstrated at each level (coefficient of variation < 7.6%), the current SWGTOX validation requirements for a valid decision point were not fulfilled. Furthermore, the urine matrix did not meet the proposed revision of the SWGTOX requirements for determining a valid decision point promulgated by the Toxicology Subcommittee of the Organization of Scientific Area Committees for Forensic Science. Cross reactivity was observed with all 29 low- and high-dose benzodiazepines analyzed. If the cross reactivity of a compound was less than 100%, then the precision for the detection of that compound was evaluated. No carryover or drift was observed. The method proved to be both rugged and robust. Case sample comparison results were comparable to those obtained when the samples were initially screened using oxazepam as the target molecule. One false negative by both the original ELISA with oxazepam and reanalysis with clonazepam was identified by confirmation with liquid chromatography/tandem mass spectrometry.

Conclusion: The Neogen® Benzodiazepine kit using clonazepam as the target molecule exhibited high cross reactivity for 29 different low- and high-dose benzodiazepines and demonstrated excellent precision and sensitivity in both whole blood and urine, making it an efficient and reliable method to screen blood and urine specimens for benzodiazepines, even though it did not fulfill current SWGTOX validation requirements for a valid decision point.

Validation, ELISA, Benzodiazepine