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Effects of filtration sterilization on the stability of ketamine, selected benzodiazepines and metabolites in female urine

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EFFECTS OF FILTRATION STERILIZATION ON THE STABILITY OF KETAMINE, SELECTED BENZODIAZEPINES AND METABOLITES IN FEMALE URINE

by

LIN ZHEN

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Approved by

First Reader

Sabra Botch-Jones, M.S., M.A.
Instructor, Program in Biomedical Forensic Sciences
Department of Anatomy & Neurobiology

Second Reader

Amy N. Brodeur, M.F.S.
Assistant Professor, Program in Biomedical Forensic Sciences
Department of Anatomy & Neurobiology

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ABSTRACT

Benzodiazepines (Benzos) and ketamine (K) are compounds that have been encountered in Drug-Facilitated Sexual Assault (DFSA) cases. Due to the intimate nature of these crimes, evidence collection is often postponed due to delays and/or reluctance in reporting these crimes. Further delays in analysis may be encountered in laboratories with large caseloads and/or backlogs. Drug identification in biological samples is important to determine whether victims knowingly or unknowingly took an impairing substance, however, the results could be negative due to chemical degradation over a long storage period. The purpose of this project was to study if degradation could be prevented with a new preservation method at the time of collection.

Urine samples were prepared by the addition of K and metabolites and selected benzos and metabolites that were subjected to different sample pre-treatment techniques, and were analyzed after storage at room temperature (25°C), refrigerator (4°C) and freezer (-20°). The samples were either pre-treated with preservative (0.5% toluene) or filtration sterilization (sterile filter kit) within two hours after sample collection, and a control group with no pre-treatment was incorporated into the study for comparison. The changes in concentrations over 50 days (Benzos group) and 210 days (K group) were

evaluated between different pre-treated methods and different temperature conditions. Sample that were treated with 0.5% toluene showed the most degradation: 44% of oxazepam and 96% of diazepam degraded over 10 days, and 80% of dehydronorketamine degraded after storage of 150 days regardless the temperature conditions. Clonazepam and flunitrazepam concentrations were reduced by 80% of the original concentration when stored at room temperature for 10 days. The major benzodiazepines evaluated in this study were stable when stored in the freezer. In K group, ketamine and norketamine that were stored at room temperature and refrigerated over 210 days were stable, however, degradation was observed after 150 days when the samples were stored in the freezer.

There was no statistically different change observed among the samples pre-treated with or without filtration sterilization. Each sample pH was measured and it was determined that those stored at room temperature had an average pH of 8.5, while samples stored in the refrigerator and freezer had an average pH of 6.7 and 6.5, respectively. This finding revealed that pH could be the major factor affecting compound degradation rather than the bacterial contamination with high pH contributing to degradation, and low pH potentially preventing sample lost.

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LIST OF ABBREVIATIONS

ACN	*Acetonitrile*
Benzos	*Benzodiazepines*
Cl^-	*Chloride ion*
CE	*Collision energy*
CXP	*Collision cell exit potential*
CUR	*Curtain gas*
CAD	*Collision gas*
DP	*Declustering potential*
Da	*Dalton*
DFSA	*Drug-Facilitated Sexual Assault*
DNK	*Dehydronorketamine*
ESI	*Electrospray ionization*
E. coli	*Escherichia coli*
FS	*Filtration sterilization*
GABA	*Gamma-aminobutyric acid*
GHB	*Gamma-hydroxybutyrate*
GC-MS	*Gas chromatography-mass spectrometry*
GS1	*Ion source gas 1*
GS2	*Ion source gas 2*
h	*Hour*
HPD	*Houston Police Department*
HPLC	*High Performance Liquid Chromatography*
HCl	*Hydrochloride acid*
IV	*Intravenous*
IS	*Internal standard*
K	*Ketamine*

K-d4	*Ketamine-d4*
kg	*Kilogram*
LASD	*Los Angeles Sheriff's Department*
LAPD	*Los Angeles Police Department*
LC	*Liquid chromatography*
LC-MS	*Liquid chromatography-mass spectrometry*
LC-MS/MS	*Liquid chromatography-tandem mass spectrometry*
LLE	*Liquid Liquid Extraction*
MS	*Mass spectrometry*
MS/MS	*Tandem mass spectrometry*
min	*Minute*
mL	*Milliliter*
MeOH	*Methanol*
μ	*Micron*
NIJ	*National Institute of Justice*
NK	*Norketamine*
NK-d4	*Norketamine-d4*
NMDA	*N-methyl-D-aspartate*
ng	*Nano gram*
psi	*Pounds per square inch*
RT	*Retention time*
RSD	*Relative standard deviation*
SAK	*Sexual assault kit*
SANE	*Sexual assault nurse examiner*
SPE	*Solid Phase Extraction*
s	*Second*
TEM	*Temperature*
V	*Ionspracy voltage*

Z	*Zolpidem*
Z-d7	*Zolpidem-d7*

1 INTRODUCTION

Drug-Facilitated Sexual Assault (DFSA), often referred to "date rape" or "drug rape" happens when a victim is impacted by alcohol and/or drugs that cause them to be unconscious and unable to give consent to sexual activity. (1, 2) There are more than 50 known or suspected substances that have been reported in DFSA crimes, and many of these substances can be obtained easier than illicit drugs. Some prescription medications or the over-the-counter pharmaceuticals impact the user's mental condition with long duration effects. (3) Gamma-hydroxybutyrate (GHB), flunitrazepam (Rohypnol®) and ketamine (K) have been reported as the most common date rape drugs by the United States (U.S.) Department of Health and Human Services, Office on Women's Health (Table 1-1). These date rape drugs are colorless, flavorless and odorless and can be easily added into any drink without the awareness of the victim. The adverse effects of these substances include drowsiness, dizziness, sedation, unconsciousness and confusion, and some of them can also cause hallucination. (4, 5) These adverse effects are detrimental to the investigation as most victims regain don't regain consciousness for hours after the event. (2) The later the victims are examined, the less evidence is likely to be collected.

Table 1-1 Examples of substances detected in urine of DFSA victims. (6)

Drug	Usual Duration of Detection in Urine[1]
Amphetamines	1-3 days
Barbiturates	2-7 days
Benzodiazepines	2-7 days
Benzoylecogonine	1-2 days
Cannabinoids	2-5 day (single use)
Carisoprodol	1-2 days[2]
Chloral hydrate	1-2 days[2]
Clonidine	1-2 days[2]
Cyclobenzaprine	1-2 days[2]
Diphenhydramine	1-2 days[2]
Ethanol	Less than 1 day
Gamma hydroxybutyrate	Less than 1 day[2]
Ketamine	1-2 days[2]
Meprobamate	1-2 days[2]
Opioids	2-3 days
Scopolamine	1-2 days[2]

[1]Estimate of the duration of detection, with the use of methods more sensitive than typical drug screening. Actual detection will depend on individual metabolism, dose, and concentration in specimen. Also, assays vary in sensitivity and specificity depending on the laboratory, so it is important to consult with the laboratory for definitive information.
[2]Specific information not available; duration given is an estimate.

There were 330,170 and 284,350 rape/sexual assault crimes in the U.S. in 2013 and 2014, but the percent of victimizations reported to police were only 45.6% and 46.0%, respectively. (7) A study from Wayne State University and Michigan State University found that only 58.6% of the sexual assault kits (SAKs) collected by sexual assault nurse examiners (SANE) in sexual assault crimes were submitted to the crime laboratory within a large Midwestern county. (8) Patterson et al. also indicated in their study that the SAKs were more likely to be submitted (a) when there were documented physical injuries; (b) if the law enforcement agency had a greater collaboration with the SANE program; or (c) if there was no evidence of any cleaning by the victims themselves.

(8) DFSA victims are less likely to have physical injuries because they can't resist due to the effects of the drugs, thus decreasing the likelihood that their case is thoroughly investigated.

Even though SAKs may be submitted, not all of them are examined. Backlog, or evidence submitted to a crime laboratory that remains untested after 30 days, can occur when agencies don't believe it will help identify the assailant, or when the agency lacks resources. (9) Backlog is a very challenging problem for the analysis of biological and chemical evidence because the evidence can degrade over time. Degradation starts at the time of sample collection, and compounds could become undetectable if the samples were highly degraded. Reports from National Institute of Justice (NIJ) revealed that the combined untested SAKs from Los Angeles Sheriff's Department (LASD) and Los Angeles Police Department (LAPD) were from 10,895 cases in the fall of 2008 and the untested SAKs from Houston Police Department (HPD) were from 6,663 cases in 2010. (10, 11)

The goal of this project was to study a new technique to preserve the samples at the time of collection, and maintain the concentration of samples over time. The new preservation method was investigated and compared with a traditional anti-bacterial preservative method, and changes in concentrations were used to determine if degradation was prevented. Ketamine (K), selected benzodiazepines (Benzos) and metabolites were used to evaluate effects of the new preservation method in this project.

1.1 Filtration Sterilization

In addition to blood, urine is an essential biological material to be collected in DFSA crimes. However, unlike blood specimens, urine specimens are more easily to be contaminated with bacteria, because: (a) they can be contaminated with naturally occurring and/or infectious bacteria; (b) their aseptic collection into a test tube is nearly impossible; (c) and they can be exposed to high ambient temperatures during collection and transportation. (12) Remarkable contamination has also been observed in postmortem cases. The contamination of bacteria can potentially degrade the drugs during the storage. Escherichia coli (E. coli) is the most common bacteria found in urine with a size of approximately 1 μm in width and 3 μm in length. (13) E. coli has a rapid growth rate such that it could grow at least 99% of the bacterial population with the mean minimum generation time of 21.7 +/- 0.6 min in the bladder model over 24 h. (14) A large population of E. coli can be accumulated within one day, which is a severe factor to the drug degradation.

Filtration sterilization (FS) is the new preservation method, which is designed to eliminate or minimize the bacterial population at the time of collection to stop or reduce the degradation caused by bacteria. A filter membrane pore size of 0.2 μm was chosen based on the overall size of E. coli, so that the filter could remove the E. coli effectively. In addition, toluene is a common antibacterial preservative used for urine specimens, and it has been found that calcium, oxalate, magnesium, phosphate, sodium, urate and pH of the urine samples could be effectively preserved with its use. (15) A set of samples pre-treated with toluene was analyzed to compare the effects between old and new

preservation methods, and another set of samples without any pre-treatment was also analyzed as a control.

1.2 Benzodiazepines

Benzodiazepines (Benzos) are a class of anxiolytic drugs that bind to the Benzos response sites located at the interface of alpha and gamma-2 subunits of GABA receptors. (4) The binding activates the chloride ion (Cl^-) channel, and the entry of Cl^- hyperpolarizes the cell. (4) This hyperpolarization can be enhanced with the binding of Benzos due to the neural depolarization that has been inhibited. (5)

The pharmaceutical effects of Benzos include sedation, hypnosis, anesthesia, anticonvulsant effects, muscle relaxation and effects on respiration and cardiovascular function that depend upon the correlation and affinity between different Benzos and the GABA receptors. (4, 16) Hence, there are many therapeutic uses in this class of drugs, including for treating anxiety disorders, insomnia, seizures, pre-anesthetic and short medical/surgical procedures, muscle relaxation, acute mania and symptoms of physical dependence. (5) Even though the effects of Benzos are various, drowsiness and confusion are the major and common adverse effects of Benzos, regardless the type of Benzos. Table 1-2 illustrates the dosage and direction of the common prescribed Benzos for sedation and hypnosis. It is easy to find that the clinical dosage of Benzos is usually very little, and that is a huge benefit to sexual assailants because it is easy to add into any drink without being caught, and the small pills dissolve quickly.

Table 1-2 Dosage common prescribed Benzos. (16)

Sedation		Hypnosis	
Drug	Dosage	Drug	Dosage (bedtime)
Alprazolam	0.25-0.5 mg 2-3-times daily	Estazolam	0.5-2 mg
Chlordiazepoxide	10-20 mg 2-3 times daily	Lorazepam	2-4 mg
Diazepam	5 mg twice daily	Quazepam	7.5-15 mg
Halazepam	20-40 mg 3-4 times daily	Temazepam	7.5-30 mg
Lorazepam	1-2 mg once or twice daily	Triazolam	0.125-0.5 mg
Oxazepam	15-30 mg 3-4 times daily		

The microsomal oxidation and conjugation occur sequentially in the Benzos metabolism, and many of the oxidation products (phase I metabolites) are pharmacologically active with a long half-life. Table 1-3 illustrates the half-life of parent compounds, which is about 1-3 h on average, but their major metabolites have an average of 40 h half-life. Most of the major metabolites are pharmaceutical active, which explains why the victims can remain unconsciousness for hours. Parent compounds would be hard to detect, and only metabolites could be found after the victims regained consciousness. This attribute makes a challenge for the analysts on the interpretation of the results. Structurally, this class of drugs shares a diazepine ring fused with a benzene ring, and the structural similarity between different Benzos explains why they have common effects of sedation and hypnosis. The structural similarity also results in Benzos sharing common metabolites, and oxazepam is one of them. The confirmation of oxazepam is often used as the indication for the use of Benzos.

Table 1-3 Pharmacokinetic properties of Selected Benzos in humans. (16)

Drug	T_{max}[1] (hours)	$T_{1/2}$[2] (hours)
Alprazolam	1-2	12-15
Chlordiazepoxide	2-4	15-40
Diazepam	1-2	2—80
Flurazepam	1-2	40-100
Lorazepam	1-6	10-20
Oxazepam	2-4	10-20
Temazepam	2-3	10-40
Triazolam	1	2-3

[1]Time to peak blood level
[2]Includes half-lives of major metabolites

The other challenge of Benzos detection is compound stability. The parent compounds are less likely to be detected due to the short half-life, but the metabolites could be found if the specimens were collected as early as the victim regained consciousness and was examined in the hospital. As described previously, the SAKs might not be submitted to the laboratory and/or analyzed without any delay. The critical point is the stability of Benzos and metabolites, and it has been found that the concentrations of Benzos decrease dramatically over time. El Mahjoub et al. studied the stability of clonazepam, midazolam, flunitrazepam and oxazepam in whole blood samples stored at four different temperatures. There was no significant change in the concentrations of samples stored at -20°C and -80°C, but they found the concentrations of the selected Benzos decreased at least 90% for the samples prepared at a low concentration (250 ng/mL) and more than 50% for the samples prepared at a high concentration (1000 ng/mL) if the samples were stored at either room temperature or 4°C. (17) Therefore, it is important to develop an effective preservation method to prevent degradation.

1.3 Ketamine

Ketamine (K), [2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one], is one of the anesthetic agents used for general anesthesia, and no specific receptor has been identified for K if administrated intravenously (IV). (16, 18) The properties of K include anesthetic and analgesic, and it is the only anesthetic agent that has both properties. (16) However, the postoperative psychic effect is severe with a high dose injection (1-2 mg/kg IV), and therefore, the use of low dose K (0.1-0.25 mg/kg IV) with a combination of preanesthetic drugs, such as Benzos, becomes favored. (16) The lower the dose, the smaller the pills, which make it easier for assailants to add them to drinks without any awareness.

The excitation of glutamate at the N-methyl-D-aspartate (NMDA) receptor complex is inhibited by K, and this inhibition triggers the open/close of the ion channels to produce anesthesia. (19) The metabolism of K occurs in the liver and is catalyzed by the cytochrome P450 enzymes that undergo N-demethylation to its active metabolite, norketamine (NK), which also contributes to the analgesic effects; the second metabolite produced is dehydronorketamine (DNK), which is formed by the oxidation of the cyclohexanone ring. (20) The bioavailability of K is about 8%-17% due to the first-pass metabolism in liver, and its half-life is only a few minutes. (20-22) However, the first metabolite NK, which has one third of potency as K, has a half-life that is much longer than K. (23) Fanta et al. found that the half-life of NK was 342 min after an initial oral dosing, which could be detected up to an average of 3 days post-dose, and DNK was confirmed to be detected up to 10 days. (21, 24) Therefore, DNK is an indicator to report

that K was very likely consumed if neither K nor NK was detected.

As mentioned previously, the dose of K needs to be high to cause the sufficient anesthetic effect. A 60-kg (132 lbs.) person will need to be injected 60-120 mg of K to experience the effect, and if a concentration of 100 mg/mL of K is available in the market, the 60-kg individual will need to consume at least 3.5 mL (solid form might be smaller in size) orally to experience the intended effects for a few minutes. The cost is also an important factor. Therefore, a combination of K and other drugs is favored not only by the surgeons but also the assailants. The analysis and interpretation of K becomes more complicated with the use of other drugs, because: (a) a sensitive screening test has to be manufactured; (b) the efficiency of the extraction method should be evaluated for the common date rape drugs; (c) a comprehensive instrumental analysis method must be developed; (d) the clinical and adverse effects should be studied for the combinational use of K and other drugs; and (e) essential training for the complex interpretation must be provided to the analysts.

1.4 Zolpidem

Zolpidem (Z) is a hypnotic substance prescribed for insomnia treatment. (25) Zolpidem targets the same receptors as Benzos, but it has no structural similarity to Benzos (Figure 1-1), which cannot be used as an anticonvulsant or muscle relaxant. (4) The therapeutical dosage of Z is 5-10 mg, which has a maximum duration time of 1-3 hrs (time to peak blood level) without active metabolite. (16) The major metabolite of Z is zolpidem carboxylic acid (ZCA), but its stability is unclear. Eighty percent of Z is

metabolized to ZCA by cytochromes P450 enzymes, and the use of Z could be confirmed if ZCA was detected. (26) The use of Z could be confirmed if M-3 metabolite was detectable. The duration time is short compared to the other compounds, but it is one of the common date rape drugs, therefore, the analysis of Z was also included in this project.

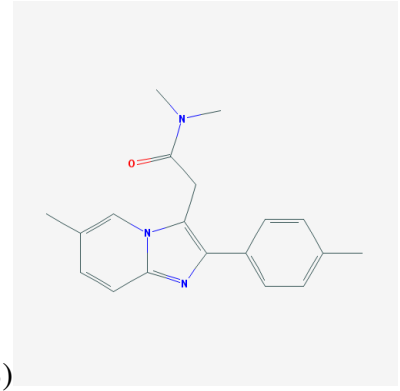
(A)  (B) 

Figure 1-1 Structures of benzodiazepine and zolpidem. (27, 28)
(A)The structure of benzodiazepine
(B)The structure of zolpidem

1.5 Purpose of This Study

The purpose of this project was to evaluate the effects of FS and toluene on preventing compound degradation in the urine specimens. To date, there are many analytical methods that have been developed using gas chromatography – mass spectrometry (GC/MS), liquid chromatography – mass spectrometry (LC-MS) or other sensitive instruments and techniques for the detection and quantification of the suspected substances, even in a trace amount. However, the prevention of degradation in the post-collection specimens have not been well studied. In this study, the analytes were divided into two groups, K group and Benzos group, to evaluate the effect of FS on the stability

of selected compounds. Ketamine (K), norketamine (NK) and dehydronorketamine (DNK) were chosen for the K group, and Benzos group included alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nordiazepam, oxazepam, temazepam and zolpidem (Z). Two groups of samples were prepared and analyzed by using different extraction and analytical methods.

2 EXPERIMENTAL

2.1 Instrument and Extraction Technique Theory

2.1.1 Liquid Chromatography-Mass Spectrometry

Liquid chromatography (LC) is an analytical technique used to separate and resolve compounds from a mixture. The components of a LC system include the mobile phase reservoir(s), a degasser, a pump, a mixer, an autosampler, a column, a detector and a data collection device. (29) The separation is based on the interaction between compounds, stationary phase and mobile phase. The polarity of the target compounds determines the choice of column (stationary phase) and the mobile phase. The column (stationary phase) is the most important element of the sample introduction in the LC system, which is a solid stationary phase packed with the adsorbent material. The selected column must be similar in polarity to the target compounds, so that the compounds can move slower due to the higher affinity to the stationary phase. The mobile phase is the key to conduct the movement of compounds in the LC column. The polarity of the mobile phase can be changed by changing the concentration of the organic solvent to alter the proportion of the aqueous and organic solutions. The compounds can

be moved faster and eluted when the polarity of the mobile phase reaches a similar polarity as the compounds. Therefore, the separation can be optimized by utilizing a gradient elution if more than one mobile phase is used.

Mass spectrometry (MS) is a sensitive analytical technique often used to identify compounds by measuring their mass-to-charge ratio (m/z) after they are separated by the LC system, and a tandem mass spectrometer (MS/MS) is a common detector used. The difference between MS and MS/MS is that one MS is attached to another MS, which can improve the sensitivity, specificity and accuracy by (a) removing matrix interferences; (b) providing reliable confirmation; (c) allowing the selective quantitation of target compounds in high background samples; and (d) getting better signal/noise in complex matrices than the single MS. (30) The compounds must be ionized before they are introduced to the MS system, and the ionization is provided by electrospray ionization (ESI). The liquid sample is injected through a capillary, and ESI produces ions while the droplets are evaporating. (31) The ionization process is to ensure the parent ions can be recognized by the MS, as well as be fragmentized later in the collision chamber.

A triple quadrupole configuration is set up linearly in the MS/MS instrument. The first quadrupole (Q1) is the mass filter to filter anything else besides the precursor ion that has been identified in the compound optimization step (detail explanation is in 2.2.6), and then the precursor ion is accelerated to the second quadrupole (Q2). The collision cell is located in Q2 where the precursor ion is dissociated into fragment ions. The fragment ions are then accelerated into the third quadrupole (Q3) which is another mass filter for the selected product ions. The product ions are also selected and identified

in the compound optimization step, and they are detected based on their m/z.

2.1.2 Solid Phase Extraction

Solid phase extraction (SPE) is a sample preparation technique used to purify compounds prior to sample introduction. In drug or toxicology analysis, plant materials, substances in the solid or the liquid form and biological samples are commonly seen. The size of the molecules in these matrices is relatively large, for example, the red blood cells have an average size of 8 μm in diameter, and an E. coli cell is approximately 1 μm in width by 3 μm in length. (32) However, a normal LC column has a particle size of less than 5 μm and a pore size of 100 Å. The matrices can cause blockage and damage to the column. In addition, they can also increase the noise in the MS detection and interfere with the results. The use of SPE can remove the impurity and extract compounds from samples based on the physical and chemical properties.

The SPE column is packed with the bonded silica gel, and the selection is based on the interactions between the sorbent and the compounds. The principle for the separation of SPE is similar to LC, and the mechanisms include non-polar (hydrophobic interactions), polar (hydrophilic interactions), ion-exchange (cation and anion) and mixed mode interactions. The affinity between the sorbent and analytes determines how tight the target analytes can be held in the column until they are eluted. The advantages of SPE include (a) solvent, and laboratory time are reduced, but the efficiency is improved compared with Liquid-Liquid Extraction (LLE); (b) it is fast and easy to perform; and (c) the matrix can be removed or minimized to protect and maintain the cleanliness of the

instrument.

An appropriate SPE method is designed based on the compounds of interest and the column as described previously, but there is a general scheme to perform SPE. The first step has been discussed in the previous paragraph, which is also called the selective extraction. In other words, the sorbent must be able to catch and hold all the compounds tight while the sample is running through the column. The second step is called selective washing, and solutions must be strong enough to remove the impurity but weak for the compounds. The last step is the selective elution, in which the compounds must be able to dissolved in the solvent so that they can be carried while the solvent is eluting. (33)

2.2 Materials & Methods

2.2.1 Reagents and Standards

Ammonium formate, ammonium hydroxide, formic acid, sodium acetate trihydrate, high performance liquid chromatography (HPLC) grade methanol (MeOH), HPLC grade acetonitrile (ACN), HPLC grade methylene chloride, HPLC grade ethyl acetate and hydrochloride acid (HCl) were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Sodium phosphate monobasic, sodium phosphate dibasic, acetic acid and HPLC grade isopropanol were purchased from ACROS® (Geel, Belgium). Beta-glucuronidase from limpets (patella vulgate) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Purified water, referred to as millipore water, was obtained from a Synergy UV water system from EMD Millipore/Merck (Darmstadt, Germany).

Nuclear fast red stain (Xmas tree stain A) and picroindigocarmine stain (Xmas tree stain B) were purchased from SERI® (Richmond, CA), hematoxylin stain was purchased from RICCA® (Arlington, TX), and eosin Y stain was purchased from ACROS® (Geel, Belgium).

Individual ampoules of K, NK, alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nordiazepam, oxazepam, temazepam, Z (at a concentration of 1 mg/mL), DNK, K-d4, NK-d4, diazepam-d5, oxazepam-d5 and Z-d7 (at a concentration of 100 μg/mL) were purchased from Cerilliant Corporation (Round Rock, TX). Stock standard solutions of these analytes were prepared in methanol or acetonitrile to make a concentration of 100 μg/mL.

2.2.2 Urine Specimen and Bacterial Contamination Determination

The urine specimens were collected from six female donors, and the presence or absence of the compounds of interest was confirmed before experiments commenced. The bacterial contamination level of the urine specimens was determined by the number of bacterial cells. Each urine specimen was mounted on a glass slide, and enhanced by hematoxylin and eosin staining (H&E) and Christmas Tree staining (KPIC) techniques. The slides were examined under 400X magnification for the presence of bacterial cells and/or epithelial cells. The number of bacterial cells per magnification field was estimated by counting the number observed in 10 representative fields, calculating the average and assigning one of these values:

0 = no bacterial cells present

1+ = few bacterial cells on entire slide; difficult to locate

2+ = at least one bacterial cell in most of the fields

3+ = several bacterial cells in most of the fields; easy to locate

4+ = many bacterial cells in most of the fields

The urine samples were examined on Day 0 and the last day of analysis to determine the original contamination level for each donor and the contamination level after aging.

2.2.3 Filtration Sterilization and Samples Preparation

The filtration sterilization (FS) was performed by using 25 mm GD/X sterile syringe filters that were purchased from Sigma-Aldrich Inc. (St. Louis, MO). The samples were pulled into the syringe, and then attached to the syringe filter which is equipped with a polyethersulfone membrane (pore size 0.2 μm, diameter 25 mm). This pore size is only half of the minimal size of E. coli, which should be sufficient enough for the bacteria based on the previous discussion (section 1.1). The sample was then pushed into the Falcon test tube by passing through the filter, and stored under three different temperature conditions: 25°C, 4°C and -20°C.

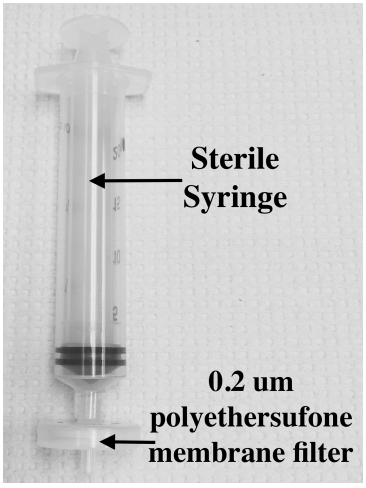


Figure 2-1 Filtration kit.

The samples were prepared by spiking the solution in the urine at a concentration of 500 ng/mL, and then the pre-treatments (FS and toluene preservative) were applied to the samples prior to storage. Next, 0.5% of toluene was added to all samples as the preservative based on the research from Wu et al. (15) On the day of analysis, all analytes and internal standards (IS) were prepared for the calibration curve and samples. For K group, the samples were analyzed on Day 0, Day 150, Day 180 and Day 210. For Benzos group, the samples were analyzed on Day 0, Day 10, Day 30 and Day 50. Table 2-1 shows the treatments and storage condition of the specimens.

Table 2-1 The treatments and temperature condition of the samples.

Temperature	Treatment		
25°C	F[1]	P[2]	N/T[3]
4°C	F	P	N/T
-20°C	F	P	N/T

[1]F – filtration sterilization
[2]P – addition of toluene preservative
[3]N/T – no treatment

2.2.4 Solid Phase Extraction

The Clean Screen Xcel®I columns were used to perform SPE for the Benzos group, and the columns were purchased from United Chemical Technologies, Inc. (Bristol, PA). The samples were pre-treated by incubating the combination of 500 μL of the urine sample, 500 μL of 100 mM acetate buffer (pH=5) and 20 μL of beta-glucuronidase at 65°C for one hour. The samples were cooled to room temperature after they were moved from the incubator, and a set of Clean Screen Xcel®I columns was inserted in the corresponding position of the column rack in the manifold. The pre-treated samples were poured into the column sequentially without the adjustment of pH and no conditioning step was required in this procedure. After the samples passed though the columns, 3 mL of acetate buffer was added to wash the columns applied with a pressure of 5 psi, and a high pressure (>25 psi) was then applied to dry the columns for 5 min. Addition of 3 mL methylene chloride was used to wash the columns applied with a pressure of 5 psi, and a high pressure (>25 psi) was applied to dry the columns for 10 min after the solvent was completely passed though. Three milliliters of the mixture of ethyl acetate and ammonium hydroxide (98:2, v:v) was used to elute the analytes. The eluate was evaporated to dryness at about 45°C, and the dried residue was dissolved with 50 μL of the mobile phase, 15 μL of the reconstituent was injected into the LC instrument.

The Clean Screen® DAU Extraction columns were used to perform SPE for the K group, and the columns were purchased from United Chemical Technologies, Inc. (Bristol, PA). A set of Clean Screen® DAU Extraction columns was placed in corresponding positions of the column rack in the manifold. The conditioning step was

required for this method, thus 1 mL of MeOH and 1 mL of phosphate buffer were added sequentially to the columns in this step. The samples were transferred into the columns and allowed to drip by gravitational flow. The wash steps included 1 mL of DI H_2O, 1 mL of 0.1N HCl and 1 mL of MeOH and were added into the columns sequentially. A high positive pressure (>25 psi) was applied to dry the samples for 5 min. The base elution solvent, a mixture of ammonium hydroxide, isopropanol and ethyl acetate (3:20:77, v:v:v), was added to elute the analytes. The 2-mL eluate was evaporated to dryness at approximately 65°C, the dried residue was reconstituted with 50 µL of a mixture of ACN and millipore water (50:50, v:v), and 5 µL was injected into the LC instrument.

2.2.4 Extraction Recovery

In each group, three samples were prepared with the analytes in negative urine at the concentrations of 50 ng/mL and 200 ng/mL, and six samples were blank urine. Twelve samples were extracted following the respective SPE procedure, and the same amount of solutions was added into the eluate extracted from the blank samples prior to the evaporation. After they were dried down, the normal reconstitution step was performed, and the samples were analyzed by LC-MS/MS.

2.2.5 HPLC (UFLC) Conditions

The LC analysis was performed on a Shimadzu Ultra-Fast Liquid Chromatography (UFLC) system consisting of Shimadzu LC-20 AD pumps and a SIL-20

auto sampler (Kyoto, Japan). The LC parameters were optimized after the compounds and the source optimization were completed, which will be discussed later (section 2.2.7). The analytical column was the 100 X 3.0 mm Phenomenex® Kinetex™ 2.6 μm Biphenyl 100 Å LC column (Torrance, CA), and the guard column was the Phenomenex® SecurityGuard™ULTRA. The same column was used for the analysis of both groups, but the analytical methods were different.

The LC method was based on work from Quintela et al. for the BPZs group. (34) The gradient separation was performed with a binary mobile phase consisting with 5 mmol/L (pH = 3) ammonium formate (mobile phase A) and a mixture of ACN and 5 mmol/L (pH = 3) ammonium formate (90:10, v:v; mobile phase B) at a flow rate of 400 μL/min. Table 2-2 demonstrates the gradient; the total run time was 7 min, and the oven temperature was set at 45°C. (34)

Table 2-2 The concentration gradient of LC-MS/MS for Benzos group.

Time (min)	Mobile phase B (%)
0	35
5	80
6	60
6.5	35

The LC method was based on the research of Parkin et al. for the K group. (24) The gradient separation was performed by using the binary flow mode with a flow rate of 400 μL/min. The mobile phase consisted of a gradient of millipore water with 0.1% formic acid (mobile phase A) and ACN with 0.1% formic acid (mobile phase B). Table

2-3 demonstrates the gradient; the total run time was 3 min, and the oven temperature was set at 40°C. (24)

Table 2-3 The concentration gradient of LC-MS/MS for K group.

Time	Mobile phase B (%)
0	30
0.3	30
2	40
2.5	30

2.2.6 MS/MS Detection

The MS/MS detection was performed on a SCIEX™ 4000 Qtrap (Framingham, MA). Data analysis was done by using SCIEX™ Analyst® software (version 1.6.2) and quantification was done by using SCIEX™ MultiQuant® software (version 3.0). The compounds of interest and source/gas parameters were optimized prior to performing analysis. Only the MS/MS system was turned on while performing the compound optimization and the manual optimization mode was used to optimize all compounds. All standards were optimized in positive-ion mode by the direct infusion at a flow rate of 10 μL/min. The standards of K, K-d4, NK, NK-d4, alprazolam, clonazepam, diazepam, diazepam-d5, flunitrazepam, midazolam, nordiazepam, oxazepam, oxazepam-d5, temazepam, Z and Z-d7 were diluted to a concentration of 10 ng/mL in a mixture of MeOH and millipore water (50:50, v:v), and the standards of DNK and lorazepam were diluted to a concentration of 10 ng/mL in a mixture of ACN and water (50:50, v:v). The first step in manual compound optimization was to confirm the presence of the precursor ion by using the Q1 MS scan type, and the syringe pump method was utilized throughout

the whole process. The molecular weight of the parent compound was searched from PubChem, and the start and stop dalton (Da) were set to be at -/+ 50 Da of the theoretical value, respectively. The most abundant ion was confirmed to be the precursor ion after a 3-min scan with a scan rate of 1000 Da/s. Once the precursor ion was confirmed, the major product ions could be searched by using the Product Ion (MS2) scan type. Collision energy of 400 v was used to fragment the precursor ion, and the search was started from 100 Da. The three most abundant product ions were continued to optimize their parameters, but the ions of 18 Da or 44 Da (water and carbon dioxide, respectively) were excluded. The parameters were optimized by monitoring the best signal for each transition while changing the ramp parameter settings, and the parameters include declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP). Table 2-4 listed the MRM transitions of the compounds from Benzos group and K group.

For source optimization, the LC system was incorporated with the MS/MS system, and the syringe pump method was also utilized. This step is to ensure the ion source and gas are set properly to protect and maintain the cleanliness of the instrument and the compounds can be introduced to MS/MS system optimally. The LC-MS/MS system setting remained unchanged but the autosampler was turned off in source optimization. The ion source and gas parameters were optimized by monitoring the most intensive signal for the individual ion extraction and the total ion extraction chromatographs for the compounds of interest while changing the parameters that followed the order of curtain gas (CUR), collision gas (CAD), ionspray voltage (IV), ion source gas 1 (GS1), ion source gas 2 (GS2) and temperature (TEM). The source optimization was performed

routinely to ensure the optimal results. The source and gas condition were optimized, and the results are listed in Table 2-5.

Table 2-4 MS conditions for the analytes in Benzos and K groups.

Compound	Q1	Q3[1]	DP	CE	CXP
Alprazolam	309	**281.1**	100	35	15
		205.2	100	55	10
Clonazepam	316	**270**	100	35	20
		241	100	50	20
Diazepam	285	**154**	100	40	13
		193	100	45	18
Flunitrazepam	314	**268**	100	40	13
		239.2	100	45	18
Lorazepam	321	**275**	70	30	15
		229	70	40	12
Midazolam	326	**291.3**	110	37	20
		223	110	50	15
Nordiazepam	271	**140.3**	90	40	10
		165	90	40	13
Oxazepam	287	**241**	90	40	10
		104	90	40	13
Temazepam	301	**255.4**	80	30	20
		177.2	80	55	16
Zolpidem	301	**255.4**	100	45	15
		177.2	100	38	15
Diazepam-d5	290	154.3	80	45	15
Oxazepam-d5	292	246.3	70	30	12
Zolpidem-d7	215	242.3	100	50	15
Ketamine	238	**125**	60	38	20
		207	60	20	33
Norketamine	224	**125.2**	60	32	20
		124.9	60	33	19.5
Dehydronorketamine	222	**141**	40	35	23
		114	40	75	18
Ketamine-d4	242	129	60	53	21
Norketamine-d4	228	211	125	53	10

[1]The transition used for quantification are in bold.

Table 2-5 LC condition for the source and gas in Benzos and K group.

	CUR	CAD	IV	TEM	GS1	GS2
Benzos group	25	medium	5500	600	90	90
K group	25	medium	5500	650	90	90

3 RESULTS & DISCUSSION

3.1 Specimen Contamination

The urine specimens from female donors were examined microscopically on the first day and the last day of analysis to determine the bacterial growth. Aseptic urine is nearly impossible due to the nature of the female excretory system and the urination process, however, the contamination level of the specimen was determined to be 0 among all donors as no bacteria cells were observed from the slides made on Day 0 and Day 210 under a magnification of 400 times. Figure 3-1 illustrates the microscopic slides made on Day 0 and Day 210 with FS under a magnification of 400 times, and there were no epithelial cells observed from the slides made from the FS samples. The negative findings could be due to two reasons: (1) the average size of epithelial cells is 64 μm but the size of E. coli is only 1-3 μm, so 400 times magnification might not be sufficient to see the bacteria, and a microscope with higher magnifying power might be helpful, and (2) a special staining technique might be required to make the bacteria easier to observe even under low magnification.

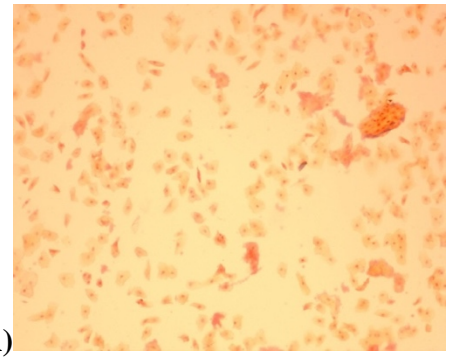
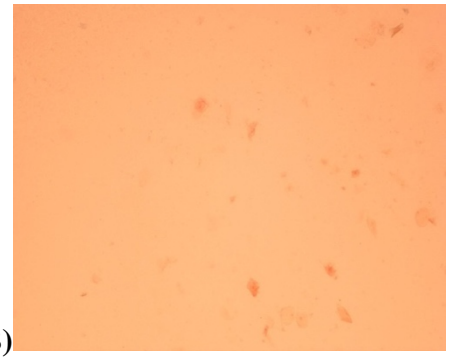
(A)  (B) 

Figure 3-1 Images of urine specimen on Day 0 and Day 210 under 400 times magnification. (A from Day 0, B from Day 210)

All donated urine specimens were negative for the presence of the target compounds prior to any pre-treatment. The solvent blanks were analyzed between samples to evaluate any potential carryover and contamination between every injection in the LC analysis.

3.2 Benzodiazepines Group

As previously mentioned, since this class of drugs shares the same benzene-diazepine ring in structure, the polarity of different Benzos can be similar, which can cause poor separation in chromatographic analysis due to the similar interaction with the stationary phase. The RT of each analyte was confirmed by running the analytes through the LC-MS/MS method individually to ensure a correct interpretation. Table 3-1 demonstrates that there was little difference in retention time (RT) between the selected Benzos, and only zolpidem could be separated apart from the other compounds, because there is no similarity in structure between Benzos and zolpidem.

Table 3-1 Compound identification of analytes in Benzos group.

Compound	RT[1] ± RSD[2]	Compound	RT[1] ± RSD[2]
Alprazolam	4.38 ± 0.000	Midazolam	3.38 ± 0.191
Clonazepam	4.36 ± 0.110	Nordiazepam	4.40 ± 0.119
Diazepam	5.24 ± 0.103	Oxazepam	3.86 ± 0.131
Flunitrazepam	4.75 ± 0.127	Temazepam	4.63 ± 0.101
Lorazepam	3.96 ± 0.118	Zolpidem	2.63 ± 0.245

[1]Retention time (min)
[2]Relative standard deviation (%)

There were 10 analytes selected for this class of drugs to prove the concept of the effects from the FS collection method. Figure 3-2 illustrates the chromatograph for this LC-MS/MS method.

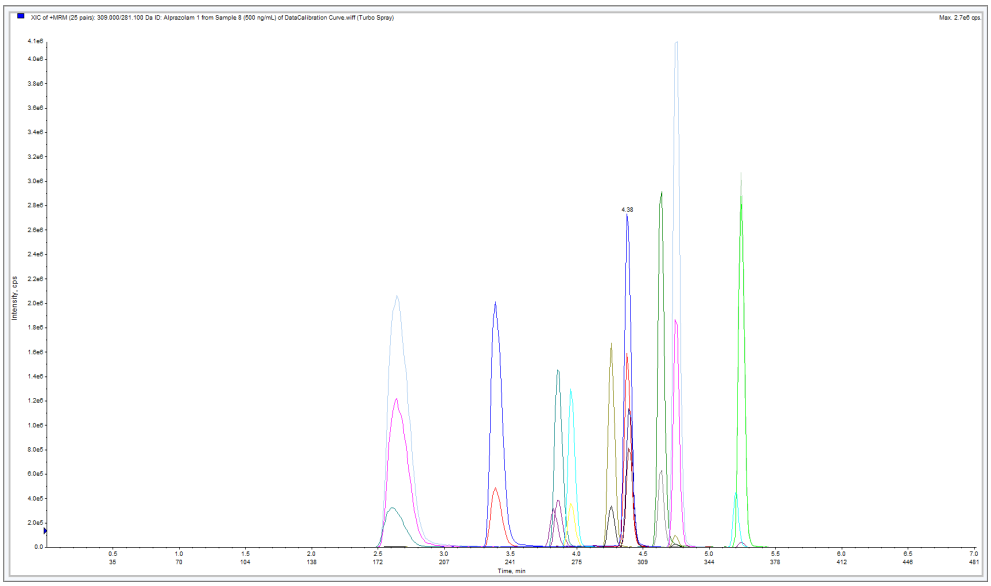


Figure 3-2 Chromatograph of the selected compounds in Benzos group.

A linear calibration curve was used for the calculation with a weighting factor of 1/x. The curve stability, data quality and assay performance could be impacted by the weighting factors. The linear regression is more likely administrated by the data at

higher concentrations, which causes errors at lower concentrations. The data from lower concentrations is not fitted with the calibration curve, and the R^2 value can be off the accepted limit. Applying an appropriate weighting factor to the calibration curve can compensate the errors at lower concentrations to improve the quality of the calibration curve. SCIEX® MultiQuant™ has a built-in analysis software that allows the operator to choose the optimal weighting factor, and 1/x gave the best results for the calibration curve of each analyte. (Figure 3-3) The accuracy was set to be +/- 20% and a calculated value greater or less than +/- 20% was considered to be inaccurate.

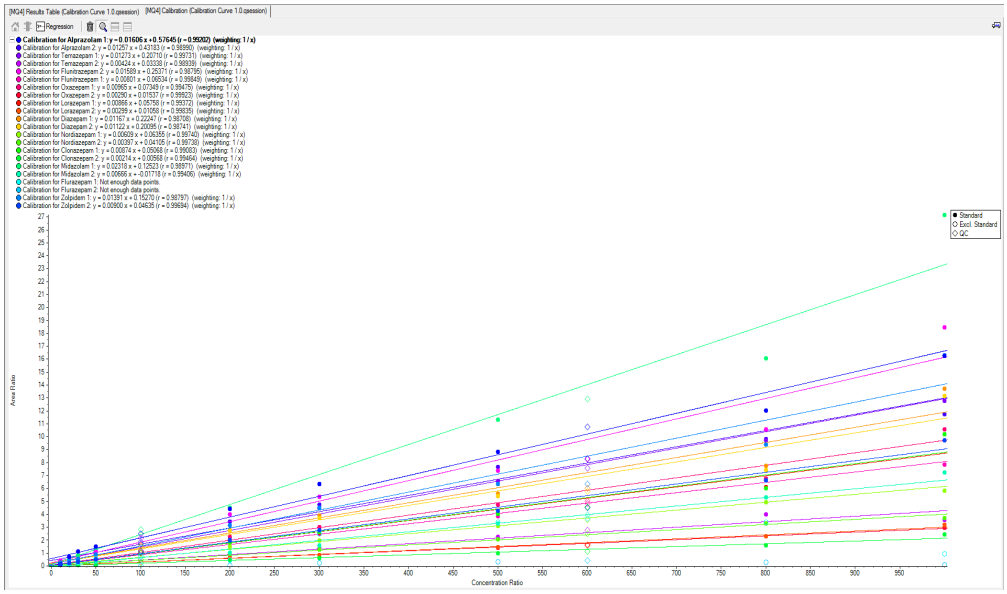


Figure 3-3 Calibration curve for all compounds with a weighting factor of 1/x. The R^2 value of each analyte was greater than the accepted limit of 0.98.

Figures 3-4 to 3-6 demonstrate that the concentrations of all analytes with the pre-treatment of toluene decreased dramatically regardless of the storage conditions with at least 50% degradation. This phenomenon indicated that 0.5% of toluene might be too high for the compounds of interest, and they were highly degraded.

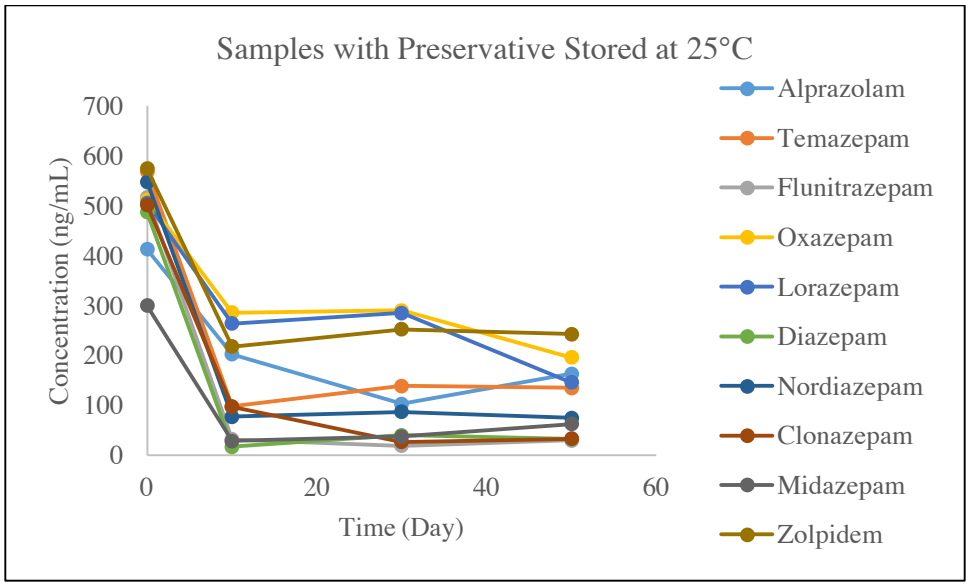


Figure 3-4 The changes of analytes in concentration with toluene stored at 25°C (target concentration was 500 ng/mL).

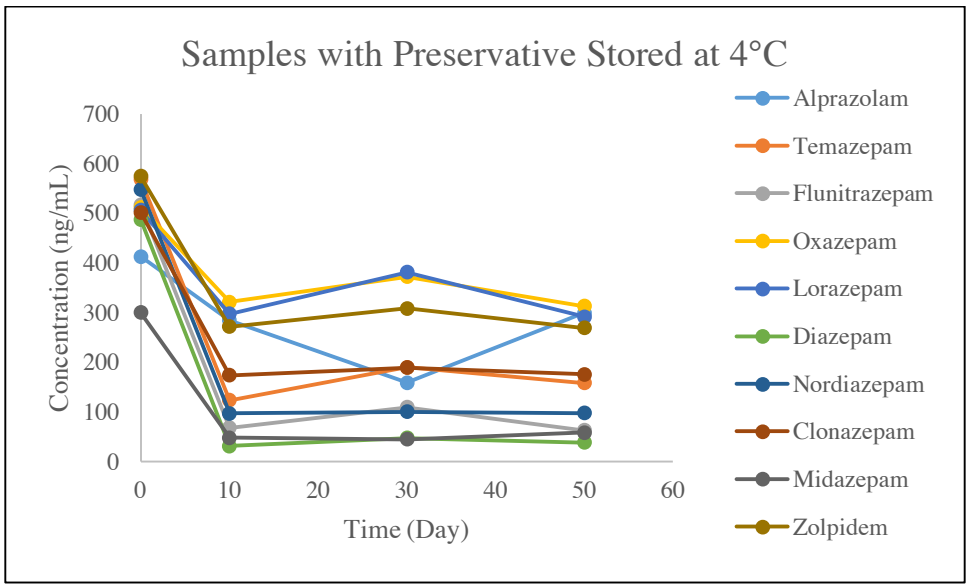


Figure 3-5 The changes of analytes in concentration with toluene stored at 4°C (target concentration was 500 ng/mL).

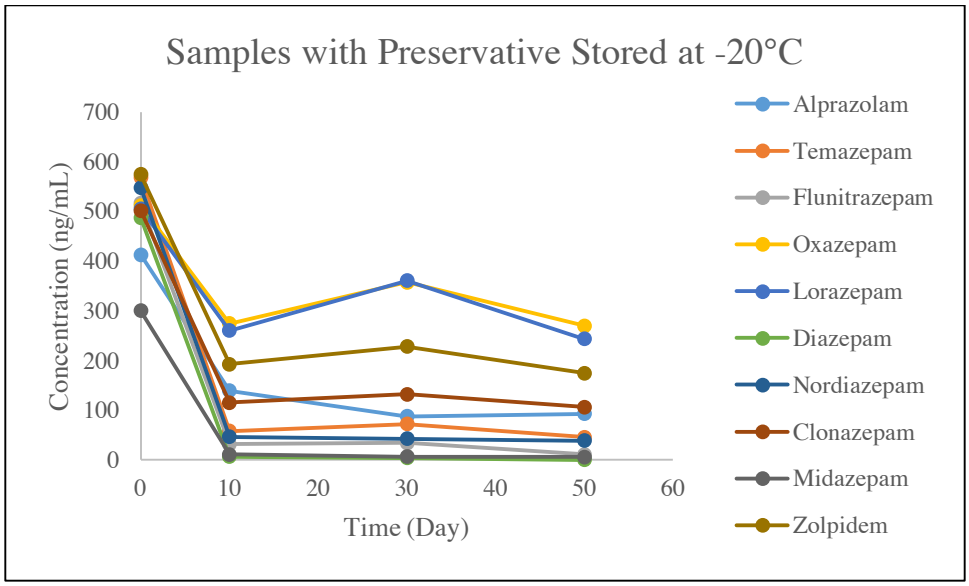


Figure 3-6 The changes of analytes in concentration with toluene stored at -20°C (target concentration was 500 ng/mL).

Figure 3-7 shows the test tube from one of the samples pre-treated with toluene, a bent line was observed, and this phenomenon was observed from all test tubes. Toluene didn't dissolve in the specimens, and it accumulated in the layer on the top of the sample. Toluene also dissolved the tube forming a mark around the test tube. Toluene and/or the organic materials dissolved from the tube might contribute the breakdown of the compounds. The samples with toluene pre-treatment will not be further discussed due to the dramatic change in concentrations among all analytes.

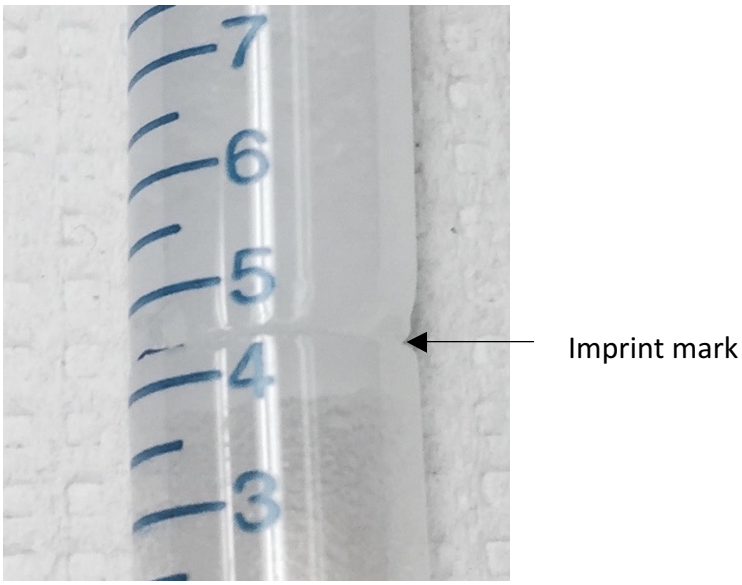


Figure 3-7 Imprint mark on Falcon test tubes with toluene pre-treatment after 10 days.

Regardless of the preparations of FS or non-treated samples, the analytes were preserved the most when the samples were stored under -20°C, and they were preserved the least when stored at room temperature. Clonazepam and flunitrazepam became undetectable after 30 days when the samples were stored at room temperature, but they were preserved when stored at 4°C. (Figures 3-8 to 3-13) These findings revealed that high temperature accelerates the degradation among the selected analytes, but also demonstrated that the freeze-thaw cycles did not cause the degradation.

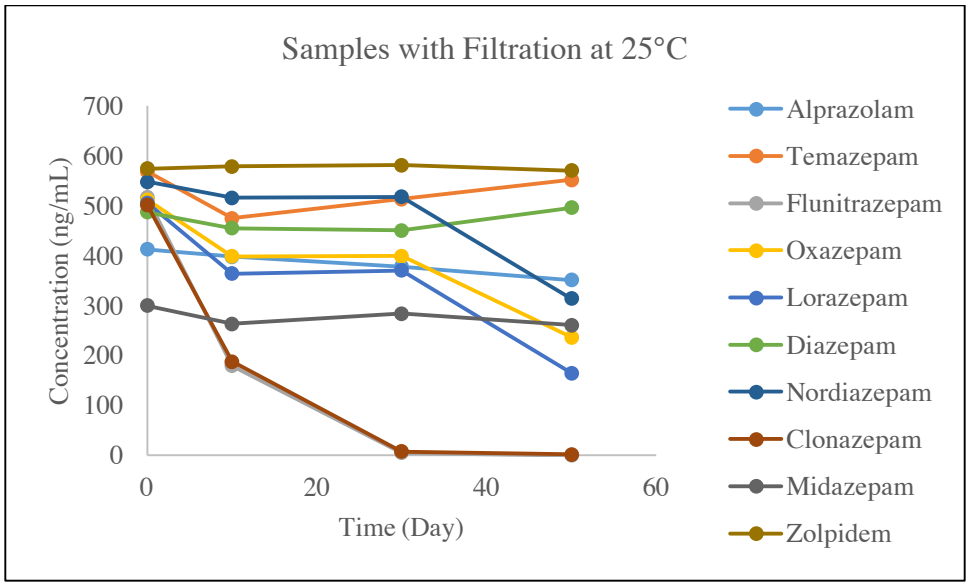


Figure 3-8 The changes in concentration of analytes with filtration pre-treatment stored at 25°C (target concentration was 500 ng/mL).

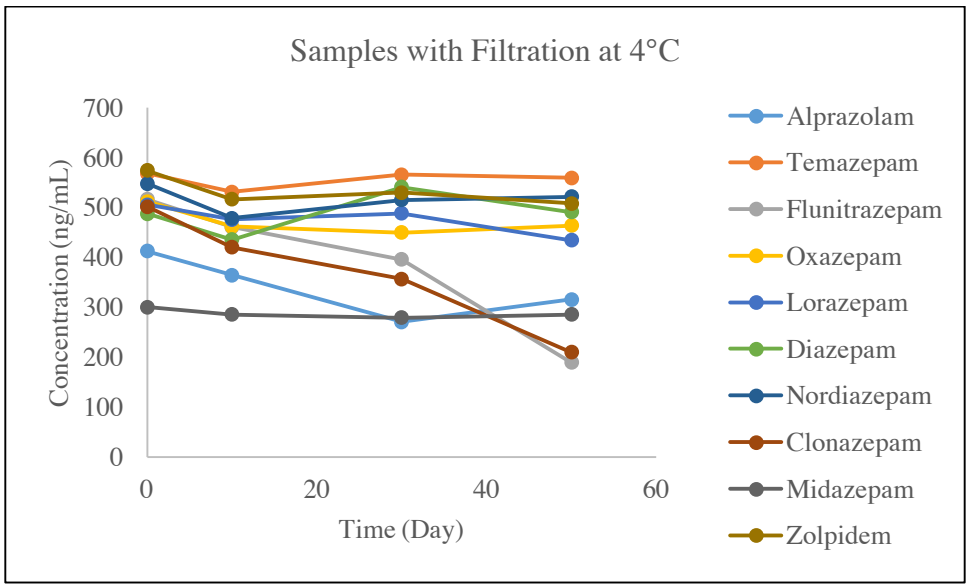


Figure 3-9 The changes in concentration of analytes with filtration pre-treatment stored at 4°C (target concentration was 500 ng/mL).

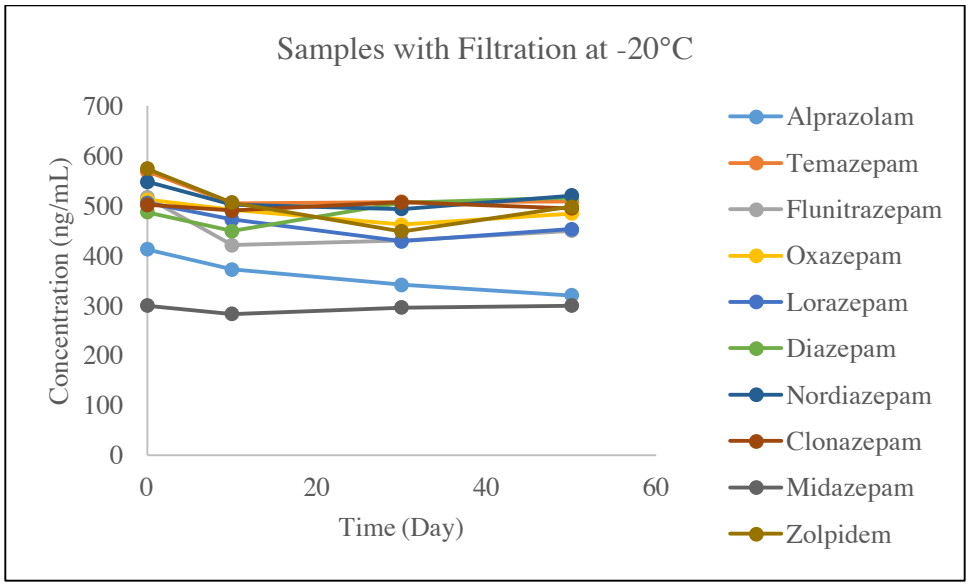


Figure 3-10 The changes of analytes in concentration with filtration pre-treatment stored at -20°C (target concentration was 500 ng/mL).

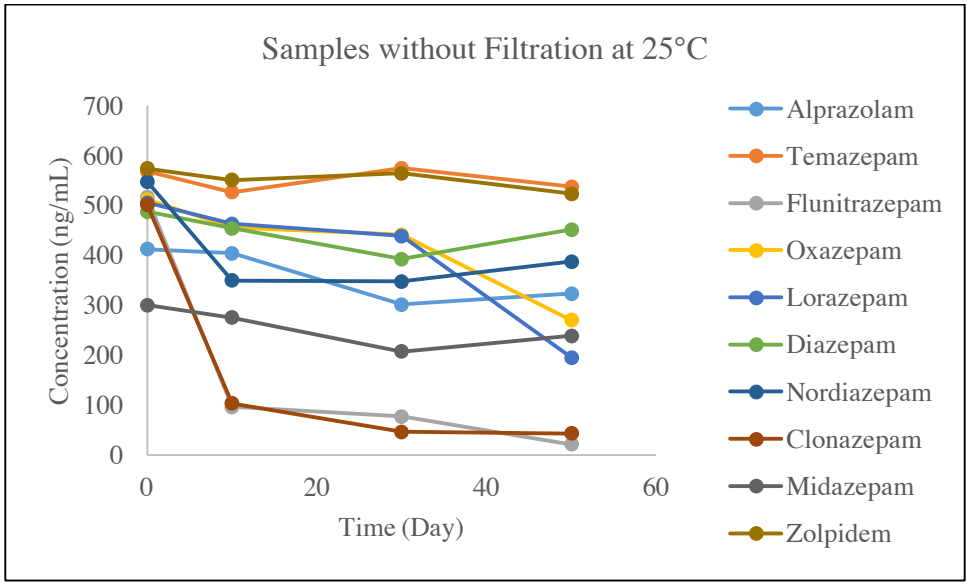


Figure 3-11 The changes of analytes in concentration without pre-treatment stored at 25°C (target concentration was 500 ng/mL).

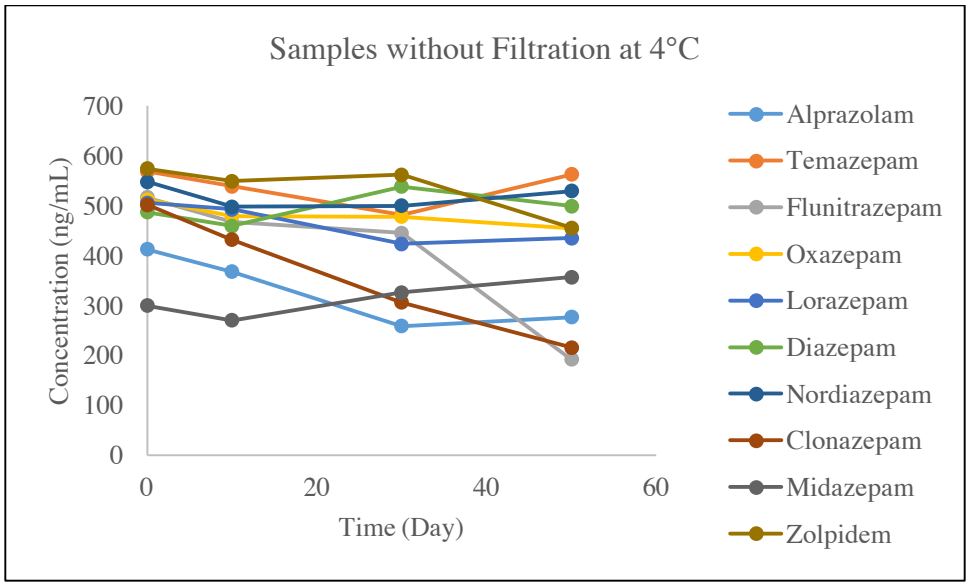


Figure 3-12 The changes of analytes in concentration without pre-treatment stored at 4°C (target concentration was 500 ng/mL).

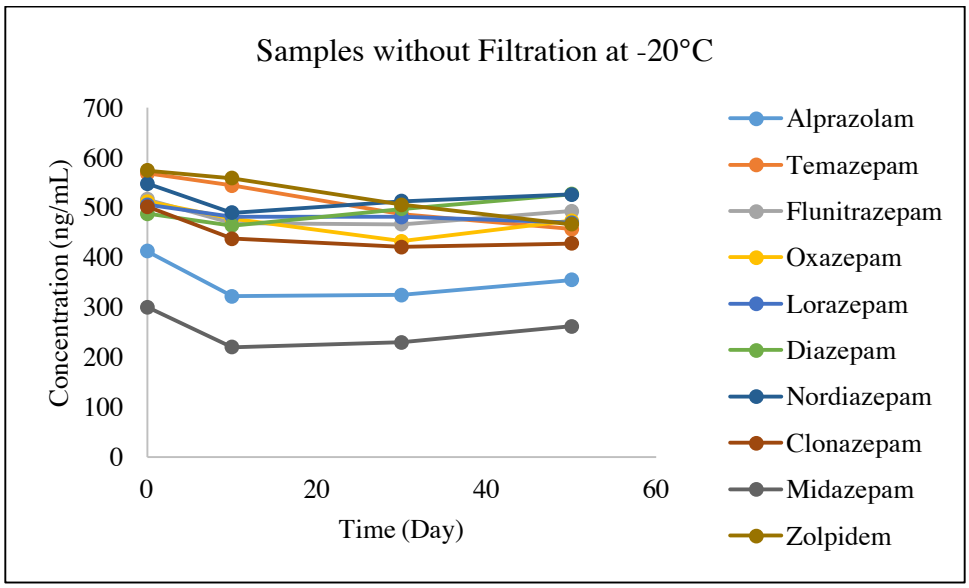


Figure 3-13 The changes of analytes in concentration without pre-treatment stored at -20°C (target concentration was 500 ng/mL).

Since the room temperature condition was not preferred for all the analytes, further analysis was performed to compare the changes between 4°C and -20°C storage

conditions. (Table 3-2) The results from student t-test suggest that there was no significant difference in changes when the samples were stored at either temperature. Best preservation can be achieved when samples are stored in the freezer, but it is acceptable to be stored in the refrigerator depending on the equipment and policy in the individual laboratory.

Table 3-2 Student t-test results for different storage conditions (target concentration was 500 ng/mL).

Analyte	Pre-Treatment	Mean		t-test[3]
		4°C	-20°C	
Alprazolam	FS[1]	341	362	59%
	N[2]	329	354	58%
Temazepam	FS	556	522	10%
	N	538	514	49%
Flunitrazepam	FS	391	454	42%
	N	405	486	31%
Oxazepam	FS	472	488	40%
	N	481	474	74%
Lorazepam	FS	476	465	63%
	N	464	485	39%
Diazepam	FS	489	490	97%
	N	496	494	91%
Nordiazepam	FS	516	516	99%
	N	519	519	98%
Clonazepam	FS	372	498	9%
	N	364	447	26%
Midazolam	FS	288	295	28%
	N	313	253	6%
Zolpidem	FS	532	507	43%
	N	535	526	81%

[1]FS – filtration sterilization
[2]N – no pre-treatment
[3]A value of 5% or greater suggests that there is no significant difference between the populations.

Table 3-3 demonstrates that there is no significant difference in concentration between with filtration and without filtration when the samples were stored at the same

temperature, except for clonazepam. The changes in concentration of each analyte behaved similarly between with and without filtration when the samples were stored at room temperature and in the refrigerator. Under the condition of -20°C, there was no degradation of clonazepam in the filtration sample, but 10.5% degradation of clonazepam was observed in the control sample. There were different degrees of degradation on each analyte, but the changes behaved similarly in the samples pre-treated with or without filtration technique, and no significant difference was observed.

Table 3-3 Student t-test results for different pre-treatments (target concentration was 500 ng/mL).

Analyte	Pre-Treatment	25°C		4°C		-20°C	
		Mean	t-test[3]	Mean	t-test	Mean	t-test
Alprazolam	FS[1]	385		341		362	
	N[2]	361	47%	329	81%	354	80%
Temazepam	FS	527		556		522	
	N	552	34%	538	43%	514	80%
Flunitrazepam	FS	175		391		454	
	N	178	99%	405	89%	486	24%
Oxazepam	FS	386		472		488	
	N	420	68%	481	64%	474	51%
Lorazepam	FS	351		476		465	
	N	401	63%	464	66%	485	31%
Diazepam	FS	472		489		490	
	N	447	30%	496	79%	494	83%
Nordiazepam	FS	474		516		516	
	N	408	40%	519	88%	519	87%
Clonazepam	FS	175		372		498	
	N	174	100%	364	93%	447	**3%**
Midazolam	FS	277		288		295	
	N	255	37%	313	23%	253	6%
Zolpidem	FS	576		532		507	
	N	553	9%	535	92%	526	61%

[1]FS – filtration sterilization
[2]N – no pre-treatment
[3]A value of 5% or greater suggests that there is no significant difference between the populations.

Table 3-4 demonstrates the difference between the extracted and unextracted samples, and the recovery was more than 50% for most of the analytes except midazolam and zolpidem. The recovery of midazolam was low for both 50 ng/mL and 200 ng/mL samples, with 46% recovery from the 50ng/mL samples and 31% recovery from the 200 ng/mL samples. The SPE procedure might not be preferred by midazolam, and further optimization is needed. However, the recovery of zolpidem for 50 ng/mL samples was 50% lower than the 200 ng/mL samples. Two 50 ng/mL zolpidem samples were undetectable, and one was only quantified at 9 ng/mL. Random error was ruled out since three samples were calculated at a very low concentration. This SPE method is specific for Benzos, because the chemical property of zolpidem is different from Benzos, and the elution solvent might not be strong enough to elute zolpidem, or zolpidem could be washed off during the washing step before elution. The other possibility is this SPE method favors zolpidem at the high concentrations, which is most likely since almost 50% recovery was achieved at the 200 ng/mL samples, and this instability can cause inaccuracy in quantification.

Table 3-4 Extraction recovery of selected Benzos.

Analyte	Mean (50 ng/mL)		Recovery	Mean (200 ng/mL)		Recovery
	Unextracted[1]	Extracted[2]		Unextracted	Extracted	
Alprazolam	60	31	52%	250	126	51%
Temazepam	62	58	93%	218	166	76%
Flunitrazepam	56	42	76%	244	205	84%
Oxazepam	51	32	62%	202	172	85%
Lorazepam	48	32	67%	149	134	89%
Diazepam	65	49	75%	189	145	77%
Nordiazepam	55	53	96%	250	204	82%
Clonazepam	52	45	85%	246	155	63%
Midazolam	59	27	46%	255	79	31%
Zolpidem	56	9	17%	205	91	44%

[1]Unextracted – analytes were added after the extraction step
[2]Extracted – analytes were spiked in the negative urine, and performed normal SPE procedure

3.2 Ketamine Group

Norketamine (NK) and dehydronorketamine (DNK) are the metabolites of ketamine (K), and the poor separation in the chromatographic analysis can be expected due to the similarity of structure, polarity and property as mentioned previously. Therefore, compound identification was the first step in analysis after the optimization steps were completed to ensure the correct interpretation. (Table 3-4)

Table 3-5 Compound identification of analytes in K group.

Compound	RT[1] ± RSD[2]
Ketamine	1.87 ± 0.289
Norketamine	1.70 ± 0.177
Dehydronorketamine	1.61 ± 0.323

[1]Retention time (min)
[2]Relative standard deviation (%)

Theoretically, the calibration curve for K was established based on the calculation of the peak area (concentration) of K-d4 (IS), and the calibration curve for NK and DNK was established based on the calculation of the peak area (concentration) of NK-d4 (IS). NK-d4 was not detected by LC-MS/MS from all the samples (Figure 3-14) and the absence of NK-d4 did not affect the detection and quantification of the K group. The elution time of K, NK and DNK were within 0.6 min in the 3-min method, and the R^2 value was greater than 0.99 for all the calibration curves. Therefore, K-d4 was sufficient for the analysis of K and metabolites by using this method.

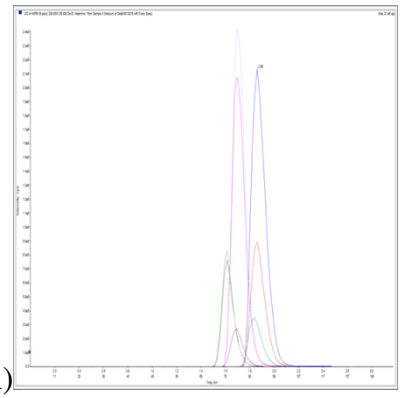
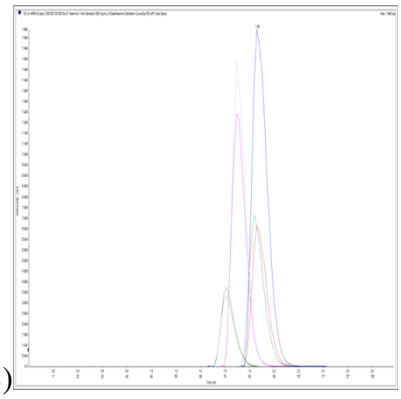
(A)  (B) 

Figure 3-14 The absence of NK-d4 in LC-MS analysis. (A is neat, B is sample)

The calculated concentrations with a range of +/- 20% of K and metabolites, and the calibration curve was established with a weighting factor of 1/x to lower the overall error. (Figure 3-15) There was a shortcoming in the analysis of K group in that the original instrumental analysis was an 8-min method, and it had been optimized to a 3-min method while the samples were aging. K is very stable in biological samples, and the initial analysis was designed to be performed after a 5-month storage. A set of samples

were prepared at the same concentration, extracted by the same SPE method and analyzed by using the 3-min method to estimate the Day 0 concentration. These initial concentrations were only used to demonstrate the changes in concentrations over time, and they did not represent the actual initial concentrations of the samples.

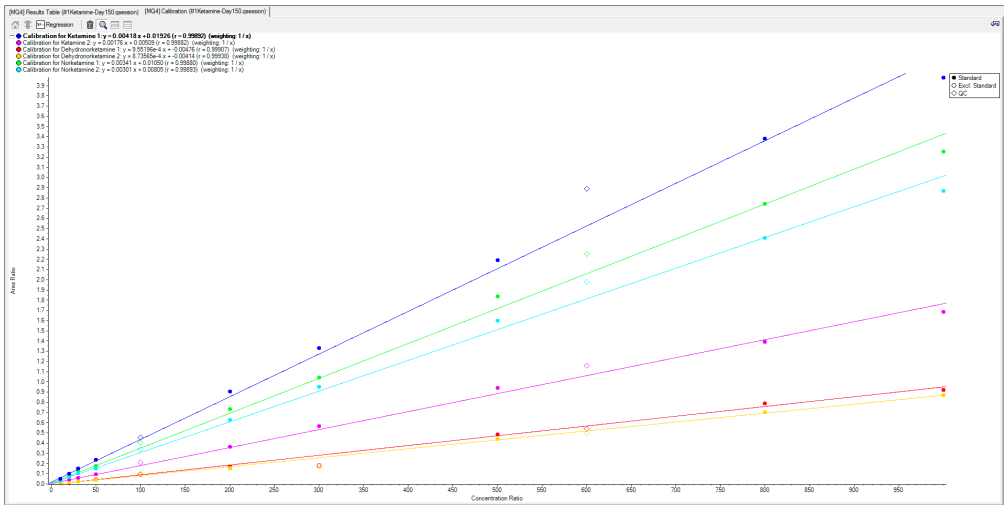


Figure 3-15 Calibration curve of all analytes with a weighting of 1/x.

The degradation caused by toluene in the Benzos group was not observed in the K group, and the high temperature condition did not contribute to the degradation. (Figures 3-16 to 3-21) However, DNK decreased dramatically after 150 days regardless of the pre-treatment methods or the storage conditions. This phenomenon revealed that K and NK are very stable in urine not only at various temperatures, but also in an organic environment. Dehydronorketamine is the dehydration product of NK, and it undergoes biotransformation to glucuronide conjugates in an acid environment; human urine is a neutral to acidic liquid that can cause the degradation of DNK.

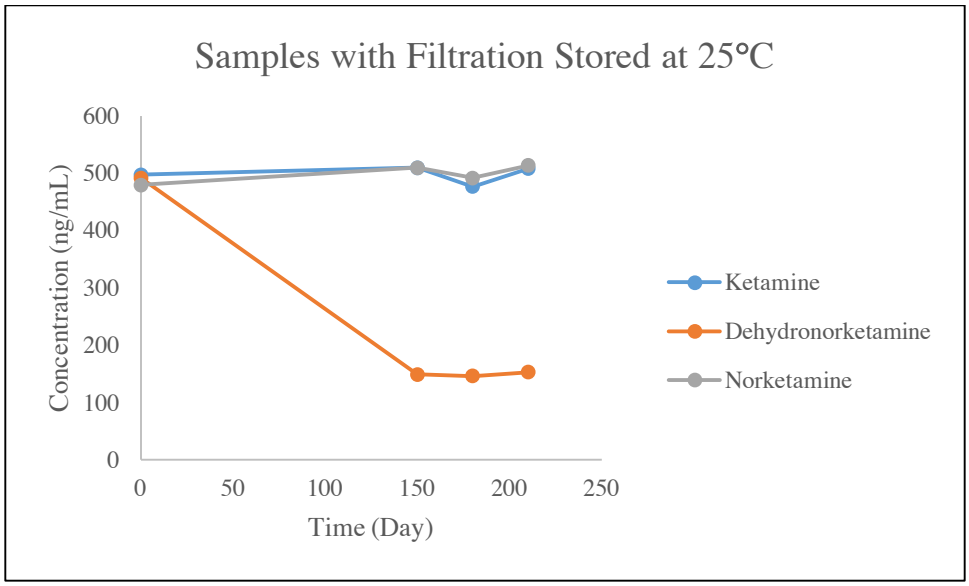


Figure 3-16 The changes of ketamine and metabolites with filtration stored at 25°C (target concentration was 500 ng/mL).

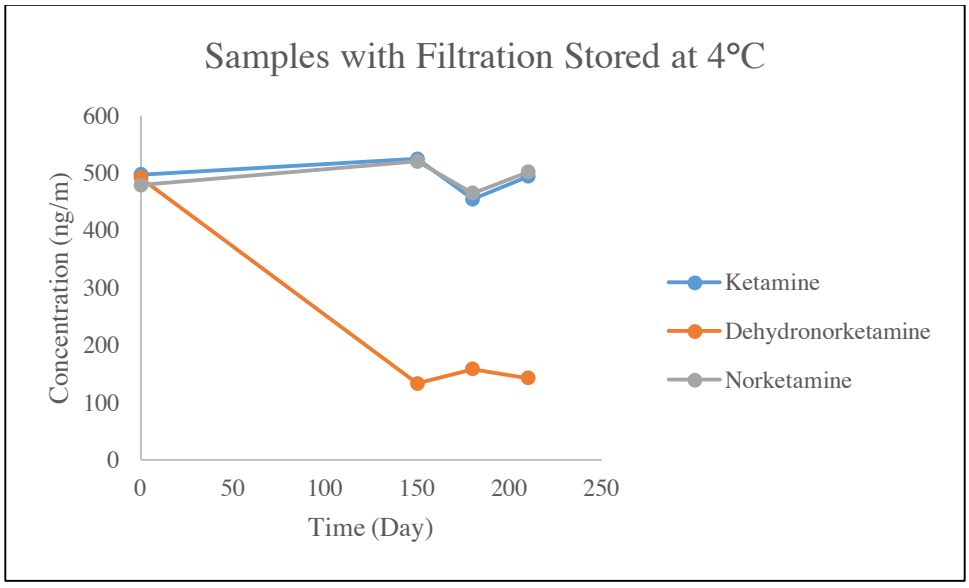


Figure 3-17 The changes of ketamine and metabolites with filtration stored at 4°C (target concentration was 500 ng/mL).

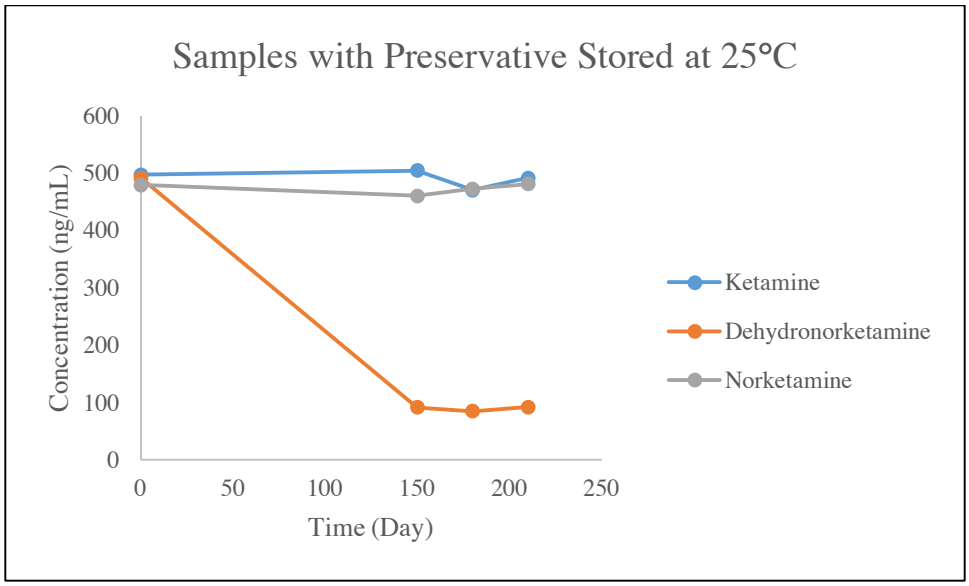


Figure 3-18 The changes of ketamine and metabolites with toluene pre-treatment stored at 25°C (target concentration was 500 ng/mL).

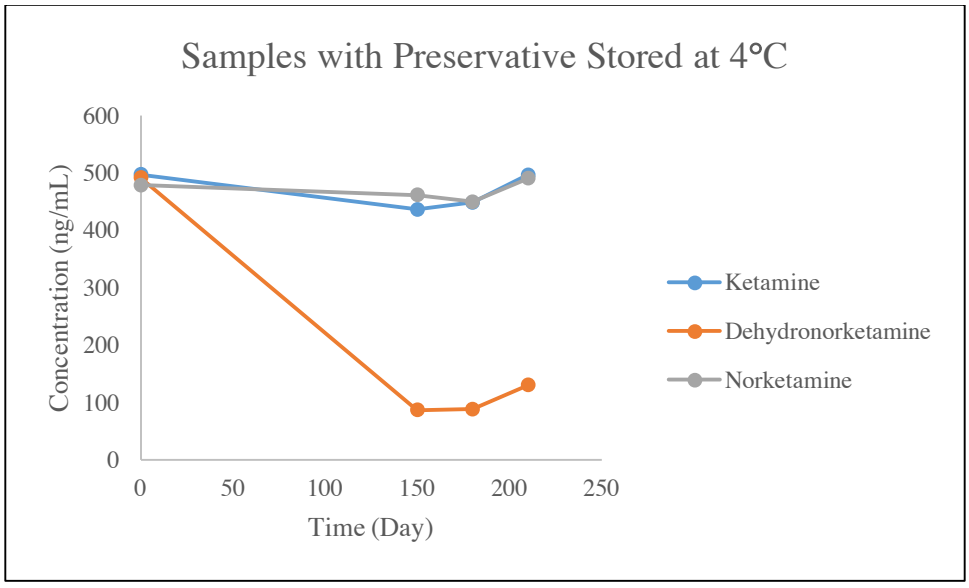


Figure 3-19 The changes of ketamine and metabolites with toluene pre-treatment stored at 4°C (target concentration was 500 ng/mL).

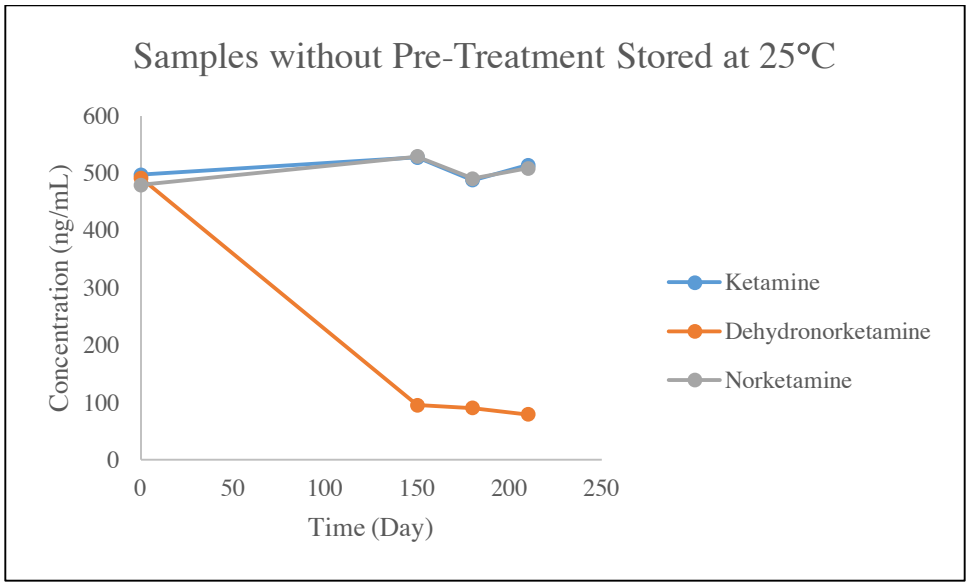


Figure 3-20 The changes of ketamine and metabolites without pre-treatment stored at 25°C (target concentration was 500 ng/mL).

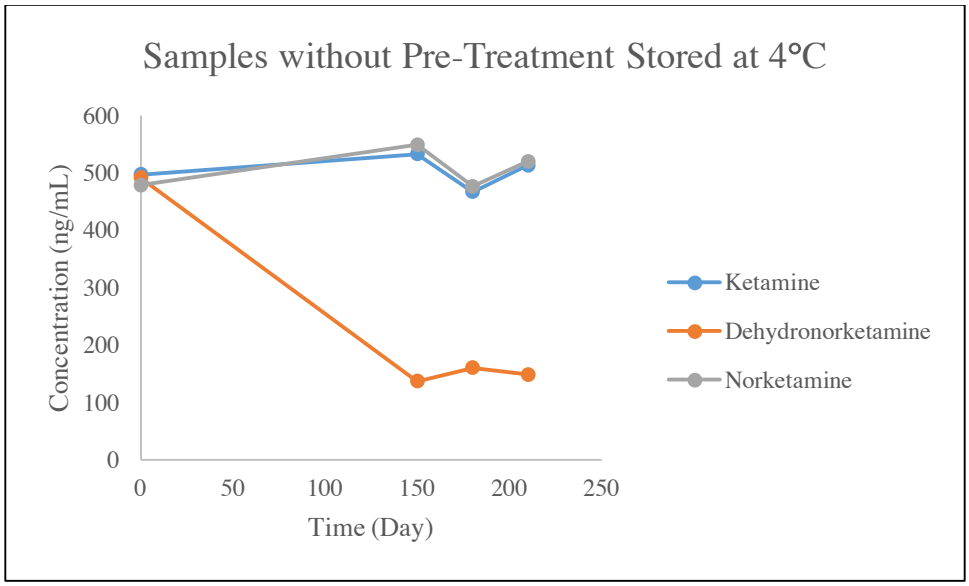


Figure 3-21 The changes of ketamine and metabolites without pre-treatment stored at 4°C (target concentration was 500 ng/mL).

However, the concentrations of K and NK from the samples stored in freezer decreased about 50% compared to the concentrations found on Day 150. (Figure 3-22 to 3-24) The freeze-thaw cycles should not cause the degradation, and this phenomenon was observed from all samples regardless of the pre-treatment methods. Further investigation on pH may explain why the pH was higher in all urine that was stored at room temperature and lower in samples stored in refrigerator and freezer. (Table 3-5) The initial pH of each urine specimen was not measured, but the changes behaved similarly under each condition regardless of donors and the time of aging. The pH increased to 8.5 when the specimens were stored at room temperature, which is outside of the pH range of normal human urine. There was no significant difference in pH between specimens stored in the refrigerator and freezer, and the pH fell within the range of normal human urine. Compounds were preserved better under low temperature conditions in general, and this phenomenon indicated that temperature affected pH, which contributed to compound degradation.

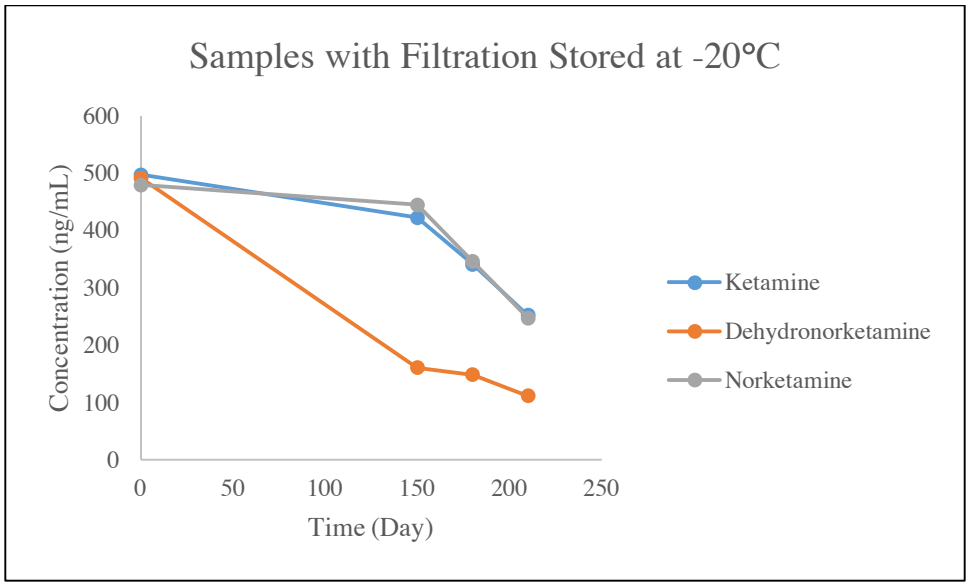


Figure 3-22 The changes of ketamine and metabolites with filtration stored at -20°C (target concentration was 500 ng/mL).

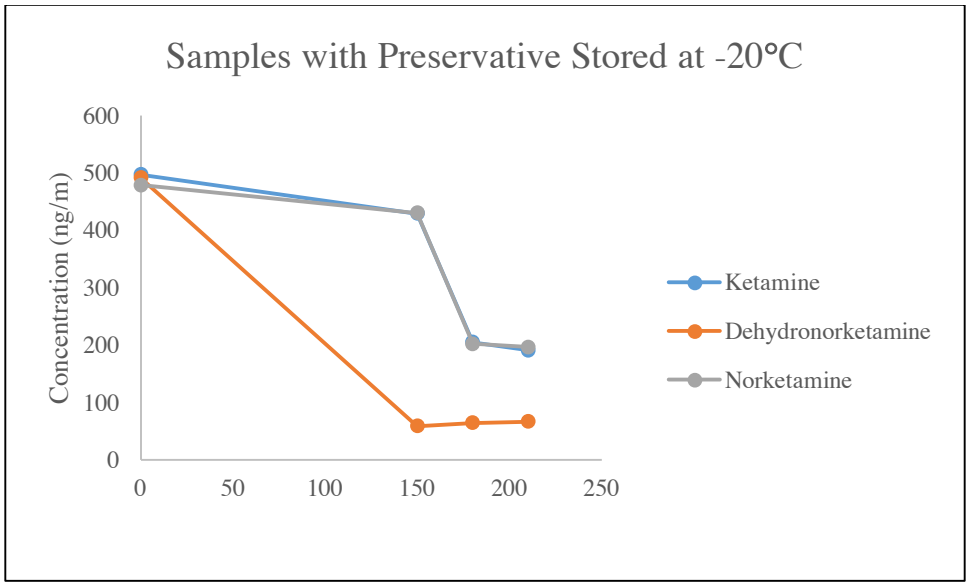


Figure 3-23 The changes of ketamine and metabolites with toluene pre-treatment stored at -20°C (target concentration was 500 ng/mL).

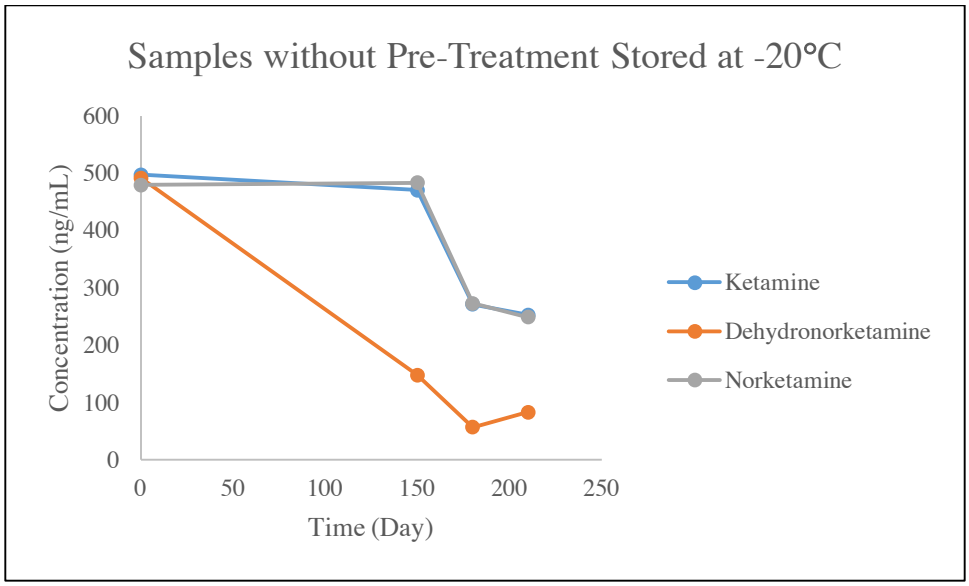


Figure 3-24 The changes of ketamine and metabolites without pre-treatment stored at -20°C (target concentration was 500 ng/mL).

Table 3-6 pH measurements.

	25°C		4°C		-20°C	
	B[1]	K[2]	B	K	B	K
	8.76	8.25	6.74	6.71	6.38	6.28
	8.35	8.66	6.86	6.78	6.64	6.62
pH	8.43	8.41	6.68	6.68	6.81	6.23
Mean	8.51	8.44	6.76	6.72	6.61	6.38
S.D.[3]	0.22	0.21	0.09	0.05	0.22	0.21
t-test[4]	69%		58%		25%	

[1]B – Urine used in Benzos group
[2]K – Urine used in K group
[3]S.D. – Standard deviation
[4]A value of 5% or greater suggests that there is no significant difference between the populations.

The recovery of this SPE procedure achieved more than 70% of recovery in K and NK, and about 40% recovery of DNK. (Table 3-6) Dehydronorketamine is less polar than K and NK due to the loss of two hydrogens, and easily washed through the column.

But the recovery was consistent between different concentrations, which would not cause inaccuracy in quantification that the Benzos group might have.

Table 3-7 Extraction Recovery of Ketamine and metabolites.

	Mean (50 ng/mL)			Mean (200 ng/mL)		
	Unextracted	Extracted	**Recovery**	Unextracted	Extracted	**Recovery**
K	63	52	82%	262	201	77%
NK	55	25	44%	220	101	46%
DNK	57	47	82%	217	189	87%

Regardless of the compounds studied in this project, compound breakdown caused by toluene and temperature effects have been discussed, but the effects of FS were unclear. The decreasing rates from the FS pre-treated samples were similar to the samples without any pre-treatment, and there are several possibilities to explain this. Theoretically, the filters were sufficient enough to filter E. coli since the pore size of the membrane is 0.2 μm compared to 1 μm, the smallest average size of the cross section of E. coli. Therefore, the first possibility is that most of the E. coli cells had smaller size than the average value, and the contamination level in the FS pre-treated samples was the same as the non-treated samples over time due to the incomplete removal of bacteria. The degradation in both samples was the same since the bacterial levels were the same, and the decreasing rate was the same among with and without FS pre-treated samples. The second possibility is that there were other types of bacteria in the urine causing compound degradation, and their size was much smaller than 0.2 μm. The last possibility is that bacterial contamination was not one of the factors contributing to

compound degradation, therefore, compounds could degrade even in a bacteria free sample.

pH could be a factor effecting degradation. The amount of bacterial contamination and the pH value are varied between individuals, and the effect of pH was not studied in this project. The pH level of human urine is between 6.5-8 depending on the time tested, ingested food substances consumed and medical history. Chemical compounds are sensitive to pH, and as discussed previously, pH did change over time with different rates under different temperatures, therefore, pH is believed to be the major reason contributing to compound degradation.

4 CONCLUSIONS

From the results of the microscopic examination, the amount of epithelial cells was reduced dramatically, which revealed that the filters functioned properly. However, no bacterial cells were observed from all of the slides from Day 0 to the last day of analysis regardless of the staining technique. Therefore, it could not be concluded that the selected filters could purify or semi-purify the urine specimens by filtrating out the bacterial cells.

In the investigation of the effects of different preservation methods on the stability of different classes of drugs, the concentrations of the samples pre-treated with toluene decreased much more than the other groups, from which it can be concluded that toluene is not suitable for the compounds studied. By comparing the samples pre-treated with

and without filtration, there was no difference found regardless of the storage conditions and the compounds. Therefore, the effect of filtration sterilization is unclear.

5 FUTURE DIRECTIONS

The microscopic results did not reveal the presence of bacteria, and the reasons have been previously discussed. Two fields can be studied to improve the results. An E. coli or bacteria specific staining technique can be utilized to make the bacterial cells more distinctive from the epithelial cells and visible under a mid-range magnification microscope. Utilization of a high magnifying power microscope (1000 times magnification) to examine the urine specimens is another option. However, a high-range microscope is expensive, and not every laboratory has access to one.

The bacterial contamination level might not be the major factor that affected the compound degradation, and pH may be the primary reason, as discussed previously. One of the future studies could be the evaluation of the pH effect on compound stability. However, it is necessary to confirm the absence of bacteria in the specimen for which filtration has been performed prior to the evaluation of the pH effect(s). Bacterial contamination must be ruled out from compound degradation, so that the concentration of target compounds could be compared with the changes in pH over time.

Benzodiazepines and ketamine are two classes of drugs with similar effects, and these effects can be enhanced when combined. It is a good interaction for patients because they can get the optimal effect with a lower dose, but this is also good to sexual assailants because the adverse effects are greater. It is common to detect more than one

class of drugs in DFSA crimes, therefore, one of the future studies can be the development of an analytical method to detect and quantify both classes of drugs, or an analytical method to detect all Benzos with better separation and resolution since there are many Benzos that have been reported in DFSA crimes.

LIST OF JOURNAL ABBREVIATIONS

Biophys J	*Biophysical Journal*
Br J Clin Pharmacol	*British Journal of Clinical Pharmacology*
Can Med Assoc J	*Canadian Medical Association Journal*
Clin Chem	*Clinical Chemistry*
Eur J Clin Pharmacol	*European Journal of Clinical Pharmacology*
Forensic Sci Int	*Forensic Science International*
Forensic Sci Rev	*Forensic Science Review*
Immunol Cell Biol	*Immunology and Cell Biology*
J Clin Microbiol	*Journal of Clinical Microbiology*
J Interpers Violence	*Journal of Interpersonal Violence*
J Pharm Biomed Anal	*Journal of Pharmaceutical and Biomedical Analysis*

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Curriculum Vitae

Lin Zhen

Year of Birth: 1988
11 Botolph Street, North Quincy, MA 02171
781-888-2866 lzhen@bu.edu

Education

Master of Science in Biomedical Forensic Sciences **Anticipate January 2017**
Boston University, Boston, Massachusetts
Bachelor of Science in Biochemistry **May 2013**
University of Massachusetts, Boston, Massachusetts

Professional Experience

Graduate Thesis Project **January 2015 – Present**
Boston University – Boston, MA

- Develop instrumental methods to detect and quantify selected compounds by using HPLC-MS/MS-ESI.

Evaluate the factors that cause compound degradation and investigate prevention methods.

Research Assistant **August 2016 – Present**
Northeastern University – Dr. Jonghan Kim's Laboratory, Boston, MA

- Study postmortem redistribution of morphine and the co-administration with fentanyl and ethanol in rats.
- Assist for animal sacrifice and specimen collection, and perform instrumental analysis by utilizing LC-MS/MS.

Intern **June 2016 – August 2016**
Massachusetts State Police Crime Laboratory – Postmortem Toxicology, Maynard, MA

- Performed method validation on a scheduled MRM LC-MS/MS method for detection and quantification of selected opioids followed by SWGTOX recommendations.

Laboratory Technician **October 2015 – May 2016**
Quintara Biosciences, Allston, MA

- Performed DNA sequencing on purified or non-purified samples by using the SimpliSeq^{TM} DNA sequencing technique, or the DirectSeq^{TM} DNA sequencing technique on the colony samples.