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ELISA Validation Method for the Detection of Ketamine in Hair

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Abstract

An ELISA method was developed and validated to detect ketamine in human hair samples. Ketamine is an anesthetic drug that causes memory loss, dissociative sensations, and hallucinations. Due to these adverse effects, ketamine is a common drug used in drug facilitated sexual assaults (DFSAs). It is very important to be able to detect the substances used in DFSAs over a longer period of time due to the delayed reporting of these crimes. Victims, often out of fear and from the sedative/memory loss effects of the drugs, tend to report these crimes when it is too late to use urine and blood for toxicological testing. Hair has a window of detection of up to 12 months, which makes it a useful matrix to use in DFSAs. The linear range of this assay was from 0 pg/mg to 1,000 pg/mg with a calibration curve returning an acceptable R^2 value of 0.9991. The lower limit of detection was calculated to be 18.1 pg/mg. Accuracy and precision of this assay was determined through replicate analysis of quality control samples. Both intraday (n = 12) and interday (n = 12) accuracy and precision data were within the acceptable limits of +/- 20% error and 10% CV. Results indicated interference with PCP, which generated a response similar to a low positive control. There was no carryover seen between samples in the wells from the plate washer or from manual pipetting. This validated method was used to analyze positive authentic hair samples from donors with reported ketamine drug use. Results indicated correlation between the ELISA screening results compared to the LC-MSMS confirmation results. However, more samples need to be tested for further research. After thorough analysis, the Ketamine Direct ELISA kit from Immunalysis is suitable to use as a screening assay to detect ketamine in hair samples.

1. Introduction

Drug-facilitated sexual assaults (DFSAs) involve the use of alcohol or drugs to compromise an individual's ability to consent to sexual activity (Department of Justice/DEA, 2020). The drugs often used in these sexual assaults gained popularity in the 1970s, '80s, and '90s for their euphoric and intoxicating effects, and became very popular at raves and parties. The Controlled Substances Act was passed in the 1970s to limit misuse of these drugs (Department of Justice/DEA, 2020). Congress, in 1996, also passed the Drug-Induced Rape Prevention and Punishment Act that established federal penalties of up to 20 years imprisonment and fines for violence committed using a controlled substance (Department of Justice/DEA, 2020). Even with these laws in place, the number of DFSAs in the United States continued to rise and is still proving to be a problem today. Having a way to detect these drugs is crucial to successful prosecutions and also brings peace of mind to the victims of these assaults.

Immunoassays are primarily used as qualitative (screening) tests that use antibodies to detect the presence or absence of drugs in biological samples. Today, the most prevalent immunoassay for screening is an enzyme-linked immunosorbent assay (ELISA). ELISA works through the interaction of a target molecule (antigen) and a corresponding antibody. Each antibody reacts only with a particular drug of interest, drug class, or drug's metabolite. Immunoassays have become more popular because they are quick and easy to administer and can detect traces of drugs. All immunoassays can produce false positives, and therefore they provide only a preliminary result, positive or negative. If the result is positive, a more specific method is used to confirm the result, such as liquid chromatography-tandem mass spectrometry (LC-MSMS).

My thesis focuses on developing and validating an ELISA test for detecting ketamine in hair. Ketamine is a powerful dissociative anesthetic drug used in both human and veterinary

surgery since the early 1960s, and is considered one of the oldest of the new psychoactive substances (Kintz et al., 2015). Because ketamine causes memory loss, as well as dissociative sensations and hallucinations, it has been abused in the United States since the beginning of the 1980s. For this reason, it is one of the more popular drugs to facilitate sexual assault and has been often referred to as a date rape drug (Department of Justice/DEA, 2020). Drugs used in sexual assault cases are hard to detect because the victim often delays reporting the crime, either from fear or from the adverse effects of the drug. This is why it is important to be able to detect and quantify the drug after a long period of time, making hair the biological matrix of choice. Hair's long window of detection makes it a useful matrix for drug-facilitated crime investigations. Hair samples can be analyzed to test for the presence of drugs after months have passed, compared to a few hours to days for urine, blood, and oral fluid.

There were no commercially available immunoassay kits for ketamine and its metabolites until around 2003 to 2005 (Huang et al., 2007). However, today these kits are still mainly used for urine and blood, not hair. My thesis will be useful because it will explore a way to detect ketamine, a drug involved often in DFSAs, in hair. These drugs are still being abused in our society and no justice is offered to the victims due to how quickly they are eliminated from the body, along with other limitations of using urine and blood as the biological specimen of choice. Using hair as a matrix will provide a longer window of detection. It can reveal traces of these drugs in the system up to 12 months after use. The combination of hair testing and immunoassays, a cost-effective and highly sensitive method for screening substances found in hair, will elevate and further develop the drug-testing field and, in turn, create a stronger judicial system.

2. Literature Review

2.1. Ketamine

Ketamine, or 2-(o-chlorophenyl)-2-(methylamino) cyclohexanone, is a phencyclidine and cyclohexamine derivative (Bergman 1999). It is a dissociative anesthetic that falls under the class of psychedelic drugs. It was first created in the 1960s because phencyclidine, which was used for anesthesia in the 1950s, was not a good choice due to poor muscle relaxation and its potential for abuse (Domino, 2010). In the 1970s the US Food and Drug Administration approved ketamine to be used as an anesthetic both in human and veterinary surgery (Yew, 2015). However, starting in the 2000s, ketamine has been used mostly as an anesthetic on animals rather than on humans (Carter & Story, 2013). Ketamine has also been used to treat depression, epilepsy, and alcohol and heroin addiction (Sha et al., 2015). Ketamine is often misused as a recreational drug at nightclubs, parties, and raves, where it is known as Special K, Vitamin K, or SuperK (Wolff & Winstock, 2006). Ketamine became widely popular at these scenes because of the dissociative effects it produces on the body and brain.

Ketamine acts as a central nervous system depressant and is known to interfere with the reception of sensory input in the brain (Mion & Villevielle, 2013). The drug primarily works on glutamatergic neurons as a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist. In other words, it works by disrupting the neurotransmitter glutamate, which is involved with learning, memory, emotion, and pain recognition (Rosenbaum et al., 2021). It can also less commonly target γ-amynobutyric acid (GABA), dopamine, serotonin, sigma, opioid, and cholinergic receptors (Zanos et al., 2018). Ketamine has two enantiomers: (*S*)- and (*R*)-ketamine. The (S)-ketamine enantiomer is involved with the acute psychotic reactions in the body and brain; the (R)-ketamine enantiomer is associated with the feeling of well-being and elevated mood (Vollenweider et al., 1997). At low doses, the effects include distortion of time and space, hallucinations, mild dissociative effects, and sedation. At high doses, a more

severe state of dissociation called K-hole occurs where a person feels completely detached from reality and themselves and are unable to control their body (Stewart, 2001). Ketamine can cause sympathomimetic activity, which lead to an increased heart rate, elevated blood pressure, and an overall increased demand on the heart (Goddard et al., 2021). In severe cases ketamine can cause respiratory depression, coma, delirium, and death from overdose if combined with another central nervous system depressant such as alcohol.

Ketamine often comes as a white powder, but it can also be in the form of a clear liquid or tablet. Ketamine tablets are administered orally. The powder is most commonly used for snorting and smoking, and the liquid form is injected either intravenously or intramuscularly (Yew, 2015). An injection yields the quickest response, with effects occurring in seconds to minutes, while intranasal ingestion leads to effects around 5 to 15 minutes later. Oral consumption takes the longest, with effects after around 20 minutes (Wang & Yang, 2015). Ketamine's half-life is around 3 hours, which means it is usually eliminated from an adult's body within 12 hours. Approximately 90% of ketamine is excreted in the urine in the form of metabolites (National Library of Medicine). These less-active metabolites include norketamine, dehydronorketamine, hydroxyketamine, and hydroxynorketamine, which are metabolized primarily by the cyctochrome P450 liver enzymes, CYP2B6 and CYP3A4 (Hijazi & Boulieu, 2002). Due to ketamine's fast elimination in the body, in combination with its sedative and memory loss effects, it is the perfect drug to be used for DFSA.

2.2. ELISA

To detect the presence or absence of these dissociative psychedelic drugs quickly and cheaply, immunoassays have been the go-to test for screening. For years radioimmunoassay was the only technique sensitive enough to detect traces of drugs in human hair (Kronstrand et

al., 2015). Today, the predominant immunoassay for screening biological samples is ELISA. This assay was developed by Peter Perlmann and Eva Engvall at Stockholm University in the 1970s and first used in the 1980s (Lequin, 2005). It was the first immunoassay that used an enzyme as the label, rather than radioactivity. All immunoassays are based on the interaction of a target molecule (antigen) with a corresponding antibody. What makes ELISA so sensitive and specific is the binding action between the antibody and molecule of interest (Tang et al., 2015). ELISA is a competitive heterogenous assay, meaning the analyte in the biological sample will compete with the labeled conjugate drug for antibody binding, thus making the concentration of the drug in the sample inversely proportional to the signal. The heterogenous nature of the assay means these assays require a physical separation of bound and unbound drug to measure the amount of analyte present (Tang et al., 2015).

ELISA typically uses a 96-well polystyrene plate with antibody bound to the bottom of the plate. A small volume of the biological matrix being tested is added to the antibody-coated wells, followed by an enzyme conjugate drug. This drug then competes with the free drug in the sample for binding sites during an incubation period. The more free drug (in this case, ketamine) present in the test sample, the less ketamine-enzyme conjugate that can be bound. After incubation, the wells are washed to remove any unbound drug. An enzyme substrate is added, a second incubation takes place, and the reaction is stopped by the addition of an acid. The resulting color intensity or absorbance is read by a spectrophotometer, where it is inversely proportional to the concentration of drug in the sample. For qualitative results, the absorbance of the sample is compared to a cutoff calibrator, where a higher absorbance is a negative result and lower absorbance is a positive result (Kronstrand et al., 2015). For quantitative results, a calibration curve is constructed and used to determine the sample concentration. Many

commercial kits are available that contain an antibody reagent, labeled drug, calibrators, and controls to perform this assay, including a kit for ketamine.

The advantages of an ELISA test is that it allows simultaneous analysis of a large number of samples with results that are easy to interpret and are ready in a few hours. In addition, these assays can be automated on large-scale automatic analyzers, which are useful for quick results and can eliminate the labor-intensive steps and minimize human error (Tang et al., 2015). With these advantages, though, come disadvantages, including cross-reactivity with compounds with similar structures that can interfere with the assay. In addition, immunoassays only provide a preliminary result, positive or negative. If a positive result is obtained, a more specific method must be used to confirm the analytical result, such as gas chromatography mass spectrometry (GC-MS) or LC-MSMS.

2.3. LC-MSMS

In LC-MSMS, the analytes are first chromatographically separated by liquid chromatography, which the separation of analytes based on their differential affinities for the stationary phase and the mobile phase. The three modes of separation are based on polarity, charge, and size. The polarity separations are based on the rule that “like attracts like” and are the most common in analytical toxicology. There can be two types of polarity separations: normal phase and reverse phase. Normal phase includes a non-polar mobile phase such as hexane and a polar stationary phase such as silica. Reverse phase is the opposite and is used with a polar mobile phase such as water/acetonitrile and a non-polar stationary phase such as an 18-carbon-long hydrocarbon (C18). The analytes will be separated and retained based on their chemical properties and, therefore, attraction to the solvents that the mobile phase and stationary phase are made up of. After this separation, the analytes are ionized in the interface

through electrospray ionization. Electrospray ionization uses electrical energy to either aid the transfer of ions from solution into a gaseous phase or convert neutral compounds into their ionic form and then into a gaseous phase by protonation (Ho et al., 2003). This occurs in three steps: dispersal of a fine spray of charged droplets, solvent evaporation, and ejection of the ions from the charged droplets.

After ionization of the analytes, they enter the mass analyzer, which contains two mass filters (quadrupoles), a collision cell, and a detector. The ions will travel through a magnetic or electrical field inside the mass analyzer, which will be the main way the ions will be separated from one another (Ho et al., 2003). The precursor ion is isolated in Q1, fragmented in the collision cell into product ions, and the product ions are then isolated in Q2 based on their mass to charge (m/z) ratio (Skoog et al., 2007). Each transformation of a precursor ion to a product ion is called a transition. These transitions are monitored through the multiple reaction monitoring mode that the LC-MSMS works in. A mass spectrum of the molecule is then produced, displaying the ion's abundance in that compound versus the m/z ratio. This alone is very specific to whatever compound you are analyzing. The compounds are further identified based on their retention time (injection to detection time), the presence of two characteristic transitions (quantifier and qualifier), and the ion ratio qualifier/quantifier being within 20% of the ion ratio of the calibrators (Concheiro-Guisan et al., 2021).

Chromatographic techniques coupled with mass spectrometry is a highly specific, sensitive, and definitive way to analyze your samples (Tang et al., 2015). LC-MSMS has become more popular compared to its counterpart, GC-MS. This is because it can analyze a more diverse sample set and does not require derivatization procedures to analyze certain types of drugs, which saves time and money. LC-MSMS can analyze inorganic and organic

molecules, large polar and non-polar compounds, ionic compounds, thermally unstable compounds, involatile compounds, and compounds with a low or high molecular mass. Finally, LC-MSMS is a form of tandem mass spectrometry, meaning there are two mass analyzers in your instrument. Having two mass analyzers allows for increased specificity of your analysis, higher accuracy and precision, and increased sensitivity of your instrument (Pitt, 2009). This all allows for lower limits of detection, definitive results, and a wider range of compounds to analyze—even ones with similar chemical compositions. LC-MSMS methods have been used and published to identify and quantify ketamine since the 1970s (Chang & Glazko, 1972). However, before any screening or confirmatory testing can occur, collection of the specimen is the first step.

2.4. Hair

Different types of specimens are used for laboratory testing, based on ease of collection, risk of adulteration, transport, stability, amount of drug, analytical methods applicable, window of detection, and other factors (Tang et al., 2015). Due to ketamine's dissociative properties, odorless and colorless nature, and 3-hour half-life, it is often used as the ideal drug in DFSAs. Victims of DFSA often cannot recall what happened because of the adverse effects of the drug. They may also be fearful no one will believe them, so it goes unreported. If they do remember, ketamine has already been eliminated from the body within about 12 hours. Due to this delayed reporting, it is important to be able to detect and quantify the drug after a long period of time, making hair the biological matrix of choice. Hair has a long window of detection. Hair samples can be analyzed to test for the presence of drugs up to 12 months after ingestion, compared to a few hours to days for urine, blood, and oral fluid. In addition, hair can also be used to test for drugs after a single exposure (Cooper, 2015). Because of these great advantages hair offers as

a matrix for drug testing, more laboratories are offering hair testing (Society of Hair Testing et al., 2008).

To understand how drugs get incorporated into hair, it is crucial to first discuss hair anatomy and physiology. The hair's purpose is to protect the skin from injury and to regulate body temperature (Cooper, 2015). Hair covers almost the entire human body, excluding the palms of the hands, soles of the feet, the outer parts of the lips, and parts of the genitalia (Buffoli et al., 2014). Hair's chemical makeup is 65% to 95% keratin, with water and lipids making up the other parts (Jenkins, 2008). The two main parts of the hair are the hair shaft and the hair bulb. The hair shaft contains the medulla, cortex, and cuticle. The hair shaft is visible above the surface of the skin and is composed of keratinized cells. The cuticle surrounds the shaft and acts as an outer protective layer (Harkey, 1993). Within the innermost region of the cortex is the medulla. The cortex is responsible for producing melanin, a pigment that gives hair its natural color (Pragst & Balikova, 2006). The hair follicle is embedded 3 mm to 4 mm below the surface of the skin, where its main function is hair growth (Cooper, 2015). The hair follicle is made up of the hair bulb, which contains the outer root sheath, inner root sheath, hair matrix, and the hair papilla (Cooper, 2015). A capillary system and glands are in close proximity to the hair follicle, including the sebaceous, apocrine, and eccrine glands. All these parts play a crucial role into how drugs get incorporated into the hair.

While the exact mechanisms by which drugs or analytes of interest are incorporated into hair are not fully understood, there are three recognized routes of incorporation. These routes are direct incorporation from the blood supply, from sebum and sweat in the hair, and from external contamination (Cooper, 2015). Amongst researchers there are different proposed models that describe how drugs are incorporated. The first is through passive diffusion directly

from the blood surrounding the hair follicle (Cooper, 2015). With this model, it is assumed that the drug concentrations in hair are correlated to the concentration of drug in the blood at the time the hair follicle was synthesized (Baumgartner et al., 1989). The fault in this model is that it doesn't account for the different metabolic profiles seen in hair and blood, with parent drugs less commonly detected in blood compared to the metabolites (Cooper, 2015). The other two models are based on drug incorporation into growing hair and the different affinities of acidic, basic, and neutral drugs to bind to the hair. Factors that are known to affect the incorporation and binding of drugs to hair include drug pKa, structure, size, lipophilicity, protein binding capacity, and melanin affinity (Joseph et al., 1996). Ketamine is a weakly basic substance (pKa = 7.5) and is mainly present in blood as a cation. However, in its neutral form, ketamine strongly interacts with melanin, facilitating its incorporation into the hair (Salomone, 2015).

The more common route of incorporation of drugs into the hair is though the blood stream, rather than through sweat (Schräder et al., 2012). Lipid solubility plays an important role in drug incorporation through the blood stream into the root bulb, with basic drugs having the advantage over acidic drugs (Cooper, 2015). Drugs get incorporated through sweat and sebum because these secretions surround and soak into the hair follicle and shaft as they grow (Huestis et al., 1999). External contamination is also a possible route. This can occur when someone is close to a person smoking drugs, as in a car or household, touching drugs and then touching the hair, or through mother-to-fetus interaction if the mother is abusing drugs (Gareri & Koren, 2010). External contamination has been a problem for hair testing but has been controlled by hair washing, using cutoff values, and determining the metabolite-to-parent drug concentration ratio (Baumgartner et al., 1989). If the drug metabolite is not present, the drug itself was likely detected from external contamination. Other problems with hair testing have been reduced

concentrations of drugs in hair after exposure to sunlight, applying heat to style the hair, and through cosmetic treatments such as dyeing or bleaching, which contains strong bases (Cooper, 2015).

Hair does not grow continually but in cycles; the three phases of hair growth are anagen (growth), catagen (transitional), and telogen (resting). Factors that affect hair growth include age, race, health status, nutritional status, and pregnancy (Concheiro-Guisan, 2021). The hair grows when it is in the anagen phase at the rate of approximately 1 cm/month (Curtis & Greenberg, 2008). This allows for segmental analysis to determine an individual's pattern of drug use whether it is a single exposure or chronic use (LeBeau et al., 2011). The predictable growth pattern acts as a calendar for an individual's drug use.-Although hair covers most of the human body, hair from the scalp in the anagen phase is most commonly used for analysis. Hair is collected close to the scalp, at the vertex posterior, where the hair growth rate is the most consistent (LeBeau et al., 2011). It is noted which is the root end (the end closest to the scalp) after it is cut. The cut hair is stored in aluminum foil and placed into an envelope that is sealed, initialed, and dated. The evidence bag can then be shipped to a laboratory for analysis, where it is stored at room temperature. Hair collection is generally a quick, easy, and noninvasive process (Kintz et al., 2015).

After collection, the hair must go through a series of steps before it can be analyzed. These steps are decontamination, segmentation, homogenization, incubation, extraction, and clean-up (Concheiro-Guisan et al., 2021). Decontamination consists of a washing step to remove any external drug interferences, hair care products, sweat, and sebum (Kintz et al., 2015). The samples are usually washed with a combination of organic solvents and aqueous solutions. The samples must then be cut into small pieces and the amount needed for analysis weighed out.

The sample size may vary from 10 to 50 mg, depending on the drug that needs to be analyzed (Cooper, 2015). Next, the hair is homogenized with a milling instrument to create a powder. This ensures that a representative portion of the hair is used for analysis (Kronstrand et al., 2015). Then, about 10 mg of the sample is incubated with a buffer solution of either organic solvents, acids, or bases to liberate the drugs from the hair matrix (Kintz, 2019). Incubation is the last step before immunoassay analysis (Usman et al., 2019). The extraction process can continue after incubation with other methods, including solid phase extraction (SPE), liquid liquid extraction, supported liquid extraction, solid phase microextraction, and headspace. The extraction methods will vary based on the laboratory, suspected drugs being analyzed, and instrumental technique.

SPE extracts compounds based on their polarity. This method uses cartridges made up of liquid chromatography stationary phases that adsorb the target molecules of interest (Concheiro-Guisan et al., 2021). The different kinds of SPE cartridges include reverse phase, ionic exchange (cation or anion), and mixed-mode, which combines reverse phase and ionic exchange (Concheiro-Guisan et al., 2021). These cartridges absorb molecules through retention mechanisms that include hydrophobic, polar, or electrostatic interactions (Supelco, 1998). The 5 steps of SPE are conditioning, sample loading, washing, drying, and elution. Once the cartridges are conditioned, the sample is loaded. The analytes of interest are retained by the cartridge, while all other unbound material will pass through and exit the cartridge. To ensure this, there is a washing step. Specific solvents are added to free the drug from the stationary phase in the cartridge so it can be used for further analysis (Supelco, 1998). SPE is the most popular extraction method because it is easily performed, automated, doesn't use a lot of solvent, and has a faster extraction time (Concheiro-Guisan, 2021). SPE not only frees the drugs

from the matrix you are working with, but it also cleans up the sample. After incubation, extraction, or a combination of both, the hair samples are ready to be analyzed through a screening technique such as ELISA. This can then be followed up by a confirmatory technique such as LC-MSMS.

2.5. Prior Research

There were no commercially available immunoassays for ketamine and its metabolites until around 2003 to 2005 (Huang et al., 2007). Before this, laboratories would use chromatographic techniques after an extraction method, such as liquid liquid extraction or SPE, to analyze ketamine (Cheng & Mok, 2004). In Hong Kong, the Urinalysis Unit of the Government Laboratory of Hong Kong performed 10 tests to detect ketamine in 1999 and 15,000 tests in 2002 (Cheng & Mok, 2004). Due to the rise in popularity of ketamine, laboratories were in need of faster methods for analysis.

The first two reports that involved the use of a commercial ELISA kit to detect ketamine and norketamine in human and monkey urine were published in 2003 and 2005, respectively (Huang et al., 2007). In the first report, Tan et al. (2003), evaluated an ELISA test kit to detect ketamine in urine samples from humans. Negrusz et al. (2005) published a study aiming to apply and evaluate a newly developed ELISA screening method to detect ketamine in primate urine samples after a single dose of ketamine.

With these two studies in mind, Huang et al., in 2007 designed a study to evaluate the performance characteristics of the only two ELISA kits commercially available. This study focused on the cross-reactivity between ketamine and its metabolites and the correlation between the ketamine concentration from the ELISA plates versus the true ketamine concentration determined by GC-MS (Huang et al., 2007). The matrix used in this study was

urine. The two ketamine ELISA kits were provided by NEOGEN Corp. (Lansing, MI) and International Diagnostic Systems Corp (IDS) (Saint Joseph, MI). This study found that the ELISA plate from IDS was too cross-reactive with ketamine's metabolites, producing false positives, while the NEOGEN plate responded specifically to ketamine (Huang et al., 2007). The NEOGEN plate also correlated to the concentrations of ketamine determined by GC-MS much better than the IDS plate (Huang et al., 2007). However, they did find that there was room for improvement with the correlation coefficient and assay formulation (reagent volumes, incubation times, etc.) to achieve better precision and separation power (Huang et al., 2007).

Another study that also used urine and the NEOGEN ELISA kits was conducted by Cheng et al., in 2006-2007. This study looked at 206 urine samples and conducted an ELISA screening and confirmation through GC-MS to detect ketamine (Cheng et al., 2007). They found that these ELISA kits and methods had high efficiency, high sensitivity, high specificity, a low false positive rate, and a moderate false-negative rate (Cheng et al., 2007). The false negative rate was around 9% because 22 urine samples were found positive for ketamine and its metabolites using GC-MS but only 20 samples were screened positive using ELISA (Cheng et al., 2007). To improve this study, the next steps would be to adjust the reagent volumes to minimize the false negative rate, to determine the cross-reactivity between ketamine and other drugs, and to determine the carry-over percentage between the wells in the ELISA plate. The researchers found that the combination of ELISA and GC-MS proved to be a favorable strategy to determine ketamine in urine specimens.

In 2009, Harun et al., conducted a study in Malaysia validating an ELISA screening method and an LC-MSMS confirmation method to identify and quantify ketamine and norketamine in urine samples (Harun et al., 2009). This study also used the NEOGEN ELISA kits for analysis.

Their results from the ELISA screen, compared to the validated LC-MSMS confirmation results, demonstrated 100% sensitivity, specificity, and efficiency (Harun et al., 2009). Therefore, this test was properly optimized and validated in 2009 and is now in current use to detect ketamine and norketamine in urine samples.

Urine is the most popular matrix used for drug testing (Tang et al., 2015). However, other matrices have been used to test for drugs of abuse, including blood, plasma, oral fluid, hair, and sweat (Tang et al., 2015). The immunoassay kits that were developed from 2003 to 2005 were originally made for urine and are not sensitive enough to be used with hair (Pragst & Balikova, 2006). Because of this insufficient sensitivity, ELISA tests for opiates, cocaine, cannabinoids, benzodiazepines, and methadone were developed and confirmed to be sensitive for hair analysis (Pragst & Balikova, 2006). Pujol et al., conducted a study using the IDS ELISA plate to detect illicit drugs in hair. The drugs of abuse they tested were cannabis, amphetamines, opiates, and cocaine (Pujol et al., 2007). From the 93 hair samples tested, there were no false negative results. All the samples that the ELISA screen detected as negative were also confirmed negative by the GC-MS. The specificity of the plate ranged from 70% to 90% because 22 of the samples screened with ELISA were false positives (Pujol et al., 2007). Meaning, the GC-MS identified the drugs but the concentrations were lower than the cutoff values, and therefore were reported as negative (Pujol et al., 2007). This study determined that the IDS One-Step ELISA kit is a highly sensitive and suitable screening method to detect illicit drugs in hair, with confirmation needed on the positive samples.

What Huang et al., Tan et al., Negrusz et al., Cheng et al., and Harun et al., have in common is that all their studies involved an ELISA screen to detect ketamine in urine samples. Not one of these studies used hair as a matrix. This demonstrates a major gap in the research when it

comes to using an ELISA screening method to detect ketamine in samples. There are earlier studies conducted that use hair as a matrix with the ELISA method, including the Pujol et al. study. However, this study and many others did not test for ketamine or its metabolites. Another gap in the research is that these earlier studies used ELISA kits from NEOGEN or IDS.

To conduct my research, I used an ELISA kit from the company Immunalysis (Pomona, CA). As a part of my research, I had to determine a cut-off value to detect ketamine in the hair samples. Because there are no published studies to detect ketamine in hair using an ELISA screen, I had to base the cut-off value on a study that detected ketamine in hair through LC-MSMS and GC-MS. Salomone et al. (2015) proposed a cut-off value of 0.5 ng/mg. Anything below this amount would be considered negative for ketamine. This cut-off value is a starting point for my research, and all assay and reagent volumes will be optimized with the ELISA kit from Immunalysis.

A final gap in the research is that most of the studies previously mentioned used GC-MS as their confirmation method. LC-MSMS has become more popular within the last decade compared to GC-MS (Needham & Williams, 2016). It can analyze more diverse samples and does not require derivatization procedures to analyze certain types of drugs, which saves time and money. Additionally, having two mass spectrometers in the instrument increases the sensitivity and specificity of the analysis (Pitt, 2009). All of these previous studies mentioned will be used as a guide and starting point to help me conduct my own research. To summarize, the combination of an immunoassay screening method and using hair as the matrix of choice will be very advantageous in detecting ketamine in samples. There is no validated method today to detect ketamine in hair samples using an ELISA screening method so the objective of my research is to do just that.

3. Materials

3.1. Chemicals and Reagents

Two ketamine HCl standards, one used for calibration and one used for quality control (1.0 mg/mL in methanol) (Lot # FE0121) were purchased from Cerilliant (Round Rock, TX) and stored in the freezer. The ketamine Direct ELISA kit (Catalog #240-0480), hair extraction buffer (HEB), neutralization buffer, and bovine serum albumin (BSA) were obtained from Immunalysis (Pomona, CA). The ketamine Direct ELISA kit contained ketamine-enzyme conjugate (ketamine derivative labeled with horseradish peroxidase), 3,3',5,5' tetramethylbenzidine and urea peroxide in buffer (TMB substrate), and stop reagent made of 1N hydrochloric acid. Dichloromethane, isopropyl alcohol, methanol, and nitrogen were obtained from Cordant Health Solutions (Long Island, NY).

3.2. Standard Solutions

Working standard solutions were prepared using the purchased certified stocks of ketamine. Ketamine working standards were prepared at concentrations of 100,000 ng/mL, 10,000 ng/mL, and 1,000 ng/mL in methanol by serial diluting the ketamine 1 mg/mL standard stock solution.

3.3. Specimen

Negative hair samples were provided by Cordant Health Solutions for drug analyses. These samples were tested negative by using a standardized LC-MSMS analytical method. Authentic de-identified donor hair samples with reported ketamine drug use were obtained from a matrix correlation study conducted by the University of Pennsylvania. This study received paired urine, blood, oral fluid, and hair samples from donors to determine drug concentration correlation across matrices.

3.4. Apparatus

The Columbus Plus Microplate Washer and Tecan Sunrise Microplate Reader were purchased from Tecan (San Jose, CA).

4. Methods

4.1. Ketamine Calibrators and Quality Controls

Ketamine calibrators and quality controls were prepared using the ketamine 1.0 mg/mL in methanol standard. The ketamine standard was serially diluted in methanol by a factor of 10, 3 different times. This produced ketamine calibrators and quality controls at concentrations of 100,000 ng/mL, 10,000 ng/mL, and 1,000 ng/mL.

4.2. Sample Decontamination

The hair samples were decontaminated through multiple washing steps in order to remove any interferences such as shampoo, hair dye products, sweat, and external contamination agents. 1 gram of negative hair was weighed out and placed inside a beaker. 100 mL of dichloromethane was added to the beaker, covering the entirety of the hair. The solution was swirled by hand for 1 minute, decanted, and aspirated under a light flow of nitrogen. Then, 100 mL of isopropyl alcohol was added to the beaker, covering the entirety of the hair. The solution was swirled by hand for 1 minute, decanted, and aspirated under a light flow of nitrogen. Next, 100 mL of methanol was added to the beaker, covering the entirety of the hair. The solution was swirled by hand for 1 minute, decanted, and aspirated under a light flow of nitrogen. The hair samples used from the University of Pennsylvania matrix correlation study were also washed with the same solvents and aspirated under nitrogen. However, only 10 mg of each sample were used so, these hair samples were washed with only 1 mL of dichloromethane, isopropyl alcohol, and

methanol. Both the negative hair and University of Pennsylvania hair samples were stored in their respective envelopes and left on the table top at room temperature until analysis.

4.3. Calibration Curve

The 1,000 ng/mL ketamine calibrator standard was used to create the calibration curve. Table 1 depicts the stock concentration, spiking volume, working concentration, and working volume used. The working concentrations were made using the specific spiking volume of the 1000 ng/mL ketamine calibrator and diluting with HEB. This resulted in the 4 working concentrations of 0 pg/mg (negative control), 250 pg/mg (low control), 500 pg/mg (calibrator cutoff), and 1000 pg/mg (high control). The working concentrations were each run-in duplicate.

Table 1. Hair Calibrator Spiking Chart

Hair				
	Stock Concentration (ng/mL)	Spiking Volume (uL)	Working Concentration (pg/mg)	Working Volume (mL)
Negative	--	--	0	--
1	1,000	50	250	10
2	1,000	100	500	10
3	1,000	200	1,000	10

4.4. Drug Extraction from Hair

10 mg of washed negative hair was aliquoted into separate test tubes and filled with 550 uL of the respective working concentration. The samples were incubated for 2 hours at 75 °C. Once cooled to room temperature, 50 uL of neutralization buffer was added and the tubes were vortexed. The samples were then centrifuged for 5 minutes. 200 uL of the supernatant was removed from each test tube and placed inside new test tubes. Then, 400 uL of BSA was added and the tubes were vortexed.

4.5. ELISA Plate

The supernatant BSA dilution mixture for the 0 pg/mg, 250 pg/mg, 500 pg/mg, and 1000 pg/mg working concentrations was then used for plating onto the ELISA 96 well Micro-plate. 25 uL of sample was added to each well, along with 25 uL of ketamine-enzyme conjugate. The plate was then swirled to ensure proper mixing. The plate was then incubated at room temperature in the dark for 1 hour. After incubation, the wells were washed with the Columbus Plus Microplate Washer in order to remove any unbound ketamine or ketamine-enzyme conjugate. The wells were washed with 350 uL of distilled water for 6 cycles. The plate was then turned over and smacked dry onto a paper towel to ensure all water was removed from the bottom of the wells. 100 uL of the TMB substrate was then added to each well. After this addition, a second incubation took place at room temperature in the dark for 30 minutes. After the 30 minutes was up, 100 uL of the stop reagent was added to each well. The plate was then read at a wavelength of 450 nm (620 nm was used as the reference wavelength) using a spectrophotometer, the Tecan Sunrise Microplate Reader, in order to determine the absorbance of each well.

4.6. Method Validation

Intraday Study

To evaluate precision, negative controls, low quality controls (250 pg/mg), calibrators (500 pg/mg), and high quality controls (1,000 pg/mg) (n =12) were run and analyzed on the same day. The % coefficient of variation (CV), also known as relative standard deviation (RSD), and % error was then calculated from the results of this run.

Interday Study

Negative controls (n =10), low quality controls (250 pg/mg), calibrators (500 pg/mg), and high quality controls (1,000 pg/mg) (n = 12) were run each day over the course of 5 days to calculate precision. The %CV and % error was then calculated from the results of the runs from each day.

Linearity and LOD Determination

Linearity and limit of detection (LOD) was determined through the results of the full spiked calibration curve with the hair samples. The 5 quality control concentrations that were used to create the curve, not including the negative control, were 125 pg/mg, 250 pg/mg, 500 pg/mg (calibrator), 750 pg/mg, and 1,000 pg/mg.

Interference

In order to make sure other compounds weren't interfering with ketamine, 21 drug standards were spiked with ketamine. Table 2 shows the list of drugs that were used in this interference study. The 21 drug standards were all placed in a 5 mL volumetric flask, according to their respective spiking volume shown in Table 2. The volumetric flask was then dried off in a concentrator using methanol. Once the volumetric flask was dried off, it was diluted with HEB. The 5 mL was then split into two test tubes, each containing 2.5 mL. One of the test tubes was then spiked with 25 uL of the 1,000 ng/mL ketamine calibrator standard. Then, 10 mg of negative washed hair was aliquoted to 4 separate test tubes. 550 uL of the HEB with the 21 drugs (negative) was added to 2 of the 4 test tubes. The other 2 test tubes received 550 uL of the HEB with the 21 drugs spiked with ketamine. The 4 test tubes were then incubated for 2 hours at 75 °C. Once cooled to room temperature, 50 uL of neutralization buffer was added and the tubes were vortexed. The samples were then centrifuged for 5 minutes. 200 uL of the

supernatant was removed from each test tube and placed inside new test tubes. Then, 400 uL of BSA was added and the tubes were vortexed. The ELISA testing was then completed following the same steps as previously stated. Once determined that compounds were interfering with ketamine, the 21 drugs were split into 4 groups and the same steps were followed above to figure out which drug or drugs were causing this interference.

Table 2. Interference Cocktail List

Drug Name	Stock Concentration (ng/mL)	Spiking Volume (uL)	Working Concentration (pg/mg)	Working Volume (mL)
Amphetamine (d-methamphetamine)	10,000	5	1,000	5
Cannabinoids	1,000	50	1,000	5
Barbiturates	1,000	50	1,000	5
Carisoprodol	10,000	5	1,000	5
Cocaine (Benzoylecgonine)	1,000	50	1,000	5
Cotinine	100,000	0.5	1,000	5
Ethyl Glucuronide (ETG)	10,000	5	1,000	5
Fentanyl	10,000	5	1,000	5
Gabapentin	10,000	5	1,000	5
Methadone	1,000	50	1,000	5
Meperidine	100,000	0.5	1,000	5
Opiates (Morphine)	1,000	50	1,000	5
Oxycodone	1,000	50	1,000	5
Phencyclidine (PCP)	1,000	50	1,000	5
Propoxyphene (PPX)	1,000	50	1,000	5
Tricyclic antidepressants (TCA)-(Nortriptyline)	100,000	0.5	1,000	5
Buprenorphine	10,000	5	1,000	5
Pentatonic Acid (Spice)	10,000	5	1,000	5
Tramadol	100,000	0.5	1,000	5
6-AM (6-acetylmorphine)	10,000	5	1,000	5
Benzodiazepine (Oxazepam)	10,000	5	1,000	5

True Positive-Negative

The hair from the University of Pennsylvania matrix correlation study was used for the true positive-negative study. 7 hair samples positive for ketamine and 5 hair samples negative for

ketamine were selected. 10 mg of each hair sample was aliquoted into their respective test tubes. All 12 hair samples were washed following the steps in the "4.2. *Sample Decontamination*" section above. 550 uL of HEB was then added to each tube and incubation took place for 2 hours at 75 °C. Once cooled to room temperature, 50 uL of neutralization buffer was added and the tubes were vortexed. The samples were then centrifuged for 5 minutes. 200 uL of the supernatant was removed from each test tube and placed inside new test tubes. Then, 400 uL of BSA was added and the tubes were vortexed. The ELISA testing was then completed following the same steps as previously stated.

Carry Over

In order to determine if there was any splashing between wells on the ELISA plate or if the plate washer was working properly, the carry over study was performed. 10 mg of negative washed hair was aliquoted into 2 test tubes. 550 uL of the 1,000 pg/mg and 550 uL of the 10,000 pg/mg quality controls were added to their respective test tubes. The samples were incubated for 2 hours at 75 °C. Once cooled to room temperature, 50 uL of neutralization buffer was added and the tubes were vortexed. The samples were then centrifuged for 5 minutes. 200 uL of the supernatant was removed from each test tube and placed inside new test tubes. Then, 400 uL of BSA was added and the tubes were vortexed. The ELISA testing was then completed following the same steps as previously stated. Surrounding both the 1,000 pg/mg and 10,000 pg/mg samples were 8 wells filled with unspiked/blank HEB. If there was no splashing between wells, the blank wells would read at a similar absorbance level to the negative control.

5. Ethics

Human hair samples were required for method development. Because human subjects were involved in this research process, informed consent and relevant information regarding the

research was required from the subjects. The hair samples were retrieved from a University of Pennsylvania matrix correlation study. All samples were de-identified and lacked any protected health information (PHI) in accordance with the Health Insurance Portability and Accountability Act (HIPAA) privacy rule. The research being conducted was performed ethically, used for scientific purposes, protected the interest of all individuals involved, and followed federal, professional, and institutional guidelines and regulations.

6. Results

Linearity and LOD Determination

Validation of this developed ELISA assay was tested using negative washed hair samples spiked over a concentration range from 0 to 1000 pg/mg, run in triplicate. The calibration curve (Figure 1) was prepared and the R^2 value was calculated to determine linearity and LOD. The calibration curve was plotted as the Ln of the concentration vs. LOGITB/B_0, where the data can be seen in Table 3. As seen in Figure 1, the curve is linear and has an R^2 value of 0.9991.

Figure 1. Calibration Curve

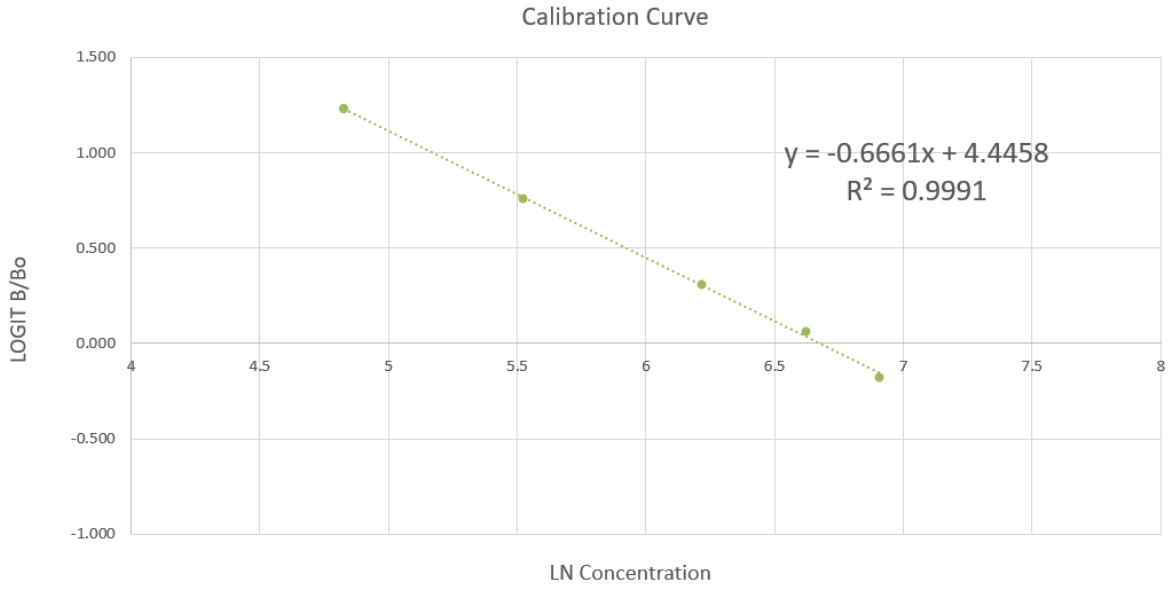

Table 3. Calibration Curve Values

Concentration (pg/mg)	Ln	Mean Absorbance	B/B_0	LOGIT
0	--	1.578	--	--
125	4.828314	1.221	0.774	1.230
250	5.521461	1.076	0.682	0.762
500	6.214608	0.910	0.576	0.307
750	6.620073	0.813	0.515	0.061
1,000	6.907755	0.720	0.456	- 0.176

The LOD was determined through the following equation:

$$LOD = A_0 – (3 \times SDneg)$$

A_0 is the mean absorbance value for the 3 negative samples run, which was 1.578, and SD is the standard deviation of those 3 absorbance values, which was calculated to be 0.039347. After solving this equation, the absorbance or OD value for the LOD was 1.460. Using the B/B_0 and LOGIT functions, the calculated concentration from this absorbance value was determined to be 18.1 pg/mg. This is the calculated LOD, which is the smallest amount of drug that can be theoretically detected by this method.

Intraday and Interday Study

The intraday (n =12) and interday (n = 12) precision and accuracy results for ketamine at the quality control concentrations of 250 pg/mg, 500 pg/mg, and 1,000 pg/mg are seen in Table 4. Figure 2 and Table 5 represent the curve and respective values from the intraday study. Figure 3 and Table 6 represent the curve and average values over the 5 days from the interday study. The intraday precision and accuracy of the 250 pg/mg, 500 pg/mg, and 1,000 pg/mg quality controls were approximately 8% and -3%, 7% and -1%, and 4% and 5%, respectively. The interday precision and accuracy of the 250 pg/mg, 500 pg/mg, and 1,000 pg/mg quality controls were approximately 9% and -0.2%, 10% and 4%, and 10% and 5%, respectively. Both the accuracy and precision data were within the acceptable limits of +/- 20% error and 10% CV.

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

Statistical Variable	Concentration (pg/mg)		
	250	**500**	**1,000**
Intraday (n =12) *			
Mean	0.6900	0.5820	0.3941
% CV (precision)	8.42	6.83	4.20
% Error (accuracy)	-3.16	-1.10	4.54
Interday (n =12) **			
Mean	0.6962	0.5887	0.4269
% CV (precision)	8.74	10.17	9.68
% Error (accuracy)	- 0.168	3.502	5.226

*Intraday results are from day 1 of the interday study

**Interday results are the average of the 12 samples over 5 days

% CV= (SD / [mean]) *100

% Error = ([calculated mean] – [expected])/ ([expected]) *100

Figure 2. Intraday Curve with Equation and R^2 Value

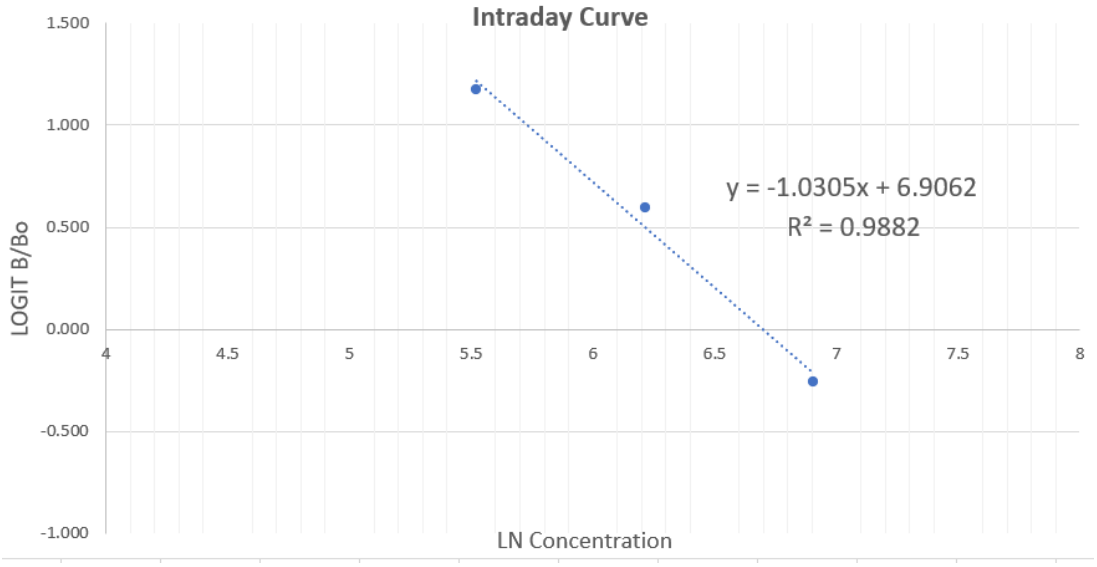

Table 5. Intraday Curve Values

Concentration (pg/mg)	Ln	Mean Absorbance	B/B_0	LOGIT
0	--	0.904	--	--
250	5.521461	0.690	0.763	1.171
500	6.214608	0.582	0.644	0.592
1,000	6.907755	0.394	0.436	- 0.257

Figure 3. Interday Curve with Equation and R^2 Value

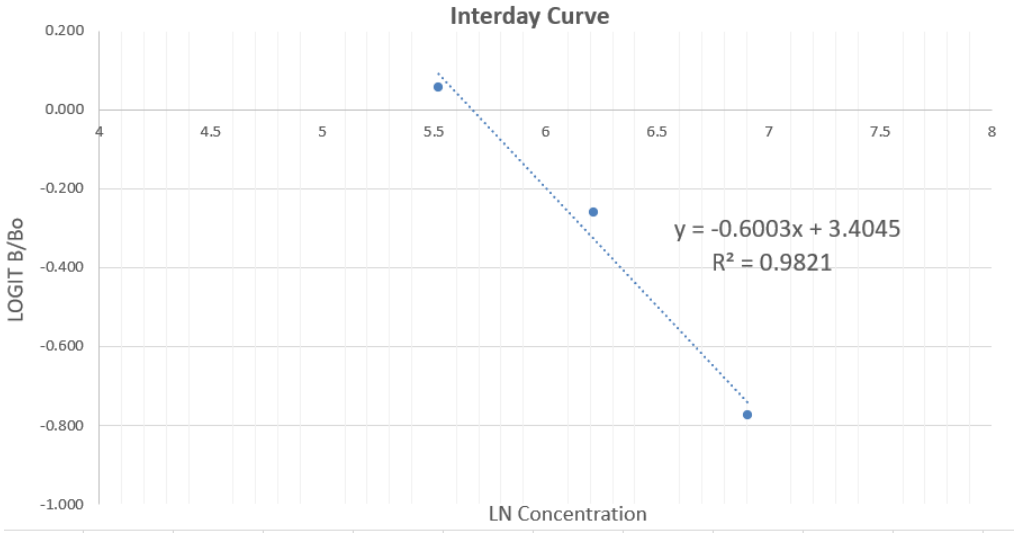

Table 6. Interday Curve Values

Concentration (pg/mg)	Ln	Mean Absorbance	B/B_0	LOGIT
0	--	1.353	--	--
250	5.521461	0.696	0.514	0.058
500	6.214608	0.589	0.435	- 0.261
1,000	6.907755	0.427	0.316	- 0.775

Interference

After testing all 21 drugs in Table 2, the absorbance of the interference cocktail was reading at an average of 0.611. This was around the same absorbance level as the 250 pg/mg quality control, which meant interference did occur. After breaking up the 21 drugs into 4 groups, group 4 showed interference while groups 1 to 3's absorbance was similar to the negative control's absorbance of 1.806. Group 4 contained PCP, buprenorphine, PPX, pentatonic acid, tramadol,

and the benzodiazepine, oxazepam. These 6 drugs were then split up individually and retested. PCP, buprenorphine, PPX, pentatonic acid, tramadol, oxazepam, and the quality controls absorbance can be seen in Table 7. PCP was the only drug out of the 6 that showed interference, with an absorbance reading of 0.786, generating a response similar to a low positive control.

Table 7. Interference Absorbance Results for Quality Controls and Group 4 Drugs

Concentration (pg/mg)	**Mean Absorbance**
0	2.020
250	0.813
500	0.686
1,000	0.555
Group 4 Drugs	--
PCP	**0.786**
Buprenorphine	1.873
PPX	1.753
Pentatonic Acid (Spice)	1.9105
Tramadol	1.836
Benzodiazepine (Oxazepam)	1.912

True Positive-Negative

Of the 7 hair samples selected that were deemed positive for ketamine by LC-MSMS from the University of Pennsylvania matrix correlation study, 4 screened positive and 3 screened negative for ketamine using the developed ELISA method. The 4 positive samples had an absorbance lower than the 500 pg/mg calibrator's absorbance of 0.610, which means they were all positive for ketamine. Sample #35 and #76 were determined to be negative for ketamine by the LC-MSMS in oral fluid, urine, and blood but positive for ketamine in hair. After screening these 2 samples with the ELISA method, both their absorbances were 0.700 and 0.980, respectively. These values were slightly higher than that of the calibrator's and would be interpreted as past ketamine use. Sample #49 was determined to be positive for ketamine in hair by the LC-MSMS. However, after screening this hair sample with the ELISA method, it came

back negative for ketamine with an absorbance of 1.635. This result could have occurred due to a sampling issue. The 5 hair samples selected that were negative for ketamine through the LC-MSMS, all came back as negative using the developed ELISA method. Their absorbance values were all higher than the calibrator's absorbance. These absorbance results, along with the paired matrices ketamine was detected in by the LC-MSMS, are summarized in Table 8.

Table 8. True Positive-Negative Absorbance Results for University of Pennsylvania Samples and Quality Control Samples

Sample #	Mean Absorbance	Matrices and LC-MSMS Results
Positive Samples		
116	**0.115**	Positive in OF/H, Negative in B, No U submitted
137	**0.249**	Positive in OF/H and Negative in B/U
217	**0.131**	Positive in OF/H/B/U
272	**0.350**	Positive in OF/H and Negative in U/B
35	**0.700**	Positive in H and Negative in OF/B/U
76	**0.980**	Positive in H and Negative in OF/B/U
49	**1.635**	Positive in H and Negative in OF/B/U
Negative Samples		
69	**1.785**	Negative in OF/H/B/U
96	**1.691**	Negative in OF/H/B/U
138	**1.550**	Negative in OF/H/B/U
141	**2.067**	Negative in OF/H/B/U
142	**1.999**	Negative in OF/H/B/U
Quality Control Concentration (pg/mg)		
0	1.801	--
250	0.708	--
500	**0.610**	--
1,000	0.506	--

OF = oral fluid, H = hair, B = blood, and U = urine

Green = positive ketamine samples, Yellow = past use, Red = negative ketamine samples

Carry Over

There was no carry over seen between the blank wells and the 1,000 pg/mg and 10,000 pg/mg quality control samples. The 1,000 pg/mg control had an absorbance of 0.464 and the 10,000 pg/mg control had an absorbance of 0.242. The 16 surrounding blank wells, which had unspiked HEB, all had an absorbance similar to the negative control's average absorbance of 1.612. This verifies that there was no splashing between the wells during the washing step or from manually pipetting samples. These results can be seen in Table 9A and 9B.

Table 9A. ELISA Plate Carry Over Study Diagram

Plate	1	2	3	4
A	250 pg/mg	Blank	Blank	Blank
B	250 pg/mg	Blank	**1,000 pg/mg**	Blank
C	500 pg/mg	Blank	Blank	Blank
D	500 pg/mg	Blank	Blank	Blank
E	1,000 pg/mg	Blank	**10,000 pg/mg**	Blank
F	1,000 pg/mg	Blank	Blank	Blank
G	Negative	--	--	--
H	Negative	--	--	--

Green = quality control samples

Table 9B. Absorbance Results from Carry Over Study

Plate	1	2	3	4
A	0.614	1.504	1.640	1.559
B	0.623	1.545	**0.464**	1.567
C	0.493	1.666	1.658	1.406
D	0.511	1.664	1.713	1.586
E	0.452	1.630	**0.242**	1.675
F	0.448	1.602	1.697	1.719
G	1.682	--	--	--
H	1.541	--	--	--

Green = quality control sample's corresponding absorbance

7. Discussion

The ELISA method developed to detect ketamine in hair samples has been efficiently validated, which can be seen from the results above. Linearity is described as an analytical procedure's ability, within a certain range, to obtain results that are directly proportional to the concentration of analyte in the sample (Minic & Zivkovic, 2021). The analytical response of this method was proven to be linear over the range of 0-1,000 pg/mg because the multi-point calibration curve depicted an R^2 value greater than the acceptable value of 0.985. The calibration curve was plotted as the Ln of the concentration of the negative, calibrator, and quality controls vs. LOGITB/B_0. B/B_0 is a ratio of the ELISA absorbance values, where B is the absorbance value of the bound calibrator and B_0 is the absorbance value of the blank calibrator (Harun et al., 2009). The higher the ketamine concentration, the lower the B/B_0 due to the lower quantity of enzyme conjugate that binds to the antibodies on the plate compared to the analyte (Harun et al., 2009). These values were then converted into log-scales through the LOGIT function, and the indirect relationship between concentration and absorbance was seen. Linearity also determines the sensitivity of a method through the LOD. The LOD was determined to be 18.1 pg/mg, which is the lowest concentration of ketamine that this ELISA method can detect. Anything below 18.1 pg/mg, this method would theoretically not be sensitive enough to identify.

Accuracy of an analytical method describes the closeness of the values determined by the method compared to the nominal concentration (Minic & Zivkovic, 2021). The accuracy of this method was determined by the percent error of the quality control samples spiked with ketamine (experimental value) compared to the actual value. Precision of an analytical method takes into account repeatability and reproducibility. Repeatability often refers to intraday precision and looks to see if the repeated measurement of the same sample on a single plate on the same day is giving

comparable results. Reproducibility often refers to interday precision and looks at whether the obtained data over the course of different days is reliable and giving comparable results. Precision describes the closeness of measurements of the same sample taking into consideration the deviation from the mean value, also known as relative standard deviation (Minic & Zivkovic, 2021). When the relative standard deviation is expressed as a percentage it is called the coefficient of variation. The intraday and interday precision and accuracy results for this ELISA method at the quality control concentrations of 250 pg/mg, 500 pg/mg, and 1,000 pg/mg were within the accepted percentage range of +/- 20% error and 10% CV. Therefore, demonstrating this ELISA method to detect ketamine in hair samples is both accurate and precise.

In the interference study, PCP, with an absorbance reading of 0.786, was the only drug out of the 21 drugs tested that demonstrated interference. PCP generated a response similar to a low positive control, which means that a sample containing PCP will be detectable in this assay. From a practical perspective, if someone was using PCP, they may screen positive for ketamine using this ELISA method. However, because an ELISA is a presumptive screen, confirmation with instrumental analysis, like LC-MSMS, would show that this said person is negative for ketamine.

The hair samples that were used for the true positive-negative study came from a matrix correlation study completed by the University of Pennsylvania. For a true positive-negative study, about 15 positive hair samples would be ideal for testing purposes. However, of the 327 samples collected from this study, only 7 hair samples positive for ketamine were available for my use. Of the 7 hair samples confirmed positive for ketamine by LC-MSMS, only 4 screened positive with this ELISA method. Sample #'s 116, 137, 217, and 272 all had an absorbance reading lower than the 500 pg/mg cutoff calibrator's absorbance of 0.610. This lower absorbance indicates a positive result for ketamine, due to the ELISA's indirect relationship between absorbance and

concentration. Sample #'s 116, 137, and 272 were confirmed positive for ketamine by the University of Pennsylvania in both hair and oral fluid. Sample #217 was confirmed positive for ketamine in oral fluid, hair, blood, and urine. Sample #49 was confirmed positive for ketamine in hair and negative in oral fluid, blood, and urine. However, after testing this sample with the developed ELISA method, it was deemed negative, with an absorbance of 1.635. This would not be considered a false positive because the University of Pennsylvania matrix correlation study was a qualitative confirmational study. Possible reasons that this sample screened negative with the ELISA method could be from a sampling issue. The donor could have been an infrequent ketamine user and the remaining hair sample that I used for testing was non-homogenous. If so, the portion I aliquoted for testing could have been drug free, where no ketamine was contained. Sample #'s 35 and 76 in the prior study were confirmed positive for ketamine in hair but negative for ketamine in oral fluid, blood, and urine. After using this ELISA method, sample #'s 35 and 76 had an absorbance of 0.700 and 0.980, respectively. These absorbance values would be interpreted as past ketamine use because in the traditional matrices of urine and blood, ketamine was not detected. Therefore, at least 7-10 days had passed since the last dose was taken.

A carry over effect occurs when a certain step in a procedure affects or influences a subject's response toward a secondary step in a procedure (O'Connor et al., 2014). For ELISA testing that could mean that something the analyst or automated instrument is doing is affecting the results of the experiment. In order to make sure that I, the analyst, wasn't contaminating the wells during the pipetting steps or the automated plate washer wasn't contaminating the wells during the washing step, this study was performed. The absorbance readings of the wells containing HEB not spiked with ketamine all showed a suspected negative absorbance value which was compared to the negative control's absorbance value of 1.612. This meant that there was no splashing between

wells during the washing step or contamination during manual pipetting steps. If carry over did occur, the 1,000 and 10,000 pg/mg quality controls would have had an effect on the absorbance of the unspiked HEB, showing absorbance values similar to the absorbance values of those 2 quality controls.

8. Future Research

For future research, I would redo the true positive-negative study with more samples positive for ketamine. This would help better correlate the results of the ELISA screen with LC-MSMS confirmation results. I would also perform a cross-reactivity study on some of the new emerging designer dissociated drugs of abuse. It would be interesting to see if this Ketamine Direct ELISA kit from Immunalysis could identify them. Examples of these designer dissociated drugs include methoxetamine, hydroxetamine, and several PCP analogs.

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