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Dose-related Distribution of Codeine, Cocaine and Metabolites into Human Hair Following Controlled Oral Codeine and Subcutaneous Cocaine Administration

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LC-APCI-MS/MS, liquid chromatography-atmospheric pressure chemical ionization tandem mass spectroscopy; LOQ, limit of quantification; C_{max}, maximum concentrations; SAMHSA, Substance Abuse and Mental Health Services Administration

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Abstract

Hair testing for determination of drug exposure has many useful applications. Drug incorporated into hair can be found for extended periods following drug exposure. There are few controlled drug administration studies investigating drug distribution into human hair. Ten volunteers participated in a ten-week controlled cocaine and codeine administration study, while residing on the secure research ward. Weekly hair samples were collected by electric razor. During the low dose week (week 4) volunteers received 75 mg/70 kg cocaine subcutaneously and 60 mg/70 kg codeine orally on alternating days, a total of three doses for each drug. Similarly, during week 7, volunteers received three doses 150 mg/70 kg cocaine and 120 mg/70 kg codeine. Maximum hair concentrations (C_{max}) were found one to three weeks after low and high doses. Dose-related C_{max} of cocaine, benzoylecgonine, ecgonine methyl ester, norcocaine, cocaethylene and codeine were found following low and high doses. Hair analysis was performed using liquid chromatography tandem mass spectrometry. A positive linear relationship was found between total melanin content of hair and C_{max} of codeine, cocaine and metabolites following high dosing. This study demonstrated dose-related concentrations of cocaine and metabolites in human hair following controlled cocaine administration. These data are the first demonstrating melanin-related incorporation of cocaine and metabolites into human hair following controlled cocaine administration.

The disposition of drugs in the body includes incorporation into growing hair, thereby making hair a useful matrix for monitoring drug exposure across a wide timeframe (Gaillard and Pepin, 1999). Several mechanisms for incorporation of drugs into hair have been proposed. Drugs diffuse from arterial capillaries near the root into hair matrix cells at the base of hair follicles (Nakahara, 1999). However, drugs also distribute into sweat and sebum, other matrices that contact hair and may play roles in drug incorporation (Joseph et al., 1998; Huestis et al., 1999; Liberty et al., 2004). Importantly, direct external contamination of hair by drugs in smoke or the environment have been shown to contribute to drug distribution into hair (Nakahara, 1999).

Of the numerous applications for hair testing, forensic and workplace drug testing programs have predominated because hair provides a longer window of drug detection than urine (Gaillard and Pepin, 1999; Caplan and Goldberger, 2001). Hair testing also is useful as an alternative to urine testing and/or self-report for objectively documenting past drug use in research studies (Tassiopoulos et al., 2004) and for monitoring relapse during drug treatment programs (Fiellin et al., 2001; Cone and Preston, 2002). Segmental hair analysis determines drug content along the length of the hair shaft and may be used to more closely approximate when drug exposure occurred. Segmental analysis is sometimes used for monitoring compliance of therapeutic drugs (Servais et al., 2001; Bernard et al., 2002; Kronstrand et al., 2002), for investigation of suspected drug facilitated sexual assault (Kintz et al., 2003; Kintz et al., 2004) and other criminal cases.

Hair testing to determine drug exposure has limitations including contamination of hair by smoked drugs (i.e. cannabis, cocaine, heroin and methamphetamine). Typically, hair samples

undergo washing in various solvents to prevent false positive results from external contamination (Paulsen et al., 2001; Romano et al., 2001; Schaffer et al., 2002).

Guidelines regarding hair testing, recently proposed by the Substance Abuse and Mental Health Services Administration (SAMHSA), stipulate cutoff concentrations and ratios for parent drug and metabolites (Substance Abuse and Mental Health Services Administration, 2004). The requirements for measuring metabolites aid in preventing false positive hair testing results due to contamination from passive drug exposure. Controlled drug administration studies in humans are needed to assist interpretation of drug concentrations in hair.

Another concern about hair testing is evidence that many drugs are preferentially incorporated into pigmented hair, such that dark colored hair tends to incorporate more drug than light colored hair. Wilkins, Rollins and colleagues conducted a series of investigations of the role of pigmentation in incorporation of drugs into hair (Gygi et al., 1996; Slawson et al., 1996; Hubbard et al., 2000). Rodent studies demonstrated higher drug concentrations in pigmented hair than nonpigmented hair of Long Evans rats following administration of cocaine, codeine and phencyclidine. Similar results were extended to humans with controlled oral codeine administration studies that demonstrated significant positive relationships between the concentrations of codeine and total melanin in human hair (Kronstrand et al., 1999; Rollins et al., 2003).

There are two types of melanin responsible for pigmentation, eumelanin, which predominates in dark hair, and yellow-red pheomelanin (Taylor, 2002). The total amount of melanin copolymers and the eumelanin/pheomelanin ratio, produces variation in hair color (Ito et al., 1993). Eumelanin, a polyanionic polymer at physiologic pH, plays the largest role in interactions between basic drugs and melanin (Slawson et al., 1998; Borges et al., 2003). The

mechanisms of drug-melanin interaction are not clearly delineated, with studies suggesting both ionic and covalent interactions (Claffey and Ruth, 2001; Claffey et al., 2001; Dehn et al., 2001; Borges et al., 2003). Additional *in vitro* studies suggest pigmented melanocytes contain a transport system responsible for drug influx and efflux (Borges et al., 2002).

The first objective of this study was to determine whether dose-related concentrations of drug and metabolites are found in human hair following controlled oral codeine and subcutaneous cocaine administration to volunteers. The second objective was to investigate whether there was a relationship between the amount of drug and metabolites incorporated into hair and total melanin content in hair.

Methods

Reagents and Standards USP grade cocaine hydrochloride administered to subjects was purchased from Mallinkrodt Chemical, Inc. (St. Louis, MO). Roxane Laboratories, Inc. supplied USP grade codeine sulfate tablets, which were encapsulated for dosing. USP grade lactose powder used as filler during capsule preparation of codeine for oral administration was purchased from Amend Drug and Chemical Company, Inc. (Irvington, NJ). Cocaine, cocaine-d_3, norcocaine, norcocaine-d_3, benzoylecgonine, benzoylecgonine-d_3, ecgonine methylester, ecgonine methylester-d_3, cocaethylene, cocaethylene-d_3, codeine, codeine-d_3, norcodeine, morphine, morphine-d_3, 6-acetylmorphine and 6-acetylmorphine-d_3 analytical standards were purchased from Cerilliant (Austin, TX). Drug free hair from healthy volunteers, verified as drug free via liquid chromatography-atmospheric pressure chemical ionization tandem mass spectroscopy (LC-APCI-MS/MS), was used for calibration and quality control samples. Ammonium acetate and formic acid were purchased from Sigma Chemical (St. Louis, MO). All solvents were HPLC grade. Fritted filters, 15mL volume, and Cleanscreen® solid phase extraction columns, part #ZSDAU020, were used in preparing hair samples for drug analysis (United Chemical Technologies, Bristol, PA). Melanin, from *Sepia officinalis*, was purchased from Sigma Chemical (catalog #M2649, St. Louis, MO). Soluene®-350 was obtained from PerkinElmer (Boston, MA). Fritted filters, 4mL volume, were used in preparing samples for determination of total melanin content (United Chemical Technologies, Bristol, PA). Albino rat hair, used for preparation of melanin calibration standards, was collected from Sprague-Dawley rats (Charles River, Raleigh, NC). Animals were housed and cared for according to "Principles of Laboratory Animal Care" (NIH publication no. 86-23, 1996).

Opiates, Cocaine and Metabolites in Hair Sample Preparation Details of the method for measuring opiates, cocaine and metabolites in hair extracts was previously published (Scheidweiler and Huestis, 2004). Twenty ± 0.2 mg of hair was pulverized using a mini bead-beater (BioSpec Products, Bartlesville, OK). Drug solution and/or deuterated internal standard solution were added to pulverized hair. 2500 µL of methanol was added and samples were sonicated at 37°C for three hours to extract drugs from hair. Following filtration, methanol was evaporated under nitrogen at 40°C. Sample residue was dissolved in three mL of 0.1M potassium phosphate buffer, pH 6.0 and analytes were extracted using CleanScreen solid phase extraction columns. Eluates were dried at 40°C under nitrogen and re-suspended in 50 µL of 10mM ammonium acetate with 0.001% formic acid (v/v).

Opiates, Cocaine and Metabolites in Hair LC-APCI-MS/MS Analysis The LC-APCI-MS/MS settings used for measuring opiates, cocaine and metabolites in hair extracts has been previously detailed (Scheidweiler and Huestis, 2004). Twenty µL of hair sample extract was injected onto a Shimadzu HPLC system (Shimadzu, Columbia, MD) consisting of a SIL-HTc auto-sampler and LC-10ADvp pumps coupled to an MDS-Sciex API 3000 triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization source operated in positive ionization mode (Applied Biosystems, Foster City, CA). Mobile phase consisted of A) 10mM ammonium acetate with 0.001% formic acid, v/v and B) acetonitrile. A Synergi Hydro-RP column (150 x 2mm, 4µm pore) with a C_{18} guard column (4 x 2mm) (Phenomenex, Torrance, CA) was utilized with a 200µL/min flow rate. Gradient elution was initiated with 90% mobile phase A and finalized with 10% mobile phase A at 20 minutes. Quantification of cocaine, norcocaine, benzoylecgonine, ecgonine methyl ester, cocaethylene, codeine, morphine and 6-

acetylmorphine was based upon multiple reaction monitoring of select fragment ions during tandem mass spectrometric (MS/MS) analysis. Recovery of opiates, cocaine and metabolites spiked into pulverized blank hair ranged from 70.9 to 96.5%. Limits of quantification (LOQ) for cocaine and metabolites were 17 pg/mg, except for ecgonine methyl ester (50 pg/mg). Opiate LOQs were 83 pg/mg. Standard curves were linear from the LOQ to 5,000 pg/mg for cocaine and metabolites, except benzoylecgonine (2,500 pg/mg). Opiate standard curves were linear from the LOQ to 12,500 pg/mg. Accuracy ranged from 84 to 115% for all analytes. Inter-assay precision, % relative standard deviation, was less than 11.0% for all analytes. Methanolic sonication produced less than 5% hydrolysis of cocaine and 6-acetylmorphine.

Assay for Determining Total Melanin Content of Hair A spectrophotometric method for determining the total melanin content of hair was developed based upon the method of Kronstrand et al. (Kronstrand et al., 1999). Calibration standards were prepared by fortifying albino rat hair with sepia melanin. Prior to pulverization in a mini bead-beater, albino rat hair was sequentially rinsed to remove bedding and feces using 2-propanol, distilled water, and methanol. All rinsings were discarded as waste. A 1 mg/mL solution of sepia melanin was prepared by incubating sepia melanin in Soluene-350: distilled water solution (9:1, v/v) at 80°C for 60 minutes in a 16x100mm glass screw-top test tube. Calibration solutions (25, 50, 100, 200 and 400 µg sepia melanin/mL) were prepared by serial dilutions of 1 mg/mL sepia melanin solutions using 90% Soluene-350 solution. Two mL of each calibrator solution was added to 25 ± 0.2 mg of pulverized albino rat hair in a 16x100mm glass screw-top test tube and incubated at 80°C for 60 min, creating calibrators of 2, 4, 8, 16 and 32 µg sepia melanin/mg of hair. Frontal head hair specimens from study subjects were pulverized in a mini bead-beater and 25 ± 0.2 mg

of pulverized hair was weighed into a 16x100mm glass screw-top test tube. Pulverized hair specimens were dissolved in 2mL of 90% Soluene-350 solution incubated at 80°C for 60 min. Following room temperature centrifugation at 500g, solutions were filtered through fritted filters under 5-10mmHg vacuum. Filtrates were aliquoted into quartz cuvettes and absorbance at 500nm measured using a Beckman DU-70 spectrophotometer (Fullerton, CA). Quantification of total melanin was determined using absorbance measurements from a 5-point calibration curve constructed using sepia melanin fortified into albino rat hair. The absorbance of 25 mg pulverized albino rat hair was subtracted from absorbance of calibrators and study specimens, i.e. background correction. Study specimens which yielded absorbance measurements greater than that of the highest calibrator were diluted 50% (v/v) with 90% Soluene-350 and re-analyzed.

Clinical Study Ten healthy volunteers participated in this Institutional Review Board (ethics committee) approved study. Inclusion criteria included self-reported history of cocaine and opiate use along with a positive urine sample for cocaine and/or opiates. All participants provided written informed consent and were financially compensated for participation. Participants resided on the secure clinical research unit located at the National Institute on Drug Abuse, Intramural Research Program in Baltimore, MD, for the duration of the nine to ten-week study and were under continuous medical supervision. The first three weeks of the study allowed for clearance of drugs self-administered prior to enrollment in the research study. Low dose cocaine and codeine administrations were conducted beginning at the end of the third week of the study. Cocaine hydrochloride was dissolved in sterile saline in preparation for subcutaneous (s.c.) administration. Codeine sulfate tablets were pulverized and added to capsules along with lactose as filler material, as required. All drug doses are expressed as the

salt. Three doses of each drug were administered on alternating days with daily doses of 75 mg/70 kg cocaine s.c. and 60 mg/70 kg codeine oral (p.o). High dose cocaine and codeine administrations were conducted beginning at the conclusion of week 7 of the study. Three doses of each drug were administered on alternating days with daily doses of 150 mg/70 kg cocaine s.c. and 120 mg/70 kg codeine p.o. Hair specimens were collected weekly throughout the course of the study via shaving the entire head with an electric razor. Head shavings were divided by region: frontal, temporal, nape, anterior vertex and posterior vertex. All hair samples were stored in glass scintillation vials at -20°C until analysis.

Statistics

Measured drug concentrations in hair were compared using a paired t-test with a significance threshold of $p<0.05$, using SPSS version 12.0 (SPSS Inc, Chicago IL). The value of the assay LOQ multiplied by 0.5 was inserted in place of samples measuring less than the assay LOQ for comparison using paired t-test. Linear regression comparing maximal concentrations of drugs measured in hair and total melanin content of hair was performed using SPSS version 12.0. Analysis of variance evaluated goodness of fit to the regression model, employing a significance threshold of $p<0.05$.

Results

Figure 1 depicts a representative profile of drug and metabolite concentrations observed during the course of the clinical study for one participant. The three-week washout phase prior to controlled dosing allowed for excretion of previously self-administered drug. Ecgonine methyl ester, codeine, morphine and 6-acetylmorphine concentrations in hair shavings collected immediately prior to commencement of drug dosing were less than the assay limits of quantification for all participants (Table 1). Morphine and 6-acetylmorphine were found in 5 of 10 subjects' initial hair shavings (data not shown), but concentrations of both analytes were less than the assay LOQ before commencement of dosing. All subjects had low but measurable cocaine concentrations the week prior to beginning low dosing (Table 1). Measurable concentrations of benzoylecgonine, norcocaine and cocaethylene were found respectively in 9, 4, and 5 participants' hair specimens (Table 1). Maximum analyte concentrations (C_{max}) occurred 1-3 weeks after drug dosing. These C_{max} were statistically different from concentrations prior to drug dosing for all analytes except cocaethylene, as evaluated with a paired t-test ($p<0.05$).

Considerable inter-subject variability was observed in cocaine and codeine hair concentrations. However, dose-concentration relationships were consistent within 9 of 10 subjects (Figure 2). There is an apparent inverse dose-relationship between the C_{max} observed after low dose and high dose. However, this subject's C_{max} occurred 3 weeks after low dosing. Since all subjects were discharged in the ninth or tenth week of the study, one to two weeks after high dosing, it appears that the hair specimen containing the C_{max} for this one subject occurred after discharge from the study. Data from this subject were included in all analyses. C_{max} observed after the high dose week were significantly greater than those observed after the low dose week for cocaine and metabolites as evaluated using a paired t-test; $p<0.05$, $n=10$ (Table 1).

Likewise, codeine C_{max} after low and high dose weeks were significantly different (Table 1). Morphine and 6-acetylmorphine did not exceed assay limits of quantification after any of the doses. Norcodeine concentrations exceeded 83 pg/mg in 5 subjects following the high dose week. Due to matrix effects and lack of deuterated norcodeine as an internal standard, only semi-quantitative measurement of norcodeine was possible (Scheidweiler and Huestis, 2004).

Cocaethylene is thought to be a specific marker indicating co-administration of cocaine and ethanol. Surprisingly, dose-related cocaethylene concentrations were observed in hair following controlled subcutaneous cocaine administration while subjects resided on the secure, clinical research ward 24 hrs/day. This was observed in all ten subjects, although the amounts of cocaethylene were small relative to cocaine C_{max} (cocaethylene C_{max} were approximately 2% of cocaine C_{max}). Cocaine hydrolysis control samples were included with each analytical run. Pulverized hair was fortified to contain 20,000 pg/mg of cocaine (without cocaine metabolites) and processed along with calibration samples to measure cocaine hydrolysis during sample processing. The average percent of cocaine converted to cocaethylene in these control samples was $0.91 \pm 0.05\%$ (n=15).

Investigation of the correlation between cocaine and codeine C_{max} in hair and total melanin content of hair revealed significant positive linear relationships (Figure 3 and Table 2). Correlations were found following both low and high dose weeks. Likewise, correlations between benzoylecgonine and cocaethylene were evident comparing hair C_{max} after low and high dose weeks of cocaine to total melanin content of hair (Figure 4 and Table 2). Ecgonine methyl ester and norcocaine C_{max} were significantly correlated with total melanin content of hair only following the high dose week (Figure 4 and Table 2).

Discussion

Development of standardized guidelines for forensic and workplace hair testing requires rigorous controlled administration studies evaluating distribution of drugs into human hair. Since guidelines currently proposed by SAMHSA utilize cutoff concentrations for classifying hair specimens as positive or negative, data demonstrating dose-related distribution of drugs into human hair are vital (Substance Abuse and Mental Health Services Administration, 2004). In an attempt to minimize positive drug test results due to externally contaminated hair from passive exposure to a smoked drug of abuse such as cocaine, currently proposed SAMHSA hair-testing guidelines for cocaine also require that cocaine metabolites, benzoylecgonine, norcocaine, or cocaethylene exceed a cutoff threshold to classify a positive cocaine hair analysis. Similar metabolite threshold concentration guidelines are proposed for opiates, amphetamines and cannabis (Substance Abuse and Mental Health Services Administration, 2004). Therefore, the demonstration of dose-related distribution of metabolites and parent drug into human hair is important for developing and implementing standardized hair-testing guidelines.

This study corroborates previous animal and human studies, which reported dose-related cocaine concentrations in hair (Joseph et al., 1999; Hubbard et al., 2000; Ropero-Miller et al., 2000). Hubbard et al. found dose-related concentrations of cocaine, ecgonine methyl ester, benzoylecgonine and norcocaine in rat hair collected 2 weeks after beginning daily 5, 10 or 20 mg/kg intra-peritoneal cocaine injections to rats on 5 consecutive days (Hubbard et al., 2000). The current study is the first to demonstrate dose-related C_{max} concentrations of benzoylecgonine, ecgonine methyl ester and norcocaine in human hair, following controlled cocaine administration.

It is unclear why cocaethylene, a marker for trans-esterification of cocaine when cocaine and ethanol are co-administered, is present in hair samples collected after controlled cocaine dosing. Subjects resided on the secure clinical research unit 24 hrs/day throughout the course of the study and did not have access to alcohol. The amount of cocaethylene C_{max} relative to cocaine C_{max} (2%) exceeds the rate of cocaethylene formation from cocaine in pulverized blank hair fortified with cocaine and carried through processing and analysis (0.91 ± 0.05% cocaine to cocaethylene). Therefore, the presence of cocaethylene in clinical specimens does not result from sample preparation and analysis. Additionally, cocaethylene demonstrated a similar concentration profile to cocaine such that cocaine and cocaethylene C_{max} occurred in the same samples of eight out of ten subjects after low and high dose regimens. In the cases for which cocaine and cocaethylene C_{max} did not occur simultaneously, cocaethylene C_{max} preceded the cocaine C_{max} by one week.

It is difficult to determine the source of ethanol responsible for trans-esterification of cocaine in these subjects. It has been shown that soft drinks and other beverages contain trace quantities of ethanol (Goldberger et al., 1996). Alternatively, the source of cocaethylene in hair potentially resulted from the interaction of endogenous ethanol with cocaine. Further controlled cocaine administration studies are necessary to investigate the appearance of cocaethylene in human hair.

Codeine hair concentrations in this study were similar to those previously reported and demonstrate dose-relationship (Rollins et al., 1996; Joseph et al., 1999; Kronstrand et al., 1999; Ropero-Miller et al., 2000). Greater than 83 pg/mg norcodeine was found in five of ten subjects after the high dose; however, only semi-quantification was possible due to matrix effects compounded by the lack of commercially available deuterated norcodeine for use as an internal

standard (Scheidweiler and Huestis, 2004). Regardless, the appearance of norcodeine in hair following our codeine dosing regimen appears to be minimal. Morphine incorporation into hair following oral codeine dosing was not significant, as morphine concentrations following codeine dosing did not exceed 83 pg/mg in any of the subjects. Similarly, Rollins et al. reported only codeine and did not detect morphine in human hair following multiple oral codeine doses of 30 mg three times daily for five days (Rollins et al., 1996).

It should be noted that in all subjects, C_{max} after low and high doses did exceed currently proposed SAMHSA requirements for confirming a hair sample as positive for presence of cocaine and codeine. Hair shavings from one of ten subjects failed to meet SAMHSA confirmatory criteria for cocaine and codeine three weeks after the beginning of low dosing. Four weeks after low dosing, seven and four subjects' hair samples were negative by SAMHSA criteria for cocaine and codeine, respectively (Substance Abuse and Mental Health Services Administration, 2004).

Numerous studies using Long Evans rats have demonstrated differential incorporation of basic drugs into pigmented vs. nonpigmented hair (Gygi et al., 1996; Slawson et al., 1996; Hubbard et al., 2000). The mechanism responsible for enhanced incorporation of basic drugs into pigmented hair is not clear. It is thought that the polyanionic nature of melanin polymers contained in pigmented hair plays an important role. Some *in vitro* studies demonstrate covalent interactions between melanin precursors and basic drugs (Claffey and Ruth, 2001; Claffey et al., 2001; Dehn et al., 2001), while another study reports ionic interactions between cocaine and melanin (Borges et al., 2003). A further report investigated amphetamine influx and efflux in pigmented and nonpigmented melanocytes (Borges et al., 2002). These studies indicated that pigmented melanocytes took up larger amounts of amphetamine than did nonpigmented

melanocytes. Pigmented melanocytes also were slower to efflux amphetamine than were nonpigmented melanocytes (Borges et al., 2002). Thus, it is possible that melanin is a marker for a melanocyte phenotype that possesses a transport system accounting for differential distribution of drugs into pigmented vs. nonpigmented hair.

Cocaine C_{max} and total melanin content were correlated after both low and high cocaine doses. This is the first study demonstrating cocaine-melanin correlations following controlled cocaine administration to humans and is consistent with Hubbard and colleagues' findings of greater cocaine in pigmented than non-pigmented hair following intra-peritoneal cocaine administration to Long-Evans rats (Hubbard et al., 2000). These data suggest a hair color (i.e. melanin content) relationship for cocaine incorporation into hair *in vivo*. This is supported by *in vitro* studies evaluating cocaine-melanin binding (Stout and Ruth, 1999; Claffey et al., 2001; Borges et al., 2003). Claffey and colleagues found tritiated cocaine was completely contained within a melanin pellet formed from tyrosine when incubated in the presence of tyrosinase (Claffey et al., 2001). Similarly, Borges et al. concluded that tritiated cocaine bound to synthetic eumelanin and mixed eumelanin/pheomelanin polymers but not pure pheomelanin (Borges et al., 2003).

Codeine C_{max} concentrations occurring after low and high codeine doses were correlated with total melanin content of hair. Our results are consistent with a series of animal studies that found higher codeine concentrations in dark vs. light hair following codeine administration, suggesting a codeine-melanin relationship for codeine distribution into hair (Gygi et al., 1996; Gygi et al., 1997; Potsch et al., 1997). Our findings confirm and expand upon previous reports that demonstrated a correlation between codeine concentrations in hair and total melanin content following oral codeine administration (Kronstrand et al., 1999; Rollins et al., 2003). Kronstrand

and colleagues conducted a study during which nine volunteers received a single 100 mg oral dose of codeine and found a positive relationship between log transformed measurements of codeine hair concentrations and total melanin content in hair samples when the data were fit to a mono-exponential model, $R^2 = 0.96$ (Kronstrand et al., 1999). Similar results were reported in a larger study by Rollins et al. in which codeine and melanin concentrations were determined in hair samples collected five weeks after 40 subjects received 30 mg codeine phosphate syrup three times a day for 5 days. Comparison of log transformed codeine concentrations in hair and total melanin content in hair revealed a positive relationship when fit to a mono-exponential model, $R^2 = 0.73$, $p < 0.001$ (Rollins et al., 2003). We propose that we failed to observe a log linear relationship for codeine concentrations in hair collected during our study since none of our subjects had light colored hair. The lowest total melanin concentration in our study was 11.9 µg/mg compared to 2.1 (Kronstrand et al., 1999) and 2.3 µg/mg (Rollins et al., 2003) reported in other studies.

This work demonstrates a correlation between total melanin content of hair and cocaine metabolites incorporated into human hair following cocaine administration. This is an important finding since previous *in vitro* studies have reported that benzoylecgonine does not bind eumelanin or pheomelanin (Borges et al., 2003). Borges and colleagues proposed that the zwitterionic characteristics of benzoylecgonine at physiologic pH limited ionic interactions between benzoylecgonine and poly-anionic melanin (Borges et al., 2003). It is possible that a transport system located on pigmented melanocytes could account for finding a melanin-benzoylecgonine correlation in this *in vivo* study, contrasting with *in vitro* studies indicating that benzoylecgonine does not bind melanin (Borges et al., 2002).

In conclusion, this work demonstrates dose-related concentrations of codeine, cocaine and metabolites in human hair after controlled oral codeine and subcutaneous cocaine administration. We observed a positive linear relationship between measured maximal concentrations and total melanin following the high dose week for codeine, cocaine and metabolites. These are the first data demonstrating dose-related cocaine metabolite concentrations in human hair and correlation of cocaine metabolites with total melanin. This controlled codeine and cocaine administration study provides insight into mechanisms of drug incorporation into hair and should be valuable when interpreting hair test results.

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Footnotes

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Figure Legends

Figure 1. Representative profile of drug and metabolite concentrations measured in weekly hair shavings collected throughout the course of the study from one volunteer. Panel A cocaine concentrations. Panel B cocaine metabolite concentrations; solid circles: benzoylecgonine, solid triangles: ecgonine methyl ester, open circles: norcocaine and open triangles: cocaethylene. Panel C codeine concentrations. Note: the first 4 specimens reflect self-administered cocaine prior to enrollment in the study. Shaded bars indicate low and high dose sessions.

Figure 2. Plot showing maximal concentrations (C_{max}) of cocaine (A) and codeine (B) observed in hair shavings following low and high doses. C_{max} from individual volunteers are connected to depict inter-subject variability of dose-relationship.

Figure 3. Correlation plots displaying the linear relationship between maximum cocaine and codeine hair concentrations (C_{max}) measured after the high dose week and total melanin content of hair. Panel A displays cocaine C_{max} and Panel B displays codeine C_{max} data.

Figure 4. Correlation between maximum cocaine metabolite concentrations (C_{max}) measured after the high dose week and total melanin content of hair. Panels display the C_{max} measured after the high dose week for A) benzoylecgonine B) ecgonine methyl ester C) norcocaine and D) cocaethylene.

Table 1. Average Maximum Codeine, Cocaine and Metabolite Concentrations in Hair Samples from 10 Participants during a Controlled Codeine and Cocaine Administration Study

		Maximum Concentrations (C_{max})	
pg/mg	**Baseline**[a]	**Low dose**[b]	**High dose**[b]
Cocaine	464 ± 176	2,997 ± 619	6,419 ± 1,698[*]
Benzoylecgonine	82 ± 19	314 ± 51	708 ± 163[*]
Ecgonine methylester	< LOQ[c]	187 ± 27	425 ± 65[*]
Norcocaine	32 ± 6.7	84 ± 21[d]	218 ± 46[*]
Cocaethylene	60 ± 20	71 ± 17[e]	143 ± 38[e*]
Codeine	< LOQ	1,291 ± 182	2,725 ± 426[*]

[a] Hair shavings collected at the end of the three week washout period, immediately preceding the start of drug dosing, representing previously self-administered cocaine (mean ± s.e.m.). Number of specimens exceeding the assay limit of quantification (LOQ) = 10, 9, 4 and 5 for cocaine, benzoylecgonine, norcocaine and cocaethylene, respectively.
[b] Maximum concentrations observed 1-3 weeks after start of drug dosing (mean ± s.e.m., n=10). [c] Concentrations did not exceed the assay LOQ. [d] One sample did not exceed LOQ, n=9.
[e] Cocaethylene concentrations were approximately 2% of cocaine C_{max}. Cocaine hydrolysis controls showed 0.91 ± 0.05% cocaethylene (n=15) formed during sample extraction and analysis. [*] $p < 0.05$ compared to low dose C_{max} using paired t-test.

Table 2. Correlation of Maximum Drug Concentrations Measured in Hair Following Controlled Subcutaneous Cocaine and Oral Codeine

Low Dose Maximum Concentrations vs. Total Melanin

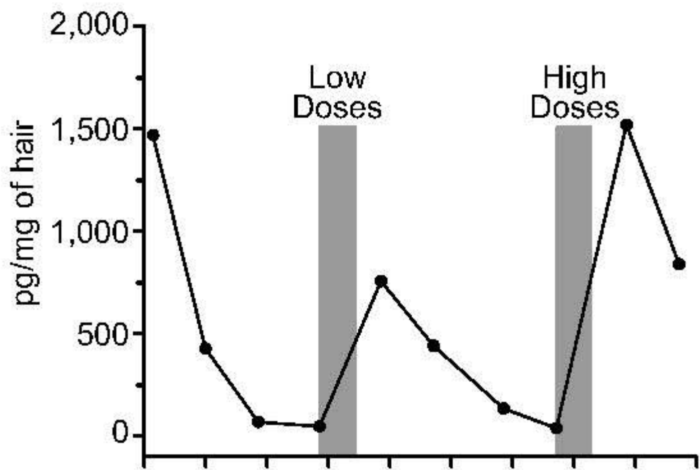
Analyte	**Fitted Equation**	**R^2**
Cocaine	y = 0.1x – 0.07	0.45*
Benzoylecgonine	y = 0.01x + 0.05	0.50*
Ecgonine methylester	y = 0.001x + 0.07	0.01
Norcocaine	y = 0.002x + 0.1	0.15
Cocaethylene	y = 0.03x – 0.02	0.58*
Codeine	y = 0.03x + 0.4	0.43*

High Dose Maximum Concentrations vs. Total Melanin

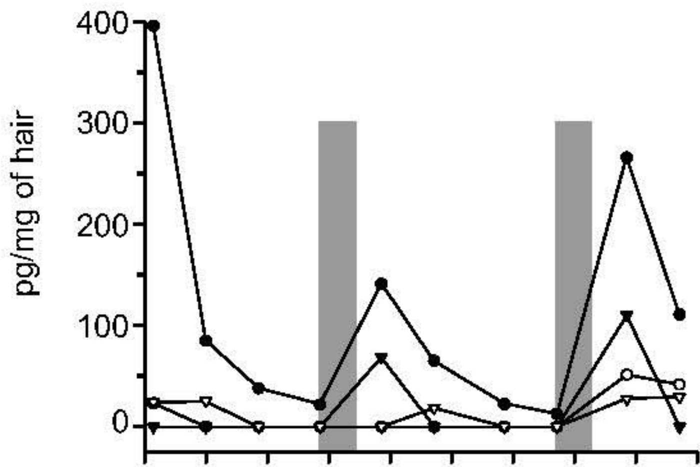
Analyte	**Fitted Equation**	**R^2**
Cocaine	y = 0.4x – 5	0.80***
Benzoylecgonine	y = 0.04x – 0.4	0.80***
Ecgonine methylester	y = 0.1x + 0.1	0.56*
Norcocaine	y = 0.01x – 0.03	0.54*
Cocaethylene	y = 0.01x – 0.09	0.70***
Codeine	y = 0.09x – 0.04	0.62**

Significance based upon analysis of variance of linear regression fit. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

A. Cocaine



B. Cocaine metabolites



C. Codeine

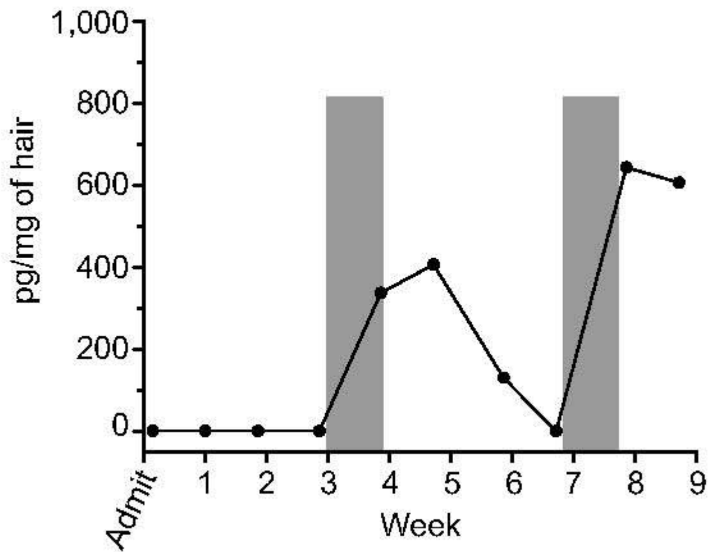
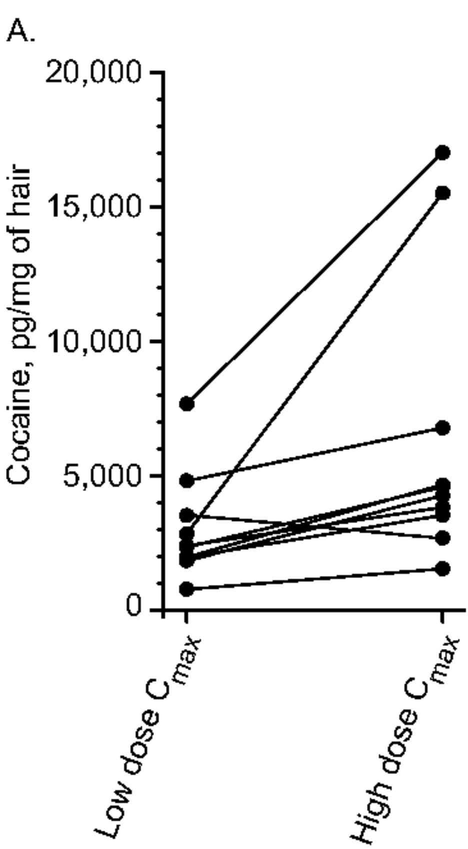


Figure 1



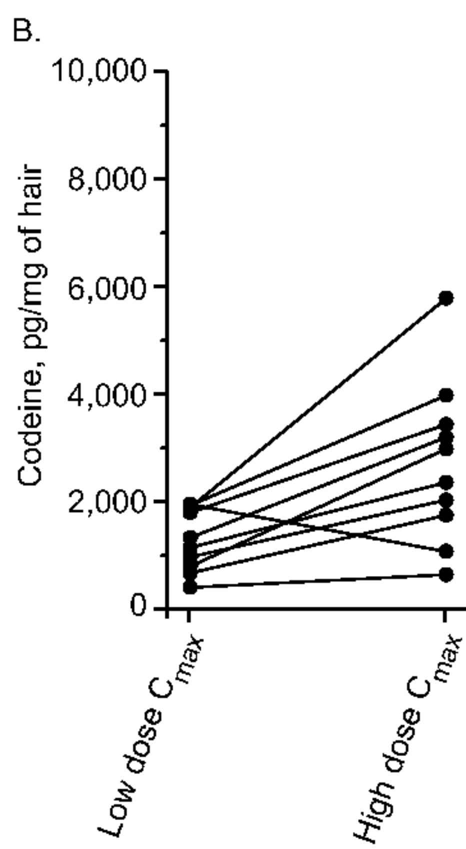


Figure 2

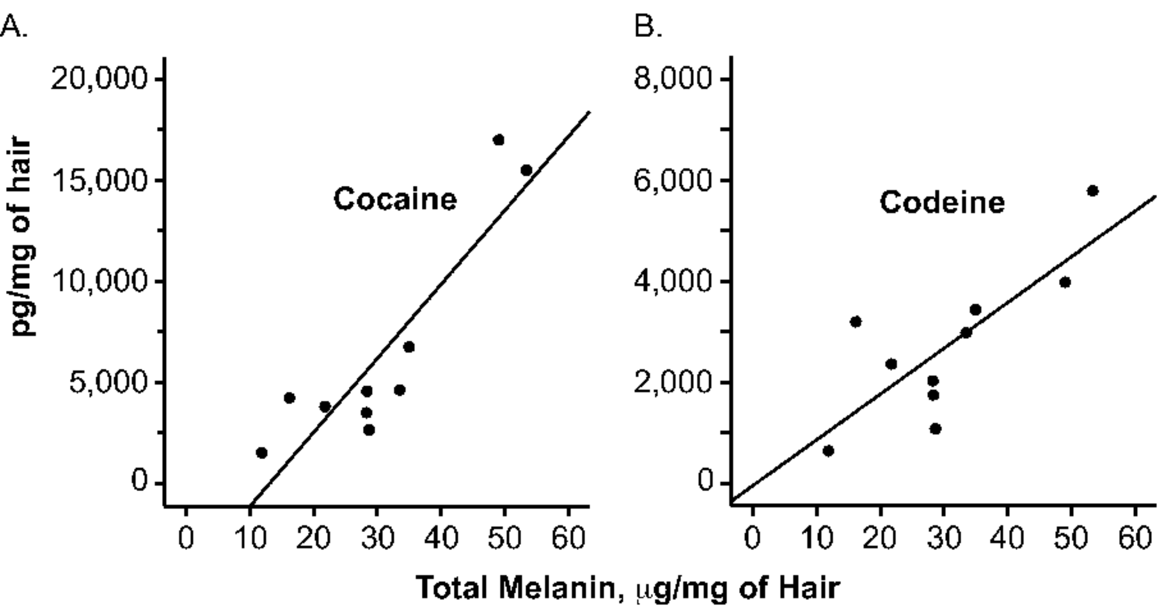


Figure 3

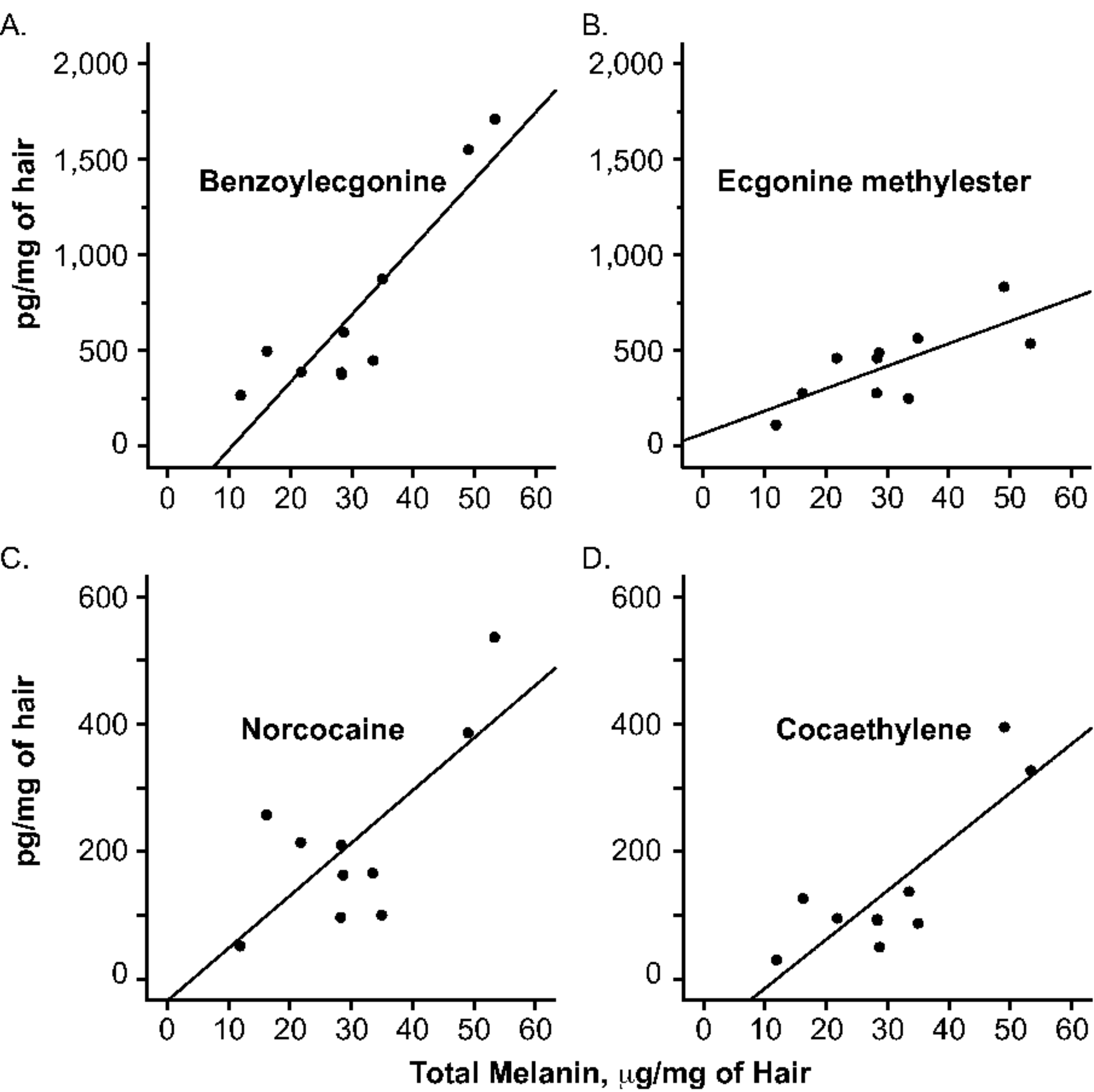


Figure 4