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A Validation of the Detection of Barbiturates in Hair by ELISA

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ABSTRACT:

An original method to extract and detect barbiturates, specifically secobarbital, in hair by enzyme-linked immunosorbent assay (ELISA), has been developed. Drug-free hair specimens were fortified with an exaggerated amount of secobarbital to produce a homogeneous, true positive sample to be utilized to test the drug extraction efficiency from hair. Drug extraction using hair extraction buffer (HEB) from Immunalysis was compared to a base digestion using 1M NaOH. After LCMS/MS analysis, HEB extraction was deemed a successful method for extracting barbiturates from hair. Using this method, the Barbiturates Direct ELISA Kit from Immunalysis was evaluated for its potential use in the detection of barbiturates in hair specimens. After thorough analysis, it appears the kit is more than suitable as a screening assay due to its sensitivity and reproducibility.

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I. INTRODUCTION:

1. Barbiturates Background

The worldwide rise in prescription drug misuse has not gone unnoticed. Over the last 15 years, misuse has been reflected in increased emergency room visits, treatments for addiction, and deaths due to overdose (NIDA, 2016). Overdose deaths alone have increased from almost 10,000 in 2001 to more than twice that amount at 25,000 in 2014 (NIDA, 2016). There are three main culprits of misused prescription drugs, one of which are central nervous system depressants. This category of drugs includes substances that can slow brain activity (i.e. tranquilizers, sedatives, and hypnotics). An example of which are barbiturates. In 2011, the Substance Abuse and Mental Health Services Administration (SAMHSA) reported that approximately 18,000 emergency calls involving the use of non-prescribed barbiturates took place in the United States (SAMHSA, 2013). Additionally, a study done in the UK from 1983-1999 found that for every million barbiturate prescriptions, 146.2 lead to fatal toxicities (Buckley, & McManus, 2004). Because of its higher risk in overdose fatalities, barbiturates have mostly been replaced by benzodiazepines, but they are still prescribed today.

Barbiturates were introduced into medicine in 1903 after the discovery of its sedative-hypnotic action; dominating the market for the first half of the 20th century. Today, there are numerous variants available, all of which are derivatives of barbituric acid – typically differing at the C5 position in structure. These modifications affect the compound's lipid solubility and, thus, duration of activity (Levine, 2013). For example, secobarbital contains an allyl and 1-methylbutyl group at the C5 position which makes it more lipid soluble and potent but, also, short acting. As stated above, this drug class is a

central nervous system (CNS) depressant that has been primarily prescribed as sedative hypnotics, anticonvulsants, and for migraine therapy (Levine, 2013). Barbiturates are most commonly administered orally, but have been known to be given intravenously or intramuscularly. They are rapidly absorbed and distribution occurs throughout the major tissues of the body (Silberstein, & McCrory, 2001). Depending on the duration of action, barbiturates are either almost completely metabolized (short-acting) by the liver or remain mostly unchanged (long-acting) (Silberstein, & McCrory, 2001). They preferentially suppress polysynaptic neuronal responses primarily by binding to a chloride ion channel, γ-aminobutyric acid (GABA) A receptor. This, in turn, activates the receptor, prolonging the opening of the channel and allowing for an influx of chloride ions (Silberstein, & McCrory, 2001). Although benzodiazepines also interact with the $GABA_A$ receptor, they potentiate different reactions. Barbiturates cause the receptor to remain open for longer, whereas, benzodiazepines increase the affinity of GABA for the receptor (Bianchi, Botzolakis, Lagrange, & Macdonanld, 2009). Because of this, the therapeutic index for barbiturates is low – increasing the likelihood of intoxication and overdose. Intoxication symptoms include: sluggishness, difficulty thinking, poor memory, slowed speech and comprehension, impaired judgement, and decreased attention span (Silberstein, & McCrory, 2001). Although barbiturates can produce tolerance, and result in dependence and addiction, some are still administered today and should be closely monitored. Below are three examples of barbiturates, ranging in different durations of action.

1.1 Secobarbital

Secobarbital is a short-acting barbiturate with a half-life of 22-29 hours (Levine, 2013). It is typically used to treat insomnia, or as a sedative before surgery.

1.2 Butalbital

Butalbital is an intermediate-acting derivative of barbituric acid. Commonly compounded with aspirin, acetaminophen, and/or caffeine, they are widely used for the treatment of migraines and tension-type headaches (Silberstein, & McCrory, 2001). 36% of patients taking prescription medicine for their headaches, take butalbital-containing analgesic combinations (Ferrari, Tifaferri, Palazzoli, Verri, Vandelli, Marchesi, Ciccarese, & Licata, 2015).

1.3 Phenobarbital

Phenobarbital is a long-acting barbiturate with limited metabolism and an elimination half-life of 2-5 days. It is one of the more commonly prescribed barbiturates today for its use in the treatment of epileptic seizures (Levine, Roveri, Paranhos, & Yonamine, 2016).

2. Hair Matrix

In recent years, analysis of the hair matrix has gained increasing importance in the determination of substances of abuse. With hair having the longest window of drug detection, as compared to the other more classic biological specimens (urine, oral fluid, and serum, in that order respectively), it has gained popularity of use in forensic cases and clinical toxicology. Typically, drug detection in hair is from weeks to months, where as in urine it is from hours to days and in blood, minutes to hours (Levine, 2013). This

allows for diagnostic information regarding the identity and concentration of drug(s) to be obtained over an extended period of time. Other advantages to using hair as a matrix include: its non-invasive collection; and its long and easy storage. With barbiturate analysis having been previously demonstrated in urine, serum, and oral fluid, this study established it for hair analysis for use in patient drug monitoring and/or forensic case work.

Hair is a non-homogeneous fiber; it is solid and durable. It consists of keratinized cells that form three concentric structures –the cuticle, cortex, and medulla (Figure 1) (Pragst, & Balikova, 2006).

Figure 1.

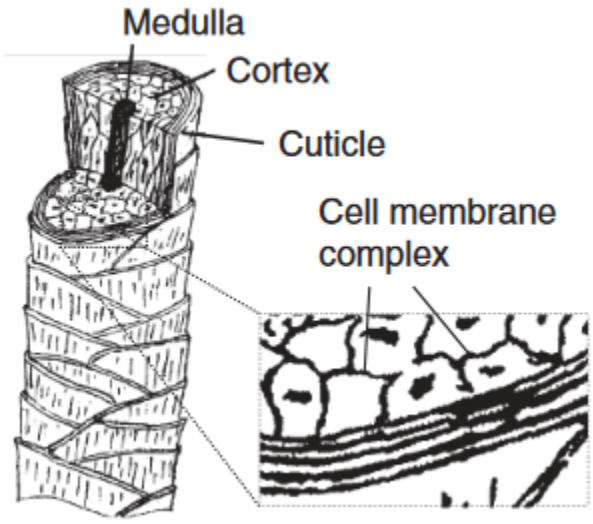


Figure 1. Structure and constituents of the human hair shaft.

Each fiber originates from the hair follicle, which resides 3-5 mm below the scalp-dermal layer and is nourished by a network of capillaries (Pragst, & Balikova, 2006). The cuticle consists of a thin layer of cells that overlap in a shingle-like fashion and make up the outside of the hair shaft (Levine, 2013). Beneath the cuticle contains the cortex which

consists of tightly packed, keratin-filled, microfibrils that make up the cell membrane complex (Levine, 2013). This part of the hair is the primary diffusion point for incorporation and diffusion of drugs, with lipophilic drugs being preferentially deposited (Pragst, & Balikova, 2006). Lastly, the most inner part of the hair structure is the medulla. Here is where ingested drugs are primarily deposited (Levine, 2013). The life cycle of human hair consists of three main phases – the anagen phase, catagen phase, and telogen phase (Levine, 2013). The anagen phase is also known as the growth phase. During this phase, the hair grows at a rate of about 0.44mm per day (0.38-0.48mm) for men and 0.45mm per day (0.4-0.55mm) for women (Nakahara, 1999). At this rate, drugs within the hair emerge above the surface about 5-7 days after ingestion (Levine, 2013). The catagen phase, or transition phase, is when the root end of the hair becomes keratinized and begins to separate from the bulb (Levine, 2013). After about 4-6weeks, the hair enters the telogen phase, or resting phase, in which the hair shaft stops growing completely and can be easily pulled out (Nakahara, 1999).

There are several mechanisms in which drugs can be incorporated into hair. One of which is through passive diffusion from the blood capillaries into the matrix cells of the growing hair (Levine, 2013). Another, is via sweat or sebum secretions (Pragst, & Balikova, 2006). In addition, substances such as smoke and powders can be deposited from the external environment, making the hair washing step prior to extraction critical (Nakahara, 1999). Experimental data has also suggested that delayed incorporation can occur from deep skin compartments during hair shaft formation (Pragst, & Balikova, 2006).

The mechanism that will predominate is strongly influenced by the structural and chemical properties of the drug, as well as, the physical and physiological characteristics of the individual. An example of this is an individual's hair pigmentation. It has been shown that pigmented hair had about a 10-fold higher concentration of basic drugs than that of non-pigmented hair, despite having the same drug concentration in blood (Rothe, Pragst, Thor, & Hunger, 1997). Additionally, it has been demonstrated that melanin, the compound responsible for hair pigmentation, has a stronger affinity for basic drugs (Claffey, Stout, & Ruth, 2001).

For analysis, a sample of hair is taken from the back of the donor's scalp in a cosmetically consealable area. This lock is about 1 inch wide by 1-2 strands deep and cut close to the scalp (Levine, 2013). It is wrapped in foil with the cut, root-end protruding and stored in an envelope in a cool dry place for as long as 5 years. Generally, approximately 4cm (or 1½ inches) of collected hair is cut from the root end for analysis. This length of hair reflects approximately 90 days (Levine, 2013). The sample of hair is then typically either cut into smaller segments, or pulverized in a ball mill, allowing for greater surface area to optimize the extraction process. Prior to the extraction process, the hair is weighed into aliquots (typically ranging from 10-50mg, depending on the assay) and then washed. Because hair is exposed to the outside environment, it may become contaminated with drugs via vapor or direct contact and, thus, an efficient wash procedure is necessary. The challenge in any wash procedure is to remove as much drug present on the surface of the hair while keeping the internalized drug within the hair for analysis. There are many wash techniques implemented today, but in general, it involves an incubation with an organic solvent (such as dichloromethane, isopropanol, or

methanol), followed by an additional one or two washes with an aqueous solvent (such as sodium phosphate buffer) (Levine, 2013). The use of an organic solvent removes any external contaminants residing on the exterior of the hair, while the aqueous solution causes the hair to swell – both allowing for the removal of any drugs that may have diffused from the exterior to the cortex of the hair. Similar to the wash procedure, there is no designated method for the extraction of drugs from hair. Typical procedures incubate the hair in an organic solvent (most commonly methanol) for extended periods of time, ranging from 5-18 hours (Pragst, & Balikova, 2006). However, studies have shown that basic drugs are well extracted using either aqueous acids or phosphate buffer (Moeller, Fey, & Wennig, 1993; Kintz, & Mangin, 1995). Whereas, compounds that are stable under alkaline conditions, typically extract well using a 1M NaOH digestion for much shorter periods of time. In this study, a commercially available hair extraction buffer (HEB) was utilized. To determine that the HEB was the optimal extraction technique for barbiturates, it was tested against 1M NaOH dissolution using in-house prepared fortified hair samples. Although some laboratories use authentic hair, fortified hair ensures homogeneity and has been used in proficiency testing programs since 1990 (Lee, Park, Han, Choe, Lim, & Chung, 2008). Because it has been shown that DMSO is a useful penetrating carrier for absorption enhancement of the compound of interest into the hair, a 50% solution of DMSO ± 0.02M HCl was utilized in the fortification process (Welch, Sniegoski, Allgood, & Habram, 1993; Welch, Sniegoski, & Tai, 2003; Lee et al., 2008).

3. Screening

The first hair analyses for drugs were performed by radioimmunoassay (RIA) (Pragst, & Balikova, 2006). Today there are more options readily available –like the enzyme-linked immunosorbent assay (ELISA), for example. The ELISA is an excellent screening technique for hair drug testing due to its sensitivity and ability to detect low levels. However, these assays are not typically validated for use in hair and thus must be before use. The principle design behind an enzyme immuoassay is the antigen-antibody interaction. A host organism is injected with a drug/compound bound to a carrier protein to induce an immune response (Levine, 2013). As a result, antibodies against the target analyte are produced and subsequently collected for use in commercial screening kits. Because the antibodies produced have different epitopes towards the target, cross-reactivity between similarly structured compounds occurs. The antibodies are then fixed to a 96-well plate, where sample and enzyme-conjugated target drug compete for antibody binding sites (Levine, 2013). The enzyme conjugate is typically horseradish peroxidase (HRP). Following the addition of a substrate, 3,3',5,5'-tetramethylbenzidine (TMB), any bound HRP-conjugated target drug will oxidize the chromogenic substrate into a blue colored product. A Stop Solution containing a dilute HCl solution is then added to quench the reaction and, ultimately, changing the color reaction from blue to yellow. In the event that a sample is positive, very little HRP-conjugated drug will bind causing a slight color change. Whereas, a negative sample will produce a greater color change. The absorbance is measured spectrophotometrically. In this study, the Barbiturate Direct ELISA Kit from Immunalysis was validated for the presumptive screening of barbiturates in hair. The target analyte is secobarbital, but the ELISA has crossreactivity capabilities.

4. LCMS/MS

A liquid chromatography tandem mass spectrometry system (LCMS/MS) is an instrument that allows for the separation, detection, and identification of compounds (Skoog, Holler, & Crouch, 2007). Liquid chromatography separates the sample components while bringing them to the mass spectrometer, where the compounds are ionized, detected, and identified (Skoog et al., 2007). The use of a calibration curve made up of known concentrations of the target analyte allows for its quantification. A deuterated form of the analyte is used to control for extraction, and LCMS/MS injection and ionization variability.

II. MATERIALS:

1. Chemicals and Reagents:

Secobarbital and secobarbital-d5 were purchased from Cerilliant (Round Rock, TX). The Barbiturates Direct ELISA kit, hair extraction buffer (HEB), bovine serum albumin (BSA), and neutralizing buffer (NEB) were obtained from Immunalysis Corporation (Pomona, CA). All HPLC grade solvents and ACS grade chemicals were purchased from VWR (Radnor, PA). All consumables were purchased from VWR (Radnor, PA) unless otherwise noted. Trace-B mixed-mode solid phase extraction (SPE) columns, autosampler vials, and autosampler snap caps were purchased from SPEware Corporation (Baldwin Park, CA).

2. Apparatus:

The Tecan-fre (used to pipette all calibrators, controls, and samples into the microplate wells), the Columbus Plus plate washer, and the Sunrise® Basic Tecan plate reader were all purchased from Tecan (San Jose, CA). Magellan software was used to analyze the OD readings. The CEREX ALD III 48 used to dispense solvents and solutions during the solid phase extraction, was purchased from SPEware (Baldwin Park, CA). All confirmational analyses were performed using a liquid chromatography/tandem mass spectroscopy system (LCMS/MS). The LC system used was an Agilent 1290 Infinity Series, coupled to an Agilent 6460 triple quadropole tandem mass analyzer equipped with a jet stream electrospray ionization source. The analytical columns used were Zorbax® Eclipse Plus C18 columns with a 2.1 mm diameter, 50 mm length, and 1.8

μm particle size (Agilent Technologies, Santa Clara, CA). The mobile phases used were 0.2% acetic acid and acetonitrile.

3. Samples:

Drug free hair was voluntarily donated from the author and stored in a sealed bag at room temperature. Prior to use in the validation process, the hair was verified to be negative.

III. METHODS:

1. Sample Preparation:

The hair was cut into 1-2 cm sections and 10 mg aliquots were placed into 13x100mm glass tubes. Hair samples were then washed with dichloromethane, isopropyl alcohol, and methanol to remove external contamination. 1 mL of each solvent was added, vortexed for 5 minutes, decanted and evaporated with a gentle stream of nitrogen at 65°C.

2. Calibration Curve for Screen:

Eight aliquots of 10 mg of washed negative hair was prepared. To this, different concentrations of secobarbital was added. Concentrations of 0 pg/mg (negative control), 250 pg/mg (low positive control), 500 pg/mg (calibrator cutoff), and 1000 pg/mg (high positive control) of secobarbital were spiked in duplicate.

3. Drug Extraction from Hair:

Calibrators, quality controls, and unknown samples were subjected to 550 µL of HEB. The samples were then incubated at 75°C for 2 hours, followed by neutralization using 50 µL of NEB. After centrifugation at 4,200 rpm for 10 min, 200 µL of supernatant was transferred to another tube and diluted with 400 µL of BSA and vortexed.

4. ELISA:

The diluted extracts were added to the microtiter plate at 10 µL per sample. Subsequently, 100 µL of the enzyme conjugate was added and the plate was incubated at room temperature for one hour. Unbound antigen was removed by washing the wells with

350 µL of dH2O (6 cycles). After the plate was pat dry, 100 µL of TMB solution was added and allowed to incubate for 30 minutes at room temperature. Immediately after this, 100 µL of Stop Solution was added to the wells and the plate was read at 450 nm using a spectrophotometer (using 620 nm as a reference wavelength) and analyzed using the Magellan software.

5. Intraday Study:

Negative, low, calibrator, and high controls (n=8 per calibrator/control) were prepared and analyzed on a single day along side the calibration curve. Intraday study results were averaged and then compared to the results of the calibration curve to determine intraday precision and accuracy using the following equations:

$$\text{Relative Standard Deviation (RSD)} = SD/x_i * 100$$

$$\text{Mean Relative Error (MRE)} = ((x_i - x)/x) * 100$$

where SD is the standard deviation, x_i is the mean of the observed concentration, and x is the true concentration.

6. Interday Study:

Negative, low, calibrator, and high controls (n=5 per calibrator/control per day) were prepared and analyzed along side the calibration curve each day over the course of 8 days. The results of the samples were then compared to that of the calibration curve to determine interday precision, accuracy and assay reproducibility. Used same equations from intraday study.

7. Carryover Study:

An aliquot of washed negative hair was spiked with secobarbital at four times the concentration of the high control to prepare a carryover sample. The calibration curve and sample were analyzed and carryover was determined by comparing the results of the negative controls, which were aliquotted before and after the carryover sample.

8. Fortified Hair Preparation:

Negative hair was soaked in a high concentration of secobarbital for several days to mimic that of authentic, true positive samples. To this end, 1g of negative hair was cut, washed and then added to a bottle containing one of two solutions. One solution was made up of deionized water (dH2O) and dimethyl sulfoxide (DMSO) at a 1:1 ratio for a total of 17mL (Welch et al., 1993).The second solution contained a 1:1 mixture of dH_20 and 0.02M HCl in DMSO for a total of 17mL (Welch et al., 2003; Lee et al., 2008). Secobarbital was spiked into these solutions at 100 times the cutoff value of 500pg/mg. Everyday the solutions were sonicated for 1 hour and every 3 days a portion of the hair was removed for analysis. The amount of hair removed from the solution was estimated to be 30mg of hair so as to analyze the sample in triplicate. The aliquot of hair was washed using the same protocol as mentioned above except 3mL of each solvent was used. Additionally, the last wash was saved to be screened alongside the fortified hair sample. Once the sample was dried down at 65°C, it was weighed and three aliquots of 10mg of hair was prepared. To each, 550µL of HEB was added and incubated for 2 hours at 75°C. Following incubation, the samples were neutralized, centrifuged, and diluted according to the same procedure stated above. The previously saved wash was prepared

similarly after having been divided into three 1mL aliquots and evaporated to dryness using a gentle stream of nitrogen. 10µL of each sample (three aliquots of extracted hair and three aliquots of last wash per each condition) was added to the Barbiturate Direct ELISA Kit plate, along with a calibrator and QCs, and the same ELISA screening procedure was carried out as mentioned above. This process was repeated until the hair samples screened positive while the last wash screened negative.

9. Drug Extraction Efficiency Study:

Secobarbital-fortified hair was used to compare the extraction efficiency of an aqueous extraction (using HEB) to that of a base digestion (using 1M NaOH). Given that a hair digestion dissolves the hair completely, it represents 100% extraction efficiency. To this end, 10 mg of fortified hair was subjected to either an aqueous extraction using 1 mL of HEB and incubated for 2hr at 75°C or a base dissolution using 1 mL of 1M NaOH and incubated for 30min at 75°C, in triplicate. Secobarbital-d5 internal standard (100 µL) was also added at a concentration of 100 ng/mL. Following incubation, the samples were neutralized. The samples treated with the aqueous extraction were neutralized using 1 mL of 0.1M phosphate buffer (pH 6.0), whereas the samples digested with the base solution were neutralized using 300 µL of 20% acetic acid. Calibration curves were analyzed concurrently with each extraction procedure at concentrations of: 0, 25, 50, 100, 250, 500, 1000, 2000, 5000, and 10000 pg/mg secobarbital. Additionally, quality control samples contained secobarbital at 100, 500, 1000, and 5000 pg/mg. All samples were processed using SPE extraction before analysis on the LCMS/MS.

10. SPE Extraction:

The extracted samples were cleaned up by using mixed mode cation-exchange solid phase extraction (SPE). These columns were used to allow for the simultaneous extraction of basic drugs, and the weakly acidic barbiturates through hydrophobic binding. The columns were conditioned with 1 mL each of methanol and dH2O. After centrifugation for 10 minutes at 4,200rpm, the sample supernatants were decanted into the conditioned SPE columns. Here, the samples were gently pushed through the column bed using a stream of nitrogen. The column beds were then washed with 2 mL of dH2O, followed by 2 mL of 0.1M acetic acid, and lastly by 2 mL of 25% methanol. Prior to elution using 1.5mL of a 80:18:2 solution of dichloromethane : isopropyl alcohol : ammonium hydroxide per sample, the column beds were dried for 14min at 45°C and with a stream of nitrogen at 50psi. Elutes were completely dried down at 40°C with the help of a gentle nitrogen stream. Samples were reconstituted in 100 µl of 0.1% formic acid.

11. LCMS/MS Parameters:

Secobarbital was analyzed using 0.2% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B). The following gradient was utilized at a flow rate of 0.5mL/min:

Time	% B
0.00	30
0.00	30
0.95	30
1.20	50
1.21	30

Mass spectral analysis was performed using multiple reaction monitoring (MRM) with two transitions per analyte (**Quantifier**/Qualifier). Below are the settings used in this study

Analyte	MRM Transition	Dwell Time (ms)	Frag. (V)	CE	Gas Temp (°C)	Gas Flow (L/min)	Neb. Pressure (psi)	Cap. Voltage (V)
Secobarbital	237.1 →194.1	30	100	8	350	10	50	4000
	237.1 →42.1	30	100	8				
Secobarbital-d5	242.1 →199.2	30	100	8				
	242.1 →42.1	30	100	8				

IV. RESULTS:

1. Intraday Study and Limit of Detection (LOD)

Eight aliquots of each of the following different concentrations of secobarbital was analyzed for the intraday study: 0pg/mg (Negative), 250pg/mg (Low QC), 500pg/mg (Calibrator), 1000pg/mg (High QC). Figure 2A is a plot of these calibration concentrations normalized to the negative control using the following equation:

$$B/B_0 \times 100$$

where B is the absorbance value of the bound calibrator and B_0 is the absorbance value of the blank calibrator (Miller, Wylie, & Oliver, 2006). As can be seen from Figure 2A, the curve is linear with an R^2 value of 0.986. Figure 2B contains the cumulative data of the eight replicates for each of the concentrations. The relative standard deviation (RSD) and the mean relative error (MRE) were well within the acceptable range of 20%.

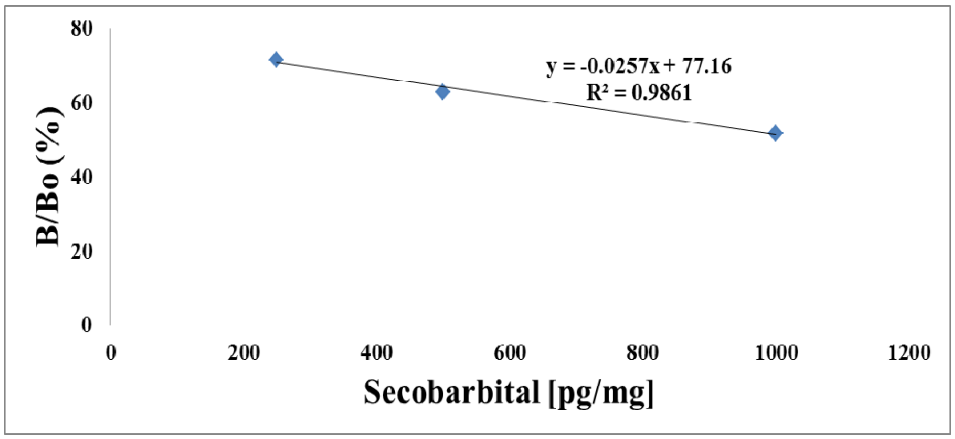
The theoretical limit of detection was determined to be approximately 90 pg/mg using the data from Figure 2 and the following equation:

$$LOD = A_0 - (3 \times SD_{neg})$$

where A_0 is the mean absorbance value for the 8 negative samples, and SD is the standard deviation of the absorbance values (Miller et al., 2006).

Figure 2 – Intraday results

A)



B)

Secobarbital				
	Negative QC	**Low QC**	**Calibrator**	**High QC**
Average (n=8)	2.43	1.74	1.53	1.26
SD	0.08	0.06	0.04	0.05
RSD	3.20	3.23	2.46	3.92
MRE	-0.16	1.87	-0.18	-0.27

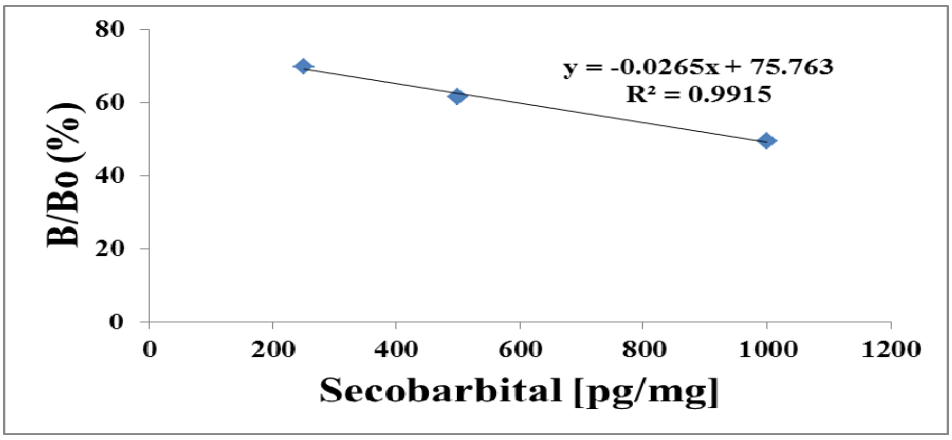
Figure 2. (A) ELISA dose response curve for secobarbital after intraday study. (B) The average absorbance obtained from all eight replicates for each concentration of secobarbital, with their respective SD, RSD, and MRE values.

2. Interday Study

For the next eight days the same procedure was followed, except the different concentrations were run in replicates of five instead of eight. The resultant data of the interday study can be seen in Figure 3. Figure 3A represents a plot of the averages of each of the points normalized to the negative control, using the same equation used for the intraday study – $B/B_0 \times 100$. The averages of all five readings obtained on all eight days for each of the calibrator and controls is represented in Figure 3B (n=40). As can be seen in Figure 3B, both the RSD and MRE were calculated to be well within the acceptable range of 20%, demonstrating the stability of the assay.

Figure 3 – Interday Study

A)



B)

Secobarbital				
	Negative QC	**Low QC**	**Calibrator**	**High QC**
Average (n=40)	2.62	1.83	1.61	1.30
SD	0.19	0.17	0.12	0.05
RSD	7.24	9.52	7.63	3.58

Figure 3. (A) ELISA dose response curve for secobarbital after interday study. (B) The average absorbance obtained from the five replicates for all eight days for each concentration of secobarbital, with their respective SD, RSD, and MRE values.

3. Carryover Study

Using the automated liquid handler, Tecan-fre, a carryover study was performed. Here, the same negative sample was aliquotted onto the ELISA plate before and after an aliquot of a highly positive sample (4x the concentration of the high QC) was taken. As can be seen from Table 1 below, there was approximately a 3% difference between the absorbance value of the negative sample taken before and after the highly positive sample.

Table 1 – Carryover Study

Negative Sample "Before"	1.98
High Positive Sample	0.96
Negative Sample "After"	1.91

Table 1. Absorbance values obtained from a negative sample before and after the addition of a high positive sample onto the ELISA plate by way of an automated liquid handler.

4. Fortified Hair –Screening

Following incubation with a high concentration of secobarbital for 6 days, the hair fortification process was complete. The extracted sample and last wash of the sample was analyzed by ELISA and the resultant absorbance values can be seen in Table 2B. In conjunction with Table 2A, it can be seen that the hair, whether soaked in a solution containing 50% DMSO or 50% 0.02M HCl in DMSO, screened positive for secobarbital –having average absorbance values of 1.27 and 1.37, respectively, which is lower than the cutoff absorbance of 1.84. Additionally, the last wash of either condition screened negative –having absorbance values well above the cutoff.

Table 2 – Fortified Hair Screening Results

A)

Calibration	
Sample	**Average Abs**
Negative	3.08
Low Control	2.27
Calibrator	1.84
High Control	1.69

B)

	DMSO Fortified Hair		HCl Fortified Hair	
	Sample	**Last Wash**	**Sample**	**Last Wash**
Absorbance Value	1.24	2.42	1.36	2.51
	1.26	2.33	1.37	2.15
	1.30	2.44	1.38	2.36
Average	1.27	2.40	1.37	2.34
SD	0.027	0.061	0.010	0.177
RSD	2.168	2.531	0.732	7.567

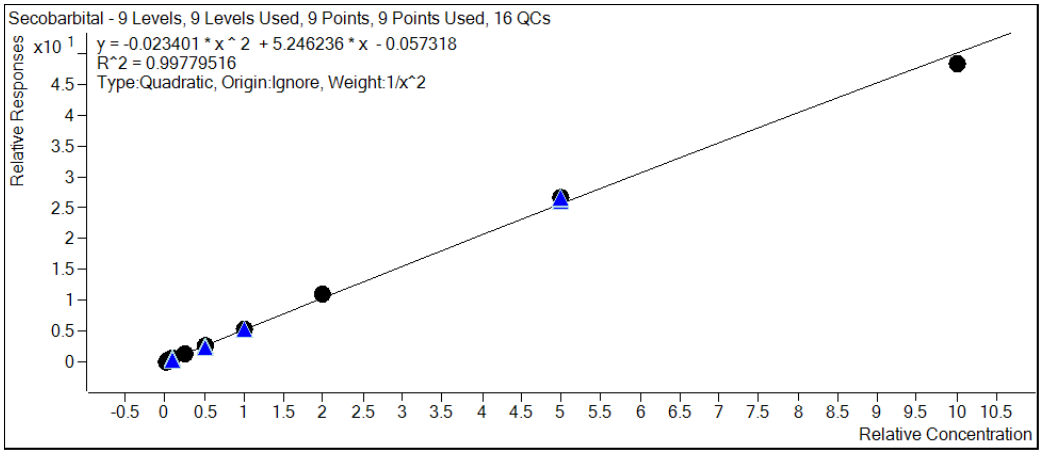
Table 2. (A) ELISA calibration points of secobarbital used to screen for positive samples. (B) Absorbance values of the secobarbital-fortified hair samples using the ELISA screen.

5. Fortified Hair –Confirmation

Known concentrations of secobarbital were analyzed to create a calibration curve to determine the concentration of secobarbital extracted from the fortified hair samples (Figure 4A). The fortified hair samples were extracted using both HEB and 1M NaOH to determine the extraction efficiency of HEB and the resultant data can be seen in Figure 4B. The fortified hair soaked in 50% DMSO (DMSO Fortified Hair) produced 3,243 pg/mg of secobarbital when exposed to HEB, whereas the use of 1M NaOH produced 2,847 pg/mg. In comparison, the hair fortified with 50% 0.02M HCl in DMSO (HCl Fortified Hair) resulted in a lower concentration of secorbarbital, producing 2,087 pg/mg of the analyte using HEB and 1,943 pg/mg using 1M NaOH. The %RSD for all is well below 20%. This demonstrates that, for either condition, HEB does, in fact, successfully extract secobarbital from fortified hair, resulting in quantities similar to that of the fortified hair extracted with 1M NaOH.

Figure 4 – Extraction Efficiency Results

A)



B)

	DMSO Fortified Hair		HCl Fortified Hair	
	NaOH [pg/mg]	HEB [pg/mg]	NaOH [pg/mg]	HEB [pg/mg]
Average	2847.44	3243.32	1943.69	2087.74
%RSD	3.36	0.57	3.81	2.12

Figure 4. (A) LCMS/MS calibration curve of secobarbital concentrations ranging from 25-10,000 pg/mg. (B) The average concentration of secobarbital obtained from the fortified samples using two different extraction solutions.

V. DISCUSSION:

The validation procedure used for this study was designed to determine if this type of assay had the capabilities to perform barbiturate screening in hair. Furthermore, extensive investigations were conducted in order to ensure that the drug extraction was optimal and that the detection results were reliable and reproducible – a critical requirement if this assay is applied in situations of routine drug testing. Based on the data generated in this study, the heterozygous immunoassay can be imployed to differentiate between the cutoff concentration of secobarbital (500 pg/mg) and its respective low (250 pg/mg) and high (1000 pg/mg) quality controls with using only 10 mg of hair. Additionally, this distinguishable separation between the concentration points is highly precise and reproducible, giving an RSD and MRE less than 20% over a course of 8 days with 5 repeats for each concentration. Although the LOD concentration was derived theoretically, the resultant value was far less than that of the low control, indicating that the assay is sensitive enough to distinguish the low control from the LOD and subsequent background noise. It was also determined that, if an automated liquid handler is to be used, carryover from a high positive sample to a negative sample is highly improbable, deeming it an assay fit for high volume throughput.

There are a vast array of methods available in the literature on how to extract drugs from hair. Because the manufacturer of the ELISA kit used in this study also provides a hair extraction buffer, it was tested on its efficiency before continual use. Although both fortification solutions prepared for this study contained DMSO – a reagent documented to help in the fortifying process by way of carrying the drug into the hair – it was not known which solution (the one with 0.02M HCl or without) would work for this

drug class. After 6 days of incubation with an exaggerated amount of secobarbital in either DMSO solution, a positive result for the ELISA screen was obtained. Three separate aliquots of fortified hair from each condition resulted in an RSD value less than 10% for both the extracted hair sample and its respective last wash – confidently solidifying its positivity was due to drug extraction and not removal of external contamination. The efficiency of the HEB extraction was then tested by comparing it to that of the 1M NaOH digestion. In concurence with the ELISA results, both fortification solutions produced hair samples positive for secobarbital. Interestingly, both conditions resulted in similar, if not, slightly greater quantities of secobarbital when using HEB than that extracted with 1M NaOH, demonstrating that HEB is an efficient buffer for the extraction of barbiturates from hair.

VI. CONCLUSION:

Screening methods for the detection of various drugs in hair have been previously validated using immunoassays. The intent of this study was to validate a method to reliably extract and detect barbiturates, specifically secobarbital, in hair to accommodate the currently prominent need to monitor patients taking potentially abused, prescription drugs. The study demonstrates that picogram quantities of secobarbital can be extracted from 10 mg of hair using HEB, with both precision and reproducibility. Additionally, it was established that, in the event that an automated liquid handler is to be utilized, no carryover is seen when a negative sample was aliquotted following a very high positive sample. Ultimately, this method is a good resource for the presumptive detection of baributrates in hair.

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