Award Number:  W81XWH-11-2-0126

TITLE: Characterization of the Human Proteomic Response to Hydrocodone: A Preliminary Study

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REPORT DATE:  March 2013

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT:  Approved for public release; distribution unlimited

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Characterization of the Human Proteomic Response to Hydrocodone: A Preliminary Study

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Lakewood, WA 96499

Non-medical use and abuse of prescription opioids is a growing problem in both the civilian and military communities. Current technologies for detecting hydrocodone use are limited. Standard drug screens do not detect hydrocodone. In order to detect the use of hydrocodone and prescription opioids for nontherapeutic purposes, it is vital to establish the excretion profile of these drugs. Currently there is no data available describing blood, urine and oral fluid profiles following administration of a 10 mg dose of hydrocodone. We will measure proteomes and metabolites in blood, urine and oral fluid samples after hydrocodone exposure. We are exposing healthy volunteers to 10 mg pure hydrocodone under controlled conditions and collecting blood, oral fluid, and urine at defined intervals up to 7 days. We included 2 subjects for control in the study giving a total of 41 participants.

Proteomic, drug abuse, opioids, metabolites, and pharmacokinetic
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INTRODUCTION:
The purpose of the overall protocol is to study the metabolism and protein expression in the urine and blood of human subjects administered hydrocodone. An opioid is prescribed as a pain medication to the patient to minimize pain. Hydrocodone will be administered to healthy volunteers. Urine and blood will be collected prior to and following administration of the drug. The three separate biofluids will be analyzed for drug and metabolites and for changes in protein expression. Changes in protein expression will provide a general understanding of opioid exposure in future studies relating to opioid abuse.

BODY:
Phase 1 – Single Dose Administration
1. Institutional Review Board (IRB) application
   - Annual Review submitted to IRB and study approved for another year, expires May 2013.
   - IRB annual report will be submitted in March 2013 for IRB review in April 2013.
   - The Clinical Research Division Quality Assurance and Education Branch conducted an assessment of this study on 11 Jul 2012. No findings were noted, however, it was noted the investigator did not scan the ICD into the subject’s AHL TA note.
   - Literature search was conducted in Feb. 2013 and no new information is available that would change the risk: benefit ratio.

2. Research Nurse coordinator
   - Research Nurse was hired in Q1 of 2011, employed via the Geneva Foundation and remains on the study.

3. Lab technician
   - Lab technician was hired in Q1 of 2011, employed via the Geneva Foundation and remains on the study.

Phase 2 – Patient recruitment
4. Drug Administration, biofluid sampling and PK Analysis
   - Forty-one Subjects signed the Informed Consent, 2 withdrew, and 39 were randomized. See Table 1 below
   - Enrollment is complete. Last Subject and last study visit occurred 07 May 2012.
   - Urine was collected for up to 5 days following administration of hydrocodone.
   - Blood was collected at specified time points throughout the first day then at 24, 48, 96, and 168 hour post dose.
   - Samples are stored refrigerated or frozen until analysis.
   - Liquid chromatography-mass spectrometry (LC-MS/MS) method validations for analysis of hydrocodone and metabolites in urine and plasma using UCT Excel I solid phase extraction columns have been completed.
   - PK analysis on plasma samples for all subject sets completed.
   - All subject sample sets have been shipped and received at PNNL for proteomic analysis on predefined intervals based on PK results.
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<td>-</td>
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<tr>
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### Local Unanticipated Problems previously reported to the IRB.

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<th>Description</th>
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<td>06 June 2011, Vomited at 12 noon, dissipated after event</td>
<td></td>
</tr>
<tr>
<td>Subject 10 (I)</td>
<td>13 June 2011, vomited at 0805, dissipated at 12 noon</td>
<td></td>
</tr>
<tr>
<td>Subject 19 (I)</td>
<td>15 Aug. 2011, vomited at 1030 &amp; 1100, dissipated after 2nd event</td>
<td></td>
</tr>
</tbody>
</table>

Unanticipated AE- Vomiting:
Each subject was closely monitored, vomiting resolved on the same day and before leaving the research facility. No Subject had to seek other medical care.
Per medical monitor’s feedback, the ICD and Protocol were amended to add vomiting as a risk to participation in the study. Amendment #8

Protocol Deviation:
02 Mar 2011 reported to IRB and Medical Monitor:
A new batch of compounded Hydrocodone drug was ordered to complete the study. The shelf life of the drug is only 6 months and replacement of 4 subjects required additional drug be ordered.
Subject’s plasma levels of HC and HM levels were analyzed as soon as feasible following individual enrollment and completion of study versus being stored and analyzed after the study enrollment has been completed for all subjects. Subject 33 was the first patient to receive the new batch of drug. Plasma analysis revealed low dose of HC and HM. Subsequently, we enrolled Subjects 34 & 35 and again plasma analysis revealed low doses of HC and HM (Deviation).
WHASC Pharmacy was then contacted and informed of low concentration detected in subjects.
The WHASC Pharmacy contacted the compounding pharmacy.
WHASC Pharmacy documentation was submitted to IRB along with deviation documentation.
An Impact Statement was submitted by the pharmacy prior to protocol approval.

Action taken:
Enrollment stopped until new verified drug was received.
New compounded drug was ordered by pharmacy.
An extra capsule was ordered by the WHASC pharmacy and analyzed by the CRD laboratory. The concentration of hydrocodone in the capsule was shown to be 10 mg.
Subject 33, 34, and 35 were not contacted since no harm had occurred to their person.
Subjects 34 and 35 repeated the study. Subject 35 was unable to repeat the study and was replaced.

5. Scientific Progress

PK Analysis:

- Analysis of hydrocodone and metabolites in Subject plasma samples by LC/MS/MS has been completed for Subjects 1-39.
- Approximately 780 plasma samples were analyzed. Peak concentrations of hydrocodone were found at 0:30 - 5:00 hours post-dose and were in the range of 12.2 - 31.7 ng/mL. Hydromorphone peak concentrations were found at 1.00 - 8.00 hours post-dose and ranged from 1.1 - 3.5 ng/mL. For norhydrocodone, peak concentrations were found at 0:30 - 6:00 hours post-dose and ranged from 2.4 to 7.4 ng/mL. Post administration, hydrocodone in plasma was first detected, and peaked at the same time or before hydromorphone and norhydrocodone in all twelve subjects. Hydrocodone was last detected for up to the same time or longer than hydromorphone and norhydrocodone with exception of one subject where hydromorphone was detected for longer than hydrocodone.

The LC/MS/MS method validation for analysis of hydrocodone and metabolites in urine using UCT Excel I solid phase extraction columns and the Phenomenex Kinetex LC analytical column was completed.
LC/MS/MS analysis of subject urine samples is currently underway. Approximate total number of urine specimens collected for all subjects was 1,167.

Proteomic analyses
Investigation of drug-induced changes in the plasma proteome is nearing completion. All plasma samples, including those from the first sample set (subjects 1-12) and the second sample set (subjects 13-37, excluding subjects 15, 19, 24, 29, 31, 34R, and 35), have been depleted of the medium to high abundance plasma proteins and analyzed by LC-MS/MS. The time points and analysis approach were modified for the second set of samples based on findings from the first twelve subjects. Data are currently being filtered and analyzed for biological insight.

Time point selection and pre-MS plasma sample processing

Preliminary metabolic analysis of the kinetics of hydrocodone and hydromorphone levels in the plasma post-treatment guided time point selection for initial proteomic LC-MS/MS analysis of the first 12 subjects (Figure 1). Selected time points (pre-treatment, 1, 2, 4, 8, and 48 hours post-treatment) captured each subject’s baseline levels, the peak of drug levels (usually between the 2-8 hour time points), and a return to baseline levels upon metabolism of the drug.

Figure 1. Plasma hydrocodone levels following drug administration demonstrate peak levels between 2-4 hours in most subjects. Time points marked were chosen for further proteomic analysis.
One goal of our initial analyses was to further assess appropriate time points and technologies for analysis of the proteome response to hydrocodone administration. Based on the observation of significant inter-individual proteome variability in our initial analyses, the pre-treatment time point was again chosen to provide a representative subject-specific, baseline plasma proteome profile. Many subjects showed peak responses at 4h, both in metabolite and protein analyses, suggesting the 4h time point should be analyzed for the second set of subjects. If a physiological response occurs around 4h, we hypothesized that protein synthesis resulting from these changes may be slightly delayed. For this reason, we also chose to include the 6h time point to capture a possible response. Finally, in order to provide an additional subject-specific “return to normal” plasma proteome assessment following treatment, we chose to analyze the much later time point of 168h. We hypothesized that the 168h sample would be largely similar to the pre-treatment sample, and may act as an internal control upon which to assess the significance of changes at the 4-6h time point.

For all plasma samples, pre-MS sample processing at PNNL included depletion of high to moderately abundant plasma proteins using human IgY14 and IgY supermix immunoaffinity columns along with tryptic digestion and isolation of the resulting peptides for LC-MS/MS analysis. Immunoaffinity depletion allows an increase in the dynamic range of detection and identification of less abundant proteins and potentially more subtle changes in the plasma proteome.

Sample analysis

All LC-MS/MS runs are complete. Samples were analyzed on a hybrid high resolution and high mass accuracy LC-MS/MS platform (ThermoScientific LTQ Orbitrap Velos) which couples peptide identification (tandem MS data) with high resolution peak intensity data for quantification. Following depletion and digestion of the second set of samples, peptides were labeled via iTRAQ (isobaric tag for relative and absolute quantitation). The iTRAQ method is based on the covalent labeling of the N-terminus and side chain amines of peptides with isobaric tags. Simultaneous identification and quantification of peptides across four (4-plex iTRAQ reagents) different samples (in this case, four different time points) can be obtained in the same analysis using MS/MS, enabling high-throughput quantitative proteomic analysis with greatly reduced sample size. As this peptide labeling technique allows for greater downstream relative protein quantification, it was employed here in order to better monitor changes in protein abundance, by subject, through time. Our goal is to identify significant proteome changes within each individual upon hydrocodone treatment, and then compare trends across individuals to assess commonalities and differences in the plasma proteome response to hydrocodone use. Finally, we will also compare trends in proteome profiles among all subjects analyzed (30 individuals total).

- For the first twelve subjects, total instrument analyses included: 12 subjects x 6 time points per subject x 2 technical replicates = 144 datasets. These data were processed via the AMT tag approach for label-free quantification of peptide abundance.
- For the second eighteen subjects, total instrument analyses included: 18 subjects x 4 time points per subject x 12 fractions (fractionation increases peptide identifications) = 216 datasets. Although we received samples from three additional subjects, they were not able to be analyzed. Samples from subjects 15 and 19 were removed from the study due to low protein recovery. Subject 24 was also removed from further study due to the absence of the 168h time point sample. These data were processed to provide relative quantitation of each peptide across all four time points for each individual.
**Data analysis and interpretation**

All datasets were analyzed to identify peptides/proteins which showed statistically significant similarities and/or differences across the plasma proteome, within individuals and across time points, in response to hydrocodone administration. Each sample set was analyzed separately, as discussed in independent sections below. Although direct comparison of peptide and protein identifications is difficult due to the use of different processing and filtering techniques, the overall distribution of peptide and protein identifications in each sample set were as follows for reference:

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<th>Datasets</th>
<th>Number of MS runs</th>
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<tr>
<td>First 12</td>
<td>144</td>
<td>12,915</td>
<td>1,074</td>
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<tr>
<td>Second 18</td>
<td>216</td>
<td>28,685</td>
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The overlap of protein identifications between sample sets is represented in Figure 2. A significant fraction of identifications from the initial sample set (83%) were also observed in the second sample set, while the second set of samples increased the overall proteome coverage. This is likely due to fractionation of peptides, which increases the depth of proteome coverage.

**First sample set**

Peptide-level data were analyzed via two complementary methods. The first method considered peptide responses to hydrocodone administration by individual subject through time. The second method considered conserved responses in multiple subjects through time. Analysis methods and findings are discussed briefly below, and were used to guide experimental design for the second set of subject samples.

**Analysis of the hydrocodone response in individual subjects:**

- Preliminary metabolite analysis revealed peak hydrocodone levels at the 1- and 2-hour post-administration time points in a most subjects (Figure 1). Therefore, we chose to assess whether blood plasma protein responses occurred following the peak drug levels by focusing our analysis on the 2- and 4-hour time points.
- The overall trend of protein expression varied by individual, meaning there was not one increase/decrease trend that occurred in everyone or at the same time post-hydrocodone administration. Figure 3 shows protein abundances through time in plasma from selected individuals, with the goal of demonstrating the heterogeneous nature of the response to hydrocodone treatment.
Figure 3. Heat map representations of protein abundance changes through time in three selected individuals. (A) We observe a significant subset of proteins increasing in abundance at the 4-hour time point in this subject (green bar), whereas (B) the changes are more subtle and occur at different time points in this individual. (C) Finally, some subjects had trends of opposite direction (note the decrease at 4-hours instead of the increase seen in (A); purple bar) or lack obvious trends (orange bar).

- As most of the more obvious proteome changes occurred at the 2- and 4-hour post treatment time points, the 2- and 4-hour time points were combined and considered “response” time points. Data from the response samples were compared to those of the pre-treatment samples to identify peptides that were altered in response to hydrocodone administration. Peptide abundances were subjected to an ANOVA test to detect significantly increasing or significantly decreasing peptides through time. This analysis revealed 1185 peptides that were significantly changing in at least one subject (p<=0.01).
- Peptide identifications were then compared among all individuals in the study, including the placebo subject. Comparison to the placebo subject allowed a better segregation of peptides that changed in response to drug treatment versus those that were altered due to fasting, eating, or other unknown variables during the study. The most confident peptide changes in response to drug administration should be largely absent in the placebo individual.
- Peptide abundance trends through time were most often observed in a subset of (not all) individuals, which was anticipated due to biological variation. Changes in peptide abundance for a selected protein of interest are compared in Figure 4.

Figure 4. Peptides derived from the protein CFAD_HUMAN (Complement Factor D) were found to be significantly changing through time in a subset of subjects. (A) In subject 10, the majority of peptides are decreasing through time (compare PRE_1 and PRE_2 with 2h and 4h time points). (B) Conversely, subject 12 does not display any significantly changing trends in peptide abundance through time.
### Analysis of the hydrocodone response in multiple subjects:

- To identify trends present in the entire dataset as a whole (i.e. in multiple subjects) through time, comparison of “pre-treatment” with each of the subsequent time points was performed. In this analysis, peptide abundance values from all subjects (including the placebo subject) were first normalized using a Rank Invariant Peptide selection routine with median centering.

- Determination of significantly changing peptides through time was accomplished by a paired t-test comparing the pre time point with each additional time point. The number of peptides that were found to be significantly changing in at least one time point was 745. When peptides were rolled up to the protein level, this analysis revealed a total of 105 proteins that were significantly changing at one or more time points (p-value cut off of 0.01).

### Second sample set

Analysis of the first set of samples revealed significant inter-individual variability in the specificity of the plasma proteome, as well as differential directionality and magnitude of responses to hydrocodone administration. Anticipating this trend would continue with the next set of samples, we chose a method that would allow us to more quantitatively compare peptide and protein abundance through time for one individual. The iTRAQ technique allows for this, as well as for deeper proteome coverage due to peptide fractionation prior to LC-MS/MS analysis (apparent in Figure 2). Upon identification of peptides/proteins that have altered abundance through time, our goal was to then look across all individuals to determine if these changes were conserved trends in response to hydrocodone. We initially hypothesized that altered responses would be identified in a fraction of the subjects as we had seen with the first sample set. We do observe that some individuals experience a significant plasma proteome response to hydrocodone administration, whereas others show a very minimal response to the drug. Although anecdotal at this stage, the observed altered response to hydrocodone in a subset of the individuals also would support the statistic that approximately 30% of individuals may have a tendency toward hydrocodone abuse if given the opportunity, suggesting there may be an inherently different physiological response to the drug.

### Analysis of protein abundance changes by individual:

Peptide abundances, as determined by iTRAQ quantification, were summed to the protein level for significance testing. Data were normalized by individual time point using the median central tendency normalization method. Ratios of protein abundance were calculated, with the pre-treatment time point used as reference for changes at the 4- and 6-hour time points. If a protein was altered by 3-fold (either increased or decreased), it was considered significant for further analysis in this study.

- Using a 3-fold change as a significance cut-off, we can gain a general overview of the heterogeneity in responses by totaling the number of proteins increased and decreased at a particular time point in the plasma of each individual. If we represent the number of altered proteins as a percentage of the total number of protein observations by individual (i.e. what percent of the proteome changes from pre-treatment to 6h post-treatment?), it becomes clear that the character of the response is varied (Figure 5).
• Interestingly, approximately one-third of the subjects display a greater number of proteome changes than the average among all subjects. These subjects may represent opioid “responders” that have an inherently different response to drug treatment. Future studies may help to elucidate the connection between plasma protein abundance alterations and the possible tendency to abuse opioids.

• Calculating confident ratios of protein abundance using iTRAQ technology relies on consistency of peptide concentration in each sample (in this case, each time point). The total concentration of peptide material must be equal in each sample that will be directly compared. For example, if the total peptide concentration of the pre-treatment sample was only half of the total peptide concentration of the 4-, 6-, and 168-hour samples, we would expect the pre-treatment sample to have lower concentrations of each individual protein. In this example, calculating the ratio of protein abundance at a later time point compared to the pre-treatment sample becomes an unfair comparison because equal amounts of total protein were not analyzed for each time point. We encountered this scenario with subject 21, where the pre-treatment sample yielded a much lower peptide concentration as compared to the other time points. As such, the number of significant changes in protein abundance shown in Figure 5 is likely inflated. We are working to establish whether the 168-hour time point might provide an alternative “baseline” sample for comparison in these cases.

• Other cases in which this technique would be useful include (1) samples that were not as completely depleted of the medium/high abundance plasma proteins during upstream processing, and (2) samples that had undergone hemolysis and display increased hemoglobin. Both circumstances will result in altered peptide abundances as compared to fully depleted or non-hemolytic samples. If observed in the baseline (pre-treatment) sample, interpretation of results can be significantly confounded. Hemolysis did occur in the pre-treatment sample from subject 20, who received a placebo treatment. For this reason, it appears as if the individual who received the placebo has a very high percentage of protein alterations (Figure 5). Again, using the 168-hour time point may prove more useful as a baseline control against which 4- and 6-hour time points can be compared. This investigation is ongoing.

Ongoing analyses
Data analysis is in progress and nearing completion, at which time a list of potential proteome markers for opioid use will be established. The role of other factors, including age, gender, height/weight/BMI, and adverse reaction to drug administration (such as nausea and/or vomiting), is also being considered. Protein abundance alterations through time and across individuals are beginning to emerge and may be pursued as markers for use/abuse of opioids in the future.

Phase 3 –
59MDW-Metabolism: Data products will include subject metabolism profile of hydrocodone for up to 7 days following hydrocodone administration. Data results and interpretation will be provided through the form of a manuscript.

PNNL-Proteomics: Data products will include all protein identifications produced during the project for each sample, a list of putative markers for opioid use through time, and preliminary biological interpretation through the form of a manuscript.

(4) Section III - Problem Areas
A description of anticipated problems that have a potential to impede progress and what corrective action is planned should the problem materialize.
- Problem: None foreseen

(5) Section IV - A description of work to be performed during the next reporting period.
- Begin pharmacokinetic analysis of the drug and metabolites in urine.
- Complete LC-MS/MS analyses and proteomics data analysis and interpretation.

(6) Section V - Administrative Comments (Optional) - Description of proposed site visits and participation in technical meetings, journal manuscripts in preparation, coordination with other organizations conducting related work, etc.

6. Manuscript preparation and results dissemination
For the Entire Study

Manuscript Submission:

Military Regional Meeting Presentations:
- Valtier, S, Mueck, R, Vargas, T, Bebarta VS, "Hydrocodone Metabolism Following Singe Dose Administration", Military Health Systems Research Symposium, Fort Lauderdale, FL, Aug 2012
Bebarta, VS, Varney, SM, Potter, J, Ramirez, S, Ganem, V, Martinez, J. "Misuse of prescribed pain medication in a military population – is there a correlation with deployment or combat illnesses or injury?" Military Health Systems Research Symposium, Fort Lauderdale, FL, Aug 2012.

International Meeting Presentations:

KEY RESEARCH ACCOMPLISHMENTS:
- Enrollment is completed.
- Preliminary data analysis is ongoing.
- Mass spectrometric analyses for proteomic data is ongoing..
- Analysis for drug and metabolites in plasma is completed.
- Quantitative method paper for analysis of hydrocodone and metabolites in plasma accepted, J Chrom b

REPORTABLE OUTCOMES:
PK Plasma Results: Peak concentrations of hydrocodone were found at 0:30 - 5:00 hours post-dose and were in the range of 12.2 – 31.7 ng/mL. Hydromorphone peak concentrations were found at 1:00 – 8:00 hours post-dose and ranged from 1.1 – 3.5 ng/mL. For norhydrocodone, peak concentrations were found at 0:30 – 6:00 hours post-dose and ranged from 2.4 to 7.4 ng/mL. Post administration, hydrocodone in plasma was first detected, and peaked at the same time or before hydromorphone and norhydrocodone in all twelve subjects. Hydrocodone was last detected for up to the same time or longer than hydromorphone and norhydrocodone with exception of one subject where hydromorphone was detected for longer than hydrocodone.

CONCLUSION:
In conclusion, a one year, no-cost extension was requested and given from USAMRAC. The proteomic steps often require interpretation and quality assessment - these steps ideally should be completed within about 3 weeks. Then interpretation of the results will be required. This interpretation includes comparisons with previous datasets, comparison with what is known from the literature, and literature follow-ups of any novel findings. These efforts would be benefited from an extension of 3 months of effort. In addition, we will compare the proteomic findings to the pharmacokinetic results to determine if there is correlation. This will take approximately six to nine months. Finally analysis will take an additional 6 weeks.

REFERENCES:


G. Zoroya, Troops reportedly popping more painkillers, USA Today, USA Today, 2008.


Supporting Uniformed Personnel by Providing Oversight and Relevant Treatment for Substance Use Disorders Act, s.459, 2009.


APPENDICES:
No appendices