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A novel screening method for glucuronidated and non-glucuronidated drugs in urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Thesis

A NOVEL SCREENING METHOD FOR GLUCURONIDATED AND NON-GLUCURONIDATED DRUGS IN URINE BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

by

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ABSTRACT

Opioids are often prescribed to patients experiencing long-term, chronic pain. Although they have many benefits to the patient, opioids are highly addictive medications and have a high abuse potential. For this reason, pain management clinics regularly send patient urine samples to toxicology laboratories to ensure that the opioids they are prescribing are not being misused.

Many labs operate under a strict budget, so being able to screen for the presence of opioids (in addition to a host of other drugs) quickly and economically is a top priority. Most labs screen bodily fluids using a form of immunoassay, but immunoassay techniques have a number of disadvantages, including their high cutoff values, low degree of specificity and selectivity, high expense, and lack of included drugs. Therefore, replacing the immunoassay technique with a more selective and sensitive technique like liquid chromatography-tandem mass spectrometry (LC-MS/MS) can prove to be a good alternative for labs looking to minimize expense.

Typical LC-MS/MS methods that analyze urine require samples to be prepared with a hydrolysis step. The hydrolysis reaction cleaves the glucuronide moiety that the liver

attaches to certain drugs (like opioids) during the metabolism process. Although hydrolysis is effective in recovering the parent drug structure, it is expensive and time-intensive.

With the goal of a more efficient, sensitive, specific, and cost-effective analysis method, a novel screening method that replaces the immunoassay instrument and eliminates the hydrolysis reaction was created. The new method screened for the glucuronidated form of many drugs with an LC-MS/MS instrument. Drugs that are glucuronidated in the liver include morphine, hydromorphone, tapentadol, codeine, oxymorphone, oxazepam, and buprenorphine.

Creating the novel method required tuning the instrument for the glucuronidated drugs, adding these glucuronidated drugs to the lab's existing LC-MS/MS method, and validating the new method. The method's validation study evaluated several parameters, including linearity, precision and bias, interference, specificity, limit of detection (LOD), limit of quantitation (LOQ), client comparisons, and carryover, to ensure that the instrument could reliably detect every drug in the LC-MS/MS method accurately.

The method validation parameters were within acceptable limits, and the validation study was submitted to the New York State Department of Health for approval. However, there were two findings that are important to mention. Morphine glucuronide and hydromorphone glucuronide were not able to be individually resolved in the LC-MS/MS method. If one or two peaks are detected at morphine glucuronide/hydromorphone glucuronide's retention time, the sample will carry a presumptive positive screen for both compounds to the confirmatory method. The confirmatory method will verify if the sample is positive for one, both, or neither drug. Second, it was not possible to detect alprazolam

at its full, known concentration. Several possible explanations, including matrix effects, stability, and the possibility of glucuronidation, were evaluated with no increase in detection; therefore, alprazolam's ion ratios require individual evaluation in order to identify a sample presumptively positive for alprazolam.

Although glucuronidated drugs have been detected in previous methods, this novel method is unique because it utilizes an LC-MS/MS to screen for six different glucuronidated drugs in addition to 21 non-glucuronidated drugs found in the urine of pain management patients. By replacing the immunoassay instrument and eliminating the hydrolysis step of the screening method, the laboratory not only saves time and expense but is able to more accurately screen for more drugs at lower concentrations.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

6-MAM	6-monoacetyl morphine
amu	atomic mass units
BEH	Ethylene Bridged Hybrid
C18	Carbon 18
CE	Collision energy
CXP	Collision cell exit potential
DP	Declustering potential
ESI	electrospray ionization
G6PDH	glucose-6-phosphate dehydrogenase
LC	Liquid chromatograph
LC-MS	Liquid chromatograph-mass spectrometer
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MDMA	3,4-methylenedioxyamphetamine
MS	Mass spectrometer
m/z	mass to charge ratio
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
rt	retention time
SIM	Selected Ion Monitoring

THC   tetrahydrocannabinol

μL   microliters

1. INTRODUCTION

1.1 Prescription Pain Medications

With more than 131 million prescriptions dispensed in 2022, prescription pain medications have become an integral part of pain management practices in the United States. Prescription pain medicines are commonly prescribed to manage chronic pain originating from both cancer and other non-cancer chronic conditions. One major class of pain relief prescription medications, opioids, have proven to be highly effective in managing chronic pain, as 70-90% of patients with cancer pain report adequate relief and an improved quality of life as a result of their use. (1) However, opioids are highly scrutinized for a number of reasons. Because they trigger the release of dopamine in the brain, opioids induce a sense of euphoria that can extend beyond pain management and lead to abuse. (2) As a result, opioids are criticized for being unsafe due to their tendency to result in dependence or addiction. (1) Dependence is defined by withdrawal symptoms upon the discontinuation of the drug use. (3) Addiction is more severe than dependence and is characterized by compulsive use and a disruption to normal life. According to Dr. Barry Levine, a board-certified toxicologist, developing an addiction occurs in 3-31% of opioid users, and individuals with previous substance abuse disorders or untreated mental illness, like depression or post-traumatic stress disorder, could potentially be at a greater risk to develop an opioid addiction. (3) As of 2010, opioid abuse was considered an epidemic by experts, with around 12 million Americans reporting non-medical use of prescription pain pills. (3)

In addition to their medical concerns, opioids are also concerning to law enforcement. Drug diversion occurs when patients that are prescribed licit drugs sell the drug instead of following their pain management protocols. These diverted drugs are often illegally sold to those that have developed opioid dependencies and/or addictions, and this action represents a large source of opioids for the illicit drug market. (3)

1.2 Champlain Toxicology Lab

Champlain Spine and Pain Management and The Surgical Pain Center of the Adirondacks make up a combined pain management program. The affiliated surgical center in northern New York treats spinal injuries and medical conditions affecting the spine. Spinal injuries are frequently accompanied by chronic pain; and therefore, a high percentage of the practice's patient pool is prescribed opioids to manage this chronic pain condition. As a result, Dr. Thierry Bonnabesse, founder and physician at Champlain Spine and Pain Management, built the Champlain Toxicology Lab to carry out routine urine drug testing on the practice's patients. Routine urine drug testing (called compliance monitoring) ensures that patients follow their pain management protocols and avoid other illicit drugs. This helps to identify cases of abuse and/or diversion of these powerful narcotic analgesics and is an added safety measure to support the continued use of prescription opioids, despite their inherent risk of dependence and addiction. A review conducted in 2008 found that 11.5% of patients prescribed opioids for chronic pain management were involved in deviant drug-related behaviors or illicit drug use, demonstrating the necessity of urine drug screening on chronic pain patients. (4,5) Ultimately, these urine drug screening results are important because they determine

whether the patient's insurance will continue pain management medication prescription coverage and/or whether an addiction recovery program is needed.

1.3 Urine Drug Testing

When selecting a matrix for toxicological analysis, it's important to consider the purpose of the testing. Typical specimen choices for antemortem toxicology testing include blood, oral fluid, or urine. These biological matrices are considered based on a drug's detection window within them as well as their ease of collection.

Blood is an important specimen for many toxicological analyses. It provides a true picture of the concentration of drugs in a person's system at the time of collection and is substantiated by extensive literature. Blood, however, requires trained phlebotomists, and its collection is invasive to the patient. It also has a short drug detection window of 1-2 days. (6) For compliance monitoring, Champlain Toxicology Lab requires a specimen with a longer detection window, such as urine, to obtain a better understanding of a patient's recent drug history as well as pain management compliance. Consequently, blood is not the ideal specimen of choice for Champlain Toxicology Lab.

Oral fluid is a popular antemortem sample due to its ease of collection, limited invasiveness, and lack of adulteration potential. (3) Oral fluid collection is unique in that the collection can be performed by the patient/suspect directly. The entire collection process can be observed by the officer/collector, thereby eliminating the risk of sample adulteration. However, oral fluid has its limitations. The detection window for drugs in oral fluid is shorter than the detection window for drugs in urine. While the detection window

for drugs in urine is 1 to 7 days, the detection window for drugs in oral fluid is only 5 to 48 hours. (6,7)

For Champlain Toxicology Lab, urine is the ideal specimen to use due to its long detection window and ease of collection. The long detection window is a result of a gradual release of the drug from body tissues. When drugs enter the bloodstream, they can bind to plasma proteins in the blood and/or leave the bloodstream to bind to body tissues (adipose, muscle, etc). If drugs bind to the body tissues, the tissues can gradually release the drug over time as the concentration of the drug in plasma decreases. (8) This results in a longer window of detection for drugs in urine and provides toxicologists with a better understanding of a patient's recent drug history.

Second, urine is simple to collect. The collection causes no pain to the patient, and there normally is enough sample volume available for testing purposes, as standard toxicological testing can be completed with less than 2 milliliters of sample. Additionally, urine collection can be observed by another member of the same sex, ensuring that the specimen isn't substituted or adulterated during the collection process. This is significant as adulteration alters the composition of the sample and impacts the results of toxicological testing. Lastly, the collection is not considered invasive to the patient and is typically able to be easily collected from a patient.

The Champlain Toxicology Lab is not limited to the analysis of prescription pain medications. The laboratory also analyzes patient urine samples for other licit and illicit drugs that patients could be taking to create a clearer picture of a patient's entire drug regimen. This includes other common prescriptions like benzodiazepines and

amphetamines, along with frequently abused drugs like cocaine, methamphetamine, fentanyl, and tetrahydrocannabinol (THC).

1.4 Drug Metabolism

After absorption, drugs are modified or broken down via metabolism. Metabolism can occur in the stomach, liver, and/or intestines. Metabolism's main function is to alter the parent drug and create a form of the drug that is easier for the body to excrete in an aqueous liquid such as urine. (3) Understanding a drug's metabolic pathway and how its metabolites sequester in urine is essential in toxicology.

Metabolism is split into two steps, Phase I and Phase II. Phase I metabolism involves the oxidation, reduction, or hydrolysis of drugs. Oxidative reactions, catalyzed by the enzyme cytochrome p450 oxygenase, are the most common and alter the functional groups of xenobiotic molecules. Phase I metabolites are more hydrophilic due to the introduction or exposure of functional groups on the drug. Phase I reactions can also result in an active metabolite from an inactive parent drug. Drugs with hydroxyl, amine, or carboxylic acid groups often skip Phase I reactions and enter straight into Phase II metabolism. (3,9)

During Phase II metabolism, conjugation reactions take place. Conjugation reactions employ an enzyme to catalyze the addition of an endogenous substance to the parent drug or to the product of Phase I metabolism. One important conjugation reaction, glucuronidation, utilizes the UDP-glucuronosyltransferases (UGTs) enzymes to attach a negatively-charged glucuronide moiety to a drug or Phase I metabolite. (3) For example, the parent drug morphine undergoes glucuronidation in Phase II metabolism. Figure 1

illustrates morphine's biotransformation into both morphine-6-glucuronide and morphine-3-glucuronide in the liver. (10)

Figure 1. Biotransformation of morphine into morphine-6-glucuronide and morphine-3-glucuronide (10)

This metabolism results in a more hydrophilic molecule which is the ultimate goal of Phase II metabolism. (11) Because urine is 95% water, hydrophilic molecules more easily partition into urine and thus are more easily eliminated. (12) As a result, many drugs are excreted in urine as metabolites. This requires toxicologists to screen for drug metabolites in addition to parent compounds.

1.5 The Homogenous Enzyme Immunoassay Technique

Champlain Toxicology Lab screens urine qualitatively then confirms positive results with a second more sensitive and specific confirmatory method. This two-step testing process is considered the gold standard in the toxicology community. The

preliminary drug screen is performed by the Olympus AU400© (Beckman Coulter, Brea, California) an automated chemical analyzer. This instrument performs a variety of techniques including homogenous enzyme immunoassay (HEIA™). (13) This instrument screens for many drugs/drug classes including amphetamines, benzodiazepines, buprenorphine, cocaine, cotinine, ethyl glucuronide, opiates, oxycodone, fentanyl, methadone, barbiturates, and THC.

The HEIA™ technique detects a drug through an enzyme/substrate reaction. This is a competitive technique in which the enzyme-labeled drug competes with the free drug in the sample. These two species compete for spots on a structure known as an antibody. Antibodies are produced by an organism in response to a foreign substance entering the body. The role of the antibody is to attach onto the foreign substance to initiate an immune response.

This attachment is mediated by structural complementarity, like a lock and key mechanism, between the antibody and the foreign substance. Antibodies are configured by the body have two areas called "Fab fragments" that bind to specific regions on a foreign substance. (14,15) However, when there are a limited number of antibodies present, and therefore a limited number of Fab fragments, the foreign substances that fit into the binding sites on the Fab fragments have to compete with one another for binding sites on the surface of the antibody. (15)

The enzyme-labeled drug and the free drug have similar structures, so they both can attach to the binding sites on the Fab fragments, but the presence of the enzyme on the enzyme-labeled drug allows for their differentiation when one is bound to the

antibody versus the other. After a sufficient amount of time has elapsed to allow the

enzyme-labeled drug and the free drug to bind to the antibody binding sites, a reagent

containing nicotinamide adenine dinucleotide (NAD) is subsequently added to the

sample. If the enzyme-labeled drug *is not* bound to the antibody's Fab fragment, the

enzyme (glucose-6-phosphate dehydrogenase (G6PDH)) remains active and will convert

NAD to nicotinamide adenine dinucleotide hydride (NADH). However, if the enzyme-

labeled drug *is* bound to the antibody, the enzyme is inactivated and will not convert the

NAD to NADH. The more free drug in the sample, the less enzyme-labeled drug will be

able to bind to the binding sites on the antibodies, which means that more of the NAD

molecules will be converted to NADH molecules by the G6PDH enzyme. Therefore, the

enzymatic activity of G6PDH can be used as an indicator for the presence of free drug in

the sample and is detected spectrophotometrically at 340 nm. Low enzymatic activity of

G6PDH indicates the presence of free drug in a sample. (16)

1.5.1 Limitations of the Homogenous Enzyme Immunoassay Technique

The Olympus AU400© instrument is effective in most cases, but one of its

significant drawbacks involves the necessity for additional testing. The limitations of the

enzyme immunoassay technique are four-fold: high cutoff values, low degree of

specificity, high expense, and lack of included drugs.

First, the Olympus AU400© uses reagent kits that have high cutoff values for

most drug classes, requiring a significant amount of analyte present in the sample to

trigger a positive result. High cutoff values increase the possibility of false negatives in

the screening phase of testing and hinder the laboratory's ability to detect low-concentration samples. (17)

Second, the enzyme immunoassay technique lacks specificity, meaning that the technique is incapable of distinguishing structurally similar compounds from each other, like amphetamine and 3,4-methylenedioxyamphetamine (MDMA). Figure 2 potrays the structural similarities between amphetamine and MDMA. The lack of specificity can be especially problematic in cases where prescription medications, like pseudoephedrine, are structurally similar to illicit substances like methamphetamine. (3)

amphetamine   3,4-methylenedioxyamphetamine

Figure 2. Structures of amphetamine and 3,4-methylenedioxyamphetamine (MDMA)

Antibodies' Fab fragments that are designed to interact with an amphetamine molecule could potentially bind to another structurally similar molecule such as methamphetamine. This is an example of cross reactivity which could occur in enzyme immunoassay screening tests. Cross reactivity can lead to low specificity, meaning that a presumptive positive result for one analyte could indicate that the sample is positive for a structurally similar yet different analyte. Specificity is a key aspect of analytical testing,

and the lack of specificity in enzyme immunoassay testing is a notable drawback for this technique.

Next, the Olympus AU400© requires two individual reagents for every drug class, an antibody/substrate reagent and an enzyme conjugate reagent, which results in an expensive technique. The expense of the reagents varies by analyte. On October 8, 2023, the cost to purchase an amphetamine reagent kit was $259.67 from Immunalysis™. (16) The Champlain Toxicology Lab analyzes approximately 70 samples per day, and the reagent kits are emptied approximately every seven business days, meaning the laboratory spends $259.67 approximately every seven days to detect just one analyte. To test for 20 analytes at $259.67 each, the laboratory would spend approximately $5,193.40 per week on the screening method alone.

Lastly, the Olympus AU400© lacks the capability to test for variety of analytes due to the absence of kits. These analytes include most glucuronidated drug metabolites and other common metabolites like 7-aminoclonazepam. The lack of reagent kits is likely due to the complexity of their production. To create a reagent kit needed for enzyme immunoassay, a drug is injected into an organism (in most cases, a rabbit), and in response, the organism produces antibodies to that drug. The antibodies are collected from the serum of the animal source to be used in the enzyme immunoassay reagent kits. (3) However, if manufacturers haven't injected a particular drug into an animal source and harvested the resulting antibodies, then those reagent kits don't exist, and as previously mentioned, this is the case for many metabolites despite their importance in urine drug testing.

The limitations of the Olympus AU400© HEIA™ method prompted Champlain Toxicology Lab to seek a more sensitive and specific as well as less expensive method for screening urine samples. There are a variety of screening techniques that a toxicology laboratory can use for drug screening, but liquid chromatography-tandem mass spectrometry (LC-MS/MS) was chosen because it is a particularly sensitive, highly specific instrument that delivers cost-effective testing (18).

1.6 Liquid Chromatography-Tandem Mass Spectrometry

LC-MS/MS combines two technologies: liquid chromatography and mass spectrometry. The liquid chromatograph (LC) is responsible for the separation of analytes, and this is process occurs prior to introduction to the mass spectrometer (MS). As the prepared urine sample travels through the column of the LC, drugs are separated out due to their varying affinities with the stationary phase and mobile phase.

The liquid chromatograph takes advantage of each drug's chemical properties, namely their affinities for the stationary phase of the column. In the case of LC, the term stationary phase refers to the particles packed inside of a short metal column. After a prepared urine sample is mixed with the mobile phase solution, the liquid is carried through the system into the particle-packed column. As analytes flow through the column, they interact with the stationary phase for varying amounts of time, depending on the properties of each analyte. Therefore, as the sample flows through the column, some analytes will come off the column and enter the MS faster than others. Ultimately, this separates the drugs in the sample from each other by their retention time, the amount of time that each drug requires to flow through the column. (3)

After the analytes exit the column, they enter the MS. The MS's role is to identify each of the separated analytes, which it accomplishes by analyzing molecular fragments. There are several configurations of MS, but the MS used in the Champlain Toxicology Lab utilizes a triple quadrupole mass analyzer. This analyzer is often referred to as a "triple quad" or "tandem mass," meaning it identifies molecules using three different sets of four rods. Figure 3 demonstrates how the three sets of four rods are aligned in tandem. The flow of the analytes through the quadrupoles is indicated by the arrow moving through the center of the three sets of quadrupoles (18).

Figure 3. Diagram of a triple quadrupole mass analyzer with three tandem quadrupoles.

The first and the third quadrupole each act as a filter. The first quadrupole filters for one particular molecular mass out of every compound in the sample using radio frequency/direct current voltages. (3) For example, for a specified mass range, the first set of quadrupoles could filter the sample for a molecular mass of 303 atomic mass units (amu). Every other molecule without a molecular mass of 303 amu will be filtered out, while molecules with a molecular mass of 303 will continue through the system to the second quadrupole. (19)

The second quadrupole acts as a collision cell. Its role is to break a molecule with a particular molecular mass into its fragments. (19) This fragmentation yields many different pieces of each molecule depending on its molecular structure. Using the previous example of the 303 amu molecule, after the collision cell, some parent molecules remain unfragmented while others fragment into smaller pieces of the original molecule.

Following the collision cell, the third quadrupole is where the final filtering process occurs. Instead of filtering out parent molecules like the first quadrupole, the third quadrupole filters molecular fragments. This last quadrupole filters the different fragments of each parent drug molecule, allowing for identification of the parent molecule.

1.6.1 Advantages of LC-MS/MS

There are many advantages to using an LC-MS/MS as a screening instrument over the traditional automated immunoassay. Not only is the instrument ultra-sensitive and specific due to its ability to recognize fragments of molecules, but it can be adapted and paired with a simple sample preparation technique that minimizes the expense of the analysis.

1.6.1.1 Sensitivity of the LC-MS/MS

Using just the third quadrupole, a MS can be programmed in two different modes—Scan or Selected Ion Monitoring (SIM) mode. With Scan mode, the instrument

searches for signals within a given mass range, not particular molecular mass-to-charge

ratios. In SIM mode, an operator inputs specific molecular masses into the instrument

method to configure the third quadrupole to filter for these particular molecular masses.

Because the mass spectrometer is scanning for a limited number of mass-to-charge ratios,

the instrument can spend a longer amount of time searching for each mass-to-charge

ratio. The increased search time results in a highly sensitive instrument with a low limit

of detection for analytes. (20)

1.6.1.2 Specificity of the LC-MS/MS

The current immunoassay technique can exhibit cross reactivity which reduces the

specificity of the screening results. While cross reactivity is permissible for a screening

technique, high specificity is a desired characteristic of toxicological analyses. In contrast

to immunoassay, an LC-MS/MS is an instrument that is regarded as exceptionally

specific. Because it can fragment and filter certain molecular masses, it can target and

identify specific analytes of interest. The third quadrupole in the LC-MS/MS can filter

for molecular mass fragments or pairs of molecular mass fragments that are unique to one

particular parent drug which lends to its high specificity. (14)

1.6.1.3 Expense of the LC-MS/MS

When considering an LC-MS/MS, it is critical to assess potential expenses

associated with sample preparation and the overall cost of analysis. Most urine LC-

MS/MS methods, including the confirmatory method at Champlain Toxicology Lab,

require extensive sample preparation procedures. These procedures typically involve a hydrolysis reaction that uses an acid or the enzyme beta-glucuronidase to cleave off the glucuronide moiety that was attached to the molecule during Phase II metabolism. (3,21–23) The standard beta-glucuronidase hydrolysis method used at the Champlain Toxicology Lab requires the analyst to add the biological sample, a buffer solution, the internal standard mixture, and beta-glucuronidase to an Eppendorf© centrifuge tube. After these components are combined, they must be incubated for thirty minutes at fifty-five degrees Celsius in order for the beta-glucuronidase to successfully cleave off the glucuronide moiety. Then curve diluent is added, and the sample is centrifuged for ten minutes. Following centrifugation, the supernatant is transferred to a flat-bottomed LC-MS/MS vial containing an insert.

This costly and extensive sample preparation process was an important consideration for the Champlain Toxicology Lab when re-examining its existing processes. A 50 mililiter bottle of beta-glucuronidase purchased from Integrated Micro-Chromatography Systems (imcs®) (IMCS, Irmo, SC, USA) lasts for around one month and costs the Champlain Toxicology Lab approximately $900 per bottle, meaning that the hydrolysis step adds approximately one dollar per sample to the technique's expense. (24) The beta-glucuronidase enzyme greatly increases the overall cost of an LC-MS/MS analysis procedure and therefore is contrary to the goals of the novel screening method.

With the combined incubation period and centrifugation times, the sample preparation technique involving a hydrolysis step requires a minimum of forty-five minutes to complete. The new LC-MS/MS screening method employs a simple dilution

without hydrolysis via beta-glucuronidase for a faster and easier sample preparation procedure.

1.6.2 Method Decision

Ultimately it was decided that the novel screening method would employ an LC-MS/MS instrument with a simple dilution sample preparation step of 100 μL of sample, 400 μL of diluent, 50 μL of internal standard. The LC-MS/MS instrument is advantageous for two major reasons. First, expenses are lowered because the LC-MS/MS does not require the expensive reagent kits used on the Olympus AU400© immunoassay instrument. This saves the lab thousands of dollars a week. Second, an LC-MS/MS allows for the detection of drug metabolites for which immunoassay reagent kits have not been created. Instead of requiring antibodies to be harvested from a donor species, any drug analyte can be added to an LC-MS/MS method with a simple tuning process.

Second, simple dilution was chosen as the sample preparation method. Many LC-MS/MS methods utilize extensive sample preparation with the beta-glucuronidase enzyme. However, the beta-glucuronidase enzyme is expensive for a clinical lab and requires a long incubation period, so the new screening method eliminated the beta-glucuronidase enzyme by testing for glucuronidated drugs. This allows for a simple dilution sample preparation, saving additional costs and time.

1.7 Creating a Novel LC-MS/MS Method

To effectively eliminate beta-glucuronidase enzyme hydrolysis, the LC-MS/MS was configured to detect glucuronidated analytes in urine samples. This configuration

adjusted many parameters in both the LC and MS portions of the instrument, as both

required optimization to detect each new analyte, and both contribute to the instrument's

abilities and efficiency.

1.7.1 Configuring the MS Method

The MS in this method will detect analytes using the multiple reaction monitoring

(MRM) technique. This technique has the ability to scan for the presence of multiple

analytes simultaneously, allowing researchers and laboratory technicians to screen

complex matrices for many analytes of interest. (19) Transition is the term used for the

pairing of a parent ion and one of its fragment ions. (25) Each analyte needs at least two

transitions in the mass spectrometer method, one corresponding to the quantitative ion

and one corresponding to the qualitative ion. The quantitative ion is the fragment that is

typically the most abundant fragment that the method will use to quantitate how much of

the analyte is present in the sample. The qualitative ion can be the second most abundant

fragment for that analyte molecule or a unique fragment ion that differentiates that

analyte from other similar analytes. (26) When the method contains molecules that are

structurally similar, a unique fragment is a good choice for the qualitative ion.

The first step of configuring a new MS method with new analytes is choosing the

quantitative and qualitative ions for each new analyte. Using SCIEX Analyst® software

(Framingham, Massachusetts), these ions are chosen by putting the MS into Q1 MS (Q1)

scan mode, injecting the analyte into the MS using a syringe and a syringe pump, and

deciphering the mass spectrum. (27) The resulting mass spectrum is a graph with peaks at

each mass-to-charge ratio (m/z) that produces a signal. Ideally, the m/z with the highest (most abundant) peaks are chosen to be the transitions for that analyte so that there is a response for the ions detectable even at low concentrations.

Once the two transitions are chosen, the instrument is tuned so that the abundances of each ion are maximized. There are three key parameters that alter how much of the ion is detected, and they must be individually configured for each transition: collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP). The CE is a measure of how much energy is used to fragment the ion in the collision cell. The DP is the voltage used to rid the sample of the last drops of mobile phase before it enters the mass spectrometer to prevent the ions from clustering together, and the CXP is the voltage used to send the ions from the second quadrupole into the third quadrupole. (28) Figure 4 shows different areas of the mass spectrometer that can be optimized, focusing on optimizing CE, DP, and CXP.

Figure 4. Voltages applied in mass spectrometry. DP, CE, and CXP are important voltages applied in mass spectrometry.

There are two ways to optimize these three parameters. This process can be done manually by altering each of the three parameters individually to optimize abundances. This process is time consuming but allows the analyst to see how each of the three parameters individually affects the abundance of ions detected.

Alternatively, the process can be completed through the Tuning mode on SCIEX Analyst® software, which is how this method was developed. This mode of the software optimizes the CE, DP, and CXP parameters in a fraction of the time of manual tuning. (27) In Tuning mode, the LC is bypassed entirely and instead the sample is introduced directly into the source of the MS using a gas-tight syringe and automated syringe pump. A syringe is filled with a concentrated solution of the analyte to be tuned. Then the syringe is connected to the MS via ferrule and PEEK™ plastic tubing. The syringe is then placed into an automated syringe pump that slowly pushes a steady amount of solution through the PEEK™ tubing and into the source of the MS. (27)

As the MS is fed a constantly supply of the analyte of interest, the CE, DP, and CXP potential are easily altered and the analyst can view results in real time. The Tuning mode of the instrument creates a graph of the abundances as they are detected, demonstrated by abundance versus voltage for each parameter. Once these three parameters are optimized for each analyte, the values are input into the method with their corresponding MRM transitions.

1.7.2 Optimizing Chromatography Parameters

Second, the LC parameters were configured in a way that maximizes the resolution of the analytes. Resolution is the instrument's ability to separate two closely eluting compounds. Factors including the choice of mobile phase and the gradient time program need to be considered to ensure that the analytes of interest are resolved.

First, the mobile phase in most reversed-phase liquid chromatography is an aqueous solvent like methanol or acetonitrile. One aqueous solvent may be able to resolve two compounds that another is unable to resolve. (3) Second, the gradient time program has a significant effect on the resolution of analytes. The gradient time program of a liquid chromatograph is a schedule of how the instrument will change the aqueous percentage of the total mobile phase over the run time of the method. As an analyte interacts with the nonpolar stationary phase of the liquid chromatography column, it will attach onto the stationary phase until its affinity for the mobile phase exceeds its affinity for the stationary phase. (29) As the aqueous percentage of the mobile phase slowly increases, analytes will lose their affinity with the stationary phase in an order determined by their physical properties and functional groups. By manipulating the aqueous percentage of the mobile phase, analysts can slow down the gradient to separate two similar analytes. (30) Even two similar molecules, like the previous example of amphetamine and MDMA, have small differences in structure that will make them lose their affinity for the stationary phase at slightly different times. Therefore, analysts can manipulate the gradient time program to separate all analytes included in an LC-MS/MS method.

1.8 Method Validation

The novel LC-MS/MS method must be validated before it can be used for casework. Method validation is a process in which the laboratory verifies that the method is configured in a way that not only detects each analyte but detects each analyte at the proper concentration. For qualitative screening techniques, like this novel method, detecting the analyte at the proper concentration refers to the method accurately determining when an analyte is above or below the cutoff value. Champlain Toxicology Lab's requirements for validation include linearity, precision, bias, specificity, interference, limit of detection (LOD), limit of quantitation (LOQ), client comparisons, and carryover.

Linearity is a study conducted to ensure that the signal detected by the instrument is linear for the range of the calibrators when graphed versus the concentration of analyte in the sample. At least 3 replicates of each calibrator were evaluated. The line also must produce an r value of greater than 0.98 to be accepted for both the quantitative and qualitative ions and this method followed these criteria. (31,32)

Precision is a study of how close the values are to one another, and bias, often referred to as accuracy, is a study of how close an experimental value is to the true value. (32) For qualitative methods, it is imperative to show that the method is robust around the cutoff value, meaning that the LC-MS/MS does not result in false positives for negative samples or false negatives for positive samples. In this research, precision and bias were evaluated by challenging the cutoff value with four concentrations, 50%, 75%, 125%, and 150% of the cutoff concentration.

The validation study also needed to prove the specificity of the method. For this study, urine was spiked with the analytes of interest, and each peak was analyzed for any distortion in peak shape or shifts in retention time to ensure that the peaks were not exhibiting interferences. (32)

Next, interferences were evaluated to ensure that no endogenous molecule or common over-the-counter medications were being detected as one of the analytes of interest. (31) Ruling out the possibility of false positives from over-the-counter medications or endogenous substances was an important faucet in bolstering the method's specificity. The interferences tested included but is not limited to: nitroglycerin, metoprolol, fluoxetine (Prozac), clopidogrel (Plavix), trelegy ellipta, docusate sodium (Colace), donazepil (Aricept), albuterol, gabapentin, atorvastatin (Lipitor), acetaminophen, aspirin, ibuprofen, hydroxyzine, penicillin, bupropion (Welbutrin), glucosamine chondroitin, and THC.

LOD is defined as the lowest concentration of analyte that can be reliably distinguished from noise. For the validation study, it was required that the limit of detection have a signal-to-noise ratio of 3:1 or greater, meaning that the height of the static surrounding the analyte of interest's peak was not greater than 1/3 of the analyte of interest's peak height. (31)

LOQ is similar to the LOD, but it is defined as the lowest concentration of analyte that the method can quantitate accurately and precisely. For the validation study, it was required that the LOQ have a signal-to-noise ratio of 10:1 or greater, meaning that the

height of the static surrounding the analyte of interest's peak was not greater than 1/10 of the analyte of interest's peak height.

Client Comparisons was a part of the method validation that compared results of the previous method to results of the novel method for at least twenty-five samples. The results for the novel method needed to correspond to what had previously been reported in at least 90% of cases in order to pass this validation study.

Last, carryover is a study done to ensure there is no analyte signal in a sample after the analysis of a highly concentrated sample. It is possible for patients to have high concentrations of a drug in their bodies in drug abuse, high tolerance, or overdose scenarios. In these cases, it must be determined how concentrated a sample can be before the highly concentrated sample begins to affect the subsequent sample. Carryover was evaluated at 500 times the highest calibrator's concentration.

2. MATERIALS AND METHODS

2.1 Instrumentation and Software

This LC-MS/MS screening method can analyze twenty-seven chosen drugs with an API 4000 Triple Quadrupole LC-MS/MS (electrospray ionization mode) with an integrated Shimadzu LC System (SCIEX Redwood City, CA, USA). Analyst® software was used to set up parameters for liquid chromatography and mass spectrometry, and quantitative peak data was analyzed with MultiQuant® software, both produced by SCIEX (Redwood City, CA, USA).

2.2 Reagents and Standards

Liquid chromatograph-mass spectrometer (LC-MS) grade water and acetonitrile was obtained from Millipore Sigma Corporation® (Burlington, Massachusetts), and methanol and isopropanol were both obtained from Honeywell® (Morristown, New Jersey). The synthetic urine solution was obtained from RICCA® (Arlington, Texas), and the formic acid used in the Mobile Phase A and Mobile Phase B was purchased from Cova Chem® (Loves Park, Illinois).

Twenty-seven certified reference standards were purchased from Cerilliant® (Table 1.) Most standards were 1.0 mg/mL solutions made in methanol, but buprenorphine-3-beta-D-glucuronide, morphine-6-glucuronide, hydromorphone-3-beta-D-glucuronide, oxymorphone-3-beta-D-glucuronide, tapentadol-beta-D-glucuronide, and oxazepam glucuronide were 100 ug/mL solutions in methanol. Tapentadol-beta-D-glucuronide was made in 1 mL of acetonitrile:water instead of methanol.

Table 1. Certified Reference Standards used in the novel screening method development and validation.

Analyte	Identifier	Concentration	Lot Number	Expiration Date
Amphetamine	A-007-1ML	1.0 mg/mL in 1 mL MeOH	FE03012217	12/2027
Methamphetamine	M-009-1ML	1.0 mg/mL in 1 mL MeOH	FE03132001	06/2025
Meperidine	M-035-1ML	1.0 mg/mL in 1 mL MeOH	FE12152103	01/2027
Meprobamate	M-039-1ML	1.0 mg/mL in 1 mL MeOH	FE03162219	06/2027
Carisoprodol	C-077-1ML	1.0 mg/mL in 1 mL MeOH	FE08312119	12/2026
EDDP perchlorate	E-022-1ML	1.0 mg/mL in 1 mL MeOH	FN06112137	06/2026
Methadone	M-007-1ML	1.0 mg/mL in 1 mL MeOH	FE06252002	06/2025
Tramadol	T-027-1ML	1.0 mg/mL in 1 mL MeOH	FE03252111	03/2026
Ketamine HCl	K-002-1ML	1.0 mg/mL in 1 mL MeOH	FE07172002	08/2025
Benzoylecgonine	B-004-1ML	1.0 mg/mL in 1 mL MeOH	FE03032102	03/2026
Oxycodone	O-002-1ML	1.0 mg/mL in 1 mL MeOH	FE08312139	09/2026
Hydrocodone	H-003-1ML	1.0 mg/mL in 1 mL MeOH	FE04241902	09/2024
Ritalinic Acid HCl	R-011-1ML	1.0 mg/mL in 1 mL MeOH	FN03242015	05/2025
7-aminoclonazepam	A-916-1ML	1.0 mg/mL in 1 mL MeOH	FN03122004	05/2025
Trazodone HCl	T-030-1ML	1.0 mg/mL in 1 mL MeOH	FE08312135	09/2026
Alprazolam	A-903-1ML	1.0 mg/mL in 1 mL MeOH	FE12172003	12/2025
6-acetylmorphine	A-009-1ML	1.0 mg/mL in 1 mL MeOH	FE07082101	08/31/2023
Norfentanyl oxalate	N-031-1ML	1.0 mg/mL in 1 mL MeOH	FC05132004	11/2025
Fentanyl	F-013-1ML	1.0 mg/mL in 1 mL MeOH	FE03252103	04/2026

PCP	P-007-1ML	1.0 mg/mL in 1 mL MeOH	FE09252108	12/2028
Buprenorphine-3-beta-D-glucuronide	B-035-1ML	100 ug/mL in 1 mL MeOH	FE05042007	08/2024
Morphine-3-beta-D-glucuronide	M-018-1ML	100 ug/mL in 1 mL MeOH	FE04251901	10/2024
Codeine-6-beta-D-glucuronide	C-126-1ML	1 mg/mL in 1 mL MeOH	FE12111805	03/2023
Oxazepam glucuronide	O-023-1ML	100 ug/mL in 1 mL MeOH	FN09231905	11/2022
Hydromorphone-3-beta-D-glucuronide	H-051-1ML	100 ug/mL in 1 mL MeOH	FE01112112	01/2026
Oxymorphone-3-beta-D-glucuronide	O-030-1mL	100 ug/mL in 1 mL MeOH	FE06072101	06/2026
Tapentadol-beta-D-glucuronide	T-060-1ML	100 ug/mL in 1 mL Acetonitrile: water (1:1)	FE03022001	04/2025

2.3 Sample Preparation

100 μL of patient urine was diluted with 400 μL of 20 mM of ammonium formate buffer in 80:20 water:methanol. 50 μL of internal standard was added to each sample. The internal standard solution was prepared with standards all purchased from Cerilliant®. Samples were subsequently centrifuged at 30,000 rpm for 10 minutes. 200 μL of the supernatant was pipetted off the top of the centrifuged sample and dispensed into a flat-bottom insert of an LC-MS glass vial for analysis.

2.4 Method Development

2.4.1 Optimizing Liquid Chromatography Parameters

The integrated liquid chromatography system utilized a Kinetex® BiPhenyl, 2.6 μm, 50 x 3.0 mm column obtained from Phenomenex® (Torrance, California). The aqueous

Mobile Phase A consistsed of LC-MS grade water with 0.1% formic acid. The organic

Mobile Phase B was composed of acetonitrile with 0.1% formic acid. The needle rinse

solution was prepared in a 60:20:20 ratio of isopropanol, methanol, and acetonitrile, and

the liquid chromatography gradient time program is outlined in Table 2.

Table 2. Gradient time program for the novel LC-MS/MS method

Time (minutes)	Module	Event	% Mobile Phase B
1.00	Pumps	Pump B Concentration	16.9
4.50	Pumps	Pump B Concentration	17.1
6.00	Pumps	Pump B Concentration	100
6.10	Pumps	Pump B Concentration	100
6.60	Pumps	Pump B Concentration	0
7.10	Controller	Stop	-

The Shimadzu® LC used an FCV-11AL valve, a flow rate of 0.700 mL/min, and an

oven temperature of 40 degrees Celsius. The autosampler was model SIL-20AC/HT and

was set with a rinsing volume of 1000 μL, a needle stroke of 49 mm, rinsing speed of 35

μL/sec, and a sampling speed of 10 μL/sec. The rinse was set to occur before and after

aspiration.

2.4.2 Optimizing Mass Spectrometry Parameters

This method development used the Tuning mode in Analyst® software. A

solution of 5000 ng/mL was created for each new glucuronidated drug to be added to the

method. The syringe was a Hamilton GASTIGHT® #1725 syringe, and it was placed onto

a Harvard Apparatus® syringe pump (Holliston, Massachusetts). This syringe pump was

connected to the mass spectrometer with silica-lined PEEK™ plastic tubing

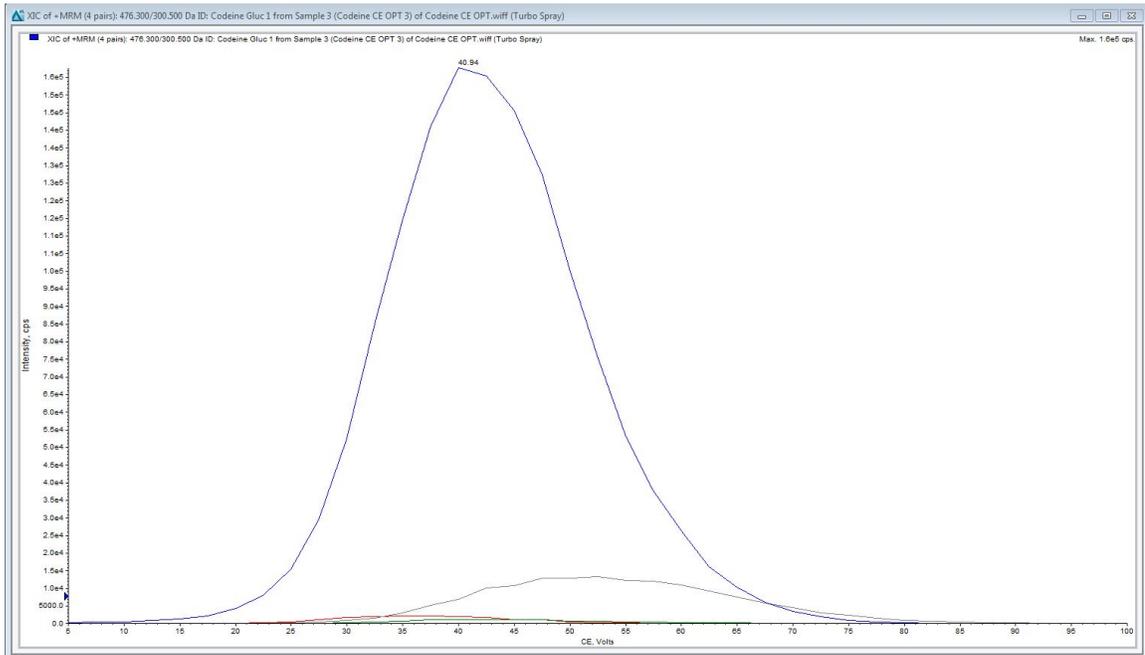manufactured by Upchurch Scientific (Oak Harbor, Washington).

3. RESULTS AND DISCUSSION

3.1 Method Development

3.1.1 Analyte Tuning

To develop a new method that eliminates the hydrolysis step of traditional urine

analysis, the mass spectrometer was tuned for the seven chosen glucuronidated analytes.

Through a literature search, it was determined that morphine, hydromorphone, codeine,

buprenorphine, oxazepam, oxymorphone, and tapentadol were common analytes

screened by the lab that exist primarily in their glucuronidated form in urine. (33) The

CE, DP, and CXP for the 21 other drugs included in the method that do not exist

primarily in their glucuronidated state in urine were copied from the previous LC-MS/MS

method.

While tuning the instrument for the glucuronidated drugs, the "Tuning" function of

the Analyst® software created graphs relating the abundance of the drug detected to the

quantitative value for each parameter (CE, DP, and CXP). The quantitative value of each

parameter that corresponded to the highest abundance of analyte was chosen for the

method optimization. To serve as an example, Figure 5 is a graph created by the Analyst®

software optimizing the CE for codeine glucuronide. The peak of the curve represents the

CE where the abundance of the drug detected is the highest (40.94 volts), so this was the

value chosen for the method. Multiple transition could be tuned at once, and this is the

reason for the presence of multiple-colored curves in Figure 5. Each colored line

corresponds to a different transition.

Figure 5. Collision energy optimization for codeine glucuronide's transitions

3.1.2 Shattering of Glucuronidated Drugs

During the tuning process, it was discovered that glucuronidated drugs demonstrated an interesting pattern when hit with increasing voltages in the collision cell. At low voltages, glucuronidated drugs stayed intact, but as the voltages increased, the glucuronidated drugs produced a shattering pattern, meaning the drug fragmented into many small pieces at low abundances instead of fragmenting into two or three high abundance fragments. The following figures (Figures 6-10) illustrate morphine glucuronide shattering as voltages increase.

Figure 6. Morphine glucuronide at collision energy 15 volts

Figure 7. Morphine glucuronide at collision energy 25 volts

Figure 8. Morphine glucuronide at collision energy 35 volts

Figure 9. Morphine glucuronide at collision energy 45 volts

Figure 10. Morphine glucuronide at collision energy 55 volts

When the morphine glucuronide molecule experienced at least 25 volts of energy, the glucuronide moiety (176 amu) was easily separated from the parent molecule. This resulted in the tall peak at 286.4 m/z, corresponding to the parent morphine molecule. However, other fragments were only present in low abundances for most collision energies. This was a problem when analyzing samples with low concentrations of morphine glucuronide because low abundances often resulted in non-Gaussian peak shapes and poor ion ratios.

To maximize abundances, one transition to the most abundant fragment and one transition to the parent molecule ("transition to itself") were chosen for the analysis of the glucuronidated analytes that produced the shattering pattern. The three analytes that produced this shattering pattern to the greatest extent were morphine glucuronide,

codeine glucuronide, and buprenorphine glucuronide. In a transition to the parent

molecule, the CE was set to a low voltage to allow some of the parent molecule to pass

through the collision cell without fragmentation.

Because a transition to the parent molecule results in an analyte being identified using

only one true fragment molecule, this strategy would not be ideal for a confirmatory

method. However, this method was a qualitative screen that required confirmation with a

different technique/method, so a transition to the parent molecule was admissible. Table 3

contains the seven glucuronidated drugs and each transition with its respective retention

time, DP, CE, and CXP. Appendix A contains the retention time, DP, CE, and CXP for

every drug included in the method.

Table 3. MRM transitions for each glucuronidated analyte and their respective DP, CE, and CXP voltages

Compound Name	Parent Ion (m/z)	Daughter Ion (m/z)	Retention Time (minutes)	Declustering Potential (volts)	Collision Energy (volts)	Collision Cell Exit Potential (volts)
Morphine-3-beta-D-glucuronide quantifier	462.5	286.4	1.50	120	40	9
Morphine-3-beta-D-glucuronide qualifier	462.5	462.3	1.50	45	25	15
Buprenorphine-3-beta-D-glucuronide quantifier	644.5	468.8	3.62	200	55	8
Buprenorphine-3-beta-D-glucuronide qualifier	644.5	644.5	3.62	200	20	8

Tapentadol-beta-D-glucuronide quantifier	398.5	222.4	1.91	110	32	16
Tapentadol-beta-D-glucuronide qualifier	398.5	121.3	1.91	110	53	8
Codeine-6-beta-D-glucuronide quantifier	476.3	300.5	1.71	158	41	8
Codeine-6-beta-D-glucuronide qualifier	476.3	476.3	1.71	158	20	15
Oxymorphone-3-beta-D-glucuronide quantifier	478.1	460.5	1.49	103	36	24
Oxymorphone-3-beta-D-glucuronide qualifier	478.1	284.5	1.49	103	35	23
Hydromorphone-3-beta-D-glucuronide quantifier	462.4	286.4	1.51	129	48	19
Hydromorphone-3-beta-D-glucuronide qualifier	462.4	185.1	1.51	129	47	12
Oxazepam glucuronide quantifier	463.2	286.9	5.59	110	20	9
Oxazepam glucuronide qualifier	463.2	269.2	5.59	110	33	7

3.1.3 Gradient Time Program

A gradient time program defines how the composition of the mobile phase changes over time. The gradient time program greatly impacted the resolution of each analyte in the method, making it a top priority for a chromatography method. Each

analyte was evaluated to ensure its resolution from other analytes in the method. Twenty-five of the 27 analytes were successfully resolved using the chosen gradient time program method, excluding morphine glucuronide and hydromorphone glucuronide that were unable to be separated. These two analytes coeluted between 1.50-1.51 minutes in the method.

Initially, this was a major concern for the method, and dozens of different gradient time programs were created to try to separate the two analytes. Efforts were unsuccessful, and the two analytes continued to coelute in the novel screening method. Although this result was not ideal, concerns were assuaged by the toxicological literature associated with the two compounds. A study by Cone et al. determined hydromorphone to be a minor metabolite of morphine. In this research, thirteen patients were dosed with morphine, and hydromorphone was detected in the blood of ten of those thirteen. (34) This relationship generates structurally similar compounds, which causes hydromorphone glucuronide and morphine glucuronide to dissociate from the stationary phase at almost identical rates, resulting in nearly indistinguishable retention times.

Ultimately, this screening method requires an additional confirmatory analysis to identify the presence of any analyte, including morphine glucuronide and hydromorphone glucuronide. Therefore, if a peak is detected at 1.50-1.51 minutes, the sample will be considered preliminarily positive for morphine glucuronide and hydromorphone glucuronide and will move forward to more specific confirmatory testing.

3.1.4 Alprazolam

During the creation of this method, alprazolam was not able to be accurately quantitated when analyzed in urine. Urine samples that had been previously analyzed by the LC-MS/MS confirmatory method were analyzed with the new LC-MS/MS screening method, and the quantitative values for each positive analyte were compared between the two methods to ensure that the new screening method could detect each analyte accurately. Because these urine samples were older samples that had been stored in the laboratory's refrigerator for various amounts of time, it was not expected that the new analysis would yield 100% of the previous quantitation, but alprazolam showed a more significant decline than was desired.

Three variables were investigated to improve the detection of alprazolam: possible glucuronidation, instability, and matrix effects. The first part of the investigation investigated whether alprazolam exists in a glucuronidated form in urine. Prior to method creation, research was completed to determine which of the drugs within the scope of this testing existed in a glucuronidated form in urine, and it was discovered that alprazolam's parent molecule exists in sufficient quantities (20% of an oral dose) in urine to be detected by the novel screening method. (33,35) However, a study by Morris et al. researched the use of beta-glucuronidase with benzodiazepines in urine and confirmed that alprazolam does exist as the parent compound in urine, but alpha-hydroxyalprazolam glucuronide is also a major urinary species. (36) With this new knowledge, urine samples at Champlain Toxicology Lab were screened to determine if the conversion to alpha-hydroxyalprazolam glucuronide was the cause of the low alprazolam abundances.

To test for the presence of alpha-hydroxyalprazolam glucuronide and other possible glucuronidated metabolites, Analyst® software was once again employed in the Tuning mode. The molecular weight of alpha-hydroxyalprazolam glucuronide and the molecular weight of an alprazolam molecule plus a glucuronide moiety were screened in the Tuning mode of the software with samples that had previously confirmed positive for alprazolam. No signal was detected for the molecular weight of alpha-hydroxyalprazolam glucuronide or the molecular weight of alprazolam plus a glucuronide moiety. It was determined from these results that glucuronidation was not the cause of alprazolam's low abundances.

Next, alprazolam's stability was assessed. The possibility that alprazolam could degrade in the samples over time in refrigerated storage was taken into consideration. However, toxicological literature indicated that when stored at 4 degrees Celsius, 95% of the original concentration of alprazolam was still present after 30 days. (37) However, urine samples at Champlain Toxicology Lab are not stored longer than one month, so it was concluded that the degradation of alprazolam was not a contributing factor to alprazolam's low abundances.

Third, matrix effects were evaluated as another possible cause of the low alprazolam detection. Matrix effects exist in two forms, ion enhancement and ion suppression. Due to the *low* abundances of alprazolam, this study evaluated whether ion suppression, in particular, was occurring in the electrospray ionization (ESI) source. Ion suppression can occur when the ability of a source to effectively ionize an analyte decreases due to contaminants within the matrix or the mobile phase co-eluting with the

target analyte. Co-eluting contaminants compete with the target analyte for charge during ionization as well as for space on the droplet's surface as the droplet approaches the Rayleigh limit. (38,39)
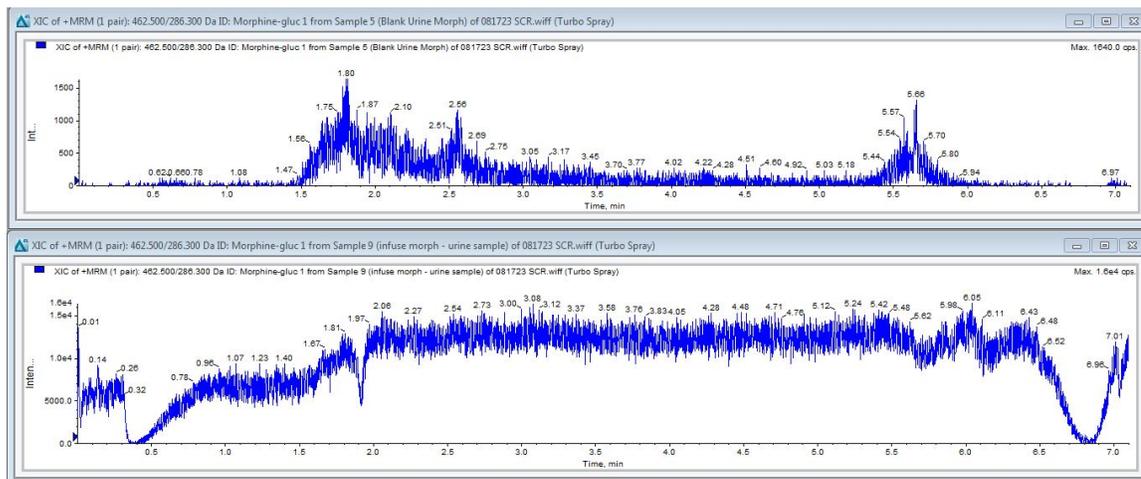
Matrix effects were also investigated as part of the method validation. Matrix effects can be avoided in a few ways, including extraction techniques and internal standards. (39,40) During extraction, contaminants like proteins, phospholipids, and inorganic salts are removed from the sample matrix, which eliminates the possibility that these contaminants could cause matrix effects during instrumental analysis. (40) Despite the benefits of an extraction method, one was not employed in this method due to efficiency and expense concerns. The main goal of this research was to reduce the cost and increase the efficiency of the lab's screening method, so adding an extraction step to the sample preparation would have been contrary to these goals. This novel method directly injected diluted urine into the LC-MS/MS system, so the sample matrix was complex, and ion suppression was considered a realistic explanation for the low abundances of alprazolam.

Another way to minimize the possibility of matrix effects is to use an internal standard. When using a mass spectrometer, analytes are quantitated by taking the ratio of the detected analyte to the internal standard, which elutes at approximately the same retention time as the analyte of interest. (41) Therefore, ion suppression is occurring in a certain retention time zone of the chromatogram, the internal standard would also be suppressed, and quantitation would not be skewed. Although this is a good fix for matrix

effects, internal standards were already a critical component of this screening method, so matrix effects were already being minimized in this regard.

Finally, the presence of matrix effects in the novel method was directly investigated. There are three main ways to evaluate the presence of matrix effects—post-column infusion, post-extraction spike, and slope ratio analysis. Post-column infusion, which is helpful in qualitative studies where blank matrix is available, was chosen to evaluate the existence of matrix effects in this investigation. (40) The post-column infusion method was developed in 1999 by Bonfiglio et. al, and it injects a sample between the LC and the MS portions of the instrument while mobile phase runs through the system. (42) The resulting graph was evaluated to determine the retention time zones that experienced ion suppression by identifying valleys in the graph of intensity versus time. (40,43) If a valley was observed in the retention time zone that alprazolam elutes, it was likely that the low abundance of alprazolam was due to ion suppression (matrix effects). (39)

After analyzing the graph produced by the post-column infusion technique (Figure 11), it was determined that valleys only existed from 1.81-1.97 and 6.52-7.01 minutes in the graph. Alprazolam eluted at 5.88 minutes in the novel screening method where no valley were present. Therefore, it was determined that matrix effects were also not likely to be the cause of alprazolam's low abundances.

Figure 11. Matrix effects present.

A literature review of other LC-MS/MS validation studies was conducted to determine if other researchers encountered a similar problem with alprazolam. It was discovered that the transitions chosen for this method and the mobile phases utilized were consistent with other research, but other studies utilized more complex sample preparation methods. These preparation methods included any combination of hydrolysis or extraction prior to analysis via LC-MS/MS, and it's possible that these preparation methods increase alprazolam's recovery during mass spectral analysis. (44–47) However, hydrolysis and sample extraction methods were not investigated by this study due to the efficiency and expense goals of this screening method.

Due to the lack of an explanation for the suppression of alprazolam in the method, it was decided that ion ratios for the quantitative and qualitative ions would be individually evaluated to determine if the sample is positive for alprazolam instead of allowing Analyst® software to quantify alprazolam in a sample. A number of calibrator

samples containing alprazolam were analyzed and evaluated to determine the ideal ion

ratio cutoffs for a positive sample. Ultimately it was determined that if the ion ratio of the

quantitative ion to the internal standard is 0.035 or above and the ion ratio of the

qualitative ion to the internal standard is 0.010 or above, the sample would be deemed

preliminarily positive for alprazolam. Any sample that had a quantitative ion ratio above

0.035 but a qualitative ion ratio of less than 0.010 was deemed a negative sample, and

similarly, any sample that had a qualitative ion ratio of greater than 0.010 but a

quantitative ion ratio of less than 0.035 was also deemed a negative sample.

3.1.5 Cutoff Values

Once the method had been created, cutoff values were chosen for every analyte

based on the cutoff values from the Olympus AU400© immunoassay method. Lower

cutoff values were used in most cases to challenge the previous Olympus AU400©

screening method. These chosen values are found in Table 4.

Table 4. Cutoff values. Cutoff for the immunoassay instrument versus the novel
screening method.

Compound Name	Immunoassay Cutoff Value (ng/mL)	New Screening Method (ng/mL)
Morphine-3-beta-D-glucuronide	200 cross reactivity with opiate reagent	150
Buprenorphine-3-beta-D-glucuronide	3,000 (cross reactivity with buprenorphine reagent)	10
Tapentadol-beta-D-glucuronide	N/A	100

Codeine-6-beta-D-glucuronide	N/A	100
Oxazepam glucuronide	1,300 (cross reactivity with benzodiazepines reagent)	50
Oxymorphone-3-beta-D-glucuronide	500 (cross reactivity with oxycodone reagent)	50
Hydromorphone-3-beta-D-glucuronide	N/A	150
Amphetamine	500	100
Methamphetamine	500	100
Meprobamate	70 (cross reactivity with carisoprodol)	100
Norfentanyl	100,000 (cross reactivity with fentanyl)	10
PCP	N/A	10
Carisoprodol	100	100
Tramadol	>10,000 (cross reactivity with carisoprodol)	50
EDDP	100	100
Benzoylecgonine	150	50
Ketamine	100	100
7-aminoclonazepam	100,000 (cross reactivity with benzodiazepines reagent)	50
Meperidine	100,000 (cross reactivity with buprenorphine and fentanyl reagents)	100
Trazodone	100,000 (cross reactivity with fentanyl)	50
Hydrocodone	4,000 (cross reactivity with opiates reagent)	50
Ritalinic acid	N/A	50
Methadone	700,000 (cross reactivity with EDDP)	100

Oxycodone	100	50
6-MAM	150 (cross reactivity with opiates reagent)	10
Fentanyl	2	10
Alprazolam	120 (cross reactivity with benzodiazepines)	50

3.2 Validation Studies

Once the method was finalized, a verification study was conducted. The study was performed in accordance with the standards put forth by the Champlain Toxicology Lab and the New York State Department of Health. The method was evaluated for linearity, precision, bias, specificity, interference, LOD, LOQ, client comparisons, and carryover.

3.2.1 Linearity

For the linearity study, at least 3 replicates of each calibrator for each analyte were analyzed, and the abundance of analyte versus calibrator concentration was graphed. Both the quantitative and qualitative ions for each analyte passed the linearity validation study, meaning that the range of calibrators for each analyte was linear with an r value of 0.98 or greater. (31) Figure 12 contains the linearity graph for 7-aminoclonazepam as an example.

Analyte Name: 7-Aminoclonazepam 1
Internal Standard: 7-Aminoclonazepam-d4

Data File	080823 SCR.wiff	Result Table	081123 SCR Linearity
Acquisition Date	8/8/2023 8:53:25 PM	Algorithm Used	MQ4
Acquisition Method	LEH Final Final.dam	Instrument Name	API 4000
Project	API Instrument		

Regression Equation: $y = 0.02377 x + -0.11310$ (r = 0.98444) (weighting: $1/x^2$)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
25.0	5 of 5	24.9	99.6	3.30	13.4
50.0	7 of 7	50.4	100.9	6.50	12.8
125.0	5 of 5	124.0	99.2	11.70	9.4
250.0	5 of 5	250.1	100.0	24.50	9.8

Figure 12. Exemplar linearity graph. Linearity graph for the quantitative ion of 7-aminoclonazepam

3.2.2 Precision and Bias

Precision and bias were determined by testing the positive/negative cutoff value with solutions made at 50%, 75%, 125%, and 150% of the cutoff value. The 50% solution and the 75% solution needed to produce a negative result, and the 125% solution and the 150% solution needed a positive result. The four concentrations were tested for every analyte in replicates of five on five separate days and were pipetted by four different analysts, creating a total of 25 replicates for each concentration and 100 total replicates for each analyte. 95 of the 100 replicates had to align with their corresponding value (positive or negative) for the analyte to pass the precision and bias study.

For many of the analytes, 100 of the 100 replicates corresponded to the correct value (the 50% and 75% solutions were negative, and the 125% and 150% solutions were positive), while other analytes did not have 100% correspondence but passed. The non-corresponding samples were as follows: PCP (1 at 75%), 7-aminoclonazepam (2 at 75%), tramadol (5 at 125%), oxymorphone glucuronide (1 at 125%), amphetamine (3 at 75%), ketamine (1 at 75% and 2 at 150%), meprobamate (2 at 75%), morphine glucuronide (1 at 125% and 1 at 75%), and hydromorphone glucuronide (2 at 75% and 1 at 125%).

3.2.3 Specificity

A solution was created that contained every analyte included in the method, and this solution was analyzed with the new method. The results were then evaluated to ensure that every analyte had a unique ion and retention time pairing. It was acceptable for two analytes to have the same retention time if each analyte had two unique transitions, and it was acceptable for two analytes to have the same transitions if they had different retention times. The solution containing every analyte is pictured in Figure 13. Every analyte, other than morphine glucuronide and hydromorphone glucuronide, passed the specificity study. Morphine glucuronide and hydromorphone glucuronide could not be separated by retention time, and they shared a common quantitative ion; therefore, they screened positive together. The individual analytes (morphine glucuronide and hydromorphone glucuronide) needed to be confirmed with the laboratory's confirmatory method before any results were reported.

Figure 13. Specificity study. All analytes are tested together to ensure specificity of the method.

3.2.4 Interference

For the interference study, six blank urine samples were evaluated for false positives. Any positive results in the blank urine samples would indicate interference from an endogenous molecule or an over-the-counter medication.

Upon analysis, the six blank urine samples weren't entirely free of peaks; however, no peak met all three criteria that constitute a positive result: correct retention time, presence of the quantifier ion, and presence of the qualifier ion. All interference graphs with explanations for each peak are found in Appendix B.

3.2.5 Limit of Detection and Limit of Quantitation

For the LOD validation study, the lowest calibrator for each analyte was analyzed with the novel method, and the signal-to-noise ratio was calculated by dividing the height

of the analyte peak by the height of the noise peaks bordering the analyte peak. Every analyte in the method passed this study as every signal to noise ratio was greater than or equal to 3:1.

For the LOQ study, the cutoff calibrator for each analyte was analyzed with the novel method, and the signal-to-noise ratio was calculated by dividing the height of the analyte peak by the height of the noise peaks bordering the analyte peak. Every analyte in the method passed this study as every signal to noise ratio was greater than or equal to 10:1.

3.2.6 Client Comparisons

The next validation study was Client Comparisons. Discrepancies between the results from the previous screening method and the novel screening method were evaluated for 25 urine samples. In the analysis of over 50 positive analyte results in 25 patient samples, 100% correspondence was observed, and the method passed the Client Comparisons study.

3.2.7 Carryover

Carryover was the last study in the method validation. Carryover ensures that each sample is independent and that no analytes from a previous sample have been introduced into a subsequent sample. For the carryover study, each analyte was spiked into blank urine at a concentration 500 times the highest calibrator. Then, a blank sample (methanol) was analyzed directly following the spiked sample. The blank sample was

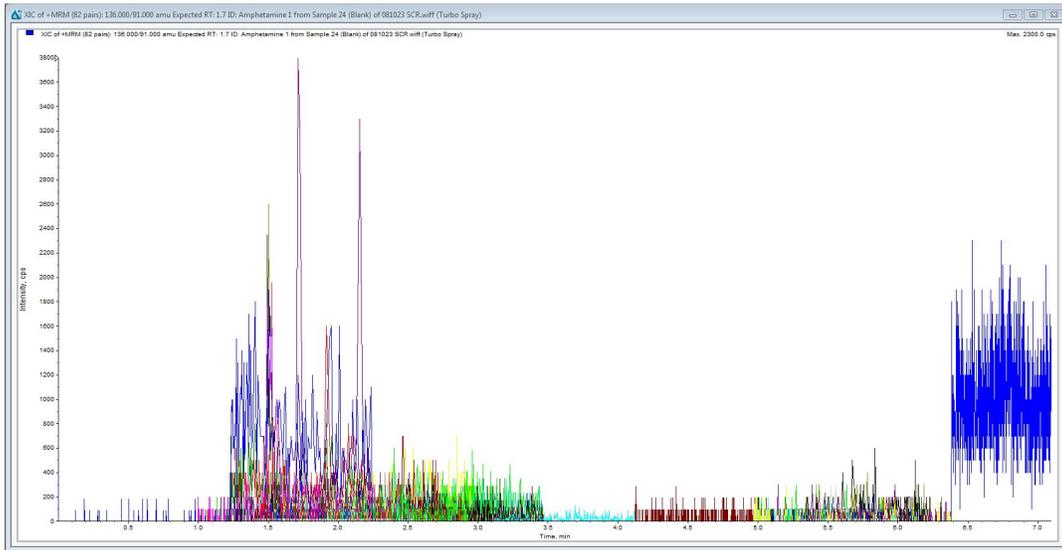evaluated for any positive results. Three separate sets of ten replicates were used to evaluate carryover.

After evaluation, none of the analytes displayed carryover into the blank samples. Peaks were observed in some of the blank samples following the spiked samples, but these peaks did not meet the criteria to be considered positive based on each analyte's cutoff value. For example, the blank sample following spiked Sample 1 showed peaks, but no peak was more abundant than 3800 counts per second, producing a negative result for every analyte. Spiked Sample 1 data can be found in Figure 14, and the blank following spiked Sample 1 can be found in Figure 15.

Figure 14. Carryover sample spiked with 500 times the cutoff value of the analytes.

Figure 15. Blank following carryover spiked Sample 1

4. CONCLUSIONS

This novel LC-MS/MS screening method for glucuronidated and non-glucuronidated drugs provides numerous advantages to a urine-testing toxicology laboratory. By using an LC-MS/MS instrument and detecting glucuronidated drugs instead of their parent compound counterparts, the method eliminated the reagent kits needed for immunoassay and the need for hydrolysis via the beta-glucuronidase enzyme. This ultimately reduced the time and expense needed for screening in this toxicological laboratory.

Although the method is highly advantageous, it is not flawless. First, alprazolam is not detected at its full concentration in human urine samples, so individual ion ratios had to be evaluated to determine alprazolam positivity in a sample. Additionally, morphine glucuronide and hydromorphone glucuronide coelute and share a common transition. These similarities between the two compounds make them indistinguishable with the new screening method, necessitating further testing.

Future areas of study include improving alprazolam's detection and successfully resolving morphine glucuronide and hydromorphone glucuronide without sample extraction or enzyme hydrolysis. One recommended route of investigation lies in the type of column used for analysis. The laboratory in which this research was performed employs a biphenyl column in its LC, but it's possible that the detection of alprazolam and/or the separation of morphine glucuronide and hydromorphone glucuronide could be achieved using a Carbon 18 (C18) column or an Ethylene Bridged Hybrid (BEH) Phenyl column instead, as different stationary phases have different resolving capabilities. (45)

Ultimately, this method is a great alternative for labs looking to lower the expense and lessen the time requirement of their current screening method. It provides high sensitivity and high specificity for twenty-seven different analytes commonly encountered in the urine of pain management patients without the use of expensive immunoassay reagent kits or beta-glucuronidase enzyme.

APPENDIX A: Method Development Parameters
Table A: MRM transitions for each analyte and their DP, CE, and CXP voltages

Compound Name	Parent Ion (m/z)	Daughter Ion (m/z)	Retention Time (minutes)	Declustering Potential (volts)	Collision Energy (volts)	Collision Cell Exit Potential (volts)
Morphine glucuronide quantifier	462.5	286.3	1.50	120	40	9
Morphine glucuronide qualifier	462.5	462.5	1.50	120	25	15
Morphine-d6	292.1	152.0	1.57	45	77	8
Buprenorphine glucuronide quantifier	644.5	468.9	3.62	200	55	8
Buprenorphine glucuronide qualifier	644.5	644.5	3.62	200	20	8
Buprenorphine-d4	472.2	59.1	5.65	20	92	15
Tapentadol glucuronide quantifier	398.5	222.4	1.91	110	32	16
Tapentadol glucuronide qualifier	398.5	121.3	1.91	110	53	8
Tapentadol-d3	225.2	106.7	2.45	40	25	10
Codeine glucuronide quantifier	476.3	300.5	1.71	158	41	8
Codeine glucuronide qualifier	476.3	476.3	1.71	158	20	15
Codeine-d6	306.2	152.2	1.77	50	58	15

Oxazepam glucuronide quantifier	463.2	286.9	5.59	110	20	9
Oxazepam glucuronide qualifier	463.2	269.3	5.59	110	33	7
Oxymorphone glucuronide quantifier	478.1	460.5	1.49	103	36	24
Oxymorphone glucuronide qualifier	478.1	284.5	1.49	103	35	23
Oxymorphone-d3	305.1	230.1	1.59	70	41	7
Hydromorphone glucuronide quantifier	462.4	286.4	1.51	129	48	19
Hydromorphone glucuronide qualifier	462.4	184.8	1.51	129	47	12
Hydromorphone-d6	292.1	185.1	1.63	50	37	6
Amphetamine quantifier	136.0	91.0	1.73	40	45	8
Amphetamine qualifier	136.0	119.1	1.73	40	20	10
Amphetamine-d5	141.1	93.0	1.73	40	23	8
Methamphetamine quantifier	150.1	119.0	1.84	30	22	10
Methamphetamine qualifier	150.1	91.0	1.84	30	60	8
Methamphetamine-d5	155.1	121.1	1.84	30	21	10
Ketamine quantifier	238.1	124.9	2.22	40	25	10
Ketamine qualifier	238.1	179.0	2.22	40	25	10

Ketamine d4	242.2	128.7	2.20	40	25	10
7-aminoclonazepam quantifier	290.1	121.1	2.36	100	42	13
7-aminoclonazepam qualifier	286.1	250.1	2.40	80	29	13
7-aminoclonazepam-d4	286.1	121.1	2.40	80	37	13
Meperidine quantifier	248.0	220.0	2.96	40	32	10
Meperidine qualifier	248.0	174.0	2.96	40	30	10
Meperidine-d4	252.1	224.1	2.94	40	30	10
Meprobamate quantifier	219.0	158.0	2.96	20	20	9
Meprobamate qualifier	219.0	97.0	2.96	20	14	6
Meprobamate-13C3	222.0	161.0	2.96	20	12	9
Norfentanyl quantifier	233.0	84.2	2.09	35	25	16
Norfentanyl qualifier	233.0	150.1	2.09	35	25	12
Trazodone quantifier	372.2	176.0	5.48	70	35	10
Trazodone qualifier	272.2	147.9	5.48	70	35	10
Trazodone-d6	378.2	182.0	5.48	70	35	10
PCP quantifier	244.1	159.0	5.50	40	31	8
PCP qualifier	244.1	86.1	5.50	40	59	8
PCP-d5	249.1	96.1	5.48	50	47	14
Carisoprodol quantifier	261.0	176.0	5.74	30	20	10

Carisoprodol qualifier	261.0	97.0	5.74	30	29	6
Tramadol quantifier	264.1	58.1	2.42	56	100	10
Tramadol qualifier	264.1	42.2	2.42	56	91	6
EDDP quantifier	278.1	186.2	5.77	50	62	10
EDDP qualifier	278.1	234.2	5.77	50	65	14
Benzoylecgonine quantifier	290.0	168.0	2.19	40	43	10
Benzoylecgonine qualifier	290.0	104.9	2.19	40	71	6
Benzoylecgonine-d8	298.0	171.0	2.17	40	27	10
Hydrocodone quantifier	300.0	199.1	1.96	50	38	12
Hydrocodone qualifier	300.0	128.1	1.96	50	75	6
Hydrocodone-d6	306.2	202.1	1.94	50	43	12
Ritalinic acid quantifier	220.1	84.1	2.02	50	60	8
Ritalinic acid qualifier	220.1	56.1	2.02	50	62	8
Ritalinic acid-d10	230.1	93.1	2.00	50	60	8
Methadone quantifier	310.1	265.0	5.81	90	35	30
Methadone qualifier	310.1	105.1	5.81	90	75	9
Methadone-d9	319.1	268.1	5.81	90	20	8
Oxycodone quantifier	316.1	241.2	1.89	45	37	22
Oxycodone qualifier	316.1	256.1	1.89	45	33	16
6-MAM quantifier	328.0	165.1	1.85	80	51	8

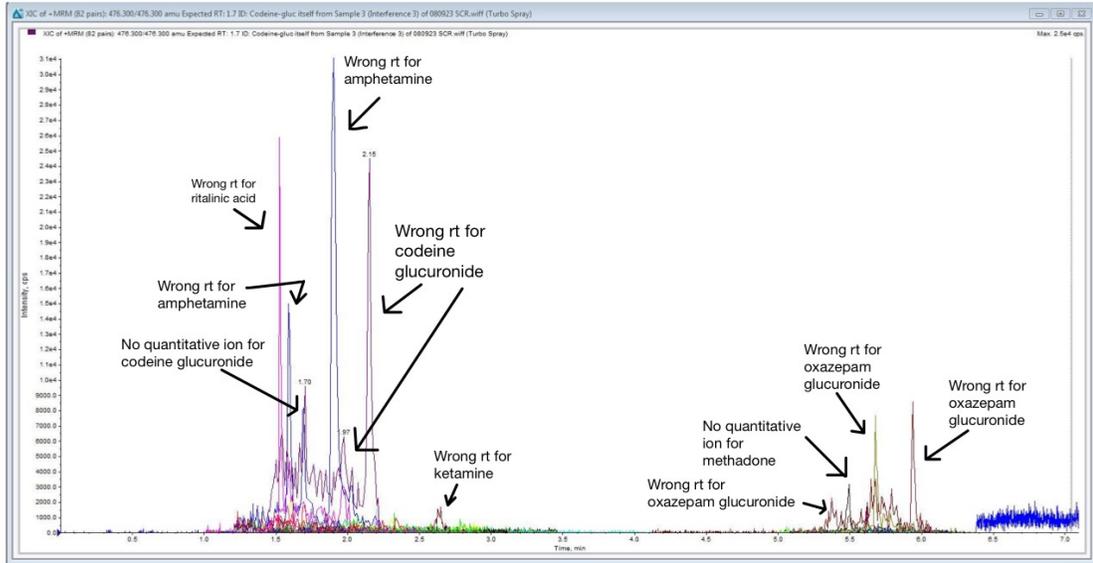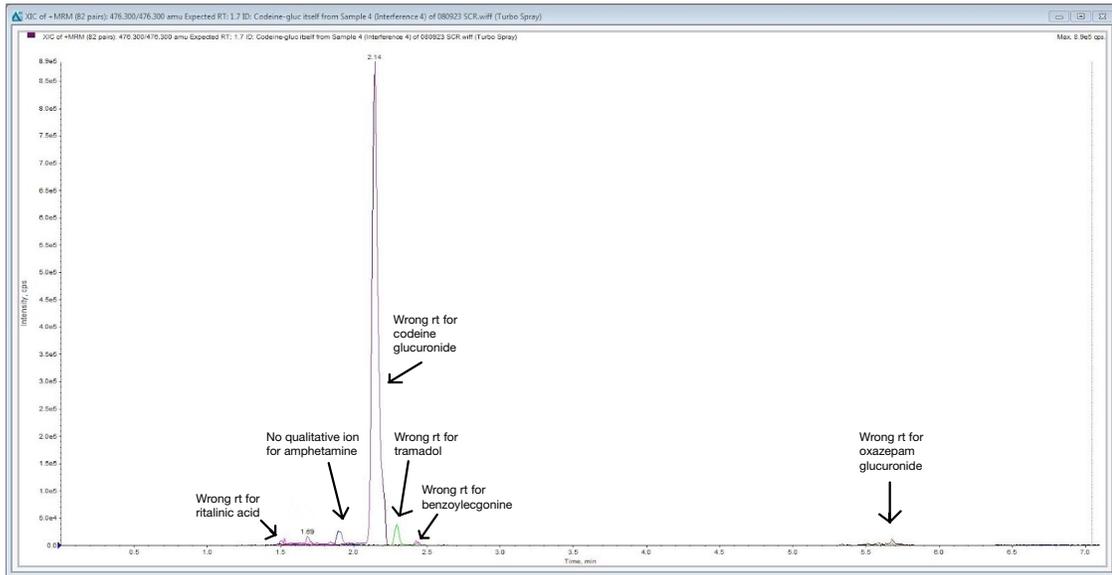| 6-MAM qualifier	328.0	211.0	1.85	80	35	8
6-MAM-d6	334.1	165.0	1.85	80	52	6
Fentanyl quantifier	337.1	188.1	5.60	50	35	18
Fentanyl qualifier	337.1	105.2	5.60	50	45	15
Fentanyl-d5	342.3	188.1	5.60	50	35	18
Carisoprodol quantifier	261.0	176.0	5.74	30	20	10
Carisoprodol qualifier	261.0	97.0	5.74	30	29	6
Methadone quantifier	310.1	265.0	5.81	90	35	30
Methadone qualifier	310.1	105.1	5.81	90	75	9
Methadone-d9	319.1	268.1	5.81	90	20	8
Alprazolam quantifier	309.8	281.3	5.88	109	38	13
Alprazolam qualifier	309.8	205.2	5.88	109	57	13
Alprazolam-d5	314.2	210.3	5.88	40	35	10

Appendix B: Interference

Figure A. Interference Sample 1

Figure B. Interference Sample 2

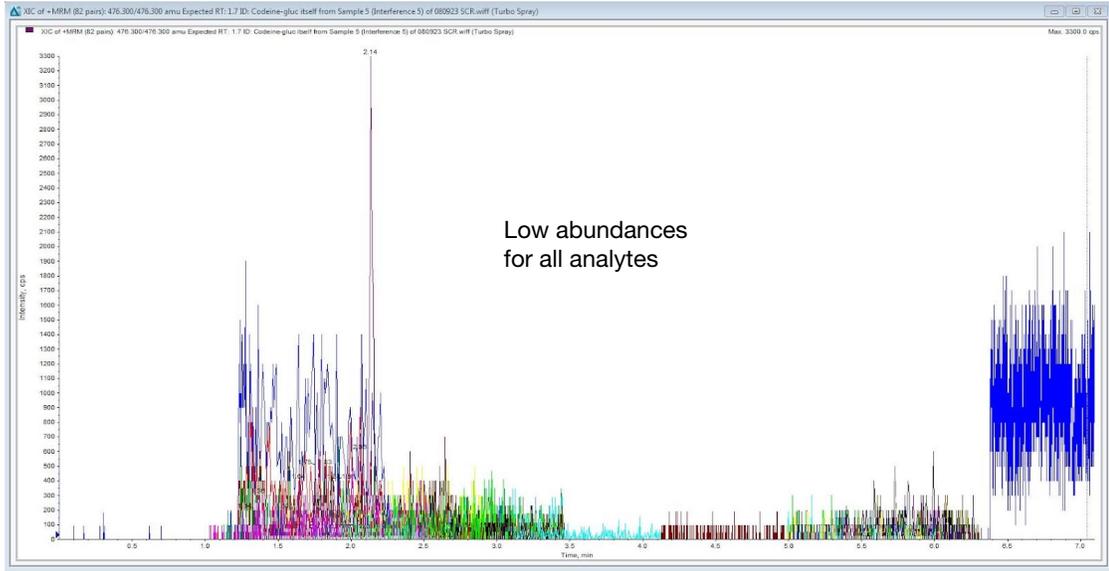**Figure C. Interference Sample 3**

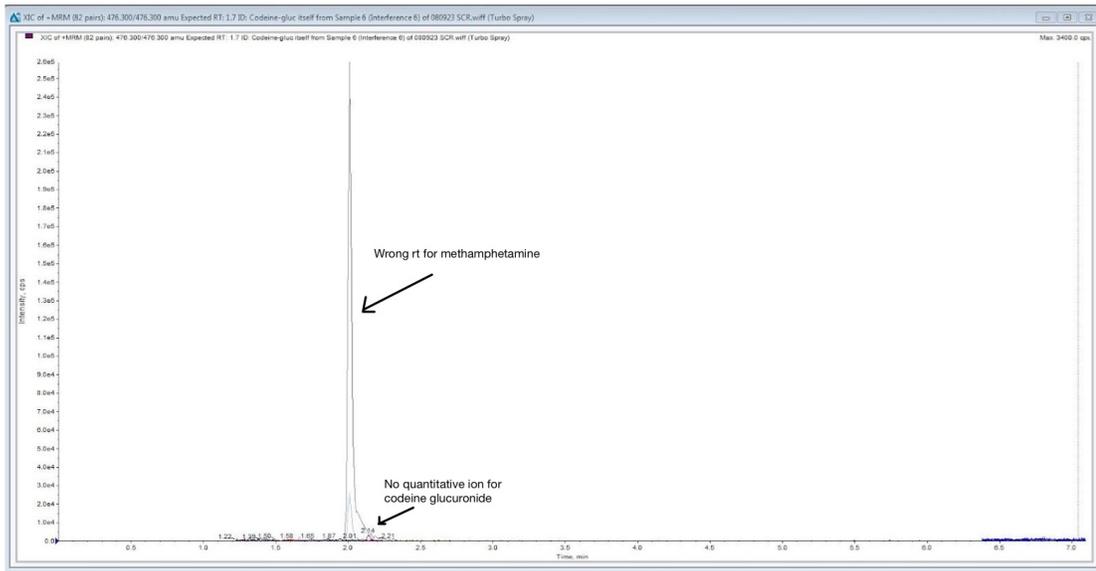**Figure D. Interference Sample 4**

Figure E. Interference Sample 5

Figure F. Interference Sample 6

LIST OF JOURNAL ABBREVIATIONS

Acc Chem Res	Accounts of Chemical Research
Clin Biochem Rev	The Clinical Biochemist Reviews
Clin Chem	Clinical Chemistry
Drug Metab Rev	Drug Metabolism Reviews
Eur J Pharm Sci	European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences
J Addict Addictv Disord	Journal of addiction and addictive disorders
J Anal Sci Technol	Journal of analytical science and technology
J Anal Toxicol	Journal of analytical toxicology
J Appl Lab Med	Journal of applied laboratory medicine
J Chromatog B	Journal of Chromatography B
J Pain Symptom Manage	Journal of pain and symptom management
J Pharm Biomed Anal	Journal of pharmaceutical and biomedical analysis
J Proteome Res	Journal of proteome research
Mass Spectrom Rev	Mass spectrometry reviews
Mol	Molecules (Basel, Switzerland)
Pain Med Malden Mass	Pain medicine: the official journal of the American Academy of Pain Medicine
Pain Physician	Pain Physician Journal
Rapid Commun Mass Spectrom RCM	Rapid communications in mass spectrometry: RCM
Sci Rep	Scientific Reports

Ther Drug Monit   Therapeutic Drug Monitoring

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