Cleveland State University
EngagedScholarship@CSU

ETD Archive

2018

Development of ESI-LC-MS Method for Drug Analysis

Kimberly Yacoub
Cleveland State University

Follow this and additional works at: https://engagedscholarship.csuohio.edu/etdarchive

 Part of the Other Medicine and Health Sciences Commons

How does access to this work benefit you? Let us know!

Recommended Citation

Yacoub, Kimberly, "Development of ESI-LC-MS Method for Drug Analysis" (2018). *ETD Archive*. 1064.
https://engagedscholarship.csuohio.edu/etdarchive/1064

This Thesis is brought to you for free and open access by EngagedScholarship@CSU. It has been accepted for inclusion in ETD Archive by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.

DEVELOPMENT OF ESI-LC-MS METHOD FOR DRUG ANALYSIS

KIMBERLY YACOUB

Bachelor of Science in Chemistry

Case Western Reserve University

May 2015

submitted in partial fulfillment of requirements for the degree

MASTER OF SCIENCE IN CHEMISTRY

at the

CLEVELAND STATE UNIVERSITY

MAY 2018

We hereby approve this thesis for

Kimberly Yacoub

Candidate for the Master of Science in Chemistry degree for the

Department of Chemistry

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies

Thesis Chairperson, Dr. Baochuan Guo

Department & Date

Thesis Committee Member, Dr. John Turner

Department & Date

Thesis Committee Member, Dr. Aimin Zhou

Department & Date

Student's Date of Defense: April 11th, 2018

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my thesis advisor, Dr. Baochuan Guo, of the Department of Chemistry at Cleveland State University. His guidance, assistance, and encouragement all helped me through my learning process of this master thesis. He offered his expert knowledge to steer the research in the right direction.

I would like to thank Raghavi Kakarla, PhD Candidate, for her support, knowledge, and expertise to undergo the development of this master thesis. Her support, assistance, and guidance aided me in achieving profound results. Thank you for all of your help and patience.

I would also like to graciously thank Dr. Turner and Dr. Zhou who took the time to participate in my final defense committee. The time you two took is valued and appreciated.

Lastly, I would like my family for supporting me during my time as a student. Your love and dedication towards helping me reach my dreams are appreciated and have not gone unnoticed.

DEVELOPMENT OF ESI-LC-MS METHOD FOR DRUG ANALYSIS

KIMBERLY YACOUB

ABSTRACT

Brain cancer, particularly astrocytomas, is one of the ten most common related deaths related to cancer. Temozolomide (TMZ), an oral alkylating chemotherapy drug, is used to treat anaplastic astrocytoma and glioblastoma multiforme. A few advantages of TMZ are its ability to cross the blood-brain barrier, its small size, and it is rapidly absorbed in the small intestine. Since this drug is advantageous to brain cancer patients, it is important to study the extraction of this drug. In addition, it is important to study administering this drug via microdialysis as the efficiency of the drug could increase.

In mice brain tumor tissue and lamb brain tissue, LC-MS was used to quantitate TMZ. TMZ has been recovered from brain tissue using a strong cell lysis protease (proteinase K) and protein precipitation using alcohol dehydration in order to increase percent recovery of TMZ and decrease matrix effects.

Most research articles have studied the plasma or urine of mice to quantitate TMZ. However, only a handful of studies focused on brain tumor tissue. Comparing with another research article that studied the recovery of TMZ from mice brain tissue, this method achieved higher percent recoveries of the chemotherapy drug, and lowered the matrix effects.

TABLE OF CONTENTS

ABSTRACT iv

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

I. INTRODUCTION 1

1.1 Astrocytomas 1

1.2 Glioblastoma Multiforme 2

1.3 Anaplastic Astrocytoma 4

1.4 Temozolomide Background 5

1.5 Mechanism of Temozolomide 7

1.6 O^6-methylguanine-DNA methyltransferase (MGMT) 9

1.7 TMZ Pharmacokinetics and Stability 10

1.8 Dacarbazine and TMZ 11

1.9 Rationale of the Work 11

1.10 Solid Phase Extraction 13

1.11 Protein Precipitation 15

1.12 Comparing Solid Phase Extraction and Protein Precipitation 16

1.13 High-Performance Liquid Chromatography (HPLC) 16

1.14 Mass Spectrometry (MS) 17

II. MATERIALS AND METHODS 19

2.1 Chemicals and Solutions 19

2.2 Sample Preparation 19

2.3 Calibration Curve Standard Preparation 20

2.4 HPLC-MS/MS Instrumentation 21

2.5 HPLC-MS/MS Optimization Parameters 21

2.6 Optimizing ZipTip Conditions 22

2.7 Optimizing Protein Precipitation 23

III. RESULTS AND DISCUSSIONS 26

3.1 Mass Spectrometry Infusion 26

3.2 Method Applicaton 30

3.3 Recovery of TMZ and Theophylline 35

3.4 Matrix Effects 36

IV. CONCLUSIONS 37

4.1 Conclusions and Future Work 37

REFERENCES 38

LIST OF TABLES

Table		Page
I.	Calibration Curve Standard Preparations for Lamb Brain	20
II.	Optimized Parameters of TMZ and Theophylline (IS)	22
III.	Varying Elution Mixtures for Optimization of ZipTips	23
IV.	Comparison of Goldwirt et al. Experimental Design for TMZ Extraction and This Experimental Design for TMZ Extraction	25
V.	Percent Recoveries of TMZ	35
VI.	Matrix Effects	36

LIST OF FIGURES

Figure		Page
1.	Magnetic Resonance Image of Glioblastoma in the Cerebral Hemisphere	2
2.	Chemical Structure of TMZ	6
3.	Mechanism of Action of TMZ and Methylation of Guanine	7
4.	DNA Methylation	8
5.	MGMT Repairing O^6-methylguanine Lesion	10
6.	MRM Transition of TMZ	22
7.	MRM Transition of IS	22
8.	Expermiental Design of Cell Lysis and Protein Precipitation	24
9.	Mass Spectrum of TMZ	27
10.	CID of TMZ	27
11.	MRM Spectrum of TMZ	28
12.	Mass Spectrum of Theophylline	28
13.	CID of Theophylline	29
14.	MRM Spectrum of Theophylline	29
15.	Chromatogram of 30 mg Mice Brain Spiked with 10 ng/mL of TMZ and 50 ng/mL of IS	30

16. Chromatogram of 30 mg Mice Brain Spiked with 50 ng/mL of TMZ and 50 ng/mL of IS 31

17. Chromatogram of 30 mg Mice Brain Spiked with 100 ng/mL of TMZ and 50 ng/mL of IS 31

18. Chromatogram of 30 mg Mice Brain Spiked with 200 ng/mL of TMZ and 50 ng/mL of IS 32

19. Chromatogram of 30 mg Mice Brain Spiked with 500 ng/mL of TMZ and 50 ng/mL of IS 32

20. Chromatogram of 30 mg Mice Brain Spiked with 1000 ng/mL of TMZ and 50 ng/mL of IS 33

21. Calibration Curve of 30 mg Mice Brain Spiked with Varying Amounts of TMZ and 50 ng/mL IS (n=2 replicates). 34

22. Calibration Curve of 30 mg Lamb Brain Spiked with Varying Amounts of TMZ and 50 ng/mL IS (n=3 replicates). 34

CHAPTER I

INTRODUCTION

1.1 Astrocytomas

Astrocytomas are primary brain tumors and one of the ten most common cancer-related deaths. Most primary brain tumors, including gliomas, originate from neuroepithelial cells. If a tumor arises from supportive tissue of the brain, referred to as glial, it is considered a glioma. Even though gliomas are rare, approximately 30-40% of all brain tumors are gliomas and approximately 50% of gliomas are glioblastomas.[1]

Between 1-5% of gliomas are considered hereditary and a majority of gliomas have unknown causes. Although these causes are unknown, a large risk factor can be attributed to ionizing radiation. Middle-aged adults are most typically affected by gliomas. The way that gliomas grow is by infiltrating into the brain, more specifically, the white matter of the brain; therefore, they are not directly visible on the surface of the brain. Most commonly in adults, gliomas can be found in the cerebral hemispheres (Figure 1).[1]

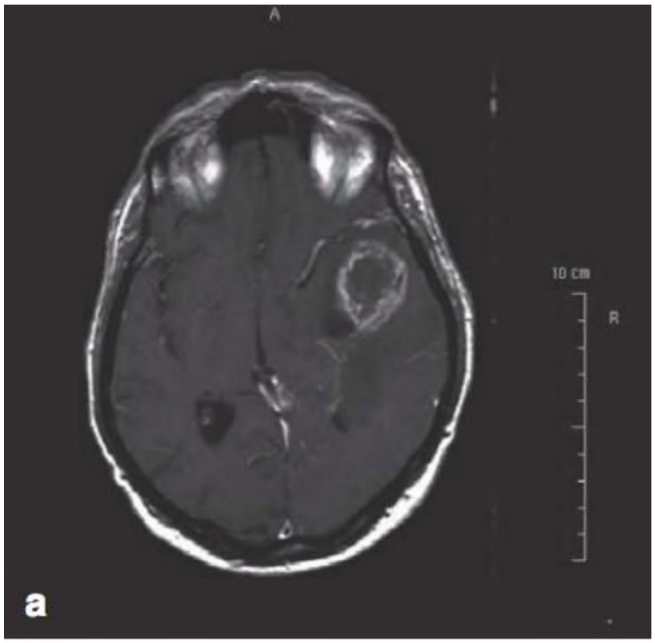


Figure 1. Magnetic Resonance Image of Glioblastoma in the Cerebral Hemisphere.[1]

With radiation therapy (RT), surgery, and chemotherapy, the average survival rate of patients with gliomas is less than 1 year.[2] During the past few decades, the main method of treating gliomas is a combination of alkylating agents and radiotherapy. With this combination of treatment, the survival rates of patients with glioblastoma multiforme has greatly improved.[3]

1.2 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is a type of high-grade astrocytoma. GBM is a type of tumor in the central nervous system (CNS). Unfortunately, GBM prognosis is poor. It is one of the most frequent types of brain tumors in adults. According to the World Health Organization (WHO), GBM is considered a Grade IV astrocytoma. There are four types of astrocytomas and grade IV is the most serious type. Primary GBMs are very aggressive and highly invasive tumors that are more common in the elderly. Secondary GBMs develop from astrocytomas that are low-grade and typically affect

people who are less than 45 years of age. Primary GBMs are more common than secondary GBMs and secondary GBMs have a much better prognosis than primary GBMs. Histologically, these two tumors cannot be distinguished, but they evolve from different genetic alterations.[4] Since GBM cells grow invasively into normal brain tissue, surgery is not possible for the resection of tumor cells.[5] Not only is GBMs invasiveness a factor in the inability to resect the tumor, but GBM tumors are also highly vascularized making surgery impractical. The high vascularization of GBM tumors is due to the overactive formation of new blood vessels also known as angiogenesis. The over-activation of these blood vessels is critical for supplying oxygen for tumor growth.[4]

The pathogenesis of GBM can be mainly attributed to an altered pathway that involves receptor tyrosine kinases (RTKs), which are receptors located on the surface of cells. Growth factors (GFs) are bound by RTKs through cross-linking. When cross-linking occurs, two adjacent receptors are dimerized, which then induces a conformational change. Once a conformational change is induced, the kinase of RTK is activated, which allows for cross-phosphorylation of tyrosine residues to prepare for signaling cascades. One of these signaling cascades is epidermal growth factor receptor (EGFR). EGFR is a transmembrane protein that plays a role in GBM pathogenesis. EGFRs normally function by dividing and proliferating normal CNS cells. When there are genetic mutations, EGFR is overexpressed. Thus, this leads to increased activity of phosphorylation and proliferation of oncogenic cells.[4]

Not only are RTK pathways altered, but the Ras pathway is too. Ras is a guanosine-binding protein (G protein). When bound to GDP, Ras is in an inactive state. When bound to GTP, Ras is in an active state (Ras-GTP). Ras-GTP leads to advancement

through the cell cycle, a cascade of downstream effectors, and survival. Like EGFR, when there is an interaction with RTK and GF, there is a signaling cascade. PI3K, phosphoinositide 3-kinase, moves to the cell membrane. Once this occurs, a secondary messenger PIP3, phosphatidylinositol trisphosphate, is created. PI3K-Akt pathway activation leads to proliferation, survival, and angiogenesis of cells.[4]

The symptoms of GBM vary based on the location of the tumor. Some of these symptoms are persistent headaches, double/blurred vision, nausea, vomiting, loss of appetite, changes in mood/personality, changes in ability to think and learn, loss of memory, new onset of seizures, muscle weakness, and speech difficulty. GBM, which may appear on any lobe of the brain, most commonly appears on the frontal and temporal lobes.[6]

1.3 Anaplastic Astrocytoma

Like GBM, anaplastic astrocytoma (AA) is a type of high-grade astrocytoma in the CNS, but it is rare.[7] AAs comprise about 30% of all astrocytomas and 7% of all primary brain tumors in adults.[8, 9] AA is considered a grade III astrocytoma according to the WHO. Over time, AA can develop into a higher grade astrocytoma, or GBM.[7] The typical treatment plan for patients with AA is to maximally and safely resect the tumor, and then further treat the tumor with RT. The combination of surgery and RT has been associated with longer survival periods.[9] AAs are most frequently found in the frontal lobe of the brain. Some prognostic factors that influence survival are Karnofsky performance score (KPS), amount of tumor resected, as well as the age of the patient at diagnosis.[10] The KPS method was first introduced in 1949. It is a widely used method in order to determine the functional status of a patient on an 11-point scale with percentages

ranging from 100% (no symptoms) to 0% (death of patient).[11] There is no link between AAs and familial inheritance, and in most cases the cause is unknown. AAs, like other cancers, are believed to occur due to factors such as genes and environment. In addition, there is a higher chance of developing AA if a patient has inherited disorders such as neurofibromatosis type I, Li-Fraumeni syndrome, tuberous sclerosis and Turcot syndrome.[12]

Like GBM, the symptoms of AA are dependent upon the location of the tumor in the brain. Some common symptoms include headaches, lethargy or drowsiness, vomiting, changes in personality and mental status, seizures, vision problems, and weakness of the arms and legs resulting in coordination difficulties.[7]

1.4 Temozolomide Background

The aim of this thesis is to develop a quantification method for the pharmacokinetic analysis of injecting temozolomide (TMZ) in the brain directly to target the drug to the tumor cells. Previous research has studied the concentration of TMZ in mice plasma; however, only a handful of studies have studied the concentration of TMZ in brain tissue. None have studied the effect of TMZ injected directly into the brain. Thus, the significance of this research is imperative to study the effects of TMZ in patients with GBM and AA to target the drug specifically to the tumor in the brain. One study attempted to create a pharmacokinetic model to determine the amount of TMZ that reaches brain tumors. Zhou et al. determined that the concentration of TMZ varied with changes in blood-brain barrier permeability as well as tumor blood volume.[13] Therefore, studying the recovery of TMZ injected in mice brain and developing a method is imperative in attempting to increase the pharmacokinetics of TMZ in patients.

In the early 1980s, Robert Stone chemically synthesized TMZ at Ashton University. TMZ is a unique compound since it contains three adjacent nitrogen atoms, as shown in Figure 2. Prior to synthesizing TMZ, other compounds were synthesized that only contained two adjacent nitrogen atoms; however, TMZ had far greater cytotoxicity when compared to the other drugs. Not only was TMZ more cytotoxic, but it was more effective than compounds with two adjacent nitrogen atoms.[2]

TMZ is an alkylating imidazotetrazine chemotherapy pro-drug approved by the Food and Drug Administration (FDA) as a first-line treatment for GBM and a second-line treatment for AA.[2] The International Union of Pure and Applied Chemistry (IUPAC) name for TMZ is 3-methyl-4-oxoimidazo [5,1-d][1,2,3,5] tetrazine-8-carboxamide.[14] It is typically administered orally and is rapidly absorbed in the small intestine. Before the synthesis of TMZ as an oral pill, it was administered intravenously. TMZ has a molecular weight of 194.154 g/mol and a molecular formula of $C_6H_6N_6O_2$. TMZ is stable at a pH less than 5 and it is labile at physiological pH. As TMZ is a pro-drug, its mechanism of action is through conversion to an active form at physiological pH.[14, 15]

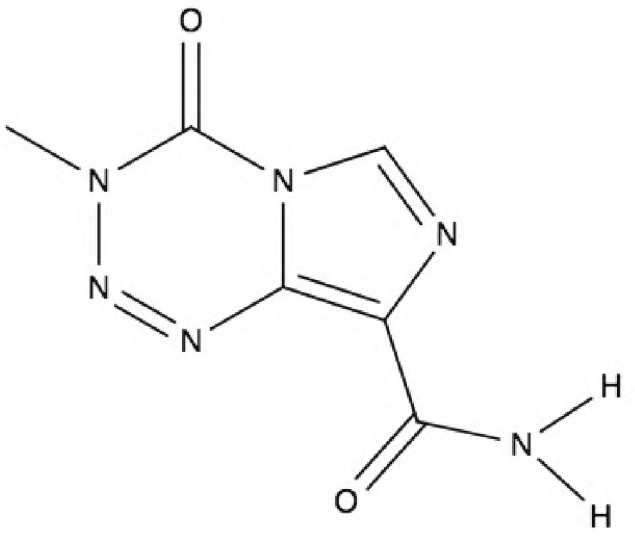


Figure 2. Chemical Structure of TMZ.[15]

1.5 Mechanism of Temozolomide

TMZ is able to readily cross the blood-brain barrier since it is small (194 Da) and lipophilic, which are a few advantageous characteristics of this drug. TMZ is hydrolyzed rapidly and spontaneously to Methyl-(trazen-1-yl) imidazole-4-carboxamide (MTIC) under physiological conditions, which is then hydrolyzed to 5-Aminoimidazole-4-carboxamide (AIC) and diazomethane (Figure 3).[14, 15] Unlike TMZ, MTIC does not readily penetrate the CNS. AIC is an intermediate in purine and nucleic acid biosynthesis and it is excreted out via the kidneys. Diazomethane is able to react with RNA, but there has been little evidence in its role in the cytotoxicity of TMZ. In comparison to other alkylating chemotherapy drugs, TMZ is spontaneously converted to MTIC at physiological pH, while other drugs require metabolism in the liver. Thus, the conversion rate of TMZ to MTIC is not hepatic-dependent and will not vary between patients.[2]

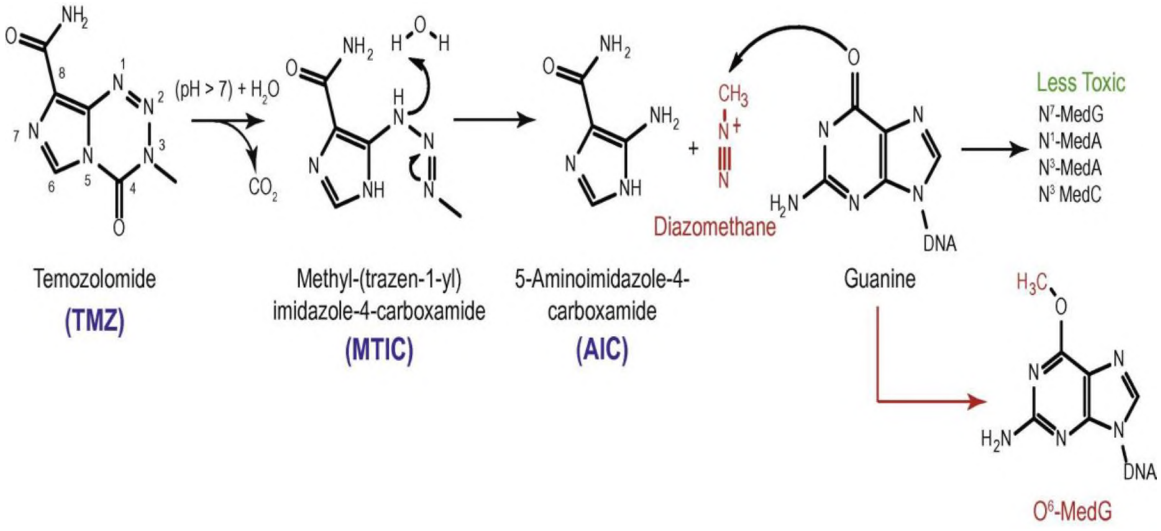


Figure 3. Mechanism of Action of TMZ and Methylation of Guanine.[16]

Since TMZ is an alkylating drug, it serves to damage DNA in order to halt cancer cell replication. TMZ undergoes this alkylation by methylating DNA at different positions of DNA, but the most common positions include the N^7 position of adenine, the

N^7 position of guanine, and the O^6 position of guanine.[2] Although only 5% of DNA is methylated at the O^6 position of guanine, this methylation is a key player in the mismatching of thymine instead of cytosine (Figure 4).[17] The constant mismatching with thymine leads to apoptosis, double stranded breaks and ultimately, cell cycle arrest after the next cycle of DNA replication.[14] O^6-methylguanine-DNA methyltransferase (MGMT) has served as the only enzyme that is able to repair these mismatched adducts. Research has been conducted on the effect of TMZ in relation to the levels of MGMT and the levels of TMZ.[18]

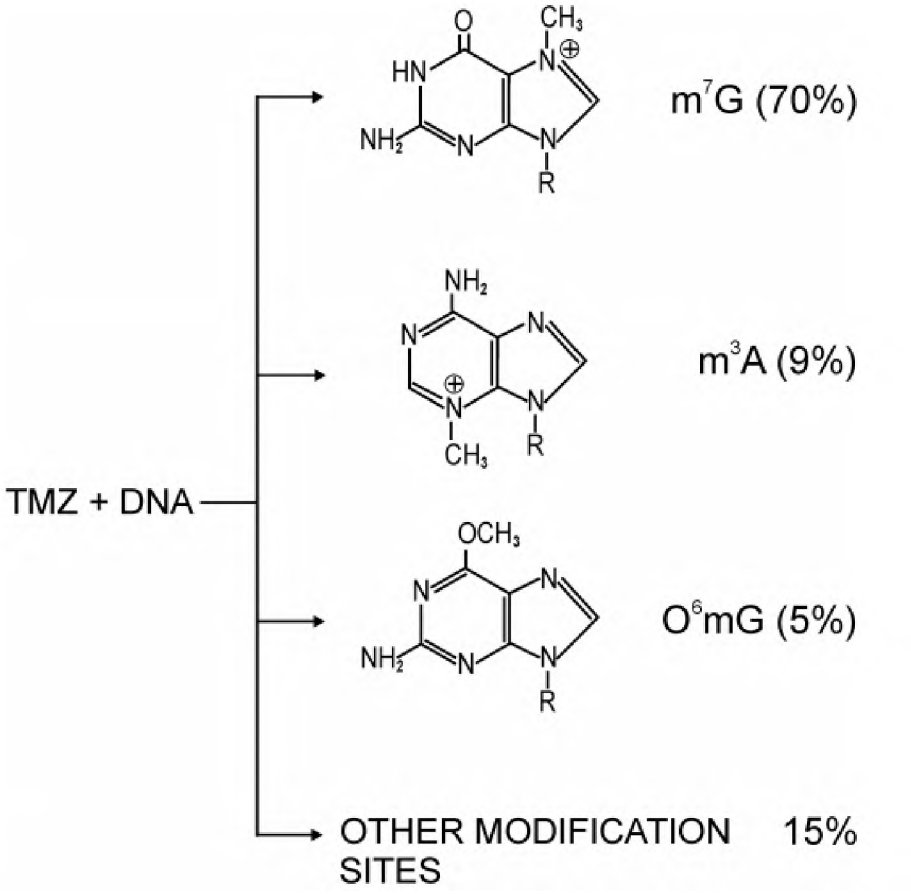


Figure 4. DNA Methylation.[17]

1.6 O^6-methylguanine-DNA methyltransferase (MGMT)

MGMT is a human protein that is critical for the stability of DNA. The DNA repair protein is 354 amino acids long with a molecular mass of 39 kDa.[19] It is encoded by chromosome 10q26.[14] MGMT is considered a "suicide enzyme" since it is able to irreversibly inactivate itself.[19] Low MGMT levels, since it is a repair enzyme, correlate with higher efficiency of TMZ.[18] A number of studies have correlated the inverse relationship between levels of TMZ and MGMT.[20]

Since TMZ serves to halt the proliferation of cancer cells by mismatching DNA adducts, repairing O^6-methylguanine is not ideal for oncogenic cells. The purpose of MGMT is to serve as a DNA repair enzyme, which hinders the effects of TMZ. MGMT is able to repair the O^6-methylguanine lesions by transferring an alkyl group from guanine to cysteine via acid catalysis allowing the protein to bind the methyl and cleave the ether bond (Figure 5).[9] Methylation of MGMT has led to improved survival in patients who were treated in combination with RT and TMZ. Although it is important to recognize that tumor cells should have depleted levels of MGMT to increase the efficiency of TMZ, MGMT is also depleted in normal cells, which unfortunately leads to hematologic toxicity.[3]

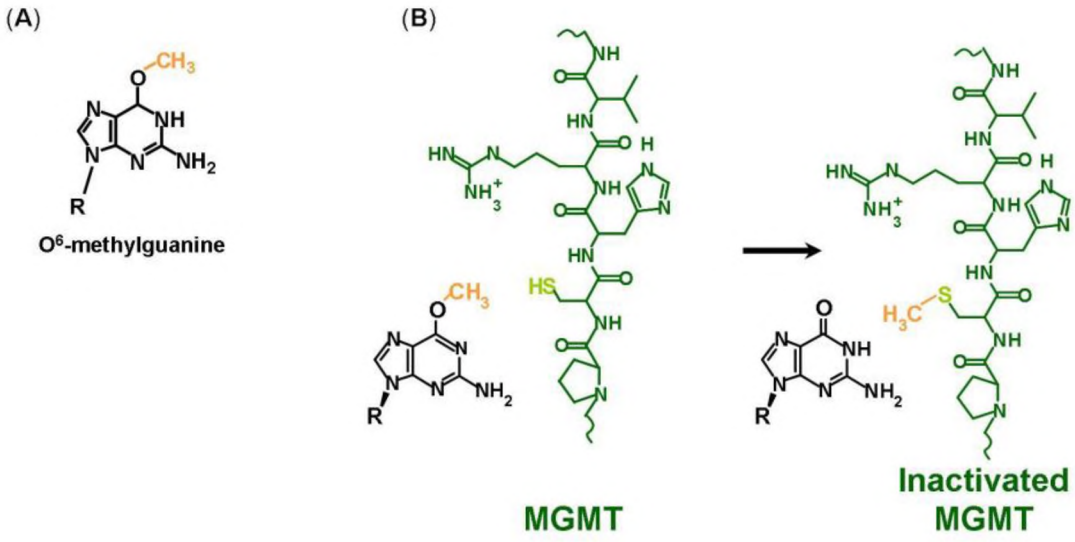


Figure 5. MGMT Repairing O^6-methylguanine Lesion.[19]

1.7 TMZ Pharmacokinetics and Stability

As stated previously, TMZ is rapidly absorbed in the small intestine. Consumption of food does delay the absorption of TMZ; however, the delay has been found to be clinically insignificant. The concentration of TMZ in plasma reaches its peak 1 hour after administration, and then decreases slowly. According to a clinical study, it was determined that the average maximum concentration of TMZ in the brain interstitium was 2 hours after administration. This clinical study concluded that levels of TMZ in the brain interstitium gradually rise over time and stayed at higher levels longer than plasma levels of TMZ.[21] In another study, it was determined that the maximum concentration of TMZ in the brain is reached at 0.75 hours.[22] Although this is different from the first study, both of these studies concluded that the maximum concentration of TMZ in brain occurs later than the maximum concentration of TMZ in plasma and the levels of TMZ in brain stayed higher for longer periods of time. The maximum concentration time differences may be attributed to the routes of administration, as well as the subjects. The

first study administered TMZ orally in humans, while the second study administered TMZ intraperitoneally in mice.

The stability of TMZ in human plasma has been studied extensively. The half-life of TMZ in vitro is 2.4 hours. At three different concentrations (0.2, 1.5, and 15 μg/mL), TMZ was stable at a pH < 4 using phosphoric acid for approximately 24 hours at room temperature (25°C). In addition, TMZ was found to be stable at a pH < 4 at -20°C for at least 30 days at the same three concentrations.[23]

1.8 Dacarbazine and TMZ

Dacarbazine (DTIC), like TMZ, is a chemotherapy drug administered intravenously to treat Hodgkin lymphoma, melanoma and soft tissue sarcoma. DTIC was approved by the FDA in the mid-1970s. Like TMZ, DTIC is also a pro-drug that is converted to MTIC. The primary difference between TMZ and DTIC is that DTIC is converted in the liver, while TMZ is spontaneously converted at physiological pH. TMZ was introduced as an alternative to DTIC because of its potential as an antitumor agent and the fact that preclinical assessments found it to be a safer drug. Not only is TMZ safer; however, it is more convenient as it can be administered orally, while DTIC is administered intravenously.[23] TMZ also has extensive tissue distribution when compared to DTIC.[23, 24]

1.9 Rationale of the Work

A number of research papers have focused on determining the amount of TMZ found in the plasma matrix. However, very few have focused solely on brain tumor tissue; therefore, we have studied TMZ extraction procedures in mice brain tumor tissue. The problems with the extraction of TMZ from plasma have been low extraction yields

and time-consuming repetitive solvent extractions.[20] The problems with the extraction of TMZ from brain tumor tissue have also been low extraction yield, as well as high matrix effects due to the high amounts of biological matrices found in brain.[25] It is worthy to note that mice brain contain between 45.0-20.1 mg/g of phospholipids, 105.8-107.4 mg/g of proteins and 14.1-20.1 mg/g of cholesterol between ages 3 months to 26 months to assist in the reduction of matrix effects.[26] Therefore, studying the method development for the injection of the drug directly targeted to the brain tumor is of utmost importance.

The two main goals of this research were to develop a method that increased the extraction efficiency of TMZ in brain and to decrease matrix effect signaling. It is known that TMZ can readily cross the blood-brain barrier if administered orally or intravenously, but studying the administration of TMZ targeted directly to the brain tumor is critical to improve the efficiency of TMZ in treating brain cancers. When determining the most useful preparation techniques for this research, two other papers were taken into consideration.

Goldwirt et al. were one of the few researchers to recover TMZ from brain tissue. A number of studies researched TMZ in plasma; however, recovering the drug from brain tissue has rarely been studied. Goldwirt et al. used small slices of mice brain (400 mg). The brain cells were lysed with 200 μL of ammonium acetate 10 mM pH 3.5 buffer and the proteins were precipitated using 200 μL of 100 mM zinc sulfate and 400 μL of methanol in order to extract the TMZ from mice brain that was administered intraperitoneally. Their results were found to be precise, accurate, and specific with low limits of detection on UPLC-MS both intra- and inter-day. For brain, the extraction recovery of TMZ was 67.7% for 125 ng/g, 61.0% for 1250 ng/g, and 61.4% for 12,500

ng/g. The matrix effect was 245% for 125 ng/g, 204% for 1250 ng/g, and 212% for 12,500 ng/g. The matrix effects were very high as expected for brain tissue due to phospholipids and a number of unwanted biological matrices.[22] Therefore, to improve this method, a stronger lysis buffer was used along with better protein precipitation solvents.

Shen et al. quantified TMZ in human plasma and urine using HPLC-UV. The difference in techniques between the two papers is that Shen et al. used solid-phase extraction (SPE) to eliminate interfering material from the biological matrices. Their results were found to be accurate and precise for both urine and plasma intra- and inter-day. The extraction recovery of TMZ ranged between 86.0-90.0% for plasma and 102.5-104.8% for urine. When using SPE, multiple solvent extractions could be avoided and higher extraction recovery of TMZ is possible when compared to liquid-liquid extraction.[20] Therefore, to extract TMZ from mice brain, ZipTips were attempted in order to recover the drug and remove unwanted biological matrices.

1.10 Solid Phase Extraction

There are a number of different techniques to prepare samples before injection into an LC-MS such as liquid-liquid extraction and protein precipitation. The reason that cleaning biological samples before injection into HPLC is important is because high protein concentration leads to a decrease in the longevity of the column, inability to separate, and a build-up of column backpressure. High protein samples injected directly in the HPLC will contaminate the system leading to electrospray instability as well as a reduction in sensitivity. The single most used technique is solid-phase extraction (SPE)

since it is able to cleanup samples, it has high throughput, extraction reproducibility, and short sampling times.[27]

The purpose of SPE is to desalt, fractionate, and purify sample before analysis for the best quality. In SPE, much like liquid chromatography, different materials will have different affinities with the stationary phase. Thus, either the impurities that are unwanted from the sample, or the analytes in the sample are bound to the stationary phase leading to the separation of wanted and unwanted sample compounds. If the wanted sample compounds are in the mobile phase that passes through the stationary phase, then this is injected into the LC-MS. If the wanted sample compounds bind to the stationary phase, then the mobile phase is discarded and the analytes are eluted from the stationary phase. The eluent is then injected into the LC-MS.[28] There are different means to perform SPE, such as normal-phase, reverse-phase, ion exchange, and cartridges. The type of SPE that was attempted to separate biological matrices from brain tissue and TMZ in this research was ZipTip.

ZipTip is a small-scale reverse-phase separation technique that is packed with resin at the end of a 10 μL pipet tip. The resin beads serve to desalt small amounts of peptides. There are different ZipTips, such as C_4 and C_{18}. C_{18} is more hydrophobic and C_4 is less hydrophobic. The carbon chains are on the surface of silica beads. SPE consists of five simple steps: wetting, conditioning, sample loading, washing, and eluting. The initial wetting step requires using a water-miscible organic solvent, such as methanol. Then, the conditioning step requires using a water of aqueous buffer that is able to displace the organic solvent in the pores. The sample is then loaded and the compounds that are attracted to the stationary phase of the ZipTip will bind. Then, the compounds that did

not bind to the stationary phase are washed away with an aqueous solvent. The sample is then eluted from the stationary phase for injection into HPLC.[28] SPE may give low recovery of the analyte. If there is low recovery in SPE, the reasoning could be due to the use of incorrect conditioning, strong loading and wash solvent, large volume of sample loading, or too weak or too small elution mobile phase volume.[27]

1.11 Protein Precipitation

Protein precipitation is a very common technique that was discovered by Franz Hofmeister over 120 years ago. Protein precipitation occurs via hydrophobic aggregation. The proteins can precipitate in two ways. First, the folded structure of proteins can be slightly disrupted thereby exposing more of the hydrophobic interior of proteins. Therefore, the proteins begin to aggregate towards one another and the amount of water per protein decreases. Second, the water molecules that begin to form over hydrophobic areas on proteins that are folded correctly can be dehydrated.[29]

Salts maintain large and stable solvent shells so salts have high protein precipitation abilities. The surface tension of the solution is then increased thereby increasing the hydrophobic effect. Thus, proteins that have a larger amount of hydrophobic surface character are able to precipitate at lower salt concentrations. Organic solvents, such as ethanol and trifluoroacetic acid, can partially denature proteins and in effect, expose more hydrophobic surfaces of the solvents. Since this research focused on removing proteins, the possible denaturation of proteins did not deter from the use organic solvents.[29]

1.12 Comparing Solid Phase Extraction and Protein Precipitation

Using the research methods Shen et al. used for extracting TMZ from plasma and urine, solid phase extraction using C_{18} ZipTips were conducted to remove phospholipids and unwanted biological matrices from the brain. Focusing on mice brain tissue, Goldwirt et al. used ammonium acetate 10 mM pH 3.5 buffer to lyse the cells and zinc sulfate and methanol to precipitate proteins to extract TMZ. Protein precipitation is a common hydrophobic aggregation method. Protein precipitation is achieved by lowering the solubility of the solute by reducing the hydration layer in order to decrease the likelihood of protein aggregation.[29] To compare, this research compared trifluoroacetic acid and ethanol to precipitate proteins to recover TMZ. To further this, proteinase K was used to digest the unwanted proteins in biological samples and lyse the cells to recover TMZ with a higher recovery. Therefore, this research compared SPE to protein precipitation solvents in order to successively extract TMZ from brain tissue.

1.13 High-Performance Liquid Chromatography (HPLC)

HPLC is a widely used separation technique of mixtures on the basis of molecular structure and composition. Specifically, reverse-phase liquid chromatography is most used as it can separate a large variety of different molecules. HPLC consists of a mobile phase and a stationary phase. The specific column used was a C_{18}, which is a reversed phase column. In reversed phase chromatography, there is strong attraction between the polar solvent and polar molecules in the mixture being passed through the column, which is the mobile phase. The stationary phase is the hydrocarbon chains of the column (C_{18}). Therefore, polar molecules of the sample will be attracted to the solvent. On the other hand, non-polar molecules in the sample will be attracted to the stationary phase, which

are the hydrocarbons of the column. Due to these interactions, the polar compounds will elute quicker leading to chromatographic separation. HPLC is different from column liquid chromatography because the high pressures allow for a much quicker separation. Some of the fixed chromatographic conditions include flow rate, temperature, and the column. Quantitative analysis of HPLC can be determined by taking the area under the peak, which is proportional to the concentration of the sample. Qualitative analysis can be determined by comparing retention times of standards and samples. The advantages of HPLC are sensitivity, accurate quantitative analysis, ease of automation, stability of separating non-volatile species or thermally fragile ones, and applicability to substances important to industry. [30, 31]

1.14 Mass Spectrometry (MS)

Mass spectrometry is a multi-purpose analytical technique in chemistry. MS can aid in the identification of compounds that are unknown in a sample, determine the concentration of known materials, and determine the structure and chemical properties of different molecules. First, gas phase ions of the compound are produced through electron ionization. The molecular ion then fragments. From the molecular ion, every primary product ion fragments and this continues. In the mass spectrometer, the ions are separated based on the mass-to-charge ratio (*m/z*) and are detected relative to their abundance. If present in a pure compound, the molecular ion appears at the highest value of *m/z*, which provides the molecular mass of the compound.[32]

The MS is composed of three key components- ion source, analyzer, and detector system. The purpose of the ion source is to produce gaseous ions of the sample. The purpose of the analyzer is to sort ions into their specific mass components according to

their *m/z* ratio. Finally, the purpose of the detector system is to detect the ions and record the relative abundance of every individual resolved ionic species.[32] Therefore, LC-MS was used in this research due to sensitivity and selectivity.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and Solutions

Temozolomide (>98%), anhydrous theophylline (≥99%), methanol (≥99.9%), ethanol (≥99.5%) and ammonium acetate (≥99%) were purchased from Sigma Aldrich (St. Louis, MO). Water was purified using the Millipore Milli-Q system (Milford, MA, USA). Formic acid was purchased from Fluka and proteinase K was purchased from Qiagen. Acidic methanol was prepared by taking 10 mL of 10 mM ammonium acetate diluted to 50 mL with methanol (20:80, v/v).

2.2 Sample Preparation

Two groups of four mice brain tumor tissues were provided. Four mice were injected with 0.9375 mg of TMZ while the other four were injected with saline solution. The mice were sacrificed 12 hours post injection and the brains were resected then sliced in half for a total of 16 samples. The brain samples were collected and kept at -80°C until use. The brain tissues were thinly sliced with a razor blade and weighed to 30 mg.

2.3 Calibration Curve Standard Preparation

The calibration curve standards were prepared as shown in Table I. 1 mg/mL of TMZ stock solution was prepared by weighing 3.9 milligrams of temozolomide diluted with 3.9 mL of acidic methanol. 1 mg/mL of the internal standard, theophylline, was prepared by weighing 10.9 milligrams of theophylline diluted with 10.9 mL of stock solution methanol. All calibration curve solutions were prepared by using Table I and spiked in 30 mg of lamb brain. For calibration standard number 1 (10 ng/mL), 6.8 µL of the TMZ stock solution was diluted to 1 mL with Mobile Phase A and 34 µL of the theophylline stock solution was diluted to 1 mL with Mobile Phase A. The final concentration of theophylline was kept at 50 ng/mL for all calibration standards.

Table I. Calibration Curve Standard Preparations for Lamb Brain.

Calibration Standard Number	Final TMZ Concentration (ng/mL)	Secondary Stock Solution from 1 mg/mL Stock TMZ (µL)	Final Theophylline Concentration (ng/mL)	Secondary Stock Solution from 1 mg/mL Stock Theophylline (µL)	Amount of Mobile Phase A (µL)
1	10	6.8	50	34	993.2
2	50	34	50	34	966
3	100	68	50	34	932
4	200	136	50	34	864
5	250	170	50	34	830
6	500	340	50	34	660
7	1000	680	50	34	320

The same calibration curve standard preparations shown in Table I were also developed using control mice brain. For further linearity, calibration standard number 5 was left out of the calibration curves for both mice and lamb brains.

2.4. HPLC-MS/MS Instrumentation

The instrumentation used was a Shaimadzu HPLC system (Kyoto, Japan) coupled to an AB SCIEX Q-Trap 5500 triple quadropole mass spectrometer (MS) with Analyst software Version 1.6.1. The HPLC was composed of a solvent reservoir, a degassing unit (DGU-20A3R), a binary pump (LC-30AD), a flow controller (CBM-20A), a column oven (CTO-10A) and an autosampler (SIL-30AC).

2.5 HPLC-MS/MS Optimization Parameters

High-performance liquid chromatographic separation was carried using a Waters Symmetry C18 column (2.1 mm x150 mm, 5 μm). The oven temperature was 30°C, the injection volume was 10 μL, while the flow rate was kept at 0.4 mL/min. Mobile phase A consisted of 10 mM ammonium acetate in ultrapure water and 0.1% formic acid. Mobile phase B consisted of 100% methanol. The chromatographic system was run on a linear gradient from 5 to 30% Mobile Phase B for 6 minutes then increased to 90% and decreased to 5% for 2 minutes.

The mass spectrometer was operated in multiple reaction monitoring (MRM) positive-ion mode. High purity nitrogen (99.99%) was used as the nebulizer, auxiliary, collision and curtain gases. The MRM transition of TMZ was *m/z* 195.1 → 138.1 and for theophylline it was *m/z* 181.0 → 124.1 as shown in Figures 6 and 7 respectively.

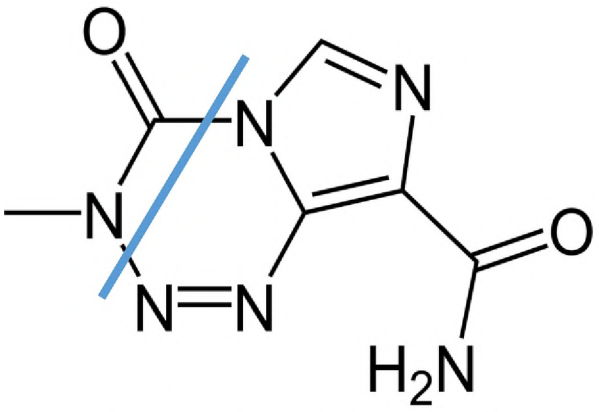


Figure 6. MRM Transition of TMZ.[33]

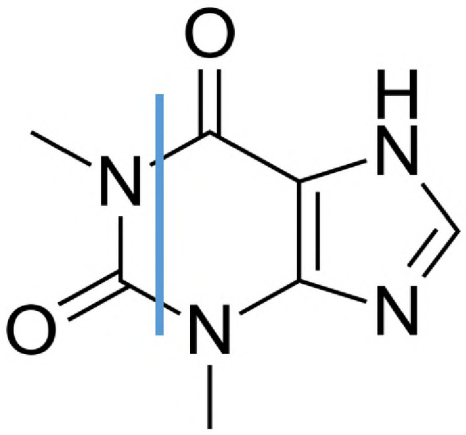


Figure 7. MRM Transition of IS.[33]

Table II shows the optimized detection parameters of the mass spectrometer for temozolomide and theophylline.

Table II. Optimized Parameters of TMZ and Theophylline (IS).

Analyte	Curtain Gas	Ion Spray Voltage	Temperature	Ion Source Gas 1	Ion Source Gas 2	Declustering Potential	Entrance Potential
TMZ	30.0 psi	5500 V	100°C	10 psi	10 psi	150 V	10 V
IS	30.0 psi	4000 V	100°C	10 psi	10 psi	150 V	10 V

2.6 Optimizing ZipTip Conditions

As TMZ is slightly soluble in water, C_{18} ZipTips Millipore were used. The wetting solvent was 50% methanol in 0.1% formic acid, and the conditioning and washing solvent was 0.1% formic acid. The loading sample consisted of 1 µL of 50 µg/mL of TMZ, 1 µL of 50 µg/mL of IS and 58 µL of Mobile Phase A. The elution solvents consisted of a varying mixture of Mobile Phases A and B (Table III). The elution

samples were further diluted so that the final amount of methanol injected in the LC/MS was 5%.

Table III. Varying Elution Mixtures for Optimization of ZipTips.

Percent of Methanol	Mobile Phase A (µL)	Mobile Phase B (µL)	Mobile Phase A Dilution (µL)
5%	47.5	2.5	0
10%	45	5	50
15%	42.5	7.5	100
20%	40	10	150
25%	37.5	12.5	200
30%	35	15	250
35%	32.5	17.5	300
40%	30	20	350
50%	25	25	450
90%	5	45	850

2.7 Optimizing Protein Precipitation

The two chosen solvents to precipitate the proteins in the brain were ethanol and TFA. These two solvents were compared to one another using lamb brain tissue. To first lyse the cells, varying volumes of proteinase K were tested to dissolve the tissue. 10 µL of 60 µg/mL of TMZ, 10 µL of 30 µg/mL of internal standard, 10 µL of formic acid and varying amounts of proteinase K (40 µL, 4 µL and 0.4 µL) were added to 30 mg of lamb brain tissue. The samples were incubated at 37°C for 1 hour. 270 µL of ethanol were added after incubation, the samples were vortexed for two minutes, incubated for 1 hour at -20°C, centrifuged at 14,000 rpm for 10 minutes, and 50 µL of supernatant was mixed with 950 µL of Mobile Phase A for injection into the LC-MS.

To compare, TFA with varying proteinase K volumes were also run. 10 µL of 60 µg/mL of TMZ, 10 µL of 30 µg/mL of internal standard, 10 µL of formic acid and varying amounts of proteinase K (40 µL, 4 µL and 0.4 µL) were added to 30 mg of lamb brain tissue. The samples were incubated at 37°C for 1 hour. 130 µL of ultrapure water

and 40 µL of trifluoroacetic acid (TFA) were added after incubation, the samples were vortexed for two minutes, incubated for 1 hour at 4°C, centrifuged at 14,000 rpm for 10 minutes, and 50 µL of supernatant was mixed with 900 µL of Mobile Phase A and 50 µL of methanol for injection into the LC-MS. As ethanol had greater recovery when compared to TFA, ethanol was the chosen protein precipitation solvent. In addition, 40 uL of proteinase K dissolved the tissue best when compared to the other volumes. The experimental design for the calibration curve for mice brain is shown in Figure 8.

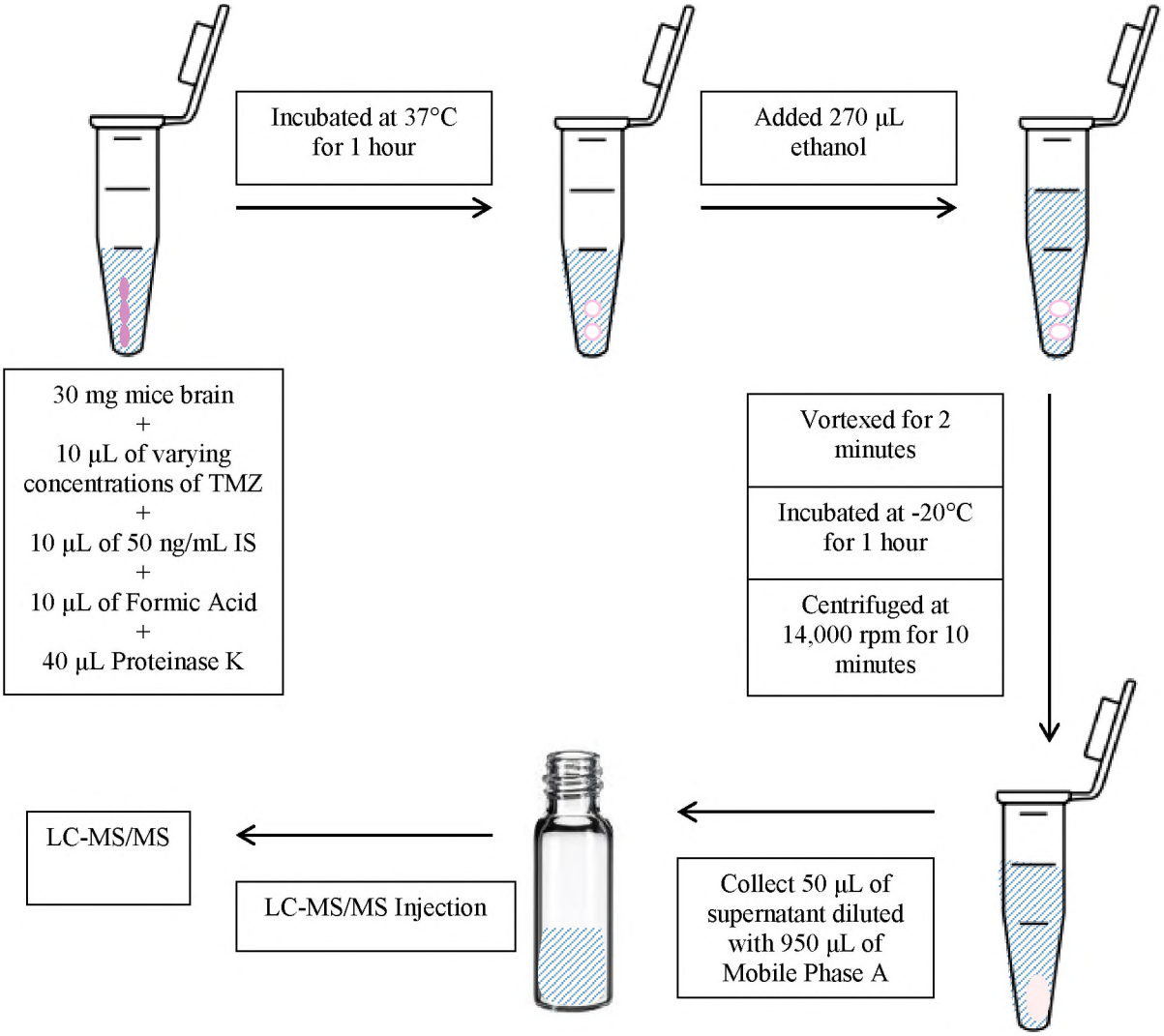


Figure 8. Experimental Design of Cell Lysis and Protein Precipitation.

The comparison of Goldwirt's current experimental design to this experimental design is shown in Table IV. In addition, Goldwirt injected their mice intraperitoneally while the mice used in this research were injected via microdialysis.

Table IV. Comparison of Goldwirt et al. Experimental Design for TMZ Extraction and This Experimental Design for TMZ Extraction.

Goldwirt et al. Experimental Design for TMZ Extraction	
Tissue	• 400 mg mice brain
Analytes	• 100 µL of 1000 ng/mL Theophylline in methanol • 100 µL of Varying Concentrations of TMZ in acid methanol
Lysis Buffer	• 200 µL of 10 mM pH 3.5 Ammonium Acetate Buffer
Protein Precipitation	• 200 µL of $ZnSO_4$ • 400 µL of Methanol
Method	• Centrifuged at 2862 g for 10 minutes • Collected supernatant and centrifuged at 21885 g for 15 minutes • Collected 5 µL of second supernatant • Injected into chromatographic system
Experimental Design for TMZ Extraction	
Tissue	• 30 mg mice brain
Analytes	• 10 µL of 50 ng/mL Theophylline in methanol • 10 µL of Varying Concentrations of TMZ in Acid Methanol • 10 µL of Formic Acid
Lysis Buffer	• 40 µL of Proteinase K
Method	• Incubated at 37°C for 1 hour
Protein Precipitation	• 270 µL of Ethanol
Method	• Vortex 2 minutes • Incubated at -20°C for 1 hour • Centrifuged at 14,000 rpm for 10 minutes • Collected 10 µL of supernatant • Added 950 µL of 10 mM Ammonium Acetate in 0.1% Formic Acid • Injected into chromatographic system

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Mass Spectrometry Infusion

For TMZ, MS infusion was done by injecting 1 μg/mL of TMZ in acidic methanol. The chromatograms for MS, CID and MRM are shown in Figures 9-11. The mass spectrum showed the precursor of TMZ to be 195.1. The collision-induced dissociation (CID) showed the product ion of TMZ to be 138.1. The MRM showed the unique fragmentation ion for TMZ used for quantification.

For theophylline, MS infusion was done by injecting 1 μg/mL of theophylline (IS) in methanol. The chromatograms for MS, CID and MRM are shown in Figures 12-14. The mass spectrum showed the precursor of IS to be 181.0. The collision-induced dissociation (CID) showed the product ion of IS to be 124.1. The MRM showed the unique fragment ion for theophylline.

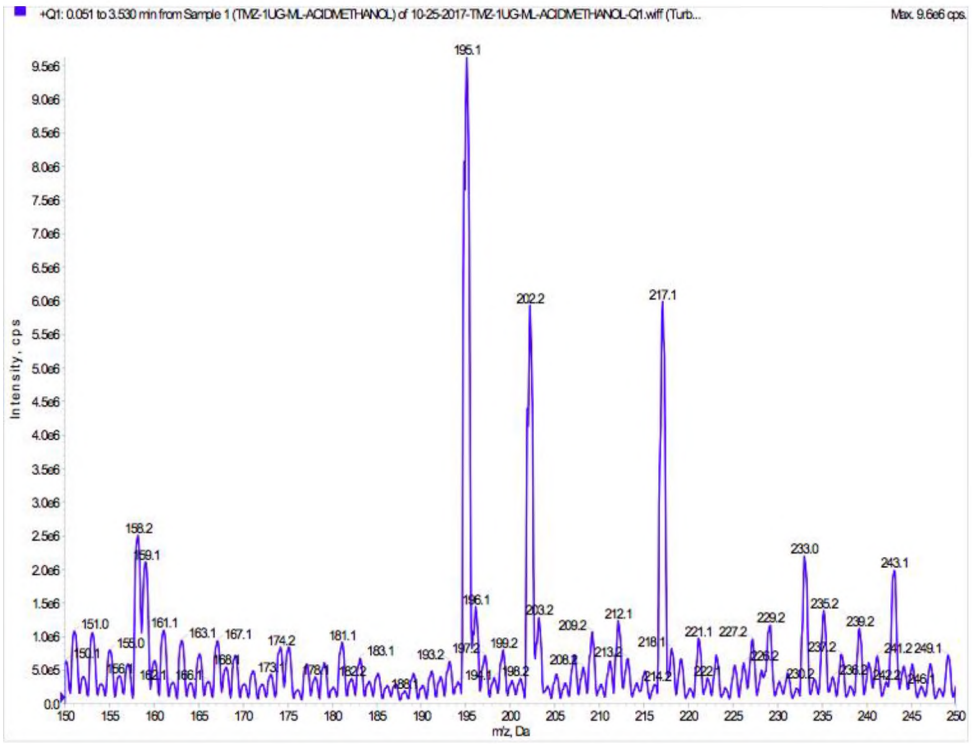


Figure 9. Mass Spectrum of TMZ.

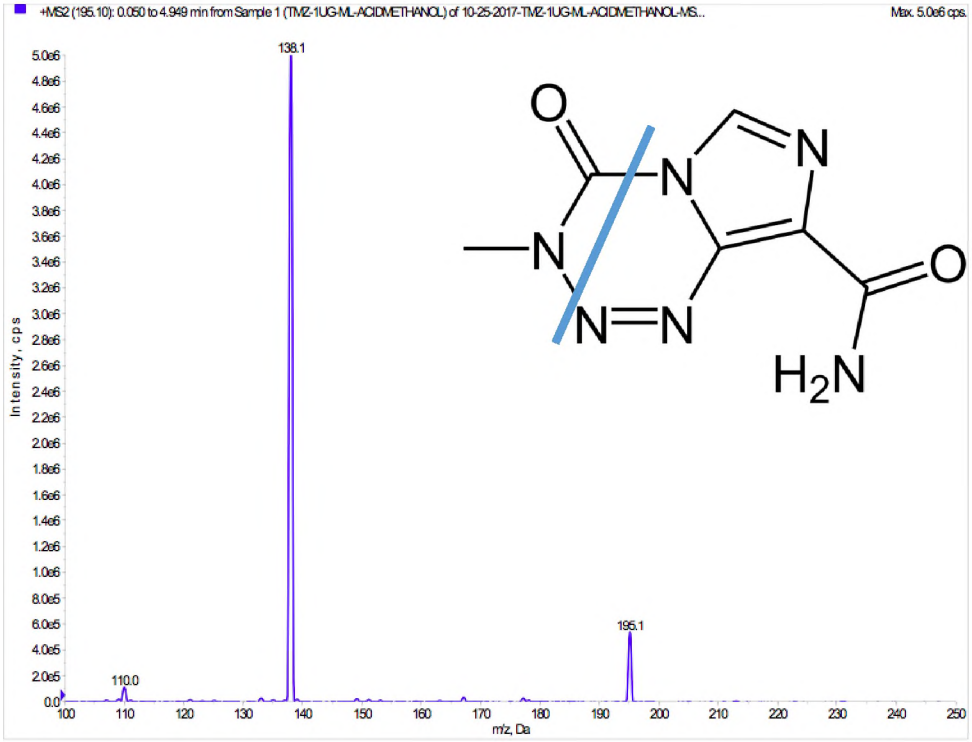


Figure 10. CID of TMZ.

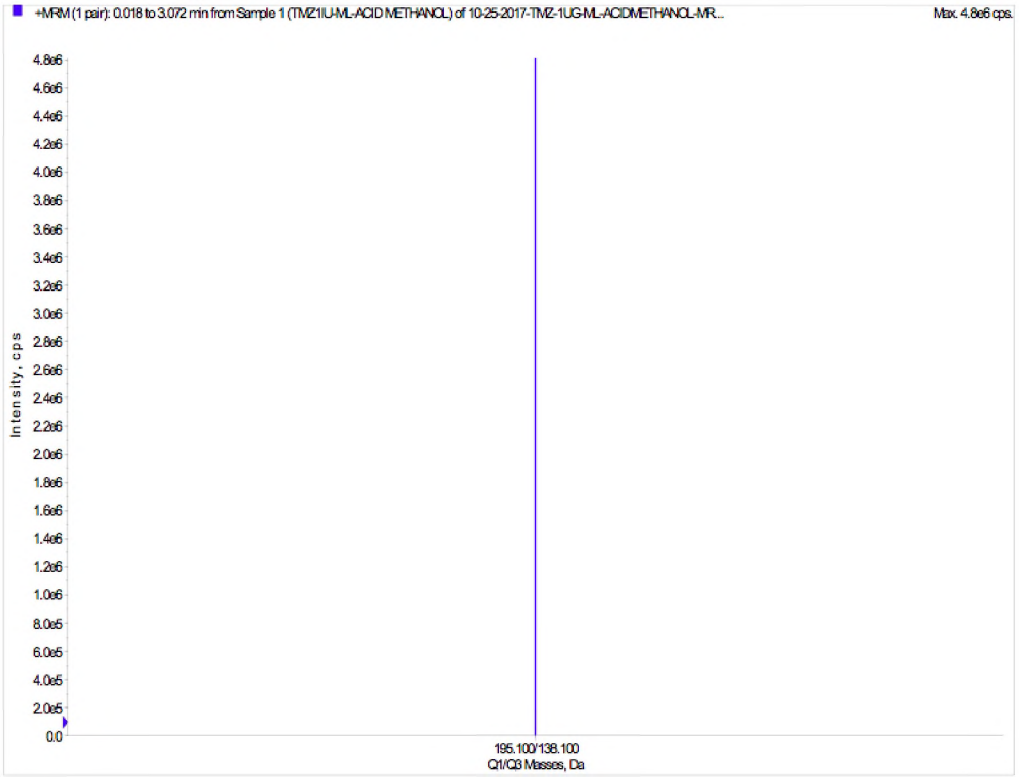


Figure 11. MRM Spectrum of TMZ.

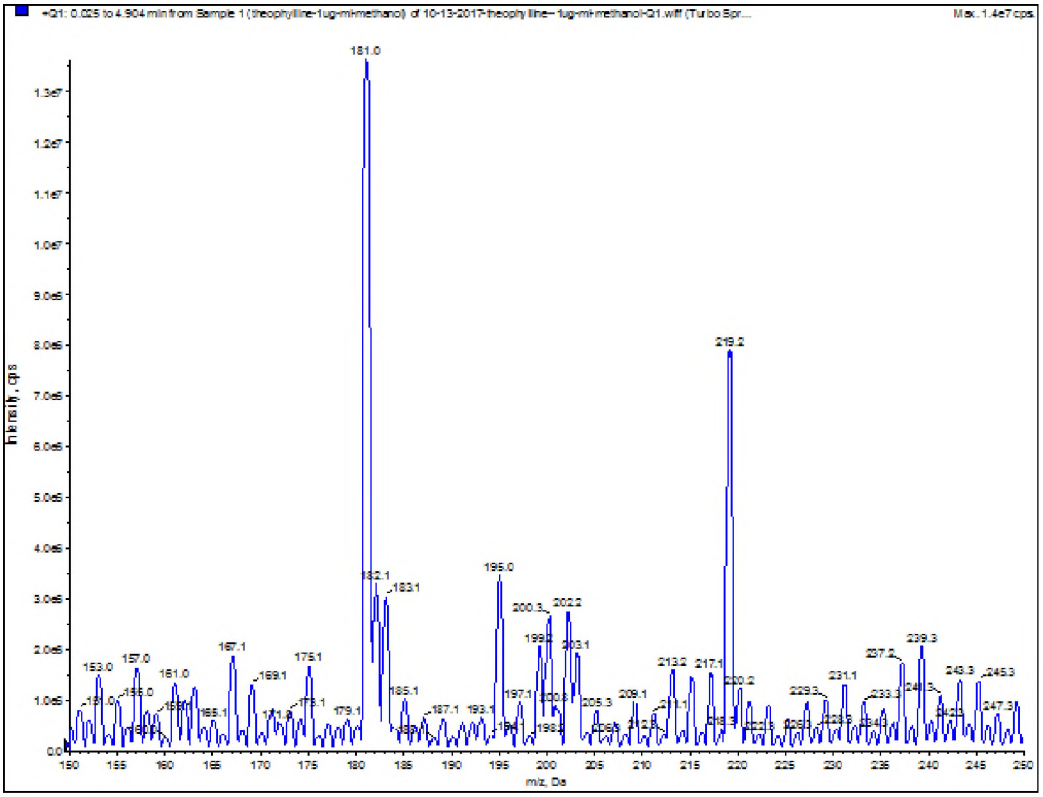


Figure 12. Mass Spectrum of Theophylline.

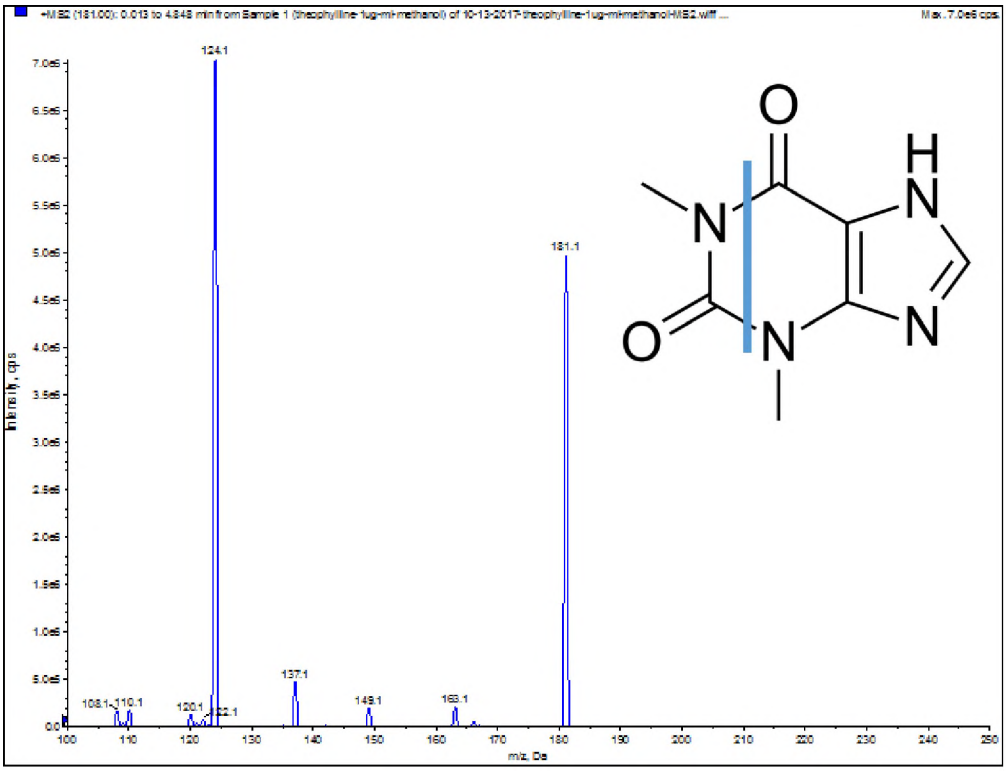


Figure 13. CID of Theophylline.

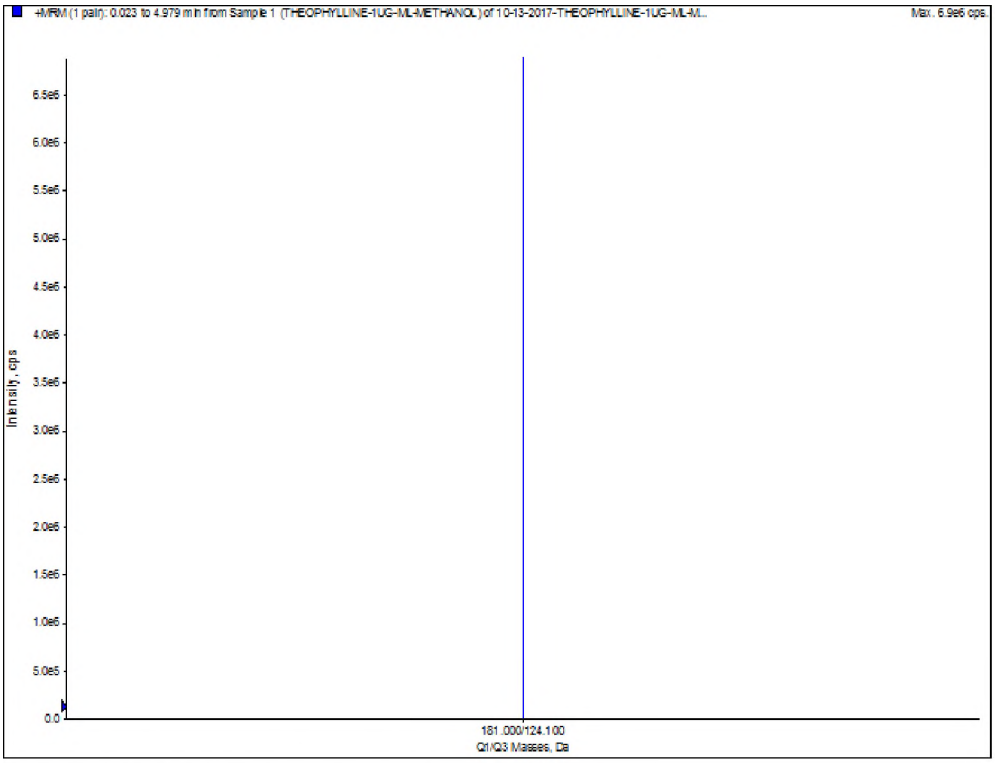


Figure 14. MRM Spectrum of Theophylline.

3.2 Method Application

As TMZ did not bind to the ZipTip columns using many different preparation techniques and ZipTip solutions, protein precipitation was chosen as the better application to recover TMZ. The LC-MS/MS method was used to quantitate TMZ in mice brain to achieve the best recovery via protein precipitation. When comparing the two solvents used for protein precipitation, ethanol was proven to be best due to fewer matrix effects and less ion suppression. Slices of mice brain without TMZ were weighed to 30 mg and used as the control standards. The resulting chromatograms are shown in Figures 15-20. TMZ had a retention time near 2.5 minutes, while the internal standard eluted later near 3.5 minutes.

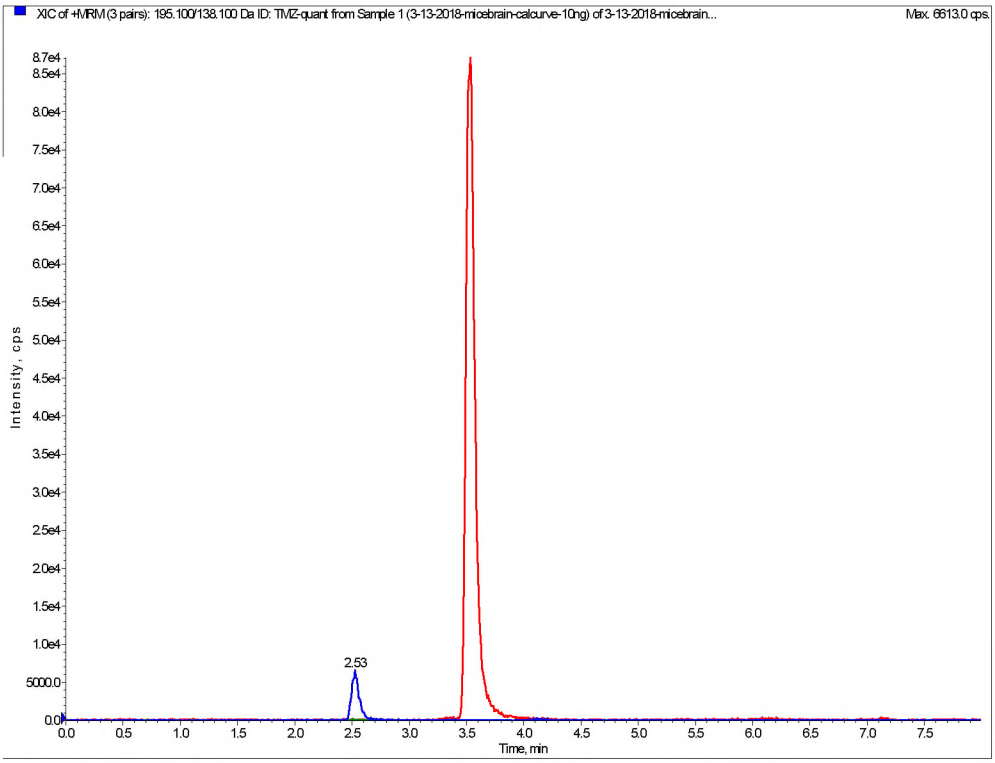


Figure 15. Chromatogram of 30 mg Mice Brain Spiked with 10 ng/mL of TMZ and 50 ng/mL of IS.

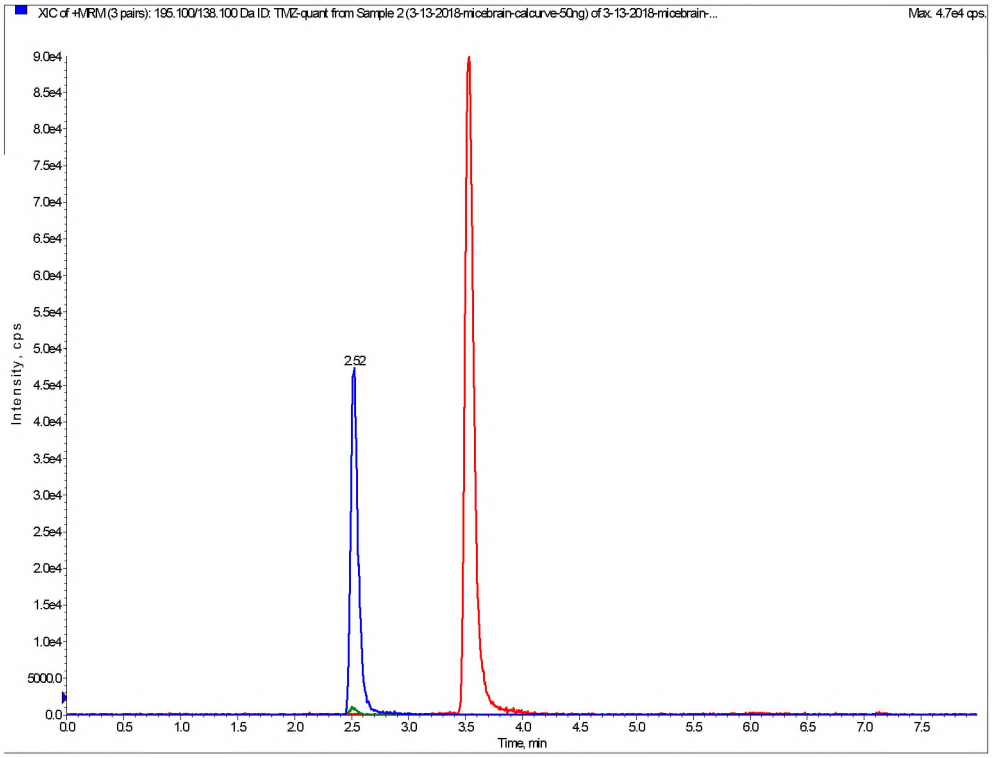


Figure 16. Chromatogram of 30 mg Mice Brain Spiked with 50 ng/mL of TMZ and 50 ng/mL of IS.

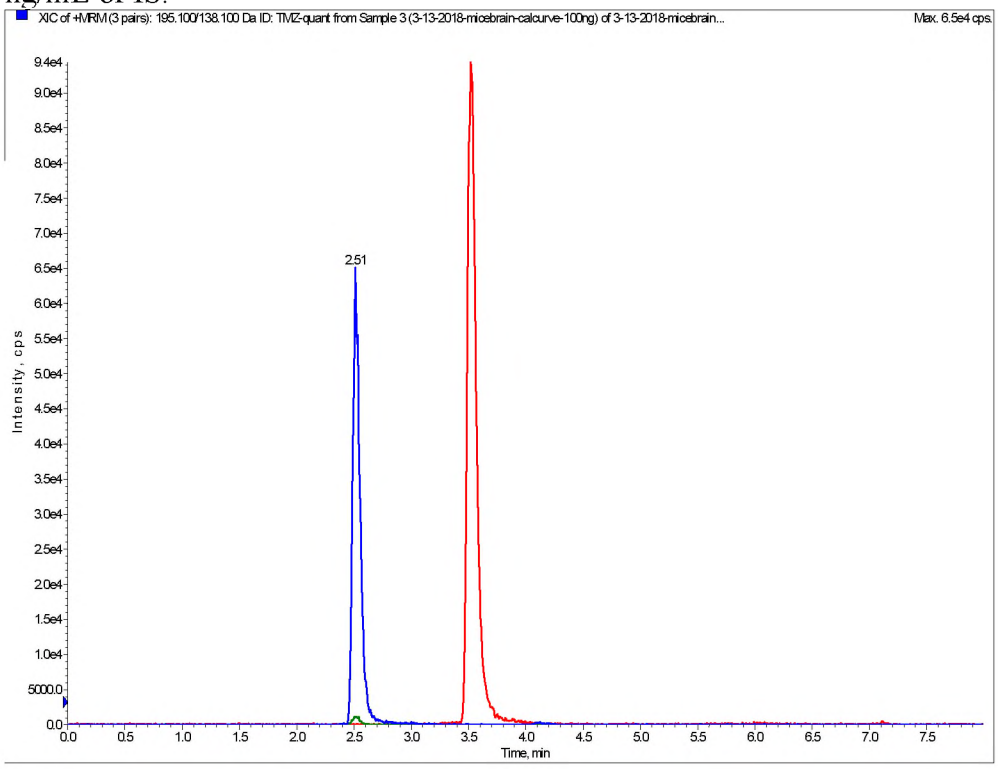


Figure 17. Chromatogram of 30 mg Mice Brain Spiked with 100 ng/mL of TMZ and 50 ng/mL of IS.

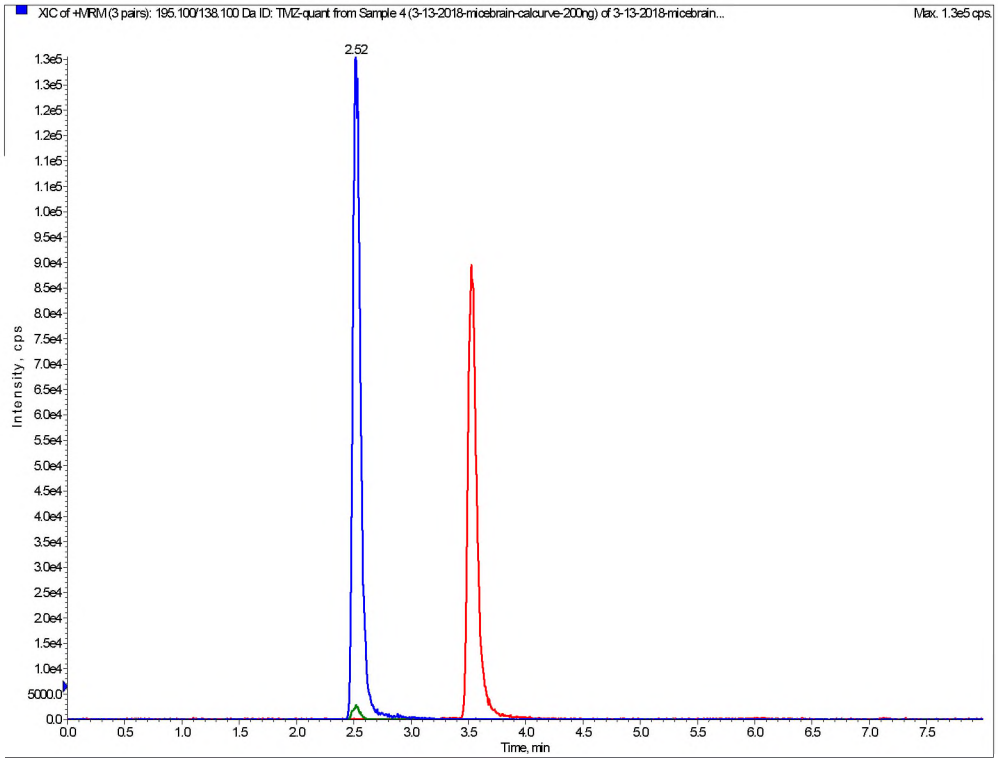


Figure 18. Chromatogram of 30 mg Mice Brain Spiked with 200 ng/mL of TMZ and 50 ng/mL of IS.

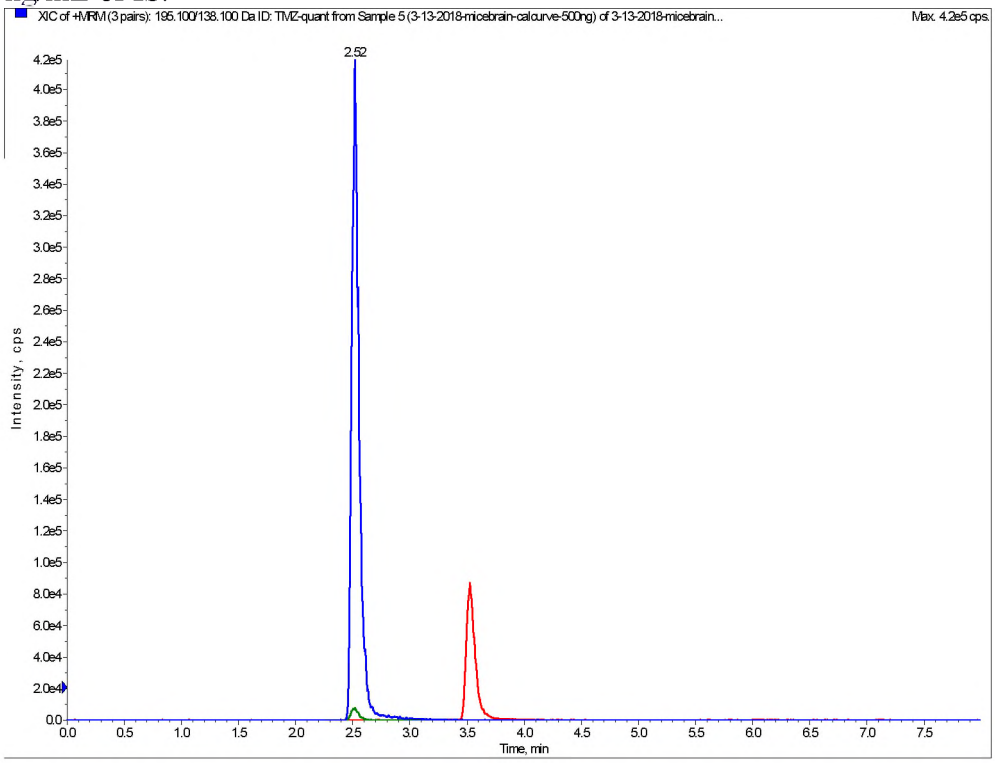


Figure 19. Chromatogram of 30 mg Mice Brain Spiked with 500 ng/mL of TMZ and 50 ng/mL of IS.

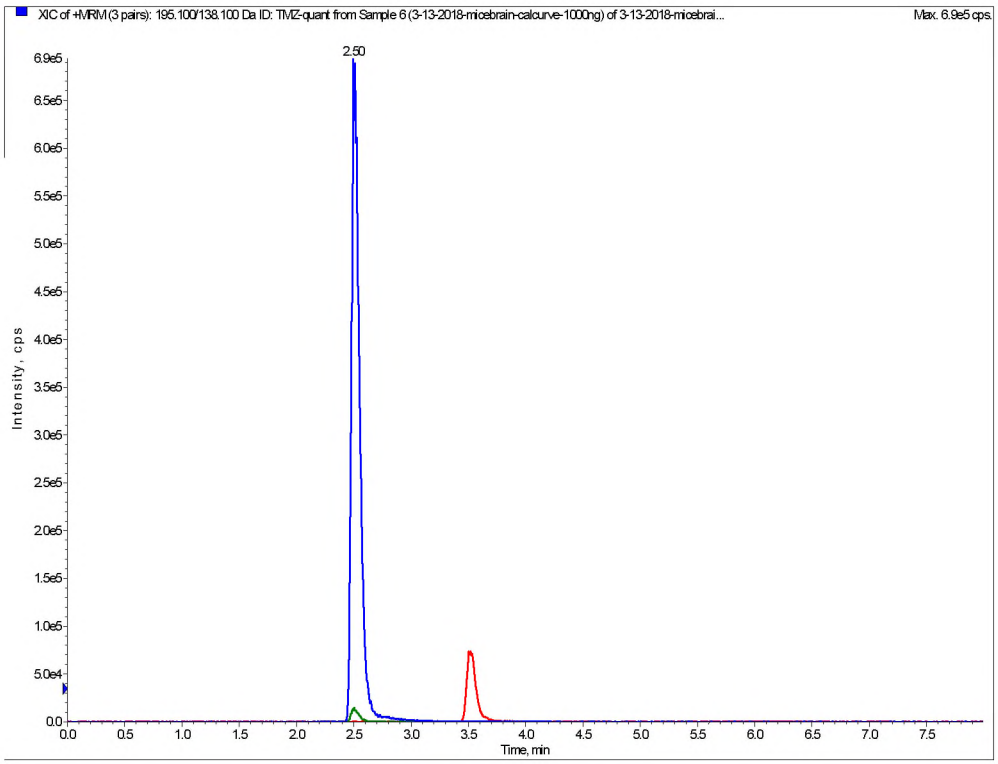


Figure 20. Chromatogram of 30 mg Mice Brain Spiked with 1000 ng/mL of TMZ and 50 ng/mL of IS.

The calibration curves for TMZ were constructed using six calibration standards. The linear calibration range was between 10 - 1000 ng/mL. The calibration curve for mice brain tissue tumors spiked with TMZ can be shown in Figure 21. In addition to mice brain calibration curve, a calibration curve was developed for lamb brain (Figure 22) consisting of six calibration standards (Table I) as 250 ng/mL was left out for further linearity.

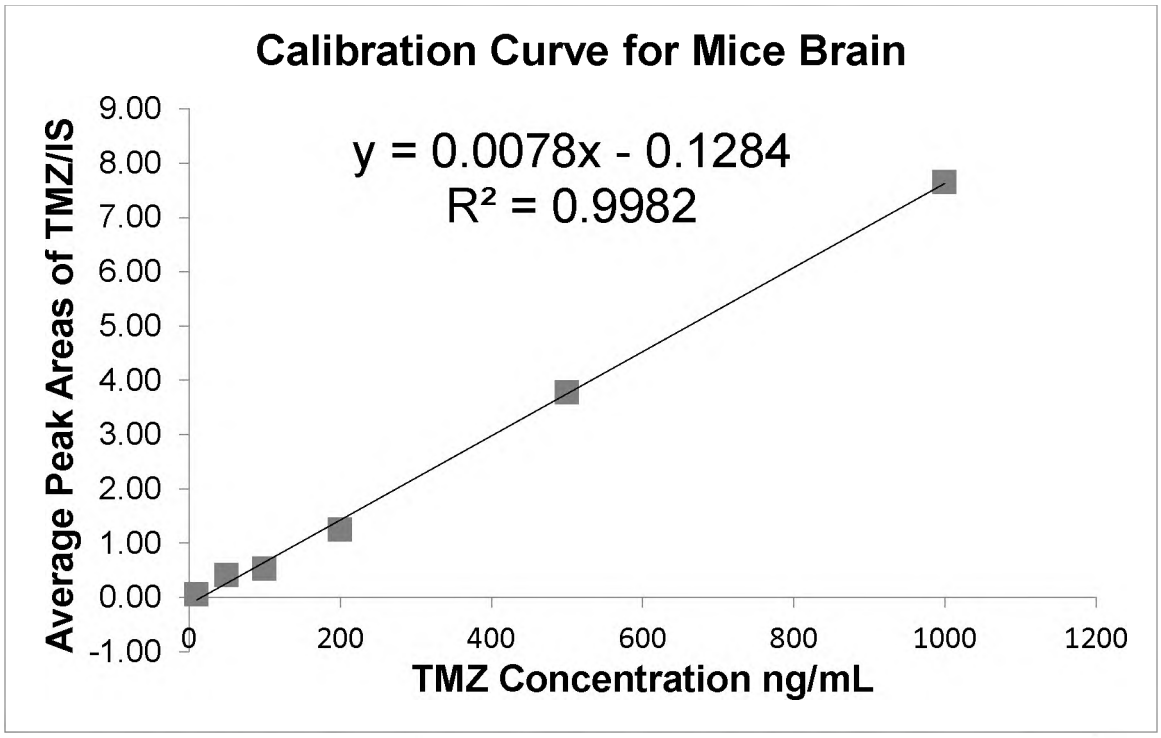


Figure 21. Calibration Curve of 30 mg Mice Brain Spiked with Varying Amounts of TMZ and 50 ng/mL IS (n=2 replicates).

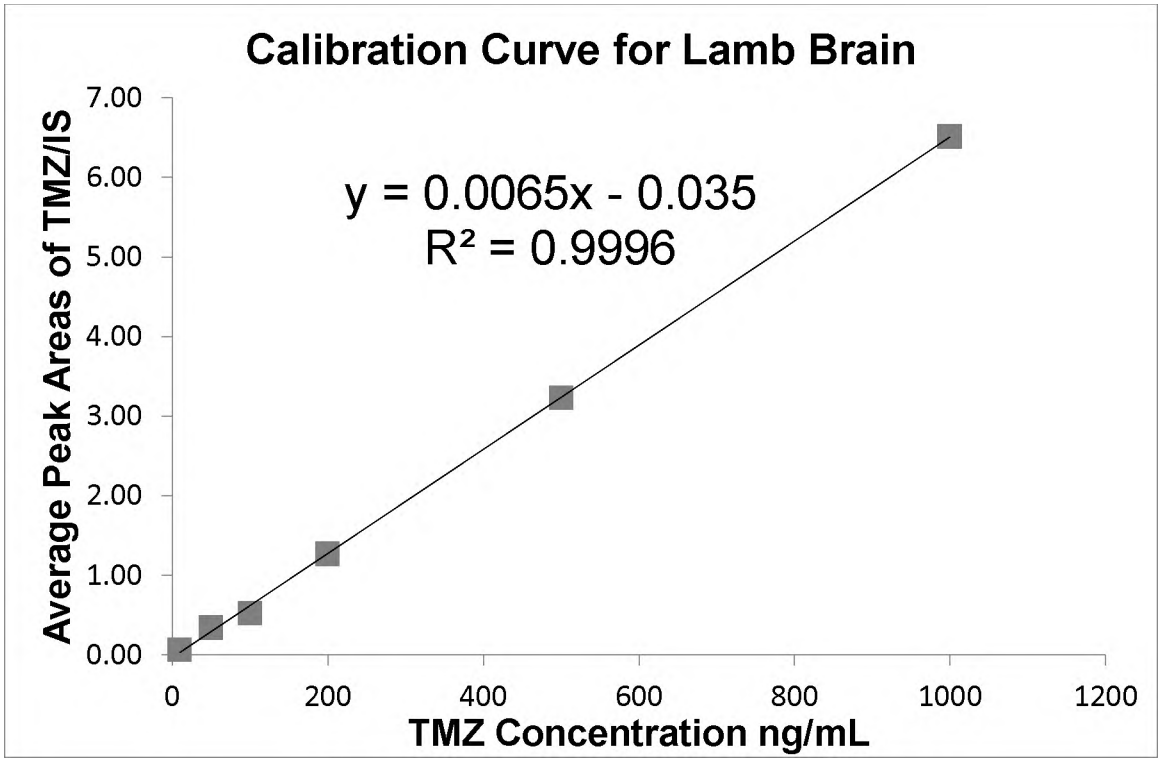


Figure 22. Calibration Curve of 30 mg Lamb Brain Spiked with Varying Amounts of TMZ and 50 ng/mL IS (n=3 replicates).

3.3 Recovery of TMZ and Theophylline

The percent recoveries of the lamb brain were determined by comparing the mean peak areas of spiking TMZ and IS before protein precipitation (n=3) with the mean peak areas of spiking TMZ and IS after protein precipitation (n=2). Table V shows the mean absolute recoveries for TMZ were consistent and ranged from 88.0%-138.6%. The percent recovery values were found by taking the values spiking the analytes before protein precipitation over the values spiking the analytes after protein precipitation and multiplying by 100.

Table V. Percent Recoveries of TMZ.

Standard Concentration (ng/mL)	Average Mean Peak Areas of Spiking TMZ/IS Before Protein Precipitation (n=3)	Average Mean Peak Areas of Spiking TMZ/IS After Protein Precipitation (n=2)	Percent Recovery
10	0.07	0.08	88%
50	0.35	0.25	139%
100	0.53	0.44	120%
200	1.27	1.29	99%
500	3.23	2.83	114%
1000	6.51	6.68	97%

When compared to Goldwirt, the percent recoveries obtained were closer to 100%. As mentioned prior, the extraction recovery of TMZ for Goldwirt was 67.7% for 125 ng/g, 61.0% for 1250 ng/g, and 61.4% for 12,500 ng/g. The differences in extraction techniques are the protein precipitation and cell lysis solvents. Proteinase K was stronger than 10 mM ammonium acetate/water/0.1% formic acid. For protein precipitation, ethanol was used in this method, while methanol and zinc sulfate were used by Goldwirt. The amount of ethanol used was about 80% of the total volume for protein precipitation, while the amount of methanol used was Goldwirt was approximately 40% of the total volume. The greater amount of alcohol used improved purification. Ethanol has a lower

dielectric constant than methanol, so it is able to reduce protein solubility leading to better protein precipitation.

3.4 Matrix Effects

The matrix effects of the lamb brain were determined by comparing the mean peak areas of the neat sample with no lamb brain (n=2) with the mean peak areas of spiking TM and IS after protein precipitation (n=3). Table VI shows the matrix effects ranged from 75%-140%. The matrix effect percentages were found by taking the values of spiking TMZ and IS after protein precipitation divided by the values of the neat samples with no lamb brain and multiplying by 100.

Table VI. Matrix Effects.

Standard Concentration (ng/mL)	Average Mean Peak Areas of Neat Samples with No Lamb Brain (n=2)	Average Mean Peak Areas of Spiking TMZ/IS After Protein Precipitation (n=2)	Matrix Effect
10	0.06	0.08	140%
50	0.33	0.25	75%
100	0.52	0.44	84%
200	1.18	1.29	109%
500	3.74	2.83	76%
1000	7.21	6.68	93%

When compared to Goldwirt, the matrix effect percentages obtained were almost half. The matrix effect for Goldwirt was 245% for 125 ng/g, 204% for 1250 ng/g, and 212% for 12,500 ng/g. The differences in matrix effects are thought to be due to the addition of proteinase K as it digests unwanted proteins. In addition, proteinase K is a strong protease.

CHAPTER IV

CONCLUSIONS

4.1. Conclusions and Future Directions

An LC-MS/MS method was developed to analyze temozolomide in mice brain tumor tissue. As many studies focused on plasma, this method focused on brain tissue. In comparison to Goldwirt et al., this method proved to obtain higher percent recoveries as well as lower matrix effects. Many different techniques were employed in order to achieve these goals. As TMZ was not able to bind to the ZipTip column, protein precipitation was utilized along with proteinase K for cell lysis.

Future work will include studying the mice brain that were injected with TMZ and quantifying the amount of drug in brain. To achieve this, larger slices of brain will be taken in order to increase the chances of the location of the drug in the brain. In addition, since 40 μL of proteinase K seemed to be most effective when compared to smaller quantities, greater volumes of proteinase K will be studied. This research will also be validated with mice brain samples.

REFERENCES

1. Schneider, T., et al. "Gliomas in adults." *Dtsch Arztebl Int,* vol. 107, no. 45, 2010, pp. 799-808.

2. Friedman, H. S., et al. "Temozolomide and Treatment of Malignant Glioma," *Clin. Canc. Res.,* vol. 6, 2000, pp. 2585-2597.

3. Wick, W., et al. "New (alternative) temozolomide regimens for the treatment of glioma." *Neuro-oncology,* vol. 11, no. 1, 2009, pp. 69-79.

4. Lombardi, Melanie, and Mahfoud Assem. *Glioblastoma*. Codon Publications, 2017. https://www.ncbi.nlm.nih.gov/books/NBK470004/ (accessed 18 January 2018).

5. Agarwal, S., et al. "Function of the Blood-Brain Barrier and Restriction of Drug Delivery to Invasive Glioma Cells: Findings in an Orthotopic Rat Xenograt Model of Glioma." *Drug Metab. Dispos.,* vol. 41, 2013, pp. 33-39.

6. "Glioblastoma multiforme." *Cancer Treatment Centers of America*, https://www.cancercenter.com/brain-cancer/types/tab/glioblastoma-multiforme/ (accessed 20 January 2018).

7. "Anaplastic Astrocytoma." *National Organization for Rare Disorders*, 2017, https://rarediseases.org/rare-diseases/anaplastic-astrocytoma/ (accessed 20 January 2018).

8. "Anaplastic Astrocytoma." *Neurosurgeons of New Jersey*, 2018, https://www.neurosurgeonsofnewjersey.com/anaplastic-astrocytomas/ (accessed 21 January 2018).

9. Barker, C. A., et al. "Survival of patients treated with radiation therapy for anaplastic astrocytoma." *Radiol. Oncol.,* vol. 48, no. 4, 2014, pp. 381-386.

10. Pan, Edward and Michael D. Prados. *Holland-Frei Cancer Medicine*. 6th ed., BC Decker, 2003.

11. Peus, D., et al. "Appraisal of the Karnofsky Performance Status and proposal of a simple algorithmic system for its evaluation." *BMC Medical Informatics and Decision Making*, vol. 13, no. 72, 2013, pp. 1-7.

12. "Anaplastic astrocytoma." *National Center for Advancing Translational Sciences*, 2018, https://rarediseases.info.nih.gov/diseases/5860/anaplastic-astrocytoma (accessed 21 January 2018).

13. Zhou, Q., et al. "Predicting Human Tumor Drug Concentrations from a Preclinical Pharmacokinetic Model of Temozolomide Brain Disposition," *Clin Cancer Res*, vol. 13, no. 14, 2007, pp. 4271-4279.

14. Liu, James K. "The role of temozolomide in the treatment of aggressive pituitary tumors," *Journal of Clinical Neuroscience*, vol. 22, no. 6, 2015, pp. 923-929.

15. "Temozolomide." *National Center for Biotechnology Information*, PubChem Compound Database, https://pubchem.ncbi.nlm.nih.gov/compound/temozolomide#section=Top. (accessed 21 January 2018).

16. Rai, R., et al. “Temozolomide analogs with improved brain/plasma ratios – Exploring the possibility of enhancing the therapeutic index of temozolomide,” *Bioorganic & Medicinal Chemistry Letters*, vol. 26, no. 20, 2016, pp. 5103-5109.

17. Barciszewska, A., et al. "A New Epigenetic Mechanism of Temozolomide Action in Glioma Cells." *PLOS One*. 2015, pp. 1-12.

18. Whitelaw, B. C., et al. "Temozolomide in the management of dopamine agonist-resistant prolactinomas," *Clinical Endocrinology,* vol. 76, 2012, pp. 877-886.

19. Canniff B., and Anderson, D. “E. coli MGMT Suicide Protein,” *Biomolecules at Kenyon*, http://biology.kenyon.edu/BMB/jsmol2016/ADAO6/ADA012.html (accessed 4 February 2018).

20. Shen, F., et al. "Determination of temozolomide in human plasma and urine by high-performance liquid chromatography after solid-phase extraction." *Journal of Chromatography B*, vol. 667, 1995, pp. 291-300.

21. Portnow, J., et al. “The neuropharmacokinetics of temozolomide in patients with resectable brain tumors: potential implications for the current approach to chemoradiation.” *Clin Cancer Res*, vol. 15, no. 22, 2009, pp. 7092-7098.

22. Goldwirt, L., et al. "Development of a new UPLC-MSMS method for the determination of temozolomide in mice: application to plasma pharmacokinetics and brain distribution study." *Biomed. Chromatogr.*, vol. 27, 2013, pp. 889-893.

23. Kim, H., et al. "High-performance liquid chromatographic analysis and stability of anti-tumor agent temozolomide in human plasma." *Journal of Pharmaceutical and Biomedical Analysis,* vol. 24, 2001, pp. 461-468.

24. Patel P. M., et al. "Extended schedule, escalated dose temozolomide versus dacarbazine in stage IV melanoma: Final results of a randomised phase III study (EORTC 18032)." *European Journal of Cancer,* vol. 47, 2011, pp. 1476-1483.

25. Li, Pei and Barlett, M. G., "A review of sample preparation methods for quantitation of small-molecule analytes in brain tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS)." *Anal Methods*, vol. 16, 2014, pp. 6183-6207.

26. *Mammalian Cell Membranes: Responses of Plasma Membranes*. Edited by G. A. Jamieson and D. M. Robinson, vol. 5., Butterworths, 1977.

27. Gilar, M., et al "Advances in sample preparation in electromigration, chromatographic and mass spectrometric separation methods," *Journal of Chromatography A*. vol. 909, 2001, pp. 111-135.

28. Aitken, A., "Sample Cleanup by Solid-Phase Extraction/Pipet-Tip Chromatography, *The Proteomic Protocols Handbook*. Walker, John M., Humana Press, 2005, pp. 307-309.

29. Plank, J., "The Ins and Outs of Protein Concentration – Protein Precipitation," *BitesizeBio*, 2015, https://bitesizebio.com/5924/the-in%E2%80%99s-and-out%E2%80%99s-of-protein-concentration-%E2%80%93-protein-precipitation/ (accessed 27 March 2018).

30. Skoog, D. A.; Holler, F. J.; Crouch, S. R. *Principles of Instrumental Analysis*, 6th ed.; Brooks/Cole Cengage Learning: Belmont, 2007; pp. 336-472.

31. Editorial Team. *High Performance Liquid Chromatography (HPLC): Principle, Types, Instrumentation and Applications,* 2015, https://laboratoryinfo.com/hplc/ (accessed 11 February 2018).

32. "Mass Spectrometry." *Premier Biosoft*, 2018, http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html (accessed 26 February 2018).

33. Peer, C. J., "Quantification of Temozolomide in Nonhuman Primate Fluids by Isocratic Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry to Study Brain Tissue Penetration Following Intranasal or Intravenous Delivery," *Separations*, vol. 3, no. 4, 2016, pp. 1-10.