K6 A Mass Spectrometric Approach to the Analysis of Covalent Modifications of Blood Proteins by Drugs of Abuse

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After attending this presentation, attendees will better understand *in vitro* formation of covalent protein modifications formed by reactive drug metabolites, as well as the Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS) analytical approach required for detection of these adducts.

This presentation will impact the forensic science community by demonstrating that covalent protein adducts formed *in vitro* provide the necessary framework for an *in vivo* detection method under development for the retrospective detection of drugs of abuse in human blood.

Hemoglobin (Hb) and serum albumin (SA), two prevalent proteins in human blood,- contain unbound cysteine thiol moieties, creating a nucleophilic site with the potential for covalent modification by reactive chemical species. These covalent modifications, called "adducts," are stable entities that accumulate during acute and chronic exposure and remain covalently bound for the life-span of the protein. Despite their current use as exposure markers for a variety of compounds, the use of adducts in assessing exposure to drugs of abuse has not yet been explored. The goal of this work is to examine the *in vitro* adduct-forming capability of selected drugs of abuse with Hb and SA to provide additional proof of principle for the development of a real-world detection and monitoring analysis method. Use of protein adducts as biomarkers of drug exposure will allow for an increased window of detection, from several days to several months, as compared to current blood analysis methods. The drugs examined in this study cover a wide range of abused drugs, including cocaine, methamphetamine, and Δ^9-THC, and have all been shown in previous work in the laboratory to form adducts with glutathione and/or other thiol-containing peptides.

For this research, a new assay procedure was created to facilitate recovery of modified protein by combining published methods with existing methodology used in the lab. The new assay utilized a dialysis membrane to maintain separation of proteins of interest from the microsomal components, while allowing for small molecules (i.e., stable and reactive metabolites) to pass through, resulting in a decrease in the number of steps required to extract the modified protein of interest. For the metabolism/adduction assay, each drug was added to a plastic microfuge tube with residual solvent removed via vacuum centrifuge. Human liver microsomes were added to the tube and combined with Nicotinamide Adenine Dinucleotide Phosphate (NADPH) in the presence of a regeneration system containing glucose-6-phosphate and glucose-6 phosphate dehydrogenase, in sodium phosphate buffer (pH 7.4). The protein of interest was then added; the tube was incubated at 37°C for 18h, then centrifuged. An aliquot of supernatant was removed and added to a clean LC/MS vial for analysis. Instrumental analysis of modified protein was performed using positive Electrospray Ionization (ESI) on an Agilent® 1290 Infinity® Ultra High-Performance Liquid Chromatography (UHPLC) coupled to an Agilent® 6530 MS and chromatographic separation utilized an Agilent® ZORBAX® Rapid Resolution HD Eclipse® Plus C8 column. Data were collected using full MS scan mode, to allow for necessary analysis of all protein components. The mobile phases used were as follows: (1) water with 0.1% trifluoroacetic acid; and, (2) 95% acetonitrile, 4.9% water, 0.1% trifluoroacetic acid. The total run time was 16 minutes with a 2-minute post-run for column re-equilibration. Initial analysis of MS data obtained was performed using Agilent's® MassHunter™ Qualitative Analysis software, followed by MassHunter™ BioConfirm software for protein deconvolution and proteomic analysis of adducts formed.

The use of protein adducts for retrospective drug detection represents a novel and useful advance in drug testing and analysis. The characterization of covalent adducts formed *in vitro* shown in this research provides the necessary framework for future examination of a more complete set of abused drugs. The confirmation and subsequent analysis of these covalent protein adducts reinforces the need for the development of a real-world applicable method to screen for drug exposure utilizing a longer window of detection than is currently available for most drugs and matrices.

Protein Adducts, Drugs of Abuse, LC/MS

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