K15 Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS) -Based Analysis of *In Vitro* Covalent Modifications of Glutathione (GSH) and Peptide Thiols by Drugs of Abuse

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After attending this presentation, attendees will better understand whether reactive drug metabolites are likely to form adducts with GSH and other thiol-containing peptides. Attendees will also understand the LC/MS/MS analytical approach required for detection of these adducts.

This presentation will impact the forensic science community by demonstrating the potential of important drugs of abuse and/or their reactive metabolites to covalently bind to thiol-containing peptides in an in vitro assay, as models for in vivo protein adduction.

The human blood proteins hemoglobin (Hb) and serum albumin (SA) have free thiol groups that can be covalently modified by reactive chemicals or their metabolites. Such reaction products ("adducts") are stable entities that accumulate during chronic exposure. The possible application of protein adducts as long-term exposure markers to facilitate forensic toxicological detection of drugs of abuse has not been explored. This work examined the capability of various abused drugs, including morphine, cocaine (COC), methamphetamine, naltrexone (NAL), methylenedioxymethamphetamine (MDMA), Δ^9-tetrahydrocannabinol (THC), buprenorphine, and methylenedioxypyrovalerone to form adducts with GSH *in vitro*. In addition to GSH, an N-acetylated thiol-containing peptide (Ac-PAACAA) was used as a model to confirm the potential of these drugs and/or metabolites to covalently bind to protein thiol residues. Acetaminophen (APAP) and clozapine (CLZ), both of which have been previously reported to covalently adduct proteins *in vitro* and *in vivo*, were also included as positive controls.

For the metabolism/adduction assay, each drug was added to a plastic vial with residual solvent removed via vacuum centrifuge. Human liver microsomes (HLM) were combined with NADPH in the presence of a regeneration system containing glucose-6-phosphate and glucose-6 phosphate dehydrogenase, in sodium phosphate buffer (pH 7.4). Contents were pre-incubated for 15 minutes at 37°C, followed by addition of GSH or peptide. The vial was reincubated at 37°C for 3 hours and then centrifuged. A 100µL aliquot of supernatant was removed and added to a clean LC/MS vial for analysis.

Instrumental analysis of GSH assay products was performed using negative ESI on an Agilent 6460 LC/QqQ/MS and an Agilent Zorbax Rapid Resolution HD Eclipse Plus C18 column was used for separation. The mobile phases used were as follows: (a) water with 0.1% acetic acid; and (b) 95% acetonitrile, 4.9% water, 0.1% acetic acid. The total run time was 14 minutes with a 2-minute post-run for column re-equilibration. Instrumental analysis of peptide assay products was performed by flow injection analysis (FIA) using positive ESI on an Agilent 6460 LC/QqQ/MS.

MS analysis successfully identified multiple stable metabolites and GSH adducts of the test drugs. Several previously unreported peaks were identified as adducts of GSH with metabolites of the test drugs, including *m/z* 482 for APAP, 470 and 481 for MDMA, and 648 and 616 for CLZ. The GSH adducts for several drugs, including COC,

NAL, and THC detected in this study are the first such reported for these drugs. Covalent adduction to the Ac-PAACAA peptide was observed for the majority of drugs tested, with eight of the drugs showing high potential for adduct formation. MS/MS data confirmed the identity of the major peak for each drug as the drug-peptide adduct. The mass difference between the adducted and unadducted peptide corresponded to the molecular mass of the drug or a metabolite, minus a proton lost by the bond formed between the drug and the cysteine thiol.

Demonstration of the capability of these drugs to covalently bind to thiol residues *in vitro* represents a critical first step in assessing their protein binding capabilities. Further studies are underway to determine if such adducts can be detected in human blood protein *in vivo* and therefore employed as long-term biomarkers for exposure to drugs of abuse.

Toxicology, Metabolite Adducts, LC/MS/MS