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Zolpidem Facilitated Sexual Assaults: A Hair Method Validation

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Zolpidem Facilitated Sexual Assaults: A Hair Method Validation

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This Thesis has been presented to and accepted by the Office of Graduate Studies, John Jay College of Criminal Justice in Partial Fulfillment of the Requirements for the Degree of Master of Science in Forensic Science.

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Abstract

A liquid chromatography tandem mass spectrometry (LC-MSMS) method was developed and validated to detect for the presence of zolpidem in human hair samples. Zolpidem is a sedative hypnotic whose adverse effects make it a common drug found in drug facilitated crimes (DFC). It is important to be able to detect and quantitate the drug after a long period of time due to the victim's delayed reporting of the crime. Hair's long window of detection makes it a useful matrix for DFC investigations. The linear range of the assay was targeted at 2 pg/mg to 1,000 pg/mg and experiments designed to test this range returned acceptable R^2 values (> 0.985) in all cases. The lower limit of linearity was established at 2 pg/mg. Accuracy and precision of the assay were determined through replicate analysis of quality control samples. Both intraday (n=3) and interday (n=15) accuracy and precision data were within acceptable limits, +/- 20% error and 15% RSD. The matrix effect, recovery, and process efficiency were determined to be 15%, 70%, and 10%, respectively. Additionally, the processed sample stability of quality control samples at room temperature and dilution integrity of spiked samples were found to be acceptable. No significant carryover was seen between samples. This validated method was used to analyze authentic hair samples from donors with reported zolpidem drug use as well as cosmetically treated samples spiked with zolpidem. Results indicate possible interferences in the analysis of zolpidem in cosmetically treated hair, but not authentic, untreated hair.

1. Introduction

Sleep-aids are commonly prescribed to help treat insomnia in the U.S. These sleep-aids have been reported to facilitate crimes such as sexual assault (DFSA). Zolpidem (Ambien) is one of these prescribed sleep-aids. Its fast-acting effects and short-duration make it an effective agent for drug-facilitated sexual assaults. Since victims do not report the crime in a reasonable amount of time, hair becomes the most important and beneficial biological matrix. Hair samples can be analyzed to test for the presence of these drugs after a period of time (months) have passed. A confirmatory liquid chromatography tandem mass spectrometry (LC-MSMS) method has been developed and validated to detect the presence of zolpidem in human hair samples.

Insomnia is a medical condition diagnosed in 5-15% of the North American and United Kingdom population (Gunja, 2013). In the past, benzodiazepines have been used to treat insomnia. Zolpidem (e.g. Ambien) has since replaced the use of benzodiazepines for treatment because they have pharmacodynamic and pharmacokinetic advantages such as a shorter duration of action and half-life. Zolpidem also does not disrupt sleep patterns or cause residual effects. There is a low risk of dependence and abuse (Gunja, 2013). Zolpidem's effects are important when it comes to determining its use in drug facilitated crimes.

Zolpidem is an imidazopyridine, a non-benzodiazepine. It exhibits sedative-hypnotic effects used to treat insomnia. A standard dosage, 10 mg for adults, is taken orally prior to bed to reduce the time for sleep on-set. The on-set is seen within 15 minutes of ingestion and has a half-life of 2-3 hours (Gunja, 2013). Once ingested, zolpidem is readily absorbed by the gastric intestinal tract (GI) and metabolized in the liver. Cytochrome p450

(CYP), metabolizes zolpidem into its inactive metabolites methoxyzolpidem and zolpidem carboxylic acid (Gunja, 2013). CYP enzymes are part of the liver microsomal drug-metabolizing system which handles endogenous and exogenous compounds (e.g. drugs). Specifically, for zolpidem, the CYP3A4 polymorph enzyme is responsible for oxidizing the molecules so that they can be removed from the body. The primary pathway for elimination is through renal excretion.

Similar to benzodiazepines, zolpidem binds to gamma-aminobutyric acid (GABA) type-A receptors and exhibits sedative and hypnotic effects (Gunja, 2013). GABA is a major inhibitory neurotransmitter in the central nervous system. It is a multi-component transmembrane protein complex with multiple ligand binding sites. GABA-A is an ionotropic receptor, with a ligand-gated chloride ion channel going across the membrane. The complex contains multiple subunits including α (1), β (2), and γ. The subunits are further divided into their own subunits (e.g. α_1, α_2, α_3, and α_5). Zolpidem is a benzodiazepine BZ1 (ω1) receptor agonist (Rust et al., 2012). Unlike benzodiazepines, zolpidem selectively binds to the α_1 subunit of the GABA-A receptor (Has et al., 2016). The α_1 subunit mediates sedation and amnesia as well as sleep regulation, while α_2 and α_3 mediate anxiety (Gunja, 2013). Once zolpidem binds to the GABA-A complex, it increases the binding of the GABA neurotransmitter, thus increasing the frequency of the opening of the chloride ion channel. When the channel is open, an influx of chloride ions enters the cell causing membrane hyperpolarization. This moves the postsynaptic potential away from the threshold, thus inhibiting propagation of an action potential, ultimately causing inhibition of the central nervous system.

There are possible adverse effects including hallucination and amnesia. Adverse effects are increased with the use of zolpidem in combination with serotoninergic and noradrenergic agents such as tricyclic antidepressants. Poisonings results in sedation and coma. Deaths are rare and tend to occur with polydrug overdoses (Gunja, 2013).

In recent reports, there have been an increase in the misuse of zolpidem especially with forensic cases, drug-facilitated crimes, and motor vehicle crashes (Gunja, 2013). Hypnotic drugs such as zolpidem are effective agents for drug-facilitated sexual assaults because they require a low dose, have a rapid on-set of action, and a short half-life (Rust et al., 2012). As mentioned, zolpidem acts on the central nervous system causing symptoms of drowsiness. This is commonly found as the means of sedation in drug facilitated sexual assault crimes.

A lot of the time, victims do not report the crime until well after the crime has taken place due to the sedative effects of the drug or their fear of reporting the crime. Thus, in order to examine the victim for exposure of the drug, the matrix studied must have a long window of detection. In comparison to oral fluid, urine, and blood matrices, hair has a longer window of detection of weeks to months. Hair is collected close to the scalp, at the vertex posterior, where the hair growth rate is the most consistent (LeBeau et al., 2011). Head hair has a known growth rate of 1 cm per month; however, the rate may change based on various factors such as age, pregnancy, and hormones (LeBeau et al., 2011). With a known rate, this allows for hair segmental analysis to determine the pattern of drug use over a period of time (LeBeau et al., 2011). A one-time use can be seen with a short drug band, while chronic use would show a longer band down the hair segment away from the

root. In addition to having a broad detection window, hair testing has a lower risk of adulteration, and an easier, non-invasive collection procedure.

Hair growth occurs it cycles. It begins at the follicle. Here, during the anagen phase, cells divide, growing new hair and pushing the club hair out from the follicle. Hair cells stop dividing, thus growth stops at the catagen phase. During the catagen phase, the outer sheath of the hair attaches to the root forming the club hair. Lastly, the telogen phase is when the hair follicle is at rest (LeBeau et al., 2011). The hair shaft is tough, yet flexible, composed of elongated keratinized cells within a membrane complex. The membrane complex is comprised of the cuticle, cortex, and medulla. The cortex provides the stability and color composition. Within the cortex, are melanocytes which produce melanin pigments within melanosomes (Pragst & Balikova, 2006). The melanin is transferred to keratinocytes where they are able to absorb light and give the hair color. Surrounding the cortex are overlaying shingle-like cells containing pigmentation to the matrix, the cuticle. The cuticle is also responsible for the chemical and physical resistance to the cell matrix (Pragst & Balikova, 2006). In some hair, a medulla is present within the middle of the hair shaft (LeBeau et al., 2011). Surrounding the outside of the hair follicle is a capillary system which provides nutrients to the growing hair as well as a means of drug incorporation.

Drugs can become incorporated into the hair through various pathways. They can enter through passive diffusion through the skin, blood capillaries, or sweat (Pragst & Balikova, 2006). Another way is through external exposure from the environment or others in the surrounding proximity. Drugs are deposited into the growing hair structure by binding to proteins, melanin, or lipids of the membrane complex (Cui et al., 2013). Melanin is the target of drug incorporation due to its ability to bind drugs. Long polymer strands of

dopaquinones form melanin. These polymers contain carboxyl groups (acidic characteristic) that are susceptible to donating a proton leaving a negative charge (Ghiani et al., 2008). Positive charged ions have a greater affinity to melanin through electrostatic interactions. The incorporation of basic drugs into hair has been seen (Kintz, 2007). There have been some suggestions that the amount of melanin within the hair is related to the amount of drug that is incorporated. Based on this understanding, it is believed that various hair colors, hair types, and cosmetic treatments will affect the drug's incorporation. The pKa of the compounds and the pH of the matrix cells are important because they determine the degree of ionization of the acidic or basic compounds following protonation or deprotonation.

In order to prevent external contaminants from causing positive hair results, the hair undergoes extensive decontamination and washing procedures prior to analysis. Since zolpidem is orally administered and not smoked, the metabolites do not need to be studied to determine if zolpidem was present due to external smoke contamination. The decontamination and washing steps remove interferences from the surface of the hair matrix such as hair products, dye, shampoo, exogenous analytes, and sweat.

Presumptive screening tests can initially be used to test for the presence of the drug based on a cut-off concentration. Enzyme-linked immunosorbent assay (ELISA) commercial kits are a common screening technique used. Positive samples need to be further analyzed using a more specific chemical method in order to confirm its presence and concentration.

Instrumental analysis is more sensitive and requires samples to be cleaned prior to analysis. Solid-phase extraction is used to free the drug from the hair matrix. Cartridges

containing a solid stationary phase retain analytes using sorbent-analyte interactions. These interactions include hydrophobic, hydrophilic, and electrostatic based on the stationary phase and the analyte. In order to activate the polymer bed, a solvent is used to condition it. Samples are loaded into the cartridges allowing for drugs to bind through various mechanisms to the sorbent bed while unbound material is removed from the cartridges. The cartridges are washed to remove excess material that is not bound to the cartridges. Solvents are added in order to break the interactions holding the drugs to the stationary phase of the column, freeing the drugs for analysis (Supelco, 1998). Solid-phase extraction not only frees drug from matrices, but they clean-up the sample prior to instrumental analysis.

Liquid chromatography tandem mass spectrometry (LC-MSMS) is the ideal technique for confirmation testing. Liquid chromatography allows for various types of samples to be analyzed as opposed to gas chromatography where the sample has to be in the gaseous phase in order to be analyzed. The separation from LC is performed at a high resolution. A solid stationary phase retains the analyte to the column, while the liquid mobile phase pushes the analyte through the column. Separation of the components is based on polarity between the solid and liquid phases. Tandem mass spectrometry allows for the sensitive detection of trace amounts of drug. An ionization source is used to remove the solvent from the sample and create small gas phase ions in a vacuum. Electrospray ionization is a common ion source used prior to mass spectrometry. In this source, the solvent and analyte compete for space inside of a droplet. Positive charge builds up and blows apart to form small ions in an aerosol. The ions are then attracted into the mass spectrometer through vacuum and magnetic fields. Once inside, the ions travel to the first

mass analyzer and are separated by their mass-to-charge ratio. Ions that meet the qualifying ratio are the "parent" ions. The "parent" ions then move to the collision cell where they come into contact with N_2 molecules and break apart to form "daughter" ions. The "daughter" ions move to the final mass analyzer where they are separated based on their mass-to-charge ratio (Skoog et al., 2007). Since multiple transitions (parent to daughter ions) are being monitored, the mode of the mass spectrometer is in multiple reaction mode (MRM). A qualifier and quantifier transition are used to detect and then quantify the analyte present. The combination of the liquid chromatography with mass spectrometry results in a rapid, specific, sensitive, and robust method for confirmatory testing.

Other studies for the detection of zolpidem in hair using LC-MSMS have been done. In one study, Kim and authors used 10 mg of washed hair (a methanol and deionized water wash) followed by a methanol digestion at 38 ºC for 16 hours. To extract the drug, the substrate was evaporated and the residue was reconstituted with methanol and mobile phase A and then filtered through a polyvinylidenefluoride (PVDF) microporous membrane prior to LC-MSMS injection (Kim et al., 2011). Their LC-MSMS method had a linear working range of 0.25 – 5 ng. For zolpidem, the LOD was 0.005 ng totoal and the LOQ was 0.25 ng total. Matrix effect was an average of 88% and 90% for the LOD and LOQ, respectively. Recovery was 93% and 89%, respectively. Process efficiency was 81% and 79%, respectively (Kim et al., 2011). Based on the results, there is a significant matrix effect seen due to ion suppression. Another hair analysis of zolpidem involved in drug facilitated crimes by Salamone and authors used 100 mg of hair, washed the hair (dichloromethane) and incubated the samples in methanol for 15 hours at 55 ºC. The organic phase was collected, evaporated, and reconstituted with the mobile phase. The

working linear range was 2 – 100 pg/mg, the matrix effect was -4.7% (compared the peak area of 25 pg/mg spiked into hair to the peak area of a methanolic standard at the same concentration) (Salomone et al., 2012).

The goal of the present project was to develop and validate a sensitive and efficient LC-MSMS analysis for the quantitative detection of zolpidem in hair. The established method would be validated using various standard procedures to ensure it is acceptable for clinical and forensic laboratory usage. This LC-MSMS method can then be applied to solving various cases including drug facilitated crimes.

2. Materials

2.1. *Chemicals and Reagents.*

Zolpidem (1.0 mg/mL in 1 mL methanol) (Z-017 Lot: FE04251603) and Sedative hypnotics internal standard, containing zolpidem-d6 (Lot: IS1603-17SED (1)) were purchased from Cerilliant (Texas, USA) and were stored in the fridge. HPLC grade methanol, dichloromethane, and isopropanol were obtained. Phosphate buffer, pH 6.0, pouches were from United Chemical Technologies (Bristol, PA). Ammonium hydroxide reagent was from Spectrum Chemical Manufacturing Corporation. Glacial acetic acid and sodium hydroxide pellets were from Sigma-Aldrich. Hair extraction buffer (HEB) was purchased from Immunalysis (Pomona, CA).

2.2. *Standard Solutions*

Working standard solutions were prepared using the purchased certified stocks of zolpidem (at 1 mg/mL in methanol) and sedative hypnotics internal standard. A working solution of internal standard at a concentration of 100 ng/mL was prepared in deionized water by the dilution of 10,000 ng/mL stock solution. Zolpidem working standards were

prepared at concentrations of 100,000 ng/mL, 10,000 ng/mL, 1,000 ng/mL, 100 ng/mL, 10 ng/mL, and 1 ng/mL in methanol by serial diluting the zolpidem 1 mg/mL standard stock solution.

2.3. *Specimen*

Negative hair including cosmetically treated samples were provided by Cordant Health Laboratory for drug analyses. They were tested negative by using a LC-MSMS analytical method. The samples were decontaminated as described later (section 3.1) to remove contamination that could interfere with the analyses. Authentic donor hair samples with reported zolpidem drug use were obtained from the University of Pennsylvania for a matrix correlation project. This project used anonymous, consented donor samples (oral fluid, urine, plasma, and hair, if applicable) to determine if there was a drug concentration correlation seen between the various sample matrices for a given drug.

3. Methods

3.1. Sample Decontamination

Hair samples were first decontaminated to remove contaminants such as dye, sebum, shampoo, and any other external interfering agents. A clump of hair was placed into a beaker with dichloromethane covering the top of the hair. The solution was swirled for 2 minutes, decanted, and dried under a light flow of nitrogen. Isopropanol was added covering the hair, the solution was swirled for 2 minutes, and decanted. Then methanol was added covering the hair, the solution was swirled for 2 minutes, decanted, and dried under a low flow of nitrogen. The hair was stored in a folded piece of paper inside of an envelope and left in a drawer at room temperature until analysis.

3.2. Method Development: Hair Digestion

Fortification of the hair was performed to ensure that the hair would be positive for zolpidem when developing the LC-MSMS method. A solution of dH_2O: DMSO (1:1) was added to 5 g of decontaminated hair. Zolpidem was spiked at 100 times the expected cut-off (20 pg/mg). In addition, to increase the total volume, 5 mL of 100,000 ng/mL, 1 mL of deionized water, and 1 mL of DMSO were added. The hair was soaked and sonicated every 2-3 days. After 5 days, the hair tested positive using a LC-MSMS method.

Hair extraction buffer (HEB) and 1M sodium hydroxide were both tested to determine which digestion solution recovered the most amount of drug. Two sets of fortified hair samples were prepared (30 mg). The samples were washed with 3 mL of methanol and twice with 3 mL of phosphate buffer (pH 6.0), each time vortexed, centrifuged, and decanted, except for the last wash with phosphate buffer. The last wash was saved to determine the efficiency of the washing procedure. In one set of samples, 1 mL 1M sodium hydroxide was added, vortexed, and centrifuged. The tubes were placed into a hot block for 30 minutes at 70 °C. After, they were cooled to room temperature and 180 µL of 20% acetic acid was added, vortexed, and centrifuged. In the second set of samples, 1 mL of HEB was added to the tubes, vortexed, and centrifuged. The tubes were then incubated for 2 hours at 75 °C. After they were cooled to room temperature, 1 mL phosphate buffer (pH 6.0) was added, vortexed, and centrifuged. Each set's supernatant was removed, spiked with internal standard, extracted with the solid-phase extraction method, dried down with nitrogen, reconstituted with 0.1% formic acid, and injected into the LC-MSMS for analysis.

3.3. Hair Digestion and Cleanup

Decontaminated hair samples (30 mg) were cut into small pieces (~1 mm) and aliquoted into 12 x 75 mm tubes. The samples were washed to ensure that all interferents were removed for a clean LC-MSMS anlysis. The hair was washed with 3 mL of methanol (vortex and centrifuged for 5 min. at 4200 rpm) and decanted, and twice with 3 mL of phosphate buffer (pH 6.0) (vortex and centrifuged for 5 min. at 4200 rpm) and decanted. This cleans the hair from any external contamination that they might have come into contact with. The washed cut hair was then spiked to create a zolpidem working range (2 pg/mg to 1,000 pg/mg) (Table 1). A sedative hypnotic internal standard (60 µL of 100 ng/mL) was also added to each sample.

Table 1. LC-MSMS Calibrator Spiking Chart.

Level	**Target Concentration (pg/mg)**	**Stock (ng/mL)**	**Spiking Volume (µL)**
Negative Control	0	----	-------
1	2	1	60
2	5	10	15
3*	10	10	30
4	20	10	60
5*	50	100	15
6	100	100	30
7*	200	100	60
8	500	1000	15
9	1000	1000	30

*Quality Control (QC)

For the digestion of the hair, a 1 mL aliquot of 1 M NaOH was added and the samples were vortex and centrifuged at 4200 rpm for 5 minutes. The samples were incubated for 30 minutes at 70°C in a hot block. Neutralization followed with 180 µL of 20% acetic acid, vortex, and centrifuged at 4200 rpm for 5 minutes. The supernatant was then removed from the tubes and the analytes were ready for extraction.

Solid-phase extraction of the drug was performed using an automated liquid dispenser (ALD) and Strata-XC cation exchange cartridges. The cartridges were conditioned with methanol and water, and then flashed with N_2 gas for 19 seconds to lightly flush out the contents. A LEUR seal was attached and 0.1 M phosphate buffer was added. The samples were then loaded and the LEUR seal was removed. Next, the cartridges were washed by first flushing out the contents with N_2 gas followed by the addition of water and 25% methanol, and another flushing with N_2 gas. The cartridges were left to dry for 14 minutes under a low nitrogen flow. After drying, an elution solvent of dichloromethane: isopropanol: ammonium hydroxide (70:26:4) was added to the cartridges to allow the free drug to elute into autosampler vials. The vials were then placed in a concentrator under a low nitrogen flow at 40°C for about 15 minutes until the vials were completely evaporated. Next, the vials were reconstituted with 50 µL of the mobile A phase: 1% formic acid and vortexed. The vials were then loaded onto the LC-MSMS for analysis.

3.4. LC-MSMS Instrumental Analysis

The LC-MSMS confirmatory analysis was performed on an Agilent 1290 liquid chromatograph with 6460 triple quadrupole mass selective detector. An LC-MSMS was used for confirmatory testing because it provides a high throughput and sensitive analysis. The Agilent instrument uses two columns simultaneously that are connected by a switch valve to avoid the use of post-column switching and the associated dead volume. Also, the use of two columns working simultaneously allows for the analysis and re-equilibration of a second column. This saves the time of switching and cleaning columns as well as increasing the throughput. A Kinetex® 2.6 µm Biphenyl 100 Å Column; 50 x 2.1 mm reverse phase column was used. The biphenyl column has strong pi-pi interactions and is

non-polar. This is good for the retention of hydrophilic aromatic compounds (Kinetex Biphenyl, n.d.). A set method "Sedative Hypnotic Hair" was optimized and used for analysis. The following are the method's parameters:

Injection: 20 µL

Mobile Phase

Mobile Phase %A: Water

Mobile Phase %B: Methanol

Mobile Phase Composition:

Time (min.)	B [%]	Flow (mL/min.)
0.00	30.00	0.700
0.30	30.00	0.700
1.20	60.00	0.700
2.00	60.00	0.700
2.01	30.00	0.700

Column

Temperature: 50 °C

Triple Quadrupole MS Parameters

Acquisition Mode: ESI (+ ion)

Multiple Reaction Monitoring Table:

Compound Name	**Internal Standard**	**Precursor Ion**	**Product Ion**	**Fragmentor**	**Collision Energy**	**Cell Accelerator Voltage**	**Polarity**
Zolpidem d6	√	314.2	65.1	175	80	7	Positive
Zolpidem		308.2	235.1	150	36	7	Positive
Zolpidem		308.2	65.1	150	80	7	Positive

ESI Source Parameters

Gas Temperature: 350 °C

Gas Flow: 10 L/min.

Nebulizer Pressure: 50 psi

Sheath Gas Temperature: 400 °C

Sheath Gas Flow: 11 L/min.

Capillary Voltage: 4000 V Positive 4000 V Negative

3.5. Method Validation

Validation of the confirmatory method using LC-MSMS contains various procedures that have to be completed in order to validate that the developed method works prior to being used in the laboratory. The analytical method was validated using spiked negative decontaminated hair and the following parameters were evaluated: accuracy and precision, linearity and sensitivity, matrix effect, recovery, and process efficiency, carryover, dilution, stability, selectivity, and proficiency testing.

First, accuracy and precision testing were performed by preparing and examining qualitative controls (10, 50, 200 pg/mg) in triplicates (intraday) and in triplicates for over five days (interday). Accuracy (bias) compares the expected result with the measured result. Systematic error can result in deviations between the two results. Precision measures the closeness of the samples performed in replicates. If deviations are seen, random errors can be the cause. The coefficient of variation (CV) is used to measure the accuracy and precision of the data. Acceptable data for each analysis is a %CV <10.

Linearity and sensitivity were examined by preparing calibration curves once per day over the course of five days to evaluate their linear range. The concentration of the

analyte in the matrix over the range of analyte concentrations being studied (the working range) has to be directly related to the response of the method in order for there to be linearity. Thus, the working range has to be tested to ensure it is in linear range. Validation was complete when all of the samples fell within 15% of their expected values and the curve r^2 values were above 0.985.

Matrix effect was examined next to determine the %recovery, the interference of the hair matrix, and the process efficiency of the extraction technique. Three groups were created at the same midway concentration (100 pg/mg) with no internal standard added: neat un-extracted (Group A), post-extraction standards (Group B), and pre-extraction standards (Group C). Group A consisted of three neat un-extracted standards, Group B consisted of prepared and extracted samples in triplicates for five different sources, and Group C consisted of prepared samples in triplicates for five different sources. Matrix effect involves the alteration or interference of an unintended analyte or substances in the sample matrix which changes the response of the LC-MSMS instrument. The intended analyte in the sample matrix has to be tested to ensure there is no interference. This is done by examining the recovery, process efficiency, and ultimately the matrix effect. Once there is high recovery and process efficiency and a low matrix effect, the data can be accepted.

The efficiency of the LC-MSMS method was looked at next by determining whether or not there was sufficient carryover taking place. High concentrations of sample remaining in the column and eluting with a lower concentration can cause problems when analyzing the data. In order to determine if this is occurring and to determine how much is being carried over, two samples containing the upper limit of linearity (ULOL) and 10 times the ULOL were prepared (1,000 pg/mg and 10,000 pg/mg respectively) as well as

two negative controls. The ULOL and the 10x ULOL samples were injected into the LC-MSMS. Following each, a negative control was injected. If there is a significant amount of carryover, the LC-MSMS method would be adjusted to correct it.

The stability of the samples at room temperature were examined next over three days. A set of QCs was prepared (doubled everything throughout the experiment to ensure that there was enough sample to examine over three days). The extraction and instrumental analysis of the samples were performed for the first day. The remaining sample aliquot was then left out at room temperature to be injected for a second day and then repeated for a third day. Stability testing is performed to show that the samples are stable throughout the procedure as well as during storage, if left at room temperature prior to analysis. Comparing freshly made samples with those that have sat out over time can demonstrate its stability.

Dilution testing is to ensure that the quantities that are being analyzed are accurate if a dilution is necessary if the concentration goes beyond the linear working range or less sample is available. The ULOL was prepared at dilution factors (DF) of 2, 4, and 10, by diluting with the 1M NaOH solution. Dilutions were added prior to digestion. All values are accepted if their quantities, with dilution factors, are within +/- 15% of the expected values.

Selectivity study is done to test the method's ability to select for only the compounds of interest and not the interferences in the matrix, standards, internal standard, or other analytes that might be present in the sample. This study uses negative hair samples from different sources. Three replicates of each source are prepared without standard or internal standard to test for the presence of any compound within the matrix itself. This

will ensure that the hair is purely negative. Next, three replicates of each hair source are prepared with only internal standard at the concentration used in the calibration curve (60 μL of 100 ng/mL). This was to see if there was a standard response present from internal standard impurities. Lastly, each of the five sources of hair were washed. In five separate tubes, one at a time, 1 mL of 10,000 pg/mg of five different analytes (benzoylecgonine, oxazepam, hydrocodone, imipramine, and lorazepam) were added and dried down with N_2. After all of the analytes were dried down, the tube residues were reconstituted with 1 mL of 2 pg/mg of zolpidem calibrator and spiked with internal standard. The reconstituted solutions were added to the source samples. The samples were then digested with 1 mL of 1M NaOH, neutralized, and extracted. The analytes were chosen from the College of American Pathologists' "Master List" of common drug facilitated compounds. The data was analyzed to see if the LOQ properly quantitates in the presence of other compounds.

Proficiency testing is performed using samples prepared by a Research and Development staff member. Hair samples are spiked with a known concentration of zolpidem by a reputable source. The concentration is unbeknownst to the scientist who will then test and analyze the sample. Extraction and proficiency testing is implemented in order to test the method as well as the scientist, to ensure that they are working properly. The scientist's quantitative results are then compared to the known reputable results to determine the efficiency of the method and the scientist.

Authentic, reported zolpidem positive, hair samples were obtained from the University of Pennsylvania in collaboration of a biological matrix correlation experiment. A study between Cordant Health Solutions and University of Pennsylvania is being conducted to quantify urine, oral fluid, plasma, as well as hair samples for a wide range of

drugs and correlate the concentrations within the four matrices. These samples were obtained from anonymous donors who also reported their medical history for the study. The samples did not contain any markers that would identify the donor. An aliquot of the positive zolpidem hair samples was analyzed using the LC-MSMS method developed. The zolpidem hair concentrations were compared to the other matrices zolpidem concentrations.

4. Ethics

Since this method development research involves human subjects, informed consent, information regarding the research, and additional protections will be required from the subjects. Cordant Health Solutions, where this research is being performed, typically request positive samples from other drug testing labs as their human subject samples for validation work. All of the samples are de-identified and lack any protected health information (PHI) in accordance with the Health Insurance Portability and Accountability Act (HIPAA) privacy rule. The research being conducted is in an acceptable manner, applied towards the planning, execution, and reporting. It is being performed ethically to protect the interest of the individuals involved and followed all federal, professional, and institutional guidelines and regulations.

5. Results

5.1. Method Validation

The developed LC-MSMS method produced a good separation and detection of the zolpidem analyte over the calibration range (2 to 1,000 pg/mg). The zolpidem and its deuterated form's structures are seen in Figure 1.

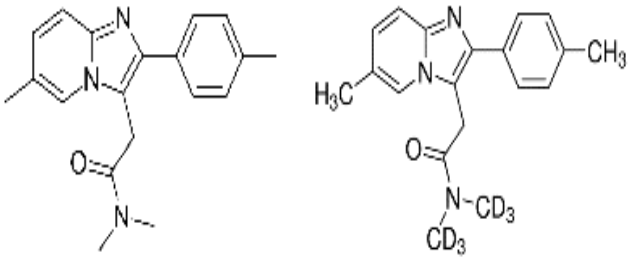


Figure 1. Zolpidem (left) and its deuterated form (right).

A representative chromatogram of zolpidem with internal standard in hair is shown in Figure 2. The peak of zolpidem is shown to elute at 1.3 ± 0.2 minutes. The parent ion of zolpidem has a mass-to-charge ratio of 308.2 m/z and two product ions of 235.1 m/z and 65.1 m/z (Figure 3).

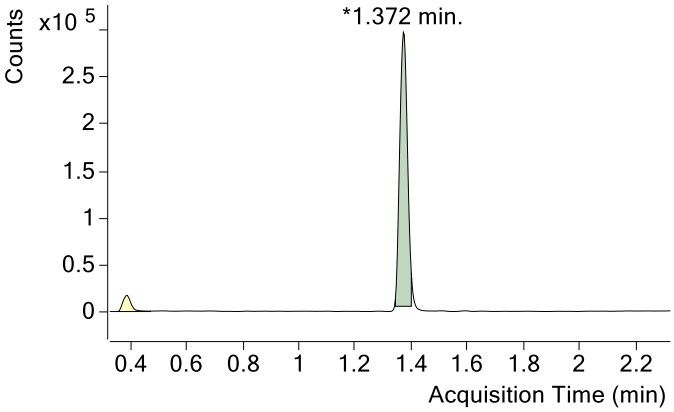


Figure 2. LC-MSMS representative chromatogram of zolpidem in blank spiked hair.

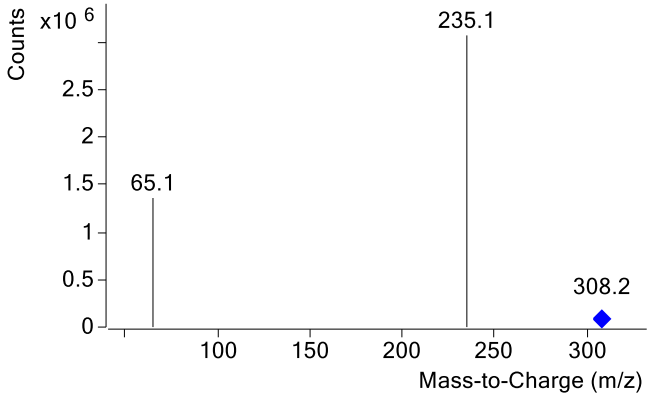


Figure 3. LC-MSMS representative spectrum showing zolpidem ion fragments (m/z 308.2 → 235.1) and (m/z 308.2 → 65.1).

A comparison between a 1M sodium hydroxide and hair extraction buffer (HEB) digestion was performed with the fortified hair to determine which had a higher drug recovery. The last wash was also saved to see how well the washing procedure worked. The wash consisted of a methanol and phosphate buffer wash. Table 2 shows the results of the washing procedure as well as the difference in recovery after both digestion methods. Zolpidem was recovered, after a 1M NaOH digestion, at a concentration five times great than that of an HEB digestion. The low concentration of zolpidem seen in the last wash indicates a good washing efficiency. Figure 4 depicts the comparison between the two digestion methods and the efficiency of the last wash.

Table 2. Wash Efficiency and Recovery After Digestion (1M NaOH vs HEB)

Sample	**Digestion Solution**	**Concentration (pg/mg)**
1 – LW	NaOH	14
2 – LW	NaOH	13
3 – LW	NaOH	14
1 – H	NaOH	5063
2 – H	NaOH	5137
3 – H	NaOH	8269
1 – LW	HEB	12
2 – LW	HEB	14
3 – LW	HEB	12
1 – H	HEB	1164
2 – H	HEB	1148
3 – H	HEB	1020

*LW- last wash, H- hair

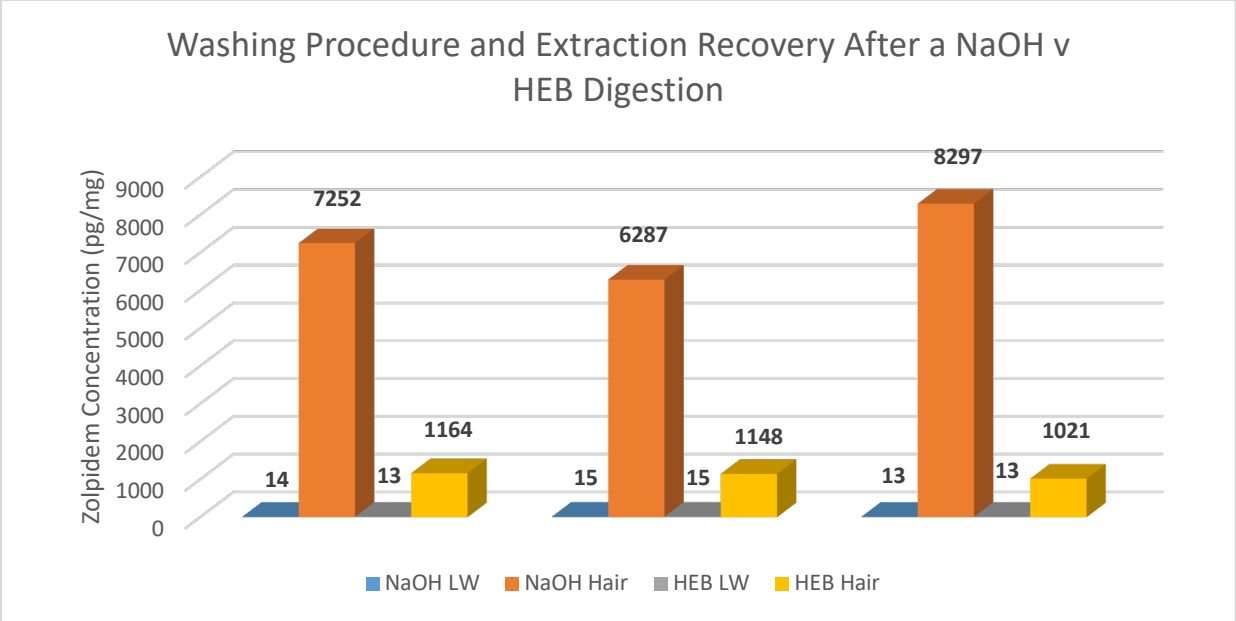


Figure 4. Washing efficiency and extraction recovery of zolpidem after a 1M NaOH and HEB digestion.

Since the 1M sodium hydroxide had a higher recovery, it was used as the digestion solution for the LC-MSMS analysis. The recovered concentration post-digestion shows how well the fortification procedure worked in incorporating the drug into the hair as well as how well the recovery of the drug from the digested matrix worked.

Validation of the developed assay was tested using decontaminated negative hair (30 mg) samples and performing various validation procedures. The hair was validated over a concentration range of 2 to 1,000 pg/mg. The calibration curves were prepared using either quadratic or linear formulas to produce a best fit line with good r^2 values. Good linearity was obtained when the r^2 was greater than 0.985 (Table 3). This was seen in all five days of linearity testing as well as in all other analyses.

Table 3. Linearity of Calibration Curves.

Day	Calibration Curve Formula	R^2
1	$y = -0.003111x^2 + 0.459669x - 0.002492$	0.9854
2	$y = -0.009311x^2 + 0.726733x - 0.002477$	0.986
3	$y = -0.002977x^2 + 0.230380 + 0.011476$	0.9973
4	$y = 0.002845x^2 + 0.250983x + 0.003026$	0.9959
5	$y = -0.002611x^2 + 0.586466x + 0.013747$	0.995

R^2 values are weighted: 1/x^2

The intraday (n = 3) and interday (n = 15) precision and accuracy (bias) results for zolpidem at the quality control concentrations (10, 50, and 200 pg/mg) are seen in Table 4. The accuracy and precision data were within acceptable limits, +/- 20% error and 15% RSD. The intraday precision and accuracy of the 10 pg/mg, 50 pg/mg, and 200 pg/mg QCs were 4% and 10%, 9% and -1%, and 2% and 2%, respectively. The interday precision and accuracy of the 10 pg/mg, 50 pg/mg, and 200 pg/mg QCs were 9% and 2%, 9% and 4%, and 11% and 6%, respectively.

Table 4. Intraday and Interday Precision and Accuracy of Zolpidem in Quality Control Hair Samples.

Statistical Variable	Concentration (pg/mL)		
	10	50	200
Intraday (n = 3)*			
Mean	11.0344	49.6598	203.7433
Precision (%RSD)	4.1165	9.3734	2.1176
Bias (%RE)	10.3437	-0.6805	1.8716
Interday (n = 15)**			
Mean	10.186	51.998	212.608
Precision (%RSD)	8.640	8.889	11.418
Bias (%RE)	1.863	3.996	6.3039

*Intraday results from Day 3
**Interday results are the average of the 15 samples over 5 days
Precision (%RSD) = ([mean]/ SD) *100
Bias (%RE) = ([mean calculated] – [expected])/ ([expected]) *100

The matrix effect, extraction recovery, and process efficiency were performed twice since the results were not expected (Table 5). The matrix effect for the five sources was observed to be on average 15% over the two days. The extraction recovery and process

efficiency of the method for five sample sources averaged 70% and 10%, respectively over the two days.

Table 5. Matrix Effect, Extraction Recovery, and Process Efficiency (100 pg/mg).

Source (Color)	Day 1			Day 2		
	% Matrix Effect	% Extraction Recovery	% Process Efficiency	% Matrix Effect	% Extraction Recovery	% Process Efficiency
1 (N/A)	19	55	10	23	56	13
2 (Black)	10	63	6	16	86	13
3 (N/A)	13	68	9	10	83	8
4 (Blonde)	17	73	13	16	81	13
5 (Light Brown)	13	97	12	14	57	8
Average	**15**	**70**	**10**	**16**	**71**	**11**

%Matrix Effect = (Post-Addition/ Neat) *100
%Recovery = (Pre-Addition/ Post-Addition) *100
%Process Efficiency = (Post-Addition/ Neat) * 100

The process sample stability of zolpidem in hair was stable over five days at room temperature after the analysis of three samples at quality control concentrations (10, 50, and 200 pg/mg). Figure 5 shows the stability of the QCs over five days. Analysis of the samples was on Day 1. The samples were then left out at room temperature and re-analyzed on Days 2 and 5.

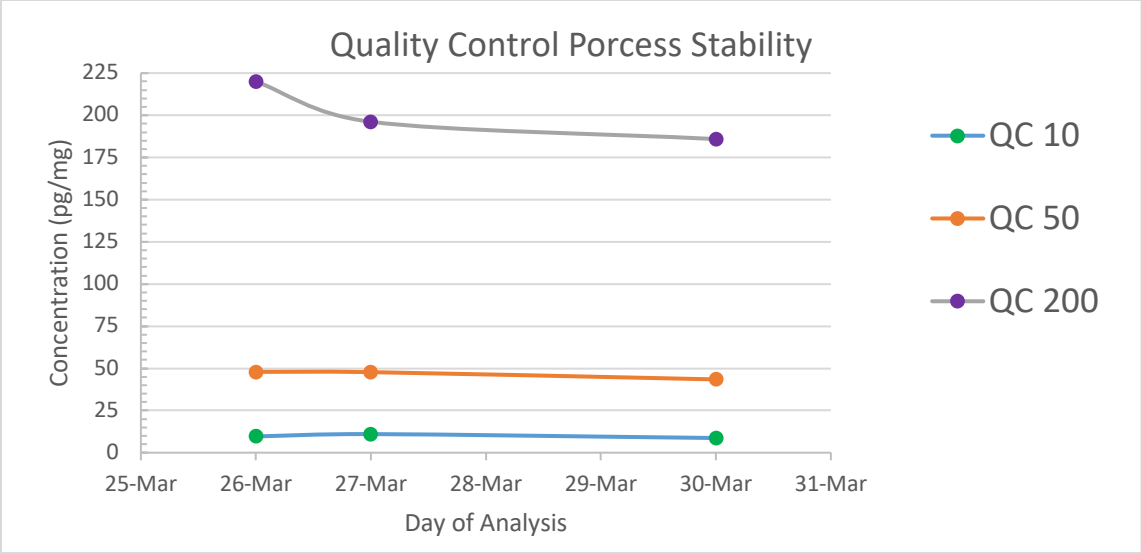


Figure 5. Stability of QCs 10, 50, and 200 pg/mg over five days. Samples were analyzed on Days 1, 2, and 5.

The dilution integrity showed accurate results between the expected and actual concentrations (Table 6). The measurements of the diluted samples were within +/- 15% of the theoretical concentration.

Table 6. Dilution Integrity.

Sample	Dilution	Expected Concentration (pg/mg)	Actual Concentration (pg/mg)	%Accuracy
ULOL	N/A	1000	1035	-3.5
DF 2	1:1	500	525	-5
DF 4	1:3	250	264	-5.6
DF 10	1:9	100	108	-8

Carryover is analyzed to ensure that there is not leftover analyte in the analytical instrument after analysis. The carryover was evaluated by analyzing two blank samples followed by high concentrations (1,000 and 10,000 pg/mg). The two blank samples showed low responses with noise compared to the upper limits'. Table 7 shows the

carryover results for the ULOL, 10x ULOL, and the negative controls between analyses. Figure 6 shows the blank chromatograms after the ULOL and 10x the ULOL. There was not a qualifier ion detected and only noise peaks were visible.

Table 7. Carryover results of the LC-MSMS.

Samples	Zolpidem Response	Internal Standard Response
ULOL	3124205	1099794
Blank 1	3858.98	1022388
ULOLx10	4.5E+07	534712
Blank 2	29757	946740

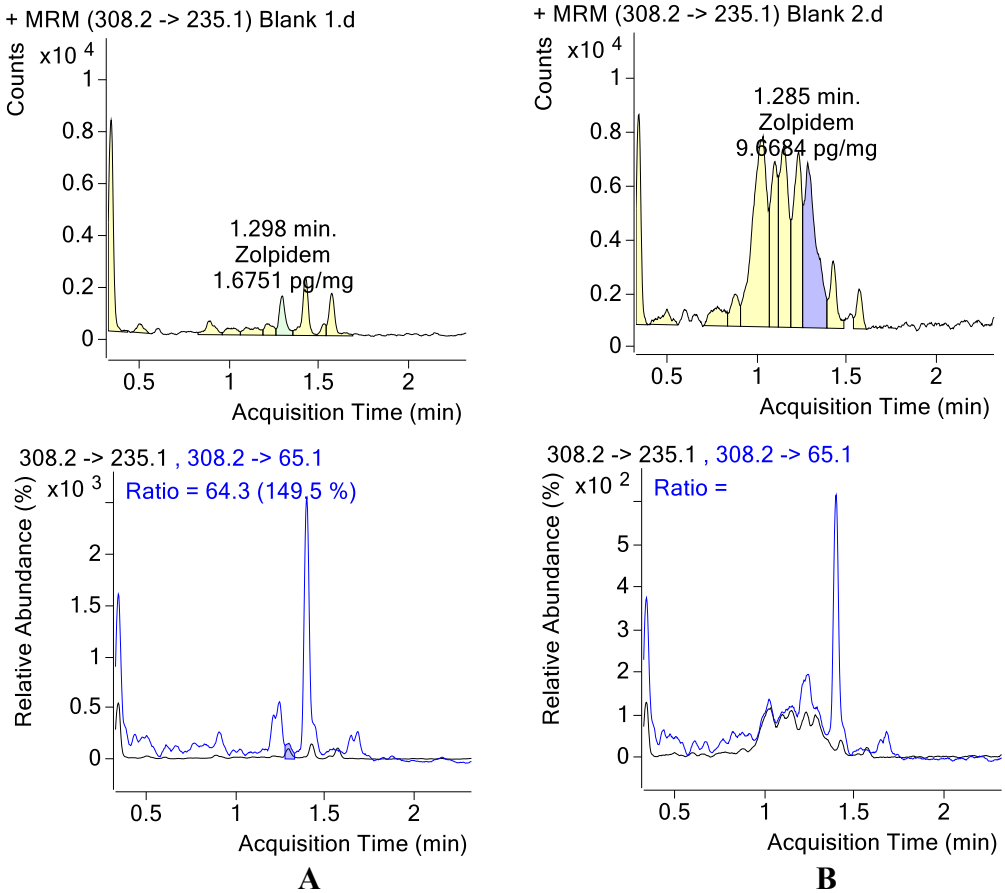


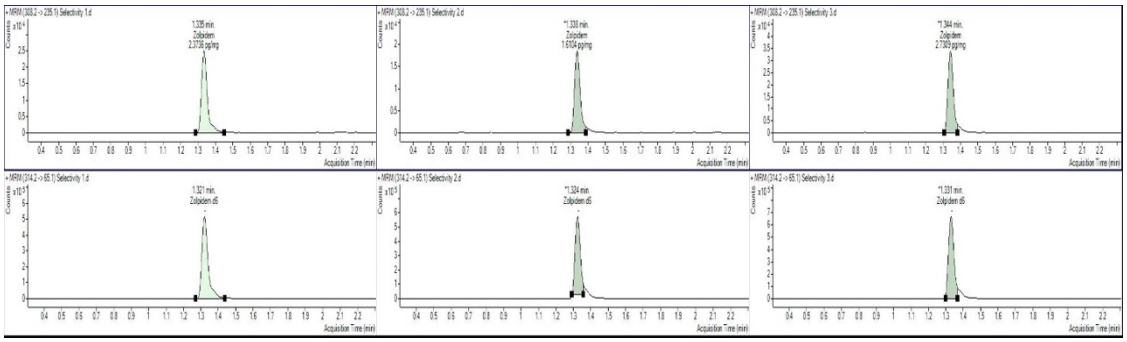
Figure 6. Blank sample chromatograms after the ULOL (A) and after 10x ULOL (B) showing noise peaks and no qualifier detected.

The selectivity study showed that the hair matrix alone did not contain any endogenous and exogenous matrix interferences. The potential of endogenous matrix interferences was assessed using five blank hair samples fortified with internal standard previous to this experiment. Exogenous matrix interferences were assessed by the analysis of five blank hair samples fortified with five common drugs used to facilitate crimes at 10,000 pg/mg, as well as 2 pg/mg of zolpidem (LOD). The five drugs that were tested were benzoylecgonine, oxazepam, hydrocodone, imipramine, and lorazepam. The analytical method was able to measure and differentiate the zolpidem analyte and internal standard in the presence of the five other compounds at high concentrations within the hair samples. Table 8 shows the analyte and internal standard response, as well as the detected zolpidem concentration after the analysis of the five samples. The 2 pg/mg zolpidem were detectable in samples 1-3, not in samples 4 and 5. The method was selective to those two samples since the analyte was not detected less than the LOD. Figure 7 depicts the chromatograms of the five samples for the zolpidem analyte m/z (308.2 → 235.1) and internal standard (m/z 314.2 → 65.1).

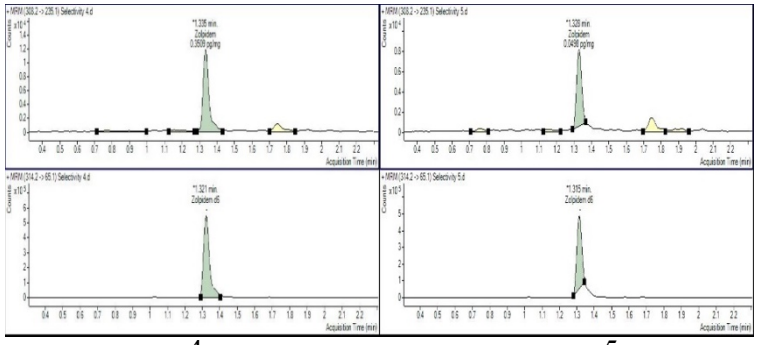
Table 8. Selectivity Study of Five Hair Samples with Five Other Compounds.

Samples*	Samples	Analyte Response	Internal Standard Response	Concentration of Zolpidem (pg/mg)
Zolpidem (2 pg/mg), Internal Standard, and Five Other Compounds	1	57700	1269040	2.3736
	2	41195	1129552	1.6104
	3	72511	1459572	2.7309
	4 (dyed red hair)	28308	1309256	0.3509
	5 (dyed red hair)	15364	850157	0.0498

*Samples were tested analytically prior to determine they were negative.



1 2 3



4 5

Figure 7. Chromatograms showing the zolpidem peak for samples 1-5.

The proficiency testing of spiked hair samples prepared by a research scientist at Cordant Health, were consistent with the expected results (Table 9). The results were +/- 15% of the actual concentrations.

Table 9. Proficiency Testing.

PT #	PT Actual (pg/mg)	PT Results (pg/mg)	% Accuracy
1	7.5	6.0147	-19.80
2	25	23.9360	-4.26
3	75	76.8704	2.49
4	250	247.8854	-0.85
5	750	752.1665	0.29

5.2. Clinical Application

Authentic hair samples, positive for zolpidem, were obtained from the University of Pennsylvania. The samples were analyzed using the developed method in collaboration with their on-going biological matrix correlation study. The study used anonymous donor patients' urine, oral fluid, plasma, and hair samples. The patients' medical history was noted. Samples 022 and 210 were weighed (30 mg and 15 mg (DF 2), respectively) and internal standard was added. The method's digestion, extraction, and LC-MSMS procedures were performed. Zolpidem was detected in samples 022 and 210 at concentrations of 578 pg/mg and 834 pg/mg, respectively (Table 10). The correlation between the biological matrices is seen in Table 11.

Table 10. University of Pennsylvania Correlation Study: Hair Analysis

Sample		Zolpidem				Qualifier	Zolpidem-d6
SID	Dil.	RT	Response	Calculated Concentration	Final Concentration	RT	Response
022	1	0.994	10139665	577.9367	577.9367	0.981	1570265
210	2	0.994	10147782	417.2332	834.4664	0.984	2172406

Table 11. University of Pennsylvania Biological Sample Correlation for Zolpidem.

SID	[Urine] (ng/mL)	[Oral Fluid] (ng/mL)	[Plasma] (ng/mL)	[Hair] (pg/mg)
022	65.6668	2.3989	56.9612	577.9367
210	4.2222	5.7796	14.7416	834.4664

6. Discussion and Conclusion

The LC-MSMS method developed to detect the presence of zolpidem, a sedative-hypnotic, in hair samples has been efficiently validated. The zolpidem was seen to elute within approximately 1.3 minutes, with a total run time of 2 minutes.

A wash using methanol and phosphate buffer has shown to successfully remove external contamination on the surface of the hair. The last wash that was analyzed from the fortified samples, showed low levels of drug compared to the amount of recovered drug. The wash though, was not tested on interferences including dye, bleach, and other common cosmetic chemicals. The influence of cosmetic treatments of hair on drug testing has been of concern for drug analysis in hair. Products that are strong bases are expected to cause damage to hair (Jurado et al., 1997). More specifically, damage to melanin within the hair follicle. Thus, drugs may be lost from the matrix.

The digestion, using sodium hydroxide was seen to show a better drug extraction than hair extraction buffer. Other digestion methods can possibly be tested that do not require a neutralization step such as methanol or an enzyme extraction. The neutralization step, can be difficult since there can be variability with pH between sources causing the amount of neutralization solution to vary. The solid-phase extraction using Strata exchange columns and the method used has shown to extract the zolpidem, basic drug efficiently.

The linearity, intraday and interday precision and accuracy, carryover, process sample stability, dilution integrity, and proficiency testing parameters yielded satisfactory results for the detection of zolpidem. Matrix effect and selectivity showed results that were not expected but are accepted since they have known causes that can be investigated.

Matrix effect was seen throughout all of the hair samples. As a result of matrix effect, process efficiency was expected to be low due to 85% ion suppression. Ion suppression is a form of matrix effect that is seen with liquid chromatography-mass spectrometry. Modifying the instrumental component parameters and sample preparation can be considered to reduce or possibly eliminate ion suppression. A cleaner extraction technique can be further investigated. This method is subject to significant matrix effects, but with the exception of dyed hair samples, the method is sensitive enough to quantitate 2 pg/mg in hair samples.

In the selectivity procedure, it was difficult to detect the LOD in samples 4 and 5. This may be due to these samples being cosmetically treated with dye. Cosmetic treatments interfere with the melanin structure, thus altering the incorporation of drugs into the hair matrix. There was limited authentic samples available for this research. Some of the samples available were cosmetically treated. Future studies are required to determine the effect cosmetically treated hair has on drug analysis.

The hair samples in the University of Pennsylvania correlation study were found to have higher zolpidem concentrations than that in the other three matrices. There does not appear to be a correlation trend between zolpidem within the various biological matrix samples.

Overall, this developed and validated confirmatory LC-MSMS method, compared to previous methods, is more sensitive with an LOQ of 2 pg/mg, quicker with a digestion of 30 minutes, and easier with a fast and automated extraction procedure (Salomone et al., 2012) (Kim et al., 2011). This method is useful for the quantitative detection of zolpidem in hair samples.

The method developed was to detect the presence of zolpidem in hair samples. A dosage study and segmental analysis was not performed. A control study is needed to analyze hair samples after a single, low-dosage of zolpidem. This will determine if the LOQ that was established in this method is acceptable for the low, single-dosage of zolpidem for a facilitated crime.

7. Future Research

A future development of an ELISA screening technique would allow for an easy initial detection of zolpidem within hair samples. The zolpidem commercial plates that are already available are not compatible with complex matrices such as hair samples. As a result, various sample sizes, digestion methods, and solution volumes have to be studied. Also, a phosphate buffered saline wash might help to remove the non-specific binding, thus lowering the possible presence of interferences and increasing the absorbance.

The effects of various cleaning agents (e.g. shampoo, conditioner) as well as chemical and physical agents (e.g. dye, bleach, heat) should be studied. Two samples that were analyzed in this experiment were red-dyed hair. The dye might have altered the melanin structure within the hair, thus affecting the ability to detect the low levels of drug. Cosmetically treated samples should be analyzed as to how to better wash and extract low levels of analytes. There have been previous studies showing the effects of cosmetic treatments on drug analysis. Jurado and authors (1997) did a study showing that the chemical composition of various cosmetic treatments, including bleaching and dyeing hair, have an effect on drug stability. They showed that strong basic treatments involving ammonium hydroxide and hydrogen peroxide (H_2O_2), affect the hair fiber causing alterations to the cuticle. "Ammonium hydroxide opens the scales of the cuticle to facilitate

the entry of the other compounds, H_2O_2, a powerful oxidant, attacks the hair pigment and decolorizes the hair" (Jurado et al., 1997, p. 161). Overall, the drug concentration in hair had decreased with the use of cosmetic hair treatments.

A segmental analysis of hair can be performed on hair samples when the root end is clearly noted. Recent and long-term drug exposure can be determined through segmental analysis. With the known rate of hair growth in head hair (1 cm/month), the identification and quantification of drugs and its metabolites along the length of the hair shaft can be determined for drug history. Small segments of hair can be cut into known lengths and analyzed for drug exposure.

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