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14. ABSTRACT

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.

15. SUBJECT TERMS

Proteomic, drug abuse, opioids, metabolites, and pharmacokinetic

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Table of Contents

	Page
Introduction……..	01
Body……..	01
Key Research Accomplishments……..	13
Reportable Outcomes……..	13
Conclusion……..	14
References……..	14
Appendices……..	17

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 February 2014 for IRB review in March 2014.
    - 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 AHLTA note.
    - Literature search was conducted in Feb. 2014 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 before randomization, 2 withdrew after randomization, 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.

Table 1

Subject Number	Male or Female	Randomization/ Enrollment Date	Age	SAE	Comment
01	M	14 March 2011	40	0	
02	M	14 March 2011	40	0	
03	F	04 April 2011	35	0	
04	M	18 April 2011	19	0	
05	M	18 April 2011	29	0	
06	M	25 April 2011	22	0	
07	F	25 April 2011	25	0	Not enough plasma taken for 2 aliquots. Only one set, set A, at 3 hour.
08	M	09 May 2011	25	0	
09	F	06 June 2011	43	0	1150 nauseated, 1200 noon vomited 400cc Dissipated after vomiting
10	F	13 June 2011	43	0	0800 nauseated, 0805 vomited 20cc Dissipated by 1200
11	F	13 June 2011	50	0	
12	F	20 June 2011	46	0	
13	M	20 June 2011	19	0	
14	F	20 June 2011	44	0	0930 itchy nose, dissipated by 1215

15	M	25 July 2011	35	0	
16	M	01 Aug. 2011	43	0	
17	M	01 Aug. 2011	48	0	0735 nauseated, dissipated by 1435
18	F	08 Aug 2011	27	0	1215 nauseated dissipated by 1350
19	F	15 Aug 2011	36	0	Nauseated at 0915 dissipated by 1600 Vomited at 1030, 200cc Vomited at 1100, 100cc
20	F	15 Aug 2011	30	0	
21	F	12 Sep 2011	48	0	
22	F	12 Sep 2011	35	0	
23	M	26 Sep 2011	18	0	No plasma collection for day 2-5 and 8.
24	F	26 Sep 2011	23	0	
25	F	26 Sep 2011	22	0	
26	F	24 Oct 2011	20	0	Vomited at 1030, 1100, 1400 and 1730
27	F	14 Nov 2011	20	0	
28	F	14 Nov 2011	55	0	
29	M	12 Dec 2011	-		Withdrew, unable access vein for IV
30	M	12 Dec 2011	25	0	
31	M	12 Dec 2011	-		Withdrew, unable to access vein for IV
32	F	23 Jan 2012	35	0	
33	F	23 Jan 2012	49	0	Repeated study Received Substandard wt. of drug (33R)

34	F	06 Feb 2012	42	0	Repeated study Received Substandard wt. of drug (34R)
35	F	06 Feb 2012	52	0	Did NOT Repeat Study Received Substandard wt. of drug
33R	F	09 Apr 2012	49	0	Repeat
34R	F	30 Apr 2012	42	0	Repeat No plasma collection Day 1 Specimen #13, 14 & 15
36	M	09 Apr 2012	52	0	
37	M	09 Apr 2012	36	0	
38	F	30 Apr 2012	41	0	
39	F	30 Apr 2012	39	0	
40	M	Not randomized		0	Signed ICD, excluded
41	F	Not randomized		0	Signed ICD, excluded

Table 2: Local Unanticipated Problems previously reported to the IRB.

UPIRSO Subject 09 (I)	06 June 2011, Vomited at 12 noon, dissipated after event
UPIRSO Subject 10 (I)	13 June 2011, vomited at 0805, dissipated at 12 noon
UPIRSO Subject 19 (I)	15 Aug. 2011, vomited at 1030 & 1100, dissipated after 2nd event

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 shelve 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.

Scientific Progress
Plasma outcomes:
This study provides a validated quantitative method for the analysis of hydrocodone, hydromorphone and norhydrocodone in plasma by LC-MS-MS. Extraction was performed using mixed mode SPE cartridges for sample preparation and a C18 column LC column for separation of the analytes. The six point calibration curve consisted of 1, 2.5, 5, 10, 50 and 100 ng/mL of the compounds; however use of a 4 or 5 point calibration curve was found to be acceptable. The limit of quantitation is 1 ng/mL and LOD is 0.25 ng/mL for all three analytes. The method provided a reliable and sensitive procedure for the quantitation of hydrocodone, hydromorphone and norhydrocodone in human plasma samples for a hydrocodone pharmacokinetic study.

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.

Proteomic analyses

Investigation of drug-induced changes in the plasma proteome is competed. 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), were 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.

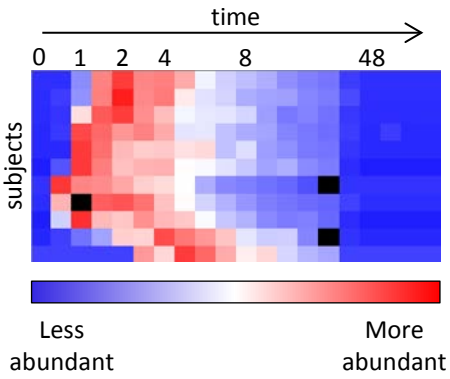

Figure 1. Plasma hydrocodone levels following drug administration demonstrate peak levels between 2-4hours in most subjects. Time points marked were chosen for further proteomic analysis.

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:

Datasets	Number of MS runs	Total number of unique peptides identified	Total number of unique proteins identified
First 12	144	12,915	1,074
Second 18	216	28,685	4,684

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.

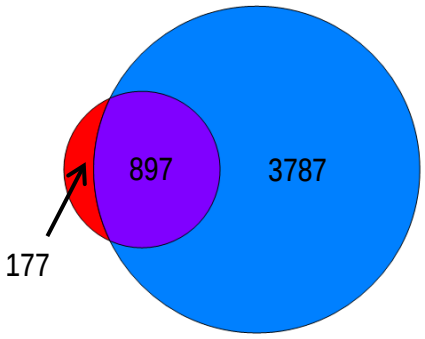

Figure 2. The total number of unique proteins identified in plasma samples from the first 12 subjects (red) and the second 18 subjects (blue) show significant overlap (purple). Increased protein identifications with peptide fractionation are observed for the second set of samples.

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.

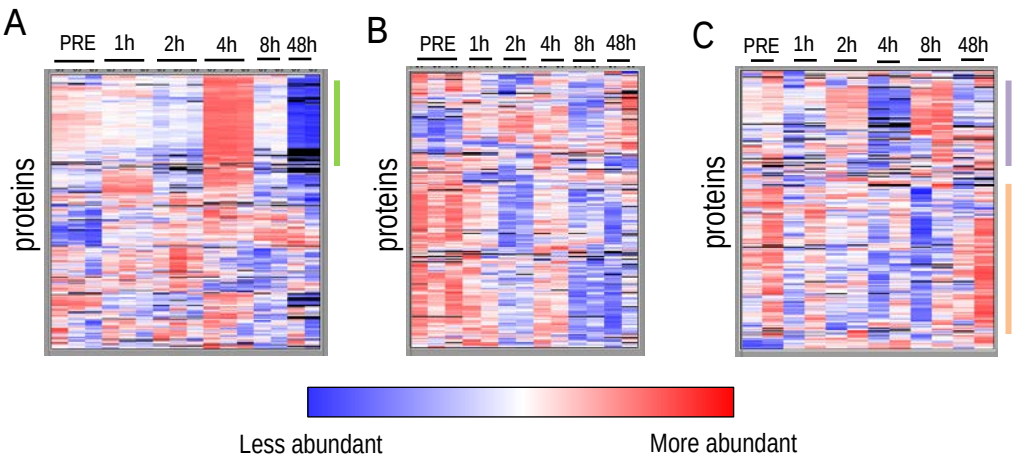

Figure 3. Heat map representations of protein abundance changes through time in three selected individuals. (A) We observe a significant subset of proteins increasing in abundance at the 4-hour time point in this subject (green bar), whereas (B) the changes are more subtle and occur at different time 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.

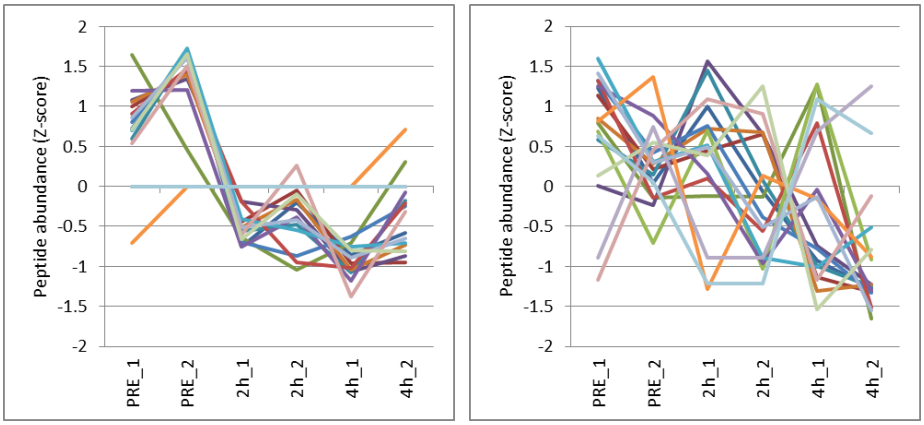

Figure 4. Peptides derived from the protein CFAD_HUMAN (Complement Factor D) were found to be significantly changing through time in a subset of subjects. (A) In subject 10, the majority of peptides are decreasing through time (compare PRE_1 and PRE_2 with 2h and 4h time points). (B) Conversely, subject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

Analysis of the hydrocodone response in multiple subjects:

- Peptide level data was log transformed, rolled up to the protein level using R rollup, and normalized by mean centering to reach protein level analysis.
- Analysis of protein expression across all subjects revealed the inherent variability of individual responses. When relative protein abundances were allowed to cluster based on similarity in protein expression profile in a heat map, it becomes clear that responses are varied (Figure 5). Trends in expression through time can still be identified, however. Data from the pre-treatment time point was compared to the data from the 4h and 8h time points (referred to here as "response"), and proteins determined to be significantly differently expressed were identified. A Principal Component Analysis (PCA) plot was generated using the abundance values of those proteins determined to be significantly changing post-treatment (Figure 6), which shows a slight delineation of "pre" and "response" data for multiple subjects.

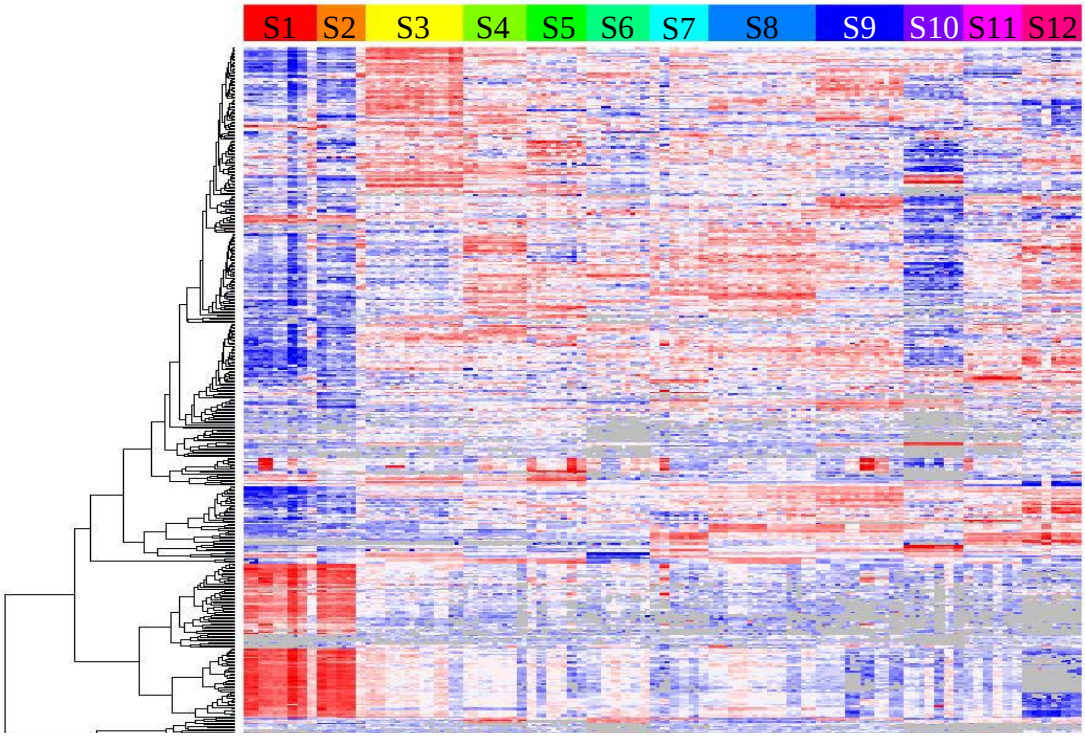

Figure 5. Heat map of protein-level data from all subjects at all time points. Subject number is noted horizontally across the top of the heat map, with individual proteins represented on the vertical axis. Note in particular subjects S1 and S2, which display significantly different protein profiles as compared to the other subjects. Red indicates the protein was more abundant, blue indicates the protein was less abundant, and gray indicates the protein was not observed in that datasets.

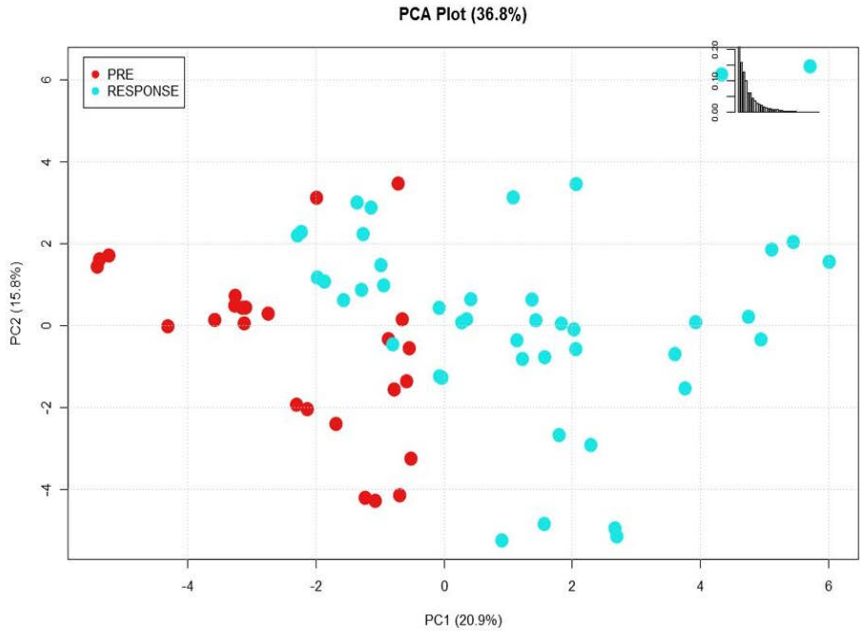

Figure 6. Principle component analysis from expression profiles of proteins identified as significantly changing between pre-treatment and post-treatment time points among a subset of subjects. Data for protein expression in all subjects was used to generate this plot, which shows the similarity among pre-treatment samples (red) and response samples (blue).

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 7).

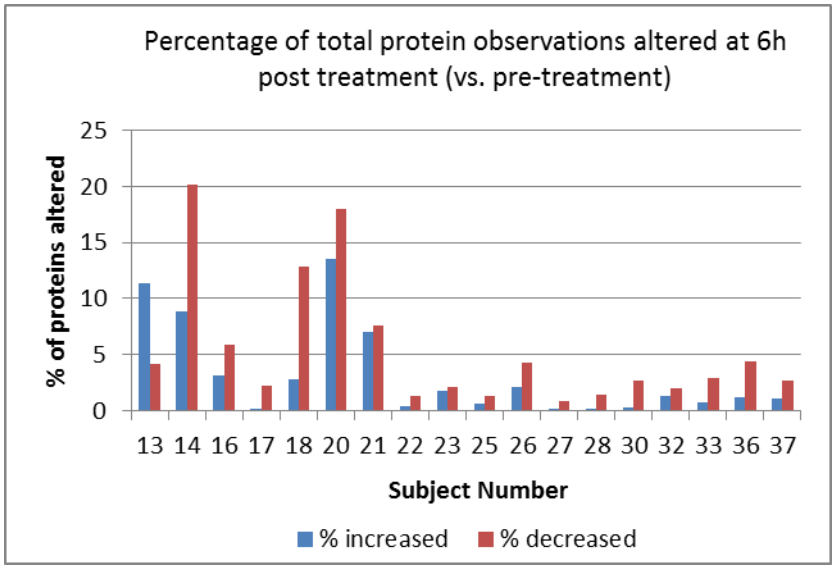

Figure 7. Percentage of total protein identifications that were determined to be significantly changed between pre-treatment and 6 hours post-treatment. Approximately one-third of subjects display a higher percentage of both increased and decreased protein abundances, indicating they may represent opioid "responders."

- 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 speculate that the 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.

Urine outcomes:

Presences of proteins in urine were in trace amounts; therefore the research team concluded that no further evaluation of proteins in urine samples would be feasible.

Manuscript preparation and results dissemination
For the Entire Study

Publications:

- Valtier, S, Mueck, R, Bebarta, VS, Quantitative method for analysis of hydrocodone, hydromorphone and norhydrocodone in human plasma by liquid chromatography-tandem mass spectrometry, Journal of Chromatography B, Apr 2013.
- Valtier, S and Bebarta VS, "Excretion Profile of Hydrocodone, Hydromorphone and Norhydrocodone in Urine Following Single Dose Administration of Hydrocodone to Healthy Volunteers", Journal of Analytical Toxicology, Sep 2012

KEY RESEARCH ACCOMPLISHMENTS:

- Enrollment is completed.
- Analysis for drug and metabolites in plasma completed.
- Mass spectrometric analyses for proteomic data completed.
- Development of critical preliminary data for future proteomics measurements to provide a basis for global and targeted analyses.
- Publications:
  - Quantitative method paper for analysis of hydrocodone and metabolites in plasma, Journal of Chromatography B
  - Excretion Profile of Hydrocodone, Hydromorphone and Norhydrocodone in Urine Following Single Dose Administration of Hydrocodone to Healthy Volunteers", Journal of Analytical Toxicology,

REPORTABLE OUTCOMES:

A selective, sensitive and accurate high-performance liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the quantitation of hydrocodone, hydromorphone and norhydrocodone in human plasma was developed. The internal standard stock solution comprised of hydrocodoned6, hydromorphone-d6 and norhydrocodone-d3 was added to 0.5 mL plasma samples. Samples were extracted using a copolymeric sorbent (mixed mode) solid phase extraction (SPE) column. Chromatographic separation was carried out using a reversed-phase C18 analytical column with a gradient mobile phase consisting of solvent A = 5% acetonitrile with 0.1% formic acid and solvent B = 100% acetonitrile. MS analysis was performed using positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode. Linearity was established over the range 1–100 ng/mL with correlation coefficients ≥0.998 for all three analytes. The coefficient of variation (CV) of intra-day samples was ≤5.6% at 10 ng/mL. The precision of inter-day (6 days) samples resulted in CVs ≤8.1% at concentrations tested at 2.5, 10 and 25 ng/mL for all three analytes. The lower limit of quantification (LOQ) was 1.0 ng/mL with signal-to-noise (S/N) ratio >10, the limit of detection (LOD) was 0.25 ng/mL with S/N ratio >3 for the drug and its metabolites. Dilution effects, extraction recovery, stability, interference, carryover and ion suppression were also evaluated. This method was successfully applied to human subject plasma samples in support of a hydrocodone pharmacokinetic study.

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.

CONCLUSIONS:

Analysis of proteins in plasma for a variety of health conditions has a long history pre-dating the concept of global analysis of protein referred to as proteomics. The fundamental question of the proteomics portion of this study was whether the use of proteomics might elucidate physiological responses to the use of opioids. Our data indicate, using two different quantification methods, that there are likely changes in plasma due to single acute exposures to opioids. This suggests and supports the idea that opioid use has broader physiological responses then binding to pain receptors. Due to typical subject to subject variability and the relatively low abundance of most proteins responding to hydrocodone administration this study provides the necessary results for targeting future studies that may need more subjects for appropriate statistical power.

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APPENDICES:

Publications

- Valtier, S, Mueck, R, Bebarta, VS, Quantitative method for analysis of hydrocodone, hydromorphone and norhydrocodone in human plasma by liquid chromatography-tandem mass spectrometry, Journal of Chromatography B, Apr 2013.
- Valtier, S and Bebarta VS, "Excretion Profile of Hydrocodone, Hydromorphone and Norhydrocodone in Urine Following Single Dose Administration of Hydrocodone to Healthy Volunteers", Journal of Analytical Toxicology, Sep 2012

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Article

Excretion Profile of Hydrocodone, Hydromorphone and Norhydrocodone in Urine Following Single Dose Administration of Hydrocodone to Healthy Volunteers

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Abuse of prescription opioids for non-medical use has been on the rise over the past decade. The most commonly abused opioid is hydrocodone, a frequently prescribed pain medication metabolized by the body to hydromorphone, norhydrocodone and other minor metabolites. This study describes the excretion profile of hydrocodone, hydromorphone and norhydrocodone in urine following a single dose (10 mg) administration of hydrocodone to human subjects ($n = 7$) and presents a validated liquid chromatography–tandem mass spectrometry method for analysis of the drug and its metabolites. Limit of quantitation was 5 ng/mL for all analytes; limit of detection was 2.5 ng/mL for hydrocodone and norhydrocodone and 5 ng/mL for hydromorphone. Peak concentrations of hydrocodone were found at 3:30–7:00 hours post-dose and were in the range of 612–2,190 ng/mL. Hydromorphone peak concentrations were found at 6:15–26:45 hours post-dose and ranged from 102 to 342 ng/mL. For norhydrocodone, peak concentrations were found at 4:20–13:00 hours post-dose and ranged from 811 to 3,460 ng/mL. Although hydromorphone was found at lower levels than hydrocodone, in six of seven subjects, it persisted for as long as hydrocodone was detected. Norhydrocodone was found at higher levels and lasted for a longer period of time than hydrocodone, thus making the nor-metabolite a valuable tool in evaluating hydrocodone use and/or misuse.

Introduction

Surveys by the National Survey of Drug Use and Health, the Drug Abuse Warning Network (DAWN) and the National Center on Addiction and Substance Abuse have shown a significant rise in the number of people reporting non-medical use of pain relievers (1–3).

Hydrocodone is a semi-synthetic opioid prescribed for pain management; however, it has a high potential for abuse. It is the most commonly abused prescribed opioid, as determined by the data reported to the American Association of Poison Centers and DAWN (4). The increased incidence of deaths attributed to hydrocodone abuse is also a major concern (4, 5).

The recommended starting therapeutic dose range for hydrocodone is 5–30 mg (one capsule) per day. Hydrocodone is metabolized by the body to hydromorphone, norhydrocodone (Figure 1) and to a lesser extent, 6-α-hydrocol and 6-β-hydrocol. Hydrocodone is sometimes referred to as a "pro-drug" because the more active compound is the metabolite (hydromorphone), not the administered drug. Opioids undergo phase I metabolism by the CYP pathway, phase II metabolism by conjugation, or both. The (phase I) metabolic pathways of hydrocodone include O-demethylation catalyzed by cytochrome P450 2D6 (CYP2D6) to its active metabolite, hydromorphone, *N*-demethylation by cytochrome P450 3A4 to form norhydrocodone, and C6-keto reduction to form approximately equal amounts of 6-α-hydrocol and 6-β-hydrocol (6–11). Hydromorphone in turn undergoes phase II glucuronidation, in which the predominate metabolite is hydromorphone-3-glucuronide. Approximately 7% of the Caucasian population possesses allelic variants of the CYP2D6 gene [poor metabolizers (PMs)], causing conversion of hydrocodone to hydromorphone occur at a slower rate (12). Urinary metabolic ratios of hydrocodone after a single dose were investigated in PMs and in extensive metabolizers (EMs) (6). Although the CYP2D6 phenotype was shown to influence the metabolic conversion of hydrocodone to hydromorphone (6, 11, 13), hydromorphone was found at relatively small amounts in both EMs and PMs. A study by Kaplan *et al.* (13) showed that PMs were equally responsive to oral hydrocodone as EMs. The study demonstrated that although hydrocodone is less potent than hydromorphone, it clearly has its own agonist actions.

To better assess the possibility of hydrocodone use, evaluation of the metabolites was considered. The metabolites hydromorphone and norhydrocodone, and the other minor metabolites have previously been identified (14); however, to our knowledge, the excretion profile of the parent drug and its two major metabolites has not yet been accomplished to this extent in urine. The nor-metabolite was detected in the absence of the parent drug in urine samples collected from chronic pain patients (14, 15), potentially making norhydrocodone a more reliable marker for hydrocodone use. Because hydromorphone is commercially available, it is difficult to determine whether the metabolite is produced from hydrocodone or is a product of another drug source. In view of this, it appeared that evaluation of the nor-metabolite might prove to be a better indicator for time of last hydrocodone use.

The goals of this study were to validate a method for quantitation of hydrocodone and its metabolites in urine and to provide the metabolism and excretion profile of hydrocodone following single dose administration of 10 mg hydrocodone to seven healthy human subjects. Use of respective internal standards and multiple reaction monitoring (MRM) by liquid chromatography–tandem mass spectrometry (LC–MS-MS) led to an accurate quantitative method with limit of quantitation (LOQ) and limit of detection (LOD) in the low ng/mL range for all three analytes. Establishing concentration levels and excretion profile for the drug and metabolites following single dose administration can provide a better understanding of hydrocodone use.

Figure 1. Chemical structure and metabolic pathway of hydrocodone, hydromorphone and norhydrocodone.

Materials and Methods

Materials

Hydrocodone, hydromorphone, norhydrocodone, hydrocodone-d3, hydromorphone-d3 and norhydrocodone-d3 were obtained from Cerilliant (Round Rock, TX). β-glucuronidase from *Helix pomatia*, Type HP-2 (aqueous solution, activity ≥ 100,000 units/mL) was obtained from Sigma Chemical Company (St. Louis, MO). Acetonitrile (Optima LC– MS), water (Optima LC–MS), methanol (HPLC grade), methylene chloride (HPLC grade), acetic acid (HPLC) isopropyl alcohol (ACS grade) and sodium acetate (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide, 28–30% (ACS) was obtained from J.T. Baker (Phillipsburg, NJ). The Kinetex 5 μ, 2.1 × 100 mm analytical column was purchased from Phenomenex (Torrance, CA) and the Clean Screen XCEL I, 130 mg/3 mL extraction column from United Chemical Technology [(UCT), Bristol, PA]. Formic acid was from Michrom Bioresources [(Ultra Pure), Auburn, CA]. Hydrocodone administered to experimental subjects, in the form of Lortab (Amneal Pharmaceuticals), was obtained from the Wilford Hall Medical Center (Lackland AFB) pharmacy.

Drug administration and sample collection

Ten milligrams of hydrocodone, in the form of a single Lortab capsule, was administered orally to each of the seven healthy volunteers (four female and three male). To avoid side effect issues from hydrocodone hypersensitivity, only subjects with a history of hydrocodone use at least one time in the past were allowed to participate. Subjects were asked to refrain from any opioid use for at least 30 days prior to study initiation. The study was approved by the Wilford Hall Medical Center Institutional Review Board. All study patients gave their written consent to participate. A pre-dose sample was taken prior to drug administration and, following administration, urine was collected at each urination for the next five days. Urine samples were collected *ad lib* to best simulate what would be seen in random drug testing. Samples were stored refrigerated or frozen until analysis.

Sample preparation and analysis

Sample pH was measured using a Fisher Accumet 50 pH meter and specific gravity determined using an AO Scientific Instruments refractometer. Creatinine levels were determined at the Wilford Hall Medical Center Clinical Laboratory using standard clinical laboratory procedures.

Table I
MRM Transitions

Compound transition 1/2*	MRM transition (*m/z*)	Dwell time (msec)	DP	CE
Hydrocodone 1	300.2 → 199.1	200	120	52
Hydrocodone 2	300.2 → 171.0	200	125	52
Hydrocodone-d3	303.2 → 199.0	150	88	44
Hydromorphone 1	286.1 → 185.0	200	100	53
Hydromorphone 2	286.1 → 157.0	200	96	40
Hydromorphone-d3	289.2 → 185.2	150	100	40
Norhydrocodone 1	286.2 → 199.1	200	80	40
Norhydrocodone 2	286.2 → 241.1	200	80	40
Norhydrocodone-d3	289.0 → 202.0	150	80	40

*Transition 1: quantifier; transition 2: qualifier.

Hydrolysis methods

Enzyme hydrolysis was used. Sodium acetate buffer (250 μL, 0.1 M) and 20 μL 10,000 μ/mL β-glucuronidase were added to 250 μL of urine. Samples were mixed, heated to 60°C for 2 h in a water bath, cooled to room temperature, transferred to a clean glass tube and then extracted.

Solid-phase extraction

A 250-μL aliquot of hydrolyzed urine was extracted with a UCT Clean Screen XCEL I 130 mg/3 mL column using a Zymark RapidTrace robotic system. The extraction protocol was as follows: the sample was loaded onto the column at 1 mL/min, dried with nitrogen for 1 min, rinsed with 2 mL 2% acetic acid in methanol, then dried with nitrogen for 5 min. The analytes were eluted with 1 mL of a freshly prepared solution of methylene chloride–isopropyl alcohol–ammonium hydroxide (78:20:2, v/v/v) at 1 mL/min. The extracts were evaporated to dryness under a stream of nitrogen in a 45°C water bath. The dry residues were reconstituted in 250 μL mobile phase (5% acetonitrile aqueous solution with 0.1% formic acid) and injected onto the LC–MS-MS system.

LC– MS-MS conditions

The LC–MS-MS configuration was comprised of an Agilent LC system coupled to an Applied Biosystems 4000 QTrap mass spectrometer. The LC mobile phase for the Phenomenex Kinetex analytical column (2.6 μ, 50 × 2.1 mm) consisted of solvent A (5% acetonitrile with 0.1% formic acid) and solvent B (100% acetonitrile); flow rate was set at 0.5 mL/min. The column oven temperature was maintained at 25°C and the injection volume was 10 μL. The gradient flow method consisted of an opening condition of 5% pump B, with a linear increase to 60% pump B over 2.2 min, then a linear increase to 95% pump B at 2.23 min, 0.27 min at 95% pump B, and then a return to the opening condition (5% pump B) via a linear gradient over 0.15 min, followed by a 2.35 min re-equilibration at opening conditions. The total run time was 5 min for each sample.

Analysis was performed using positive ion electrospray MS-MS in MRM mode. Two MRM transitions, precursor ion (transition 1: quantifier) and product ion (transition 2: qualifier) were used, and the declustering potential (DP) and collision energy (CE) were optimized as shown in Table I. The

detector conditions were as follows: polarity mode, ESI positive; scan type, MRM; curtain gas 15 psi; collision-activated dissociation (CAD) gas, high; ion source gas 1, 40; ion source gas 2, 40; ion spray voltage, 5,000 V; temperature, 600°C; entrance potential, 10.

Quantitative analysis

Quantitation and detection were based on a six-point calibration using calibration standards containing 5, 10, 50, 100, 500 and 1,000 ng/mL of hydrocodone, hydromorphone and norhydrocodone prepared in drug-free urine with each standard containing 100 ng/mL of deuterium-labeled isotopomer internal standards (hydrocodone-d3, hydromorphone-d3 and norhydrocodone-d3). To test dilution effects and linearity range, concentrations at 1, 2.5, 25, 750, 2,500 and 5,000 prepared from a 10,000 ng/mL urine stock solution of the three analytes were evaluated on seven consecutive runs. Ions monitored (Table I) for the analytes and respective internal standards were as follows: hydrocodone, m/z 300.2 > 199.1, 300.2 > 171.0; hydrocodone-d3, 303.2 > 199.0; hydromorphone 286.1 > 185.0, 286.1 > 157.0; hydromorphone-d3, 289.2 > 185.2; norhydrocodone, 286.1 > 199.1, 286.2 > 241.1; and norhydrocodone-d3, 289.0 > 202.0. Ion ratios from the precursor/product ions derived from the analytes in controls and subject samples had to be within ±20% of the ion ratio calculation; the calculated range was based on the average of ratio ranges obtained for the six standards.

Twelve samples were analyzed to determine the efficiency of the extraction procedure. Drug and internal standards were added to six samples before extraction. To the remaining six samples, internal standard was added after the samples were extracted. The mean and standard deviation were calculated for each set of samples and extraction efficiency determined. The linear range was determined for this procedure by the analysis of at least seven different runs with concentrations ranging from 5 to 10,000 ng/mL hydrocodone, hydromorphone and norhydrocodone. Within-run precision was measured by testing replicates ($n = 6$) of the three analytes at 100 ng/mL and respective internal standards at 100 ng/mL. Between-run precision was measured by testing concentrations at 25, 100 and 2,500 ng/mL of the three analytes and respective internal standards on seven separate runs. The LOD was assessed by testing analyte concentrations at 1 and 2.5 ng/mL on at least six runs. Six random urine specimens collected from human volunteers were analyzed to check for potential endogenous interferences with the analytes of interest. Stability of the drug and metabolites in urine was also assessed. Aliquots of a 12-h post-dose urine from one subject and of a spiked urine sample at 500 ng/mL of the analytes were stored refrigerated (2–8°C) or frozen (−20 and −70°C) and were tested at one week, one month and three months from storage date.

Ion suppression

Because matrix effect can influence the extent of analyte ionization, an ion suppression experiment was conducted. Drug-negative urine was hydrolyzed and extracted in the same manner as test samples to best mimic matrix complexity. A 200-ng/mL solution of the analytes in acetonitrile was prepared and was loaded into the infusion pump syringe. The infusion pump was connected post-column via a tee connector; the solution was infused at 10 μL/min. Once the baseline was stable, injections of drug-free urine extract were made in the same manner as standard acquisitions.

Results and Discussion

Method evaluation

Gas chromatography mass spectrometry (GC–MS) and LC–MS-MS methods for analysis of opiates such as hydrocodone and hydromorphone in urine and blood have been described (16–23); however, those methods did not include the analysis of norhydrocodone. In a study of chronic pain users (14), the evaluation of the nor-metabolite in urine was reported in which the LOQ was 50 ng/mL. In the current study, the LOQ and LOD are significantly lower, making it possible to detect the drug and metabolites for a longer period of time.

The most viable product ions were selected based on their characteristic fragments of the precursor ion and their maximal response. One of the advantages of LC–MS-MS is the ability to spectrally separate these opioids. An LC gradient with run time of 5 min was instituted to achieve better separation of the compounds. Retention times were 2.55 min for hydrocodone, 2.11 min for hydromorphone and 2.50 min for norhydrocodone (Figure 2). Hydromorphone and norhydrocodone share the same precursor ion, but were separated chromatographically in time. Hydrocodone and norhydrocodone were not totally separated, but were spectrally distinguishable due to the difference in precursor ion between the two analytes. The hydroxy metabolite exists in both the free and glucuronide-conjugated forms. The samples were subjected to enzyme hydrolysis prior to extraction to provide measurement of total (free and conjugated) hydromorphone.

For our purpose, linearity was defined as quantitative values within ±20% of the target concentration with proper qualitative identification, as determined by ion ratios for quantifying and qualifying ions within ±20% of calibrator ratio (average of six calibrators). The linear range was determined for this procedure by the analysis of at least seven different runs on concentrations ranging from 1 to 10,000 ng/mL hydrocodone, hydromorphone and norhydrocodone. Using 5, 10, 50, 100, 500 and 1000 ng/mL calibrators ($r > 0.99$ for all analytes), samples ranging from 5 to 10,000 ng/mL for hydrocodone and hydromorphone and 5 to 5,000 ng/mL for norhydrocodone provided results within ±20% of their target value. Dilution integrity, defined as the accuracy of the calculated quantity to the true value of the diluted sample, proved to be 88.7 to 109.8% for concentrations at 25, 750, 2,500 and 5,000 ng/mL for all three analytes. The LOD [acceptable ion ratios, presence of all MRM transitions and signal-to-noise ratio (S/N) ≥ 3] was shown to be 2.5 ng/mL for hydrocodone and norhydrocodone and 5 ng/mL for hydromorphone. At 2.5 ng/mL hydrocodone and norhydrocodone, S/N ratios of greater than 13:1 for all transitions were achieved. However, the ion mass ratios were not always within range. The S/N ratio for hydromorphone transition 2 (286.1 > 157.0) at 2.5 ng/mL was < 3, and therefore failed to meet ion ratio acceptance criteria. At 5 ng/mL, S/N > 10 was achieved; the LOD for hydromorphone was defined

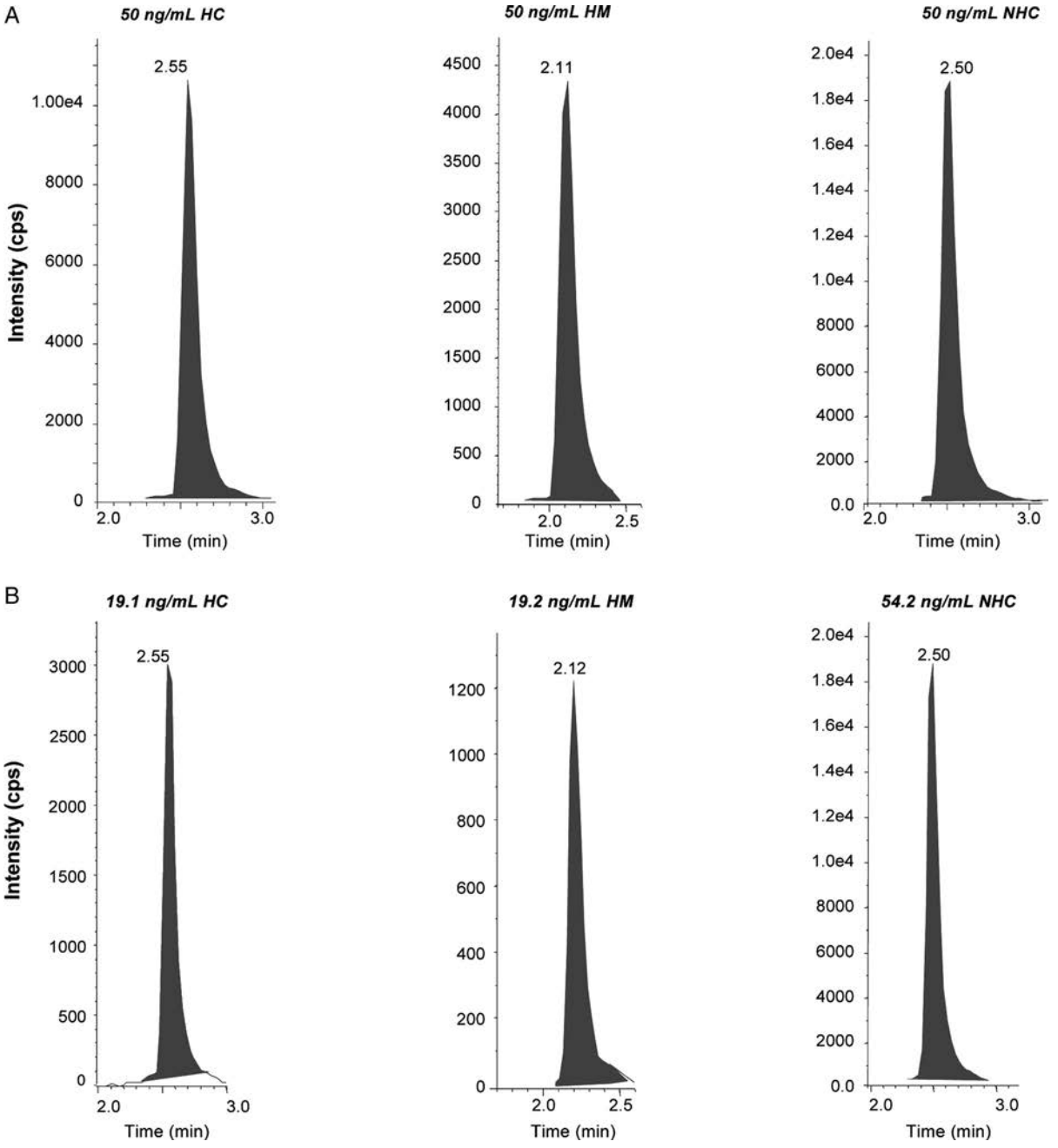

Figure 2. Chromatography of hydrocodone (HC), hydromorphone (HM), and norhydrocodone (NHC) from calibration standard containing 50 ng/mL each analyte (A); chromatography of HC, HM and NHC from a urine sample collected following use of hydrocodone (B).

as 5 ng/mL. The lower and upper limit of quantitation (LLOQ and ULOQ) were defined as the lowest and highest concentration for the analyte that could be quantified with an acceptable level of precision and accuracy and for which ion ratios fall within 20% of the established range and S/N ratios are at least ≥ 10. The LLOQ was 5 ng/mL for all analytes; the ULOQ was 10,000 ng/mL for hydrocodone and hydromorphone and 5,000 ng/mL for norhydrocodone. Six different random urines were evaluated to check for any indication of interference with the ions of interest. No interference was observed at the retention time of peaks for hydrocodone, hydromorphone, norhydrocodone or respective internal standards in any of the negative urines. Within-run precision was determined by testing replicates ($n = 6$) of the three analytes at 100 ng/mL and internal standards at 100 ng/mL in a single assay. The relative standard deviation (RSD) for within-run precision was 5.3, 4.4 and 3.2 % for hydrocodone, hydromorphone and norhydrocodone, respectively. Between-run precision was determined by calculating the mean, standard deviation and RSD at 25, 100 and 2,500 ng/mL of the analytes and internal standards on seven consecutive runs. The RSD for between-run precision at 25, 100 and 2,500 ng/mL was 7.9, 7.8 and 5.3% for

hydrocodone; 7.3, 6.8 and 5.8% for hydromorphone; and 5.1, 8.4 and 4.3% for norhydrocodone, respectively.

Samples stored refrigerated (2–8°C) or frozen (−20 and −70°C) were shown to be stable for at least three months. Samples were considered to be stable if quantitative values obtained for the stored samples were within ±20% of the initial values acquired when first analyzed. No significant loss or deterioration (≤20%) for any of the analytes of interest was observed.

Extraction efficiency of hydrocodone, hydromorphone and norhydrocodone showed recoveries to be 69, 65 and 66%, respectively. Although the extraction efficiency for these analytes appears at first glance to be low, the compounds were readily extracted and found in high abundance during MS analysis; consequently, detection and quantitation of the metabolites was easily accomplished, even at low ng/mL concentrations.

Injections of drug-free urine along with post-column infused analyte solution were made to evaluate ion suppression. No ion suppression was observed at the retention times of interest for any of the analytes. Figure 3 demonstrates a typical ion suppression pattern for a blank urine sample.

Excretion profile in urine

Hydrocodone was administered to seven healthy subjects. Following administration of the drug, the subjects were asked if they noticed any subjective effect from the drug. Three subjects reported mild to moderate drowsiness 30 min to 2 h following drug administration; one of those subjects also reported dry mouth at 2 h. Subject 4 reported nausea and impaired motor skills approximately 1.5 h following administration. As expected, all three analytes were detected in subject urine samples. A typical chromatogram of a 50-ng/mL calibrator and a subject urine sample containing 19.1, 19.2 and 54.2 ng/mL hydrocodone, hydromorphone and norhydrocodone, respectively, are shown in Figure 2. The data presented here are on a controlled single dose administration of hydrocodone with no other drug use. In previous studies (14, 17, 24), hydrocodone, hydromorphone and norhydrocodone were detected in human urine; however, those studies consisted of samples from chronic pain patients for whom multiple drug use is common. Excretion of hydrocodone and hydromorphone in urine from human volunteers following administration of hydrocodone has been described; in those studies, either measurement of the metabolites was not provided or urines were pooled over a four-hour period (6, 13). Consistent with our data, Smith *et al.* (25) reported hydrocodone and hydromorphone concentrations of less than or equal to 300 ng/mL in urine within 24 h after single dose administration of hydrocodone, however, no data on norhydrocodone were provided in that report. To our knowledge, no studies have shown the excretion profile, to this extent, of hydrocodone, hydromorphone and norhydrocodone following single dose administration of hydrocodone to human subjects. In the present study, only the parent drug and the *O*-demethylated and *N*-demethylated metabolites were measured by LC–MS-MS. Specimens were hydrolyzed by

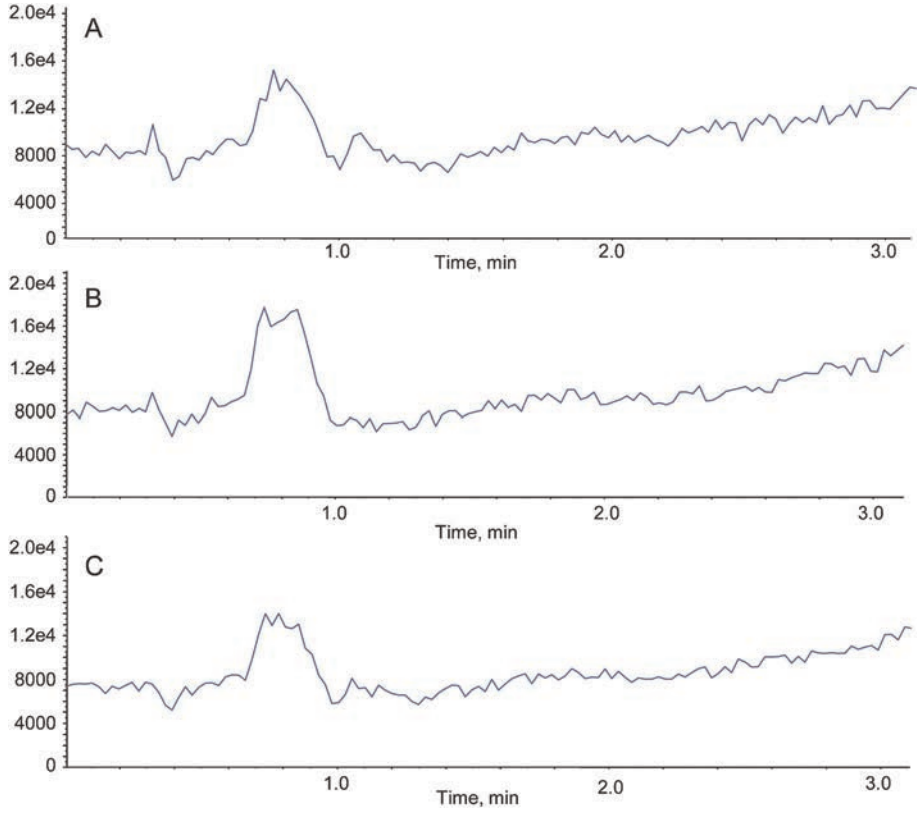

Figure 3. Illustration of ion suppression evaluation. Infusion at 200 ng/mL while injecting extracted blank matrix (drug negative urine): hydrocodone (A); hydromorphone (B); norhydrocodone (C).

enzymatic treatment before assay to provide measurement of total drug. Hydromorphone was detected at lower concentrations than hydrocodone and norhydrocodone and for a shorter period of time than the nor-metabolite. Norhydrocodone was detected at higher concentrations and for a longer period of time than hydrocodone. In view of these findings, the inactive metabolite, norhydrocodone, may serve as a valuable indicator of hydrocodone use.

A summary of hydrocodone, hydromorphone and norhydrocodone results from the study samples is given in Table II and metabolism profile in Figure 4 (four of seven subjects). Only four of seven subject excretion profiles are shown in Figure 4. The excretion profiles of Subjects 5–7 were comparable to those of Subjects 1–4, and therefore were not depicted. The first detectable levels of hydrocodone, hydromorphone and norhydrocodone (LOD = 5 ng/mL hydrocodone, norhydrocodone;

Table II
Summary of Hydrocodone (HC), Hydromorphone (HM) and Norhydrocodone (NHC) Detection

Subject	HC, HM, NHC first detected (≥ 2.5; 5; 2.5 ng/mL) (h post dose)	HC last detected (≥ 2.5 ng/mL) (h post dose)	HM last detected (≥ 5 ng/mL) (h post dose)	NHC last detected (≥ 2.5 ng/mL) (h post dose)	Maximum concentration HC (ng/mL)	Maximum concentration HM (ng/mL)	Maximum concentration NHC (ng/mL)
1	3:30	51:00	84:00	84:00	919	163	1,440
2	5:40	68:00	68:00	71:30	2,190	342	1,790
3	1:45	49:50	49:50	56:10	1,160	102	1,240
4	7:03	98:00	98:00	102:35	1,390	310	3,460
5	4:45	91:45	91:45	129:40	612	261	811
6	3:15	96:45	59:15	84:15	1,380	329	2,050
7	2:00	46:00	46:00	74:00	947	239	1,420

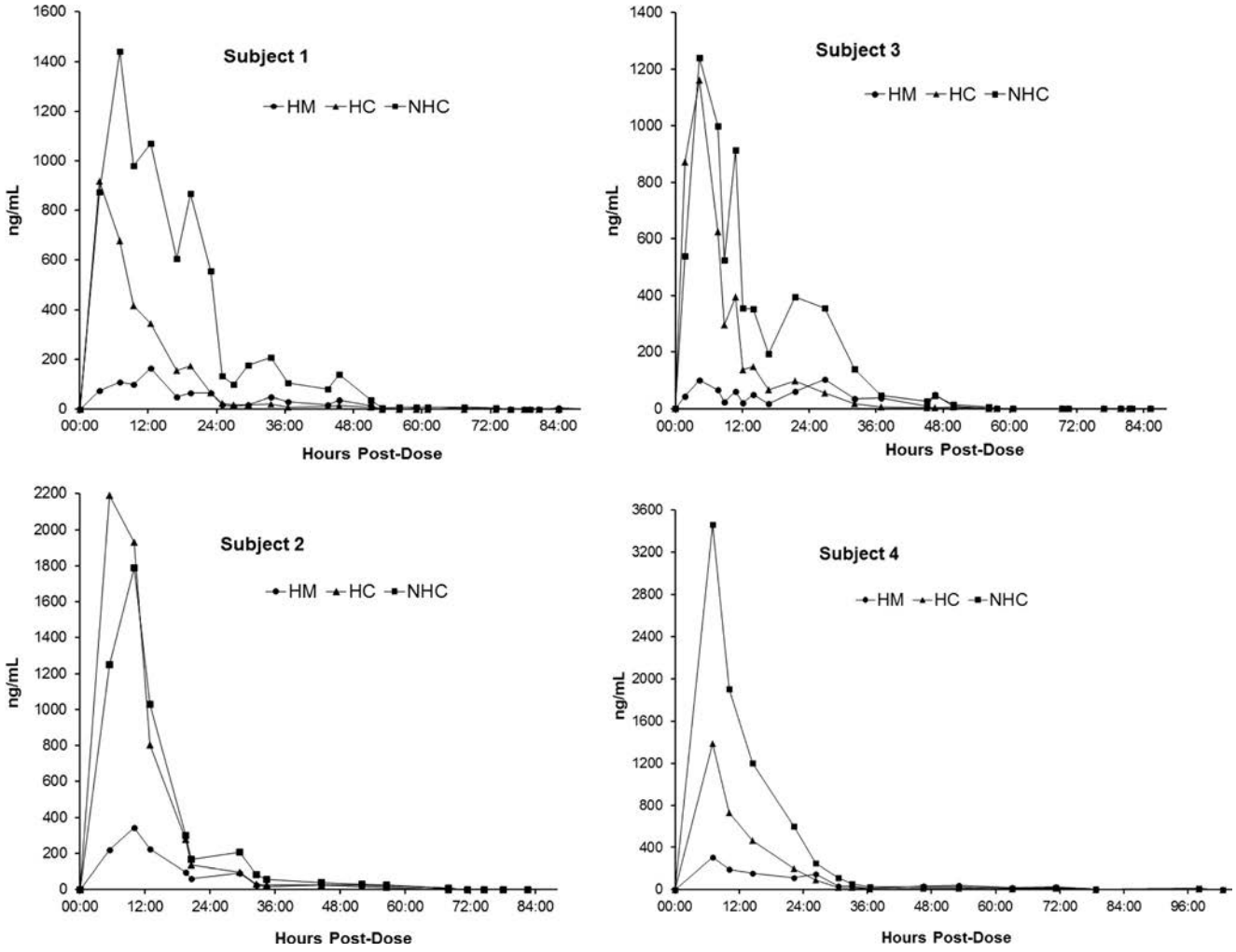

Figure 4. Metabolism profile for hydrocodone (HC), hydromorphone (HM) and norhydrocodone (NHC) following single dose hydrocodone administration.

2.5 ng/mL hydromorphone) ranged from 1:45 to 7:00 h post-dose. Peak concentrations of hydrocodone were observed in samples collected 3:30 to 7:00 h post-dose where the concentrations reached 612 to 2,190 ng/mL. Hydrocodone was last detected from 46:00 to 98:00 h post-dose. Hydromorphone peak concentrations ranged from 102 to 342 ng/mL and were observed 6:15 to 26:45 h post-dose. It was last detected from 46:00 to 98:00 h post-dose. Norhydrocodone was detected at much higher concentrations and lasted for a longer period of time than the parent drug. Peak concentrations were observed in samples collected 4:20 to 13:00 h post-dose, when concentrations reached 811 to 3,460 ng/mL. It was last detected from 56:10 to 129:40 h post-dose.

Hydrocodone peaked at the same time or before norhydrocodone in every subject. With the exception of one subject, norhydrocodone reached higher concentrations than hydrocodone in the early hours following administration of the drug. Another significant finding in this study was that the nor-metabolite was found for a longer period of time than hydrocodone. In all subjects, norhydrocodone could be detected in samples after hydrocodone dropped below detection limits. In all but one subject, hydromorphone was detected for at least as long as hydrocodone was detected. Norhydrocodone was observed at higher concentrations for the first 3:15–13:00 h post-dose, with peak concentrations up to 2.5 times that of hydrocodone. In every case, once the norhydrocodone exceeded hydrocodone concentrations, norhydrocodone was always found in greater amounts than the parent opioid, and was detected for a longer period of time.

The rate of excretion varies depending on differences in metabolism and urinary function. Urinary drug and metabolite concentrations can fluctuate depending on daily fluid intake. Excessive fluid intake can cause dilute urine that may result in a false-negative drug test. To cope with dilute urine specimens, measurement of specific gravity and creatinine is recommended for urine testing programs (26, 27). According to the United States Department of Health and Human Services drug testing guidelines, dilute urines are those with specific gravity < 1.003 and creatinine concentration < 20 mg/dL (28). In the current study, subjects were allowed to drink fluids *ad libitum* throughout the study. All urines were collected for up to five days post dose, and pH, specific gravity and creatinine measurements were performed on each sample. Specific gravity was ≥ 1.003 and creatinine was > 20 mg/dL for all subject samples.

Conclusion

Samples obtained from a controlled single dose study involving the administration of hydrocodone were analyzed for the presence of hydrocodone, hydromorphone and norhydrocodone. A procedure for the quantitation of hydrocodone, hydromorphone and norhydrocodone was evaluated and shown to be suitable for the detection of this drug and its metabolites in subject urine samples. Administration of hydrocodone resulted in urinary excretion of substantial amounts of the parent drug along with norhydrocodone and smaller amounts of the active metabolite, hydromorphone. Hydrocodone was found at relatively high concentrations, reaching peak values as high as 2,190 ng/mL, and with detectable levels observed for up to 98 hours post dose. Although hydrocodone was detected at rather high concentrations, hydromorphone was detected at very low concentrations, and for the most part was detected for as long as hydrocodone. A notable finding is that all samples containing hydrocodone at or above 5 ng/mL showed detectable amounts of hydromorphone and norhydrocodone. However, detection of hydromorphone in urine does not clearly demonstrate the use of hydrocodone because of its availability as a commercial analgesic, and detection of both hydrocodone and hydromorphone does not indicate whether an individual consumed one or both drugs. The nor-metabolite was detected in all seven subjects for a longer period of time and in samples containing no detectable amount of hydrocodone. Thus, the absence of parent drug in the urine does not exclude the possibility of its use. Future studies evaluating hydromorphone-3-glucuronide will be undertaken, albeit the metabolite of significance appears to be the unique nor-metabolite. Norhydrocodone proved to be an excellent indicator and may serve as an interpretative biomarker for hydrocodone use. The data provide a clearer understanding for interpretation of hydrocodone use.

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The voluntary fully informed consent of the subjects used in this research was obtained as required by AFI 40-403.

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the US Government.

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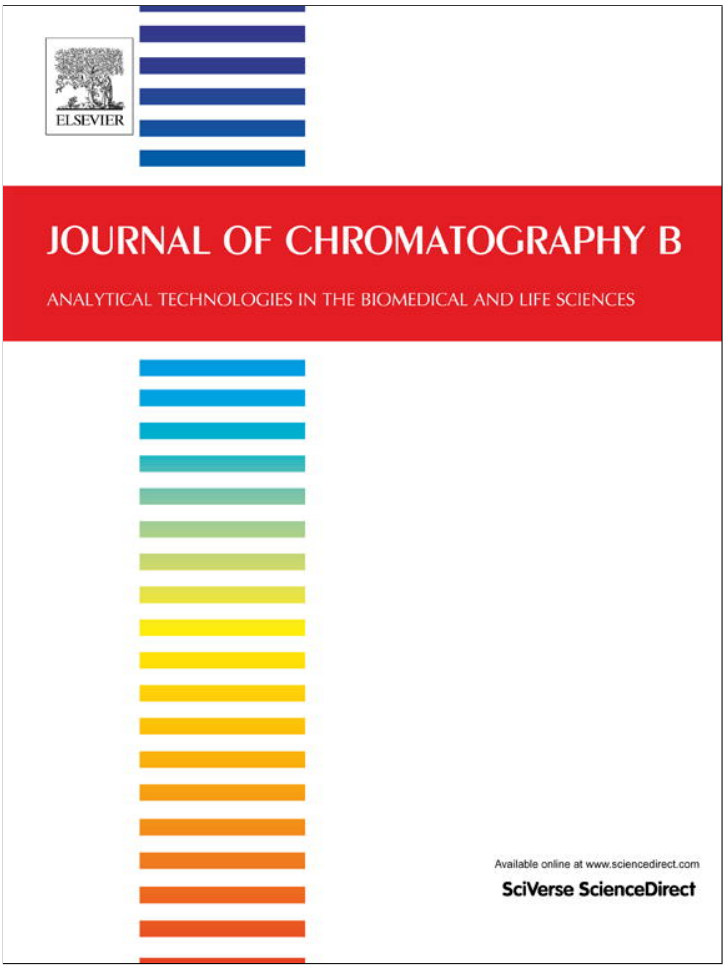

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Short communication

Quantitative method for analysis of hydrocodone, hydromorphone and norhydrocodone in human plasma by liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

A selective, sensitive and accurate high-performance liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the quantitation of hydrocodone, hydromorphone and norhydrocodone in human plasma was developed. The internal standard stock solution comprised of hydrocodone-d6, hydromorphone-d6 and norhydrocodone-d3 was added to 0.5 mL plasma samples. Samples were extracted using a copolymeric sorbent (mixed mode) solid phase extraction (SPE) column. Chromatographic separation was carried out using a reversed-phase C18 analytical column with a gradient mobile phase consisting of solvent A = 5% acetonitrile with 0.1% formic acid and solvent B = 100% acetonitrile. MS analysis was performed using positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode. Linearity was established over the range 1–100 ng/mL with correlation coefficients ≥0.998 for all three analytes. The coefficient of variation (CV) of intra-day samples was ≤5.6% at 10 ng/mL. The precision of inter-day (6 days) samples resulted in CVs ≤8.1% at concentrations tested at 2.5, 10 and 25 ng/mL for all three analytes. The lower limit of quantification (LOQ) was 1.0 ng/mL with signal-to-noise (S/N) ratio >10, the limit of detection (LOD) was 0.25 ng/mL with S/N ratio >3 for the drug and its metabolites. Dilution effects, extraction recovery, stability, interference, carryover and ion suppression were also evaluated. This method was successfully applied to human subject plasma samples in support of a hydrocodone pharmacokinetic study.

1. Introduction

There has been a substantial increase in prescribing and misuse of the opioid pain medication, hydrocodone. Hydrocodone is a semi-synthetic opioid that has been used for decades as an analgesic [1–3] and is prescribed frequently for patients suffering from acute and chronic pain. It is highly addictive and has a high potential for abuse. Hydrocodone is metabolized by the body to hydromorphone, norhydrocodone and other minor metabolites. Although hydrocodone has been shown to have some activity, the more active compound is hydromorphone [4]. The cytochrome P450 (CYP) isoforms involved in the oxidative metabolism of hydrocodone have been well characterized. Hydrocodone is O-demethylated by cytochrome P450 2D6 (CYP2D6) to its active metabolite, hydromorphone and N-demethylated by cytochrome P450 3A4 to form norhydrocodone [4–9]. Because hydrocodone is biotransformed into another commercially available prescription opiate, hydromorphone, detecting the active metabolite in biological matrices may not be the best indicator for hydrocodone use. On the other hand, norhydrocodone is not available as a prescription drug thus may be the more useful metabolite in monitoring and understanding hydrocodone use.

Several quantitative methods for hydrocodone and/or hydromorphone in blood or plasma have been reported [5,10–17]; however, detection or quantitation of the nor-metabolite was not accomplished. In other studies, description of quantitative methods for hydrocodone, hydromorphone and norhydrocodone have been published; however, the matrix in those studies was human liver microsomes [18] or urine [19]. In the current study, a quantitative method for analysis of hydrocodone, hydromorphone and norhydrocodone in plasma using SPE and LC–MS–MS was validated and

☆ The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the US Government.

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applied to a pharmacokinetic study in plasma of human subjects administered hydrocodone.

2. Experimental

2.1. Chemicals and reagents

Hydrocodone, hydromorphone, norhydrocodone, hydrocodone d-6, hydromorphone-d6 and norhydrocodone-d3 were obtained from Cerilliant (Round Rock, TX). β-Glucuronidase from *Helix pomatia*, Type HP-2 (aqueous solution, activity ≥100,000 U/mL) was obtained from Sigma Chemical Company (St. Louis, MO). Acetonitrile (Optima® LC/MS), water (Optima® LC/MS), methanol (HPLC grade), methylene chloride (HPLC grade), acetic acid (HPLC grade) isopropyl alcohol (A.C.S.), and sodium acetate (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide, 28–30% (A.C.S.) was obtained from J.T. Baker Inc. (Phillipsburg, NJ). The Kinetex 2.6μ, C18, 50 mm × 2.10 mm analytical column was purchased from Phenomenex (Torrance, CA) and the Clean Screen XCEL I, 130 mg/3 mL extraction column from United Chemical Technology ([UCT], Bristol, PA). Formic acid was from Michrom Bioresources, Inc. ([Ultra Pure], Auburn, CA).

2.2. Sample preparation

A stock solution was prepared by adding 1000 ng/mL hydrocodone, hydromorphone and norhydrocodone to opioid negative human pooled plasma. Further dilutions were made at the following concentrations: 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL. Enzyme hydrolysis was used. Twenty-five microliters of 1 μg/mL d6-hydrocodone/d6-hydromorphone/d3-norhydrocodone, 500 μL 0.1 M sodium acetate buffer, pH 5 and 20 μL 10,000 U/mL β-glucuronidase were added to 500 μL of plasma. Samples were mixed, heated to 60 °C for 2 h in a water bath, cooled to room temperature, centrifuged at 2500 rpm for 15 min then transferred to a clean glass tube and then extracted. A 500 μL aliquot of hydrolyzed plasma was extracted with UCT Clean Screen XCEL I 130 mg/3 mL column using a Zymark RapidTrace robotic system. The extraction protocol was as follows: the sample was loaded onto the column at 1 mL/min, dried with nitrogen for 1 min, rinsed with 2 mL 2% acetic acid in methanol, then dried with nitrogen for 5 min. The analytes were eluted with 1 mL freshly prepared solution of methylene chloride/isopropyl alcohol/ammonium hydroxide (78:20:2, v/v/v) at 1 mL/min. The extracts were evaporated to dryness under a stream of nitrogen in a 45 °C water bath. The dry residues were reconstituted in 100 μL mobile phase (5% acetonitrile aqueous solution with 0.1% formic acid) and injected onto the LC–MS–MS system.

2.3. LC–MS–MS conditions

The LC–MS–MS configuration was comprised of an Agilent LC system coupled to an Applied Biosystems 4000 QTrap mass spectrometer. The samples were analyzed using a Phenomenex Kinetex analytical column (2.6μ, 50 mm × 2.1 mm). The LC mobile phase consisted of solvent A = 5% acetonitrile with 0.1% formic acid and solvent B = 100% acetonitrile; flow rate was set at 0.5 mL/min. The column oven temperature was maintained at 25 °C and the injection volume was 10 μL. The gradient flow method consisted of an opening condition of 5% solvent B, with a linear increase to 60% solvent B over 2.2 min, then a linear increase to 95% solvent B at 2.23 min, 0.27 min at 95% solvent B, and then a return to the opening condition (5% solvent B) via a linear gradient over 0.15 min, followed by 2.35 min re-equilibration at opening conditions. The total run time was 5 min for each sample. Analysis was performed using positive ion electrospray MS–MS in multiple reaction monitoring (MRM) mode. Two MRM transitions (transition 1: quantifier; transition 2: qualifier) per analyte were used, and the declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized as shown in Table 1. The detector conditions were as follows: polarity mode, ESI positive; Scan type, MRM; Curtain gas 15 psi, Collision–activated dissociation (CAD) gas = high; ion source gas 1 = 40; ion source gas 2 = 40; ion spray voltage 5000 V; temperature 600 °C; entrance potential 10.

2.4. Validation

Quantitation and detection were based on a six-point calibration using calibration standards containing 1, 2.5, 5, 10, 50 and 100 ng/mL of hydrocodone, hydromorphone and norhydrocodone prepared in drug free plasma with each standard containing 50 ng/mL of deuterium-labeled isotopomer internal standards (hydrocodone-d6, hydromorphone-d6, norhydrocodone-d3). To test dilution effects and linearity range, concentrations at 1, 2.5, 25, 50, and 100 prepared from a 1000 ng/mL pooled plasma stock of the three analytes were evaluated on six consecutive runs. Transition ions monitored (Table 1) for the analytes and their respective internal standards were as follows: hydrocodone m/z 300.2 > 199.1, 300.2 > 171.0; hydrocodone-d6, 306.2 > 174.0; hydromorphone 286.1 > 185.0, 286.1 > 157.0; hydromorphone-d6, 292.2 > 185.2; norhydrocodone, 286.2 > 199.1, 286.2 > 241.1; and norhydrocodone-d3, 289.0 > 202.0. The mass spectra of the three opioids and deuterated internal standards are shown in Fig. 1. Acceptance criteria were: quantitative values within ±20% of the target concentration with proper qualitative identification as determined by transition ion area ratios within ±20% and retention time within ±2% of the calibration standards while exhibiting acceptable chromatography. The calculated ion area ratio range was based on the average of ratio ranges obtained for the six standards.

Twelve samples were analyzed to demonstrate the efficiency of the extraction procedure. Drug and internal standards were added to 6 samples prior to extraction. To the remaining 6 samples, internal standard was added after the samples had been extracted. The mean and standard deviation were calculated for each set of samples and extraction efficiency determined. The linear range was established for this procedure by the analysis of at least seven different runs with concentrations ranging from 1 to 100 ng/mL hydrocodone, hydromorphone and norhydrocodone. Within run precision was measured by testing replicates (n = 6) of the three analytes at 10 ng/mL and respective internal standards at 50 ng/mL. Between run precision was measured by testing concentrations at 2.5, 10, and 25 ng/mL of the three analytes and respective internal standards on six separate runs. The LOD was assessed by testing analyte concentrations at 0.25 and 0.5 ng/mL on at least six runs. Six random plasma specimens collected from human volunteers were analyzed to check for potential endogenous interferences with the analytes of interest. Stability of the drug and metabolites in plasma was also evaluated. Plasma stock solutions at concentrations of 25 and 50 ng/mL of the analytes were stored refrigerated (2–8 °C) or frozen (−70 °C). Refrigerated samples were tested after 2 months of storage; frozen samples were tested for up to 1 year from storage date. The freeze–thaw stability was evaluated by analyzing three subject samples with known drug concentrations and two quality control samples at concentrations of 7.5 and 25 ng/mL after undergoing three freeze–thaw cycles. Samples remained unfrozen for a minimum of 2 h per cycle with at least 20 h between cycles. An autosampler stability (room temperature) determination of six quality control sets was performed at a 24 h period.

Since matrix effect can influence the extent of analyte ionization, an ion suppression experiment was conducted. Drug negative

Table 1
MRM transitions.

Compound transition 1/2[a]	MRM transition (*m*/*z*)	Dwell time (ms)	Declustering potential (DP)	Collision energy (CE)	Collision cell exit potential (CXP)
Hydrocodone 1	300.2 → 199.1	100	90	42	13
Hydrocodone 2	300.2 → 171.0	100	90	54	13
Hydrocodone-D6	306.2 → 174.0	100	97	42	13
Hydromorphone 1	286.1 → 185.0	100	94	42	11
Hydromorphone 2	286.1 → 157.0	100	94	57	11
Hydromorphone-d6	292.2 → 185.2	100	100	44	12
Norhydrocodone 1	286.2 → 199.1	100	85	39	14
Norhydrocodone 2	286.2 → 241.1	100	85	34	14
Norhydrocodone-d3	289.0 → 202.0	100	80	40	10

[a] Transition 1: quantifier and transition 2: qualifier.

plasma was hydrolyzed and extracted in the same manner as test samples to best mimic matrix complexity. A 200 ng/mL solution of the analytes in acetonitrile was prepared and was loaded into the infusion pump syringe. The infusion pump was connected post-column via a tee connector; the solution was infused at10 μL/min. Once the baseline was stable, injections of drug-free plasma extract were made in the same manner as standard acquisitions.

3. Results and discussion

3.1. LC–MS–MS

One of the advantages of LC–MS–MS is the ability to spectrally separate these opioids. An LC gradient with run time of 5 min was instituted to achieve better separation of the compounds. Retention time for hydrocodone was 2.49 min, 1.48 min for hydromorphone and 2.42 min for norhydrocodone. Hydromorphone and norhydrocodone share the same precursor ion but were separated chromatographically in time. Hydrocodone and norhydrocodone were not totally separated but were spectrally distinguishable due to the difference in precursor ion between the two analytes.

The hydroxy metabolite exists in both the free and glucuronide-conjugated forms. The samples were subjected to enzyme hydrolysis prior to extraction to provide measurement of total (free and conjugated) hydromorphone. Evaluation of conjugated versus unconjugated metabolite in plasma samples from subjects administered hydrocodone was assessed. Results showed that total hydromorphone was clearly generated by hydrolysis. Specimens were hydrolyzed by a 2 h enzymatic treatment prior to assay to provide measurement of total drug.

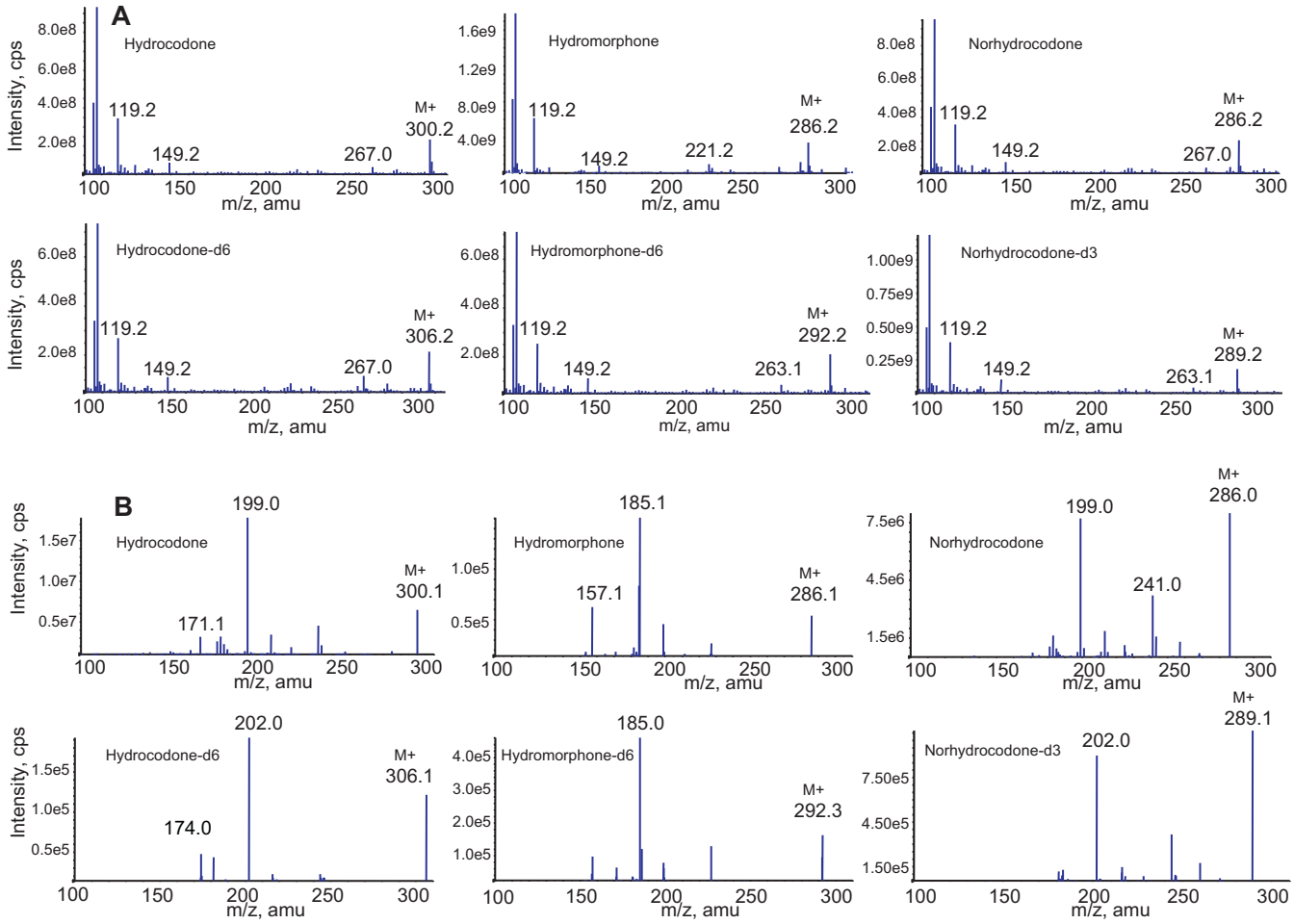

Fig. 1. (A) ESI-mass spectra and (B) MS–MS spectra of the opioids, and their deuterated internal standards.

3.2. Dilution integrity

Samples diluted from a stock prepared in opioid negative human plasma were analyzed to study dilution effects. The 100 ng/mL concentration was diluted directly from the 1000 ng/mL stock. Concentrations diluted directly from the 100 ng/mL stock were the 75, 50, and 10 ng/mL; the 7.5, 5, and 1 ng/mL were diluted from the 10 ng/mL stock. The 25 ng/mL was diluted from the 50 ng/mL stock; 2.5 ng/mL from 5 ng/mL; 0.5 from 1 ng/mL; 0.25 ng/mL from 0.5 ng/mL and 0.1 ng/mL from 1 ng/mL. Concentrations were analyzed on 6 different runs. The assay yielded good reproducible results (±20% of target concentration) and were within the linear range of the standard curve with exception of the 0.1 and 0.25 ng/mL concentrations. In addition, 1:40 and 1:20 dilutions of the 1000 ng/mL concentration were prepared in opioid negative human plasma, results were within acceptable range of target value. At dilution factor of 40×, quantitative results were 1024, 1020 and 976 ng/mL; at 20× dilution factor, quantitative results were 986, 996 and 1004 ng/mL for hydrocodone, hydromorphone and norhydrocodone, respectively.

3.3. Linearity and recovery

A multi-level calibration was used; the curve was constructed by calculating the amount ratio and the response ratio for the calibrator levels using the instrument's data system software. The average correlation coefficient and standard deviation for the curves was calculated to determine the minimum acceptable value for the calibration curve (1–100 ng/mL). Based on these data, the minimum allowable value for a calibration curve for all analytes is 0.995. Acceptable linearity was defined as a set of concentration values with a linear-regression correlation coefficient (r) of ≥0.995 and ion ratio values within ±20% of average standard values; all standard curves over the range from 1 to 100 ng mL hydrocodone, hydromorphone, and norhydrocodone were within acceptable calibration criteria. Quantitation was based on a six-point calibration curve: 1, 2.5, 5, 10, 50, 100 ng/mL with final internal standard concentrations of 50 ng/mL. The linear range for hydrocodone, hydromorphone and norhydrocodone is 1–100 ng/mL. The linear range was established for this procedure by analysis on 6 different runs on concentrations ranging from 0.1 to 100 ng/mL of the analytes prepared in opioid negative plasma. Values were considered within acceptable range if the measured amount was within ±20% of target concentration and ±20% of ion ratio calculation. Originally, 12 concentration points (0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 25, 50, 75 and 100 ng/mL hydrocodone, hydromorphone and norhydrocodone) were tested; however, the measured amount for 0.1, 0.25 and 0.5 ng/mL of each analyte was not always within acceptable limits.

For extraction efficiency evaluation, twelve samples were either subjected to addition of internal standard pre-SPE or to addition of internal standard post-extraction. Drug (10 ng/mL hydrocodone, hydromorphone, norhydrocodone) and 50 ng/mL internal standard (hydrocodone-d6, hydromophone-d6, norhydrocodone-d3) were added to 6 samples prior to extraction. To the remaining 6 samples, internal standard was added after the samples had been extracted. The mean and standard deviation were calculated for each set of samples and extraction efficiency determined. The extraction efficiency for hydrocodone, hydromorphone, and norhydrocodone was 74, 72, and 73%, respectively.

Table 2
Between run precision.

Compound	Concentration (ng/mL)	Mean (n = 6)	Std. dev.	% CV
Hydrocodone	2.5	2.43	0.10	4.2
	10.0	9.84	0.79	8.1
	25.0	24.18	1.23	5.1
Hydromorphone	2.5	2.52	0.16	6.3
	10.0	10.0	0.72	7.2
	25.0	23.88	0.81	3.4
Norhydrocodone	2.5	2.61	0.11	4.1
	10.0	10.18	0.35	3.4
	25.0	24.15	1.27	5.3

3.4. Precision

Intra-assay (within run) precision was assessed by testing replicates (n = 6) of the analytes and internal standard in a single assay. Inter-assay (between run) precision was determined by testing replicates (n = 6) of the analytes and internal standard in at least six consecutive runs. The mean, standard deviation and CV were calculated. Within-run precision CV was ≤5.6% for all analytes. Between run precision CVs were ≤6.3% at 2.5 ng/mL, ≤8.1% at 10 ng/mL and ≤5.3% at 25 ng/mL for all three analytes (Table 2).

3.5. Sensitivity

The limit of detection (LOD) is the minimum concentration at which the analyte can be identified (signal-to-noise [S/N] ratio >3). The lower limit of quantitation (LLOQ) by this procedure was defined as the lowest concentration of the analyte that can be quantified with an acceptable level of precision and accuracy and for which ion ratios fall within 20% of the established range and S/N ratios are at least >10. To determine LOD and LOQ, concentrations at 0.1, 0.25 and 0.5 ng/mL hydrocodone, hydromorphone, and norhydrocodone were assayed on six runs. Under these conditions, the LOD (acceptable ion ratios, presence of all MRM transitions and signal-to-noise [S/N] ratio ≥3) was 0.25 ng/mL. Representative chromatograms of the LOD sample for the analytes are shown in

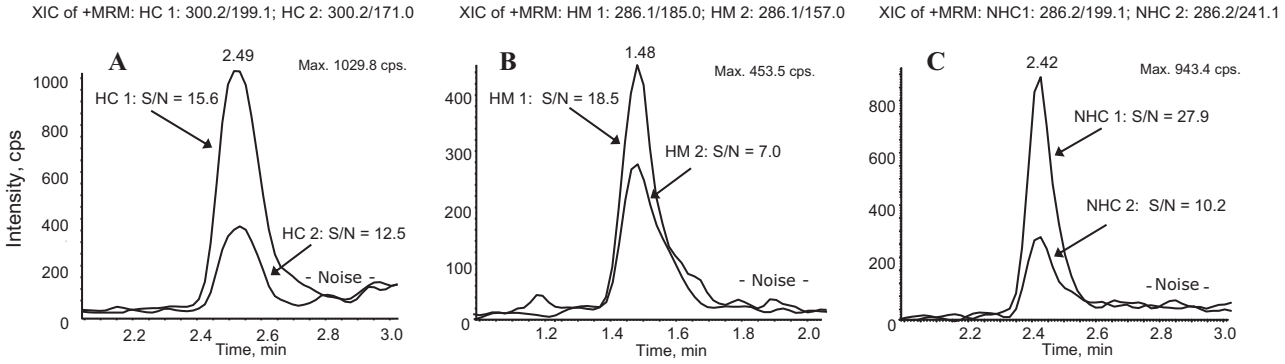

Fig. 2. Signal-to-noise ratio (S/N) for 0.25 ng/mL: (A) HC, (B) HM and (C) NHC MRM transitions 1 and 2.

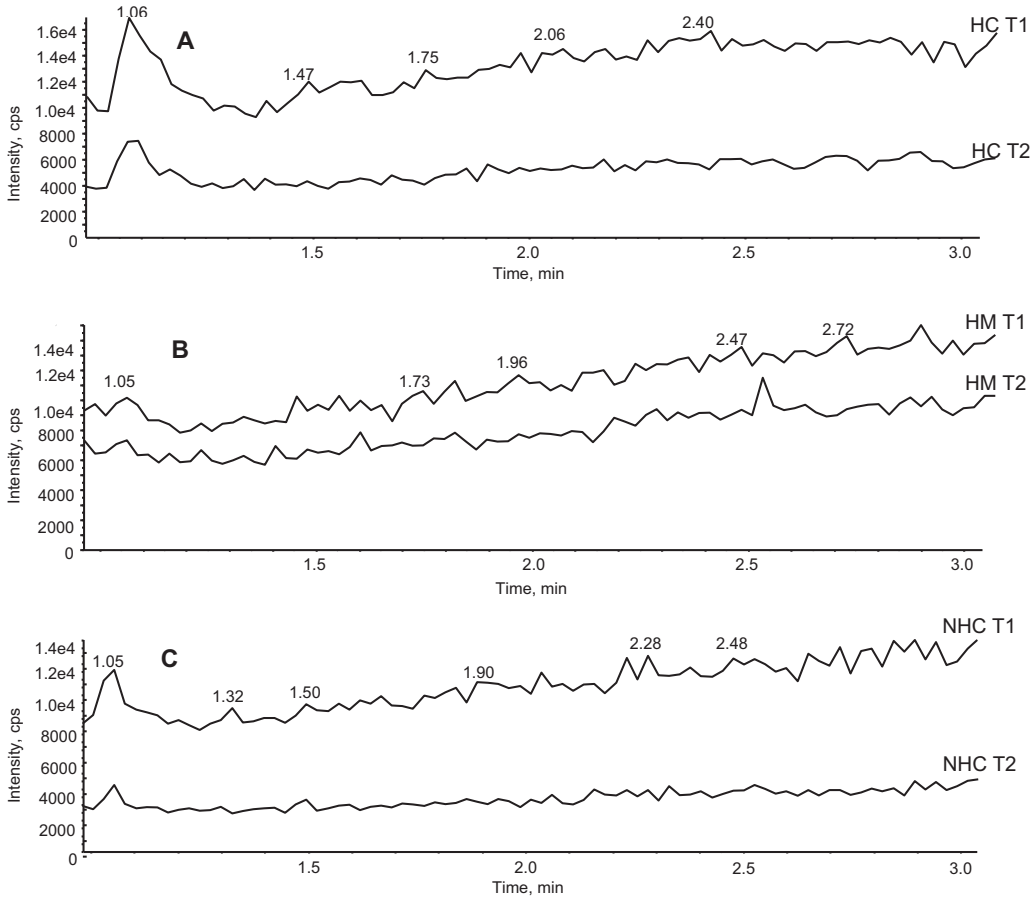

Fig. 3. Ion suppression evaluation. (A) HC MRM transition 1 (300.2/199.1) and 2 (300.2/171.0), (B) HM MRM transitions 1 (286.1/185.0) and 2 (286.1/157.0) and (C) NHC MRM transitions 1 (286.2/199.1) and 2 (286.2/241.1).

Fig. 2. The LOQ was 1.0 ng/mL for all three analytes with S/N ratios greater than 12:1.

3.6. Stability.

Hydrocodone, hydromorphone and norhydrocodone plasma spiked samples and samples from human subjects administered hydrocodone were aliquoted into several sets and stored either at 2–8 °C for 2 months or −70 °C for up to 12 months. No statistically significant difference ($p > 0.05$) was seen between results for first run vs. refrigerated or freezer stored samples, therefore, under these conditions, the analytes are stable for at least 2 months at 2–8 °C and up to 12 months at −70 °C. The analytes were found to be stable in plasma through three freeze (−70 °C)–thaw (4 °C) cycles, and for at least 24 h in the reconstitution solution on the autosampler tray (room temperature).

3.7. Interference and carry-over

Six different random opioid-negative plasma samples were evaluated to check for any indication of interference with the monitored ions for the analytes or internal standards under the conditions used in this study. No interference was seen; endogenous components in plasma did not interfere with any of the analytes at the retention time of peaks for hydrocodone, hydromorphone, norhydrocodone and respective internal standards in negative plasma samples.

Carry-over was evaluated by injection of blank sample (mobile phase) or known opioid negative plasma following high concentration (100 and 1000 ng/mL) samples. Solvent and negative plasma injections following the high concentration injections showed no significant carryover. Although no significant carryover was seen following high concentration samples, one solvent injection was placed between each sample injection.

Ion suppression was tested by infusing a 200 ng/mL solution of the analytes in acetonitrile. Once the baseline became stable, injections of drug-free plasma ($n = 3$) were then made as normal acquisitions. No ion suppression was seen at the retention times of interest (see Fig. 3).

4. Conclusion

This study provides a validated quantitative method for the analysis of hydrocodone, hydromorphone and norhydrocodone in plasma by LC–MS–MS. Extraction was performed using mixed mode SPE cartridges for sample preparation and a C18 column LC column for separation of the analytes. The six-point calibration curve consisted of 1, 2.5, 5, 10, 50 and 100 ng/mL of the compounds; however use of a 4 or 5 point calibration curve was found to be acceptable. The limit of quantitation is 1 ng/mL and LOD is 0.25 ng/mL for all three analytes. The method provided a reliable and sensitive procedure for the quantitation of hydrocodone, hydromorphone and norhydrocodone in human plasma samples for a hydrocodone pharmacokinetic study.

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