Document Title:	**Novel Blood Protein Modification Assay for Retrospective Detection of Drug Exposure – Development and Validation**
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Document Number:	**305942**
Date Received:	**February 2023**
Award Number:	**2017-MU-BX-0002**

Federal Award Number: 2017-MU-BX-0002

Project Title: Novel Blood Protein Modification Assay for Retrospective Detection of Drug Exposure – Development and Validation

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Project Period: 01/01/2018 – 12/31/21

Award Amount: $467,885

Summary of the Project

Major goals and objectives: Detection and confirmation of human exposure to drugs typically relies on measurement of parent compounds or specific metabolites in blood, urine, or an alternative sample matrix. This “biomonitoring” approach is widely employed for forensic toxicological applications and has been standardized for many xenobiotics. However, with the exception of highly lipophilic compounds, most drugs and their metabolites are cleared from these matrices within a week. As a result, blood or urine measurements alone generally cannot provide data on past episodic exposure, cumulative exposure, or time-dependent exposure profiles for drugs. Nevertheless, such data may be critically important in forensic toxicology, for example as evidence in drug facilitated crimes and for measurement of drug compliance or abstinence in pain drug management, addiction rehabilitation programs, and probation/parole criminal justice situations.

Currently, retrospective biomonitoring of drug use or exposure is limited to analysis of hair, for which numerous methods and a large literature database exists. While clearly useful for this purpose, hair analysis does have technical and interpretive challenges. Another potential technology for longer-term monitoring of drug exposure involves measurement of the products of covalent modification of free thiol moieties of blood proteins, such as hemoglobin (Hb) and serum albumin (SA), by reactive metabolites (RM) of drugs. Since they typically persist for the life of the protein, such protein “adducts” can provide a much longer window of detection of exposure than is generally possible by direct measurement of parent compound or a metabolite. While widely used in human exposure assessment for environmental and occupational

chemicals, applications of protein adducts as markers of illicit drug exposure are virtually nonexistent.

Our previous NIJ funded proof-of-concept study (2015-NE-BX-K001) demonstrated *in vitro* modification of glutathione by RM of 16 drugs selected from various physicochemical and pharmacological classes, confirming the basic feasibility of this approach. In the present project, we conducted additional research to support further development of a robust blood protein adduct detection technology for retrospective monitoring of drugs of abuse as an alternative or complement to hair analysis. We examined additional *in vitro* assay systems for identifying both stable metabolites (SM) and RM of a variety of drugs and characterized their ability to target a specific reactive thiol group within Hb that could be employed as an analytical target for assessing retrospective drug exposure.

Research questions: The first part of this project was designed to detect and characterize thiol modifications of human Hb and SA by RM of selected drugs following *in vitro* incubation in a human liver microsome (HLM) based assay. This task included confirmation of covalent protein modification and LC-QTOF-MS based structural analysis of thiol adduction products. The primary research question was to assess which specific protein adducts for each drug could be identified as targets to monitor in an LC-QqQ-MS based MRM screening/confirmatory assay. Subsequently, in order to further improve detection and identification of potential drug-protein adducts to ultimately target, two additional *in vitro* approaches to generation of SM and RM, *i.e.,* electrochemical oxidation and biomimetic chemical catalysts, were added to the project.

For this *in vitro* work, two thiol containing target molecules, glutathione (GSH) and a relevant tryptic peptide containing the reactive Hb β^{93}Cys moiety (*i.e.,* GTFATLSELH^{93}CDK), were utilized.

In the second part of the project, a technique for enrichment of adducted protein species by selective removal of unadducted protein for enhancement of assay sensitivity and selectivity was explored. The specific question addressed here was whether a published method for enrichment of adducted SA could be adapted to Hb, as this had not previously been reported in the literature. These experiments were conducted to further facilitate the goal of eventual development of a sensitive and selective LC-QqQ-MS MRM based targeted adduct screening/confirmatory method.

The third planned phase of the project was to finalize an LC-QqQ-MS method and to screen of a set of authentic blood specimens from drug users for the presence of the targeted drug modifications in SA and Hb, as a final proof-of-principle. However, due to study delays primarily associated with pandemic issues, these experiments could not be completed during the project period but are the subject of continuing work.

Research design, methods, analytical and data analysis techniques:

Drugs examined in the project - Specific drugs to be investigated were chosen to provide a range of licit and illicit abused compounds with consideration of previous data generated in the PI laboratory and other published work. In addition, the well characterized adduct-forming drugs acetaminophen and clozapine were utilized as positive controls to ensure active mechanisms necessary for biotransformation-induced adduct formation. Table 1 summarizes the drugs under study and experiments

conducted for each.

In vitro metabolism/peptide trapping assays - Relevant *in vitro* assays are widely employed by the pharmaceutical industry and basic researchers to assess the extent and rate of xenobiotic metabolism and examine the formation of both SM and

Table 1: Drugs under study and assays conducted.

Drug	Assay					
	HLM Metabolites	**EC Metabolites**	**BMO Metabolites**	**GSH Trapping**	**β^{93}Cys Peptide Trapping**	**Hb Protein Trapping**
APAP	X	X	X	X	X	X
Clozapine				X		X
Methamphetamine	X	X	X	X	X	X
MDMA	X	X	X	X	X	X
Δ^9-THC	X	X	X	X	X	X
Oxycodone						X
Cocaine				X	X	X
Diazepam						X

X = Assay performed for that drug.

RM of lead drugs.[1,2] RM can often be identified by the presence of stable hydrolysis products. Alternatively, the models can be extended to provide assessment of the covalent binding potential of RM by the inclusion of nucleophilic “trapping” molecules such as GSH and synthetic peptides containing Cys, Lys, or other nucleophilic moieties.[3-6] The most common *in vitro* model systems utilize whole human hepatocytes or microsomes (HLM) as the primary contributor of Phase I metabolic activity.[7,8] Less explored alternatives include electrochemical (EC) oxidation assays and synthetic metalloporphyrin catalysts that mimic cytochrome P450 activity to generate SM and RM.[9,10]

In vitro trapping assays utilized GSH and a custom synthesized peptide containing

the reactive Hb β^{93}Cys moiety (*i.e.,* GTFATLSELH^{93}CDK), that is generated following tryptic digestion of Hb. Before performing an incubation with the drugs of abuse using the Hb β^{93} Cys peptide, an incubation with iodoacetamide (IAM) was done to confirm site of adduction and to provide a positive control with stoichiometric modification of the cysteine thiol. For this synthesis, 0.1 mg/mL of β^{93}Cys peptide and 6 mM of IAM were prepared in 25 mM ammonium bicarbonate buffer, pH 7.4. Incubation then ensued at 37°C for 1 h in the dark. After the incubation period, the samples were analyzed using the LC-QTOF-MS in FIA mode.

An HLM based *in vitro* assay already in place in the PI's laboratory for generation and analysis of SM and RM of drugs of abuse (Figure 1A) was optimized for selective covalent modification of GSH and β^{93}Cys peptide by drug RM. We previously reported the use of this model to investigate adduct formation by nitrogen mustards and other xenobiotics.[11,12] The assay components included the drug of interest, HLM to initiate P450-mediated metabolism, and an NADPH regenerating system to generate SM and RM. For assessment of covalent modification potential by RM, the thiol-containing target peptide was also added. Negative controls, in which NADPH or drug were omitted from the incubation mixture, were also prepared. All controls were run in parallel with positive samples and processed and analyzed in the same manner.

The EC oxidation assay (Figure 1B) was performed on an eDAQ ER 466C Integrated Potentiostat System. The electrode system used consisted of glassy carbon as the working electrode (WE), calomel standard as the reference electrode (RE) and Pt/Ti as the counter electrode (CE). For all the EC experiments, a 1 mg/mL solution of drug was prepared in 100 mM ammonium bicarbonate (AmBic) buffer, pH of 7.4. Initial

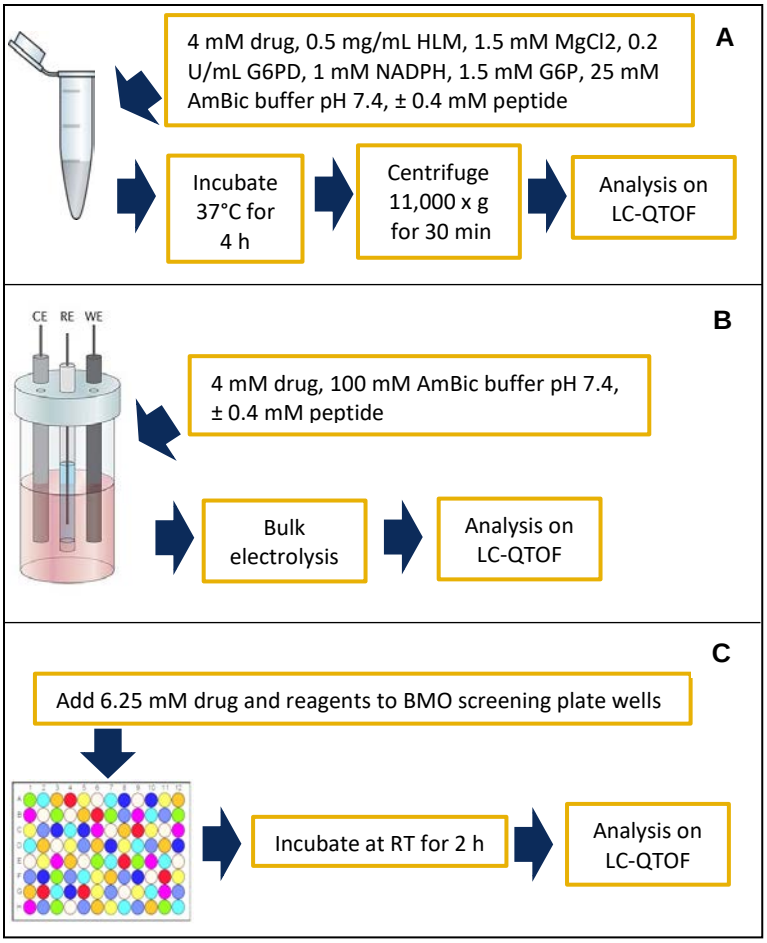

Figure 1: *In vitro* metabolism assays based on A) HLM, B) electrochemical oxidation, and C) biomimetic catalysts.

studies were conducted to determine the ideal voltage for the oxidation of each drug using cyclic voltammetry. Then a bulk electrolysis was performed, where a two-compartment cell vial was used with 2 mL of the working solution in one compartment with the W.E. and buffer solution in the second compartment with the C.E. and R.E. The solutions were maintained under magnetic stirring for the entire duration of the experiment. The oxidation voltage was fixed with the ideal voltage for each drug and a bulk electrolysis was performed, with aliquots being collected at 5, 10, 20, 30 40, 50, and 60 min to determine the ideal oxidation time. Finally, the aliquots collected were

analyzed on the LC-QTOF-MS/MS system (see below).

For the synthetic metalloporphyrin catalysts approach (Figure 1C), commercial biomimetic kits (BMO kit; HepatoChem, Inc., Beverly, USA) were employed. In the first step, a BMO “screening kit” with 50 different reaction conditions and two different reaction solvents was utilized. Optimal conditions for metabolite generation were determined by analyzing the variety and yield of metabolite formation for each condition. For further analysis with selected compounds, a BMO “optimization kit” was also employed, where the optimal reaction conditions found with the screening kit were further optimized. For this purpose, 12 additional reaction conditions were tested. Instructions provided with the BMO screening kit and optimization kits were followed. Optimal conditions for metabolite generation were determined by analyzing the variety and yield of metabolite formation for each condition.

The drug solution was prepared with two solvents provided with the kit at a concentration of 6.25 mM; then the drug solution and provided reagent solutions were added to the 96 well plate, each well of which contained a different mixture of catalyst reagents. After all the components were added, the plate was incubated at room temperature on a shaker for 2 h. Then, 2 µL aliquots were collected from each well and transferred to LC vials containing 200 µL of acetonitrile. The diluted samples were then ready to be analyzed by LC-QTOF-MS/MS to determine the most effective one to produce the desired metabolites.

For some of the biomimetic catalyst studies, MetaSite software was also employed to predict the metabolism of the drugs under study and potential RM. MetaSite predicts Phase I metabolic conversions via CYP450 and FMO biotransformation. The software

allows the user to choose the site of metabolism (*e.g.,* liver, skin, brain) and specific CYP and FMO enzymes. This allows accurate prediction of metabolites formed by assigning liver as the metabolic pathway in the software. The chemical structure of each drug is input to the software and a prediction of hotspots and metabolites is performed using both metabolic enzyme systems. The predictions made are then be compared with the *in vitro* experimental results to allow a broader understanding of potential stable and reactive drug metabolites.

In vitro protein trapping assay - For studies of specific modification of the Hb β^{93}Cys moiety by RM of the selected drugs, the trapping assay previously developed for peptide modification was adapted for use with intact protein. Figure 2 shows details of the assay, where Hb was utilized as the trapping agent with various concentrations of drug. Centrifugal filters were used to remove assay components and collect protein, which was then subjected to an enrichment assay (see below) to selectively remove unadducted protein. Adducted Hb was then either directly analyzed by LC-QTOF-MS (top-down analysis) or digested with trypsin for peptide mapping (bottom-up analysis) to identify β^{93}Cys peptide modifications.

Enrichment of adducted protein - Sepharose 4B thiol affinity resin was prepared according to manufacturer protocol as a slurry solution of 75% elution buffer and 25% settled resin. The elution buffer consisted of 0.1 M Tris HCl, 0.5 M NaCl, pH 7.4. Thiol resin was incubated at 2°C for 4 h to hydrate. Stock solutions of 2 mg/mL Hb, 150 mM NEM, 0.2 mg/mL $CaCl_2$, and 0.5 mg/mL trypsin were prepared daily. All stock solutions

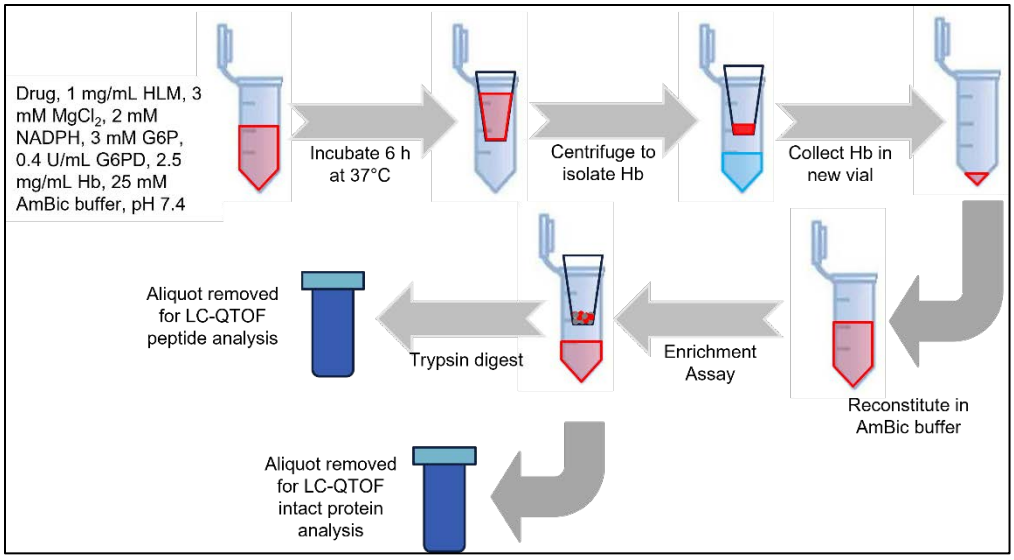

Figure 2: Enrichment/trapping assay for Hb adduction studies.

were prepared in a 25 mM ammonium bicarbonate buffer (AmBic) at biologic pH 7.4.

To generate Hb with stoichiometric modification of β^{93}Cys for use as a positive control, control Hb was treated with the thiol modifying reagent N-ethylmaleimide (NEM). Aliquots of stock Hb and NEM solutions were incubated for 1 h at 37°C. After incubation, the positive covalent adduct control was serial diluted in fresh AmBic buffer to a concentration of 0.4 mg/mL. Each test mixture was then generated by adding aliquots of unadducted Hb control and the Hb-NEM modified protein to create mixtures with various molar ratios of control and modified Hb. Equal volumes of the test mixture and hydrated Sepharose 4B thiol affinity resin were then added to a 1 mm cellulose acetate spin filter, briefly vortexed, and incubated for 18 h to trap unadducted Hb. Samples were then centrifuged for 15 min at 15,000 x g. After centrifugation, the flow through fraction containing thiol-adducted Hb was transferred to a 3 kDa molecular weight cutoff filter (MCWF) for a series of buffer exchange into fresh AmBic buffer. Aliquots were split to two vials for whole protein and tryptic peptide analysis, respectively.

For tryptic digestion, supernatant was collected and transferred to a clean 3 kDa spin filter. Protein was isolated and reconstituted in a 1 mg/mL $CaCl_2$ buffer at pH 7.4. Protein sample was then incubated for 18 h at 37°C with an aliquot of 0.5 mg/mL sequencing grade trypsin solution. Once the incubation period was complete the sample was processed in a 3 kDa spin filter for 30 min to remove trypsin digestion enzymes. Peptides in the filtrate were collected and added to clean LC vial for LC-QTOF-MS and MS/MS analyses.

LC-QTOF-MS analyses and data processing - High sensitivity and selectivity were identified as being crucial to success of this study and for the ultimate use of this approach in forensic toxicological applications. Quantitative data on blood protein modifications induced by RM of typical drugs of abuse in human subjects are not currently available, except for the special case of acetaldehyde adducts in Hb from ethanol consumption. However, based upon published determinations for licit drugs and other xenobiotics, adduct levels are typically in the high ppb to low ppm range (*i.e.,* 1 adduct per 10^5-10^8 protein molecules).[5,13-15] For example, acetaminophen adducts in SA induced by the reactive quinone imine metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI) were estimated to be in the range of 60 ppm in subjects exhibiting drug-induced hepatotoxicity.[16]

Critical instrumentation utilized in the PI's lab for the project included an Agilent 1290 Infinity UHPLC coupled to an Agilent 6460 Triple Quadrupole MS with Jet Stream ESI technology and diode array detector and Agilent 1290 Infinity UHPLC coupled to a model 6530 QTOF MS with Jet Stream ESI technology, both running version B.06.00

Mass Hunter Software. The QTOF instrument was capable of high resolution (20,000) and high mass accuracy (<5 ppm), making it ideal for HRMS based proteomics. The QqQ instrument performed both dynamic and triggered MRM at high sensitivity, with up to 10 transitions for each analyte.

Mass spectrometric analyses of metabolites and adducted GSH and β^{93}Cys peptide products formed in the *in vitro* assay systems were performed on an Agilent 1290/6530 liquid chromatography/quadrupole time-of-flight (QTOF) mass spectrometer, with a Dual Jet Stream Electrospray Ionization (ESI) as the ionization source. All chromatographic separations were performed using an Agilent Zorbax Rapid Resolution HD Eclipse Plus C18 column (3.0 x 100 mm; particle size 1.8 μm). A biphasic elution system consisting of eluent A: Optima grade water with 0.1% formic acid and B: acetonitrile with 0.1% formic acid was utilized. The gradient started at 5% B and was ramped to 95% B over 19 min. Initial analysis by QTOF-MS was performed using full scan mode. In this mode, the mass range was *m/z* 50-1000 with fragmentor voltage set to 100 V and no collision induced dissociation. Data were collected for this mass range over the entire run time and any prominent peaks of interest were recorded and then analyzed using targeted MS/MS. In targeted mode, MS/MS data were collected only for the molecular ion peaks of interest, with the mass range set to *m/z* 50–1000 and collision energies of 10, 20 and 40 eV to allow for full visualization of fragments formed.

Data analysis was done using Agilent MassHunter Qualitative Analysis B.07.00 software, Agilent MassHunter BioConfirm software (version B.08.00), MetaSite software (Molecular Discovery, Version 6), and Protein Prospector software (http://www.prospector.ucsf.edu). Adduct structures to be targeted were proposed

based on accurate mass data for the molecular ion of each drug-peptide adduct and for major MS/MS fragments. A list of metabolites potentially formed *in situ* was compiled using published metabolism data as available and MetaSite for prediction of reactive and unstable metabolites. The structures for each possible metabolite modification were added to MassHunter Sequence Manager.

Initial analyses by QTOF-MS were performed using full scan mode. Analysis of the data collected for the peptide adducts was performed using MassHunter BioConfirm. The workflow used was the “peptide digest” method, the condition was “reduced”, the sequence selected was the Hb β^{93}Cys peptide (GTFATLSELHCDK), the enzyme selected was trypsin, and the modifications were oxidation, hydroxylation, methylation, and the predicted drug/metabolite modifications previously added to Sequence Manager. For the MS studies, once the workflow was run, the predicted modifications were analyzed, and any potential drug adducts were recorded. For the targeted MS/MS studies, the spectra were collected, compared to a theoretical peak list generated by Protein Prospector, and confirmed peaks were recorded. Peaks were considered a positive match if the observed mass differential was <0.1 Da of the hypothetical mass reported in the peak list and a possible match if the mass differential was 0.1 - 0.5 Da. All peaks with a mass differential ≥0.5 Da were excluded.

Characterization of adducted Hb was performed by both whole protein and bottom-up peptide mapping HRMS analysis. Whole protein analysis was used as an initial confirmation of adduction, followed by tryptic peptide analysis to assess specificity of binding sites. For whole protein MS analysis, the mass range was set to *m/z* 100-1700 with the fragmentor voltage set to 175 V and no collision induced dissociation. Data was

collected over the entire range of *m/z* and deconvoluted to give whole protein data. For the bottom-up tryptic peptide analysis, initial studies were performed in full scan mode with the same parameters as the whole protein analysis. This untargeted approach allowed for all possible covalent adducts at the β^{93}Cys to be initially identified. Peptide fragments containing the β^{93}Cys tryptic peptide containing the covalent adduct were then examined by Auto-MS/MS for peptide fragment identification.

After the enrichment assay and tryptic digestion, Hb samples were subjected to a full scan MS screen. This screening indicated if any potential covalent modifications were present on specific peptide fragments. BioConfirm and Mass Hunter Qualitative software were used for the identification of potential covalent Hb adducts. BioConfirm allows users to select specific workflow conditions such as the specific proteins and the type of digestion enzymes that was used. The user enters selected standard modifications and custom modifications that encompass all potential RM for the specific target drug that was used during the *in vitro* trapping assay that may potentially covalently adduct to the cysteine thiol moieties of interest. Figure 3 shows the BioConfirm sample processing method that was utilized for the analysis of enzymatically digested tryptic peptides with target drug cocaine. The left panel allows the user to select the specific protein sequences for the α and β subunits of Hb, trypsin enzyme used for digestion, and all modifications that should be considered. The right panel is the selected screen for the modifications. Drug specific modifications, such as cocaine and all metabolites, are custom modifications that were inputted into the software along with standard modifications oxidation, hydroxylation, and methylation.

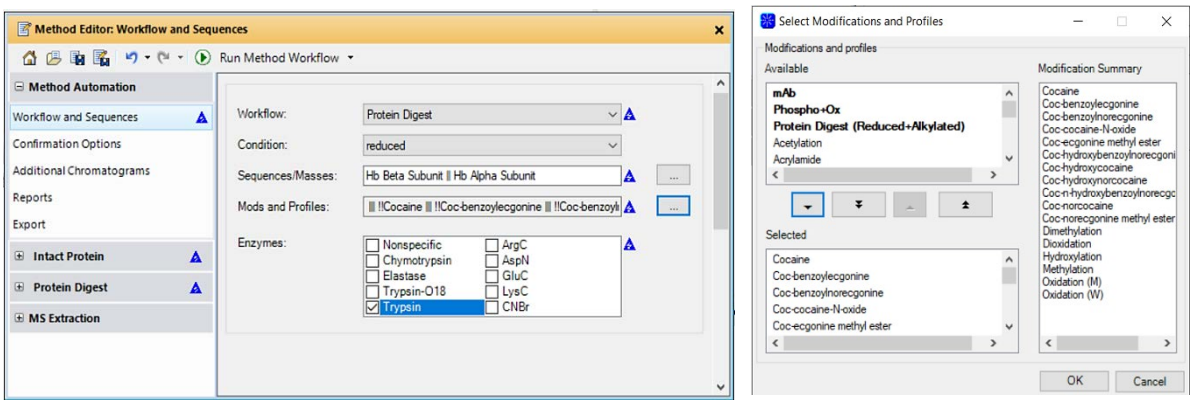

Figure 3: Examples of BioConfirm data processing inputs for tryptic digested hemoglobin with target drug cocaine (left panel) and standard and drug specific modifications that were input during the data analysis process (right panel).

For the identification of potential covalent adducts, modifications were considered a confirmed hit if the mass error was ≤5 ppm and the modification match score generated by the software was ≥85%. If both of these criteria were met, it was considered a high likelihood that the identification of the covalent adduct was accurate. In those cases, the sample was re-analyzed using AutoMSMS or TgtMSMS methods in order to collect fragmentation data for the covalent modification of interest.

MS/MS fragmentation data were cross referenced with Protein Prospector, which allows a user to enter specific peptide fragments and custom modifications. The example in Figure 4 shows the tryptic peptide containing the β^{93}Cys moiety and a custom entered modification of a covalent adduct for the reactive metabolite of APAP (NAPQI) to the Cys thiol moiety.

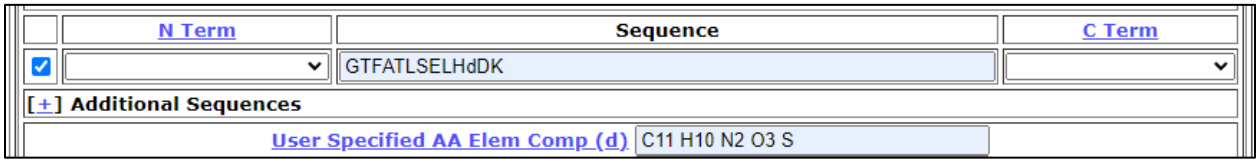

Figure 4: Tryptic peptide containing β^{93}Cys with annotation for NAPQI adduct modification ($+C_{11}H_{10}N_2O_3S$).

The software then generates a theoretical peak table containing all possible fragments of the peptide that contain the custom modification. A sample of a section of the theoretical peak table can be seen in Table 2. Experimentally collected data is then mined peak by peak to identify all fragments found.

Table 2: Protein Prospector theoretical peak table for the analysis of the Hb β[93]Cys tryptic peptide covalently adducted to APAP reactive metabolite (NAPQI).

User AA Formula 1: **C11 H10 N2 O3 S1**
Elemental Composition: C69 H102 N17 O23 S1

MH^{+1}(av)	MH^{+1}(mono)	MH^{+2}(av)	MH^{+2}(mono)	MH^{+3}(av)	MH^{+3}(mono)
1569.7437	1568.7050	785.3755	784.8561	523.9195	523.5732

[–] **Theoretical Peak Table**

60.0444	S	C2 H6 N1 O1	392.1781^{+3}	y_9-H_2O^{+3}	C51 H76 N13 O17 S1	697.8059^{+2}	a_{12}^{+2}	C62 H88 N15 O20 S1
65.5468^{+2}	y_1-NH_3^{+2}	C6 H12 N1 O2	392.5061^{+3}	y_9-NH_3^{+3}	C51 H75 N12 O18 S1	702.7981^{+2}	b_{12}-H_2O^{+2}	C63 H86 N15 O20 S1
74.0600	T	C3 H8 N1 O1	394.6552^{+2}	x_5^{+2}	C34 H46 N9 O11 S1	705.8216^{+2}	y_{11}^{+2}	C63 H92 N15 O20 S1
74.0600^{+2}	y_1^{+2}	C6 H15 N2 O2	398.1816^{+3}	y_9^{+3}	C51 H78 N13 O18 S1	711.8034^{+2}	b_{12}^{+2}	C63 H88 N15 O21 S1
84.0808	K	C5 H10 N1	406.8414^{+3}	x_9^{+3}	C52 H76 N13 O19 S1	718.8112^{+2}	x_{11}^{+2}	C64 H90 N15 O21 S1
86.0964	L	C5 H12 N1	415.8571^{+3}	y_{10}-H_2O^{+3}	C54 H81 N14 O18 S1	720.8086^{+2}	b_{12}+H_2O^{+2}	C63 H90 N15 O22 S1
87.0497^{+2}	x_1^{+2}	C7 H13 N2 O3	416.1851^{+3}	y_{10}-NH_3^{+3}	C54 H80 N13 O19 S1	744.3134	y_5-H_2O	C33 H46 N9 O9 S1
88.0393	D	C3 H6 N1 O2	421.8607^{+3}	y_{10}^{+3}	C54 H83 N14 O19 S1	745.2974	y_5-NH_3	C33 H45 N8 O10 S1

If the differentials between experimental and calculated masses for each peptide as generated by Protein Prospector were <1.1 Da, they were considered a positive match for the identified covalent modification.

Expected applicability of the research: The principal purpose of this study was to continue development of a validated assay for analysis of protein adducts as retrospective exposure biomarkers for drugs of abuse. Such tools would allow for exposure assessment for these compounds over a much longer period of time than is currently possible and provide an alternative or complement to hair analysis. A longer window of detection for drugs of abuse is critically important in forensic toxicology, for

example as evidence in drug facilitated crime cases or to identify individuals suspected of illicit synthesis of drugs. In addition, longer windows of detection are valuable for measurement of drug compliance or abstinence, such as in pain drug management, rehabilitation programs, and probation/parole criminal justice situations. Consequently, this novel approach is anticipated to significantly benefit forensic science for criminal justice research by providing additional tools for detecting and quantifying important agents of forensic interest in biological specimens over longer periods of time.

Participants and Other Collaborating Organizations

Anthony P. DeCaprio, Ph.D.

Project Role: Principal Investigator

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Project Role: Graduate student (Ph.D.)

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Project Role: Graduate student (Ph.D.)

William Morrison, B.S.

Project Role: Graduate student (Ph.D.)

Changes in approach from original design and reason for change

Substantial delays were encountered during the project period due to Covid-19

pandemic issues which necessitated extended lab shutdowns and which also impacted scheduling of instrument repairs for the LC-QTOF-MS instrument. Two one-year no cost extensions were requested and granted to help ameliorate these delays. Nevertheless, planned final development of the LC-QqQ-MS based screening method for routine detection of drug adducted hemoglobin and serum albumin and testing with authentic specimens could not be completed. However, progress on this final goal is continuing with funding under NIJ award 2020-R2-CX-0023 and other sources.

Outcomes

Activities/accomplishments:

Activities and accomplishments under this project included the following achievements to support the ultimate goal of developing a reliable routine screening assay for blood protein modifications as retrospective biomarkers for abused drug exposure:

- Successful development and adaptation of three *in vitro* assay systems and LC-QTOF-MS analysis to generate and identify stable and reactive metabolites of eight licit and illicit drugs.
- Confirmation of covalent thiol modification of a specific hemoglobin tryptic peptide by four of five drugs examined and identification of adduct structure by LC-QTOF-MS analysis.
- Development of a novel procedure to enrich drug modified hemoglobin and remove unmodified protein, an approach that yields orders of magnitude increases in sensitivity for detecting drug modified protein species.

- Demonstration of covalent modification of the reactive β^{93}Cys thiol moiety in human hemoglobin for six of eight drugs tested *in vitro* under model physiological conditions.
- Presentation of project results at national forensic science conferences and publication of peer-reviewed articles.

Results and findings:

Identification of stable and reactive drug metabolites by in vitro HLM assay.

The *in vitro* HLM enzymatic assay using HLM and NADPH was applied to generate SM and RM of APAP, MDMA, METH and THC. A summary of MS data for identified metabolites in the HLM assay is shown in Table 3. All mass errors for identified metabolites and fragments were <5 ppm.

Table 3: MS/MS data and identification of metabolites formed with the HLM enzymatic assay.

Drug	Metabolite	Formula	RT (min)	Observed *m/z*	Mass Error (ppm)	Fragments (*m/z*)
APAP	NAPQI	$C_8H_8NO_2^+$	4.02	150.0546	-2.7	108.0444, 80.0502
MDMA	MDA	$C_{10}H_{14}NO_2^+$	4.05	180.1028	1.7	163.0759, 135.044, 105.0702
	HHMA	$C_{10}H_{16}NO_2^+$	2.82	182.1168	-4.4	151.0754, 123.0446, 105.0702
	4-OH-3-formate AMP	$C_{11}H_{16}NO_3^+$	5.54	210.1121	-1.9	192.1030, 163.0754, 133.062, 105.0702
METH	Amphetamine	$C_9H_{14}N^+$	3.87	136.1126	0.0	119.0859, 91.0549
	N-OH-METH	$C_{10}H_{16}NO^+$	5.51	166.1226	-3.6	148.1126, 119.0857, 91.0541
	Methcathinone	$C_{10}H_{14}NO^+$	3.96	164.1074	-0.6	148.1126, 135.0803, 107.0490, 91.0537
	p-OH-AMP	$C_9H_{14}NO^+$	5.10	152.1072	-1.9	119.0859, 91.0552
THC	11-OH-THC	$C_{21}H_{31}O_3^+$	15.22	331.2281	2.4	313.2172, 271.1688
	11-COOH-THC	$C_{21}H_{29}O_4^+$	11.50	345.2074	2.3	327.1938, 285.1842, 255.1420, 229.1256
	11-oxidocarbonate-THC	$C_{21}H_{29}O_3^+$	12.38	329.2095	-4.9	311.2009, 245.1531, 269.1535

The well-characterized reactive metabolite of APAP (N-acetyl-p-benzoquinone

imine; NAPQI) was detected in the HLM assay. The MS/MS spectrum for NAPQI is shown in Figure 5, with the molecular ion at 150.0546 *m/z*.

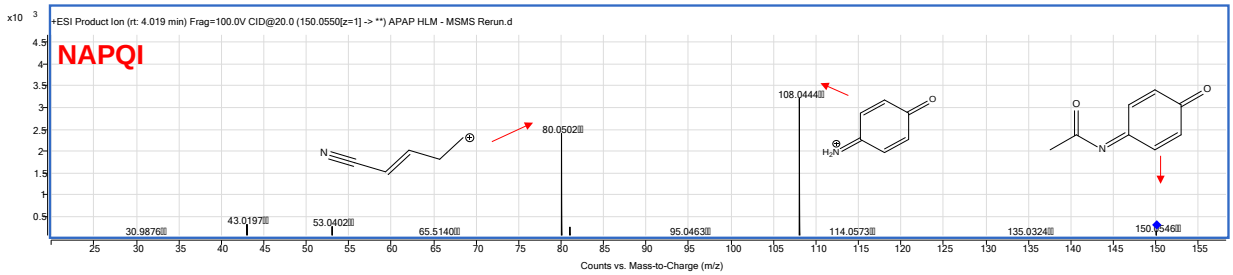
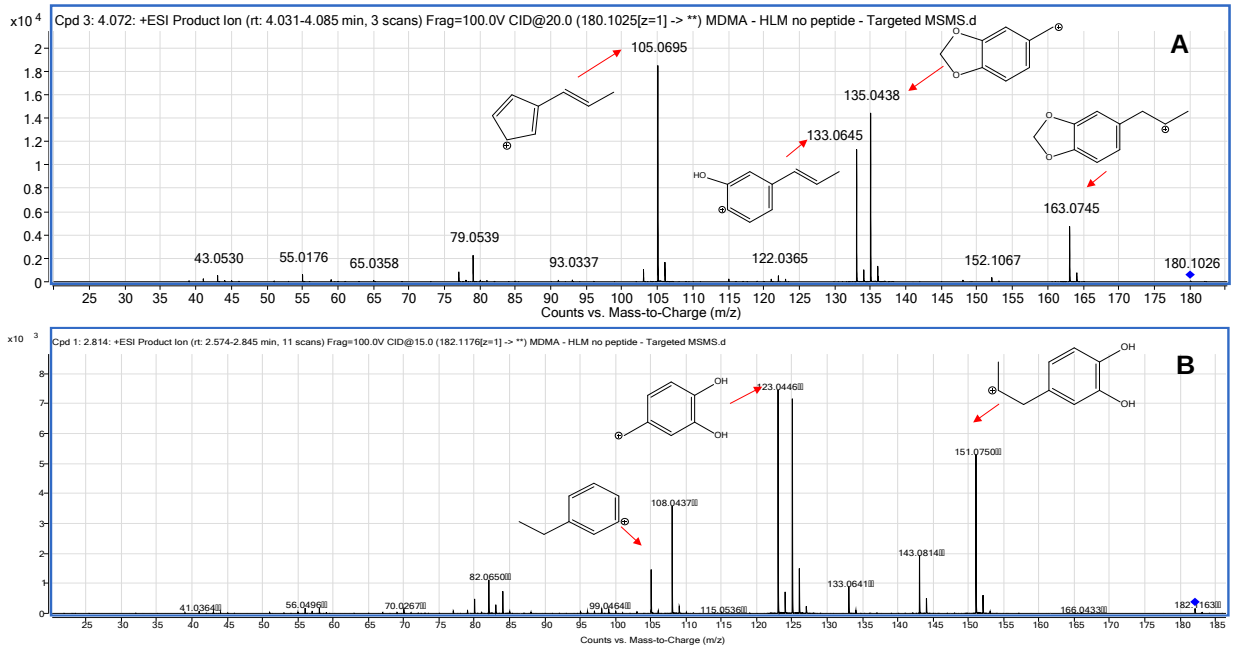


Figure 5. MS/MS spectrum of NAPQI generated with HLM enzymatic assay of APAP.

MS/MS results for MDMA in the HLM assay can be seen in Figure 6. Three metabolites were identified; MDA, HHMA, and a formate derivative of 4-OH-AMP, with molecular ions at 180.1028, 182.1168, 168.1029 *m/z*, respectively. The first two metabolites have previously been reported for MDMA, while the latter metabolite is novel.



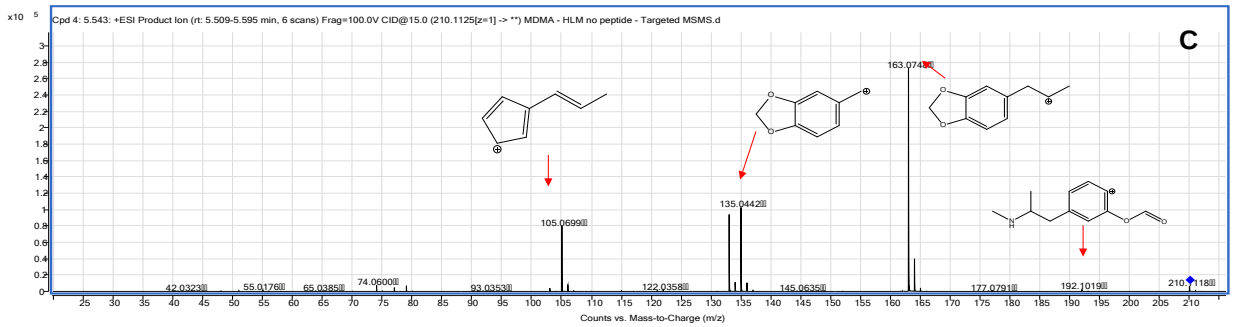
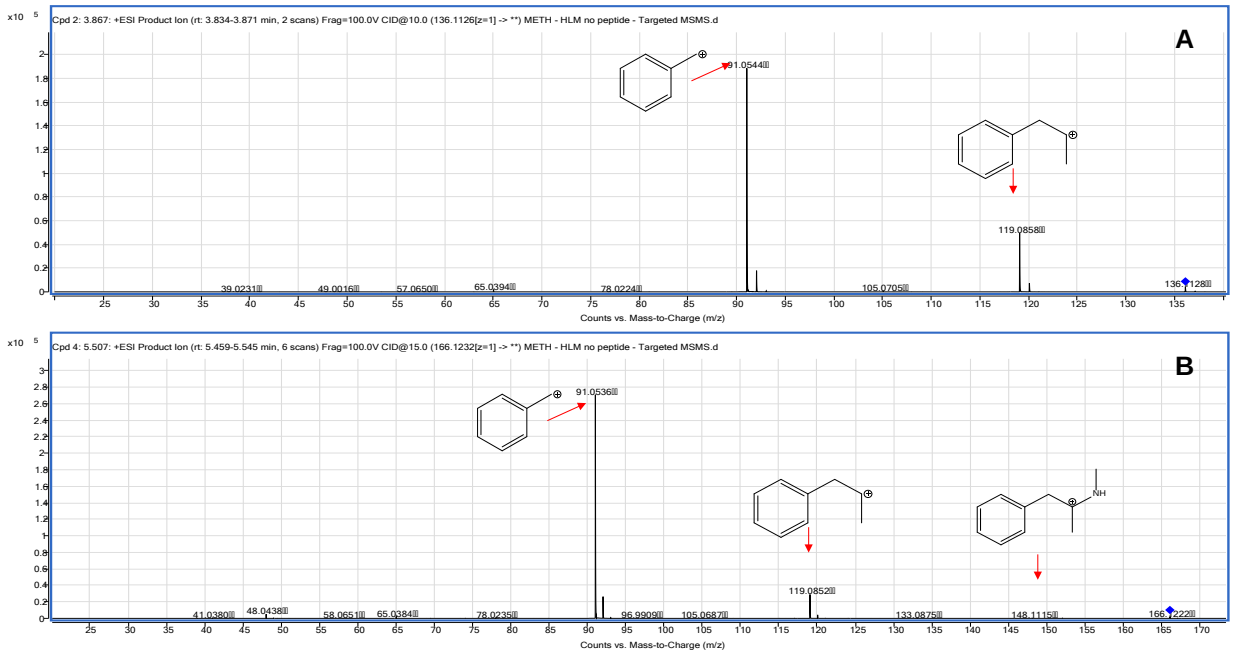


Figure 6. MS/MS spectra of A) MDA, B) HHMA, and C) a 4-OH-3-formate amphetamine derivative generated with HLM enzymatic assay of MDMA.

The MS/MS spectra of the metabolites identified for METH can be seen in Figure 7. These included amphetamine, N-hydroxymethamphetamine, N-hydroxyamphetamine, and methcathinone, with molecular ions at 136.1126, 166.1226, 164.1074, and 152.1072 *m/z*, respectively.



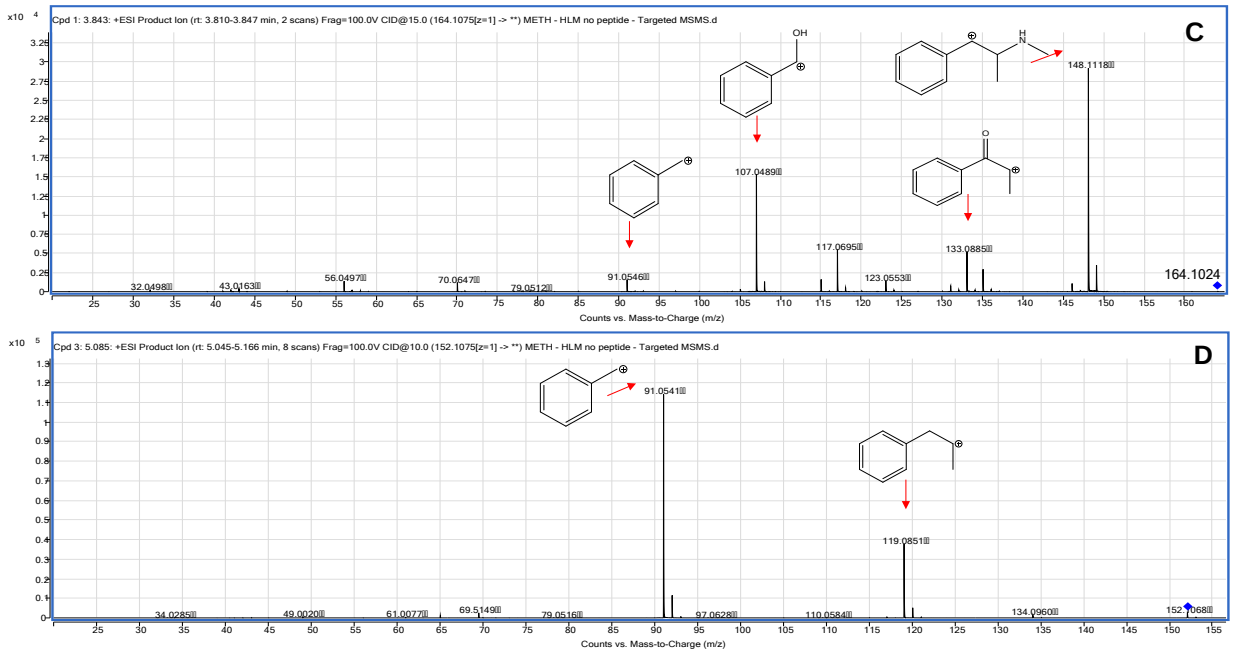


Figure 7. MS/MS spectra of A) amphetamine, b) N-hydroxymethamphetamine, C) methcathinone, and D) N-hydroxyamphetamine generated with HLM enzymatic assay of METH.

The MS/MS spectra of the metabolites identified for THC can be seen in Figure 8. These included the well-known THC metabolites 11-OH-THC and11-COOH-THC, with molecular ions at 331.2281, 345.2074, and 329.2095 *m/z*, respectively.

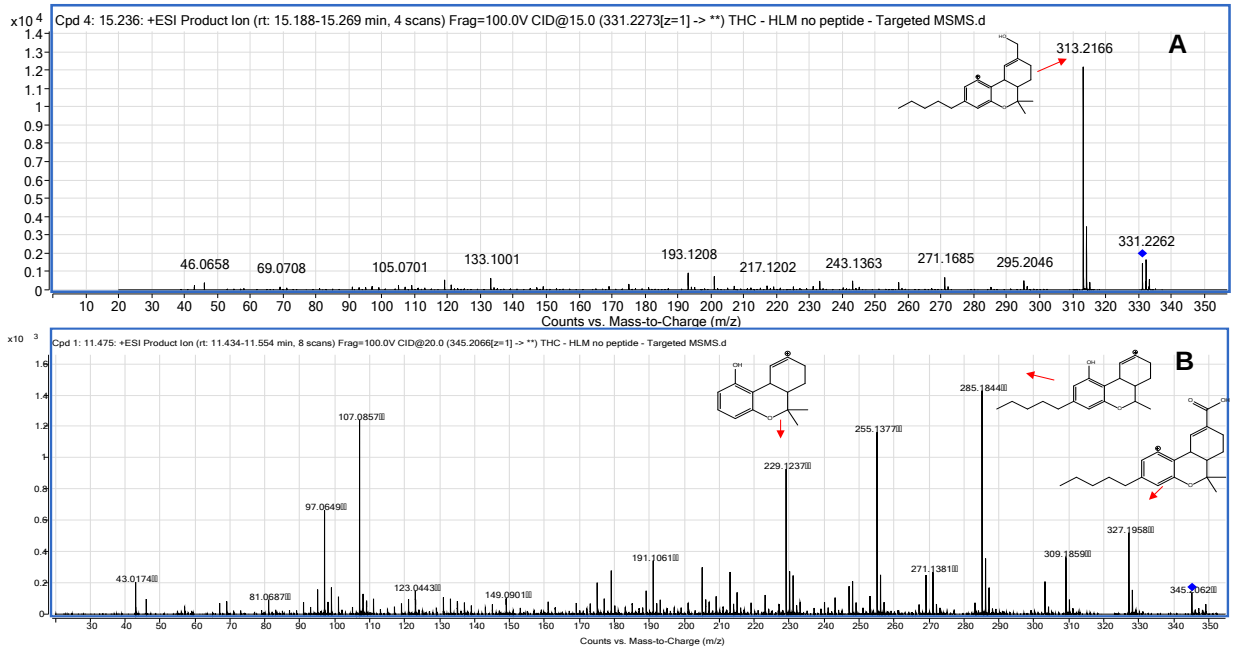


Figure 8. QTOF MS/MS spectra of A) 11-OH-THC and B) 11-COOH-THC generated with HLM enzymatic

assay of THC.

***Generation of reactive drug metabolites by electrochemical oxidation*:**

MS and MS/MS data for identified metabolites in the EC assay are shown in Table 4. All mass errors for identified metabolites and fragments were <5 ppm. For the EC assays, a 1 mg/mL solution of drug was prepared in 100 mM ammonium bicarbonate buffer with pH of 7.4. Then, 2 mL of the solution was transferred to a one compartment cell vial and the solution was maintained under magnetic stirring for the entire duration of the experiment. Initial studies were conducted to determine the ideal voltage for the oxidation of the drug using cyclic voltammetry. The cyclic voltammogram obtained for APAP is shown in Figure 9, where two primary oxidation events at specific voltages (marked with arrows) can be seen. Based on the voltammogram results, the voltages chose for the electrolysis of APAP were 380 mV and 500 mV. The next step was to perform a bulk electrolysis, where the voltage was fixed and the drug was oxidized over a specified time period. Both optimal voltages for APAP oxidation were tested during the bulk electrolysis, with similar results.

The full scan MS spectra for the 380 mV oxidation experiment with APAP is shown in Figure 10. In the full scan spectrum, it can be noted that the most abundant peak is unoxidized APAP at *m/z* 152.0704. However, if the spectrum is expanded, it is also possible to see the formation of two potential RM of APAP; 1,4-benzoquinone (*m/z* 110.0601) and NAPQI (*m/z* 150.0534). This initial test demonstrated that higher yields of RM of interest are formed with EC as compared to the HLM approach.

Table 4: MS/MS data and identification of metabolites formed with the EC assay.

Drug	Metabolite	Formula	RT (min)	Observed m/z	Error (ppm)	Fragments (m/z)
APAP	NAPQI	$C_8H_8NO_2^+$	4.060	150.0549	-0.7	108.0413, 80.0493
MDMA	MDA	$C_{10}H_{14}NO_2^+$	4.185	180.1017	-4.4	163.0767, 135.0380
	HHMA	$C_{10}H_{16}NO_2^+$	2.801	182.1176	-1.1	151.0755, 133.0646, 123.0434, 105.0675
	4-OH-3-formate AMP	$C_{11}H_{16}NO_3^+$	4.119	210.1123	-0.9	192.1025, 177.0778, 91.0543
	Quinone intermediate	$C_9H_{14}NO_2^+$	3.647	166.0868	0.0	135.0437, 77.0383
METH	Amphetamine	$C_9H_{14}N^+$	3.715	136.1121	0.0	119.0864, 91.0539, 105.0664
	N-OH-METH	$C_{10}H_{16}NO^+$	3.965	166.1223	-1.8	148.1120, 133.0893, 107.0484
	Methcathinone	$C_{10}H_{14}NO^+$	7.861	164.1057	-7.3	91.0532, 86.0594
	METH imine derivative	$C_{10}H_{14}N^+$	4.159	148.1134	5.4	133.0891, 119.0791, 91.0530
THC	11-COOH-THC	$C_{21}H_{29}O_4^+$	17.172	345.2062	-1.2	299.2066, 317.2050, 287.1612, 259.1698
	11-dihydroxy-THC	$C_{21}H_{31}O_4^+$	12.493	347.2202	-4.3	329.2117, 311.2005, 271.1690
	11-oxidocarbonate-THC	$C_{21}H_{29}O_3^+$	15.288	329.2116	1.52	311.2001, 287.1932, 271.1694, 231.1380
	11-vinyl-THC	$C_{21}H_{29}O_2^+$	8.796	313.2162	-0.6	231.1344, 217.1214, 201.0886, 193.0861

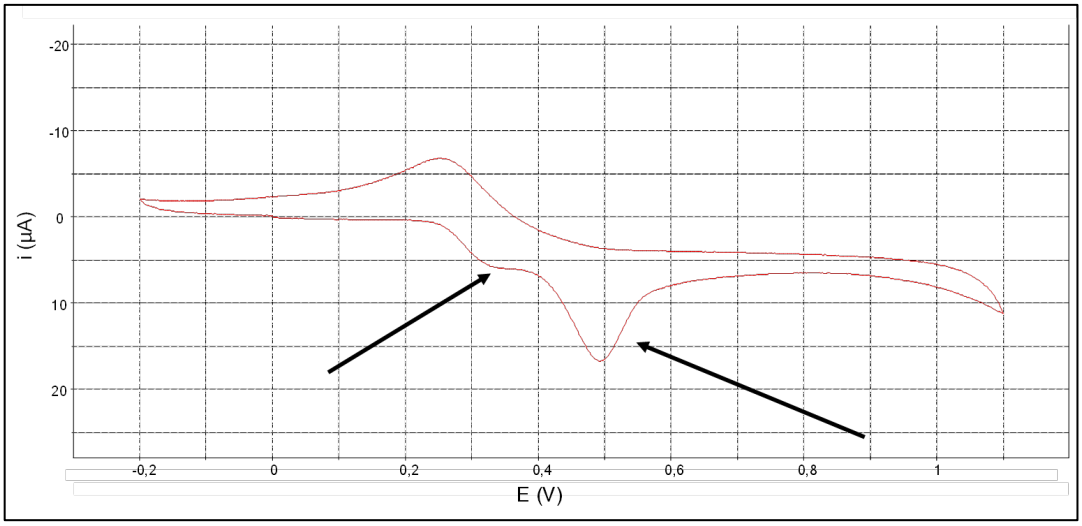


Figure 9. Cyclic voltammogram for APAP using glassy carbon as working electrode, Ag/AgCl as reference electrode, and Pt as counter electrode.

The MS/MS spectrum for NAPQI formed with EC oxidation of APAP is shown in Figure 11. Ion intensities for the 1,4-benzoquinone metabolite noted in full scan were too low for MS/MS confirmation.

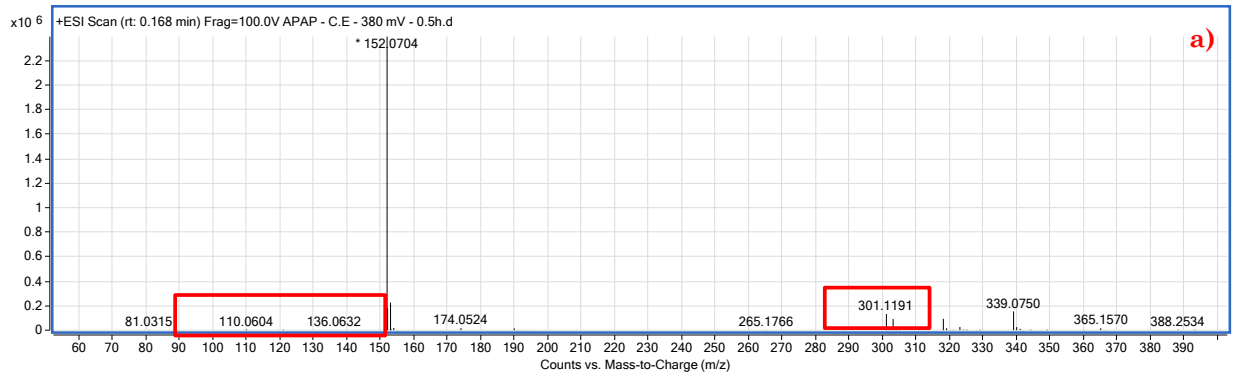


Figure 10. Full scan MS spectrum of APAP after 30 min of bulk electrolysis at 380 mV, using glassy carbon as working electrode, Ag/AgCl as reference electrode, and Pt as counter electrode.

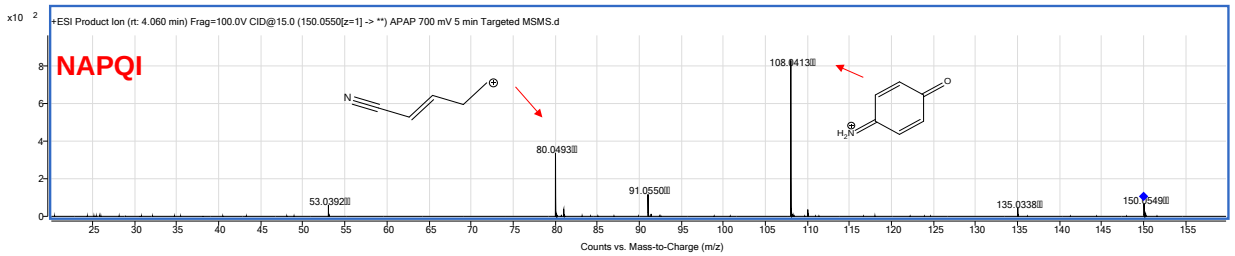


Figure 11. MS/MS spectrum of NAPQI obtained with EC oxidation of APAP.

The EC assay for MDMA, METH and THC followed the same experimental procedure as for APAP. The best experimental conditions for MDMA were an oxidation voltage of -1.045 V for 30 min with the glassy carbon working electrode. The MS/MS spectra for metabolites found with bulk electrolysis of MDMA are shown in Figure 12. These included MDA, HHMA, and two novel metabolites: a formate derivative of 4-OH-AMP and a quinone derivative.

For METH, the best experimental conditions included an oxidation voltage of 480 mV for 5 min with the glassy carbon working electrode. MS/MS spectra of metabolites found in the bulk electrolysis of METH are shown in Figure 13. These included methcathinone, p-OH-METH, AMP, and an amphetamine imine (methanimine) derivative.

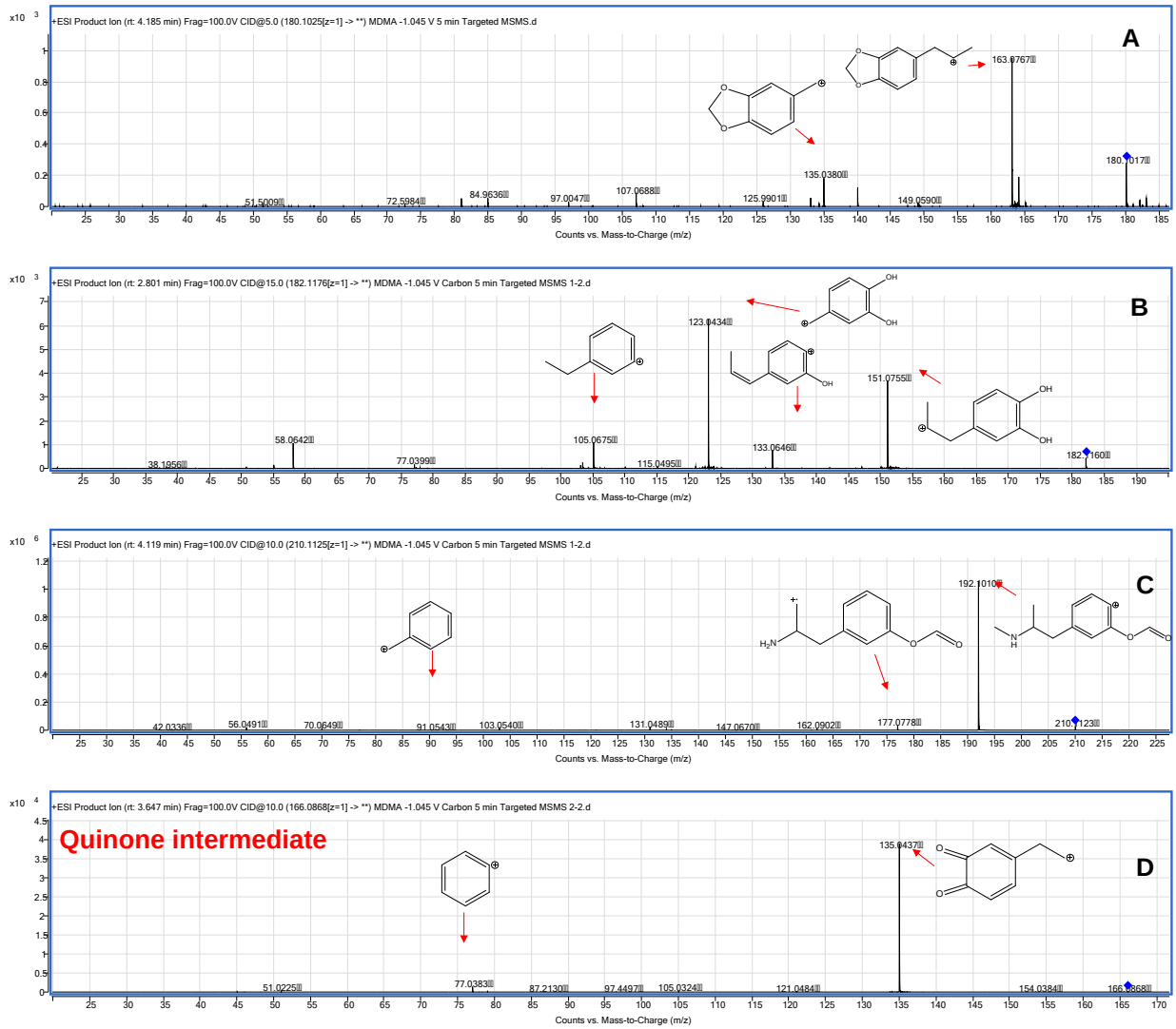
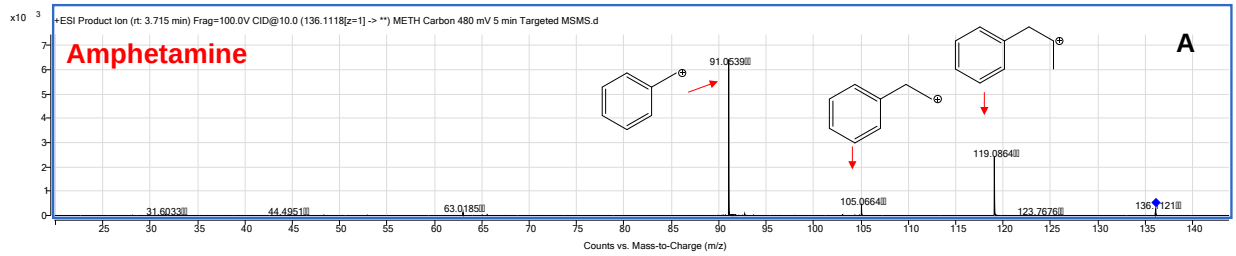


Figure 12. QTOF-MS/MS spectra of A) MDA, B) HHMA, C) a formate AMP derivative, and D) a quinone intermediate obtained with the electrochemical oxidation of MDMA.



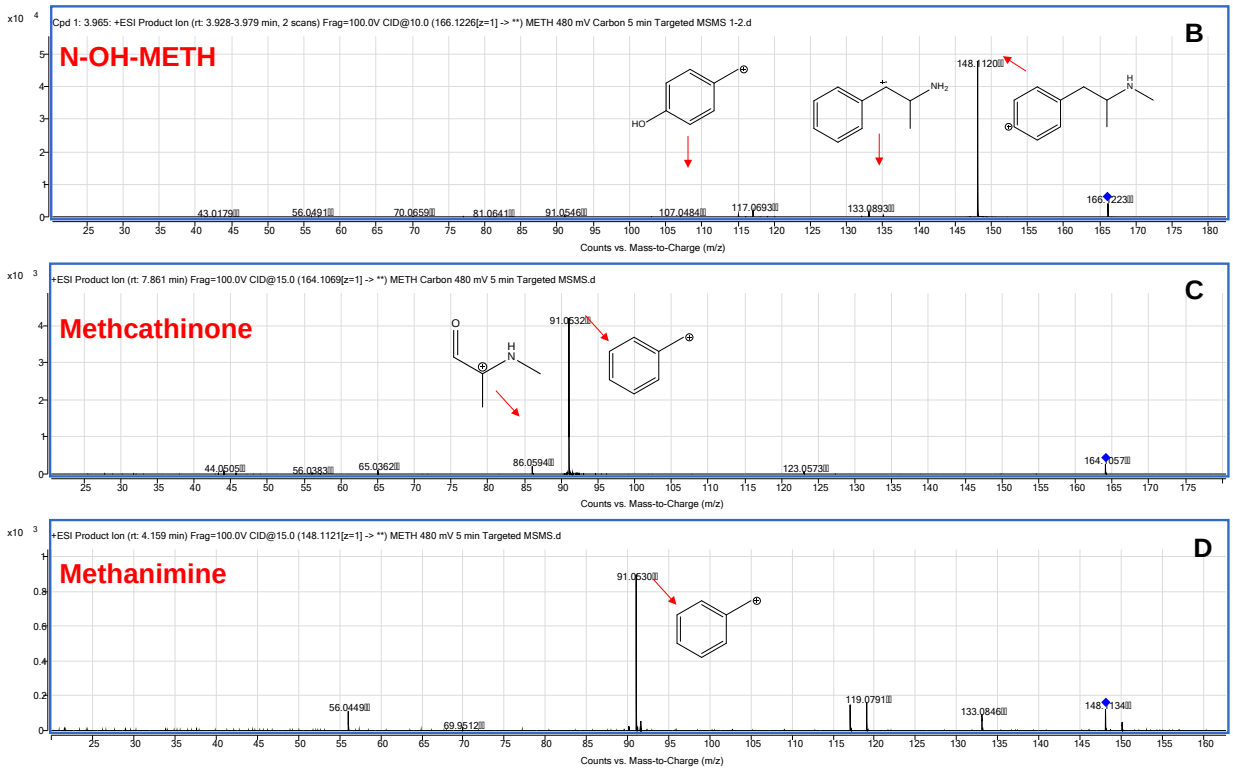
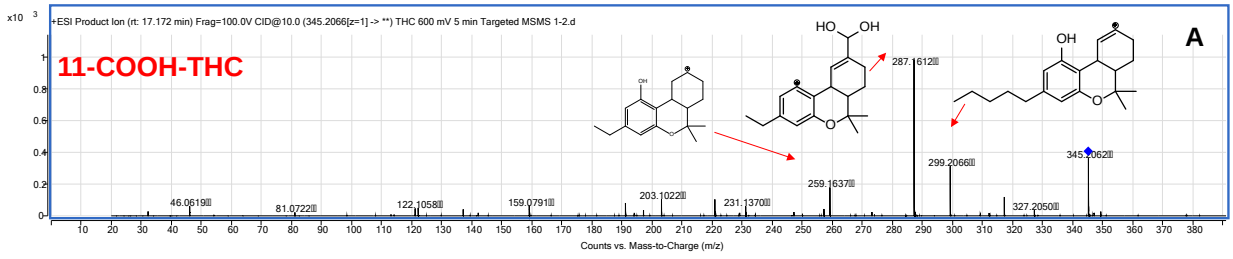


Figure 13. MS/MS spectra of A) amphetamine, B) N-OH-METH, C) methcathinone, and D) imine derivative (methanimine) obtained with EC oxidation of METH.

The optimal results for EC of THC were found with oxidation voltage of 600 mV and oxidation time of 5 min. The MS/MS spectra of metabolites found for THC are shown in Figure 14. These included 11-COOH-THC, 11-dihydroxy-THC, an 11-oxidocarbonate-THC derivative (carbaldehyde-THC), and an 11-vinyl-THC derivative (methylidene-THC).



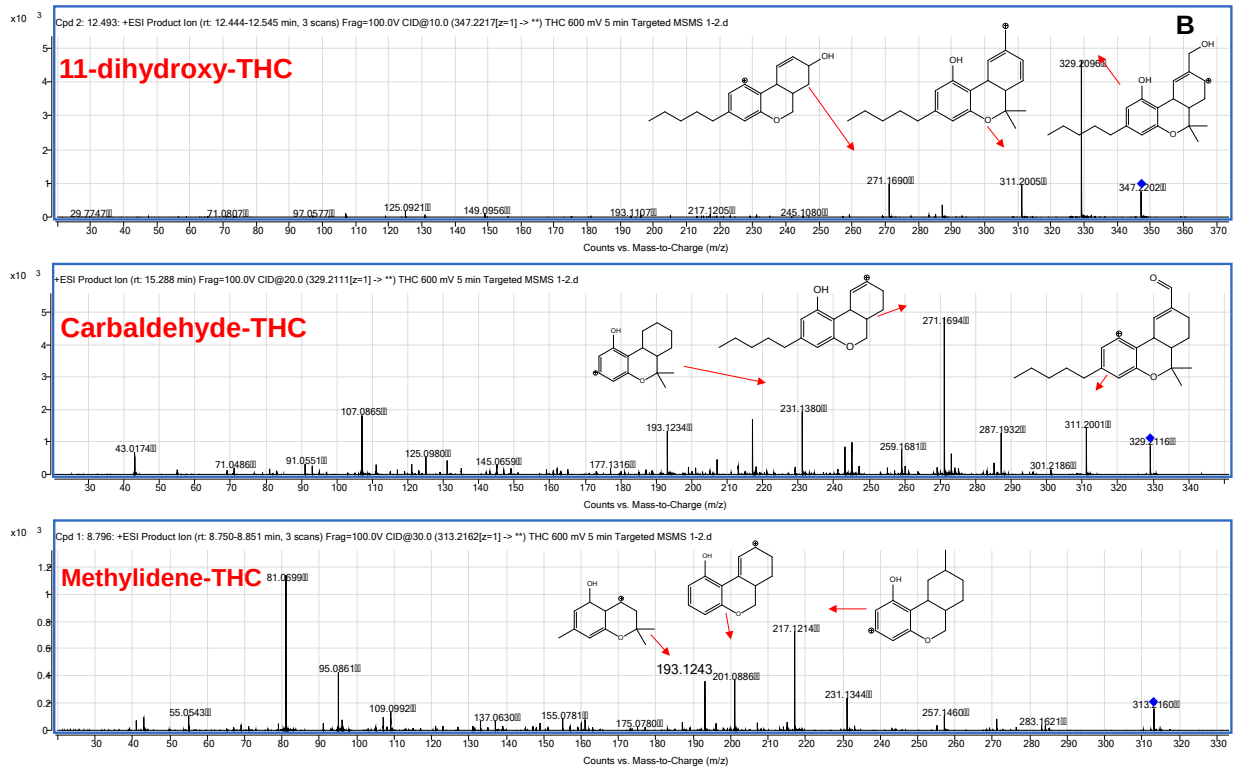


Figure 14. MS/MS spectra of A) 11-COOH-THC, B) 11-dihydroxy-THC, C) a carbaldehyde-THC derivative, and D) a methylidene-THC derivative obtained with the electrochemical oxidation of THC.

***Generation of reactive drug metabolites by biomimetic chemical catalysts*:**

A final approach for the generation of SM and RM of the selected drugs was oxidation by biomimetic chemical catalysts. For this purpose, commercial screening and optimization kits (BMO kits) developed by HepatoChem, Inc., that utilize synthetic metalloporphyrin catalysts to mimic the oxidative *in vivo* metabolism mediated by liver enzymes (cytochrome P450) were used as described in Methods. A summary of MS and MS/MS data for identified metabolites in the BMO assay is shown in Table 5. All mass errors for identified metabolites and fragments were <5 ppm. In addition, as described under Methods, MetaSite software was employed in some cases to predict the metabolism of the drugs under study and the potential RM.

Table 5: MS data and identification of metabolites formed with the biomimetic catalyst (BMO) assay.

Drug	Metabolite	Formula	RT (min)	Observed m/z	Error (ppm)	Fragments
APAP	NAPQI	$C_8H_8NO_2^+$	3.729	150.0550	0.0	108.0445, 80.0501
MDMA	MDA	$C_{10}H_{14}NO_2^+$	4.565	180.1051	14.4	163.0717, 135.0433, 105.0697
	HHMA	$C_{10}H_{16}NO_2^+$	1.766	182.1176	-1.1	151.0753, 133.0649, 123.0442, 105.0699
	4-OH-3-bicarbonate AMP	$C_{11}H_{16}NO_4^+$	3.888	226.1076	-1.3	195.0876, 166.0865, 136.0726
	4-OH-3-formate AMP	$C_{11}H_{16}NO_3^+$	3.575	210.1117	-3.8	179.0702, 151.0749, 123.0441, 105.0697
METH	Amphetamine	$C_9H_{14}N^+$	4.417	136.1111	-11.0	119.0887, 91.0547
	N-OH-METH	$C_{10}H_{16}NO^+$	1.693	166.1214	-7.2	148.1115, 135.0800, 107.0490
	Methcathinone	$C_{10}H_{14}NO^+$	1.686	164.1075	0.6	148.1102, 135.0805, 107.0491
THC	11-OH-THC	$C_{21}H_{31}O_3^+$	16.963	331.2236	-11.2	313.2113, 271.1726, 231.1357, 193.1225
	11-COOH-THC	$C_{21}H_{29}O_4^+$	16.400	345.2062	-1.6	327.1956, 303.1613, 285.1544
	11-dihydroxy-THC	$C_{21}H_{31}O_4^+$	15.079	347.2217	0.0	329.2101, 311.2001, 271.1673
	11-oxidocarbonate-THC	$C_{21}H_{29}O_3^+$	16.400	329.2109	-0.6	311.1997,287.1966, 271.1698, 231.1373
	11-vinyl-THC	$C_{21}H_{29}O_2^+$	15.485	313.2162	-7.9	271.1710, 231.1358, 217.1231, 81.0694

For APAP, the set of conditions that best produced metabolites in the BMO optimization kit were those with reagent A6 and solvent 1. The full scan and MS/MS spectra for APAP are shown in Figures 15A and 15B, respectively, where NAPQI is identified as the primary metabolite.

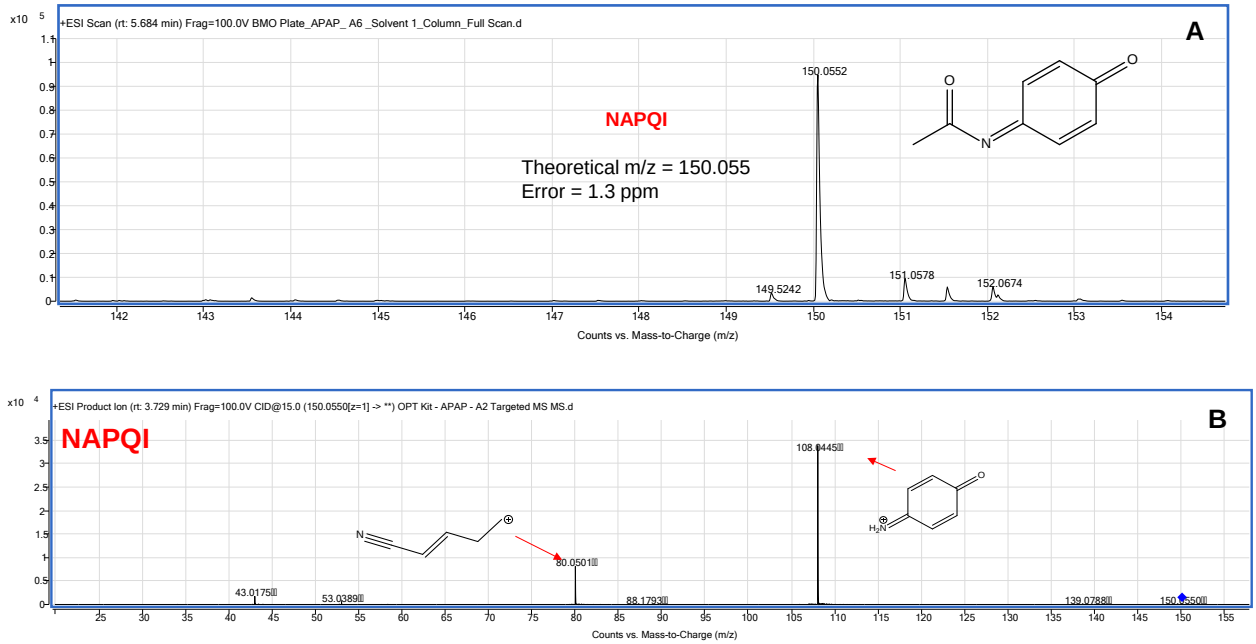


Figure 15. A) Full scan MS and B) MS/MS spectra of NAPQI generated with BMO screening with APAP.

For MDMA, the set of conditions that best produced metabolites in the BMO screening kit were those with reagent A5 and solvent 1. The full scan mass spectrum of these products is shown in Figure 16. Three metabolites of MDMA were identified, including 3,4-dihydroxyamphetamine (α-MeDA), 3,4-methylenedioxyamphetamine (MDA), and 4-hydroxy-3-methoxyamphetamine (HMA).

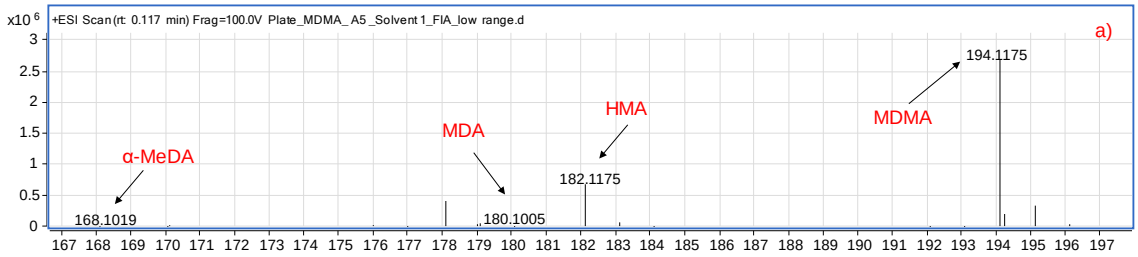
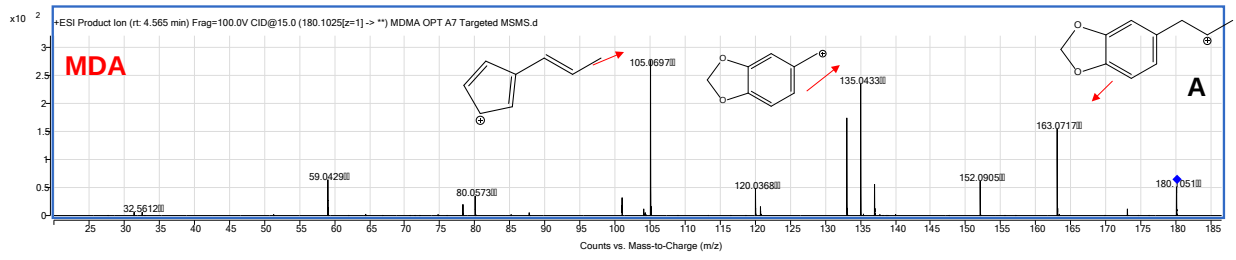


Figure 16. Full scan MS spectrum of metabolites generated with BMO screening of MDMA.

The set of conditions that best produced metabolites of MDMA in the BMO optimization kit were those with reagent A5. The MS/MS spectra for these products are shown in Figure 17. In addition to HMA and MDA identified with the screening kit, two MetaSite predicted metabolites were also detected using the optimization kit. These included an aldehyde derivative (formate AMP) and a carboxylic acid derivative (4-OH-3-bicarbonate AMP).



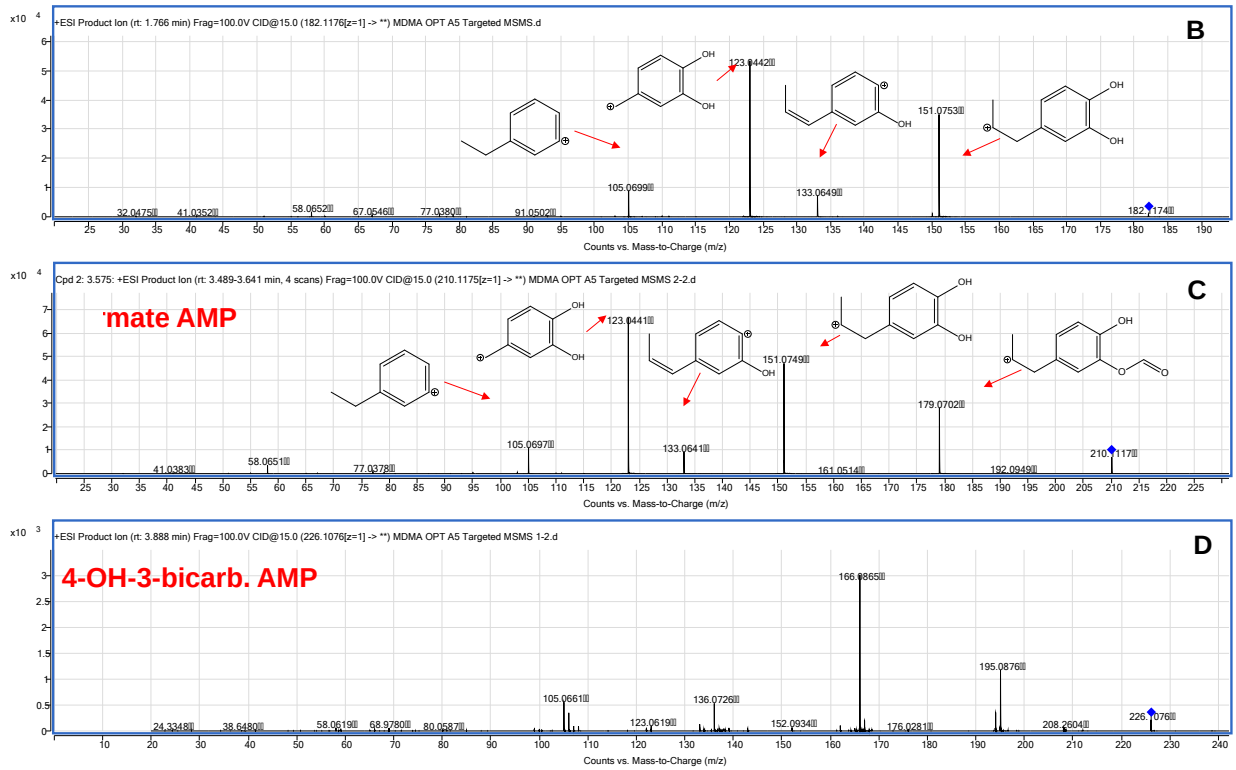


Figure 17. MS/MS spectra of A) MDA, B) HMA, C) an AMP aldehyde derivative, and D) an AMP carboxylic acid derivative obtained with the BMO optimization kit for MDMA.

The set of conditions that best produced metabolites of MET in the BMO screening assay were reagent A2 and solvent 2. The full scan mass spectrum for these products is shown in Figure 18. Two metabolites of METH were identified as AMP and p-hydroxymethamphetamine.

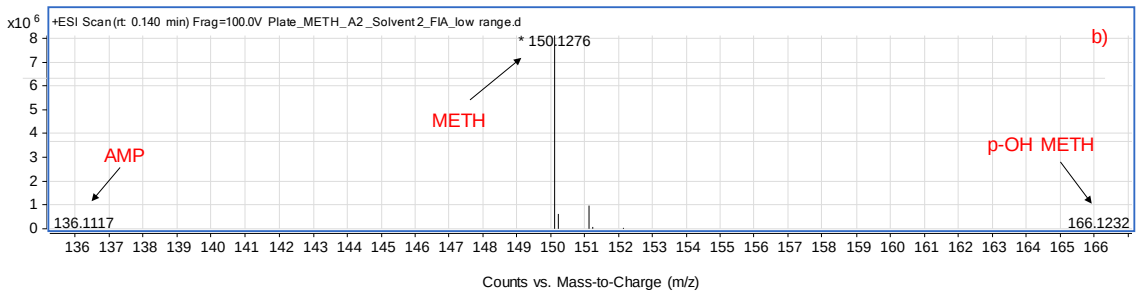


Figure 18. Full scan MS spectrum of BMO screening kit with methamphetamine.

The set of conditions that best produced metabolites of MET in the BMO

optimization assay were those with reagent A11. The MS/MS spectra for these products are shown in Figure 20. In addition to AMP and N-OH-MET identified with the screening kit, methcathinone was also identified using the optimization kit.

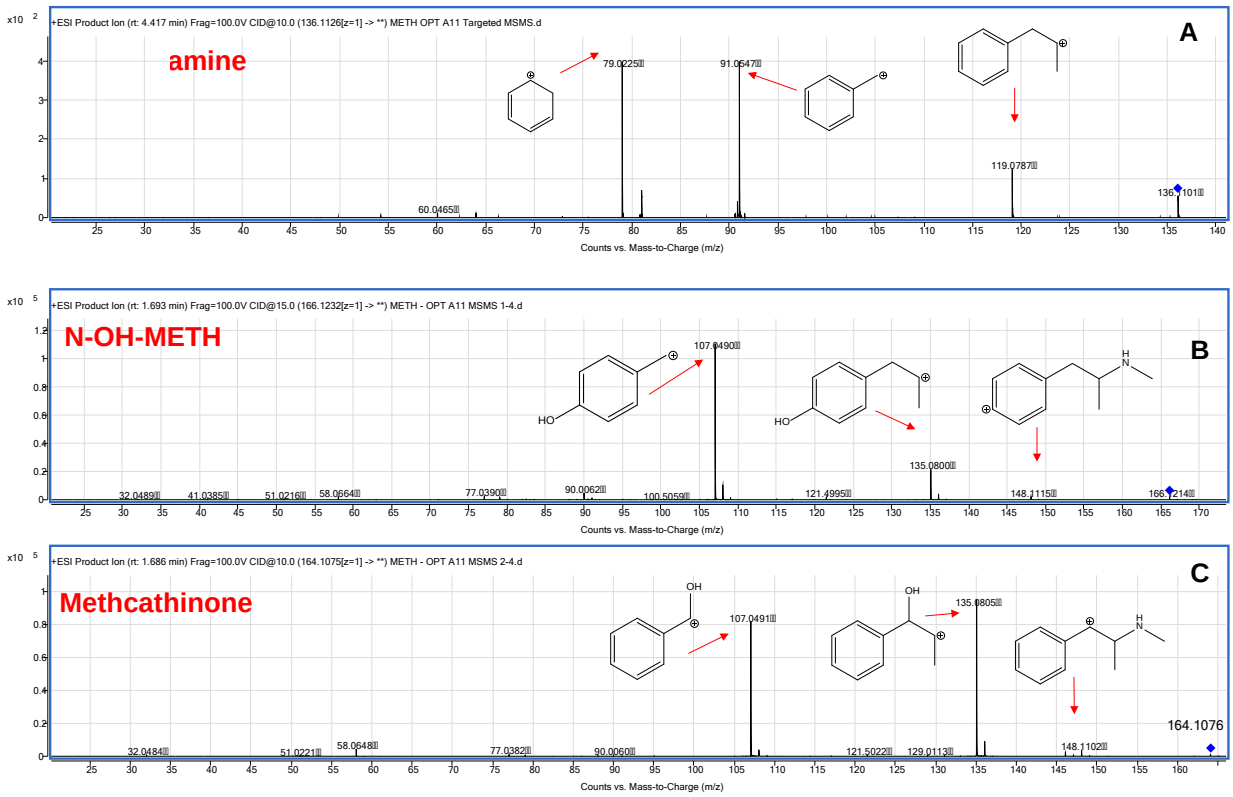


Figure 19. MS/MS spectra of A) amphetamine, B) N-OH-METH, and C) methcathinone obtained with the BMO optimization kit for METH.

For THC, the reaction condition that optimally produced metabolites in the BMO screening kit were those with reagent D4 and solvent 1. The full scan mass spectrum for these products is shown in Figure 20. Two known metabolites of THC were identified; 11-OH-THC and 11-COOH-THC. Two additional products representing previously unreported metabolites consistent with those predicted by the MetaSite software were also identified.

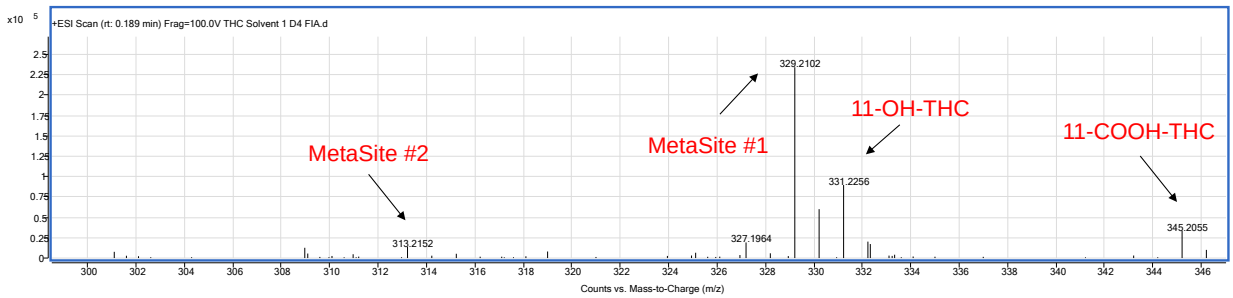
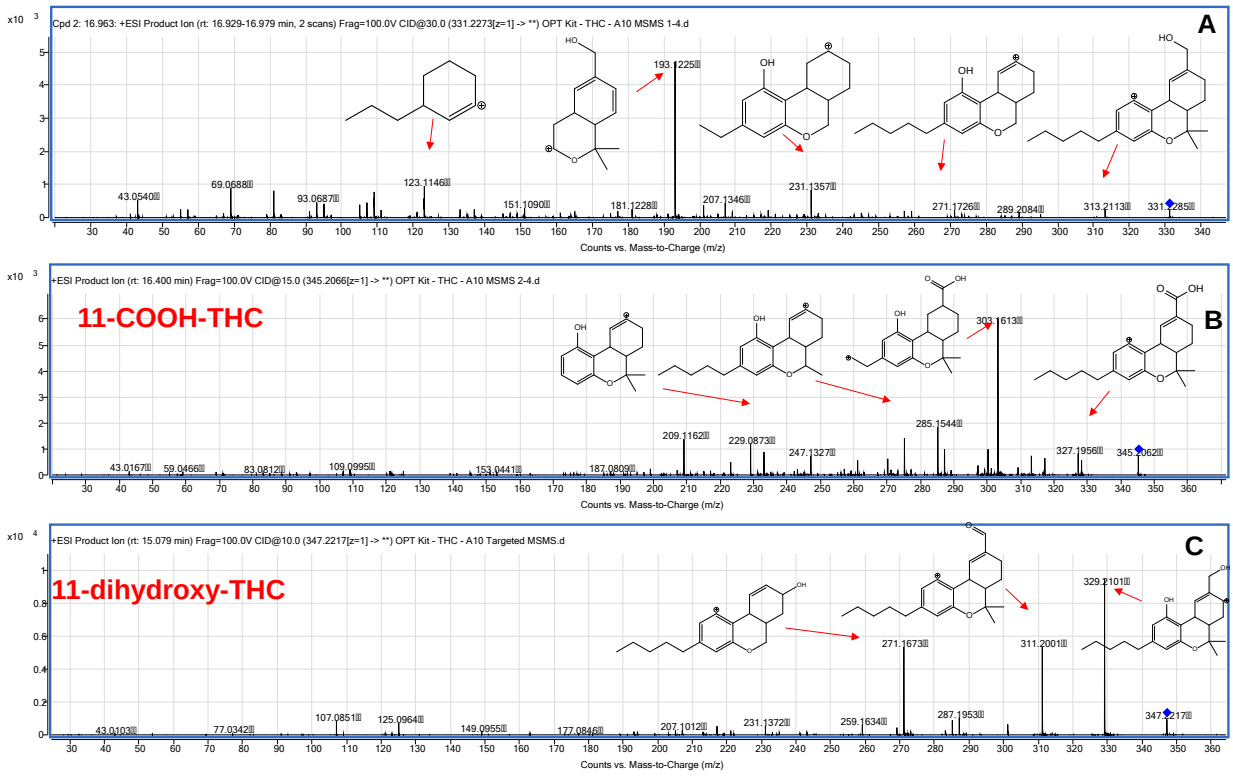


Figure 20. FIA QTOF-MS full scan spectrum of BMO screening kit of THC.

The set of conditions that best produced metabolites of THC in the optimization assay were those with reagent A10. MS/MS spectra for the generated metabolites can be seen in Figure 21. Five metabolites were noted; 11-OH-THC, 11-COOH-THC, 11-dihydroxy-THC, a carbaldehyde-THC derivative, and a methylidene-THC derivative. The latter two metabolites, which are novel, were also predicted by the MetaSite software.



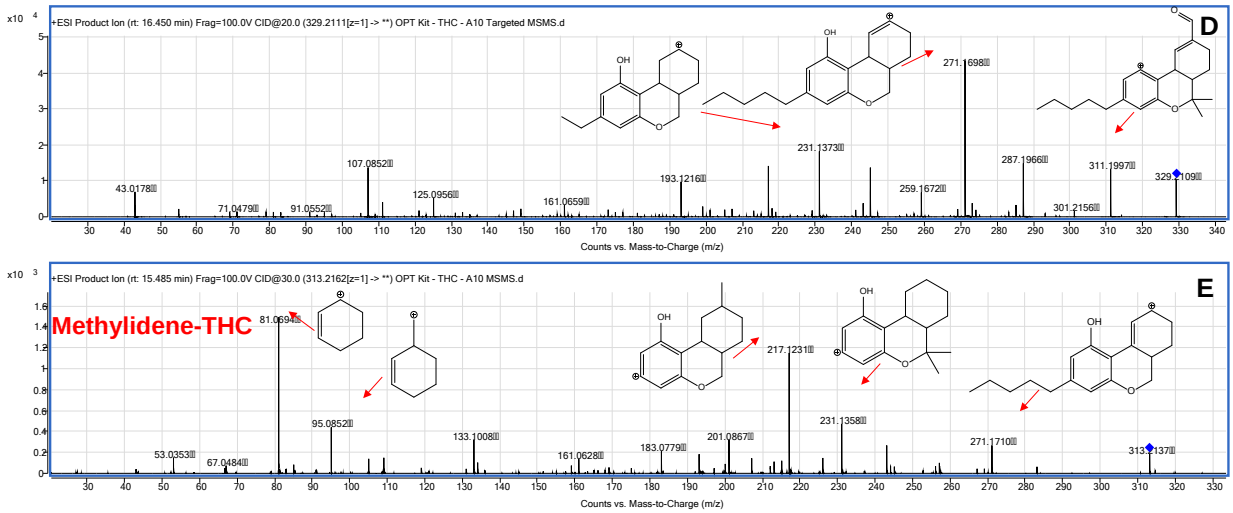


Figure 21. MS/MS spectra of A) 11-OH-THC, B) 11-COOH-THC, C) 11-dihydroxy-THC, D) a carbaldehyde-THC derivative, and E) a methylidene-THC derivative obtained with the BMO optimization kit and THC.

In summary, results of the *in vitro* SM and RM assay studies revealed that the metabolites obtained with the three different *in vitro* systems exhibited a few common derivatives but also compounds unique to each system. In addition, major reported *in vivo* metabolites for each drug were also found with all three *in vitro* systems, *i.e.,* NAPQI, MDA, amphetamine, and 11-COOH-THC. The synthetic metalloporphyrin and electrochemical oxidation systems appeared to generate a wider variety of metabolites than encountered with human liver microsomes, including stable and potentially reactive derivatives such as formate AMP, 4-OH-3-bicarbonate AMP, methanimine, carbaldehyde-THC derivative, and methylidene-THC. Results indicate that use of all three *in vitro* systems may provide a more complete profile of potential Phase I oxidative SM and RM for a variety of drugs that may be targeted for analysis in forensic toxicological studies and that reveal possible adduct forming species.

Identification of peptide-drug adducts by in vitro trapping assay.

In the initial part of this study, positive mode MS ionization was employed to analyze drug-GSH adducts. This was performed to identify additional adduct structures that may have been missed in previous work utilizing negative mode ionization. Adducts were detected with positive mode ionization for acetaminophen, cocaine, clozapine, and MDMA. Table 6 summarizes the MS/MS data collected for these drugs. These specific adducts had been previously reported using negative mode ionization by the PI of this project and/or other investigators, as indicated in the table. No novel adducts were identified in these studies.

Table 6. Summary table of the positive mode MS/MS data collected for glutathione adducts.

Drug	Parent Drug $[M+H]^+$	Adducted GSH $[M+H]^+$	Mass error (ppm)	Previously Reported
APAP	152.0706	457.1393	1.53	Zhu et al. (2007)[17]
COC	304.1543	627.2331	-1.75	Schneider and DeCaprio (2013)[12]
CLZ	327.1371	632.2058	1.42	Zhu et al. (2007)[17]
MDMA	194.1176	487.1863	-2.87	Meyer et al. (2014)[18]

In the next part of the study, the specific tryptic peptide containing the reactive ^{93}Cys residue of β-globin (*i.e.,* GTFATLSELH^{93}CDK; β^{93}Cys peptide) was custom synthesized and used as trapping agent in *in vitro* assays. As a positive control, β^{93}Cys peptide with stoichiometric conversion of the thiol was prepared by incubation with IAM, as described under Methods. Samples were analyzed on the LC-QTOF-MS in FIA mode. Figure 22 shows the MS spectrum of the control peptide (A) and adducted peptide (B). The observes mass differential of +91.4614 Da on the doubly charged peptide is consistent with covalent modification of the β^{93}Cys by IAM. The sequence of the Hb peptide with the IAM modification was also confirmed by MS/MS analysis.

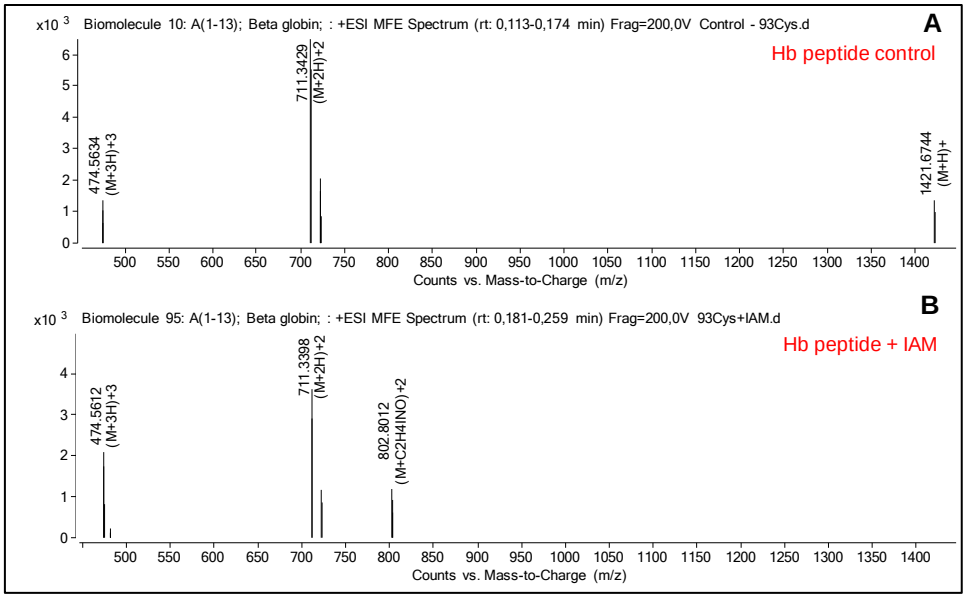


Figure 22. MS spectra of A) control and B) IAM adducted β^{93}Cys peptide.

The β^{93}Cys peptide was then utilized in the HLM metabolic assay as a trapping agent for RM of selected drugs. Theoretical covalent modifications for selected drugs and/or their metabolites were added as target ions to the BioConfirm software via Sequence Manager. MS/MS analysis was then performed to confirm the location of the putative adducts at the Cys moiety. Results for APAP, cocaine, and MDMA are shown in Table 7 and Figure 23.

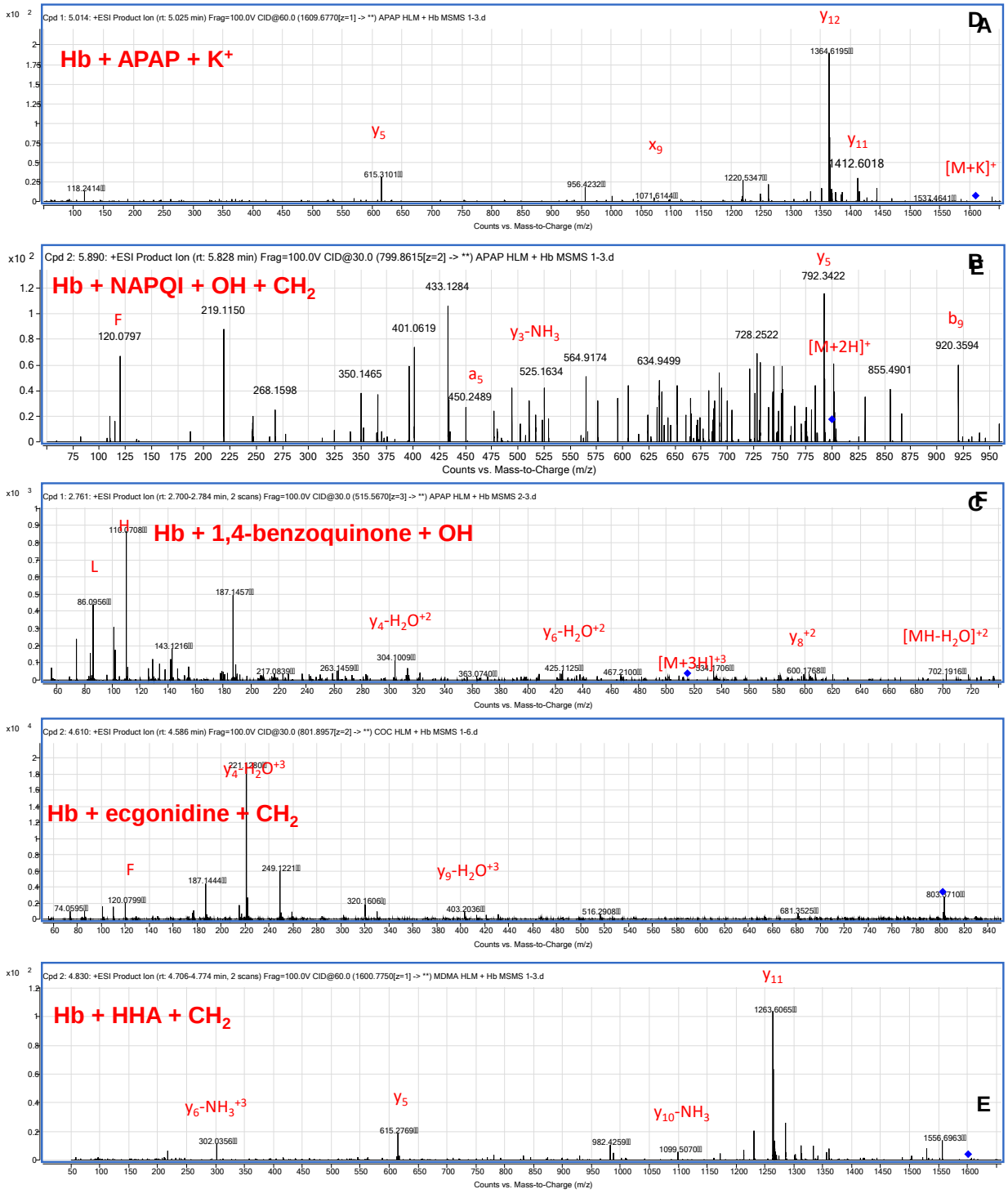
Table 7. MS/MS results the *in vitro* enzymatic metabolism of APAP, cocaine, and MDMA, including proposed composition and major fragment ions detected.

Drug	Molecular Ion (m/z)	Composition	Fragments (m/z)	Fragments (ID)
APAP	1608.6776	Peptide + APAP + K	1412.6018, 1364.6195, 1071.6144, 615.3101	y_{11}, y_{12}, x_9, y_5
	799.8614	Peptide + NAPQI + OH + CH_2	920.3594, 792.3422, 525.1634, 450.2489, 120.0797	b_9, y_5, y_3-NH_3, a_5, F
	515.9012	Peptide + 1,4-benzoquinone + OH	600.1768, 425.1125, 304.1009, 110.0708, 86.0956	y_8^{+2}, y_6-H_2O^{+2}, y_4-H_2O^{+2}, H, L
Cocaine	801.9225	Peptide + ecgonidine + CH_2	403.2036, 221.1280, 120.0799	y_9-H_2O^{+3}, y_4-H_2O^{+3}, F
MDMA	1600.7676	Peptide + HHA + H + CH_2	1263.6065, 1099.5070, 615.2769, 302.0356	y_{11}, y_{10}-NH_3, y_5, y_6-NH_3^{+3}
	539.5802	Peptide + Aminochrome + O + OH	647.2571, 504.1562, 414.1393, 362.1625, 245.1183, 110.0708, 84.0788	y_{10}-H_2O^{+2}, y_7-H_2O^{+2}, y_9^{+3}, x_4^{+2}, y_2-NH_3, H, K

Three peptide modifications were found for APAP. The first exhibited a mass differential of *m/z* +188.0108 and was consistent with addition of APAP plus K^+ ion. With further analysis by Protein Prospector, four diagnostic fragment ions were found (y_{11}, y_{12}, y_9, and y_5) that contained the adducted thiol. The second modification exhibited a mass differential of *m/z* +177.0426, consistent with a NAPQI modification at ^{93}Cys, a hydroxylation at ^{95}Lys, and a methylation at ^{92}His. Three characteristic fragments were found (y_5, y_3-H_2O, and y_9) that contained the adducted thiol. The third adduct found with APAP exhibited a mass differential of *m/z* +124.0160, identified as a 1,4-benzoquinone modification on ^{93}Cys and a hydroxylation on ^{95}Lys. Three diagnostic fragments were found (y_8^{+2}, y_6-H_2O, and y_4-H_2O) that contained the adducted thiol.

For cocaine, one peptide adduct was found, with a mass differential of *m/z* +178.0868, consistent with an ecogonidine modification at ^{93}Cys and a methylation on ^{95}Lys. Two diagnostic fragments of the adducted Hb molecule were found (y_9-H_2O^{+3} and y_4-H_2O^{+3}) that contained the adducted thiol.

Finally, for MDMA, two peptide adducts were found, the first with a mass differential of *m/z* +179.0946 consisting of an HHA modification ^{93}Cys and a methylation on ^{95}Lys. Four diagnostic fragments were found (y_{11}, y_{10}-NH_3, y_5, and y_6-NH_3^{+3}) that contained the adducted thiol. The second peptide adduct exhibited a mass differential of *m/z* +195.0532 consistent with an aminochrome plus oxygen modification on the ^{93}Cys and a hydroxylation on ^{95}Lys. Three diagnostic fragments were found (y_{10}-H_2O^{+2}, y_7-H_2O^{+2} and y_3^{+3}) that contained the adducted thiol.


Cpd 1: 5.014: +ESI Product Ion (rt: 5.025 min) Frag=100.0V CID@60.0 (1609.6770[z=1] -> **) APAP HLM + Hb MSMS 1-3.d
Hb + APAP + K+
y12
y5
x9
y11
[M+K]+
Cpd 2: 5.890: +ESI Product Ion (rt: 5.828 min) Frag=100.0V CID@30.0 (799.8615[z=2] -> **) APAP HLM + Hb MSMS 1-3.d
Hb + NAPQI + OH + CH2
F
y3-NH3
a5
[M+2H]+
b9
Cpd 1: 2.761: +ESI Product Ion (rt: 2.700-2.784 min, 2 scans) Frag=100.0V CID@30.0 (515.5670[z=3] -> **) APAP HLM + Hb MSMS 2-3.d
H
L
Hb + 1,4-benzoquinone + OH
y4-H2O+2
y6-H2O+2
y8+2
[M+3H]+3
[MH-H2O]+2
Cpd 2: 4.610: +ESI Product Ion (rt: 4.586 min) Frag=100.0V CID@30.0 (801.8957[z=2] -> **) COC HLM + Hb MSMS 1-6.d
y4-H2O+3
Hb + ecgonidine + CH2
F
y9-H2O+3
Cpd 2: 4.830: +ESI Product Ion (rt: 4.706-4.774 min, 2 scans) Frag=100.0V CID@60.0 (1600.7750[z=1] -> **) MDMA HLM + Hb MSMS 1-3.d
Hb + HHA + CH2
y11
y6-NH3+3
y5
y10-NH3
Counts vs. Mass-to-Charge (m/z)

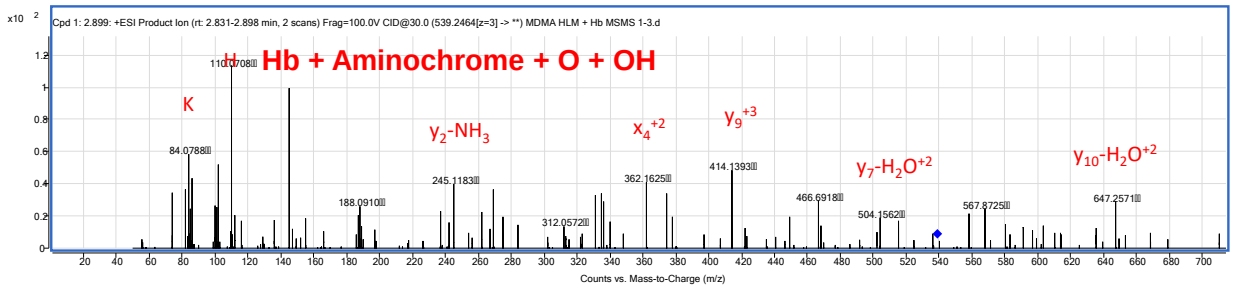


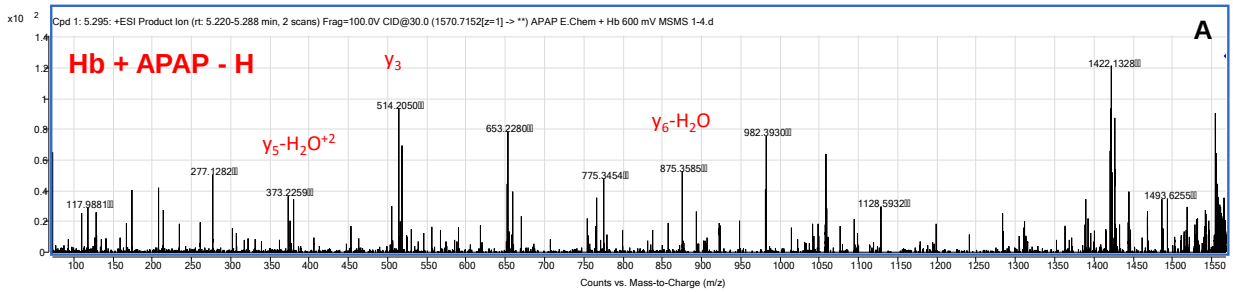
Figure 23. MS/MS spectra of β[93]Cys peptide modified by APAP (A-C), cocaine (D), and MDMA (E and F) obtained with the *in vitro* enzymatic trapping assay.

The *in vitro* enzymatic metabolism studies were also performed for METH and THC; however, no peptide adducts were detected.

Electrochemical oxidation trapping assay MS/MS results for the β[93]Cys peptide treated with APAP, cocaine, and THC are shown in Table 8 and Figure 24.

Table 8. MS/MS data for [93]Cys peptide adducts observed with the electrochemical oxidation of APAP, cocaine, and THC.

Drug	Molecular Ion (*m/z*)	Composition	Fragments (m/z)	Fragments (ID)
APAP	1570.7206	Peptide + APAP - H	875.3585, 514.2050, 373.2259	y_6-H_2O, y_3, y_5-H_2O
	793.8614	Peptide + APAP - H + OH	706.8351, 573.3008, 460.2171, 377.1816, 288.1333, 262.1353, 147.1118	a_{12}^{+2}, b_6-H_2O, b_5-H_2O, b_4, b_3-H_2O, y_2, y_1
	1569.7128	Peptide + NAPQI + OH	1425.6047, 390.1187, 288.1302	y_{11}, y_5^{+2}, b_3-H_2O
Cocaine	534.9326	Peptide + ecgonidine + H + CH_2	1012.4773, 796.3858, 454.7086, 120.0796	y_7, y_5, y_6-NH_3^{+2}, F
THC	882.4317	Peptide + Carbaldehyde-THC - H + OH	573.2977, 377.1789, 288.1344	b_6-H_2O, b_4, b_3-H_2O



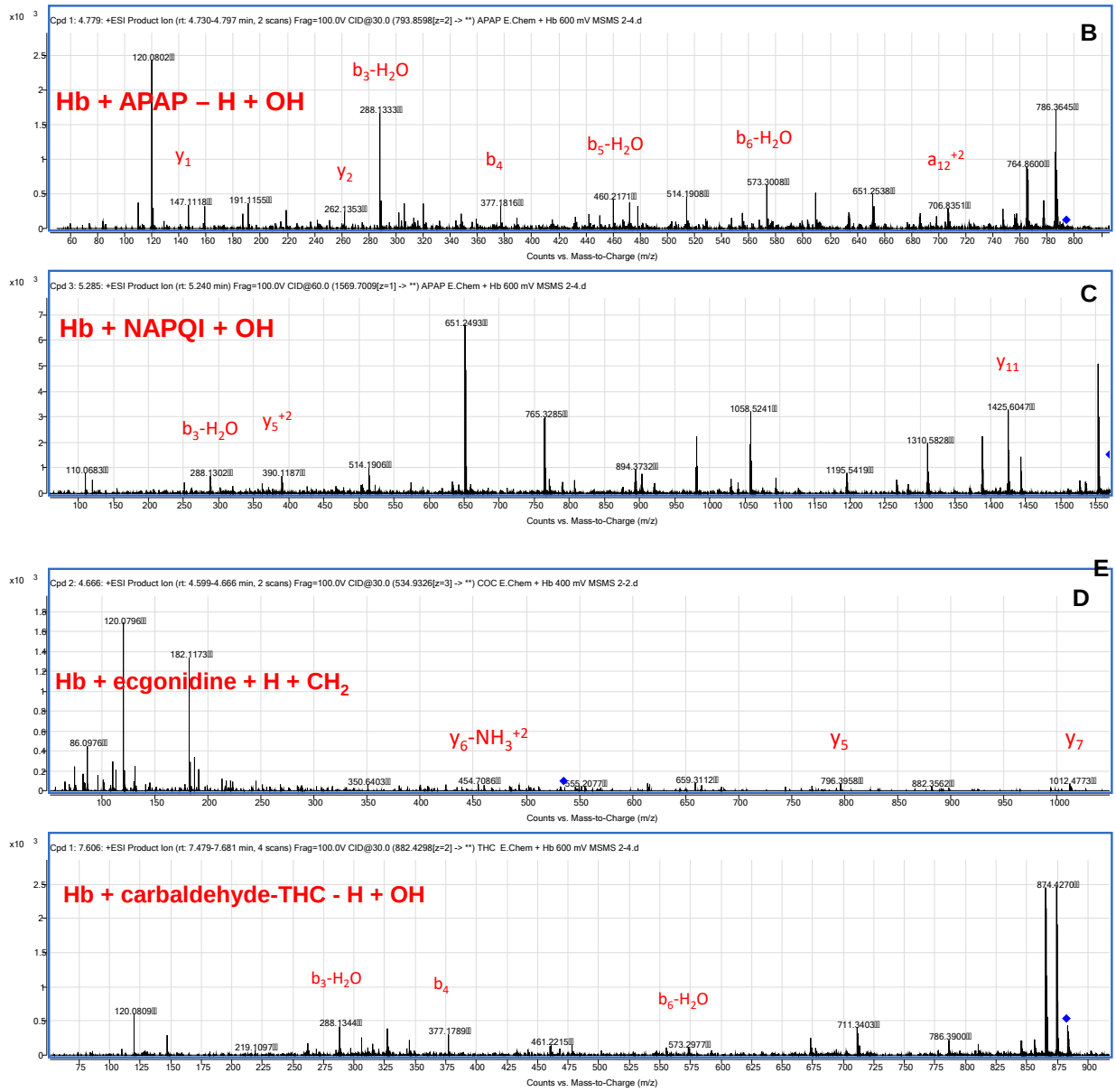


Figure 24. MS/MS spectra of β[93]Cys peptide modified by APAP (A-C), cocaine (D), and THC (E) obtained with the electrochemical oxidation trapping assay.

Three peptide adducts were found with APAP. The first exhibited a mass differential of *m/z* +149.0477, consistent with addition of APAP and loss of H. Protein Prospector analysis indicated three diagnostic fragments (y_6-H_2O, y_3, and y_5-H_2O) that contained the adducted thiol. The second exhibited a mass a mass differential of *m/z* +165.0426, characteristic of addition of APAP at [93]Cys, loss of H, and hydroxylation on [95]Lys. A

single diagnostic fragment (a_{12}^{+2}) that contained the adducted thiol was noted. The third adduct found with APAP exhibited a mass differential of *m/z* +164.0348, corresponding to a NAPQI modification on ^{93}Cys and hydroxylation on ^{95}Lys. Two diagnostic fragments (y_{11} and y_5^{+2}) that contained the adducted thiol were identified.

For cocaine, a single modified peptide species was found, with a mass differential of *m/z* +181.1103, consistent with addition of an ecogonidine moiety and H on ^{93}Cys and methylation of ^{95}Lys. Three diagnostic fragments (y_7, y_5, and y_6-NH_3^{+2}) that contained the adducted thiol were identified.

For THC, a single modified peptide species was found, with a mass differential of *m/z* +342.1831, consistent with addition of a carbaldehyde-THC moiety on ^{93}Cys and hydroxylation on ^{95}Lys. Three diagnostic fragments (b_6-H_2O, b_4, b_3-H_2O) were found.

The *in vitro* EC oxidation studies were also performed for MDMA and METH; however, no peptide adducts were detected.

In summary, results of the *in vitro* trapping assay studies with GSH and the β^{93}Cys peptide revealed that the electrochemical oxidation can be employed in the generation and trapping of metabolites by peptides containing reactive thiol moieties. The ability of abused drugs and/or metabolites to covalently modify the β-Hb peptide containing the reactive ^{93}Cys suggests that such modifications could be monitored as an alternative to clinical and forensic hair analysis, which is the current approach to long-term and retrospective exposure assessment for abused drugs and could be usefully applied in areas of drug testing and forensic toxicological analysis.

Enrichment assay for adducted Hb

Prior to developing the adducted Hb enrichment protocol, studies were conducted

to duplicate in the PI's lab a previously published method for enrichment of adducted SA.[19] For this purpose, SA stoichiometrically modified with iodoacetamide (IAM) at the ^{34}Cys free thiol moiety was prepared as a positive control.

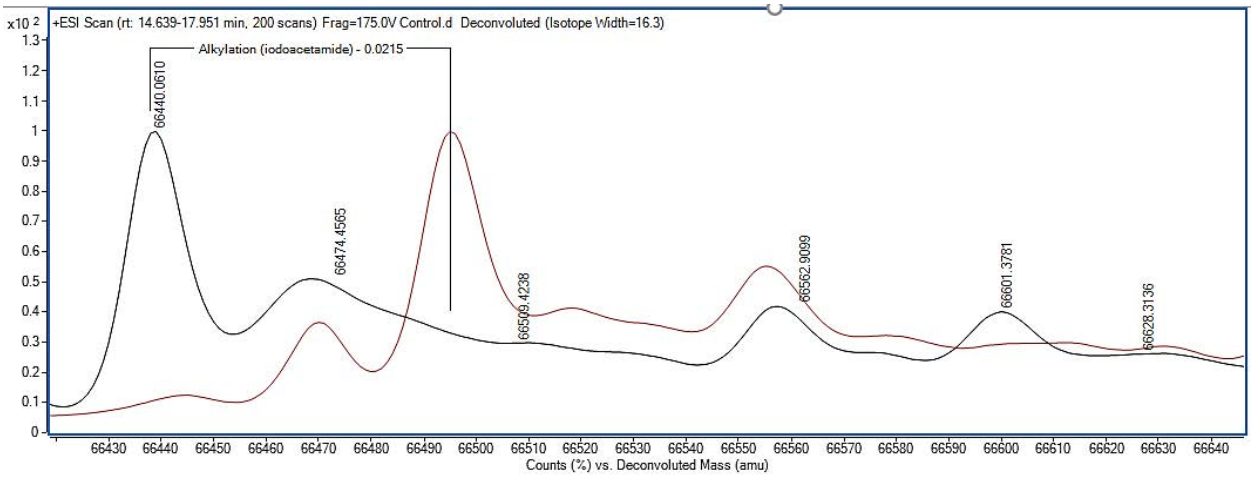


Figure 25. QTOF-MS deconvoluted mass spectrum containing whole SA treated with IAM post enrichment assay.

Figure 25 shows the deconvoluted whole protein mass spectrum of unadducted SA overlaid with IAM adducted SA following the enrichment procedure. BioConfirm reported unadducted SA control (black MS trace) at the correct *m/z* of 66,440 Da. The enriched preparation of IAM adducted HSA (red MS trace) exhibited a peak present at *m/z* 66,497 Da, with a mass differential of +57 Da consistent with the presence of a single IAM modification at the free thiol moiety. The absence of unadducted SA in the enriched sample (red MS trace) confirmed that the enrichment assay was successful in removal of all unmodified protein.

Further SA enrichment studies were done to test the sensitivity of the method. The protocol stated above was followed with a series of serial dilutions of adducted and unadducted SA mixtures (up to a 1:16 molar ratio). Figure 26 shows the deconvoluted

mass spectrum of control SA overlaid with a series of adducted SA dilutions. BioConfirm software again reported unadducted SA control (green MS trace) at the correct *m/z* of 66,439 Da. The diluted mixtures of control and adducted SA treated with the thiol affinity resin show average *m/z* of 66,496 Da, corresponding to a +57 Da modification, with the absence of unmodified product further indicating that the enrichment method was successful, even at high dilution with unadducted protein.

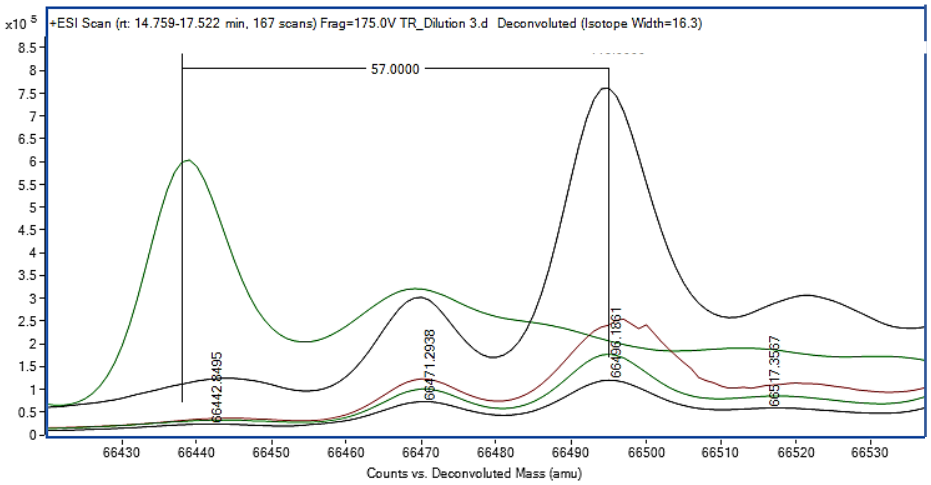


Figure 26. QTOF MS deconvoluted mass spectrum containing whole human SA treated with iodoacetamide post thiol enrichment assay.

This method was then adapted for enrichment of adducted Hb. For these experiments, a positive control adducted Hb was synthesized using N-ethylmaleimide (NEM) as the thiol modification reagent, rather than IAM as was done for SA. NEM was used as the alkylating agent for these experiments, as literature indicated that it is a good Michael acceptor for the nucleophilic addition reaction necessary for adduction to the thiol residue. In addition, the mass increment of the NEM modification is +125 Da, allowing the software to more easily identify any covalent modifications that have occurred as compared to IAM. Finally, the optimal pH condition for NEM is in the 6.6 -

7.6 range, which is ideal for the experiments that were performed at a biologic pH 7.4.

Figure 27 shows the deconvoluted mass spectrum of unmodified control Hb (orange) overlaid with NEM adducted Hb (blue) which had been subjected to the optimized thiol enrichment assay. BioConfirm analysis for Hb control (orange) produced a confirmed match for the β-subunit of Hb with *m/z* of 15,867.7265 Da. The 2:1 mixture sample containing both Hb control and alkylated Hb+NEM (blue) produced a confirmed match for the β-subunit of Hb plus one addition of NEM, with *m/z* of 15,992.7574 Da. The delta mass caliper tool was used to determine that there was a mass differential of +125 Da between the adducted and control samples. The enrichment of the mixture sample was deemed successful, due to the complete removal of unadducted Hb that was added to the mixture. This result is the first instance of successful enrichment of adducted Hb using this type of approach.

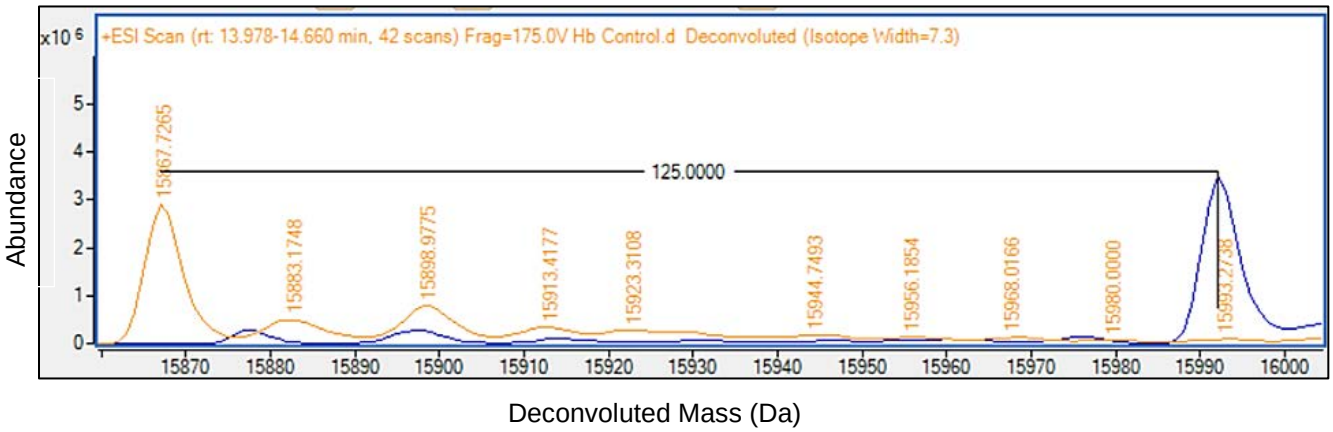


Figure 27. QTOF mass spectrum overlay of unadducted Hb (orange) and a 2:1 mixture of Hb control:alkylated Hb (blue) that was subjected to the thiol enrichment assay.

After successful initial studies on the enrichment of covalently modified β-subunit of Hb, experiments were conducted to determine which of the free thiols on the β-subunit were being covalently modified by NEM. This was done by following the Hb enrichment

protocol as above with added trypsin digest steps for bottom-up proteomic analysis. Figure 28 shows the mass spectra of the tryptic peptide GTFATLSELCDK that contains the reactive β^{93}Cys moiety for control Hb (panel A) and adducted Hb-Cys-NEM (panel B).

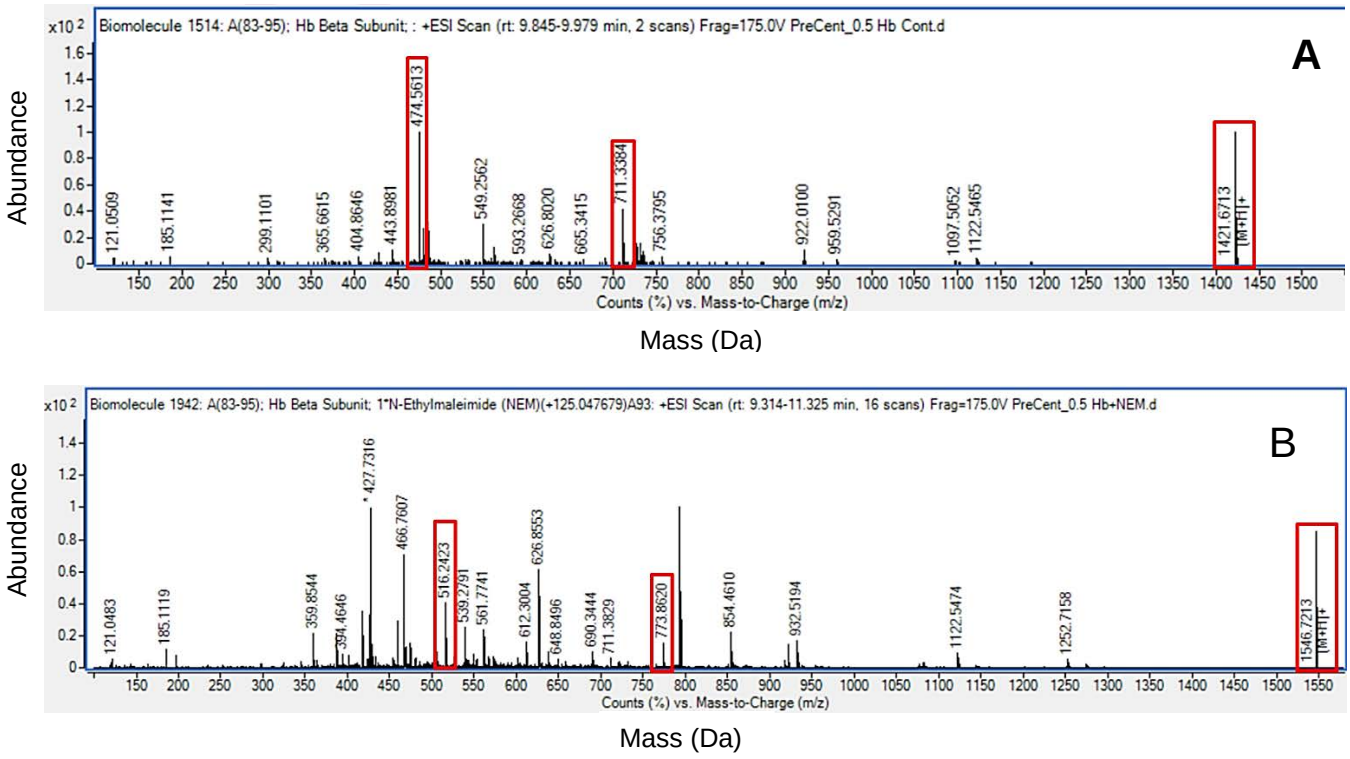


Figure 28. (A) QTOF-MS/MS spectrum of unadducted (control) Hb tryptic peptide, (B) QTOF-MS/MS spectrum of NEM modified Hb tryptic peptide GTFATLSEHCDK following the enrichment procedure.

These mass spectra were generated using BioConfirm B.08.00 software. Spectrum A of unmodified Hb control resulted in a confirmed match for the unadducted tryptic peptide GTFATLSEHCDK and contained three primary ions of interest. The first ion at 1421.6713 Da represented the $[M+H]^+$ protonated molecular ion of the peptide, with a mass error of 1.48 ppm. Additional ions at 711.3384 Da and 474.5613 Da represented the $[M+H]^{+2}$ and $[M+H]^{+3}$ multiply charged states, respectively. Both of ions also exhibited mass errors well below the set 5 ppm acceptance limit.

Spectrum B shows a confirmed match for the tryptic peptide with one covalent modification by NEM. The $[M+H]^+$ of this modified peptide is 1546.7213 Da and has a mass error of -1.69 ppm. This is significant because there is a delta mass differential of 125.05 Da from the unmodified Hb control to the enriched adducted sample, indicating that there is only one NEM adduct modification in this peptide.

Following up on this finding, MS/MS analysis of the covalently adducted peptide was performed. Figure 29 shows an Auto-MS/MS spectrum of the NEM adducted tryptic peptide at the ^{93}Cys moiety. Peak annotation of the MS/MS spectrum was done using

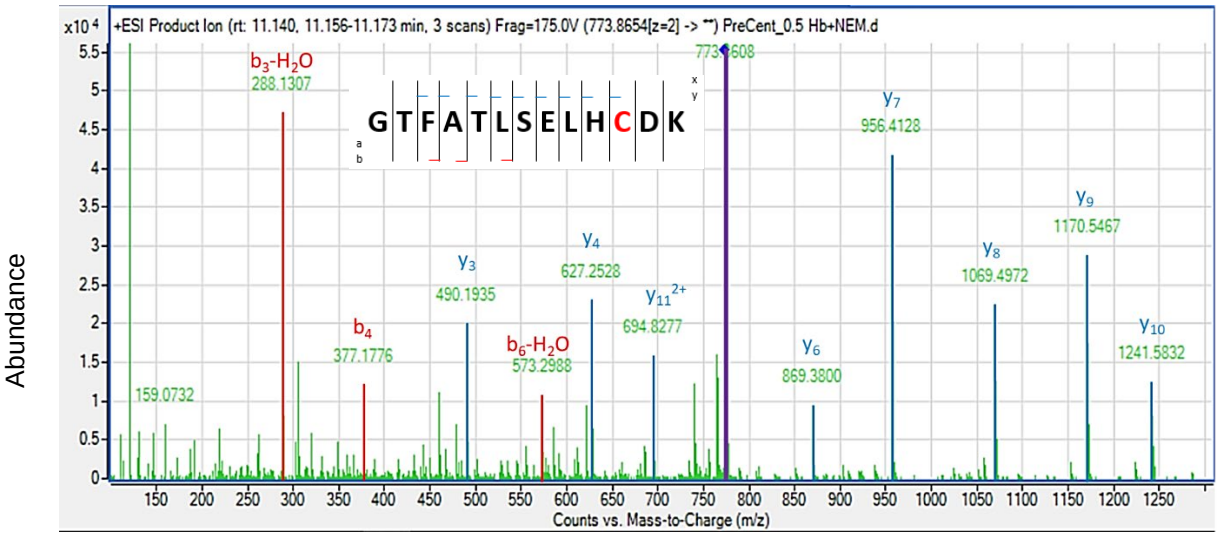


Figure 29. QTOF-MS/MS mass spectra of positive Hb-Cys-NEM adduct highlighting fragments associated with cysteine adduction of NEM. Inset shows peptide fragmentation template using standard notation.

BioConfirm and Protein Prospector. The labeled ions use the standard notation of a,b,c/x,y,z for peptide fragmentation of peptides in MS/MS analysis (Figure 29, inset). The y series ions that are denoted are of significance because they contain fragments of the tryptic peptide containing the NEM modified β^{93}Cys. These results confirm that covalent adduction occurred specifically at the β^{93}Cys moiety of interest.

Following up on these experiments, additional sensitivity tests of the enrichment method for Hb were conducted. Protocol for the sensitivity tests followed the method described in the previous section on enrichment of adducted human Hb. Aliquots of unadducted control Hb and covalently modified Hb-NEM were mixed in 100:1, 1000:1, and 10,000:1 unadducted:adducted molar ratios and then subjected to enrichment to assess the sensitivity and selectivity of the procedure. Figure 30 shows the whole protein deconvoluted mass spectra of unenriched control Hb (Panel A) and enriched

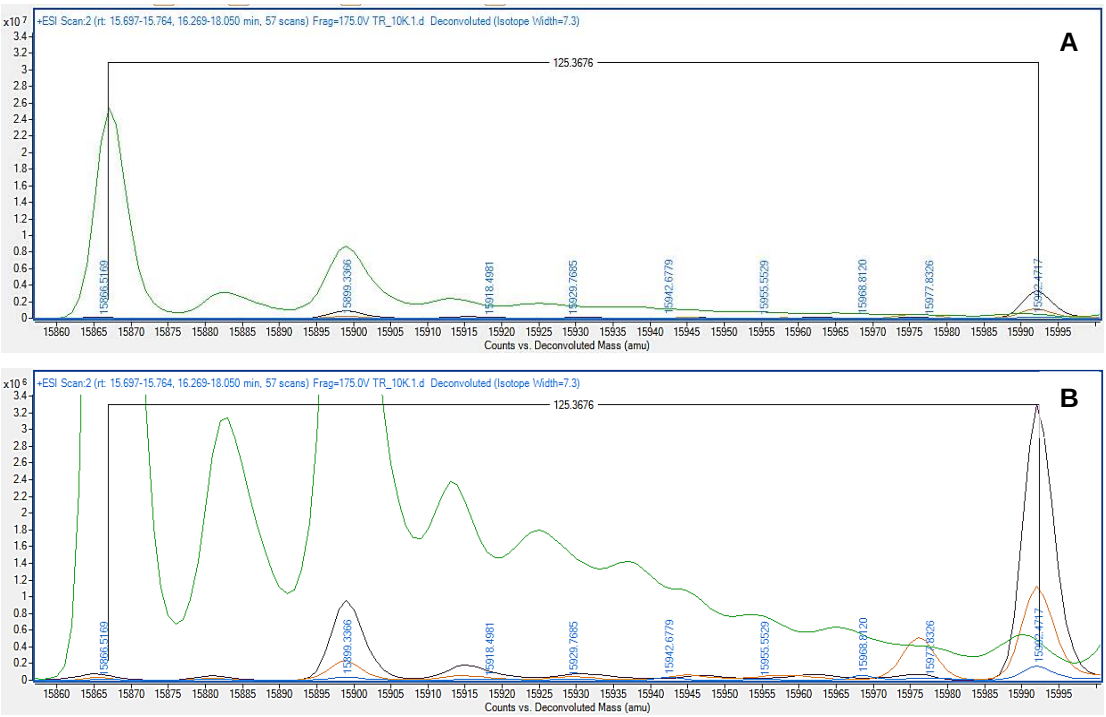


Figure 30. (A) QTOF-MS whole protein deconvoluted mass spectra overlay of unenriched control Hb (green) and enriched mixtures of control and NEM-adducted Hb mixtures at 100:1 (black), 1000:1 (orange), and 10,000:1 (blue) molar ratios. (B) 10X scaling of spectra in (A).

mixtures.

BioConfirm analysis for unadducted Hb control produced a confirmed match for the β-subunit with a *m/z* of 15,867.5795 Da. This result is consistent with the literature

reported mass of the β-subunit of unadducted Hb. The enriched mixtures all exhibited *m/z* values of 15,992.1 to 15,992.7 Da and were confirmed to have a mass differential of +125.3676 Da, confirming that they contain a single NEM modification. The enrichment for these mixtures was successful in that complete removal of unadducted Hb was achieved, leaving only the Hb-Cys-NEM covalently adducted product. In addition, adducted Hb was readily enriched even when at present at very low abundance (10,000:1) as compared to unadducted species. These experiments indicate that analyses of authentic Hb from drug users are likely to have sufficient sensitivity to detect adducted β^{93}Cys moiety.

Identification of Hb adducts by top-down and bottom-up proteomics

Initial studies focused on the identification of covalent thiol modifications by selected drugs in unenriched Hb treated in the HLM trapping assay. In these experiments, drug treated Hb was subjected to tryptic digestion and peptides analyzed by full scan QTOF-MS. These preliminary studies demonstrated promising adduction data for APAP, METH, THC, oxycodone, naltrexone, and α-PVP. Table 9 summarizes the MS data collected for these drugs.

For both APAP and METH, the adducted peptide mass corresponded to addition of unmodified parent drug to β^{93}Cys, a finding in agreement with our previous data on GSH adduction for these compounds. The adducted peptide mass observed for THC corresponded to addition of an 11-hydroxy-THC metabolite moiety to β^{93}Cys, also in agreement with earlier GSH studies. In addition to the highly reactive β^{93}Cys, APAP was found to produce an adduct at the β^{112}Cys residue, while METH produced adducts at

both the β^{112}Cys and α^{104}Cys residues. Two of the observed METH adducts and the single observed THC adduct had additional modifications to the peptide chain (*i.e.,* hydroxy and/or acetyl moieties) as determined by the BioConfirm software. For oxycodone, naltrexone, and α-PVP, the adducted peptide mass corresponded to addition of unmodified parent drug at β^{93}Cys, also in agreement with our previous GSH data.

Table 9. Summary table of the MS data collected for Hb adduction in unenriched preparations for selected drugs, with site of modification and mass difference from calculated exact mass.

Drug	Adduction Site	Other Mods	Δ*m/z* (ppm)	Adducted Peptide Mass (Da)
APAP	β^{93}Cys	none	2.93	1569.7180
APAP	β^{112}Cys	none	-3.29	1868.0069
α-PVP	β^{93}Cys	none	-3.63	1651.8220
METH	β^{93}Cys	none	-4.63	1597.7632
METH	α^{104}Cys	hydroxy + acetyl	3.36	3171.7261
METH	β^{93}Cys	acetyl	5.14	1609.7728
NAL	β^{93}Cys	none	-0.61	1759.8117
OXY	β^{93}Cys	none	3.57	1733.8033
OXY	α^{104}Cys	hydroxy + acetyl	7.84	3337.7682
THC	β^{93}Cys	hydroxy + acetyl	4.34	2914.4338
THC	β^{93}Cys	hydroxy	-7.16	1780.8466

For MS/MS analysis of adducted peptides, enrichment of adducted Hb followed by tryptic digestion was required, as MS/MS of unenriched preparations lacked the sensitivity required for these determinations. These studies also focused on detection of thiol modifications in the tryptic peptide (*i.e.,* GTFATLSELHCDK) containing the β^{93}Cys moiety. Table 10 summarizes the MS/MS data for the covalent adducts of acetaminophen, clozapine, oxycodone, cocaine, Δ^9-THC, and diazepam at β^{93}Cys in this peptide. Covalent adducts were detected using BioConfirm B.08.00 software. Of the drugs examined, only metabolites (rather than parent drugs) were found to form covalent adducts at the β^{93}Cys. Based upon literature review, proposed binding

sites/structures are proposed for some of the adducted species.

Table 10. Summary table of MS data collected for peptide containing β^{93}Cys Hb adducts with acetaminophen, clozapine, oxycodone, cocaine, Δ^9-THC, and diazepam.

Drug	Adduct Moiety	Molecular Ion	Observed Mass (Da)	Target Mass (Da)	Mass Error (ppm)
APAP	NAPQI	$[M+H]^{+2}$	785.9110	785.3755	4.32
clozapine	clozapine-N-oxide	$[M+H]^{+}$	1761.1689	1761.7820	-1.76
clozapine	desmethylclozapine	$[M+H]^{+}$	1731.7722	1731.7715	-3.57
oxycodone	noroxycodone	$[M+H]^{+}$	1721.6820	1721.9381	-4.63
cocaine	hydroxybenzoylnorecgonine	$[M+H]^{+}$	1710.1374	1710.7680	-1.63
Δ^9-THC	11-oxo-Δ^9-THC	$[M+H]^{+}$	1747.8640	1747.8611	0.62
Δ^9-THC	9α,10α-epoxy-Δ^9- THC	$[M+H]^{+}$	1749.2137	1749.8768	5.83
diazepam	hydroxydiazepam	$[M+H]^{+}$	1719.7244	1719.7238	-4.21

An auto MS/MS method was used to collect data for APAP and cocaine before transitioning to a targeted MS/MS method for clozapine and oxycodone. The example MS/MS spectrum in Figure 31 shows the fragments that correspond to an acetaminophen metabolite (NAPQI) adduct located at β^{93}Cys in the tryptic peptide. Four MS/MS fragments (Figure 32) were deemed significant, as they contain the cysteine-NAPQI covalent adduct. The labeled peaks use the *a,b,c* and *x,y,z* nomenclature, common for peptide fragmentation in MS/MS. All significant fragments were cross referenced with Protein Prospector.

The proposed binding mechanism of NAPQI to Hb β^{93}Cys according to literature review would be a S_n2 nucleophilic conjugate addition reaction that occurs at one of the double bonds in the ring structure. The nucleophilic thiol attacks the π-antibonding orbital along the ring structure generating the covalent bond between NAPQI and thiol of β^{93}Cys. The p-benzoquinone imine is the reactive electrophile that covalently adducts to the nucleophilic thiol. Literature suggests when the nucleophilic attack occurs by the process of 1,4 Michael-type addition, the p-benzoquinone imine is rearranged into the

covalent adduct shown.

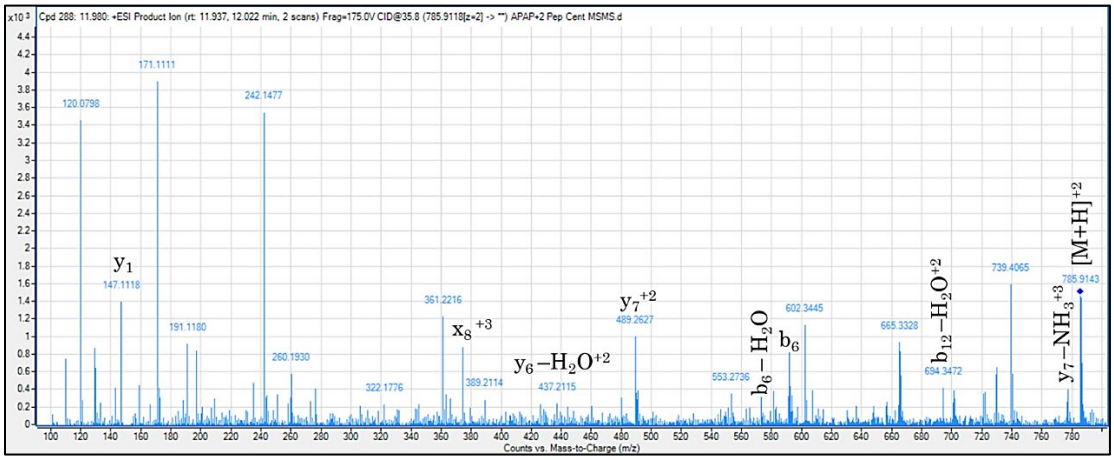


Figure 31. QTOF-MS/MS mass spectrum of β[93]Cys peptide from APAP treated Hb, with significant fragments associated with cysteine adduction by NAPQI.

Fragment	Mass (Da)
y_1	147.0455
y_4^{+2}	325.1635*
x_6^{+2}	458.2591*
$y_8\text{-}H_2O^{+2}$	535.3231*
x_8^{+2}	561.2907*
$y_{12}\text{-}H_2O^{+2}$	748.6831*
x_{12}^{+2}	769.3889*
$y_7\text{-}H_2O$	959.7971*
$[M+H]^+$	1570.7770

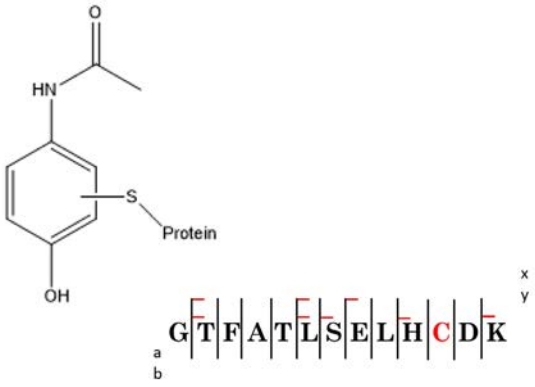


Figure 32. Significant MS/MS fragments for the adducted β[93]Cys peptide in Hb treated with APAP. Fragments that contain the Cys-NAPQI adduct are shown with asterisks. Proposed adduct structure and link to β[93]Cys moiety are shown.

Figure 33 shows the MS/MS spectrum for the clozapine N-oxide adduct located at β[93]Cys in the tryptic peptide GTFATLSELHCDK. Figure 34 shows the two significant MS/MS fragments that contain the cysteine-clozapine-N-oxide adduct.

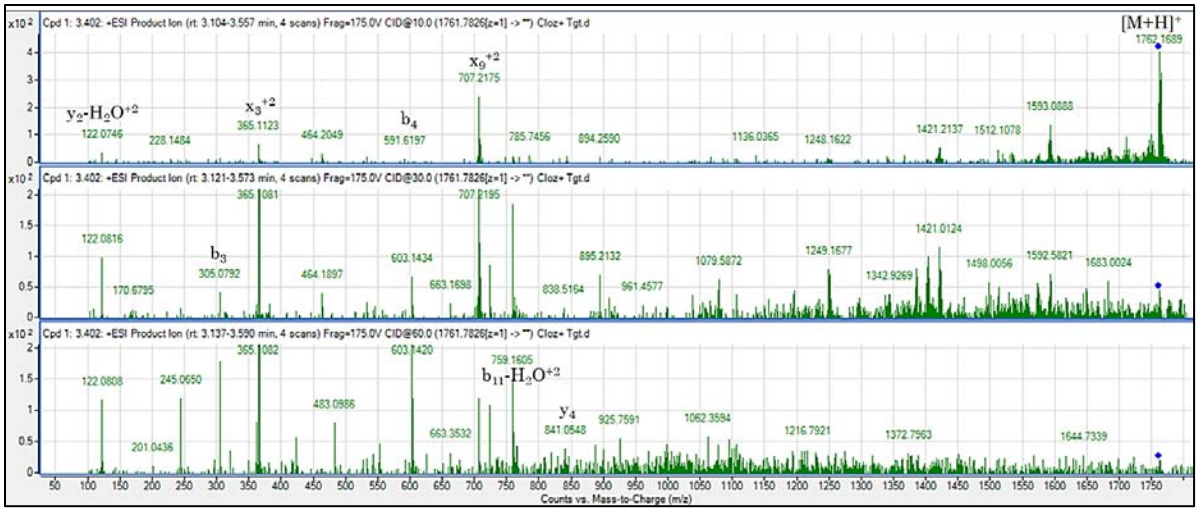


Figure 33. QTOF-MS/MS mass spectrum of β[93]Cys peptide from clozapine treated Hb, with significant fragments associated with cysteine adduction by clozapine-N-oxide.

Fragment	Mass (Da)
y_2-H_2O^{+2}	122.0746
b_3	305.0792
b_4	591.6197
b_{11}-H_2O^{+2}	759.1605*
y_4	541.0548*
$[M+H]^+$	1761.7820

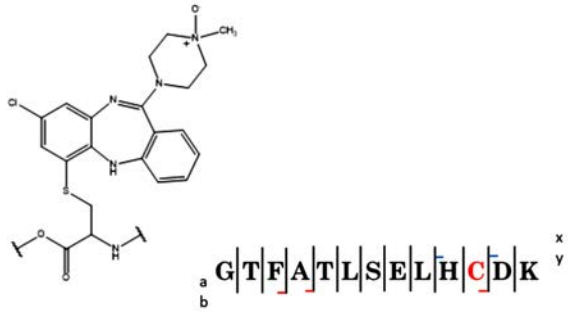


Figure 34. Significant MS/MS fragments for the adducted β[93]Cys peptide in Hb treated with clozapine. Fragments that contain the Cys-clozapine-N-oxide adduct are shown with asterisks. Proposed adduct structure and link to β[93]Cys moiety are shown.

The proposed binding mechanism of clozapine-N-oxide to Hb β[93]Cys according to literature review would involve initial Phase I enzyme oxidation of clozapine to the electrophilic metabolite clozapine-N-oxide. The charge on the nitrogen in the piperazine ring causes a shift in the delocalized π electron cloud that pulls towards the charged nitrogen allowing for the cysteine thiol to nucleophilic attack a double bond of the chloro-ring structure on the opposite side of the molecule.

Figure 35 shows the fragments that correspond to the desmethylclozapine adduct

located at β[93]Cys in the tryptic peptide GTFATLSELHCDK. Seven significant MS/MS fragments were found that contain the cysteine desmethylclozapine covalent adduct.

Fragment	Mass (Da)
y_2-NH_3	243.6292
a_4	348.9530
x_7^{+2}	391.1648*
y_9-H_2O^{+3}	446.6723*
x_8^{+2}	583.2885*
x_9^{+2}	692.2335*
y_{12}^{+2}	837.9591*
y_7-NH_3	1124.5067*
$[M+H]^+$	1731.7722

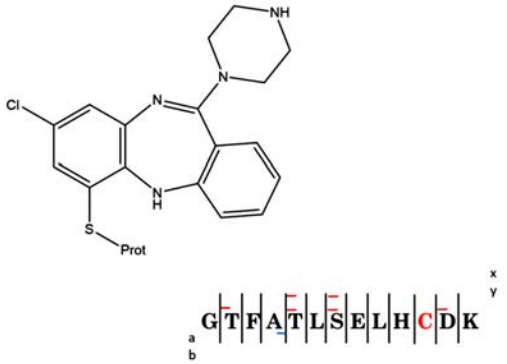


Figure 35. Significant MS/MS fragments for the adducted β[93]Cys peptide in Hb treated with clozapine. Fragments that contain the Cys-desmethylclozapine adduct are shown with asterisks. Proposed adduct structure and link to β[93]Cys moiety are shown.

Figure 36 shows the fragments that correspond to the oxycodone metabolite noroxycodone adduct located at β[93]Cys in the tryptic peptide GTFATLSELHCDK. Six significant MS/MS fragments were found that contain the cysteine-noroxycodone covalent adduct.

Fragment	Mass (Da)
y_4-NH_3^{+3}	271.1994*
b_4-H_2O	360.2157
y_4-H_2O^{+2}	404.2197*
b_5-H_2O	461.2661
y_9^{+2}	686.0073*
b_9	921.5186
y_8	1270.7776*
M-NH_3	1730.0706*
$[M+H]^+$	1747.8620

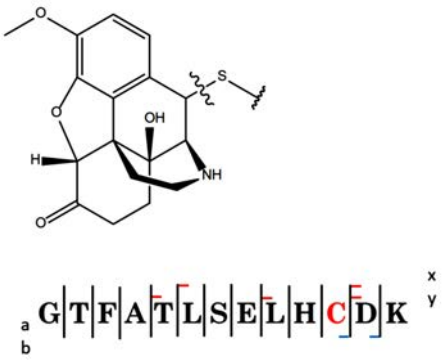


Figure 36. Significant MS/MS fragments for the adducted β[93]Cys peptide in Hb treated with oxycodone. Fragments that contain the Cys-noroxycodone adduct are shown with asterisks. Proposed adduct structure and link to β[93]Cys moiety are shown.

Oxycodone metabolites have not previously been reported to covalently adduct Hb β^{93}Cys. The proposed binding linkage is based on previous work in the laboratory examining oxycodone covalent adduction to GSH. While the proposed binding places the thiol linkage at the benzylic carbon, further confirmation is needed.

Figure 37 shows the fragments that correspond to cocaine metabolite hydroxybenzoylnorecgonine adduct located at β^{93}Cys in the tryptic peptide GTFATLSELHCDK. Two significant MS/MS fragments were found to contain the cysteine-hydroyxybenzoylnorecgonine adduct.

Fragment	Mass (Da)
y_2-H_2O^{+3}	122.0796
y_2-NH_3	245.0633
y_5-H_2O^{+3}	295.0427*
y_{10}-NH_3^{+3}	464.1739*
b_9-H_2O	901.3006
b_9	920.0733
y_9	1338.4512*
x_{10}	1430.4621*
y_{11}-H_2O	1532.1430*
y_{12}	1651.8248*
$[M+H]^+$	1710.7685

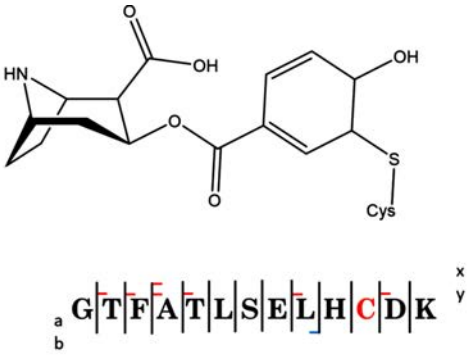


Figure 37. Significant MS/MS fragments for the adducted β^{93}Cys peptide in Hb treated with cocaine. Fragments that contain the Cys hydroxybenzoylnorecgonine adduct are shown with asterisks. Proposed adduct structure and link to β^{93}Cys moiety are shown.

Figure 38 shows the fragments that correspond to Δ^9-THC metabolite 11-oxo-Δ^9-THC adduct located at β^{93}Cys in the tryptic peptide GTFATLSELHCDK. Six significant MS/MS fragments were found to contain the cysteine-11-oxo-Δ^9-THC adduct.

Fragment	Mass (Da)
y_2-H_2O^{+2}	122.0746
x_2^{+2}	145.0483
y_2-NH_3	245.0544
y_5^{+3}	305.0726*
x_8^{+3}	423.0495*
y_9^{+2}	671.1932*
b_{12}-H_2O^{+2}	778.0978*
b_{12}^{+2}	808.0811*
b_{11}	1460.7274*
$[M+H]^+$	1721.6820

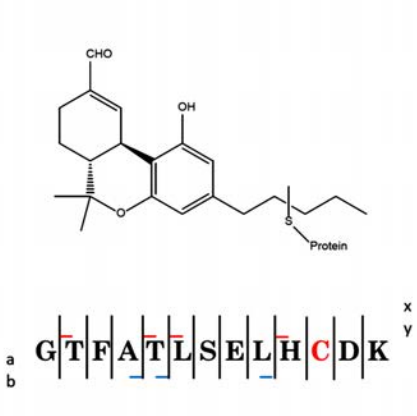


Figure 38. Significant MS/MS fragments for the adducted β^{93}Cys peptide in Hb treated with THC. Fragments that contain the Cys 11-oxo-Δ^9-THC adduct are shown with asterisks. Proposed adduct structure and link to β^{93}Cys moiety are shown.

Figure 39 shows the fragments that correspond to an adduct located at β^{93}Cys in the tryptic peptide GTFATLSELHCDK, proposed to form via the Δ^9-THC metabolite 9α,10α-epoxy-Δ^9-THC. Four significant MS/MS fragments were found to contain this adduct.

Fragment	Mass (Da)
y_1	147.3245
b_3-H_2O	289.0891
y_9-H_2O^{+3}	451.1328*
y_{12}^{+3}	565.1329*
x_8^{+2}	649.2131*
x_8	1299.4117
a_{11}	1460.5058*
$[M+H]^+$	1749.8033

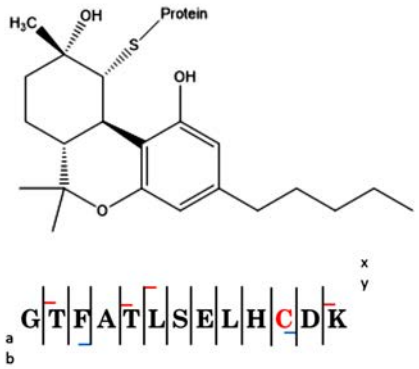


Figure 39. Significant MS/MS fragments for the adducted β^{93}Cys peptide in Hb treated with THC. Fragments that contain the Cys 9α,10α-epoxy-Δ^9-THC derived adduct are shown with asterisks. Proposed adduct structure and link to β^{93}Cys moiety are shown.

Figure 40 shows the fragments that correspond to diazepam metabolite

hydroxydiazepam adduct located at β[93]Cys in the tryptic peptide GTFATLSELHCDK. Three significant MS/MS fragments were found to contain the cysteine hydroxydiazepam adduct.

Fragment	Mass (Da)
y_2-H_2O^{+2}	122.0746
y_2-NH_3	245.0544
b_3-H_2O	289.0876
x_8^{+2}	424.0728*
b_{11}-H_2O	1475.6543*
y_{12}	1662.5126*
$[M+H]^+$	1719.7244

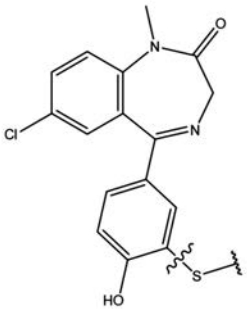


Figure 40. Significant MS/MS fragments for the adducted β[93]Cys peptide in Hb treated with diazepam. Fragments that contain the Cys hydroxydiazepam adduct are shown with asterisks. Proposed adduct structure and link to β[93]Cys moiety are shown.

In summary, results of the *in vitro* trapping assay studies with Hb protein revealed a number of drug specific adducts that could be targeted in a screening assay. A protocol for tryptic digestion of whole Hb protein and selective identification of β[93]Cys modifications by selected drugs of abuse was developed and optimized. Adduct detection at high sensitivity was facilitated by the development of a novel approach to enrich adducted Hb protein by removal of unadducted protein species from the preparation.

Limitations:

Results from this project provide additional proof of concept for the development of a blood protein modification assay for characterization of retrospective abused drug

exposure. These results are limited in that data were generated using *in vitro* models and HRMS analysis. Final development of a routine screening assay will require assessment of authentic blood specimens and transfer of the analytical method to an LRMS platform, which is in more common use in forensic toxicology labs. These studies are currently underway.

Artifacts

List of products:

Peer-reviewed publications:

Gilliland, R.A., Möller, C., and DeCaprio, A.P. (2018). LC-MS/MS based detection and characterization of covalent glutathione modifications formed by reactive drug of abuse metabolites, *Xenobiotica,* 49, 778-790*.*

Four additional manuscripts are in preparation.

Invited platform presentations:

DeCaprio, A.P. and Gilliland, R.A. (2018). Analysis of drug-protein modifications in forensic toxicology. *Invited talk presented at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon),* Orlando, FL; February 26 - March 1.

DeCaprio, A.P., Gilliland, R.A., Tavares, L., and Morrison, W. (2019). Development and validation of a blood protein modification assay for retrospective detection of abused drug exposure. *Invited talk presented at the Pittsburgh Conference on Analytical*

Chemistry and Applied Spectroscopy (Pittcon), Philadelphia, PA; March 17 - 21.

DeCaprio, A.P. (2020). Retrospective exposure assessment for drugs of abuse. *Invited seminar presented to the Department of Environmental Health Sciences, Florida International University,* December 10.

DeCaprio, A.P., Morrison, W.J., and Tavares, L.S. (2022). Blood protein modification assay for retrospective detection of abused drug exposure. *NIJ Forensic Science and Research and Development Symposium (virtual),* March 1-2.

Research poster presentations:

Gilliland, R.A. and DeCaprio, A.P. (2018). Analysis and characterization of in vitro glutathione adducts formed with drugs of abuse. *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon),* Orlando, FL; February 26 - March 1.

Gilliland, R.A. and DeCaprio, A.P. (2018). A mass spectrometric approach to the analysis of covalent modifications of blood proteins by drugs of abuse. *American Academy of Forensic Sciences 70th Annual Meeting,* Seattle, WA; February 19-23.

Morrison, W.J. and DeCaprio, A.P. (2021). Enrichment of hemoglobin covalently adducted at β^{93}Cys by reactive xenobiotics as potential biomarkers of drug exposure. *Annual Meeting of the Society of Forensic Toxicologists,* Nashville, TN; September 26 - October 1.

Tavares, L.S. and DeCaprio, A.P. (2021). Comparison of *in vitro* systems for the generation of drug metabolites in forensic toxicology. *Annual Meeting of the Society of Forensic Toxicologists,* Nashville, TN; September 26 - October 1.

Morrison, W.J. and DeCaprio, A.P. (2022). Analysis of human hemoglobin covalently adducted at β^{93}Cys by reactive metabolites as potential biomarkers of abused drug exposure. *Annual Meeting of the Society of Toxicology,* San Diego, CA; March 27 - 31.

Tavares, L.S. and DeCaprio, A.P. (2022). *In vitro* generation of reactive drug metabolites and trapping by a thiol-containing hemoglobin peptide. *Annual Meeting of the Society of Toxicology,* San Diego, CA; March 27 - 31.

Data sets generated:

Nothing to report.

Dissemination activities:

Six research posters and four invited talks were presented at national toxicology and forensic science conferences. One peer-reviewed article was published and four articles are in preparation.

Literature cited:

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liquid chromatography high-resolution mass spectrometry/mass spectrometry and studies on their glutathionyl transferase inhibition potency. *Anal. Chim. Acta 822*, 37-50.

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