Utilizing Liquid Chromatography Tandem Mass Spectrometry and Direct Analysis in Real Time to Assess the Metabolism of Fentanyl Derivatives

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Abstract of Thesis

Fentanyl and fentanyl analogs are drugs of abuse that can also serve as common adulterants in drugs of abuse, and their potency causes many drug overdose deaths in the United States. In an effort to better understand the opioid crisis plaguing the country, fentanyl-related compounds must be studied to understand their impact in opioid-related overdoses and deaths, and on the crisis overall. Fentanyl-related compounds are best understood by studying their metabolism, and while previous research has focused primarily on drugs of abuse and their metabolites, this research focuses on an untouched idea with substantial development potential – drug adulterants and their metabolites. In this research, Liquid Chromatography – Triple Quadrupole Tandem Mass Spectrometry (LC-MS/MS) and Direct Analysis in Real Time – Mass Spectrometry (DART-MS) were used in conjunction with Agilent MassHunter software and Biomimiks™ technology to analyze the metabolism of fentanyl-related compounds. Fentanyl and fentanyl analogs were analyzed neat and in mixtures, before and after the addition of a biomimetic catalyst with an oxidizing agent. The LC-MS/MS method developed was quantitatively able to identify all of the expected MRM transitions in a mixture of four fentanyl-related compounds, and the DART-MS method developed functioned as a high throughput screening approach to identify norfentanyl, a known primary metabolite of fentanyl. These results can guide future efforts to develop an LC-MS/MS method that can identify unanticipated metabolites of fentanyl-related compounds, such as those produced by Biomimiks™ technology. Additionally, new biomimetic catalysts can be tested to maximize the efficiency of Biomimiks™ technology in producing recognizable metabolites. This project will guide the development of methodologies for the analysis of actual postmortem samples, to aid forensic and clinical toxicology laboratories nationwide.
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List of Symbols and Abbreviations

DART  Direct Analysis in Real Time
DEA   Drug Enforcement Agency
CDC   Centers for Disease Control and prevention
CRM   Certified Reference Material
FeOCOB octa-chloro octa-bromo porphyrin iron III-chloride
FeOCSulfonate iron octa-chloro tetra-sulfonic acid porphyrin chloride
GC-MS Gas Chromatography – Mass Spectrometry

$g/mol$ grams per mole
IMS   Ion Mobility Spectrometry
LC-MS Liquid Chromatography – Mass Spectrometry
MCPBA 3-chloroperoxybenzoic acid
MeOH methanol
mg milligram
mg/mL milligram per milliliter
min minute
mL milliliter
mm millimeter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MMPP</td>
<td>magnesium monoperoxyphthalate hexahydrate</td>
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<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Mass Spectrometry / Mass Spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>ng/mL</td>
<td>nanogram per milliliter</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole Time of Flight</td>
</tr>
<tr>
<td>QqQ</td>
<td>triple quadrupole</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid Phase Micro Extraction</td>
</tr>
<tr>
<td>SRM</td>
<td>Single Reaction Monitoring</td>
</tr>
<tr>
<td>SVP</td>
<td>Standardized Voltage and Pressure</td>
</tr>
<tr>
<td>TIC</td>
<td>Total Ion Chromatogram</td>
</tr>
<tr>
<td>Triple Quad LC/MS</td>
<td>Liquid Chromatography / triple quadrupole Mass Spectrometry</td>
</tr>
<tr>
<td>μg/mL</td>
<td>microgram per milliliter</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance (Pressure) Liquid Chromatography</td>
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1.1 Introduction

1.1.1 Opioid Crisis: The Problem to be Addressed

The opioid crisis is an epidemic plaguing much of the United States, and has been an increasingly studied and debated topic. It has garnered an alarming number of users and caused a staggering number of deaths, especially in recent years. A contributor to this crisis is the drug fentanyl, which was originally synthesized in 1959 as a legal painkiller, but began to be produced clandestinely in the 1990’s. The illicit fentanyl produced was cut into heroin, and was believed to be responsible for over 100 deaths at the time. As the years went on, more secret fentanyl production laboratories were discovered, but between 2005 and 2007, the number of fentanyl-related deaths had grown to over 1,000 people\(^1\). By 2013, the number of overdose deaths was alarming, and could confidently be attributed to fentanyl, fentanyl-related substances, and other synthetic opioids in fake pharmaceutical products. From 2014 to 2015, opioid death rates increased by 15.6%, and 72.2% of these deaths were from synthetic opioids other than methadone, making them due to illicitly manufactured fentanyl\(^2\). As the years went on, the opioid crisis continued to become a severe problem, to the extent that it was declared a public health emergency by the White House in October 2017. Efforts to remediate the opioid crisis cost the United States $504 billion in 2015, or 2.8% of the year’s gross domestic product, making it a political and socioeconomic issue as well\(^3\).

The exponential rate at which this crisis is growing can be seen visually in Figure 1. In the figure, the darker the color of each state, the greater the number of deaths, with the lightest shade corresponding to 6.3 – 11.0 deaths and the darkest shade corresponding to 21.0 – 35.5 deaths per 100,000 population. The graphs from 2014 to 2016 indicate a distinct increase in overdose deaths, as the graphs become visually darker in color as time goes on.
Figure 1. Drug Overdose Death Data by State from 2014 to 2016. Adapted from Centers for Disease Control and Prevention website\textsuperscript{4}
Although this epidemic is a national issue, some regions of the United States are seeing higher usage than others. Although there are several theories behind this, one definitive explanation does not exist. It is possible that fentanyl is more prevalent in the northeast because it originated abroad and entered the United States from Massachusetts, continuing into New Hampshire. Fentanyl is thought to have been manufactured in China and Mexico, and distributed through routes in Massachusetts. Because it is so potent, it may have been circulated in smaller quantities and distributed discreetly. It is also possible that some states had higher rates of opioid prescriptions than others, which led to an addiction for some of the population. After depleting the quantity of drug they were prescribed, some people craved more of the drug, and found means to obtain it. Some states may not have had the funding for preventative measures to be taken, and once the addiction began, certain regions may not have had the treatment facilities necessary. The last theory is that the drug is more popular in regions where there are economic challenges or fewer public activities available, and drug use becomes a social phenomenon.

Figure 2. Regional Trends in Fentanyl from January 2001-December 2016. Obtained from National Forensic Laboratory Information System: 2016 Annual Report
This crisis is a complex issue with many moving parts. Apart from the obvious problem of thousands of deaths, other worries include the prevention of illicit fentanyl production, the tracking and eventual capture of those who are manufacturing and selling fentanyl-related compounds illegally, and the treatment of those who are in deadly danger from using these drugs. And although fentanyl is still being legally prescribed in cases where extreme pain management is necessary, it is a schedule II drug according to the Controlled Substances Act, so its activity is highly regulated. This means illicitly produced fentanyl and fentanyl analogs are responsible for the majority of overdose deaths, which once again vary by region according to Figure 3.

Figure 3. Synthetic Opioid Overdose Death Rates by Region. Obtained from Centers for Disease Control and Prevention website
Because of the relative ease at which fentanyl derivatives can be produced and the numerous unidentified or unregulated compounds that exist and are constantly being created, it is extremely difficult to prevent illicit production and trace those who are involved. Fentanyl itself is dangerous, but some analogs, such as carfentanil, can be up to 100 times more potent than fentanyl\(^9\). Generally, what contributes to the difficulty of tracking the sale of fentanyl and related compounds are their potency – because these compounds are so many times stronger than morphine and other common painkillers, they can be consumed in smaller quantities for the same effect. This makes packages so small that they can be nearly impossible to track.

Additionally, people are getting more and more creative with ways to cut and sell fentanyl and its analogs. Synthetic opioids are being sold on the internet through the “dark web”, where drugs can be purchased anonymously and shipped without tracking. In 2013, authorities found and dismantled the Silk Road, a famous online drug marketplace, but since then many similar shops have emerged and the online drug trade continues\(^10\). Fentanyl is often cut into heroin, oxycodone, MDMA, percocet, and other drugs, many times without the user knowing that he or she is taking an adulterated drug\(^11\). The original drugs may be cut with fentanyl or fentanyl-related compounds for several reasons – because fentanyl is cheap to produce, because it is easier to synthesize than the original compound, or to create the illusion of quality. The preparation of fentanyl for non-chemists is a 4-step process with non-prescription ingredients that are easily available, making it an attractive adulterant\(^12\).

Those who are addicted to opioids are typically more concerned about obtaining their desired drug of abuse than they are about their own safety, or the origin or purity of the drug. This is why adulterants such as fentanyl are especially dangerous; because drug users will use drugs cut with dangerous compounds if they cannot acquire a purer substance. On the other hand, people who
are not typically fentanyl users could die by inadvertently ingesting fentanyl while assuming they are using another opioid drug. Because fentanyl and fentanyl-related compounds are so prevalent in drug overdose deaths, focusing research on them and their metabolites will attack the issue from a different angle, offering a new perspective for solving the opioid crisis.

1.1.2 Scientific Relevance

Analytical and forensic chemistry go hand in hand, and the field of analytical chemistry is constantly improving. New advancements in technology mean more efficiency in performing research, and it is important to utilize these advancements to try to solve problems such as the opioid crisis. This crisis is so complex that it cannot be tackled by one person or group, and will take years to combat completely. However, by working towards smaller goals, parts of it can be solved. The overall goal of this project is source origin determination, and can be explained in the following manner.

Although fentanyl was originally developed as a legal painkiller, it was later synthesized illegally to be cut into heroin for numerous reasons. As various analogs of fentanyl were synthesized, they were also cut into heroin, along with typical diluents such as baking soda and typical adulterants such as caffeine$^{13}$. Cutting agents are divided into two categories – diluents, which are pharmacologically inactive and generally readily available, and adulterants, which are pharmacologically active and have a biological effect. Because of its effects, fentanyl is considered an adulterant$^{14}$.

Eventually, each heroin seller created his or her own specific mixture of heroin, fentanyl-related compounds, and other adulterants and diluents to sell as “heroin”. Although these mixtures probably varied according to the availability and price of the drugs and cutting agents at the time, every batch was distributed to other sellers or to users once it was prepared.
The fact that many unique drug mixtures exist can be useful in tracking those who develop and sell them. In certain counties within the United States, people are dying of drug overdoses at a greater rate than people from other counties, an example of which is shown in Figure 4.

Although the deaths are considered heroin overdose deaths, if fentanyl or a fentanyl-related compound has been cut into the heroin, this compound could potentially be causing the death faster than the heroin itself. If autopsy samples could be profiled by fentanyl metabolites, it could be determined what exactly the user overdosed on, whether it was heroin, fentanyl, or a different adulterant. As more fentanyl derivatives are illegally synthesized and begin to appear in postmortem sample analysis, a library can be created of all known compounds. This can be used to analyze the total metabolite profile of multiple postmortem samples to see if a pattern develops – if samples with similar metabolite profiles were obtained from the same region, it may help to find who exactly makes or sells a specific batch of “heroin”.

Figure 4. Reports of Acetyl Fentanyl by County in Massachusetts. Obtained from NFLIS Fentanyl Brief 2015-2016
In the long term, if postmortem urine samples of known or suspected heroin overdose deaths could be obtained and analyzed by previously developed LC-MS/MS methods, the metabolite profile of the samples could be confirmed. Currently, forensic toxicologists aim to identify metabolites of only drugs of abuse in toxicology study samples. However, it should be noted that adulterants present within samples are also metabolized. The complete characterization of metabolites of both primary drugs and drug adulterants present in these samples would create a more in-depth identification profile. This would then allow for the correlation of urine samples to different counties where the drug was obtained in order to trace the drug, provided consistency was found between the samples. The urine samples could be cleaned up by an established SPE method, and the mass spectral data from each sample could be compared to previously collected metabolite data to determine exactly what was present in each victim’s urine. Then, samples with similar data could be grouped according to region, since the origin of the post-mortem samples would be known. Through the higher level of characterization created by analyzing drug adulterants and their metabolites, people synthesizing and selling these deadly cocktails of drugs could be tracked down and caught.

1.1.3 Approach

To begin, fentanyl and fentanyl-related compounds were studied. An extensive literature review was performed, along with instrument and software familiarization. Then, fentanyl and fentanyl analogs were ordered through Cayman Chemical (Ann Arbor, MI), as the company is a collaborator on the project and can provide almost any known fentanyl analog. A variety of samples were ordered, including those that have not previously been studied extensively by other research groups. The compounds were selected based on their availability and their prevalence in actual forensic casework, and those that fit this criteria were ordered. The compounds ordered
were limited to those that were sold as exempt substances, as Northeastern University did not possess a license for controlled schedule I substances.

Some samples were available as solutions and others as solids, so dilutions were performed as explained in section 1.5.1. In the future, biological samples or synthetically produced samples might be cleaned up by SPE to isolate the compound of interest.

The LC-MS/MS analytical method was developed in Agilent MassHunter software, and the same software was used to analyze samples on the Agilent Triple Quad LC/MS. Data was obtained for each compound separately, then for a mixture of the compounds. The method was modified so that the compounds could be distinguished in the mixture.

After method optimization and interpretation of the chromatograms of the fentanyl-related compounds neat and in mixtures, Biomimiks™ technology was used with the compounds, as explained in section 1.1.5. This involved the addition of a catalyst, co-catalyst, oxidizing agent, and solvent. Samples were prepared following the outline in section 1.5.3.

Since the goal was to analyze fentanyl-related compounds before and after the Biomimiks™ catalyst addition, it was necessary for the developed method to be able to identify unanticipated fragments induced by the catalyst. By comparing the data before and after catalyst addition, the anticipated metabolites from the fentanyl-related compounds could have been determined. Although the method developed on the Triple Quad LC/MS could identify all the expected MRM transitions in the fentanyl compound mixtures, it could not identify unanticipated fragments, such as those produced by Biomimiks™ technology. Instead, the DART-QTOF-MS was used with Agilent MassHunter software as a screening method, providing real time results after the addition of the Biomimiks™ catalyst. This instrument consisted of an IonSense DART-SVP source interfaced to an Agilent Q-TOF LC/MS. In the future, the DART-QTOF-MS data can
help guide LC-MS/MS method development for accurate quantitation, as an optimized LC-MS/MS method will provide quantitative results.

1.1.4 Prior Research

In recent years, a plethora of methods have been developed for analyzing fentanyl and its analogs by LC-MS, GC-MS, IMS, DART, and other analytical instrumentation. Because fentanyl and its analogs can be extremely dangerous upon exposure, IMS and DART are being developed as screening methods. In some instances, the instruments are being made portable to detect illicit drugs at airports and crime scenes\textsuperscript{16}. However, for quantitative results, LC-MS/MS is a popular choice. Many groups have developed methods for the analysis of fentanyl analogs and metabolites by tandem mass spectrometry from blood, plasma, or urine. Some methods involve liquid-liquid extraction or solid phase extraction as part of the sample preparation, and some experiments require dosing rat models as opposed to using human samples. While analytical methods like IMS and DART can provide high throughput screening, methods like LC-MS and GC-MS are required for quantitative accuracy. For this reason, many approaches use a combination of analytical tools for the most informative results.

Because the opioid crisis is relatively recent, new fentanyl analogs are constantly being synthesized. Some new analogs have not yet been officially named, and therefore have certainly not yet been analyzed by analytical methods. Fentanyl-related compounds have also been rarely analyzed as mixtures, the way they would likely appear in the human body. Additionally, many efforts have been made to study drugs and their metabolites, but drug adulterants and their metabolites have not been studied with the same intensity. Since drug adulterants have become very toxic, some more toxic than the drug of abuse itself, studying adulterant metabolites is of
great interest. Focusing on developing methods for this purpose will breach a previously untouche\nt area of study, and has great potential for advancing research to solve the opioid crisis.

To effectively tackle the crisis will take many years as all possible fentanyl analogs are developed and universally named, analyzed by various forms of instrumentation individually and in mixtures, and eventually identified in bodily fluid samples from those who have overdosed. Then, the drugs can be tracked by region, eventually to the distributors and manufacturers of the illicit fentanyl compounds, to attack the issue from its start. This research helps to complete one of the preliminary goals to eventually solve the crisis.

1.1.5 BiomimiksTM Technology

A close collaboration with Empiriko, a local biotechnology company, allowed for the use of BiomimiksTM technology. The unique biomimetic platform uses a chemical catalyst to accurately simulate the oxidative function of the liver, allowing for a rapid and precise prediction of the liver’s metabolic reaction to drugs such as fentanyl. This “chemosynthetic liver” technology mimics cytochrome P450, the enzymes found in the liver, to replace animal testing and predict metabolic pathways and patterns in the human body\textsuperscript{17}.

The technology has been used effectively on several drug substrates in the past, and was used with fentanyl and its analogs for the first time during this project. For this reason, some trial and error was necessary for the catalyst to perform at maximum efficiency. Typically, the catalyst is incubated overnight with a co-catalyst, a solvent, an oxidant, and the drug substrate, which in this case is the selected fentanyl compound\textsuperscript{18}. During incubation, the catalyst performs functions that would naturally occur in the liver, such as demethylation or hydroxylation, but cannot perform secondary metabolism functions like glucuronidation because the additional necessary compounds are not added to this reaction\textsuperscript{18}. 

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In typical scenarios involving Biomimiks™ technology, the starting substrate is never fully metabolized, and yields about 10% of metabolized product\textsuperscript{18}. This is where the Biomimiks™ technology differs from actual biological function, since the body metabolizes substrates much faster, and the catalyst might produce metabolites that would not be prominently seen in the body. The catalyst does not go into solution, has a high molecular weight, and is used in very low concentration, so it is not seen in the chromatographic analysis\textsuperscript{19}.

Biomimiks™ technology was utilized in this project for several reasons. First, it is relatively simple to use, once the correct combination of drug substrate and oxidizing agent is discovered depending on the catalyst. Second, it has accurately predicted the metabolites of other drug compounds in 50 drug metabolism studies, proving its efficacy\textsuperscript{18}. And finally, this technology bypasses the need for animal models, which are typically used to the same effect but require animal sacrifice and can be ineffective\textsuperscript{17}.

Since the goal of this technology is to mimic the oxidative function of the liver, it will provide valuable information about the metabolism of fentanyl and fentanyl derivatives. Figure 5 shows the major proposed routes of fentanyl and fentanyl-related compound metabolism, using several biotransformation pathways. If the Biomimiks™ technology is successful, it will be able to produce some of the compounds in Figure 5, such as the phase 1 metabolites labeled 2 through 7.
1.2 Experimental Conditions

1.2.1 Safety

Extreme precaution was taken while handling fentanyl and related compounds. During sample preparation, proper personal protective equipment was worn, and care was taken not to work with any drug substances outside of a small area in the laboratory.

1.2.2 Instrumentation

The Agilent Technologies 1200 Series Liquid Chromatography system (Santa Clara, CA) was used in conjunction with the Agilent Technologies 6460 Series Triple Quadrupole LC/MS to analyze fentanyl-related compounds neat and in mixtures. Additionally, the IonSense DART-SVP source (Saugus, MA) interfaced with the Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS was used to analyze the fentanyl compound mixture after the addition of and incubation with the Biomimiks™ catalyst.

Figure 5. Major Metabolism Routes of Fentanyl-Related Compounds. Obtained from Cayman Chemical website
1.2.3 Software

Several components of the Agilent MassHunter software were used with the Triple Quad LC/MS: Data Acquisition for monitoring and controlling data acquisition for the LC/MS instrument, Optimizer as a method development tool for generating and optimizing MRM transitions, and Qualitative Analysis for qualitatively analyzing data. Optimizer was used first with each compound individually to determine transitions, then Data Acquisition was used to collect data to confirm these transitions, and finally Qualitative Analysis was used to examine the chromatograms of each compound individually and in a mixture of compounds. The Optimizer results are in section 1.5.1, the Data Acquisition results are in section 1.5.2, and the Qualitative Analysis results are in section 1.5.3.

On the DART-QTOF-MS instrument, Agilent MassHunter software was also used. The Data Acquisition portion of the software was used to edit the method parameters and collect data, and the Qualitative Analysis portion was used to analyze the data. Specifically, the DART-QTOF-MS was used to analyze the fentanyl-related compounds after catalyst addition. The Data Acquisition results are in section 1.5.4, and the Qualitative Analysis results are in section 1.5.5.

1.3 Materials and Methods

1.3.1 Chemical Compounds

Fentanyl-related compounds were ordered from Cayman Chemical. Para-methoxy butyryl fentanyl (hydrochloride) and Benzyl fentanyl (hydrochloride) were purchased in solid formulation while Acetyl fentanyl (hydrochloride) and Fentanyl (hydrochloride) were purchased as 100 μg/mL solutions in methanol. Compounds were diluted with Fisher Chemical HPLC grade Methanol prior to instrument analysis.
Table 1. Fentanyl-Related Compounds with Chemical and Structural Information

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formulation</th>
<th>Compound Molecular Mass (g/mol)</th>
<th>Hydrochloride Compound Molecular Mass (g/mol)</th>
<th>Structure</th>
</tr>
</thead>
</table>
| Para-methoxy Butyryl fentanyl (hydrochloride) | Neat solid (about 1 mg)            | 380.52                         | 417.0                                       | ![Structure](image1)
| Acetyl fentanyl (hydrochloride) (CRM) | 100 μg/mL solution in MeOH         | 322.45                         | 358.9                                       | ![Structure](image2)
| Fentanyl (hydrochloride) (CRM) | 100 μg/mL solution in MeOH         | 336.50                         | 372.9                                       | ![Structure](image3)
| Benzyl fentanyl (hydrochloride) | Crystalline solid (about 1 mg)     | 322.45                         | 358.9                                       | ![Structure](image4) |
As part of Biomimiks™ technology, several other compounds were used, and this process is further explained in section 1.4.3. Acetonitrile was used as a solvent, octa-chloro octa-bromo porphyrin iron III-chloride (FeOCOB) and iron octa-chloro tetra-sulfonic acid porphyrin chloride (FeOCSulfonate) were used as catalysts, and imidazole, ≥ 99% purity (Sigma-Aldrich, St. Louis, MO), was used as a co-catalyst. The oxidizing agents used were iodoso benzene, hydrogen peroxide 35% w/w aqueous solution (Alfa Aesar, Haverhill, MA), diacetoxyiodo benzene 98% purity (Sigma-Aldrich, St. Louis, MO), magnesium monoperoxyphthalate hexahydrate (Sigma-Aldrich, St. Louis, MO), and 3-chloroperoxybenzoic acid 70-75% wet with water (Alfa Aesar, Haverhill, MA).

1.3.2 DART Technology

Direct Analysis in Real Time Mass Spectrometry (DART-MS) is a relatively new analytical technique that utilizes thermal desorption and chemical ionization to provide real-time results. Unlike classical atmospheric pressure ionization techniques, DART is a form of “ambient mass spectrometry”, meaning sample ionization occurs outside of a vacuum environment. With other forms of mass spectrometry, sample preparation is required, as the sample must be pretreated and introduced to the mass spectrometer in a specific manner. With DART, molecules from the sample are ionized and directly enter the mass analyzer, without dissolution or extraction\textsuperscript{21}.

The instrument is comprised of a DART-SVP source, which is an enclosed cylinder containing the ionization source and a reaction zone. The SVP, or standardized voltage and pressure, provides reliable desorption ionization, and this source can be interfaced directly to a mass spectrometer\textsuperscript{22}. In the ionization source, helium gas flows through and is treated so that upon exiting the source, it contains only electronically excited neutral species. The helium is heated to between 50 °C and 550 °C, and flows at about 3.5 L/min as it exits the source. Because the gas is
electronically excited, it will immediately ionize any surrounding gas upon emission from the DART-SVP source\textsuperscript{21}.

Provided the analyte of interest is placed between the DART source and the mass spectrometer, gas-phase reactions will occur due to the excited helium ions – ions of the analyte will form from chemical ionization processes and be carried into the mass analyzer by the helium gas. This process will work as long as analyte molecules are present in the gas phase, and even works with compounds with very low vapor pressure. As long as the compound will not thermally degrade at high temperatures, it can be analyzed by DART-MS\textsuperscript{21}. Figure 6 shows the DART-SVP source and mass spectrometer used during the course of this research.

![DART-SVP Ion Source Interfaced to the Front End of an Agilent 6520 Accurate Mass Q-TOF LC/MS](image)

Figure 6. DART-SVP Ion Source Interfaced to the Front End of an Agilent 6520 Accurate Mass Q-TOF LC/MS
1.3.3 Liquid Chromatography Triple Quadrupole Mass Spectrometry

Triple Quad LC/MS is a powerful analytical tool that has many applications in forensic and analytical chemistry. The instrument consists of several parts – an electrospray ion source where the analyte of interest is ionized and desolvated, the first quadrupole analyzer which filters selected ions based on their mass to charge ratio, the collision cell or “second quadrupole” in which the ions are fragmented, and the third quadrupole which receives the fragmented ions in order to isolate the product ions from the precursor ions and investigate them. A theoretical model of the instrument is shown in Figure 7.

![Figure 7. Theoretical Model of Triple Quadrupole Mass Spectrometer. Obtained from Agilent QQQ Concepts Manual](image)

While single quadrupole systems only contain one quadrupole that filters ions by their mass to charge ratio, triple quadrupole systems have three – Q1 and Q3 operate as mass filters while Q2 functions as a collision cell. This allows for multiple reaction monitoring (MRM), which is the most common use of a triple quadrupole system for qualitative analysis. MRM can be
described as performing several single reaction monitoring (SRM) processes at once, for the same set of precursor ions\textsuperscript{23}. In the MRM process, Q1 filters specific precursor ions of interest, Q2 produces product ions by collision with a neutral collision gas, and Q3 filters out all of the product ions that do not match the selected m/z. By the time the sample reaches the detector, it has passed through two mass filters, Q1 and Q3, and a collision cell, Q2, as shown in Figure 8. In this way, the MRM mode of operation reduces noise and increases selectivity, enabling the quantitation of many selected analytes in a single experiment\textsuperscript{24}.

![Cutaway View of Agilent 6460 Series Triple Quad LC/MS](image)

Figure 8. Cutaway View of Agilent 6460 Series Triple Quad LC/MS. Obtained from Agilent QQQ Concepts Manual\textsuperscript{23}

\section*{1.4 Sample Preparation}

\subsection*{1.4.1 Dilution}

Compounds in solid formulation (para-methoxy butyryl fentanyl and benzyl fentanyl) from Cayman Chemical arrived as 1 mg samples and compounds in solution (fentanyl and acetyl fentanyl) arrived as 100 μg/mL solutions in methanol. Both were diluted to 10 ng/mL prior to utilizing MassHunter Optimizer.

For the solid compounds, 1 mL of methanol was added to each solid sample to create a 1 mg/mL solution, and then several dilutions were performed to obtain the required 10 ng/mL
concentration of para-methoxy butyryl fentanyl or benzyl fentanyl in methanol. For the compounds in solution, one dilution was performed to achieve the desired solution of 10 ng/mL fentanyl or acetyl fentanyl in methanol. All of the solutions were stored in the freezer at 5°C to preserve their longevity.

To perform data acquisition, new samples were prepared from the original solutions on the day of analysis. The 10 ng/mL concentration was kept consistent for all samples throughout data acquisition, even in mixtures of fentanyl-related compounds. During catalyst addition, the 100 μg/mL solutions of fentanyl and acetyl fentanyl and the 1 mg/mL solutions of para-methoxy butyryl fentanyl and benzyl fentanyl were used without further dilution.

1.4.2 Catalyst Addition and Incubation

The samples that arrived as 100 μg/mL solutions in methanol were treated slightly differently than those that arrived as solids. 10 μL of the 100 μg/mL fentanyl and acetyl fentanyl solutions were used while 5 μL of the previously prepared 1 mg/mL benzyl fentanyl and para-methoxy butyryl fentanyl were used as the substrate in the sample. In a glass vial, a small amount of catalyst, co-catalyst, and oxidizing agent along with 100 μL of acetonitrile were added to the drug substrate. A disposable glass Pasteur pipette was used to estimate the amount of catalyst, co-catalyst, and oxidizing agent used – a similar amount of each of these was used in each sample, and was not measured exactly as the quantity was not crucial to the reaction. Instead, the tip of the pipette was dipped into each catalyst, co-catalyst, and oxidizing agent until the compound was visibly forced into the tip, then the pipette was tapped into each vial to deposit the compound. This method was used with of all the compounds that were solid powders, with the exception of the oxidizing agent hydrogen peroxide, as it was a liquid. Instead, 2-3 drops of
hydrogen peroxide were added to the requisite vials with a glass Pasteur pipette and rubber pipette bulb.

The first catalyst used was octa-chloro octa-bromo porphyrin iron III-chloride (FeOCOB), a charcoal colored powder, and the co-catalyst used was imidazole, a granular off-white powder. While the catalyst, co-catalyst, and acetonitrile stayed consistent in each sample, several oxidizing agents were evaluated with each of the drug substrates. These oxidizing agents were iodoso benzene, hydrogen peroxide, diacetoxyiodo benzene, magnesium monoperoxyphthalate hexahydrate (MMPP), and meta-chloroperoxybenzoic acid (MCPBA). Because four fentanyl related compounds were mixed with these five oxidizing agents, a total of 20 pairwise combinations were prepared.

Figure 9. Structure of Octa-Chloro Octa-Bromo Porphyrin Iron III-Chloride (FeOCOB) Catalyst

Each mixture was incubated at ambient temperature for about 24 hours prior to instrumental analysis, stirring constantly. After the incubation period, the samples were washed with acetonitrile and filtered, as explained in section 1.5.4. To determine the ideal combination of substrate, solvent, catalyst, and oxidant, all 20 of the samples were analyzed by DART for a real time analysis of the ions produced.
Table 2. Combinations of Drug Substrates and Oxidizing Agents for DART Sample Preparation

<table>
<thead>
<tr>
<th>Drug Substrate</th>
<th>Oxidizing Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μL of 100 μg/mL Fentanyl in Methanol</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>10 μL of 100 μg/mL Acetyl Fentanyl in Methanol</td>
<td>Diacetoxyiodo Benzene</td>
</tr>
<tr>
<td>5 μL of 1 mg/mL Benzyl Fentanyl in Methanol</td>
<td>Magnesium Monoperoxyphthalate Hexahydride (MMPP)</td>
</tr>
<tr>
<td>5 μL of 1 mg/mL Para-methoxy Butyryl Fentanyl in Methanol</td>
<td>3-Chloroperoxybenzoic Acid (MCPBA)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Co-Catalyst</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>100 μL Acetonitrile</td>
</tr>
</tbody>
</table>

After analyzing the data produced by the FeOCOB catalyst, another similar catalyst, iron octa-chloro tetra-sulfonic acid porphyrin chloride (FeOCSulfonate), was used. Because all of the fentanyl-related compounds had similar structures, the efficacy of this catalyst was tested with only 5 pairwise combinations – by using the drug substrate fentanyl with all 5 oxidizing agents, and the co-catalyst and solvent as previously used. The same incubation time and sample analysis method was used for these samples.

Table 3. Five Pairwise Combinations with Fentanyl and FeOCSulfonate Catalyst

<table>
<thead>
<tr>
<th>Drug Substrate</th>
<th>Oxidizing Agent</th>
<th>Catalyst</th>
<th>Co-Catalyst</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>Hydrogen Peroxide</td>
<td>FeOCSulfonate</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>Diacetoxyiodo Benzene</td>
<td>FeOCSulfonate</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>MMPP</td>
<td>FeOCSulfonate</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>MCPBA</td>
<td>FeOCSulfonate</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>Iodoso Benzene</td>
<td>FeOCSulfonate</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
</tbody>
</table>
1.5 Method Development

1.5.1 MassHunter Optimizer

MassHunter Optimizer, an automated method development tool used to generate and optimize MRM transitions, was used as a starting point to create methods. The desired compounds were input into the Compound Setup portion of the program, and the default method for opioids was chosen in the Method section. Once the compound name and molecular formula were selected, the software filled in the mass, and the appropriate sample position was chosen. Parameters such as the collision energy range and injection volume were input into the Optimizer Setup and Precursor Ion Setup portions of the program. All parts of the instrument were powered on and the optimization was started.

After optimization, the program showed the product ions, the collision energy, and the abundance for each ion, as shown in Table 4. This data was acquired for each compound, and was then imported into the Data Acquisition portion of the software. MassHunter Optimizer provided a valuable starting point for method development as it predicted the transitions of each compound, or where the compound would fragment after being exposed to a certain collision energy.
Table 4. MassHunter Optimizer Data for Fentanyl and Fentanyl-Related Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor Ion (m/z)</th>
<th>Fragment Voltage (kV)</th>
<th>Product Ion (m/z)</th>
<th>Collision Energy</th>
<th>Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>337.2</td>
<td>140</td>
<td>105</td>
<td>41</td>
<td>447690</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>188.1</td>
<td>17</td>
<td>461880</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>79.1</td>
<td>60</td>
<td>88667</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>77.1</td>
<td>60</td>
<td>81600</td>
</tr>
<tr>
<td>Acetyl Fentanyl</td>
<td>323.2</td>
<td>135</td>
<td>105</td>
<td>37</td>
<td>1218978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>188.1</td>
<td>17</td>
<td>1180954</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>79.1</td>
<td>60</td>
<td>279675</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>77</td>
<td>60</td>
<td>261760</td>
</tr>
<tr>
<td>Benzyl Fentanyl</td>
<td>323.2</td>
<td>130</td>
<td>91.1</td>
<td>41</td>
<td>11042</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>174.1</td>
<td>17</td>
<td>7318</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.1</td>
<td>60</td>
<td>1472</td>
</tr>
<tr>
<td>Para-methoxy</td>
<td>381.3</td>
<td>160</td>
<td>105</td>
<td>45</td>
<td>6477</td>
</tr>
<tr>
<td>Butyryl Fentanyl</td>
<td></td>
<td></td>
<td>188.1</td>
<td>21</td>
<td>6634</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>134.1</td>
<td>29</td>
<td>1033</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>162.1</td>
<td>33</td>
<td>1588</td>
</tr>
</tbody>
</table>

1.5.2 MassHunter Data Acquisition

After the MassHunter Optimizer data was transferred to the Data Acquisition portion of the software, the method was further optimized. The method was created by including the transitions from the optimization data of each compound and modifying the appropriate parameters, and was modeled after several methods in literature\textsuperscript{25, 26}.

Table 5. Instrumentation Parameters for Data Acquisition by LC-MS/MS

<table>
<thead>
<tr>
<th>LC System</th>
<th>Agilent Technologies 1200 Series</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS System</td>
<td>Agilent Technologies 6460 Triple Quad LC/MS</td>
</tr>
<tr>
<td>LC Column</td>
<td>Raptor Biphenyl 5 μm, 50 x 2.1 mm</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>500 μL/min</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>10 μL</td>
</tr>
</tbody>
</table>
Table 6. Mobile Phase Gradient Parameters for Data Acquisition by LC-MS/MS

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Mobile Phase A 0.1% Formic Acid in Water</th>
<th>% Mobile Phase B 0.1% Formic Acid in Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>2.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>7.5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>8.0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>8.1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10.0</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Several iterations of the method were developed as part of the optimization process. To achieve better chromatography, the flow rate was increased from 300 μL/min to 500 μL/min, and the gradient was altered with a faster ramp and a longer hold. Although some methods provided poorer chromatography than others, all of the methods were able to identify all of the transitions in each compound. Even when the method was shortened from a total time of 10 minutes to a total time of 6 minutes, all of the expected MRM transitions were present. Once each chromatogram was extracted and integrated, it was clear that all of the anticipated ionization and fragmentation had occurred, as all of the anticipated fragment ions were present.

Figure 10 shows the data produced from the conditions in Table 5 and Table 6. The first chromatogram is the TIC, or total ion chromatogram, and the rest are the extracted and integrated chromatograms of the fentanyl-related compounds. The chromatograms are grouped by compound according to molecular weight and transition – for example, the first 4 chromatograms after the TIC correspond to para-methoxy butyryl fentanyl and its related transitions. Table 4 shows the predictions of MassHunter Optimizer while Table 7 shows the actual precursor and product ions detected by MassHunter Data Acquisition, that are seen in Figure 10.
Figure 10. MRM Transitions in Chromatogram of Fentanyl-Related Compound Mixture
1.5.3 MassHunter Qualitative Analysis

Table 7. MRM Transition Data with Retention Time and Most Abundant Product Ions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ion 1 (m/z)</th>
<th>Product Ion 2 (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>3.6-3.7</td>
<td>337.2</td>
<td>105.0</td>
<td>188.1</td>
</tr>
<tr>
<td>Acetyl Fentanyl</td>
<td>3.4-3.6</td>
<td>323.2</td>
<td>105.0</td>
<td>188.1</td>
</tr>
<tr>
<td>Benzyl Fentanyl</td>
<td>3.5-3.6</td>
<td>323.2</td>
<td>91.1</td>
<td>174.1</td>
</tr>
<tr>
<td>Para-methoxy Butyryl Fentanyl</td>
<td>3.8-3.9</td>
<td>381.3</td>
<td>105.0</td>
<td>188.1</td>
</tr>
</tbody>
</table>

As seen in Table 7, the LC-MS/MS data provided all of the anticipated product ions, matching the transitions predicted by MassHunter Optimizer. Since the LC-MS/MS method was not further optimized to be able to detect unanticipated fragments, the instrument was not used past detecting the MRM transitions predicted by the MassHunter Optimizer software. After the use of Biomimiks™ technology, unanticipated fragments were created. As a result of the Triple Quadrupole LC/MS being utilized in MRM mode, these unanticipated fragments could not be identified by this method, but the fragments could be identified by DART-MS. Therefore, instead of using LC-MS/MS to analyze the fentanyl-related compounds after catalyst addition, the DART-QTOF-MS was used. Using the DART data as a guide, the LC-MS/MS method can be further developed to detect unanticipated metabolites created by Biomimiks™ technology.

1.5.4 DART-QTOF-MS Data Acquisition

The DART-QTOF-MS was used as a screening approach to analyze fentanyl and fentanyl analogs after the catalyst reaction. This provided results in real time, showing possible metabolites produced by Biomimiks™ technology.
First, the run was set up in the MassHunter Data Acquisition software. A previously created method similar to the default method was used, the source temperature was set to 300°C, and the m/z range was adjusted to 150-400. An unlimited run time length was chosen, and all of the other default settings were kept the same, including using the dual ESI ion source setting in positive ion mode. It should be noted that the “dual ESI” mode setting within the MassHunter software is required to allow for the DART source to correctly interface to the front end of the Q-TOF mass spectrometer.

To prepare samples for DART analysis, they were washed with acetonitrile, filtered, and placed into vials. Approximately 100 μL of acetonitrile was added to each of the incubated samples, the samples were briefly mixed, and a glass Pasteur pipette was used to deposit the washed samples through a filter into separate vials. No additional sample cleanup was required because of the negligible amount of Biomimiks™ catalyst added.

Figure 11. DART-SVP Ion Source, With Arrow Indicating Sample Introduction Location
During data collection, a capillary tube was dipped into each washed and filtered sample and held in front of the ion source for 0.4 min. – for example, the first sample was held from 0.3-0.7 minutes, the second from 1.3-1.7 minutes, and so on. It was necessary to be precise with the timing of data collection so that the chronogram could be analyzed accurately in MassHunter Qualitative Analysis. The capillary tube was held between the DART-SVP source and the entrance to the mass spectrometer, as indicated by the arrow in Figure 11. All 20 combinations of drug substrate and oxidizing agent with the FeOCOB catalyst were analyzed by DART, as well as the 5 combinations with the FeOCSulfonate catalyst.

Table 8. Pairwise Combinations of Drug Substrate and Oxidizing Agent with FeOCOB Catalyst

<table>
<thead>
<tr>
<th>Drug Substrate</th>
<th>Oxidizing Agent</th>
<th>Catalyst</th>
<th>Co-Catalyst</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>Hydrogen Peroxide</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>Diacetoxyiodo Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>MCPBA</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>Iodoso Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Acetyl Fentanyl</td>
<td>Hydrogen Peroxide</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Acetyl Fentanyl</td>
<td>Diacetoxyiodo Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Acetyl Fentanyl</td>
<td>MCPBA</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Acetyl Fentanyl</td>
<td>Iodoso Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Benzyl Fentanyl</td>
<td>Hydrogen Peroxide</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Benzyl Fentanyl</td>
<td>Diacetoxyiodo Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Benzyl Fentanyl</td>
<td>MCPBA</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Benzyl Fentanyl</td>
<td>Iodoso Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Para-methoxy Butyryl Fentanyl</td>
<td>Hydrogen Peroxide</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Para-methoxy Butyryl Fentanyl</td>
<td>Diacetoxyiodo Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Para-methoxy Butyryl Fentanyl</td>
<td>MCPBA</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Para-methoxy Butyryl Fentanyl</td>
<td>Iodoso Benzene</td>
<td>FeOCOB</td>
<td>Imidazole</td>
<td>Acetonitrile</td>
</tr>
</tbody>
</table>
1.5.5 DART-QTOF-MS Qualitative Analysis

Samples were analyzed in sets depending on the number of samples prepared each day and the samples that were due for analysis after the 24-hour incubation period, as explained in section 1.4.3. The specific timing of 0.4 min. per sample allowed for the distinction of different samples within a chronogram, as explained in section 1.5.4. Figure 12 shows a chronogram of 4 samples, with the brackets shown indicating the 0.4 minute time intervals.

![Figure 12. Chronogram of Four Drug Substrate/Oxidizing Agent Samples.](image)

From each chronogram, the spectrum of each sample was derived. First, the 20 spectra from the use of the FeOCOB catalyst were observed. A cursory glance at the spectra showed that this catalyst was not creating many known metabolites that would naturally occur in the human body. However, one known primary metabolite of fentanyl, norfentanyl, seemed to be present in low abundance. This was a result of oxidative N-dealkylation of the carbon nitrogen bond on the fentanyl molecule, or when the phenethyl alcohol moiety from the fentanyl molecule was removed by the catalyst and a bond between nitrogen and hydrogen was formed. N-dealkylation is a typical biotransformation pathway in reactions involving enzymes that exist in the liver,
showing that the Biomimiks™ catalyst performed similarly to actual liver function. From Figure 13, it can be observed that fentanyl and norfentanyl are both present, albeit in low abundance. The molecular weight of fentanyl is 337.2, N-dealkylation causes a loss of 104, and the molecular weight of norfentanyl is 234.2.

![Figure 13. DART Spectrum Showing N-Dealkylation of Fentanyl to Norfentanyl](image)

Because the FeOCOB catalyst produced such a low abundance of norfentanyl and produced many other unidentifiable peaks, a different catalyst, FeOCSulfonate, was tried next. To reduce the sample preparation and analysis time, only 5 samples were prepared, with fentanyl and all 5 oxidizing agents. Because the structures of the fentanyl-related compounds were so similar, analyzing just one of the compounds was a good indicator of the efficacy of this catalyst. Unfortunately, after analysis, it was found that this catalyst was not much more effective than FeOCOB. As explained in section 1.7, the next step will be to try a different catalyst, with a central atom of manganese instead of iron. Other reaction parameters such as the use of a different co-catalyst or solvent can also be modified for the combination of catalyst, co-catalyst, oxidizing agent, and solvent that will yield results that most closely resemble actual reactions of the liver inside the human body.
1.6 Experimental Conclusions

Going into this project, it was known that the opioid crisis would not be solved by one scheme and a narrow, concentrated topic of research. However, the progress made created the basis for future research on this topic.

Four fentanyl-related compounds, specifically fentanyl, acetyl fentanyl, benzyl fentanyl, and para-methoxy butyryl fentanyl, were analyzed neat and in mixtures by Triple Quadrupole LC/MS. The method developed using MassHunter Optimizer software and other sources in literature was able to identify all of the expected MRM transitions, even in a mixture of all the compounds. The fentanyl-related compounds were then used with Biomimiks™ technology to see if oxidative liver function could be replicated, so that the metabolism of fentanyl analogs could be predicted and studied. MassHunter software was used once again with the DART-QTOF-MS to analyze the fentanyl-related compounds in mixtures with a catalyst, co-catalyst, oxidizing agent, and solvent. The technology was able to identify norfentanyl, a known, primary metabolite of fentanyl, in low abundance. To create reactions where more metabolites are present and identifiable, the technology will be modified to use a different catalyst and potentially a different co-catalyst or solvent in the future.

In addition to the generated data, an extensive literature review was conducted. This provided insight into the opioid crisis at large, the history and specific role of fentanyl and its analogs, and the methods that are being developed to analyze these compounds. In addition to generating additional data, the literature review must be continued with the same vigor to build on what is already known, and to learn more about the progress being made from other research groups and other fields. Many of the sources making up this literature review are cited in section 1.9.
1.7 Future Directions
Moving forward, the steps are quite clear as to what must be done to accomplish the overall goal of this project. Because the developed LC-MS/MS method could not identify unanticipated metabolites of fentanyl-related compounds, it must be modified to do so, using the DART-QTOF-MS data as a guide. The current method was effective for the objective of identifying the expected MRM transitions because it was created to do so, but the new method must be a “scan” method that will look for more than just known fragments. Although DART is a high throughput, high resolution screening method, it was not designed to provide the quantitative results that LC-MS/MS can produce. Eventually, when actual postmortem samples are received, they will need to be analyzed in a quantitative fashion, so the LC-MS/MS method will need to be developed in advance of that time.

Although the FeOCOB catalyst from Empiriko was effective in producing norfentanyl, a known metabolite of fentanyl, it did not produce other recognizable metabolites and the DART spectra were complicated many other unidentifiable m/z species. Even within the spectra of all 20 combinations of drug substrates and oxidizing agents, other recognizable metabolites were not present, indicating that FeOCOB might not be the best catalyst to use with fentanyl-related compounds. Since the Biomimiks™ technology does not use one standard method for simulating the function of the liver, another approach should be taken with this technology for best results. The catalyst used had iron as a central atom, but the next catalyst utilized will have manganese as a central atom in place of iron. In other words, instead of FeOCOB, MnOCOB can be used – the catalysts both have the same porphyrin structure, with a different central atom, as shown in
The use of this catalyst is recommended as the next logical step by Empiriko, in order to maximize the potential of Biomimiks™ technology.

Additionally, instead of using acetonitrile as the solvent during sample preparation, a biphasic mixture can be tried, such as 2 parts dichloromethane to 1 part water. This non-miscible solvent combination can be used in conjunction with a manganese-based catalyst and the same 5 oxidizing agents to create many new pairwise sample combinations. If additional recognizable metabolites are not seen with this combination, additional co-catalysts may be tried as well.

In the long term, once the methods for analyzing fentanyl analogs before and after catalyst addition have been optimized, an SPE approach will need to be developed to isolate the metabolites of opioid adulterants. Postmortem urine samples from fentanyl-related overdoses will contain compounds that must be filtered out prior to analysis by LC-MS/MS, as the instrument is sensitive and sample contaminants will create confounding issues. Additionally, fentanyl analogs and other diluents and adulterants may deliberately be injected into synthetic urine during the method development stage. Synthetic urine is a substance that simulates the
appearance and chemical properties of urine, and is used in toxicology or as a calibrant. The SPE approach will likely be based off of methods in literature, and will selectively isolate metabolites of opioid adulterants\textsuperscript{28, 29}.

Afterwards, a SPME-DART-MS approach will be developed to analyze samples of bodily fluid at the scene of a crime. Using the previously developed SPE method as a guide, the SPME-DART-MS method will isolate and detect fentanyl-related compounds and their metabolites in real time. Although SPME is a technique often paired with LC/MS or GC/MS, using it with DART will allow for its use as a screening technique in order to generate data almost immediately\textsuperscript{30}. Eventually, once many postmortem samples have been analyzed by this new approach, regional trends can be assessed. This will lead to clues explaining why some counties experience higher death rates than others, and will hopefully allow for the tracking of those manufacturing and selling illicit drugs.

1.8 Bibliography


https://www.cdc.gov/mmwr/volumes/65/wr/mm655051e1.htm (accessed February 2018).


1.9 Additional References

**News, Reviews, and General Information**


Methods of Analysis


