Investigation of Microdialysis Sampling to Monitor Pharmacodynamics

By

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Abstract

Microdialysis sampling is a technique commonly used to monitor a variety of processes in a tissue-targeted fashion. This sampling technique has several advantages over other methods since it allows continuous sampling throughout the entire experiment. Because microdialysis can be implanted in virtually any tissue, the application of the microdialysis sampling technique can be applied in many areas, including monitoring enzyme activity mechanisms or monitoring the effects of chemotherapy agents on brain activity.

To expand the application of microdialysis sampling, two different enzymes were monitored and tested with this tissue-specific sampling technique. The use of microdialysis sampling to monitor the activity of 11β-HSD1 *in vitro* and *in vivo* would increase the throughput for screening novel inhibitors. Microdialysis sampling was successful in monitoring the conversion of cortisone to cortisol *in vitro* human microsome experiments, but not so in rat microsome *in vitro* or *in vivo* experiments. This was because rat enzymes generate a different metabolite profile than humans. In a separate study, microdialysis was used to monitor the production of cGMP by the enzyme activation of GC-C in the colon submucosa. The location of the microdialysis probe was verified by comparing the concentration of cGMP in a probe implanted in the submucosa and lumen microdialysis probe. These results indicated a clear correlation with probe placement. Upon the activation of a GC-C agonist, a consistent increase in cGMP levels was seen in the submucosa probe. This allows further determination of the mechanisms responsible for pain reduction upon the administration of GC-C agonists in the submucosa, which has not been performed before due to the inability of obtaining this tissue using conventional methods.

The second studies utilized microdialysis sampling to continuously monitor neurotransmitters and an oxidative stress biomarker (MDA) before, during and after the administration of anticancer compounds. Both short- and long-term studies were performed. Initial studies monitored the direct effects of these chemotherapy compounds in short-term experiments using direct perfusion. An increase in glutamate and GABA was seen upon the administration of doxorubicin and cyclophosphamide through the microdialysis probe. This demonstrated the toxicity of these compounds on brain activity. These anticancer compounds were then administered in long-term experiments to obtain a complete time-profile of the changes on several neurotransmitters and MDA upon chemotherapy administration. A detectable change was not seen in the amino acid neurotransmitters or MDA in the samples analyzed. An increase was seen in glutamate with i.p. administration; however this was also seen in the control rat. To alleviate an increase in glutamate from handling, several intravenous cannulations were attempted and no difference was seen in any of these experiments. During these experiments, the longest microdialysis studies were performed, to the best of our knowledge, and the technique was optimized to allow further investigation of these compounds or other disease states that occur over months instead of days.

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Introduction to microdialysis sampling

1.1 Introduction

In 2011, 263 billion dollars were spent for prescription drugs at retail outlets in the United States according to the National Health Expenditures[1]. This results in 45 billion dollars of out-of-pocket expenses for Americans, which then leads to patients skipping doses to lower costs[2]. This is especially true if there are side effects that reduce the quality of life. To improve the patients health status, continual improvements of pharmaceuticals are made by either developing new prescription drugs or adjusting the prescriptions already on the market. Prior to consumer availability, a drug must undergo extensive studies in order to obtain FDA approval. *In vitro* studies are commonly performed first to determine the basic action of the novel drug. However, before a drug moves to human clinical studies, *in vivo* studies are performed in at least one non-clinical safety testing animal species, since *in vitro* studies do not accurately reflect the absorption, distribution, metabolism, elimination (ADME) and toxicology of a drug in an *in vivo* system (pharmacokinetics/pharmacodynamics (PK/PD))[3].

If the appropriate sampling techniques, animal models, and analytical systems are used, the cost of drug development decreases by thoroughly testing and only advancing the drugs that will likely succeed in clinical studies. Continual advancements in these three areas increase the efficiency and efficacy of the drug in order to further improve the health of patients. While all three areas are important for the drug development process, the main focus of this research is the application of novel microdialysis sampling techniques.

1.2 Sampling approaches

Choosing an appropriate sampling technique is extremely important for determining the PK/PD of a drug. In order to monitor these processes, several tests are performed *in vitro* to determine the preliminary data on the drug's actions, and then move on to *in vivo* studies to obtain more information about each drug in animal experiments. *In vivo* studies provide information based on the collection of samples by blood, urine, tissue, or microdialysis. To determine the appropriate technique, several factors play a vital role. For each method, temporal and spatial resolution, animal or human perturbation, sample collection, and necessary preparation prior to analysis are all important parameters to keep in mind. First a brief description of *in vitro* studies will be discussed. Then the advantages and disadvantages of each of the sampling techniques *in vivo* will be discussed in the following sections.

1.2.1 In vitro sampling

Typical *in vitro* screening methods consist of cell-based assays to determine the metabolic profile of a drug. *In vitro* screening is commonly performed to determine the molecular mechanism of the drug of interest. This screening method is highly beneficial due to economical feasibilities and efficient monitoring capabilities for identifying novel drug candidates. The most common *in vitro* experiments utilized for monitoring initial metabolism of drugs are incubation with microsomes and incubation with intact cells for peripheral studies, while neurochemical mechanisms are studied with endothelial cells, which represent the blood brain barrier (BBB).

Microsomes are easy to obtain and maintain for short experiments; incidentally, the information obtained from *in vitro* microsome experiments is only for phase one metabolism. The activities of phase one typically only represents the catabolic processes, such as oxidation,

reduction or hydrolysis[4]. During *in vitro* tests, more reactive products are often formed than an *in vivo* studies, thus more toxic products can be formed in these reactions than in a system using intact cells or an *in vivo* experiments[4-5]. Therefore, only a limited amount of information can be obtained from microsome experiments. Due to the feasibility of collection and storage of microsomes, while providing excellent drug screening capabilities, these are commonly used, especially in liver enzyme studies.

While intact cells are desirable to use for *in vitro* experiments, since the metabolic pathways and cofactors/enzymes are highly preserved, the ability to perform experiments in an environment conducive to keep the cell viable with the same properties as *in vivo* is extremely difficult, thereby limiting their use. Cell cultures can also be used; however, the ability to duplicate the highly specialized network of an *in vivo* system such as the BBB is extremely difficult. Current BBB models typically use multiple cell lines, including brain endothelial, brain pericyte, and astroglial cells[6-7]. Due to the complexity of the BBB, there is not one cell culture model that incorporates all of its characteristics. While there are several cell cultures available for screening potential drug candidates, "no ideal BBB cell culture model has been developed yet" Toth *et al.*[6].

1.2.2 In vivo sampling

Once the metabolic profile of the newly tested drug are determined to be promising, experiments typically advance to *in vivo* studies. Sampling *in vivo* can occur by various methods, which will be discussed in detail throughout this section.

1.2.2.1 Blood sampling

Blood sampling is one of the most common techniques to monitor drug pharmacokinetics. This technique is popular since sampling from blood represents the amount of drug circulating throughout the body. This is the most common technique to monitor several PK characteristics by determining the concentration of the drug (i.e. area under the curve) and its half-life, toxicity and metabolite formation in the circulatory system. Unfortunately, these samples do not demonstrate the efficacy of the drug in certain tissues (spatial resolution) and are limited in sample collection (temporal resolution) to minimize a change in blood volume of the animal.

Blood volume collection is restricted due to the perturbation of the system upon withdrawal. For example, the typical blood volume of a rat is 78 mL kg^{-1}, which corresponds to a total blood volume of 30 mL for a 0.385 kg rat. To minimize the change in blood volume, only 10% of the volume is typically withdrawn in a two week period. Therefore, only three milliliters can be collected during the course of a study. This limits the number of time points that can be collected throughout the duration of the experiment, which is in turn dependent upon the volume requirements of the analytical technique utilized. Typical conventional analytical systems require approximately 100 to 400 µL of plasma for each time point, unless a technique such as dried blood spot (DBS) sampling is utilized, which requires approximately 10 to 100 µL. Therefore for a method requiring 100 µL of plasma sample per analysis, a total blood volume of approximately 250 µL must be withdrawn, since the plasma only constitutes 55% of the blood volume and additional fluid is required to prevent blood contamination during plasma separation[8]. Therefore, a study will be limited to approximately 10 sample points for a two week time period.

Furthermore, obtaining a blood sample is typically invasive and labor intensive. Blood samples are usually acquired by a needle or an indwelling catheter (cannula). Since the samples are typically collected by an attending person, this makes it labor intensive. In animal experiments, blood sampling requires extensive monitoring. Automatic blood sampling systems are available, such as the Culex® system. However, these systems are extremely expensive and monitoring is still recommended to ensure successful sampling. Due to the intricate nature of blood sampling, several things can lead to sample failure such as cannula malfunction from the movement of an animal or any blood accumulation (e.g. clotting) in the catheter. Either of these events will prevent future blood collection. If this happens, the animal and/or cannula may need to be adjusted/flushed in order for the sample to be collected successfully. Hence, you need to be watching the animal carefully to ensure consistent blood sampling throughout the experiment.

Moreover once the sample is obtained, it must be processed immediately to preserve the stability of the sample and to prevent degradation of the analyte of interest by enzymatic reactions, such as oxidation. To prevent degradation or further enzymatic reactions, the blood sample can be processed by a variety of techniques. One of the most common methods is the addition of an acid or organic solution and then centrifugation to separate the plasma from the protein components. This is considered one of the most effective and efficient methods[9]. With this method, many of the analytes that were bound to proteins in the blood typically become free. This method represents the total concentration of the biomarker/drug (protein bound + free, unbound biomarker/drug). In reality, only the free fraction of a drug is typically of interest, since it is the therapeutically active portion. Furthermore, even after the plasma is obtained by centrifugation, additional sample cleanup is often necessary. Other methods such as solid phase extraction or liquid-liquid extraction are also used since these extraction methods produce

cleaner samples; however these methods are more complex, thus requiring more time for sample preparation[9]. Since sample preparation for conventional methods of biological fluid analysis, including blood, take 80% of the total analysis time, processes that add to the analysis time are not used unless it is absolutely essential[9]. Therefore new techniques to minimize sample preparation and increase sampling efficiency are continually being developed.

1.2.2.2 Dried blood spot sampling

Dried blood spot (DBS) sampling in PK/PD studies has grown in interest over the past five years[10]. This technique is advantageous since it utilizes minimal blood collection of approximately 10 to 100 μL by a simple needle prick of the finger or a heel, and the blood is then applied to the DBS card[11-12]. This technique is extremely beneficial since it allows human patients to collect, prepare and ship samples from their home. In addition, DBS enables more samples to be collected from both human patients and animal experiments, if serial sampling is preferred. Unfortunately, the sample collection and preparation procedure has essentially the same drawbacks as conventional blood sampling technique. For example, if multiple samples are collected over a 24-hour period, even if needle pricks are used, this sampling method would still be labor intensive and time consuming, especially in animal experiments. However this sampling technique would allow a higher temporal resolution, even if the same sampling collection (e.g. intravenous cannula) method is used.

Another drawback of DBS is the application of sample on the DBS card has to be done consistently and accurately to increase the precision of the quantitation of the compound of interest[10]. To distribute the blood sample evenly on the DBS card and improve precision, the sample is often applied by pipet. If the user does not perform several steps accurately (apply, dry or ship), the reproducibility of this sampling method decreases substantially[10]. One way to

overcome these drawbacks is the addition of an acid to increase drug stability or the addition of an internal standard to normalize differences in applications into account[10].

Furthermore, sample analysis throughput and reliability diminishes due to the complexity of the sample extraction procedure and the stability of the compound of interest[10]. The sample extraction method typically involves organic solvents to extract the analytes. The sample is then evaporated and diluted in the desired matrix. To increase throughput of DBS samples, perforated DBS (PDBS) cards have been developed, but throughput decreases from the necessity of extracting the compound from the DBS card by an organic solvent. This not only decreases throughput but is costly and labor intensive. While there are many analytical methods capable of analyzing DBS samples, such as mass spectrometry and immunoassays, the ability to monitor trace analytes in these small samples is extremely difficult[10]. In addition, analyte stability must be kept in mind and is dependent upon the DBS card, temperature, humidity, and storage time[10].

Other applications of DBS sampling are still in development, including employing this technique for the collection of other biological fluids, such as cerebral spinal fluid[12]. Unless this technique is further developed for sampling extracellular fluid in tissues, it does not provide any site-specific information, and therefore represents the total concentration of the compound in circulation throughout the entire body.

1.2.2.3 Urine

Sampling of urine is another common method to monitor drug metabolism. This technique is popular since it is one of the easiest samples to collect, and it gives a good representation of the overall metabolite production in the body. Urine samples are collected in humans and rats using a cup. In animals, this is typically achieved by putting the animal in a metabolism cage

with a cup below the cage. While the collection of urine is easy and useful, it has the drawback that the profile generated represents the end point of the metabolic process (elimination) and not necessarily what occurs at the site of interest. Thus the profile produced can actually give a misinterpretation of the mechanism of interest (poor spatial resolution). In addition, the samples can only be collected when a void occurs, which does not typically occur more than every three hours (poor temporal resolution). Due to the volume of the sample being limited, especially in animal experiments, a collection period typically occurs over six to 24 hours[13].

Variation in volume excretion and analyte concentration is an important consideration for urine collection as well. Since the excretion volume is dependent upon water intake and other physiological factors, normalization procedures are typically performed to account for this disparity[13]. Methods to account for this deviation requires the monitoring of the volume, creatinine content, and/or osmality to adjust the concentration of the compound of interest[13]. Due to the extreme variation in these factors, it is common for multiple components to be utilized to provide an accurate representation. The favored technique is monitoring the creatinine content, but this requires another analysis[13]. Even though the total volume would be the easiest normalization procedure to use, this is not typically done since it is difficult to obtain accurate volume levels, especially for animals in a metabolism cage, since the urine is contaminated with food and feces[13]. This contamination of food and feces also increases the sample preparation procedures that are needed as typically a sample clean-up step is necessary to eliminate these particles before sample analysis. Furthermore, to increase sample stability, a bacteriostatic agent is also typically added to the urine sample (such as sodium azide) to prevent bacterial growth. If osmality is being measured, the agent needs to be added after the osmality measurement[13]. While urine samples are more stable than blood samples, at least a couple

normalization procedures are advised and sample treatment and clean-up may be necessary for reproducible results.

1.2.2.4 Tissue

Tissue collection is commonly used to obtain tissue specific PK/PD information. This is generally performed using tissue biopsy. In this case, multiple animals (3 to 15) are typically euthanized for each time point desired. Due to several processes occurring during the euthanasia process, some experiments require the animal to be euthanized quickly. This can be accomplished by cervical dislocation, decapitation, anesthesia overdose, carbon dioxide, isoflurane inhalation, or by focused microwave radiation[14]. The method chosen depends upon several factors, such as the effects of the euthanasia on the tissue of interest and the technique that is allowed by an appropriate committee[14].

An advantage of tissue sampling is that it does have good spatial resolution, as long as the specific tissue can be located and isolated accurately. A disadvantage of this technique is that the temporal resolution is dependent upon the number of animals sacrificed for the experiment. Due to the high cost of this practice, the temporal resolution is usually limited. This technique is further complicated by the inter-animal variability that occurs using multiple animals for different time points in the study and the processes that occur during sample collection.

Once the tissue is obtained, the sample is immediately treated to prevent a cascade of biological reactions from occurring[14]. Typical treatments consist of a buffer to quench any reactions and/or flash freezing by liquid nitrogen or dry ice[14]. Flash freezing is common since it keeps the sample stable and viable; however, once the tissue is thawed reactions can still occur if a buffer is not applied to the tissue to prevent further enzymatic processes[14]. This typically

requires multiple tissue sample preparation methods to be tested extensively and quickly to determine the best technique to minimize these further reactions.

Just like blood sampling, the extraction of an analyte from the tissue sample is often complex and time consuming due to the multiple components present in the biological sample[9]. During tissue sample preparation, the analytes that were bound to proteins in the tissue typically become free. This is not beneficial since only the unbound portion is of primary interest, since it is the therapeutically active portion. A sampling technique monitoring drug biomarkers continuously with a minimum number of animals would enhance the knowledge obtained for PK/PD studies.

1.2.2.5 Microdialysis Sampling

Using microdialysis sampling to obtain information for PK/PD studies has gained considerable attention in the past few decades[15-17]. While typical microdialysis sampling experiments last a few hours to a few days, experiments have been monitored for up to a month[18-19]. For this sampling technique, a microdialysis probe is implanted in the specific tissue or area of interest. Any analyte below the molecular weight cut-off (MWCO) will diffuse through the membrane of the microdialysis probe. This allows the continuous collection (temporal resolution) of only the free fractions of endogenous compounds at the site of probe placement (spatial resolution) over a period of time.

Monitoring these compounds by microdialysis sampling not only allows a complete time-profile, but also minimizes animal variability since each animal can serve as its own control. This reduces the number of animals necessary for each experiment. In addition, microdialysis samples only collect the free (non-protein bound) molecules in the extracellular fluid. Any molecule that is bound to a protein will not penetrate the membrane, unless a large MWCO

membrane is used. Therefore only the therapeutically relevant free fraction will be obtained in the dialysate sample. With all of these benefits of microdialysis sampling for PK/PD studies, this technique has been utilized to obtain information regarding biomarkers of enzymatic reactions and neurotransmitters following drug administration. Since this technique was utilized for all of the studies in the following chapters, it will be discussed thoroughly.

1.3 Microdialysis Sampling

Microdialysis sampling was developed over 40 years ago, and has been widely used in a variety of tissues. Initially microdialysis sampling was used to monitor amino acids in the brain and subcutaneous tissue by Bito *et al.*[20]. This preliminary study implanted dialysis sacks with 6% dextran in dogs and left them implanted for 10 weeks. At the end of the study, these sacks were removed, and blood was withdrawn to compare the concentration of amino acids obtained from the microdialysis sacs and the blood samples. Several years later, Delgado *et al.* developed the *dialytrode* sampling method similar to the push-pull method with a permeable membrane at one end[21]. This method held two rods next to one another with a dialysis bag at the end of the rods. This *dialytrode* was implanted in rhesus monkeys for several weeks before the perfusion of glutamate to the amygdala and the collection of several amino acids from the same brain region. The perfusion was initiated by creating positive or negative pressure to create an exchange rate of 2 to 4 μL min^{-1}[21]. In the 1970's and early 80's, Ungerstedt's group further developed and then patented the continuous flow microdialysis sampling method as it is known today[22-25]. With the development of commercially available probes, the number of articles published using this technique exponentially increased in the subsequent decades[15-16].

At first microdialysis sampling was most commonly employed for brain studies[26-27], it is now used to sample the ECF of a variety of peripheral tissues including liver[27-30], eye[31],

heart[32-33], kidney[34-35], muscle[36-37], skin[38], and solid tumors[39-40]. While microdialysis sampling is ordinarily used in animal experiments, some human studies have utilized this technique as well[38, 41].

1.3.1 Principles of microdialysis sampling

A microdialysis probe consists of a semi-permeable membrane that allows analytes to diffuse across the membrane based on their concentration. Equilibrium is not usually achieved across the membrane due to the continuous flow of perfusion fluid through the probe. A typical flow rate of the perfusion syringe pump is 1 μL min^{-1}, although flow rates between 0.5 and 5 μL min^{-1} have been used[42]. Lower flow rates can be used (i.e. 50 nL min^{-1}) to obtain near equilibrium conditions, although factors such as sample volume, sample evaporation, and flow stability are important considerations before using such low flow rates[43].

Another essential component to microdialysis sampling is the selection of a perfusate solution that matches the ionic composition and pH of the sample matrix. A proper perfusate selection will eliminate the loss or gain of ion and water molecules across the membrane from the perfusate[16]. Perfusate solutions range from Ringer's solute to artificial cerebral spinal fluid (aCSF) for brain experiments, to saline (0.9% sodium chloride) for peripheral or intravenous sampling. Once the perfusate solution exits the microdialysis probe, it is known as the dialysate, which is collected at the microdialysis probe outlet. Due to the MWCO of the membrane, only the molecules below the MWCO diffuse through the microdialysis membrane. If a low MWCO membrane is selected, all proteins will be excluded from the sample, thus eliminating or minimizing sample clean-up. If protein sampling is desired, recent developments have improved the recovery of these large molecules by using large MWCO membranes, but this is still an emerging field[44].

1.3.2 Microdialysis probe designs

All microdialysis probes consist of an inlet, outlet, and membrane material. The geometry of the microdialysis probe (rigid/flexible) depends upon the tissue being sampled. The probe type that is used is highly dependent on the tissue composition at the site of interest. For example, the brain tissue is protected by the skull and moves as one unit, therefore a rigid microdialysis design works great to sample from the brain tissue. Conversely, blood sampling is usually performed in peripheral tissue and in a vessel; therefore a flexible microdialysis probe is utilized to minimize tearing the critical vessel tissue. Microdialysis probes are configured in many different geometric designs to make it applicable to the tissue of interest.

1.3.2.1 Pin-style, rigid cannula

The most common probe design utilized is the pin-style probe (Figure 1.1A & Figure 1.2A) design for brain and blood sampling. This probe consists of the membrane on the tip of a pin-style cannula and then the inlet and outlet on the opposite side of the probe. The concentric pin-style is primarily used for brain sampling experiments. This design consists of one end of the inlet (smaller) tube placed in the lumen of the membrane near the tip. At the other end of the membrane (typically one to four millimeters from the tip) the outlet tubing is connected to the membrane. The outlet tubing is generally made of rigid material (e.g. stainless steel) to prevent damage during insertion and experimentation. This rigid style works well in the brain since the brain moves consistently in the skull, whereas in peripheral tissue, this rigid design would tear the tissue and/or vessel.

1.3.2.2 Pin-style, flexible cannula

For peripheral tissue and vessel implantations, a flexible pin-style probe (Figure 1.1B & Figure 1.2B) was developed by our laboratory[45]. This design has the same pin-style geometry as the concentric probe for brain sampling, where the membrane is on the opposite end from the inlet and outlet. The pin-style flexible cannula consists of a side-by-side design for the inlet and outlet tubing instead of the concentric tubing. This added flexibility minimizes tearing and perturbation of peripheral tissues (including vessels) since pliable tubing is used for the side-by-side design. As with the concentric design, the inlet extends a few millimeters into the membrane near the tip. The outlet is then attached to the end of the membrane window. The inlet and outlet tubing on the opposite side of the membrane are extended in the same direction, thus allowing a single entry point for blood sampling in vessels or peripheral tissue.

1.3.2.3 Linear probes

Another probe commonly used for peripheral tissues is a linear microdialysis probe (Figure 1.1C & Figure 1.2C). These probes have the inlet and outlet tubing stuck inside each side of the membrane lumen. Since the probe membrane is the only material in the middle, these probes are extremely supple making them ideal for peripheral tissues. Originally these microdialysis probes were used for brain experiments[26, 46], but now they have been implanted in several peripheral tissues[27, 29-30, 33, 36-37, 39-40, 47-49]. This allows the direct sampling of these peripheral tissues over several days or weeks with minimal tissue perturbation.

1.3.2.4 Shunt probes

Lastly, another probe designed by our laboratory is the shunt probe (Figure 1.1D)[50-51]. This probe is essentially a linear probe with another tube over the membrane. The inlet and

outlet of the tube over the membrane is then inserted into the bile duct and the small intestine[51]. This allows the continuous sampling of bile from an animal without removing the bile salts. In conventional methods, removal of the bile salts from an animal changes the physiology of the animal, just like removing too much blood. With the utilization of the shunt microdialysis probe, the bile flow is conserved by returning it to the small intestine[52]. This allows liver metabolism studies to be performed by monitoring the direct metabolism of a drug for PK/PD studies[52].

1.3.3 Probe implantation

Proper implantation of a microdialysis probe is important to obtain consistent data. Successful implantation of the microdialysis probe is dependent upon the degree of difficulty of the implantation and the skill of the surgeon. Microdialysis brain probes are typically simple and reproducible to implant due to the utilization of a stereotaxic apparatus and the rat atlas map, thereby minimizing the degree of difficulty. In contrast, the implantation of microdialysis probes in peripheral tissue can be quite complicated. This is especially true in small, heterogeneous spaces such as the submucosa of the ascending colon. While the latter implantation is more difficult, both techniques require intricate precision to minimize tissue damage.

Minimal tissue damage is critical in obtaining valid results. Even though the brain surgery is extremely straightforward, care must be taken to minimize tissue trauma. For example, three or four holes are drilled through the skull for the insertion of the bone screws and the brain cannula. While holes are necessary in the skull, the tissue below should be minimally affected during the drilling procedure. This is directly related to the 'skill' of the surgeon. The slow insertion of the cannula is also critical to minimize tissue injury or destruction.

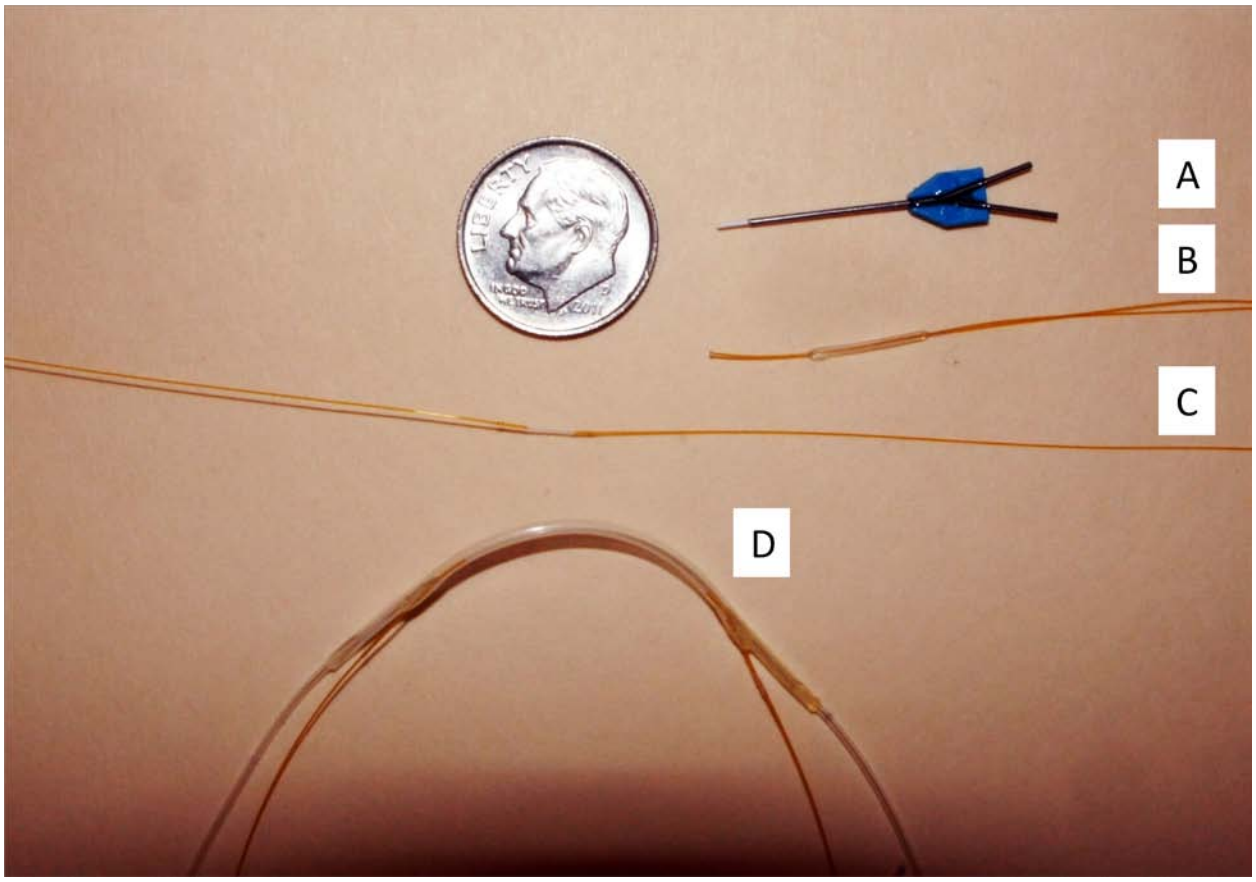

Figure 1.1: Probe designs for microdialysis sampling shown with a dime for size comparison. From top to bottom: A) pin-style (rigid-cannula), B) pin-style (flexible cannula), C) linear probe, and D) shunt probe.

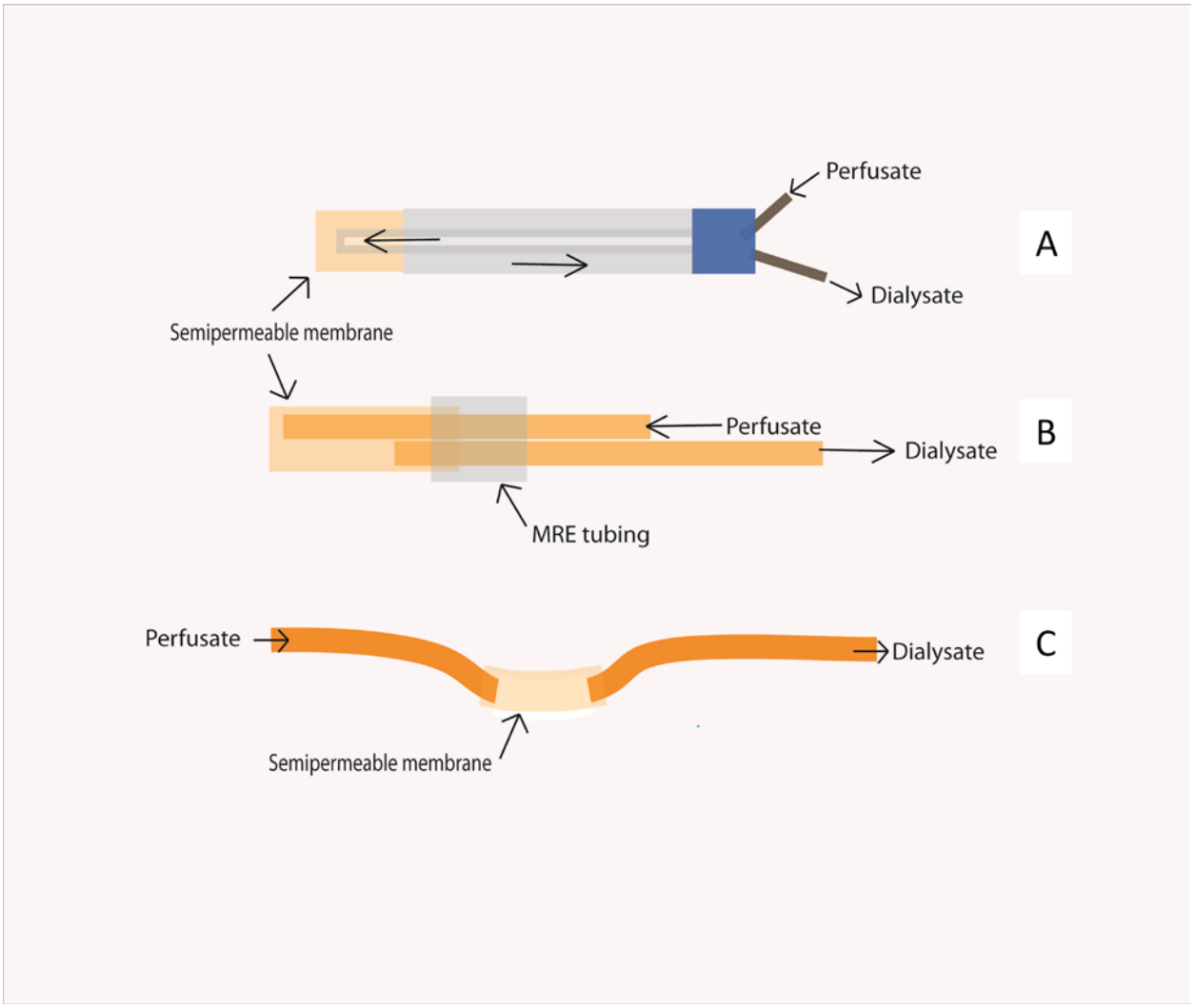

Figure 1.2: Diagram of microdialysis probes. From top to bottom: A) pin-style (rigid-cannula), B) pin-style (flexible cannula), and C) linear probe. MRE: micro-renathane.

Implantation of a microdialysis probe in peripheral tissue can be complicated and must be done with care, especially in tissues such as the colon submucosa. This area has an added degree of difficulty due to the small space available for implantation (60 μm) and the number of blood vessels present in the submucosa layer of the colon. To increase the precision of the implantation and to prevent destruction of the probe, tubing, or tissue, a needle is used to insert the probe into the tissue. The smallest needle possible is used to minimize tissue damage. Several other procedures must also be followed in order to keep the colon tissue viable, as described in the experimental procedures discussed in Chapter 3. While it is more difficult to implant microdialysis probes in peripheral tissues, especially in tissues such as the submucosa of the colon, these techniques provide valuable information that cannot be obtained by any other information.

1.3.4 Tissue response to probe implantation

While it is best to minimize tissue damage to reduce the severity of the immune response, the latter will occur with all microdialysis implantations. This immune response is important to keep in mind when designing the experimental protocol and during implantation (Table 1.1). Fortunately, this immune response has been well-documented and is consistent between most tissues[53].

The initial response is the dilation of the vasculature system which can cause bleeding and hemorrhaging. This increase in blood flow to the area allows the infiltration of neutrophils [polymorphonuclear leukocytes (PMNs)] to reach the foreign material (i.e. microdialysis probe) between six hours to three days after implantation[54]. As the neutrophils encompass the foreign material, monocytes (macrophages) will start to appear within one to three days to remove any necrotic tissue or bacteria. As the macrophages appear, the neutrophil presence will diminish

Cell type or event	Initial	Peak	Decline
Neutrophils (PMNs)	6 hours	days 1-3	after day 4
Monocytes (macrophages)	24 hours	days 2-4	slowly after day 4
Fibroblasts	2nd day	day 6	days 6-10
Collagen production	4th day	increasing until at least day 12	
Capillary formation	4th day	day 8	after day 8

Table 1.1: Inflammatory response. The cell types and events at wound site.[53-54]

and fibroblasts will appear and produce collagen around day four. Blood vessels will also form around this time as well. As is to be expected, all of these processes can affect the extraction efficiency of the microdialysis probe. The severity of the immune response can be substantially decreased by meticulousness in the surgeon's techniques. Therefore it is in the best interest of the surgeon to perform all procedures as precisely as possible to maximize the amount of useful data that will be obtained from the experiment.

1.3.5 Microdialysis probe extraction efficiency

In order to use microdialysis sampling, it is important to determine the extraction efficiency (EE) of the compound of interest. Typically, the EE is established in an *in vitro* experiment first with a certain set of parameters, and then it can be utilized for *in vivo* sampling. In order to determine the EE, the concentration (C) of all the parameters in equation 1.1 must be known.

$$EE = \frac{C_{perfusate} - C_{dialysate}}{C_{perfusate} - C_{sample}} \qquad \textbf{(1.1)}$$

Different applications can utilize the EE equation, which can be manipulated depending upon the microdialysis sampling experiment that is performed. For example, in a delivery experiment a select concentration of the compound can be delivered through the microdialysis probe in the perfusate ($C_{perfusate}$). The delivery of a compound can be utilized to determine the local effects of a drug delivered to the targeted-tissue. In order to determine the delivery of the compound, the EE for delivery uses equation 1.2, when the concentration in the tissue is zero (C_{tissue}=0).

$$EE_{delivery} = \frac{C_{perfusate} - C_{dialysate}}{C_{perfusate}} \quad \textbf{(1.2)}$$

In preliminary *in vitro* experiments, a recovery experiment is also typically performed under the same set of conditions to determine the EE. In general, the recovery and delivery EE are equal under the same set of conditions. To calculate the relative recovery, equation 1.3 is used, and $C_{perfusate}$=0.

$$EE_{relative\ recovery} = \frac{C_{dialysate}}{C_{tissue/sample}} \quad \textbf{(1.3)}$$

1.3.5.1 Factors affecting extraction efficiency

Since microdialysis is a dynamic technique, several factors can change the extraction efficiency of an analyte. There are primarily three distinct regions an analyte must diffuse through in order to be collected in the dialysate. These regions are the perfusion fluid, the microdialysis membrane, and the sample solution. All of these microdialysis parameters must be carefully considered and optimized in each experiment in order to obtain the temporal resolution desired for the experiment, but yet enable the detection of the analyte with the selected analytical system.

1.3.5.1.1 Perfusion fluid

It has been well established that the linear velocity of the perfusate affects the EE of an analyte[55]. The two main components that affect the linear velocity of the perfusate is the flow rate set on the syringe pump and the geometry of the microdialysis probe. The relative EE of the microdialysis probe increases as the flow rate decreases. The relative EE is the common term used to determine the concentration of analyte obtained. Absolute EE can also be used to describe the delivery or recovery of an analyte. Since the perfusate acts as a sink, as the linear

velocity of the perfusate increases the mass recovery of the analyte increases as well, and the absolute EE increases[55]. However, the absolute EE is not commonly used since it is difficult to determine the total mass of an analyte present in the tissue surrounding the probe[55]. As shown in Figure 1.3, the EE of relative and absolute recoveries are opposite in value regarding the flow rate used.

Linear velocity of the perfusate has also been shown to be influenced by the probe geometry used in a microdialysis sampling experiment. A linear microdialysis probe demonstrated a higher recovery than a pin-style flexible probe with the same length of membrane, as determined by Zhao *et al.*[42]. The inlet of the pin-style flexible probe extends into the lumen of the microdialysis probe and decreases the volume of the 'dialysate' solution in the membrane. This also increases the linear velocity of the perfusate, as shown in Figure 1.4A[42]. In the linear probe, the entire 'dialysate' solution is exposed to the membrane. Since there are no objects in the active membrane window, the linear velocity should be equal to the flow rate of the pump. This allows the concentration of the analyte to increase uniformly across the membrane window, as shown in Figure 1.4B.

1.3.5.1.2 Membrane

In addition to the linear velocity of the perfusate, the type of membrane utilized can also affect the EE of an analyte. A microdialysis membrane is chosen based on MWCO, hydrophobicity and charge. There are several microdialysis membranes that are commonly used and commercially available. Each of these membranes has different characteristics, which can change the EE of an analyte. The property of the membrane is an important factor to keep in

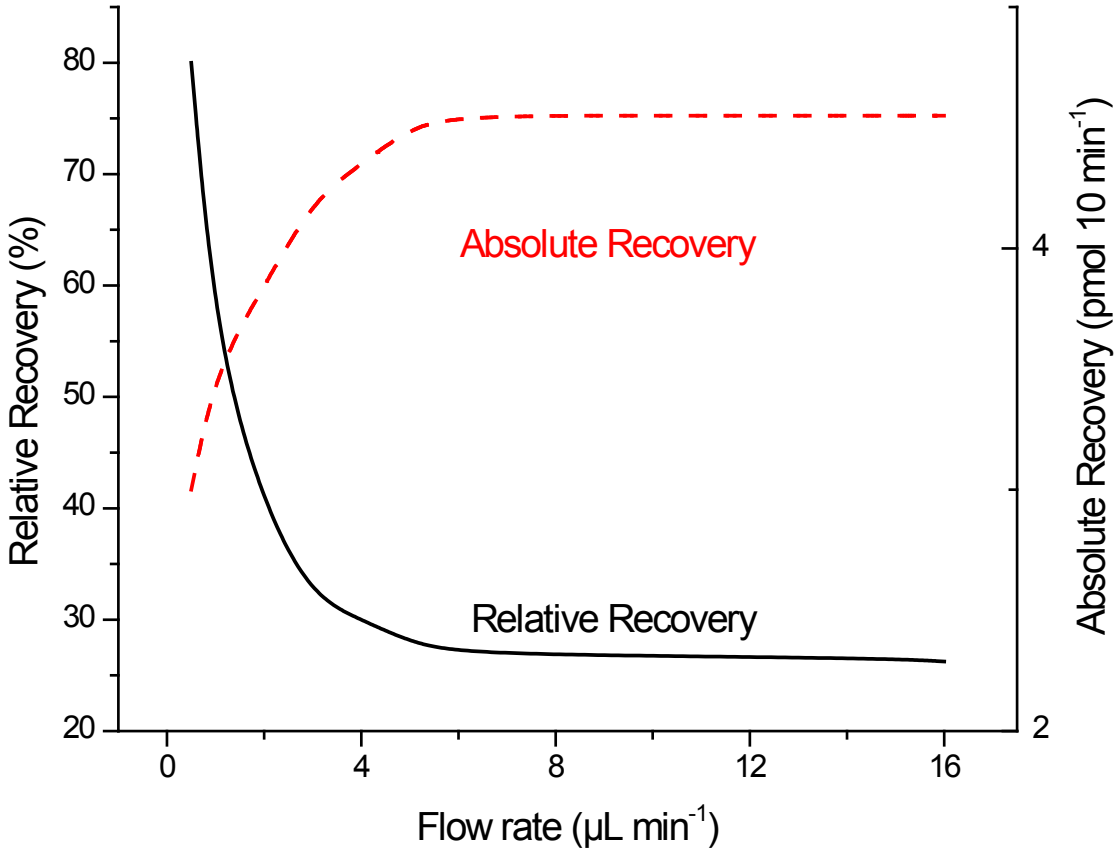

Figure 1.3: Recovery percentage illustration of relative (_) and absolute (- -).

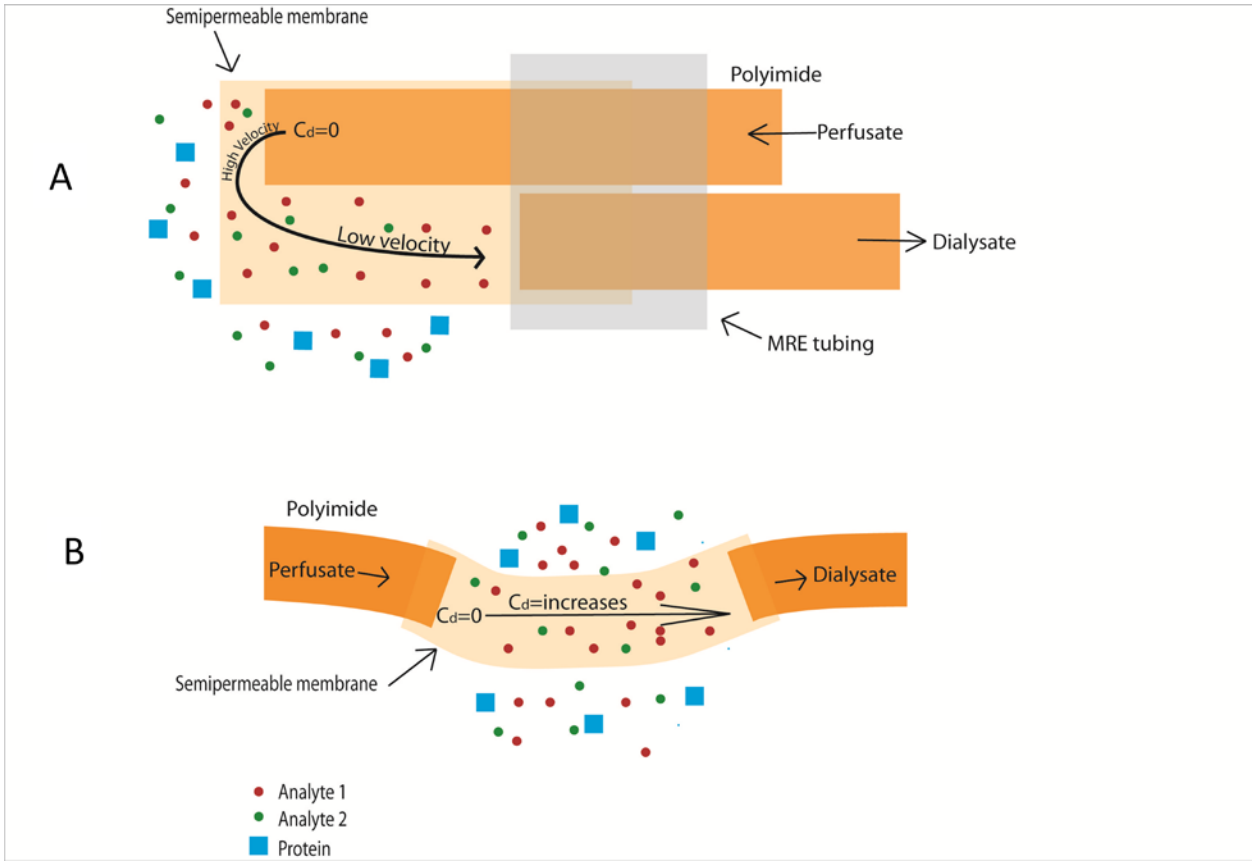

Figure 1.4: Illustration of linear velocity and concentration differences in microdialysis probes A) pin-style (flexible cannula) probe, and B) linear microdialysis probe.

mind for the analyte of interest. The most common membrane materials are regenerated cellulose, cellulose acetate, and polyacrylonitrile (PAN), polycarbonate, and polyarylethersulfone (PES)[42].

The PAN membrane is commonly used in linear microdialysis probes since it is robust and has a large surface area, with an outer diameter (o.d.) of 340 μm and an inner diameter (i.d.) of 240 μm[42]. It is important to keep in mind that the EE of different analytes can vary because this membrane carries a negative charge due to the cyanide groups. This can cause compounds that are polar or those that have a negative charge to have lower EE due to the hindered diffusion of the analyte through the membrane[55]. Therefore, due to different membrane characteristics, most compounds are tested with the selected membrane to optimize the EE under a given set of parameters.

In addition to the different membrane materials, membrane lengths are also tested since longer membrane lengths can increase the EE. While increasing the membrane length can increase the EE of an analyte, the desired spatial resolution also plays a role in the length of the membrane utilized. A great example is brain tissue. Each brain region possesses different spatial characteristics, such as the hippocampus versus the striatum. Sampling the hippocampus requires more spatial resolution, requiring a microdialysis membrane approximately 3 mm or less, while the striatum only requires approximately 6 mm or less resolution since it consists of a larger area[56]. While shorter membranes provide better spatial resolution, the EE usually decreases as well. In heterogeneous tissue (e.g. brain), typical membrane lengths are one to four millimeters. While in homogenous tissue (e.g. muscle), membrane lengths can be up to one centimeter. Membranes greater than one centimeter are not typically utilized since the extraction efficiency approaches 100%, thus increasing the membrane length does not increase the recovery

or delivery of an analyte[42]. The desired spatial resolution and its importance to the experiment is the greatest factor in determining membrane length, and is determined by the tissue in the desired species (e.g. mice, rats or humans).

1.3.5.1.3 Sample environment

The sample environment also plays a role in the EE of the microdialysis probe. The EE of an analyte is influenced by the diffusion of the compound in the surrounding tissue, the linear velocity of fluid surrounding the microdialysis probe, and the uptake of the compound by mechanisms adjacent to the microdialysis probe[57].

As the linear velocity around the probe increases, the diffusion layer around the probe decreases. This decrease in the diffusion layer makes the EE of the compound dependent upon the movement of the analyte into the lumen of the membrane. When the linear velocity of the extracellular fluid (ECF) is below 0.211 cm sec^{-1}, the rate-limiting step is dependent upon the diffusion of the compound in the sample matrix. The only time the rate-limiting step of the EE is based on the diffusion through the membrane is when the linear velocities are above 0.211 cm sec^{-1}. This is only typical in hydrodynamic systems, such as *in vitro* studies, blood vessels or the bile duct[58]. The EE of the compounds for these systems can typically be determined *in vitro* since the recovery and delivery rate is determined by the membrane. Whereas in tissues where the linear velocity of the sample matrix is below 0.211 cm sec^{-1}, the tissue or the uptake mechanisms near the probe determine the EE of an analyte. Thus, calibration methods must be utilized to determine the EE of the compound *in vivo.*

1.3.5.2 Probe Calibration

Once the other parameters are determined *in vitro* to obtain the highest EE for the compound of interest, probe calibration methods must be performed if quantitative measurements are desired *in vivo*. There are several techniques that can be used to determine the *in vivo* concentration of the sample. The calibration technique utilized depends upon the type of information desired for a given experiment. If a percent change is of utmost importance, then no calibration technique is necessary. Retrodialysis with antipyrine is a typical technique that can be performed to ensure the EE of the probe remains constant throughout the experiment for percent change studies. However, if it is critical to quantitatively determine the concentration in the sample, then one of the other calibration methods is necessary to attain the information of interest. If this is the case, calibration by the delivery of an analyte or no-net flux (NNF) are the best techniques to utilize in these instances. The advantages and disadvantages of each technique are described in detail.

1.3.5.2.1 Retrodialysis

The retrodialysis method consists of a marker that simulates the compound of interest and is included in the perfusate throughout the entire experiment. Ideally the chosen marker is similar in chemical structure and has similar EE properties as the analyte without having any physiological effect in the tissue of interest. Radiolabelled compounds can be utilized as markers, but they are usually toxic with chronic exposure and expensive. A common marker used in our laboratory is antipyrine[30, 59]. While it is not the compound of interest, it does enable the determination of the EE of the probe to be monitored throughout the experiment If the exact concentration of the sample is necessary, then the other two techniques are commonly used for the precise determination of the EE of the specific analyte of interest.

1.3.5.2.2 Calibration by delivery of an analyte

The calibration by delivery of an analyte method is similar to retrodialysis, except that it delivers the compound of interest before and/or after the experiment. The benefit of this calibration technique is that the EE for the specific compound is determined for each probe, which is effective since the recovery and delivery of an analyte should be equal. If the compound of interest is perfused through the probe before and/or after the experiment, the viability of the probe will be verified through the entire experiment. However, if the EE of a probe is altered during the experiment due to administration of a vasodilator or constrictor, this calibration technique will not detect this change in the EE. Therefore, multiple calibration techniques may be utilized for analyte quantitation. In addition, if perfusion of the compound interferes the physiological system, perfusion of the compound after the experiment may only be an option to prevent the compound from affecting the results of the experiment.

1.3.5.2.3 No-net flux

The NNF method calibrates the probe based on the point at which the perfusate concentration is equal to the ECF, thus representing no loss or gain of analyte from the perfusate. This situation is obtained by changing the concentration of the perfusate several times throughout the experiment. Several perfusate concentrations above and below are typically tested to determine the loss or gain of an analyte in the perfusate to determine the point at which NNF occurs. Since this method requires both the delivery and recovery of the compound of interest, this is the most accurate method for obtaining the EE of the compound.

This information is then plotted with the change of the dialysate concentration (C_p-C_d) represented on the y-axis and the concentration of the perfusate (C_p) plotted on the x-axis. The EE is then determined by the slope, and the concentration of the ECF (C_s) is obtained by the x-

axis intercept. While this method is beneficial since it determines the EE for the particular analyte, it requires that the concentration of the sample remains stable throughout the experiment. Additionally, it takes an incredibly long time to complete, if accurate data is desired. In order to get enough points regarding the EE of the compound's EE, this method typically takes a minimum of eight hours, whereas determining the EE by the delivery method might take an hour or two.

1.3.5.2.4 Advantages/disadvantages

Microdialysis offers a site-specific and continuous sampling method. Since animals are monitored continuously, a minimal number of animals are used and each animal can serve as its own control. This decreases inter-animal variability and allows a full picture of PK/PD events to be obtained. If a low MWCO probe membrane is utilized, sample clean-up is not usually necessary. In addition, no fluid is lost or gained with the utilization of an appropriate perfusate in the microdialysis probe. Between this and the small size of the microdialysis probe, microdialysis samples result in minimal system perturbation, especially in comparison to conventional methods.

Despite all of these, microdialysis sampling does have several limitations as well. Due to the continual flow of a perfusate, animal physiology, animal husbandry, and analyte extraction efficiency all influence the results obtained. Due to the implantation of a foreign device, the body exhibits an immune response that can change the animal's physiological state. While there are other site-specific techniques (such as biosensors or electrochemical voltammetry) that offer higher spatial resolution, and will potentially result in less tissue damage, these techniques are often difficult to fabricate and are limited to certain analytes.

Due to the constant flow rate required through the microdialysis probe, animals are typically housed in a Raturn® bowl. The Raturn® allows the animal to move freely during sampling. The animal is typically kept in a harness with a tether connected to it to prevent the tubing from tangling or kinking. In short-term experiments, there is a fine line between keeping the harness tight enough while ensuring the harness is not too tight to cause discomfort. However, in long term experiments this proves to be an extremely difficult task, especially after a couple of weeks. This will be discussed further in Chapter 5.

Moreover, the constant removal of all small endogenous analytes through the microdialysis membrane during the entire experiment can alter the animal's physiology, since all endogenous compounds below the MWCO of the membrane are removed. In addition, because the perfusate is usually flowing at a high linear velocity, the compound of interest never truly equilibrates with the ECF. This requires the meticulous calibration of the microdialysis probe if the exact concentration of an analyte is desired. If a percent change is of interest, a retrodialysis compound may be utilized, but this is not necessary. While all of these drawbacks of microdialysis sampling are important to consider, microdialysis still offers many advantages over other conventional *in vivo* sampling methods, such as blood, urine, and tissue collection.

1.4 Conclusions

Microdialysis sampling is a useful tool for many applications including PK/PD studies. The goals of this research project were to monitor enzymatic reactions in peripheral tissue upon drug administration and to monitor neurotransmitters in the brain following administration of chemotherapeutics. In the first studies, novel applications of microdialysis sampling was used to monitor enzymatic activity in peripheral tissue. In the first enzyme study, microdialysis experiments were performed in rats, as the ability to monitor 11β- hydroxysteroid dehydrogenase

(11β-HSD) in a tissue-targeted manner in a non-human species is necessary for efficient progression of novel enzyme inhibitors to clinical studies. In the second enzyme study, a microdialysis probe was implanted in the submucosa of the ascending colon to observe the activation of an enzyme, guanylate cyclase-C, *in vivo.* The effects of the administration of an enzyme agonist, STcore was then observed. These studies, upon the administration of an inhibitor or agonist, determined the response of the enzyme to the drug in the peripheral tissue of interest. The enzyme activity of 11β-HSD in rat microsomes by microdialysis sampling was performed. The difference in 11β-HSD metabolism of cortisone between two species (humans versus rats) was compared in these studies, demonstrating the importance of species comparison. In the second enzyme activity study, the implantation of the microdialysis probe was optimized in the submucosa allowing the first ever experiment performed in colon submucosa tissue. This allowed the comparison of cGMP levels between the colon lumen and submucosa.

While microdialysis sampling is commonly used to monitor neurotransmitters, it has never been utilized to monitor the effects of the administration of chemotherapy compounds on neurotransmitters and biomarkers of oxidative stress. In order to elucidate possible mechanisms that cause cognitive dysfunction in chemotherapy patients, short- and long-term studies were performed. Moreover, the application of long-term experiments lasting over one month was needed, since cognitive effects in human cancer patients are typically noticed a few weeks to years after chemotherapy administration. Typical microdialysis experiments last a few days to a couple of weeks[60-62]. The optimization of a long-term microdialysis study (months) is a novel approach to have a continuous time-profile over this length of time. To the best of our knowledge, only a couple literature articles monitored endogenous analytes in a microdialysis experiment with a duration of a month[18-19]. The development of a long-term microdialysis

sampling study (months) may reduce the animal variability that is observed in other chemotherapy studies performed using conventional sampling methods.

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~Chapter 2~

Monitoring 11β-HSD1 by microdialysis *in vitro* and *in vivo*

2.1 Introduction

Monitoring enzyme activity with a tissue-targeted sampling method provides the metabolite profile of the substrate in an efficient and continuous fashion. Amgen is a pharmaceutical company interested in screening novel inhibitors of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). This enzyme converts the inactive glucocorticoid (cortisone) to the active glucocorticoid (cortisol) as shown in Figure 2.1. In order to develop a new technique to reflect *in vivo* activity, Amgen contacted Craig Lunte to perform studies using microdialysis *in vitro* and *in vivo* studies. Interestingly, when these studies were initiated, microdialysis sampling had been performed in human studies to monitor 11β-HSD1 activity after inhibition, but not in rats or in any *in vitro* studies[1].

The ability to monitor 11β-HSD1 activity *in vitro* and *in vivo* in an efficient manner is critical to rapidly screen novel inhibitors. While there are several screening methods for monitoring 11β-HSD1 activity *in vitro* and *in vivo,* most of them are discontinuous sampling methods and require extensive sampling preparation. The *in vitro* methods commonly used to monitor 11β-HSD1 activity include cell culture systems, microsomes, or hepatocyte baths. These *in vitro* methods involve quenching the reaction at different times to determine the enzyme activity. This requires more solutions and substrates, which is extremely detrimental if supplies are limited. The same thing is true for *in vivo* sampling. The two main sampling techniques used to monitor 11β-HSD1 activity consist of sampling blood and urine[2]. As discussed in Chapter 1, blood and urine sampling have several limitations. For instance, monitoring 11β-HSD1 activity by blood sampling includes both the free and the bound form of cortisone

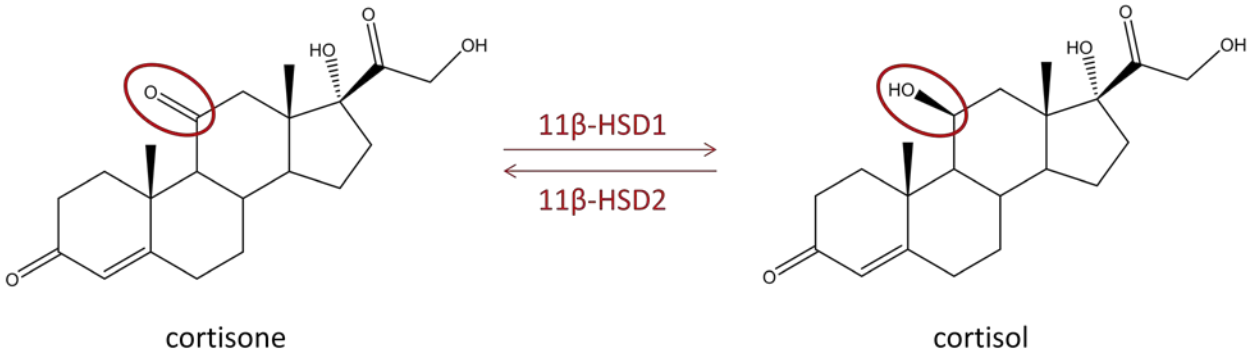

Figure 2.1: 11β-HSD human conversion of cortisone and cortisol

and cortisol, and only the unbound form is the one of interest since it is the active portion[2]. Due to the sample preparation method used for blood samples, the total amount of reactant and product (bound and unbound) are typically represented, limiting its clinical value. While urine samples do only represent the free fraction, the temporal and spatial resolution is extremely limited with this sampling technique. Due to the limitations of the current sampling methods, a better procedure to monitor 11β-HSD1 activity *in vitro* and *in vivo* would speed up the development of inhibitors for this important enzyme.

This conversion of cortisone to cortisol is of interest since continual exposure to high levels of cortisol may result in metabolic syndrome, obesity, and type II diabetes mellitus with insulin resistance[3-6]. To prevent these deleterious events, selective inhibition of only type 1 of this enzyme (11β-HSD1), while not inhibiting the other isoenzyme, 11β-HSD type 2 (11β-HSD2), is an emerging treatment route[7-9]. Inhibition of 11β-HSD type 2 is not desirable since this enzyme catalyzes the reverse conversion of cortisol to cortisone, leading to the deleterious side effects or diseases[10-11]. Currently, the only FDA approved inhibitors for this enzyme are non-selective and will inhibit both isoforms of 11β-HSD. Therefore, several companies are trying to develop a selective inhibitor to provide an effective treatment for people with abnormally high levels of cortisol. In order for potential novel inhibitors to progress to clinical trials in a more cost effective and efficient manner, the development and validation of a better screening method would increase the throughput of these inhibitors.

2.1.1 Effect of inhibitors on 11β-HSD

The current treatment for patients not responding to insulin injections is carbenoxolone, a non-selective inhibitor for 11β-HSD, which increases insulin efficacy[1, 6, 12-13]. However, carbenoxolone cannot be used for long periods of time without causing multiple serious side-

effects. This has led several companies to pursue selective inhibitors for 11β-HSD1 that improve insulin sensitivity, glucose tolerance, lipid profiles, and decrease atherogenesis[1]. Moreover, only a few compounds have been tested in clinical trials, with a high failure rate due to the limited screening methods and reliable animal models[1].

In order for these compounds to be clinically useful, they must inhibit 11β-HSD1 in the target tissue, which is primarily adipose tissue. In a study by Tomlinson *et al.*, they measured the generation of cortisol in adipose tissue by each of the following sampling techniques: local activity from microdialysis sampling, hepatic activity from serum samples, and global activity from urine samples following the administration of cortisone acetate with and without the administration of carbenoxolone[1]. This study examined the effectiveness of carbenoxolone inhibiting the conversion of cortisone to cortisol in adipose tissue. Utilizing microdialysis, carbenoxolone was determined to decrease 11β-HSD1 activity in adipose tissue by 45% compared to only 25% in serum[1]. To validate these results several other parameters were also investigated, including the concentration of carbenoxolone in both microdialysis samples from adipose tissue and plasma samples. By comparing the concentration of carbenoxolone to the concentration of the glucocorticoids, including cortisol, in the microdialysis and blood samples, the effectiveness of carbenoxolone was determined in each sampling fluid. Even though the concentration of carbenoxolone was 5000-fold greater in the serum than in the ECF of adipose tissue, the concentration of carbenoxolone in the adipose tissue was still greater than the glucocorticoid concentration, thereby allowing competitive inhibition to occur at a higher rate.

In this study, the ratio of the component of tetrahydro-metabolites of cortisol (tetrahydrocortisol (THF) + 5α-THF) to that of cortisone (tetrahydrocortisone) in the serum, and the free cortisol to free cortisone ratio in urine agreed with other literature sources. The

Tomlinson study confirmed, with microdialysis sampling, the effectiveness of carbenoxolone in the adipose tissue. This emphasizes the need to implement this sampling technique *in vivo* in non-clinical species and *in vitro* systems to monitor 11β-HSD activity.

2.1.2 Monitoring enzyme activity by microdialysis

The application of microdialysis sampling has not been fully explored for monitoring enzyme activity and would provide several advantages, including the ability to monitor activities *in vitro* and *in vivo,* so long as the appropriate system was found and validated. Considering microdialysis has already been used in human studies to monitor 11β-HSD1 activity, using microdialysis sampling in an *in vitro* system and *in vivo* in a non-human species would make it possible to use smaller amounts of solutions/tissues and increase throughput of inhibitor testing. In addition, sampling could be performed in a continuous manner in the same system, increasing precision.

Currently, most experiments monitoring enzyme activity do so *in vitro*[14]. The information obtained from these *in vitro* experiments are critical in the preliminary development of novel inhibitors for clinical applicants. However, if a novel inhibitor is really promising it is necessary to perform tests in a non-human species due to the regulations of the US Center for Drug Research (CDER)[15]. According to CDER regulations, at least one non-human species that has a comparable metabolite production (>10% of the total drug-related material) must be tested before large clinical trials are conducted in humans[15].

Currently, most *in vivo* studies monitoring enzyme activity employ imaging methods, such as magnetic resonance imaging (MRI)[16-17], positron emission tomography (PET)[18-19], or near-infrared imaging (NIRF)[20-21]. While these techniques are successful in monitoring enzyme activity in specific tissues there are still issues. For example, NIRF raises

concerns of specificity of the fluorochrome to the enzyme of interest and the limited penetration of light into the tissue (<10mm). Both of these analytical limitations require further studies or the need for another technique if deeper tissues are relevant[21]. Another limitation with imaging techniques is requirement of restraint to minimize animal movement. Thus anesthesia is typically used, which could potentially change the results of the enzyme study. The only *in vivo* study, to the best of our knowledge, for monitoring the activity of 11β-HSD1 in subcutaneous tissue in the previously mentioned Tomlinson *et al.* study[1].

2.1.3 Specific aims of research

The objective of this research was to directly monitor the analytes of interest (cortisone and cortisol) in the desired tissue (adipose tissue) by microdialysis before and after an inhibitor is given. Interestingly, this method has been shown to work *in vivo* in humans, but it has not been tested in an *in vitro* system or an *in vivo* system in a non-human species. This method would be highly beneficial given that current methods rely on the indirect determination of cortisone and cortisol using their metabolites, tetrahydrocortisone (THE) and tetrahydrocortisol (THF), in urine or plasma. However, circulating concentrations of THE and THF are not representative of the concentration of cortisone and cortisol in specific tissues such as visceral fat. The development of this technique in an *in vitro* and an *in vivo* system (with a non-human species) with the ability to monitor cortisone and cortisol directly would expedite the development of potential inhibitors to clinical studies, given that the inhibitor's efficacy will be determined directly in the tissue of interest and not based on the global effects of the inhibitor. To accomplish this, a method was first tested *in vitro* to obtain a profile of the products that are formed (i.e. cortisol) and was also tested *in vivo* in rats to determine if they are an appropriate animal model for pre-clinical testing. If the same metabolites are produced by rats and humans, then this would allow a direct

determination of cortisone and cortisol in specific tissue in an animal model to determine the enzyme activity of novel inhibitors to progress only the most potential inhibitors to expensive clinical studies..

2.2 Chemicals and solutions

Cortisone (E), cortisol (F), antipyrine, corticosterone, β-nicotinamide adenine dinucleotide phosphate sodium salt (β-NADP), D-glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase from leuconotstoc mesenteroides (Type XXIV), and rat liver microsomes (female and male) were obtained from Sigma Aldrich (St. Louis, MO, USA). Dipotassium hydrogen phosphate (K_2HPO_4), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), sodium hydroxide (NaOH), methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). 11-Dehydrocorticosterone (11DHC or 4-pregnen-21-ol-3, 11, 20–trione) was acquired from Steraloids, INC. (Newport, RI, USA). Ultrapure water was filtered (18 MΩ resistivity) with a Labconco system (Kansas City, KS, USA) or a Milli-Q system (Millipore, Bedford, MA, USA). Ringer's solution composition was 145 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$,and 1.2 mM $MgCl_2$ (pH of 7). Solutions were filtered through a 0.22 μm nylon filter prior to use.

Ketamine hydrochloride injection, USP (Phoenix; St. Joseph, MO), acepromazine maleate injection (Phoneix; St. Joseph, MO), xylazine sterile solution (Lloyd Laboratories; Shenandoah, IA), isoflurane (MWI; Boise, ID), 0.9% sodium chloride injection USP (Abbott Laboratories; North Chicago, IL), artificial tears ointment (Rugby Laboratories, INC.; Duluth

GA), and Webglue (formulated cyanoacrylate surgical adhesive) were purchased from Patterson Logistics (Webster Veterinary; Kansas City, MO).

2.3 Microdialysis methods

2.3.1 Microdialysis probe fabrication

A linear microdialysis probe was constructed for all experiments in the following manner. A 30 cm piece of polyimide tubing, with a 122 μm inner diameter (i.d.) with 163 μm outer diameter (o.d.) was cut in half with a razor blade making two 15 cm pieces. The polyacyrlonitrile (PAN) membrane was cut to approximately 14 mm long. One piece of polyimide was inserted approximately 2 mm into the PAN membrane. Once the polyimide was in the appropriate position, ultraviolet glue was applied and activated with ultraviolet light for approximately 30 seconds when the glue was near the end of the polyimide. The other piece of polyimide was then inserted into the PAN membrane to create an active window of 10 mm and then held in place with UV glue and light. A piece of tygon tubing, approximately 10 mm in length, was put on one end of the polyimide tubing and glued in place with the UV glue.

2.3.2 *In vitro* experimental system

Liver microsomes from rats (Xenotech, Kansas City, MO USA) and humans (Xenotech, Kansas City, MO USA) were utilized in these studies and kept frozen at -80°C until use. Microsomes were added at time zero to a regenerating NADPH solution. This NADPH regenerating solution (3 mL) consisted of 1.3 mM β-NADPH, 6.6 mM D-glucose-6-phosphate, 250 mM K_2HPO_4, 3.3 mM $MgCl_2$, and 15 units of glucose-6-phosphate dehydrogenase at pH 7.4

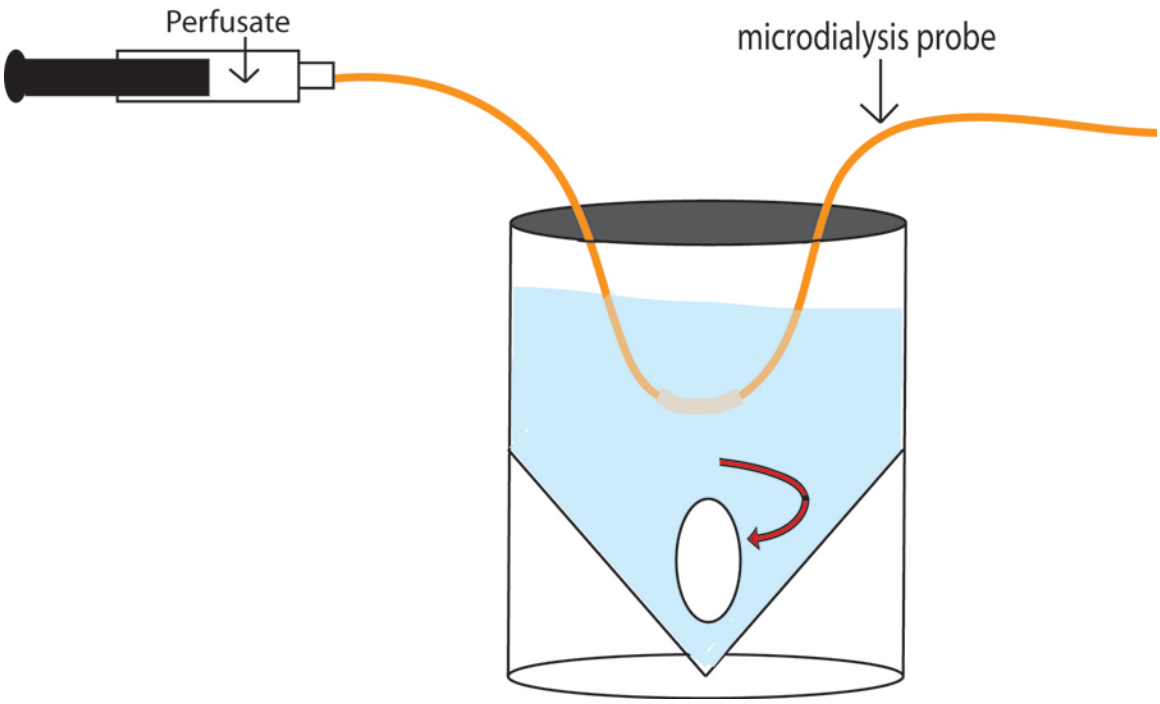

Figure 2.2: *In vitro* experimental design.

in a v-vial, as shown in Figure 2.2. This solution was continuously stirred and kept at 37° C. The linear microdialysis probe was constructed as described in section 2.3.1. The perfusate consisted of 250 mM K_2HPO_4 and 3.3 mM $MgCl_2$ at a pH 7.4. The perfusate was set at a flow rate of 1 μL min^{-1}. Samples were collected continuously in 15 minute intervals throughout the experiment. For delivery experiments, antipyrine, the microdialysis probe internal standard, and cortisone (50 μg mL^{-1}) were included in the perfusate. For recovery experiments these compounds were put in the NADPH regenerating solution. Antipyrine and cortisone were put in the appropriate solution at the beginning of the experiment, and it remained that way until the end of the experiment.

2.3.3 In vivo experimental system

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) animal protocols. Sprague-Dawley rats (300-400 g) were obtained from Sasco (Wilmington, MA, USA). A microdialysis probe with a 10 mm window was implanted in visceral fat or the left hind leg fat. The rats were pre-anesthetized with isoflurane and then administered an anesthetic cocktail of ketamine, acepromazine, and xylazine (80 mg kg^{-1}, 1 mg kg^{-1}, and 5 mg kg^{-1}). Once the microdialysis probe was implanted, the tissue surrounding the probe was kept moist with saline using a gauze pad. The microdialysate perfusate consisted of antipyrine and cortisone (both 50 μg mL^{-1}) in Ringers solution. The perfusate was set at a flow rate of 1 μL min^{-1}. Samples were collected continuously over 15 minute intervals. At least four basal samples were collected prior to IP administration of an inhibitor of 11β-HSD (carbenoxolone or 18-α-glycyrrhetinic acid). At the end of the experiment, the rat was euthanized by an overdose of isoflurane.

2.4 Sample analysis

2.4.1 LC-UV system

Microdialysis samples were analyzed by a liquid chromatography system with ultraviolet detection (HPLC-UV). This system consisted of a Shimadzu LC-10AD pump, a Shimadzu SPD-10AV UV-Vis spectrophotometric detector and a Shimadzu SCL-10Avp system controller (Columbia, MD, USA). Sample injections of 10 μL were made using a Rheodyne model 7125i injector. Separation of antipyrine, cortisone, cortisol, 11DHC and corticosterone was achieved on a Phenomenex Gemini C18 (150 mm x 2 mm, 5 μm particle) column with mobile phase of methanol/acetonitrile/water (50/3/47 v/v). The flow rate was set at 0.3 mL min^{-1}. The primary wavelength used for the detection of these compounds was 242 nm. A wavelength of 210 nm was also utilized. To detect other metabolites of 11βHSD1 that have been reported in literature, the mobile phase for this separation was also modified to 45/3/52 (v/v) MeOH/ACN: nano pure water in order to separate the unknown peaks.

2.4.2 LC-MS system

Microdialysis samples were also analyzed by a Waters 2690 high-performance liquid chromatograph with a Waters Micromass Quattro micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The HPLC system consisted of a refrigerated autosampler and a column oven as well as an external diverter valve. The software utilized was MassLynx 3.5. Sample injections of 30 μL were made into a Rheodyne model 7125i injector. Separation of antipyrine, cortisone, cortisol, THE, THF, 6β-hydrooxycoritsone (6β-OHE), 6β-hydrooxycoritsol (6β-OHF), 20β-dihydrocrotisone (20β-DHE), and 20β–dihydrocortisol (20β-DHF) was achieved on a Phenomenex Gemini C18 (150 mm x 2mm, 5 μm particle) column. The mobile phase for

this system was methanol/1mM ammonium acetate pH 6.7 (65/35 v/v). The flow rate was 0.3 mL min^{-1}. The transitions monitored for the analytes of interest are listed in Table 2.1.

2.5 Results and discussion

2.5.1 Microdialysis probe calibration

Due to the hydrophobic nature of cortisone and cortisol, the first objective was to determine the extraction efficiency (EE) of the microdialysis probe for these compounds. The EE of cortisone and cortisol was determined at three different concentrations using a 5 mm microdialysis probe for the delivery and recovery of cortisone and cortisol. The average EE of cortisone was 80 ± 2.4% for delivery, and 81.6 ± 8.9% for recovery (Table 2.2). The EE for recovery (EE_R) and delivery (EE_D) were not statistically different for cortisone for five out of six studies, as confirmed by a t-test assuming equal variance after the determination of the variance by the F-test. Cortisone was only significantly different from the average EE for recovery at the concentration of 180 ng mL^{-1}, thus demonstrating acceptable extraction efficiency reproducibility overall. The average EE for cortisol was 77.7 ± 2.0% for delivery and 79.7 ± 5.7% for the recovery. All six of the cortisol experiments (three concentrations for recovery and delivery studies) were not significantly different than the average of the delivery and recovery of cortisol. With the high and similar extraction efficiencies, the PAN membrane was utilized in all further studies

	Parent (m/z)	Daughter (m/z)
Antipyrine	189.1	55.9
Cortisone	361.0	163.0
Cortisol	363.2	121.0
THF	367.1	252.9
THE	365.3	149.1
6β-OHE	377.1	161.0
6β-OHF	379.1	239.0
20β-DHE	363.3	163.0
20β-DHF	365.3	269.10

Table 2.1: MS analysis transition of each analyte.

Cortisone (ng mL^{-1})	Delivery	Recovery
180	80.0 ± 3.2%	90.2 ± 5.8%
360	80.5± 2.6%	76.6 ± 5.4%
540	79.9± 1.6%	80.0 ± 5.9%
Average	80.0± 2.4%	81.6 ± 8.9%

Cortisol (ng mL^{-1})	Delivery	Recovery
180	77.0 ± 2.6%	84.5 ± 5.0%
360	78.5± 2.0%	75.4 ± 3.8%
540	77.8± 1.1%	79.4 ± 4.4%
Average	77.7± 2.0%	79.7 ± 5.7%

Table 2.2: Extraction efficiency of cortisone and cortisol at three different concentrations

2.5.2 Cortisone and cortisol

2.5.2.1 *In vitro* studies

Utilizing cortisone as a substrate, female Sprague-Dawley rat microsomes (Xenotech, Kansas City, MO USA) were tested in an *in vitro* delivery experiment to monitor 11β-HSD1 activity with microdialysis sampling. To simulate an *in vivo* experiment in a rat, cortisone was delivered through the probe. Because cortisone is not endogenous in the rat, the amount of cortisol produced should reflect the activity of 11β-HSD1. In the delivery experiment, cortisone and antipyrine were continuously delivered by the microdialysis probe to the NADPH regenerating solution in the v-vial (Figure 2.2). *In vitro* basal dialysate samples were analyzed until a steady state of cortisone delivery was achieved for a minimum of one hour (n=4). Microsomes from female rats were then added directly to the NADPH regenerating solution in the v-vial at time zero. Upon the addition of microsomes at time zero, no change was observed in cortisone delivery (Figure 2.3), as expected.

A significant decrease in cortisone was not anticipated due to the hydrodynamics of an *in vitro* system. The continuous stirring of the solution creates a linear velocity higher than 0.211 cm s^{-1} surrounding the microdialysis membrane, therefore the extraction efficiency is determined by the diffusion of the analyte across the membrane instead of the diffusion through solution[22]. It has been reported in the literature that the mass transfer coefficients show no change in linear velocities above 0.211 cm s^{-1}[22]. Since the removal of the substrate (cortisone)from the region surrounding the microdialysis membrane was faster than the enzyme kinetics, the delivery of cortisone did not change once microsomes were added. In order to see a change in cortisone delivery upon the addition of the microsomes, an extremely low linear velocity would be needed such that the diffusion layer surrounding the microdialysis membrane would be large enough to

make the enzyme kinetics dominate the removal of cortisone. While a change in cortisone delivery was not expected, especially *in vitro,* observation of a product (cortisol) is necessary to determine the enzyme activity, if the delivery percentage of cortisone is does not change the substrate can not be used to determine enzyme activity. To ensure cortisone was metabolized by the NADPH regenerating solution and/or the microsomes, an *in vitro* recovery experiment was performed.

An *in vitro* recovery experiment was performed in a similar fashion as the delivery experiment, with the exception of antipyrine and cortisone being in the NADPH regenerating solution versus the perfusate. Upon addition of the microsomes to the v-vial containing the NADPH regenerating solution at time zero in the presence of cortisone, a significant decrease was seen immediately in cortisone in all three experiments, as shown in Figure 2.4a. This decrease demonstrated that the microsomes were metabolizing cortisone. Interestingly, cortisol was present in the beginning of the beginning of the experiment and increased slightly until the addition of microsomes. The presence of cortisol was determined to be a contaminant in the cortisone standard that increased with heat, as determined in a comparison study of warm microsome buffer bath continuously stirred versus a room temperature microsome buffer bath. Since cortisol was not observed, the identification of at least one product is necessary considering the delivery of cortisone was insensitive to enzyme activity in this hydrodynamic system.

Due to the inability to see any products with female rat microsomes, male rat microsomes were also tested in the same manner as the female rat microsomes. Again, no significant change

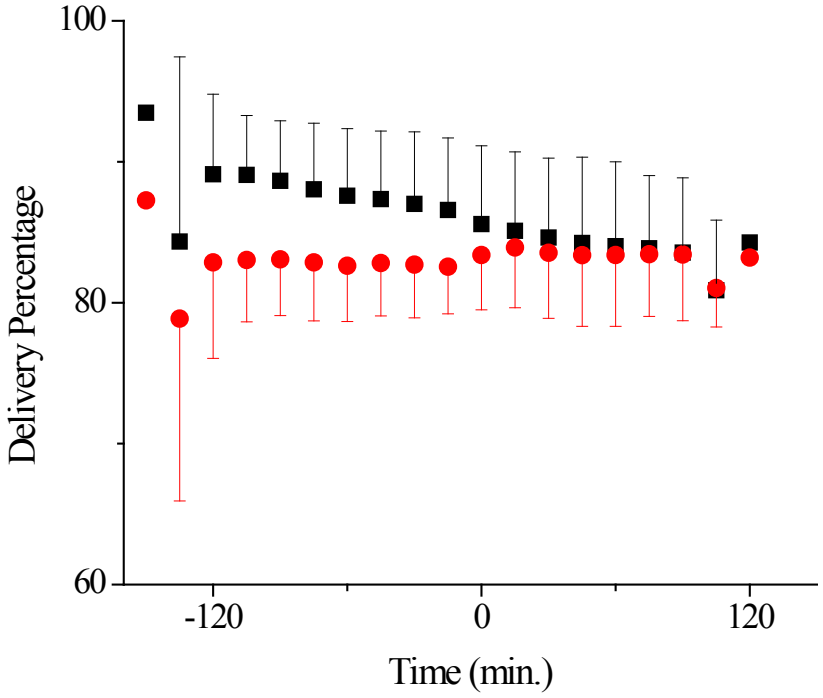

Figure 2.3: *In vitro* delivery experiment with the addition of female rat microsomes at time 0 (n=3). Delivery of antipyrine (■), cortisone(●).

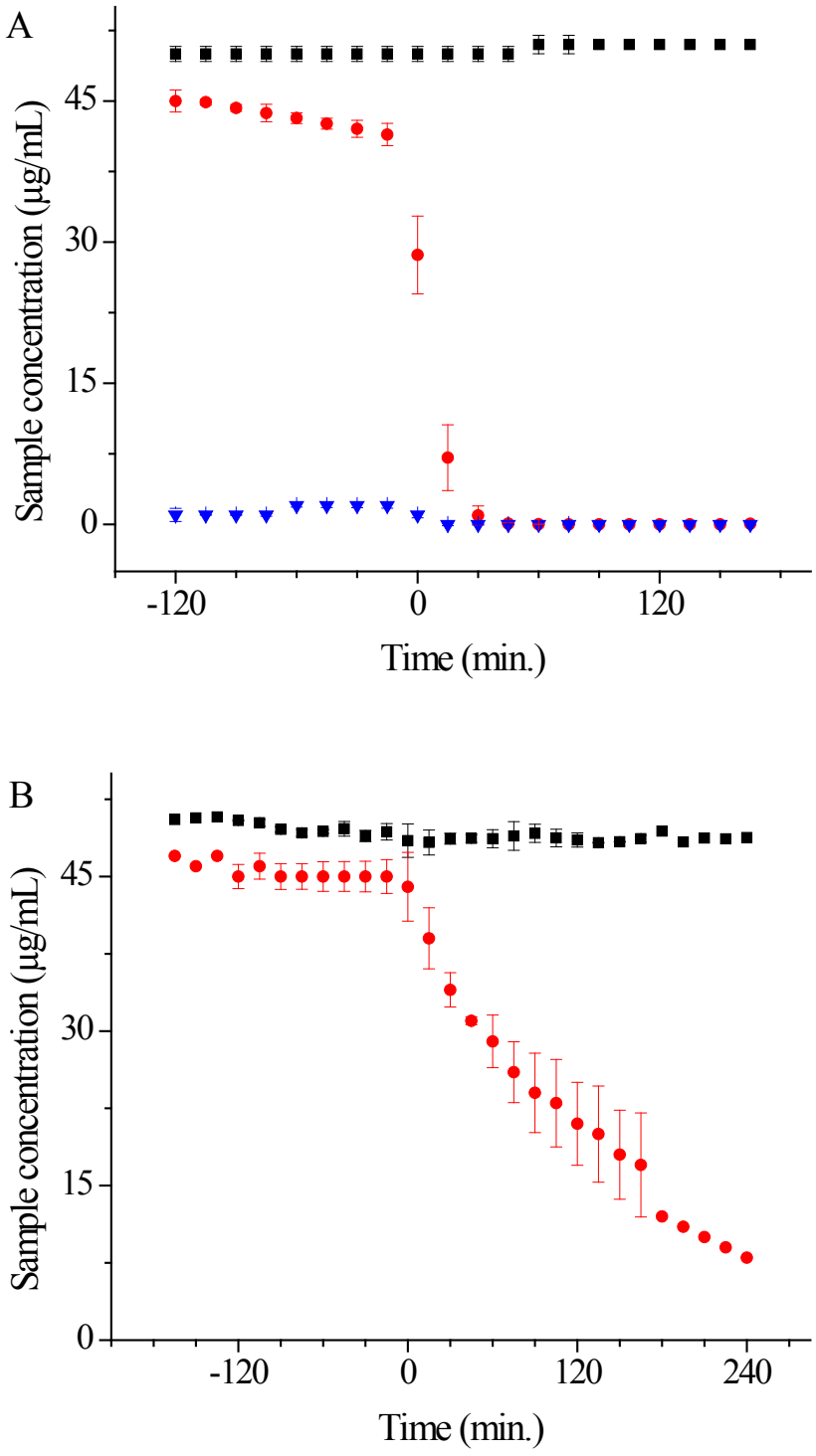

Figure 2.4: *In vitro* recovery experiment with the addition of A) female rat microsomes (n=3), and B) male microsomes (n=3) added at time 0. Concentration of antipyrine (■), cortisone(●), and cortisol (▼) in dialysate sample.

was observed in cortisone delivery in the *in vitro* delivery experiments, while a significant decrease in cortisone was observed in the *in vitro* recovery experiments, as shown in Figure 2.4b. As with female microsomes, no production of cortisol was observed. Interestingly, the rate of cortisone disappearance occurred at a much slower rate with the male rat microsomes than the female rat microsomes. This confirms the ability of microdialysis to be a viable technique for monitoring enzymatic processes in rat microsomes, since the rate of enzymatic activity was different between the two genders. However, the determination of a product similar to one produced in humans is essential in order to monitor 11β-HSD1 activity *in vivo* and be relevant to drug screening.

2.5.2.2 *In vivo* studies

To determine 11β-HSD activity *in vivo,* preliminary experiments were performed to monitor the conversion of cortisone to cortisol in rats. While no change in delivery was observed under hydrodynamic conditions (*in vitro* system) mimicking a well-perfused tissue, the experiments were performed in adipose tissue (*in vivo* system), which represents a poorly perfused tissue. In hopes that a change in delivery could be observed in tissue, preliminary tests were performed to monitor the 11β-HSD activity *in vivo* in rats. A microdialysis probe was then implanted in visceral, IP, and leg fat of Sprague-Dawley rats. In these experiments, cortisone was delivered with antipyrine to the tissues of interest, visceral and leg fat. Carbenoxolone was administered IP at time zero, and the effects were monitored in adipose tissue as shown in Figure 2.5a. Along with carbenoxolone, another inhibitor of 11β-HSD, 18α-glycyrrhetinic[4, 11], was administered IP and monitored by microdialysis in leg fat (Figure 2.5b). From these preliminary experiments, it was determined that neither inhibitor caused any reproducible or significant changes in the delivery of cortisone in either tissue. The only changes that were seen with

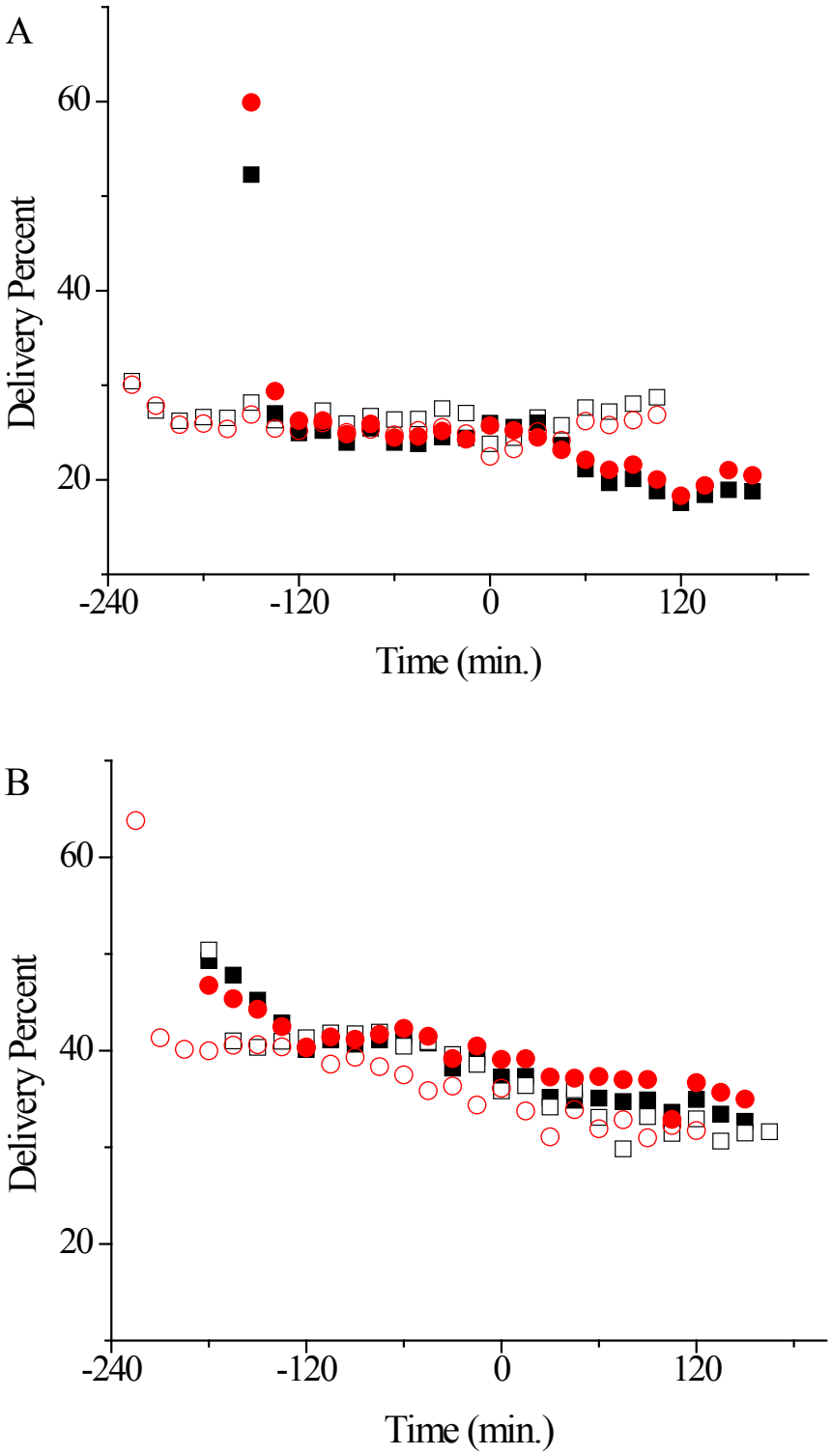

Figure 2.5: *In vivo* delivery experiment in A) IP fat and B) leg fat. Delivery percentage of antipyrine (■), and cortisone (●) in rat one (□) and rat two (○) with microdialysis sampling.

cortisone were also seen with antipyrine, demonstrating only an EE change and not a change in cortisone concentration. A change in delivery of cortisone was a possibility *in vivo*, since the removal of cortisone from the area surrounding the probe would be limited by the resistance of the tissue, thereby possibly allowing the enzyme kinetics to dominate instead of diffusion. However, Figure 2.5 shows that, the delivery percentage changes observed in cortisone was also seen in antipyrine, thus demonstrating that enzyme kinetics was not the predominant mechanism of transport in the tissue. In order to monitor 11β-HSD activity, at least one product must be seen. Therefore, further tests were necessary in order to obtain a viable system.

2.5.2.3 Cortisone product determination

To look for other products of cortisol metabolism, a less-selective wavelength of 210 nm was utilized to enable the detection of all organic compounds with a C=O bond, along with the original detection wavelength of 242 nm, which is selective for cortisone and cortisol. Chromatograms obtained at 210 nm and 242 nm are shown in Figure 2.6 and Figure 2.7, respectively. From these experiments, it was determined that no new products were detected at the 210 nm upon microsome addition that was not seen at 242 nm. In addition, due to the high background at 210 nm, the limits of detection were much worse at this wavelength.

After investigating the literature regarding other enzymatic processes reported for rats, the most common metabolites, as shown in Figure 2.8, were obtained and investigated. These metabolites were detected and identified using LC-MS (Figure 2.9). All of the metabolites were determined to have acceptable linearity in the concentration range of 10 ng ml^{-1} to 500 ng ml^{-1} with correlation efficiency above 0.99. The precision was better than 10% for all analytes, except for 6β-OHF and 6β-OHE with 20% (n=5 for each standard concentration). After the linearity and reproducibility of the metabolites were determined on the LC-MS/MS,

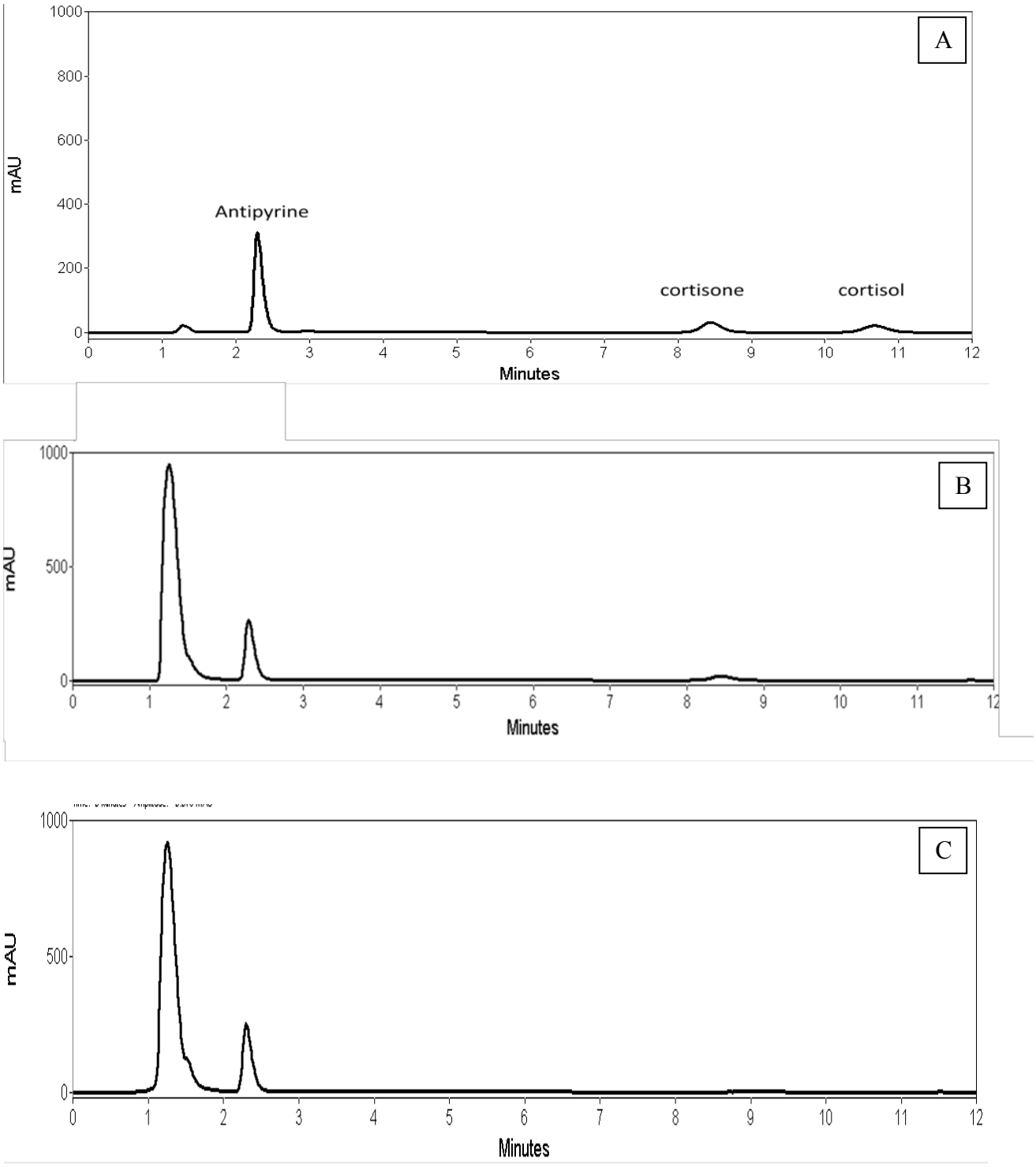

Figure 2.6: Typical chromatogram at 210 nm wavelength of antipyrine, cortisone and cortisol in a 50 µg mL^{-1} standard (A), microdialysate sample from an *in vitro* recovery experiment at 30 minutes before (B), and after microsome addition (C).

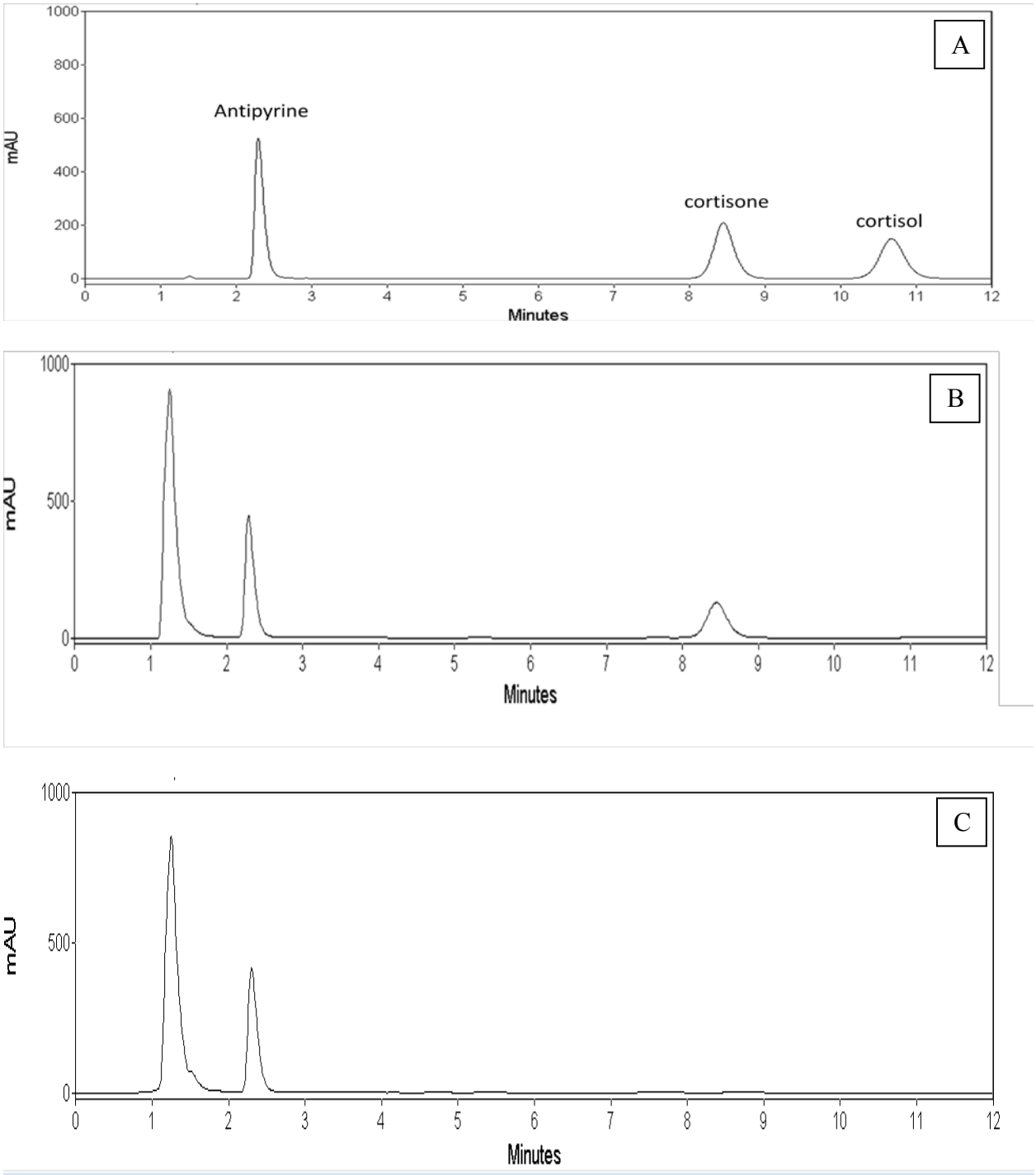

Figure 2.7: Typical chromatogram at 242 nm wavelength of antipyrine, cortisone and cortisol in a 50 μg mL^{-1} standard (A), microdialysate sample from an *in vitro* recovery experiment at 30 minutes before (B), and after microsome addition (C).

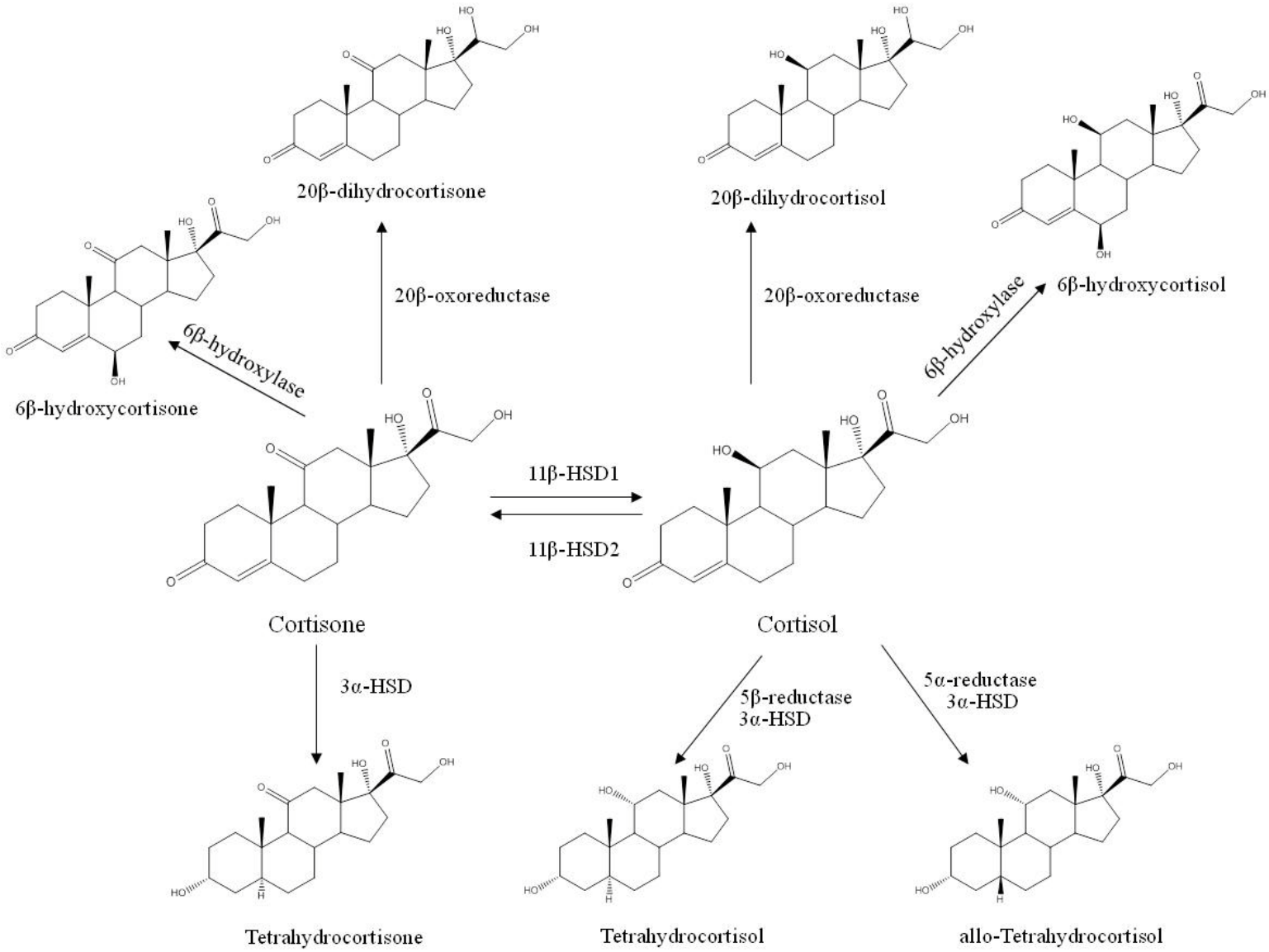

Figure 2.8: The metabolites associated with 11-βHSD1. Adapted from[23].

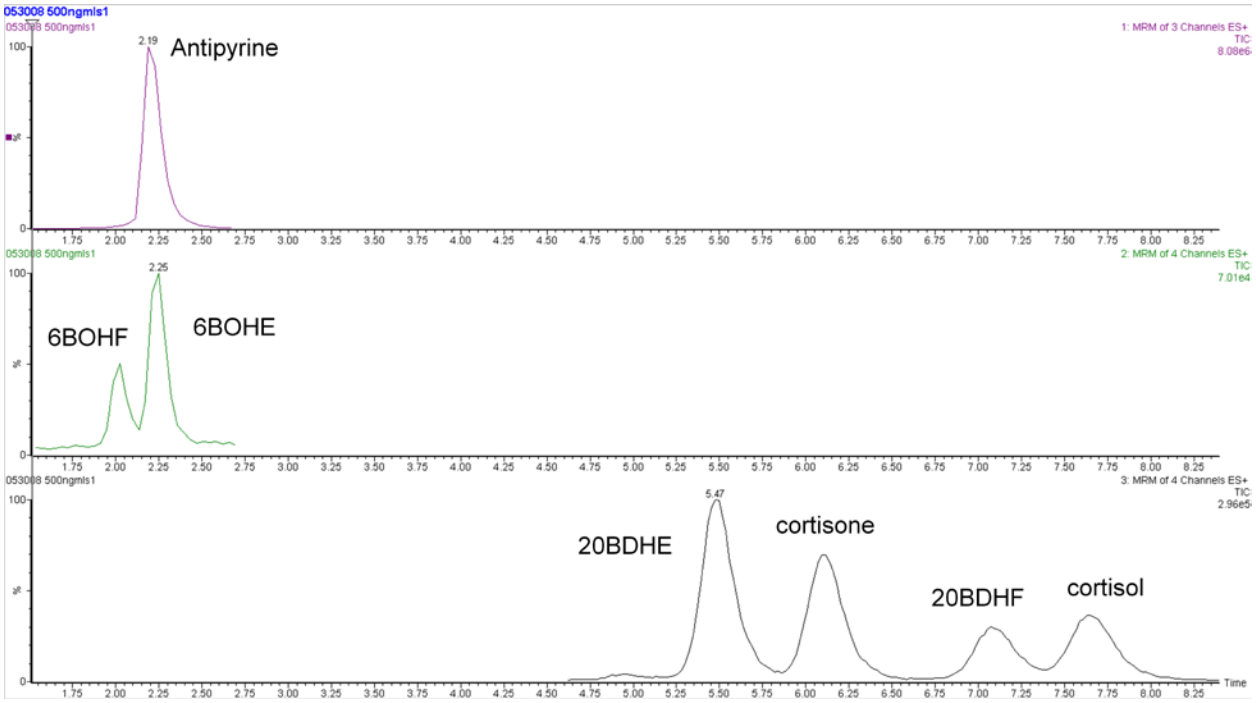

Figure 2.9: Common metabolites of 11-βHSD1detected with LC-MS/MS**.**

an *in vitro* recovery experiment was performed with female Sprague-Dawley rat microsomes, as determined in earlier experiments. None of the potential metabolites were detected, as seen in previous experiments. Before extensive metabolite profiling was performed for rats, the metabolite profile for humans was determined first. This is because a similar metabolite profile must be obtained between species in order for rats to be a viable animal model to test novel inhibitors for human pharmaceutical screening.

2.5.3 Human microsome *in vitro* studies

The activity rate of 11β-HSD1 was determined in an *in vitro* recovery experiment using human microsomes with and without carbenoxolone, shown in Figure 2.10. The concentration of cortisone decreased significantly immediately after microsome addition at time zero (Figure 2.10A). The decrease in cortisone concentration upon the addition of human liver microsomes was expected to result in an increase in a product, such as cortisol (Figure 2.10b). An increase in cortisol upon the disappearance of cortisone enabled 11β-HSD1 activity to be monitored with microdialysis sampling. While cortisol was expected in the previous studies in rat experiments, microdialysis sampling can examine the direct conversion of cortisone and cortisol determining the activity of 11β-HSD1 by the substrate and products instead of monitoring the activity indirectly, by markers such as NADPH[24]. In clinical studies, the ability to monitor the metabolite profile is critical to proceed with larger clinical trials. Direct sampling methods, such as microdialysis sampling, is a novel approach to directly monitor the enzyme kinetics. With this sampling method, the determination of 11β-HSD1 enzyme kinetics of a well-known inhibitor, carbenoxolone was determined in humans, which was not possible in rats. From these studies, it was determined that rats have a different metabolic profile. Now that a metabolite was

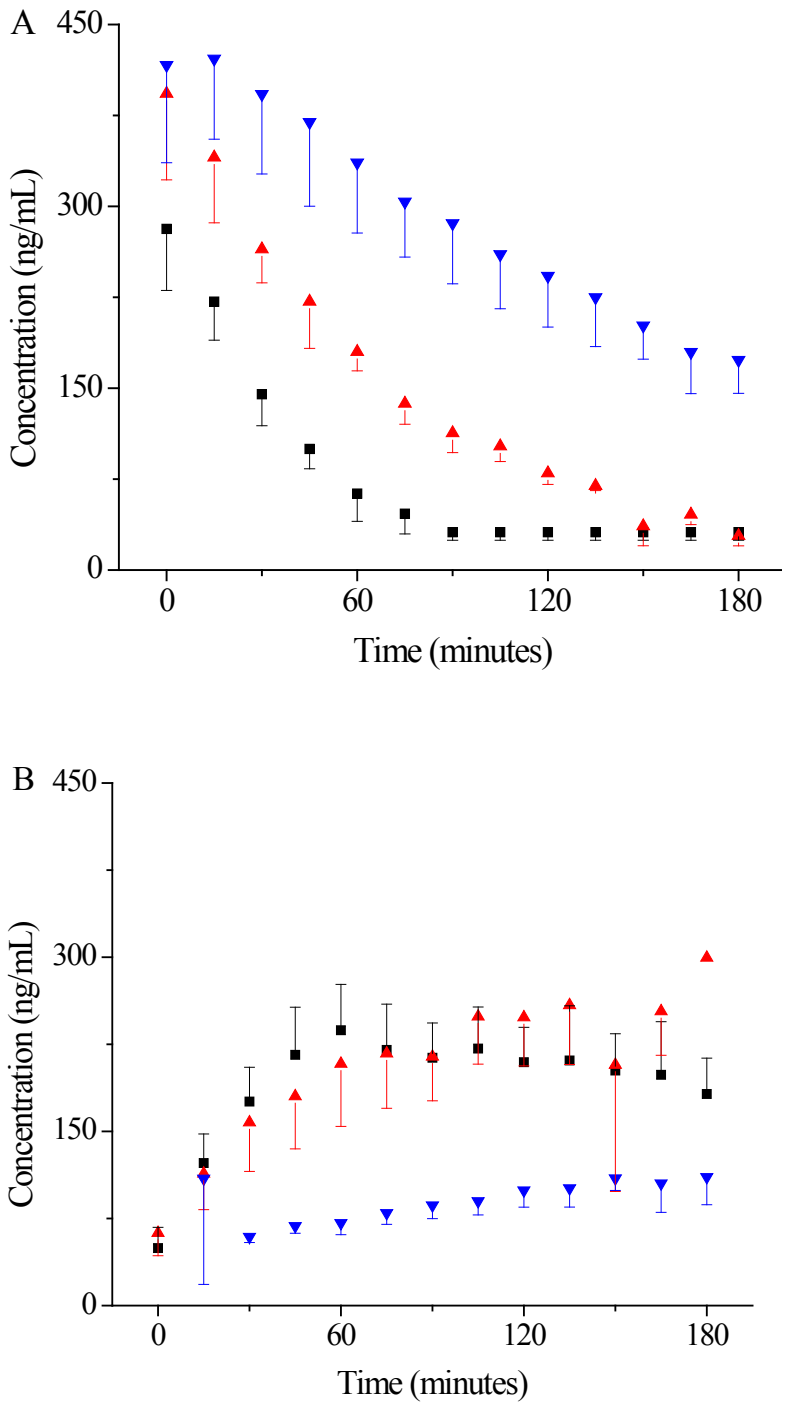

Figure 2.10: *In vitro* recovery experiment with human microsomes with different concentrations of inhibition. Effect of 500 ng ml-1 substrate cortisone (n=5), and no inhibition (■), 100 µg mL^{-1} (▲), and 500 µg mL^{-1} (▼) of carbenoxolone (n=3) added to human microsome buffer at zero minutes. This shows the disappearance of (A) cortisone and the appearance of (B) cortisol due to 11β-HSD1 enzyme activity.

identified in the human microsome studies, several concentrations of carbenoxolone were tested along with different concentrations of cortisone.

The results obtained from this experiment (Figure 2.11) match previous results collected regarding the inhibition kinetics of 11β-HSD1 in humans[9]. Both graphs clearly illustrate that carbenoxolone expressed competitive inhibition with the 11β-HSD1 enzyme. Competitive inhibition has been reported in literature in both humans and rats[9-10]. As shown in Figure 2.11A, a Lineweaver-Burk plot exhibits a straight line also demonstrating that this enzyme follows Michaelis-Menton, which agrees with other published literature of this enzyme in kidney and liver tissues[9, 25]. The second graph, a Dixon plot, as shown in Figure 2.11b, demonstrates the reproducibility of microdialysis sampling to monitor enzyme kinetics. These studies confirmed the ability to monitor 11β-HSD1 activity by microdialysis sampling *in vitro.*

The ability to monitor 11β-HSD1 activity by microdialysis *in vitro* was expected, since this technique was previously performed in human *in vivo* experiments first by Tomlinson *et al.* [1]. During these studies, another group performed *in vitro* microdialysis studies using isotope labeled cortisone and cortisol, and compared the metabolism in rats, dogs, and monkey microsomes[26]. This same group then performed further *in vivo* studies in monkeys since this species had a similar profile as humans[27]. Due to the expense and extensive requirements of utilizing monkeys, further *in vivo* testing could be performed in other species to find a smaller non-human species that matches the metabolic profile obtained in humans. Interestingly, Amgen did recently publish an article demonstrating that a primary metabolite was produced in human serum that was observed in rat serum after the administration of a novel inhibitor[28]. With the production of a similar metabolite in rats as that found in humans, further investigation of this metabolite could be monitored using microdialysis sampling to monitor the production of this

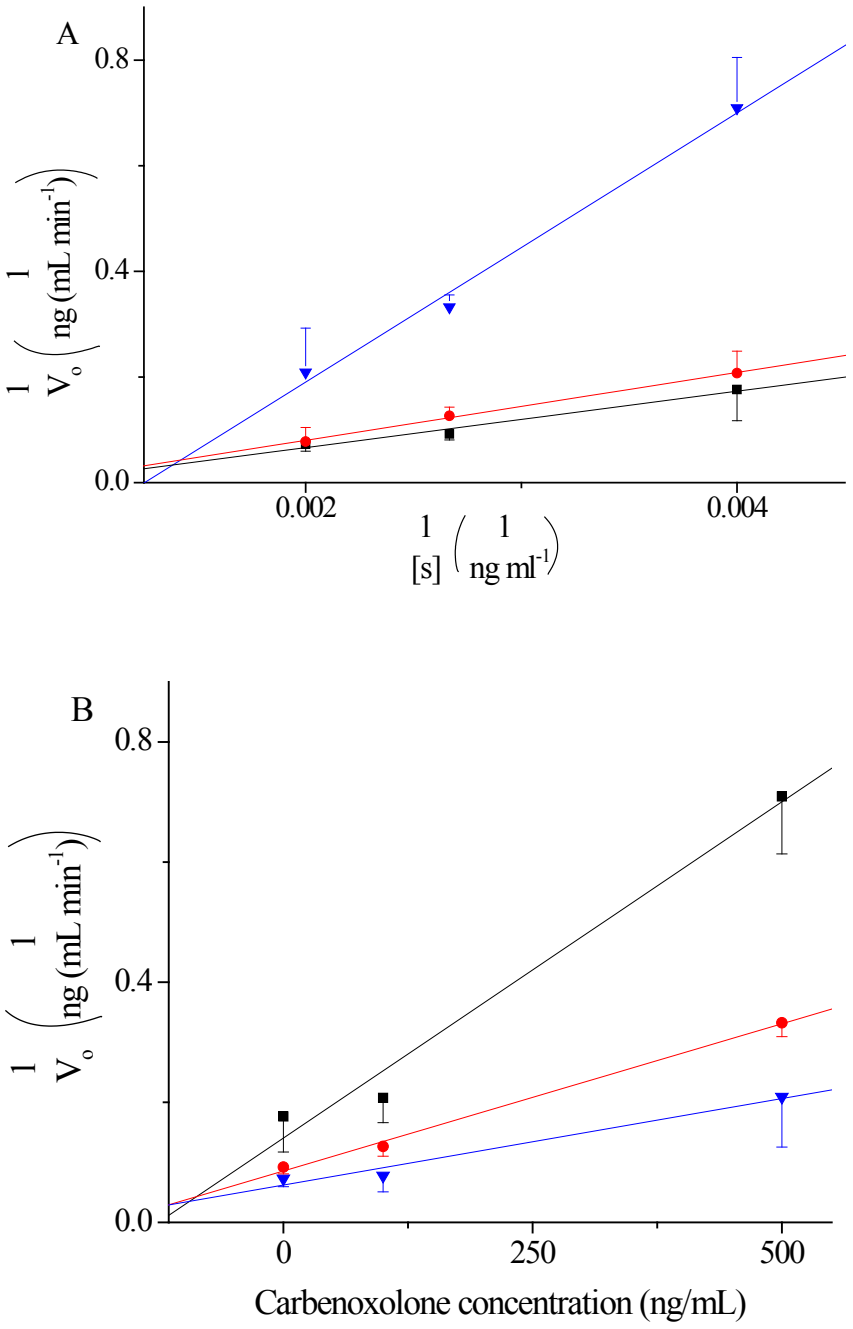

Figure 2.11: Graphs of *in vitro* recovery experiments with different concentrations of inhibition. Effect of carbenoxolone in human microsomes in (A) double-reciprocal plot with no inhibition(■), 100 ng mL^{-1} carbenoloxone (●), and 500 ng mL^{-1} carbenoxolone (▼), and (B) a Dixon plot with different cortisone concentrations of 250 ng mL^{-1} (■), 375 ng mL^{-1} (●) and 500 ng mL^{-1} (▼).

metabolite in the tissue of interest upon the administration this novel inhibitor. In the study performed by Amgen, the metabolite was only monitored in the plasma of the rat, which represents the whole-body metabolism of the novel drug. The ability to monitor the metabolism of this novel inhibitor directly in the adipose tissue would provide beneficial information regarding the activity of 11β-HSD1, instead of monitoring the whole-body activity.

2.6 Conclusions

Microdialysis sampling is a viable technique to monitor enzyme kinetics, as originally shown by Tomlinson in humans[1]. Several *in vivo* and *in vitro* experiments were performed in Sprague-Dawley rats and microsomes with no product observed of any significance. Interestingly, a kinetic difference in cortisone disappearance *in vitro* was observed between male and female Sprague-Dawley microsomes. Unfortunately, no product was observed as needed for *in vivo* use of the method. Therefore, human microsomes were tested *in vitro,* and the corresponding appearance of cortisol was observed.

The results of the microdialysis *in vitro* recovery experiment utilizing human female liver microsomes were as expected. Upon the disappearance of cortisone, the appearance of cortisol was observed utilizing the LC-UV method developed in human female liver microsomes. This illustrates that microdialysis is useful and a beneficial tool for monitoring 11βHSD1 enzyme activity in female human liver microsomes, along with measuring enzyme kinetics in the presence of the selected inhibitor. This was possible due to the production of a detectable metabolite observed in the buffer containing human female liver microsomes. This was not observed utilizing microdialysis *in vitro* with rat female liver microsomes or *in vivo* in rats with probes implanted in the visceral fat and leg muscle monitored by LC-UV. With microdialysis sampling it was determined that rats are not a good model for these studies. As shown by

Tomlinson in humans, microdialysis is a good technique for monitoring enzyme kinetics of this enzyme; however in order for this to be applied *in vivo* a smaller mammal would be ideal for preliminary testing to increase throughput of testing these novel inhibitors.

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~Chapter 3~

Monitoring cGMP by microdialysis in the submucosa of the colon upon agonism of GC-C in rat intestinal loops

3.1 Introduction

The development of a technique to monitor enzyme activity, such as guanylate cyclase-C (GC-C), in the submucosa tissue would provide a direct way to monitor the production of cGMP in the intestinal tract. An increase in cyclic guanosine- 3', 5' – monophosphate (cGMP) decreases the transient time of fecal material and reduces the symptoms of Irritable Bowel Syndrome (IBS) with chronic constipation (IBS-C) and IBS with chronic idiopathic constipation associated (IBS-CIC)[1]. A technique to sample directly from the submucosa would enable the development of novel drugs to treat diseases, such as IBS.

IBS diagnosis is based on the recurrence of abdominal pain or discomfort for more than three days per month that is associated with changes in bowel movement/frequency[2]. The movement of fecal material comprises the subclassifications of IBS, which consists of constipation, diarrhea, or the alternation between the two[3]. These conditions affects millions of people worldwide (i.e. 1 in 10), and typically causes abdominal pain causing a decrease in the quality of life (QOL)[2]. Patients typically seek treatment to eliminate the abdominal pain associated with these symptoms[2].

One approach to treat IBS is to increase the levels of the intracellular second messenger cGMP by activating the enzyme GC-C on the apical surface of intestinal epithelial cells[1, 4]. GC-C is activated by ligands such as endogenous peptide hormones (guanylin and uroguanylin), which then catalyze the production of intracellular cGMP[5]. Several physiological processes are regulated by cGMP through the intracellular interaction with three groups of target proteins, namely cGMP-dependent protein kinases, cyclic nucleotide-gated ion channels, and cGMP-

regulated phosphodiesterases[4]. Several studies have monitored the secretion of cGMP in the colon by a variety of methods, including *in vitro* studies, fluid collected from intestinal loops, or by associating the transient time of the fecal material to the secretion of cGMP[1].

3.1.1 Effects of GC-C agonists

While the GC-C activity in the colon is well characterized, the decrease in pain upon GC-C agonist administration is a new revelation[4]. These effects were noted by Ironwood Pharmaceuticals upon the administration of a novel GC-C agonist, linaclotide. This new drug elicited a reduction in abdominal pain in addition to relieving the constipation of the patients in a recent clinical trial[2, 4]. Other medications prescribed for pain, such as anti-depressants (tricyclic agents), typically exacerbate the constipation, minimizing the utilization of these medications[2]. Linaclotide, a 14-amino acid peptide, has been recently approved by the FDA, and is the first drug on the market to increase the quality of life (QOL) and treat the problem of IBS-C and IBS-CIC[2, 4].

The decrease in abdominal pain caused by linaclotide administration may be from the effects of cGMP on the decreased activity of pain-sensing fibers[6-8]. The effects on neurotransmitter release is possibly due to the efflux of intracellular cGMP from the epithelial cells to the submucosa[4]. Once cGMP is in the extracellular space, it is postulated that the colonic nociceptors are inhibited, thus reducing pain (Figure 3.1)[4]. Due to these findings, animal studies were performed to confirm that GC-C agonists or direct cGMP administration reduced pain. These studies correlated the presence of cGMP on pain tolerance, now additional studies are necessary to elucidate the mechanism of interest. In animal studies, a reduced sensitivity to pain in stress-induced and chemical-induced models was found with cGMP administration[4].

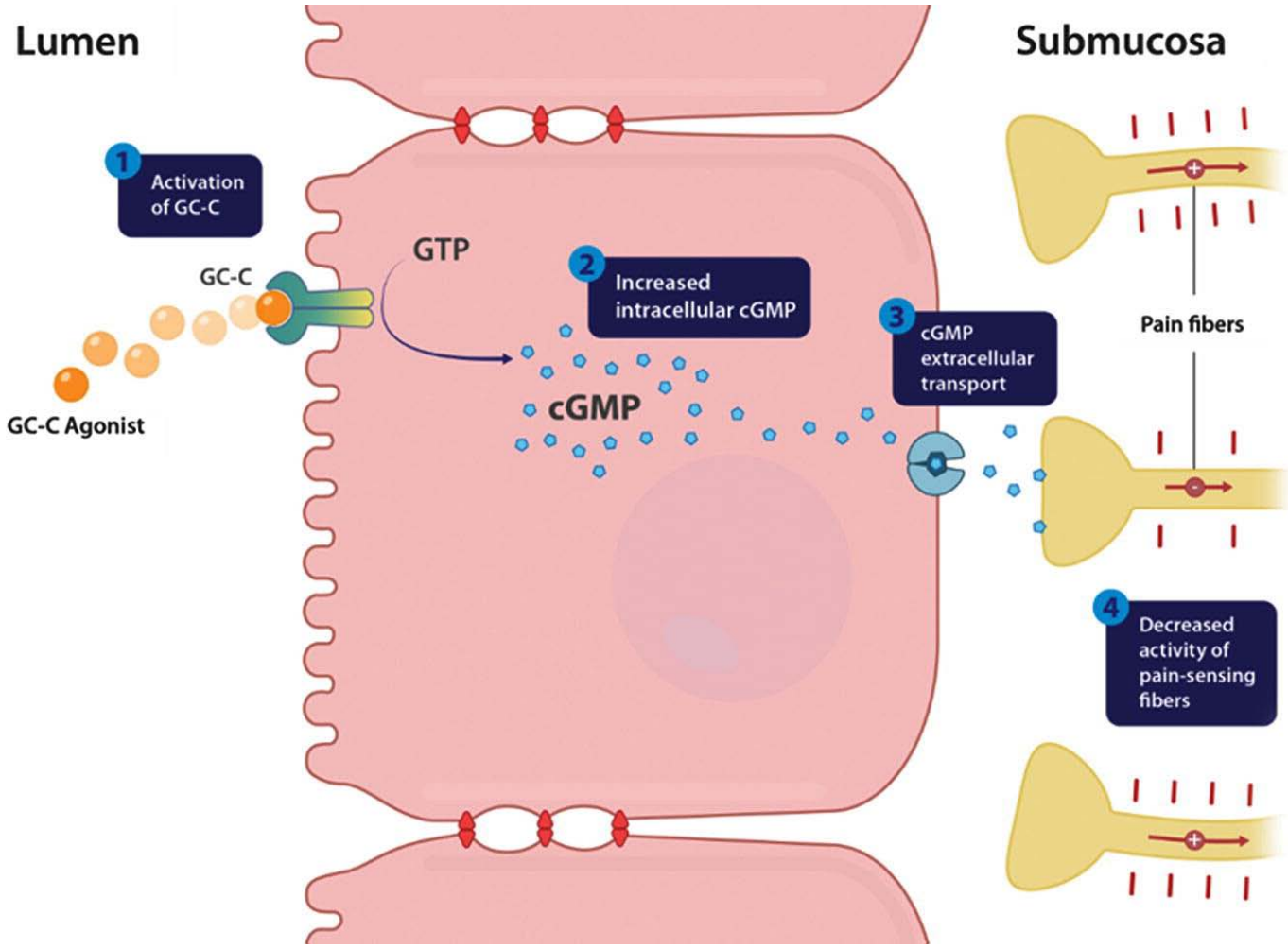

Figure 3.1: Proposed mechanism of pain reduction by action of GC-C agonism through the production of cGMP. Reprinted with permission of [4].

A technique to sample continuously in the submucosa to monitor the biological effects of novel therapeutic drugs or events associated with IBS would be highly beneficial. In order to monitor the levels of cGMP in the submucosa layer *in vivo*, a reliable sampling/screening technique is needed. An attempt was made to homogenize the tissue from the submucosa in order to determine the cGMP levels in this region; however, due to this layer being so thin, this was not possible without the contamination with cells from the mucosa, as shown in the literature[9]. In order to eliminate tissue collection, another experiment was performed by Cibicek *et. al.*. They implanted a microdialysis probe in the submucosa of the descending colon in male Wistar rats[10]. These experiments tested the permeability of trace compound ^{51}Cr-EDTA before, during, and after the perfusion of ethanol to the lumen of the colon to monitor the change of mucosa permeability[10]. The exact location of the microdialysis probe was confirmed in the control experiments, but was not discussed regarding the ethanol perfusion experiments for the chemical induction of IBS.

One goal of this research was to successfully implant a microdialysis probe in the submucosa of the colon and sample cGMP while the integrity of the colon was maintained throughout the experiment. Therefore, the implantation of the entire microdialysis probe in the submucosa was critical to obtain valid results. A former graduate student in our laboratory optimized this implantation in several layers (lumen, mucosa, and submucosa) of the stomach[11]. As reported by Woo, the thickness of the stomach submucosa layer is 500 μm ± 420, and the mucosa layer is 690 μm ± 280[11]. The implantation of the microdialysis probe was first attempted in this region of the digestive tract since these layers in the stomach are thicker than the layers in the ascending colon (approximately 60 μm).

3.1.2 Specific Aims of Research

The purpose of this research was to implement microdialysis sampling by the implantation of the probe in the submucosa to monitor cGMP release before and after the administration of STcore, a potent GC-C agonist derived from E. coli that is similar in structure to guanylin and uroguanylin. The development and implementation of this sampling technique will help elucidate the roles of cGMP released in the submucosa in causing other peripheral events, such as a change in neurotransmission. In these experiments, the reproducibility and tissue damage associated with microdialysis probe implantation was determined.

3.2 Chemicals and reagents

3-Isobutyl-1-methylxanthine (IBMX) and cGMP sodium salt were obtained from Sigma Aldrich (St. Louis, MO, USA). STcore was purchased from American Peptide Company (Sunnyvale, CA, USA). Linaclotide was purchased from Polypeptide Group (Torrance, CA, USA). Buprenorphrine hydrochloride (Reckitt Benekiser Pharmaceuticals INC.; Richmond, VA), and Webcryl 3/0 sterile violet braided coated polyglycolic acid synthetic absorbable suture were purchased from Patterson Logistics (Webster veterinary; Kansas City, MO, USA). Medical oxygen (99.8%) was purchased from Matheson Tri-gas (Basking Ridge, NJ). All other chemicals/solutions were as described in section 2.2 in Chapter 2.

3.2.1 Microdialysis methods

3.2.1.1 Microdialysis probe fabrication

Linear microdialysis probes were constructed as described in section 2.3.1, with the exception that a microdialysis membrane window of 5 mm was used.

3.2.2 In vitro recovery experimental system

An *in vitro* recovery experiment was performed to monitor cGMP behavior with a linear microdialysis probe. A solution of cGMP (2.4 μM) in saline was continuously stirred and kept in a warm bath (37°C). A linear microdialysis probe with a 5 mm PAN membrane window was perfused with saline. Three to five samples were collected at the flow rates of 0.5, 1, and 2 μL min^{-1}.

3.2.3 In vivo experimental system

3.2.3.1 Animal preparation

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) animal protocols. Female Sprague-Dawley rats (190 to 250 g) (Harlan Laboratories, Inc.; Indianapolis, IN, USA) were used for all the experiments unless stated otherwise. Initially, all animals were pre-anesthetized with isoflurane followed by an intraperitoneal (i.p.) injection of the anesthetic cocktail of ketamine, acepromazine, and xylazine (40 mg kg^{-1}, 1 mg kg^{-1}, and 5 mg kg^{-1}). Buprenorphrine (0.1 mg mg kg^{-1}) and saline was then administered subcutaneously. Due to deaths from anesthesia overdose, later experiments (microdialysis probes implanted in the lumen and submucosa) only utilized isoflurane for anesthesia of the animals. Eye ointment was applied to the eyes to prevent them from drying out during the experiment. The abdomen of the rat was then shaved as close as possible. Isoflurane via delivery with 99.8% medical oxygen was used as the supplemental anesthesia for the duration of the experiment. An oval hole was then cut in the middle of a three inch square piece of the following items on top of one another: surgical drape, gauze and a soft absorbent wipe. The drape was then placed on top of the animal with the other two on top of it. The gauze and

absorbent wipe, including any exposed tissue, were kept moist throughout the surgery and experiment by the application of saline.

3.2.3.2 Animal surgery

The implantation technique was optimized extensively to minimize colon handling to prevent tissue damage or a change in the tissue physiology. The optimized colon ligation technique was based on the technique utilized at Ironwood Pharmaceuticals, with a few modifications for the implantation of the microdialysis probe in the submucosa and lumen of the ascending colon. With the rat lying on its back, an incision approximately two centimeters long was made along the midline of the abdomen, located approximately one centimeter posterior from the urethra (Figure 3.2A). The cecum and ascending colon were located and exposed onto the moist wipe. A ligation loop, using 3/0 suture, was placed proximal to the cecum around the ascending colon (Figure 3.2B). The suture for the ligation loop was inserted with forceps between the blood vessels and the ascending colon. Once the suture was around the colon, it was loosely looped. Another ligation, performed in the same manner as the first one, was placed 2 cm distal from the first one, and loosely tied around the colon. Bulk fecal material was then removed by administration of 4.5 mL of saline (Figure 3.2C). The colon was allowed to naturally move the lumen contents (including the saline) from the area of interest. After a few minutes, a bulldog clamp was then applied proximal to the cecum prior to administration of an additional 0.5 mL of saline. Once the saline was injected into the area, the other bulldog clamp was applied to the colon approximately 3 cm away from the first clamp (Figure 3.3A).

The implantation of the microdialysis probe was performed by the following procedures. Magnifying glasses were utilized to aid in the visualization of blood vessels and to keep the

needle tip at a consistent level in the colon tissue to achieve the microdialysis probe implantation in the submucosa layer. In the colon of male Sprague-Dawley rats, the submucosa layer is approximately 120 μm thick, making precise implantation a necessity[12]. The antimesentric side of the colon was placed on top in a straight position. Saline was applied topically to the colon immediately prior to the insertion of the microdialysis guide in the submucosa, using a 25 gauge 1.5" long needle (Figure 3.3B). If the needle placement was satisfactory, the microdialysis probe was threaded into the needle tip until polyimide was seen at the hub of the needle. Once the polyimide was through the needle, the needle was removed from the colon. The microdialysis probe was placed in the colon tissue accordingly. Next, the lumen guide needle was inserted into the colon (Figure 3.3C). Tissue glue was applied to each end of the polyimide at the entrance and exit site on the serosa of the colon. Ideally, 1 cm or more of tissue was above the needle to ensure that the entire dialysis membrane was in the colon, and to prevent any glue being on the membrane window. Prior to the application of the glue, a q-tip applicator was used to dry the tissue at the entrance and exit site in order for the tissue glue to adhere securely to the serosa. Tissue glue was then employed on the entrance and exit for each microdialysis probe (Figure 3.4A). Bulldog clamps were then removed, and the colon contents were cleared by peristaltic movement. A few minutes later, the ligation sutures were tightly tied approximately 2 cm apart (Figure 3.4B). Once the sutures were ligated securely around the colon, all tissues were put back into the abdomen except the ligated portion of the colon.

In the preliminary experiments, the colon was massaged several times to remove the fecal material. In addition, the bulldog clamps were left on, since ligation proximal to the cecum would be loosely tied around the colon, until the injection of STcore (a 14-amino acid custom peptide made for Ironwood Pharmaceuticals) or saline (the vehicle). The colon was then covered

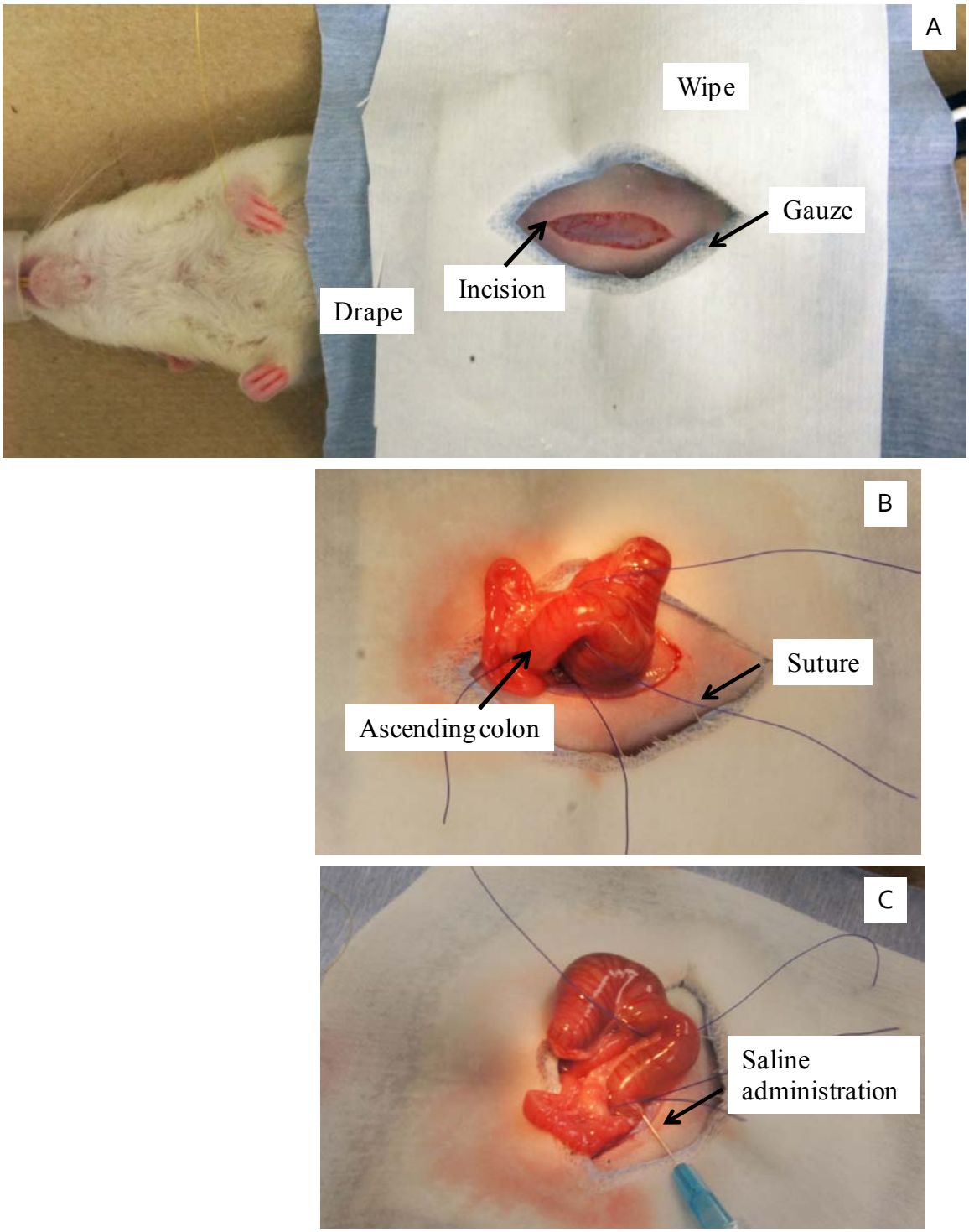

Figure 3.2: Abdomen incision and colon preparation for the implantation of microdialysis probes. A) Incision of abdomen, B) suture placement around ascending colon, and C) administration of saline in the ascending colon.

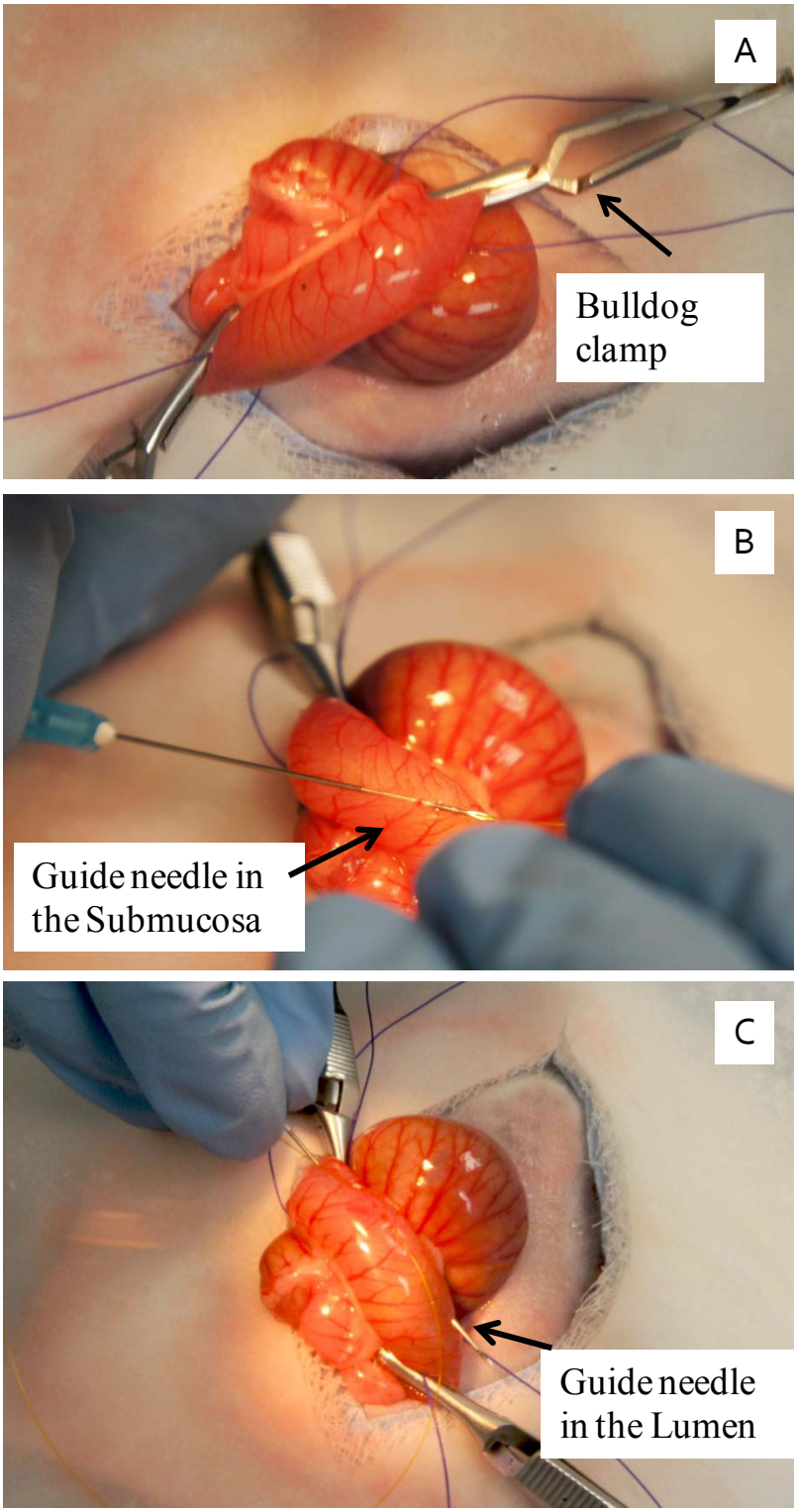

Figure 3.3: Microdialysis implantation. A) Bulldog clamps locations, B) guide needle implantation for the submucosa probe, and C) guide needle implantation for the lumen probe.

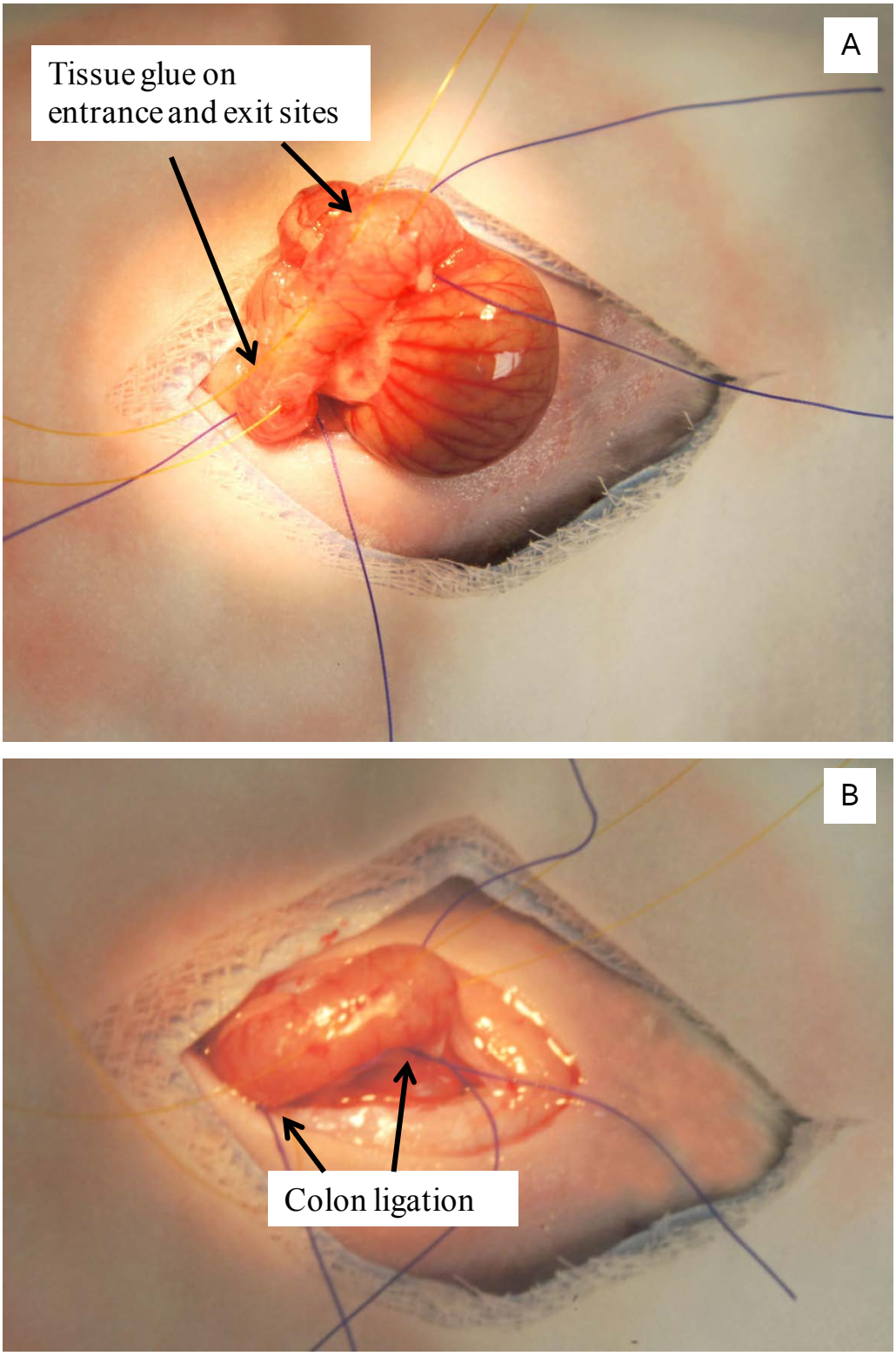

Figure 3.4: Experiment preparation. A) Tissue glue application on the entrance and exit sites of the colon to prevent probe movement, and B) colon ligation with only the ligated portion of the colon exposed throughout experiment.

with gauze surrounding the colon until solution administration. The gauze was kept moist by continually applying saline throughout the experiment.

3.2.3.3 Compound administration

For the administration of STcore or the vehicle, the colon would be uncovered, and 200 µL of the appropriate dosing solution was administered into the ligated colon. A 30-gauge needle was inserted between the bulldog clamp and the suture proximal to the cecum. The needle was inserted only 4 mm and the solution was carefully dispensed into the colon. The ligation proximal to the cecum was then tied after squeezing the section between the suture and bulldog clamps to ensure no solution was in this portion of the colon. Once the ligation was secured tightly, the colon was covered with gauze for the remainder of the experiment.

For the final experiments (microdialysis probes implanted in both the lumen and submucosa), STcore was administered directly into the ligation loop three minutes before sample three collection. A 30 gauge needle was inserted directly between the two ligation sutures.

3.2.3.4 Microdialysis sampling parameters

A Hamilton syringe (1 mL) was filled with saline with the flow rate set at 1 µL min^{-1} using a BAS bee hive syringe pump. A linear microdialysis probe, with a 5 mm PAN membrane window, was attached to a Hamilton syringe (1 mL) prior to the implantation of the microdialysis probe. Sample collection was started a minimum of 20 minutes after probe implantation.

In the preliminary experiments, one basal sample was collected for 40 minutes, and then the rest of the samples were collected in 30 minute intervals with 3-Isobutyl-1-methylxanthine (IBMX) put in the vial prior to sample collection (to give a final concentration of 1 mM in each vial) unless stated otherwise. IBMX was put in the vials to increase cGMP stability in the samples. At the end of the experiments, 1000 ng ml^{-1} of cGMP was delivered through the microdialysis probe to determine the extraction efficiency of the microdialysis membrane. This sample was collected for 15 minutes after a 5 minute equilibration period.

In final experiments (microdialysis probes implanted in the lumen and submucosa), samples were collected in 15 minute intervals. Two basal samples were collected, and then four samples after the administration of the compound. STcore administration occurred three minutes before the collection of sample three. IBMX was put in all vials and cGMP was delivered at the end of the experiments, as described above.

3.2.4 Sample analysis

3.2.4.1 HPLC-MS (Ion trap) detection of cGMP

Microdialysis samples and standards were analyzed by liquid chromatography with mass-spectrometry detection (LC-ion trap). The liquid chromatography system consisted of two Shimadzu LC-20ADxr pumps and a Shimadzu CBM-20A system controller (Columbia, MD, USA). Sample injections of 5 µL were made with a Rheodyne model 7125i manual injector installed with a 10 µL sample loop. Separation of cGMP was achieved on a Phenomenex Kinetex XB-C18 (100 mm x 2.1 mm, 1.7 µm particle) column. Solvent A was water with 0.1% formic acid and solvent B was acetonitrile/water (95/5 v/v) with 0.1% formic acid. The flow rate was kept at 0.2 mL min^{-1} with 98% solvent A and 2% solvent B throughout the entire run.

Analysis was performed on a ThermoScientific LTQ XL ion trap monitoring cGMP at 346.1 mass to charge ratio (*m/z*) with multiple reaction monitoring (MRM) of daughter ion 152 *m/z* between 2 and 5 minutes. The system was set to divert the flow of the liquid chromatography to waste before two minutes and after 5 minutes.

3.2.4.2 HPLC-MS (triple-quad) detection of cGMP

All microdialysis samples were kept at -20°C. The samples were then shipped overnight on dry ice to Ironwood Pharmaceuticals for analysis. These microdialysis samples were analyzed by a Waters Acquity AB/Sciex 5500 at Ironwood Pharmaceuticals. A volume of 10 µL was injected onto a Hypersil Gold (C18), 2.1 x 50 mm, 3 µm particle column at 25^{o}C with an Acquity autosampler set at 6°C. The flow rate was set at 0.4 mL min^{-1}. Solvent A consisted of 0.1% formic acid in water with Solvent B 0.1% formic acid (v/v) in 95/5 acetonitrile/water. The gradient was set at the following:

Time (min)	**% A**	**% B**
0	100	0
1.0	100	0
1.2	80	20
1.8	80	20
2.5	5	95
2.75	5	95
3.0	100	0

Ionization was performed with positive mode electrospray with the source temperature set at 550°C. The transition of 346.1 *m/z* to 152 *m/z* was monitored by MRM. cGMP standards were prepared in saline at concentrations ranging from 0.1 to 25 ng mL^{-1}. A volume of 10 uL of standard or microdialysate sample was added to a 96-well plate. A volume of 2 uL of 50 ng

mL^{-1} of $^{13}C_2$,^{15}N-cGMP was then pipetted into each well as an isotopic analytical standard (349.1 *m/z* to 155 *m/z*).

3.3 Results and discussion

3.3.1 Method validation results

Analysis of cGMP was initially evaluated using an ion trap system in our laboratory. Using this method, the linearity of cGMP was excellent between 9 ng mL^{-1} and 880 ng mL^{-1} with a correlation coefficient of 0.9997. The limit of detection (LOD) (S/N≥ 3) was determined to be 3.7 ng mL^{-1} and the limit of quantitation (LOQ) (S/N≥10) was 9.2 ng mL^{-1}. After the validation of cGMP analysis on the LC-ion trap system, microdialysate samples obtained from a male Sprague-Dawley rat were analyzed using this method, as shown in Figure 3.5. Unfortunately, the basal levels for both samples were below the LOD, thus the analysis of cGMP was not feasible on this system. Therefore, all further samples were analyzed at Ironwood Pharmaceuticals by LC-MS/MS. The LC-MS/MS instrument was optimized and tested for cGMP detection with linearity in the range of 0.1 to 25 and a LOQ of 0.1 ng mL^{-1}.

3.3.2 In vitro extraction efficiency determination

As with every compound, cGMP was first tested *in vitro* to determine its extraction efficiency with a microdialysis probe. In this experiment, the extraction efficiency was tested at different flow rates. As shown in Figure 3.6, the relative extraction efficiency of cGMP decreased with increasing flow rates, as expected. At a slow flow rate, such as 0.5 µL min^{-1}, cGMP had more time to equilibrate with the fluid inside the lumen of the microdialysis probe. Thus a higher relative extraction efficiency of 85% ±11% was achieved. Whereas at 2 µL min^{-1}, cGMP did not have as much time to equilibrate with the saline inside of the microdialysis probe,

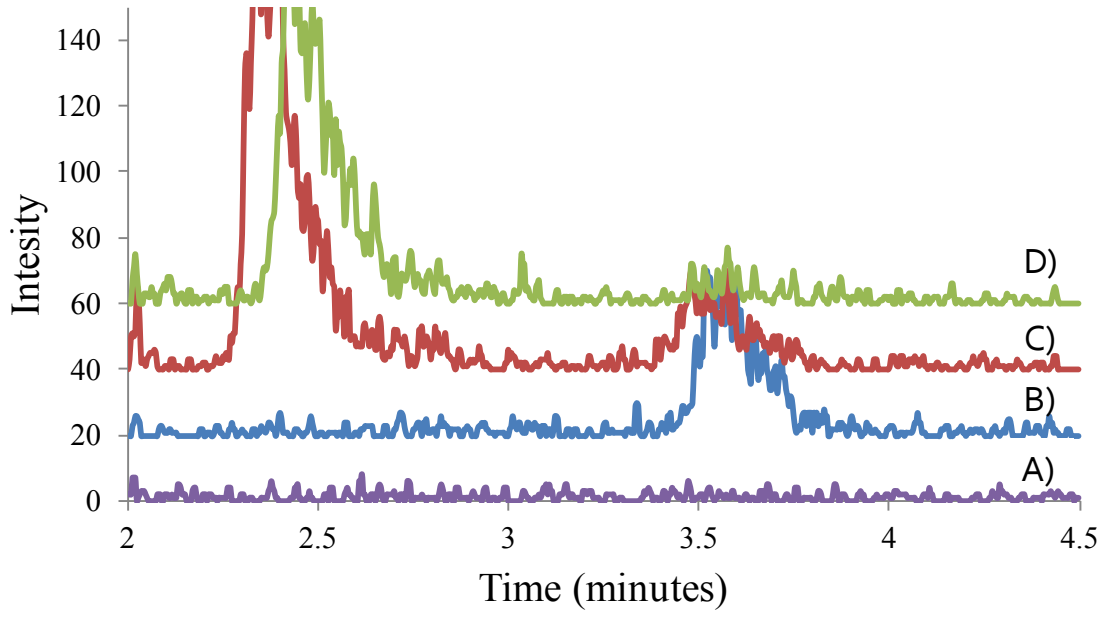

Figure 3.5: LC-MS chromatograms of cGMP in A) saline, B) 3.7 ng mL^{-1}, C&D) colon dialysate samples.

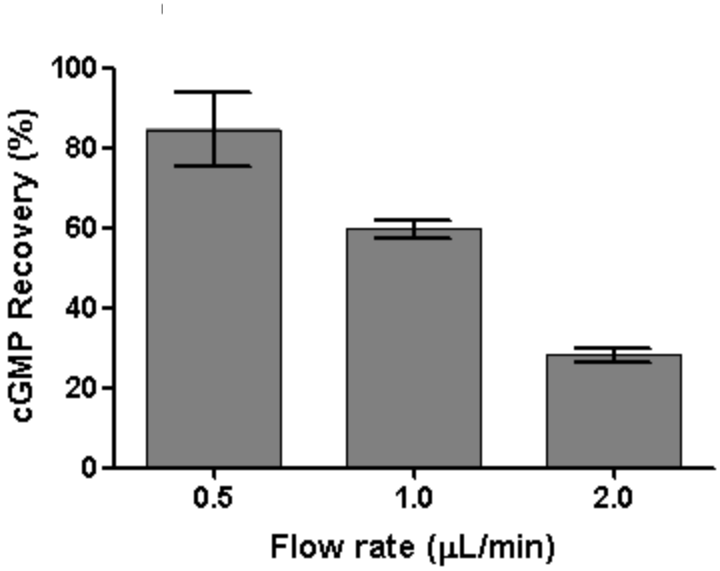

Figure 3.6: *In vitro* extraction efficiency of cGMP.

resulting in a lower relative extraction efficiency of 28% ±2%. Due to the good extraction efficiency of 60%±4% obtained at 1 μL min^{-1}, this flow rate was used for all further experiments.

3.3.3 Implantation of microdialysis probes in the submucosa

In vivo experiments were performed to determine the feasibility of implanting the microdialysis probe in the ascending colon. Initially implantation trials were conducted on both male and female rats. Interestingly, it was observed that the submucosa was significantly thicker in female rats (Figure 3.7) relative to male rats (Figure 3.8). This resulted in the implantation being more reproducible in female rats. For this reason, and because female rats had been used in previous studies, all further experiments of GC-C agonists were performed in female rats. In addition, during these initial experiments it was also established that the basal levels of cGMP seemed to vary with age (Figure 3.9). To minimize cGMP variability, all rats were utilized within two weeks after their arrival.

3.3.4 Administration of STcore in vivo

Preliminary *in vivo* studies were then performed to determine the concentration of cGMP in the submucosa of the ascending colon upon activation of GC-C by STcore. Upon STcore administration between 0.5 to 5 μg, a significant increase in cGMP (compared to the vehicle) was seen in these experiments as determined by unequal variance t-test (Figure 3.10). Interestingly, cGMP concentrations exhibited two distinct populations (low versus high cGMP concentrations) existed in the majority of groups administered STcore, as seen in Figure 3.10.

To determine possible factors in the split response, the histology of colon tissue collected from these rats was critically examined to determine if a correlation existed between the probe location and the cGMP response. The histology results with the location of the microdialysis

Figure 3.7: Submucosa of female Sprague-Dawley rat A) whole colon (2x magnification), and B) 10x magnification of colon with a) intestinal crypts, b) microdialysis probe in submucosa, c) muscularis externa.

Figure 3.8: Submucosa of male Sprague-Dawley rat A) whole colon (2x magnification), B) 100x magnification of colon with a) intestinal crypts, b) microdialysis probe in submucosa, c) muscularis externa.

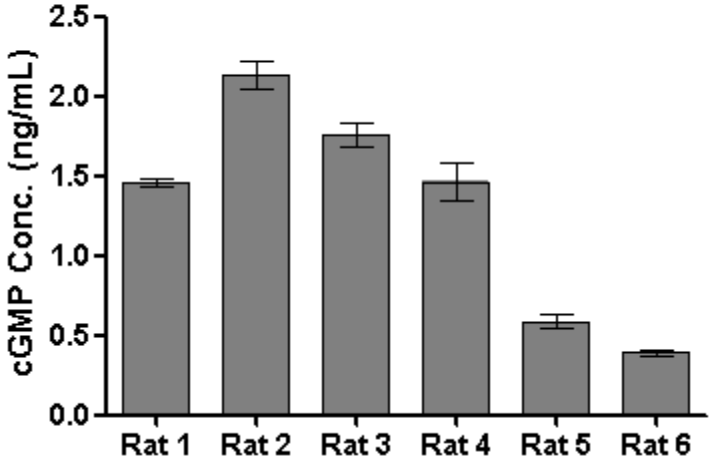

Figure 3.9: Basal concentrations of cGMP in dialysate of different rats (age in weeks, F:Female and M: male)

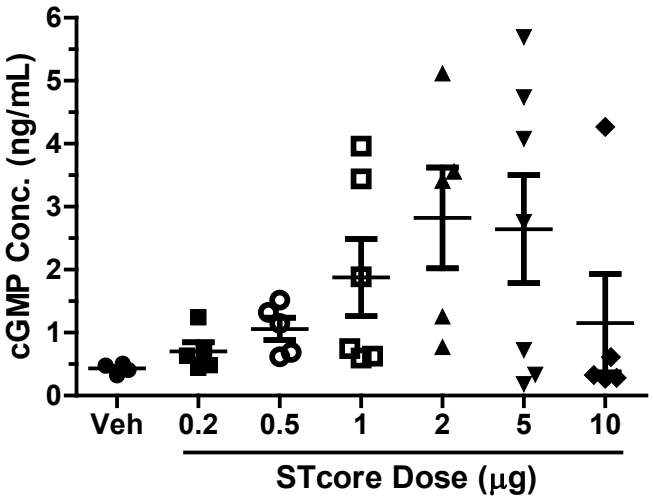

Figure 3.10: Dose response of cGMP in microdialysate sample implanted in submucosa of colon in preliminary experiments upon STcore administration. Each point represents the cGMP levels of pooled samples from an animal after STcore administration of 0.2 μg (■), 0.5 μg (○), 1 μg (□), 2 μg (▲), 5 μg (▼), 10 μg (◆), and sham (●).

A)

STcore administration (ug)

(cGMP concentration (ng mL^{-1}))

Vehicle	0.2	0.5	1	2	5	10
0.48	0.482	1.52	1.89	0.773	4.08	0.326
0.41	1.24	1.15	0.749	3.41	5.69	0.28
0.51	0.445	0.69	0.625	1.26	4.74	4.27
0.322	0.717	0.616	3.44	5.12	2.76	0.612
	0.636	1.32	0.596	3.56	0.334	0.269
			3.96		0.181	
					0.722	

B)

STcore administration (ug)

(Rat ID key)

Vehicle	0.2	0.5	1	2	5	10
7	31	36	42	20	8	26
9	32	38	43	21	12	27
10	33	39	44	22	13	28
18	34	40	45	23	14	29
	35	41	46	24	15	30
			47		16	
					17	

C)

Rat	Basal	ST
42	0.465	1.89
43	0.375	0.749
44	0.426	0.625
45	0.849	3.44
46	0.462	0.596
47	0.771	3.96

Table 3.1: cGMP concentration in dialysate (ng mL^{-1}). Samples taken from each rat after the administration of STcore (A), along with the rat ID key shown below (B). High cGMP concentrations are highlighted in blue. Probe location determined by the histology evaluated by Dr. Thompson at LMH by color coding of the rat ID. Comparison of cGMP concentrations in dialysate samples from the colon of the rat before (basal) and after STcore administration of 1µg (C).

Legend for table B:

Submucosa, Submucosa and Lumen, and Lumen

probe did not correspond to the concentrations of cGMP (Table 3.1). Some rats, such as 8, 24, 32, and 47 were found to have the microdialysis probe implanted in the submucosa of the colon, and the cGMP levels were extremely high upon the administration of STcore. Whereas some rats, such 13, 23, and 45, were found to have the microdialysis probe implanted in the lumen of the colon, and also had high levels of cGMP. This split in cGMP response lead to further investigations to determine if the response was from the administration of STcore or from experimental parameters (i.e. location of the microdialysis probe).

One advantage of microdialysis is the ability to compare the levels of cGMP throughout the experiment. As shown in Table 3.1, in rat 45 the probe was reported to be in the lumen, while in rat 47 the probe was reported in the submucosa; however the same cGMP levels were reported in the basal and post STcore administration samples for both rats. Due to the similar levels of cGMP, histology used to determine the location of the microdialysis probes was questioned. This apparent discrepancy in the histology results could be from the movement of the microdialysis probe after the animal was euthanized. Therefore several dyes were obtained that could be perfused through the probe immediately after the experiment. Unfortunately, no dye was found that would diffuse through the microdialysis probe into the tissue. In addition to the evaluation of the dyes, to minimize colon handling and tissue damage, the implantation procedure was modified.

Since the split response was still seen after surgical modifications, more experiments were performed. In the final experiments, microdialysis probes were implanted in both the lumen and submucosa to determine if the higher levels of cGMP were from the location of the probe, or a true response from the administration of STcore. Upon the implantation of both probes, only one rat, number 66, had a high level of cGMP after the administration of 5µg

STcore in the "submucosa" probe. Interestingly, the cGMP levels measured in the lumen microdialysis probe in this rat were consistent with the "submucosa" probe (Table 3.2), indicating that the probe location was most likely in a different layer than the submucosa, such as the mucosa or lumen. If the cGMP levels correlate between the two probes, this allows the elimination of these experiments from the dose response, thereby minimizing cGMP variance from inconsistent implantation in the "submucosa". While a few rats may need to be eliminated, the success rate of the implantation of the submucosa probe was 83%, (5/6 rats) with the new implantation technique. This technique demonstrated a highly reproducible method to monitor cGMP levels in the submucosa, which has never been monitored previously. Further experiments with the administration of STcore directly in the colon loop, led to a two fold increase in cGMP levels in the submucosa microdialysis probe (Figure 3.11A) and luminal microdialysis probe (Figure 3.11B) upon the administration of STcore.

3.4 Conclusions

This study was the first study to utilize microdialysis sampling in the submucosa of the ascending colon to determine the enzyme activity of GC-C before and after the administration of an agonist. During the initial implantation studies, female and male rats of the same strain demonstrated different submucosa thicknesses. The male rat submucosa was thinner than the female rat submucosa. The submucosa layer was approximately 60 μm in female Sprague-Dawley rats. Female rats were used in further studie, since the implantation of the microdialysis probe in the submucosa was successful with better reproducibility, and were used in other studies monitoring the effects of STcore and linaclotide using conventional sampling techniques. This novel sampling technique was then applied to experiments monitoring the concentration of cGMP in the submucosa before and after administration of an agonist of GC-C, e.g. STcore.

A)

Submucosa cGMP

	Rat	basal	5 ug ST	%Delivery
Vehicle	70	0.08	0.17	46
Vehicle	71	0.65	0.61	56
ST dosed	72	0.06	0.24	51
ST dosed	74	0.19	0.67	45
ST dosed	65	0.50	1.10	33
ST dosed	66	2.86	6.61	37
ST dosed	67	0.48	0.72	47
ST dosed	68	0.78	0.97	

B)

Lumen cGMP

	Rat	basal	5 ug ST	%Delivery
Vehicle	70	0.19	0.23	49
Vehicle	71	1.72	3.25	42
ST dosed	72	1.76	3.25	42
ST dosed	74	1.05	0.42	54
ST dosed	65	4.35	1.82	50
ST dosed	66	2.90	10.40	65
ST dosed	67	4.77	10.40	47
ST dosed	68	7.72	9.36	76

Table 3.2: Comparison of cGMP concentration in dialysate samples from A) submucosa and B) lumen microdialysate probes in the colon before and after the administration of 5 µg STcore.

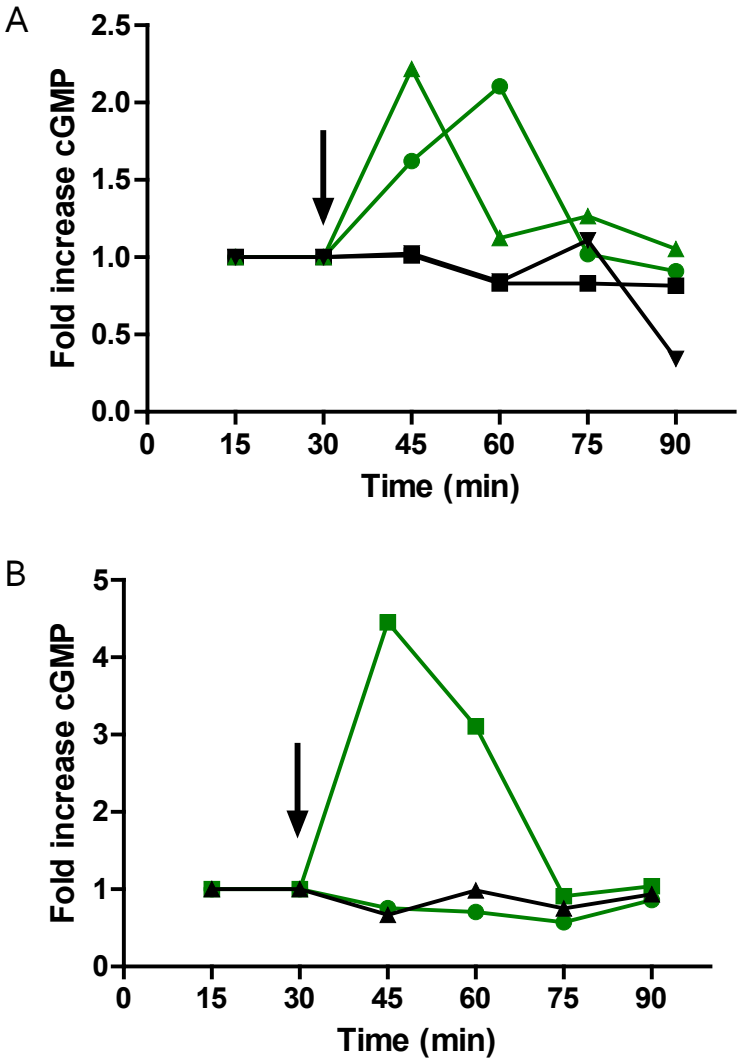

Figure 3.11: cGMP fold increase in A) submucosa and B) luminal dialysate samples upon the administration of 5 µg STcore (green) and vehicle (black), indicated by arrow, directly in the colon loop. STcore administered rats: one (—●—), two (—▲—) and vehicle administered rats: three (—■—), and four (—▼—).

In the preliminary experiments, a split response was observed in the cGMP concentration obtained from the "submucosa" of the colon. Histology was performed in all of these experiments to determine the location of the probe, but the concentrations of cGMP did not correlate with the tissue region of the microdialysis probe in histology.

Due to the possible movement of the microdialysis probe between the experiment and the histology evaluation, microdialysis probes were implanted in both the submucosa and the lumen of the colon. This allowed the comparison of cGMP concentration between the submucosa microdialysis probe and the lumen microdialysis probe. So far the high cGMP concentrations extracted via the "submucosa" microdialysis probe correlates with the lumen microdialysis probe. In the six final experiments, the microdialysis probe appears to be placed in the submucosa for five of them. This procedure will be utilized in future experiments monitoring the cGMP level in the submucosa tissue of the colon, which to the best of our knowledge is the first time this has ever been done.

3.5 References

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~Chapter 4~

Monitoring chemobrain in short-term experiments by microdialysis

4.1 Introduction

Survival after cancer diagnosis has improved drastically in the past few decades. In the early 1970's, the five-year survival rate of invasive cancer was approximately 40 percent[1]. Since then, the survival rate has nearly doubled, mostly due to the improvement in therapies such as adjuvant chemotherapy and radiation therapy (Figure 4.1)[1]. The increase in survival rate after the administration of multiple chemotherapeutic compounds has resulted in more patients reporting a decline in the quality of life, partly due to a decrease in cognitive function. This decline in cognitive function is reported more frequently in patients receiving chemotherapy than in patients who only received radiation, thus the derived name of "chemobrain". Many symptoms have been reported in articles and on the nightly news, including the loss of memory, concentration, the ability to learn new material, and reading comprehension[2-3]. These symptoms have been widely reported in cancer patients starting in the 1980's and have been particularly well studied in breast cancer patients[4-5]. Now that cancer patients are living for decades post-diagnosis and adjuvant therapy is commonly used, the neurotoxicity that occurs from chemotherapy is becoming more of a problem.

Neurotoxicity could result from several factors, such as the synergistic effects from the administration of several chemotherapeutic compounds, and through a variety of different mechanisms. One of the main mechanisms of interest is the generation of radical oxygen species (ROS) and/or radical nitrogen species (RNS), which leads to oxidative damage[4, 6]. Other

possible mechanisms causing a decline in cognitive function are changes in the levels of neurotransmitters (such as norepinephrine, dopamine, serotonin, and 5-hydroxyindoleacetic acid)[7]. These changes may be caused directly by the chemotherapy compounds or from other possible changes to neurogenesis (neuronal generation), the blood brain barrier (BBB), white matter, grey matter, the immune system, blood flow, and/or the hypothalamus-pituitary-adrenal (HPA) (Figure 4.2).

4.1.1 Chemotherapy studies

In order to determine the effects of chemotherapy on cognition, the neurochemical processes causing a decrease in cognitive function are important to study to improve the quality of life of cancer survivors. Changes in cognitive function can be monitored in both humans and animals, although both have their advantages and limitations.

In humans, the most common methods used are neuropsychological, such as cross-over and longitudinal studies[8-15]. Neuroimaging is now frequently performed during psychological examination using structural and functional magnetic resonance imaging methods to monitor the brain directly[9-11, 14, 16-22]. The less common method to study cognitive function in humans is by using neurophysiologic tests. To the best of our knowledge, electroencephalogram is the only physiological test capable of being performed on humans.

With the utilization of animals, critical physiological information can be obtained through tissue biopsy, homogenation, or microdialysis sampling. These tests, especially homogenation, are usually performed only on animals since they are more invasive. However, through these animal studies, the exact physiological responses that various chemotherapy treatments elicit can

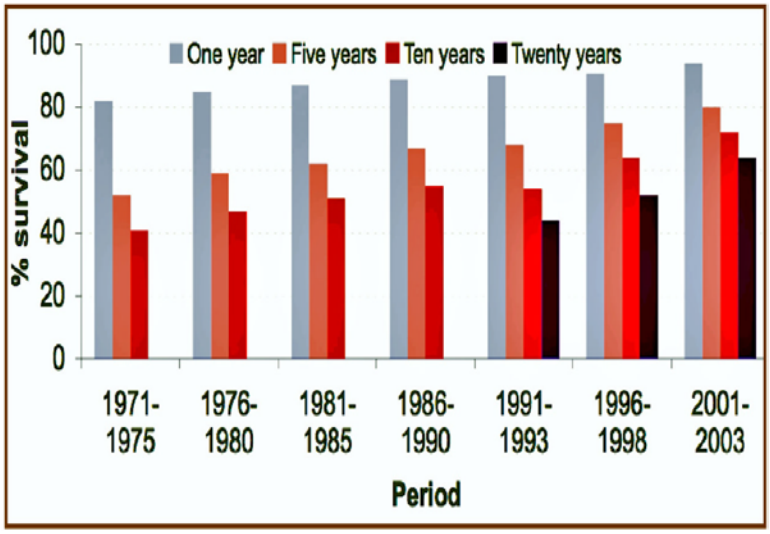

Figure 4.1: Cancer survival rate for the years of 1971 through 2003 with permissions[1].

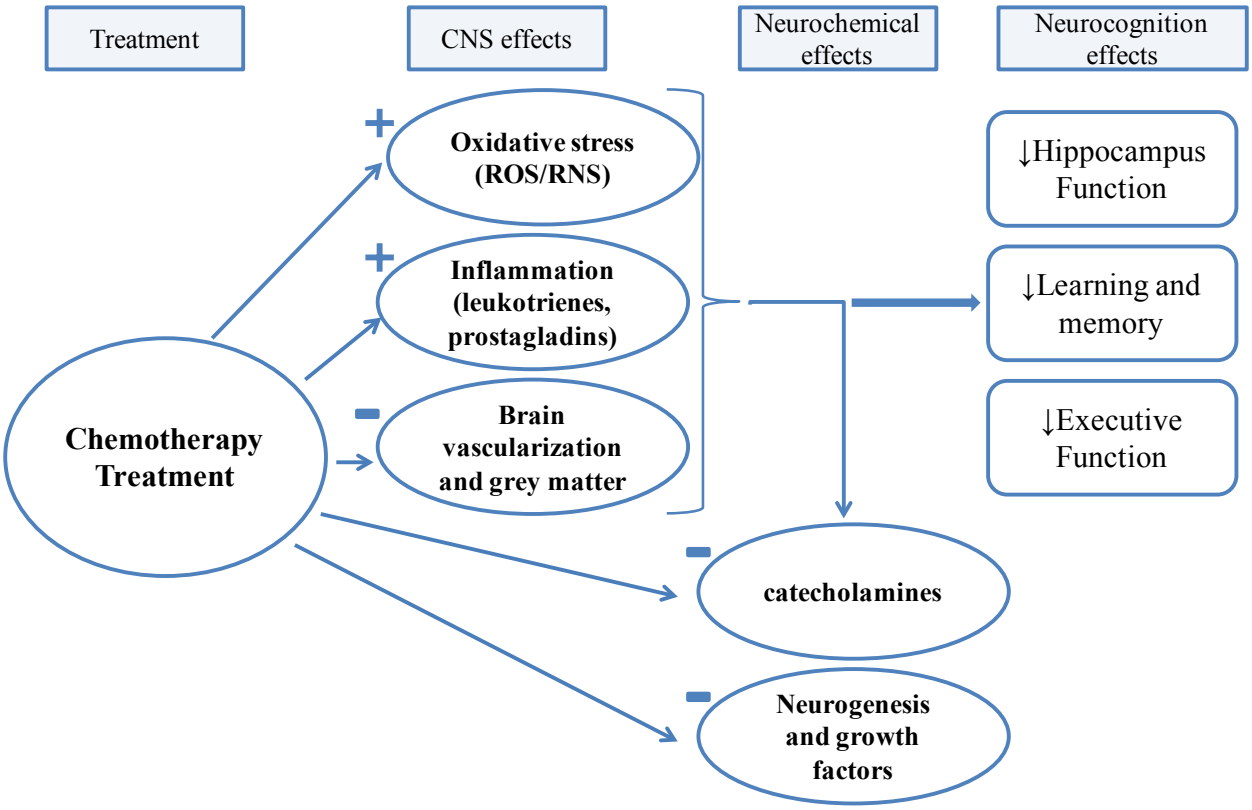

Figure 4.2: Chemotherapy mechanisms associated with cognitive impairment. Adapted from [23] with permission.

be determined. In addition to the determination of the physiological mechanisms, behavioral and neuroimaging tests can also be utilized on animals.

The ability to perform the appropriate tests in each species (rats and humans) separately and then combine the results will lead to translational benefits. This collaboration will potentially elucidate the mechanisms responsible for chemobrain which could lead to higher cognitive function and quality of life for patients that have undergone chemotherapy. The different tests that are currently performed in humans and animals will be briefly discussed with the emphasis on the following areas of interest: neuropsychological, neuroimaging, and neurophysiological applications in human and animal research.

4.1.1.1 Human research

4.1.1.1.1 Neuropsychological

Neuropsychology, also known as the study of cognitive function, has been used in a number of human studies, especially in breast cancer patients. In these studies, humans are monitored with a variety of experimental designs. The three most common studies are self-reported, cross-sectional, and longitudinal. Self-reported cognition reports are the easiest for the patient, since the patient typically fills out a questionnaire at home or makes a list of issues as they occur. Conversely, cross-sectional and longitudinal evaluates cognitive function using psychology tests administered by a professional. Cross-sectional study patients undergo psychological evaluation once for each group (e.g. control, chemotherapy with or without radiation), while longitudinal studies go through several psychological assessments over the

course of the treatment to the same patient (e.g. once before chemotherapy administration and up to several times afterwards).

The results of the self-reported studies are directly correlated with mood-related symptoms (such as depression and anxiety), along with physiological characteristics (such as fatigue and a patient with a lower quality of life)[11, 22, 24]. Since the results obtained from the self-reported studies have not correlated with a decline in cognitive function based on professionally administered psychological tests, studies from cross-sectional and longitudinal studies are the preferred method of choice for this determination.

In preliminary studies, cross-sectional psychological evaluations were performed by researchers to determine cognitive function after chemotherapy treatment and to compare them to a control group[8]. The majority of cross-sectional studies observed that there was a higher incidence of cognitive dysfunction in patients who had received chemotherapy relative to their respective controls. The range of cognitive dysfunction reported in this type of study has been reported to be anywhere from 17 to 75%[25]. This wide range of cognitive dysfunction was partially attributed to the basal differences of humans in intelligence, age, gender, etc.

To minimize these human factors, longitudinal studies have been utilized to monitor cognitive function. These experiments allow the cognitive function of each individual to be tested before and after receiving chemotherapy and then at a later time as well, thus allowing the direct effects of the chemotherapy itself to be monitored without the influence from the vast range in native intelligence present in the population. Using longitudinal studies, cognitive function has typically affected up to34% of patients receiving chemotherapy[26]. Interestingly, some studies have found significant cognitive impairment prior to any chemotherapy

treatment in patients diagnosed with cancer as compared to the controls. For example, Wefel *et al.* found 33% of patients exhibited cognitive impairment prior to any chemotherapy, indicating possible cognitive decline due to the stress from the cancer diagnosis[27]. A more pronounced decline in cognitive function (61%) was observed after chemotherapy treatment, in this same study, indicating that chemotherapy lead to an even further decline of cognitive function[25, 27].

Even if a decline in cognitive function was not observed with psychological evaluations, differences in neurochemical mechanisms or differences in certain brain regions were observed in neuroimaging tests, as discussed in the neuroimaging section coming up[28]. These results indicated that self-reported test results correlate more with changes in brain structure and function than the decline in cognitive function reported by professionally administered psychological tests[28].

Human neuropsychological tests are beneficial, although repeating psychological tests in longitudinal studies also raises concerns about the adjustments made to the administration of the psychology test to take practice effects into account, since the psychology tests are administered numerous times. In a longitudinal study performed by Shagen *et al.*, three different groups with breast cancer were compared: a high dose of chemotherapy consisting of cyclophosphamide, thiotepa and carboplatin (CTC; n=28), a standard dose of chemotherapy consisting of 5-fluorouracil, epirubicin, and cyclophosphamide (FEC; n=39) and a group with stage I breast cancer who did not receive any systemic chemotherapy (no CT; n=57)[29]. A group of healthy controls were also enrolled (n=60). A cognitive difference was found in the high dose CTC group compared to the control group, with the CTC group four times more likely to experience cognitive dysfunction. Interestingly, no difference of cognition was observed between

any of the other groups, including the ones receiving FEC chemotherapy (13%) versus those who did not (17.5%). Due to these effects determined by the healthy controls, the test was modified using the reliable change index. Since adjustments in the psychological evaluations are made in the healthy controls and then the modified test was given to the chemotherapy patients, the estimates of cognitive dysfunction taken from longitudinal studies may be lower than actual due to the repetition of the same cognitive tests. In addition, the subjectivity and variety of cognitive tests makes interpreting the results and comparing them across multiple studies extremely difficult, which makes more objective tests, such as neuroimaging and neurophysiology tests, beneficial.

4.1.1.1.2 Neuroimaging

In order to monitor the multiple effects of chemotherapy administration, it is ideal to use complementary imaging techniques, both functional and structural. Taking measurements using both allows the determination of functional aspects of the brain, such as chemical activity, blood flow, and electrical impulses, along with the structural aspects such as bone, tissue, and blood vessel composition. Functional measurements can be made with positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and proton magnetic resonance spectroscopy (MRS). On the other hand, structural measurements are taken with structural MRI (sMRI) and diffusion tension imaging (DTI).

For example, PET can be used to monitor glucose levels[22], neurotransmitter levels[19, 30], cerebral blood flow, mild depression[22] and chemotherapeutic compound distribution by the administration of the appropriate tracer or radiolabelled compound[31]. The

chemotherapeutic drugs cisplatin, 1,3- bis (2-cholroethyl)-1-nitrosourea (BCNU) and paclitaxel were found to cross the BBB by PET studies[31]. After this initial determination, further physiological studies to determine the processes that control the concentration and deviation of chemotherapeutic agents in the brain are important to perform. For instance, a multidrug resistance protein might increase the concentration of a compound in the brain, causing a decrease in cognitive function due to the chemotherapeutic agents. Due to the sophistication of other instruments, such as MRI, high resolution maps of the entire brain can be used to correlate brain function to the anatomy[21].

MRI instruments offer multiple functional and structural measurement capabilities that are unmatched by any other neuroimaging technique, especially for human neurochemical measurements used for studying the effects of chemotherapy administration. The ability to combine the fMRI and sMRI measurements increases the ability to correlate structure to neurochemical activation. This may elucidate the mechanisms responsible for cognitive impairments in certain conditions, such as mild cognitive impairment induced by chemotherapeutic agents.

The most common fMRI measurement uses an endogenous biomarker, deoxymyoglobin, to monitor neuronal activity. Deoxymyoglobin is converted to oxyhemoglobin, making it sensitive to blood oxygenation. This is commonly known as blood oxygen level-dependent (BOLD) contrast[21]. BOLD contrast is used to monitor the neuronal activity by monitoring the changes in the microvascular system, since these two systems are tightly coupled in normal systems[21]. The measurement of cerebral blood flow and cerebral blood volume are correlated to the structures of the brain taken by sMRI. This method has the ability to monitor atrophy

of cerebral gray matter and demyelination of white matter. Changes in gray and white matter can also be monitored by DTI, which has been found to decrease in chemotherapy patients[6].

One of the first studies using both fMRI and sMRI was performed by Ferguson *et al.* to monitor a set of monozygotic twins, one treated with doxorubicin, cyclophosphamide, and docetaxel while the other was healthy and did not receive any chemotherapy[28]. Both structural and functional differences were found for the twin treated with chemotherapeutic agents with an increase in white matter lesion volume (structural) and a broader spatial activation profile (functional). Interestingly, the cognitive performance of the twins was not significantly different in standardized neuropsychological tests (such as short- and long-delay recall, and California Verbal Learning Test-Total Score), thereby demonstrating the need for more neuroimaging studies.

In another study utilizing sMRI, fMRI, and DTI, Conroy and collaborators, found a decrease in activation profile during a common psychological evaluation technique (3-back working memory task) and a decrease in gray matter density (GMD) in the breast cancer patients mainly receiving doxorubicin plus cyclophosphamide (AC) or doxorubicin, cyclophosphamide, and a taxane (AC-T)[16]. Interestingly, the cognitive performance of the breast cancer patients was lower in the memory domain and the Functional Assessment of Cancer Therapy-Cognitive Function (FACT-COG) test, but performance accuracy and reaction times were not lower in the 3-back task. This study was the first to correlate the results obtained from sMRI, fMRI, DTI, and oxidative stress tests to demonstrate that GMD was inversely proportional to oxidative stress. Oxidative damage was assessed by two comet assays for the detection of both direct and oxidative DNA damage. In this test, as GMD decreased, oxidative stress was more

prevalent. Performing more studies that monitor brain activation and composition at the same time will elucidate the processes causing a decrease in cognitive function.

4.1.1.1.3 Neurophysiology

The main technique used to monitor neurophysiology in humans is an electroencephalogram (EEG)[30]. This allows the monitoring of brain function by electrophysiological determination and can be used in conjunction with fMRI and PET to map brain function. One of the main advantages of this technique over the neuroimaging techniques previously described is that EEGs have a temporal resolution of approximately 1- to 10-ms versus the minutes to hours required for the neuroimaging techniques. This makes this technique ideal to monitor rapidly changing neurophysiological processes and is widely used for events such as seizures[32]. Because it is a highly sensitive, convenient, and a relatively inexpensive technique, it is also growing in other applications such as monitoring neuropathy during chemotherapy administration[33]. This technique allows clinicians to monitor a patient's nerve conduction, muscle function, and autonomic function to determine if any adjustments are necessary for further treatment while keeping the side effects of the compounds to a minimum[33].

A few studies with chemotherapy administration have also monitored neurotoxicity by EEG and found similar results as were determined in the neuropsychological studies by Schagen *et al.* [34-35]. In the preliminary study performed by Schagen *et al.*, asymmetric alpha rhythm (>0.5 Hz), which is indicative of cortical and subcortical dysfunction, in the brain was found in 41% of the high dose chemotherapy, 13% of the standard dose, and 0% in the controls [34].

Interestingly, deviations in the neuropsychological scores correlated with the latency of the P3 physiological levels. P3 latency determines the initiation timing of neurons. The latency and amplitude were also tested in further tests to determine the degree of neuron firing in the P3 component. In these studies, the N1 component was also studied in both latency and amplitude to determine the perceptual processing effects of different chemotherapy dosing regimens. P3 amplitude was decreased in all chemotherapy treatments in comparison to the controls (stage 1 breast cancer patients who did not receive chemotherapy). A difference in P3 latency was only observed between the cyclophosphamide, methotrexate, 5-fluoruracil (CMF) and cyclophosphamide, thiotepa, carboplatin (CTC) groups, with CMF showing a shorter latency. No difference was observed for the N1 region, in amplitude or latency, with the exception of some shifts in the maximum negative values. However, the CMF patients also had a different testing interval after treatment (over a year difference) than the CTC group; this change in testing interval could lead to a difference in the results.

By combining different testing practices (neuropsychological, neuroimaging, and neurophysiological) in humans, many interesting results regarding the effects of chemotherapy on brain structure and function have been determined. However, obtaining more chemical information would be extremely beneficial. One extremely useful application of determining many mechanisms upon chemotherapy administration is the utilization of animals.

4.1.1.2 Animal research

Animal studies allow for a systematic determination of the mechanisms responsible for chemobrain. In human studies, the primary objective is for the patient to survive the cancer and

chemotherapy treatment. This prevents systematic experiments from being performed. For example, in the previous section, most human studies were divided into three groups: high dose chemotherapy, standard dose chemotherapy and a control group. To obtain a better comparison, the high dose and standard dose should use the same chemotherapeutic compounds at different concentrations. This was not necessarily the case in human studies. This then poses the question of whether the difference was attributed to the higher dose or the different chemotherapeutic compounds. Multiple combinations can be administered in animal experiments to answer questions that are not ethically feasible in human trials. In addition, multiple other enlightening possible studies can be performed with animals, which will be discussed in more detail throughout this section.

4.1.1.2.1 Neuropsychology

Animals can be psychologically tested by various mechanisms, including cued learning (food, shock, etc.), spatial memory (mazes, novel object recognition, etc.), and recognition memory (novel object location, levers, etc.). As highlighted by Leuner *et al.*, there are multiple facts in favor and against most cognitive studies for the function the test represents [36]. The cognition tests mainly focus on the hippocampus region, since it is responsible for memory, learning, and the ability to recall events. This is further emphasized by recent developments in animal studies that propose certain tasks require an intact hippocampus or dentate gyrus, while some tasks do not[36]. For example, an object recognition task does not require the hippocampus, while recognition of the placement of the object does. This was demonstrated by rats that had an irradiated hippocampus(causing a disruption in signal transmission between the

hippocampus and other structures e.g. 'not intact')[36]. As with human cognitive tests, each animal cognitive test has its advantages and disadvantages.

The ability to compare the results obtained from different studies for animal cognitive function after administration of chemotherapy agents is often difficult, just as it is in humans. An impairment of memory retention was found in male Wistar rats using an inhibitory avoidance conditioning test [37], while a test using passive avoidance task in male and female albino Swiss mice found no impairment [6]. Both of these tests are considered cued learning tests which require an intact hippocampus. Since so many different variables exist between these studies, including different species, a concrete comparison is extremely difficult. The results from these experiments indicate that mice are possibly resistant to cognitive decline while rats are susceptible to cognitive dysfunction following doxorubicin administration. Interestingly, in two other studies using a contextual fear test, no changes were observed in cognitive function with the administration of doxorubicin and cyclophosphamide in a mouse study[38], while a decrease in memory was observed in a very similar study using Sprague-Dawley female rats[39]. Due to its role in explicit memory, contextual fear requires an intact hippocampus. This provides more affirmation of the difference in response between species (mice versus rats) since similar tests were performed.

Even using the same test as performed with human studies, animal based psychological tests have added complications of interpreting the results. Different parameters are often used for the same test, thereby making the ability to compare the results of the tests with one another difficult. The ability to compare the results between the species (humans versus animal) is an even harder task. Therefore, other tests are also typically performed in animal studies to

correlate multiple changes observed in animals and relate them to those observed in humans.

4.1.1.2.2 Neuroimaging

Neuroimaging in animals is not very common as it is in human applications, but there have not been any neuroimaging studies to monitor physiological changes upon the administration of chemotherapy in animals. One study did use dMRI to monitor the effectiveness of BCNU administration for brain cancer[40]. Upon BCNU administration to male Fischer 344 rats, water diffusion in the tumor increased, thus causing the tumor in the brain to regress. After success in the animal model was achieved, dMRI was then used to monitor the progression or regression of the brain tumor in humans. This illustrates the importance of animal experiments, as usually tests in animal experiments can then be applied to humans. Neuroimaging is not widely used in animal cognitive studies since the animal is generally asleep during the imaging process, limiting the information gained in this area. Therefore, most animal studies utilize other methods to monitor physiological responses.

4.1.1.2.3 Neurophysiology

Neurophysiology is routinely monitored during animal studies and is important to gain an understanding of the mechanisms that are responsible for cognitive dysfunction following chemotherapy. Most of the emphasis in chemotherapy studies has been the investigation of neurobiological mechanisms that are causing changes in cognitive function which can include: oxidative stress, effects on BBB or HPA, demyelination of white matter or gray matter, immune system response (neuroinflammation), blood flow changes, neurogenesis and neurotoxicity[6]. These processes are typically monitored in animals by the collection of biofluids (such as

blood, cerebral spinal fluid, urine and saliva), tissue biopsy, and other sampling techniques (such as microdialysis), which were discussed in more detail in chapter one.

Currently, the hippocampus is the most commonly studied brain region to monitor chemobrain events, since it is the main area of the limbic system that is responsible for learning, memory, locomotion, and exploratory activity due to neurogenesis, in addition to many chemical modulators, such as neurotransmitters[7]. Neurogenesis primarily occurs in the subventricular and subgranular zone of the hippocampal dentate gyrus. These two regions contain precursor neuronal cells (neuronal stem cells), which can undergo cell proliferation, forming new neurons or glial cells[41-42]. This is of particular interest in chemobrain, because multiple studies have shown a decrease in neurogenesis and hippocampal (e.g. glial) cell proliferation following administration of various chemotherapy agents, such as carmustine[42-43], cyclophosphamide [44], 5FU [45-46], and methotrexate[47-49]. The decline in cell proliferation in the brain is to be expected, since most chemotherapeutic agents cause DNA damage or are cytotoxic, thus preventing cell division[5-6, 50]. This is especially true for chemotherapy agents that cross the BBB.

Chemotherapy compounds that do not cross the BBB must change/damage the brain by other processes, such as oxidative stress (ROS/RNS) or alterations in cytokines, chemokines, neurotrophic factors, steroids, or other hormones (e.g. oestrogens)[5, 51-52]. These compounds then cause changes in the brain through the release of other chemical modulators such as neurotransmitters (such as glutamate, ϒ-aminobutyric acid (GABA), dopamine (DA) and/or serotonin (5-HT)) and oxidative stress biomarkers (such as 8-oxoguanine (8-oxoG),

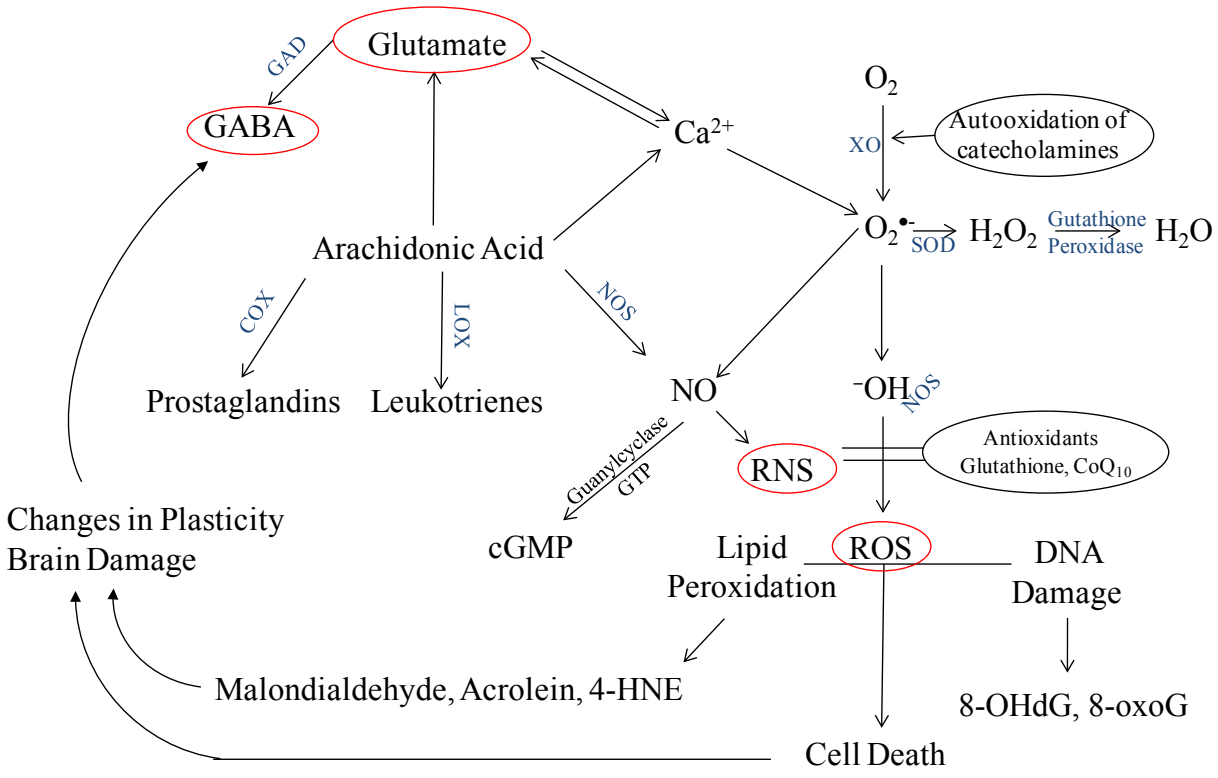

Figure 4.3: Potential neurotransmitter and oxidative stress mechanisms involved in chemobrain toxicity.

8-hydroxy-2'-deoxyguanosine (8-OHdG), and malondialdehyde (MDA), as shown in Figure 4.3[42, 51, 53]. Due to these chemical modulators causing changes in neuronal and glial plasticity, measurement of neurotransmitter release after chemotherapeutic administration could provide valuable information in determining the mechanisms responsible for cognitive impairment due to chemotherapeutic agents.

Several studies have shown chemotherapy causes changes in several endogenous compounds of the brain (e.g. neurotransmitters and neuromodulators) particularly in the hippocampus. In a study performed by Madhyastha *et al.*, male Wistar rats were given intracerebroventricular injections of methotrexate[7]. These rats performed cognitive tests a week after the implantation of a microdialysis probe to monitor the following neurotransmitters: norepinephrine (NE), DA, 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT. A decrease in all three neurotransmitters (DA, 5HT, and NE) was observed in this study as well as a loss of cognitive function. This observation is similar to that observed in other diseases, such as Parkinson's and Alzheimer's, that also result in decreases in these neurotransmitters that correlate to a decline in cognitive function[54-55].

Several neurotransmitters of the hippocampus are also influenced by oestrogen, including GABA, 5-HT, and DA[53, 56]. Chemotherapeutic administrations, such as alkylating agents, can cause early menopause, indicating a change in a decrease in hormones levels, such as oestrogen and ovarian function[2]. Tamoxifen, an antagonist of estrogen, has been shown to influence cognitive function and decrease neurotransmitters concentration (such as DA and 5-HT) in the brain[57]. Hormones and neurotransmitters can have a profound effect on many systems (cytokines, HPA, etc) and multiple studies have been performed monitoring

numerous physiological mechanisms (e.g. neurogenesis) throughout the body after chemotherapeutic administration[6, 57-58]; however, most of these studies collected samples at discrete time points, which could increase the variation in the results.

As discussed above, many mechanisms exist that cause memory impairment, and numerous studies have found possible factors causing cognitive impairment. Most studies monitor the effects of chemotherapy drugs either by monitoring neuropsychology, neuroimaging, or different physiological changes at discrete time points. With the use of microdialysis, it is possible to simultaneously monitor changes of several analytes before, during and after chemotherapeutic administration, therefore allowing a complete continuous picture of the effects of the compound.

4.2 Specific aims of research

The purpose of this research was to monitor several analytes over a short period of time employing brain microdialysis sampling to correlate the changes in neurotransmitters (glutamate and GABA) and oxidative stress levels (MDA) with the administration of different chemotherapeutic compound at a particular concentration. With of microdialysis sampling, an animal can be monitored before, during and after compound administration, which allows the same animal to serve as its own control in both short and long-term studies. The initial experiments delivered the chemotherapy agent directly into the brain through the microdialysis probe. Short-term animal experiments were conducted to determine the immediate effects of different chemotherapeutic compounds on brain chemistry by monitoring glutamate, GABA, aspartate, arginine, and alanine, along with MDA as a marker for oxidative stress via lipid

peroxidation. In addition to the delivery studies, the chemotherapy agents were intravenously infused to determine the short-term effects of these chemotherapy compounds. These administration studies will further elucidate the roles of the neurotransmitters and oxidative stress on cognitive impairment in chemotherapy.

4.3 Chemicals and solutions

5-Fluorouracil (5FU), carboplatin and all of the standards, unless mentioned otherwise, were obtained from Sigma Aldrich (St. Louis, MO, USA). Pharmaceutical grade doxorubicin (TEVA, Irvine, CA, USA) and cyclophosphamide (Baxter Healthcare Corporation, Deerfield, IL, USA) were obtained from Watkins pharmacy at the University of Kansas. Tetrahydrofuran, sodium phosphate dibasic anhydrous (Na_2HPO_4), and sodium phosphate monobasic ($Na_2H_2PO_4$) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Artificial cerebral spinal fluid (aCSF) composition was 145 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 1.2 mM calcium chloride ($CaCl_2$), 1 mM magnesium chloride ($MgCl_2$), 2.33 mM sodium phosphate dibasic anhydrous (Na_2HPO_4), and 0.45 mM sodium phosphate monobasic $Na_2H_2PO_4$ (pH of 7). Solutions were filtered through a 0.22 μm nylon filter prior to use.

Chemotherapy solutions were prepared by the following methods. All stock solutions were made up in water, except doxorubicin, carboplatin, and 5FU. Pharmaceutical grade doxorubicin was only available in diluted saline and was used directly for all experiments as requested by the Animal Care Unit veterinarian. Carboplatin was dissolved directly in aCSF to obtain the desired concentration for brain perfusion studies due to its low solubility. Dimethyl sulfoxide solution (DMSO) with 50% water was necessary to dissolve 5FU. After the stock

solutions were made, cyclophosphamide and 5FU were made up in the appropriate solutions (aCSF for brain perfusion or saline for intravenous infusion). All other solutions and chemicals used were described in section 2.2.1 of chapter two or 3.2.1 of chapter three.

4.4 Microdialysis methods

4.4.1 Surgical procedures

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) animal protocols. All experiments utilized male Wistar rats (Charles-River, Portage, ME, USA) weighing 250-500 grams, unless otherwise specified. All animals were kept on a 12 hour light/dark schedule at all times, with free access to food and water. Rats were pre-anesthetized with isoflurane and then given an anesthesia cocktail of ketamine (80mg kg^{-1}), acepromazine (1mg kg^{-1}), and xylazine (10 mg kg^{-1}) diluted to 2.5 milliliters with saline and then administered intraperitoneally (i.p). Booster doses of ketamine were given as needed by intramuscular (i.m.) injections. The body temperature of the rat was maintained at 37°C by an automated heating pad (CMA Microdialysis AB, Sweden).

4.4.1.1 Brain cannula implantation

After the head was shaved, the animal was positioned in the stereotaxic with the skull level between the lambda and bregma. An incision was made to the left of the midline of the skull, approximately 1 cm in length. The adventitious tissue was then carefully removed from the top of the skull to allow the determination of the bregma and the position of the probe implantation. The position of the bregma was determined by the CMA 12 guide cannula (CMA

Microdialysis AB, Solna, Sweden) in the stereotaxic (Stoelting, Wood Dale, IL, USA). The hole for the guide cannula was then marked (stereotaxic coordinates: posterior 5.6, lateral 4.8, and vertical -5.0, the CA3 region of the hippocampus). A series of two or three holes were then drilled in a radius of approximately 3 to 5 mm surrounding the region marked for the guide cannula. Immediately after drilling each hole, the bone material would be removed via a cotton swab and then a bone screw (BAS, West Lafayette, IN, USA) was put in place. The final hole, approximately 3 mm, was then drilled for the guide cannula. The guide cannula was then lowered to the top of the skull for the stereotaxic measurement, and then it was slowly lowered to the desired location. Dental cement (BAS, West Lafayette, IN, USA) was then applied to the guide cannula shaft where it would flow down to the skull and below the bone screws to provide a good anchor. After each application of dental cement, cotton swabs were used to smooth any rough edges to minimize tissue damage and bleeding. Tissue glue or sutures (Dexon 3-0) were used to loosely close the incision around the dental cement. For probe insertion, the steel dummy was removed from the guide cannula to allow probe placement. A CMA 12 MD Elite probe was used with a 2 mm polyarylethersulpone (PAES) membrane cannula (CMA Microdialysis AB, Solna, Sweden).

4.4.1.2 Jugular vein implantation

An incision, approximately 1 cm in length, was made based on the location of the jugular vein and pectoral muscle observed through the skin. The vein was then isolated from all the adventitious tissue via forceps or cotton swab with saline on it. A flat metal spatula was then put under the vein, and two ligation sutures were put around the vessel. One ligation was positioned

near the pectoral muscle, while the other was approximately 5 mm inferior to the first one. The cannula tubing (MRE-033 or BAS cannula) was prepared by cutting a beveled edge, if this had not already been done. A small nick was made in the jugular vein by spring scissors (Fine Science Tools, Foster City, CA, USA) distal to the proximal muscle, while ensuring the metal spatula was held firmly against the vein. Otherwise the spring scissors may cut the entire vessel, which makes the insertion of the tube very difficult. Immediately after the cut in the vessel, the tubing was inserted into the vessel. If necessary, a dental pick, a metal guide, was used to get the cannula into the vein. The cannula was inserted approximately 3 cm toward the heart. After the tubing was in place, the ligation sutures (3-0 Dexon) were tightened around the vessel and/or cannula. The animal's skin was then sutured (3-0 Dexon) shut for the remainder of the experiment.

4.4.1.3 Short-term microdialysis system

A CMA 12 MD Elite probe with a 2 mm PAES membrane (CMA Microdialysis AB, Solna, Sweden) was set up to perfuse at 1 μL min^{-1} with aCSF prior to surgery. This was done using 10 cm of FEP Teflon microdialysis tubing (BAS, West Lafayette, IN, USA) for the inlet and 15 cm of FEP Teflon tubing for the outlet. The outlet was then connected to the BAS Fraction Collector needle (BAS, West Lafayette, IN, USA). As soon as the surgery was completed, the rat was positioned on a platform above the BAS Fraction Collector (BAS, West Lafayette, IN, USA), and the CMA microdialysis probe was inserted into the CMA 12 guide cannula (CMA Microdialysis AB, Solna, Sweden). The amino acid neurotransmitters were then monitored to determine a steady state for a minimum of one hour prior to dosing, typically using

a two hour basal microdialysis sampling period prior to compound administration.

4.4.1.3.1 Direct brain perfusion

For the specific compound administration another syringe with the chemotherapeutic compound was equilibrated at 1 μL min^{-1} for several minutes prior to the connection to the CMA 12 brain microdialysis probe with PAES membrane. In these studies a 1 mL Hamilton syringe was used, and the BAS syringe pumps were set at 1 μL min^{-1}. Samples were collected continuously over 10 minute intervals after the neurochemical levels remained at consistent levels (flush), which was typically a minimum of 60 minutes. Basal samples were typically collected for two hours before the perfusion of the selected compound. The compound was administered immediately after the start of sample collection. After 60 minutes of the compound perfusion, the perfusate would be changed back to the syringe containing the aCSF for the remainder of the experiment. Control perfusion experiments were performed with aCSF, except for doxorubicin (saline) and 5FU (50% DMSO), as detailed earlier.

4.4.1.3.2 Intravenous infusion

The intravenous infusion was performed in a similar manner as the brain perfusion experiments, except for the following changes. The infusion rate of the chemotherapy compounds was 5 μL min^{-1}. Otherwise saline was infused at 5 μL min^{-1} throughout the entire experiment to keep the neurotransmitters at a consistent level. All other parameters, such as sampling intervals and infusion period (60 minutes), were identical to the brain perfusion experiments. The control experiments were performed with saline. In addition, samples were collected continuously over 10 minute intervals after the neurochemical levels

remained at consistent levels (flush) was collected for a minimum of 60 minutes. Basal samples were typically collected for two hours before the perfusion of the selected compound. The compound was administered immediately after the start of sample collection. After 60 minutes of the compound perfusion, the perfusate would be changed back to the syringe containing the saline for the remainder of the experiment. Control perfusion experiments were performed with saline, except for 5-flurouracil (50% DMSO).

4.4.2 Analysis methods

4.4.2.1 Derivatization scheme for amino acid neurotransmitters

Amino acids, such as glutamate and GABA, are not natively fluorescent or electrochemically active. Therefore, in order to detect these compounds they must first be derivatized. Naphthalene-2,3-dicarboxaldehyde (NDA) (442nm) and o-phthalaldehyde (OPA) (325 nm) are the most common fluorescent derivatizing agents used for the analysis of amino acid neurotransmitters[59-65]; however there are some other possible derivatizing agents, such as fluorescein isothiocyanate (488 nm), imidazole naphthalene-2,3-dicarboxaldehyde (488 nm), and 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA) (488 nm)[66-69]. NDA forms a fluorescent N-substituted-1-cyanobenz[f]isoindole (CBI) adduct in the presence of cyanide, a nucleophile, and a primary amine, as shown in Figure 4.4[59]. OPA forms an N-alkyl-1-isoindole sulfonate in the presence of β-mercaptoethanol (βME), a reducing agent, and a primary amine. Due to the NDA adduct CBI being more stable than the isoindole adduct produced by the OPA derivatization, NDA has been implemented in our lab. The CBI adduct has been shown to be stable for over 11 hours[59], while the isoindole is only stable for five hours[70].

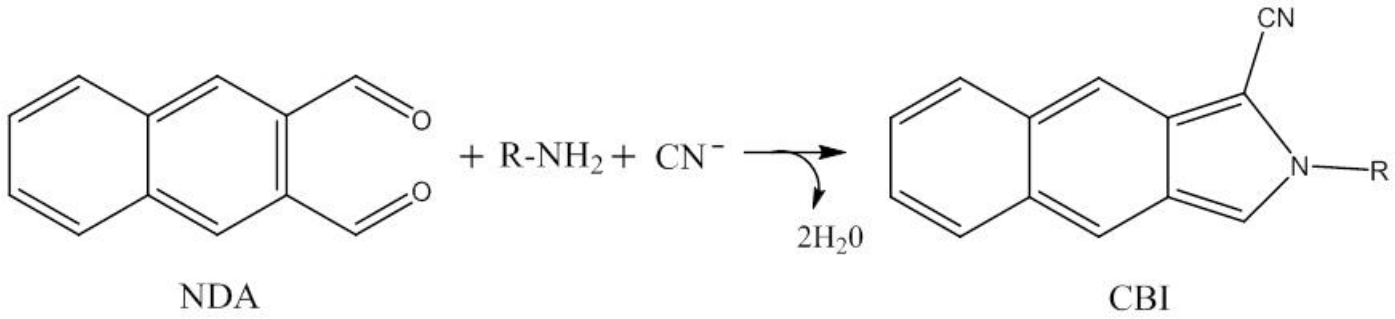

Figure 4.4: NDA/CN^- reaction scheme with primary amines

Once derivatized with NDA/CN, the amino acid neurotransmitters can be detected by electrochemical or fluorescent detection, as first published by Matuszewski *et al*.[59].

Initially, a borate buffer was made according to the following procedure: a 240 mM of sodium tetraborate solution was prepared and then the pH adjusted with 500 mM boric acid to a pH of 8.7, adapted from Siri *et al.*[71]. Each day a fresh solution of borate cyanide solution (100:20, v/v) was made by adding 87 mM of sodium cyanide (33.3 µL) to 125 mM borate buffer (161.6 µL), along with 200 µM of DL-2-aminoadipic acid (5 µL), as the internal standard. This derivatization mixture with the internal standard was then added to the microdialysate sample using the following mixture: 1 µL borate-cyanide with internal standard, 1 µL of 3 mM NDA, and 3 µL of microdialysis sample. This sample mixture was derivatized and then mixed by vortexing, then allowed to set at room temperature (approximately 25 °C) for a minimum of 30 minutes before analysis with high performance liquid chromatography using fluorescent detection (HPLC-Fl).

4.4.2.2 Amino acid neurotransmitter analysis

The mixture described in the previous section (4.4.2.1 derivatization scheme for amino acid neurotransmitters) was directly injected into a liquid chromatography system consisting of: a Shimadzu SCL-10A_{VP} System Controller, two Shimadzu LC-20AD prominence LC pumps, a Shimadzu 100 µL mixer, a Rheodyne 7725i stainless steel sample injector connected to a Phenomenex C18 guard cartridge before the Phenomenex Synergi 4 µ Hydro-RP column (150 x 2.0 mm, 4 µ, Phenomenex, Torrance, CA), and a Jasco FP-2020 Plus Intelligent Fluorescence Detector with a 5 µL flow cell. Injection volumes were 4 µL, which was less than half of the

loop size of 10 µL. The detector was set at an excitation wavelength of 442 nm and an emission wavelength of 490 nm. The two pumps were set to run a gradient, with mobile phase A consisting of 50 mM ammonium acetate at pH 6.8 with 5% THF, and mobile phase B consisting of 100% methanol at a flow rate of 0.35 mL min^{-1}. The data was collected with Chrom & Spec software version 1.5 (Ampersand International Inc. Beachwood, OH, USA). A typical chromatogram is shown in Figure 4.5.

4.4.2.3 Malondialdehyde derivatization

Malondialdehyde (MDA) is typically converted to a fluorophore by derivatization with thiobarbituric acid (TBA), as shown in Figure 4.6[72-73]. TBA was made daily in nanopure water to obtain a concentration of 0.4% (v/v). In order to make the solution acidic, a 0.4% (v/v) solution of sulfuric acid was also prepared in nanopure water. The TBA and sulfuric acid solutions were then combined in a 2:1 ratio (40 µL of TBA and 20 µL of sulfuric acid), and the volume doubled with the addition of water (60 µL). Half of the microdialysate sample collected (5 µL) was then put in a vial. Microdialysis samples collected during the perfusion of doxorubicin and 5FU employed solid phase extraction (SPE) by using Ziptips to adsorb the compounds from the dialysate sample before the addition of the derivatization solution (Millipore, Billerica, MA USA). Ziptips was useful for removing the interferences for 5FU detection, but not the doxorubicin. Once all the samples were ready, the derivatization solution (3.75 µL) was added to the top of the vial. The vials were then tapped on the bench and stirred by vortexing for approximately 3 seconds. The vials were then put in a warm bath (90-95 $^{\circ}$C) for 20 minutes. The samples were then tapped prior to being flash frozen by liquid nitrogen.

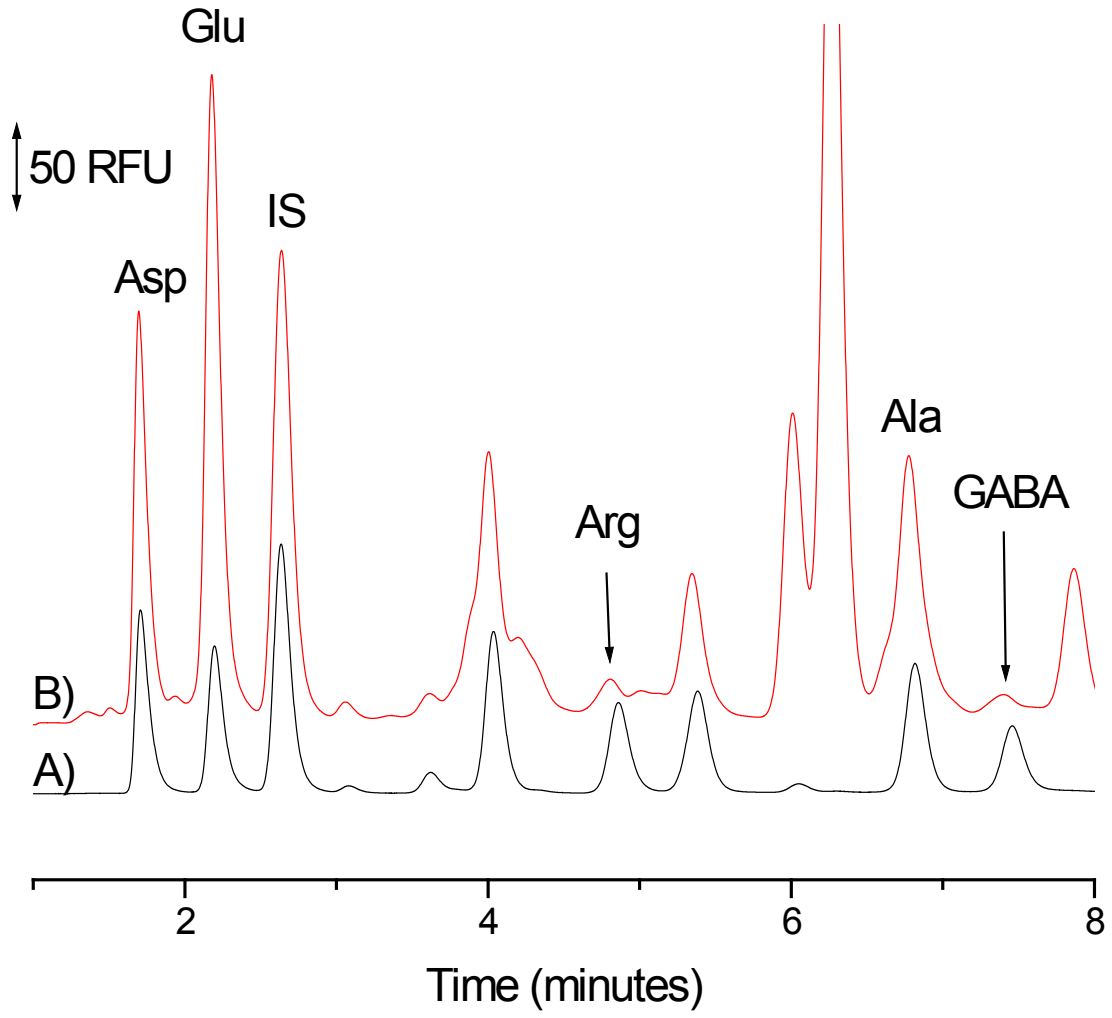

Figure 4.5: Typical chromatograms of glutamate, GABA and 2-aminoadipic acid (IS) in a) 750 nM standard mix (with aspartate, arginine, and alanine), and b) brain dialysate sample. Glu and GABA are clearly baseline resolved. λ_{em}= 442 nm λ_{ex}=490 nm

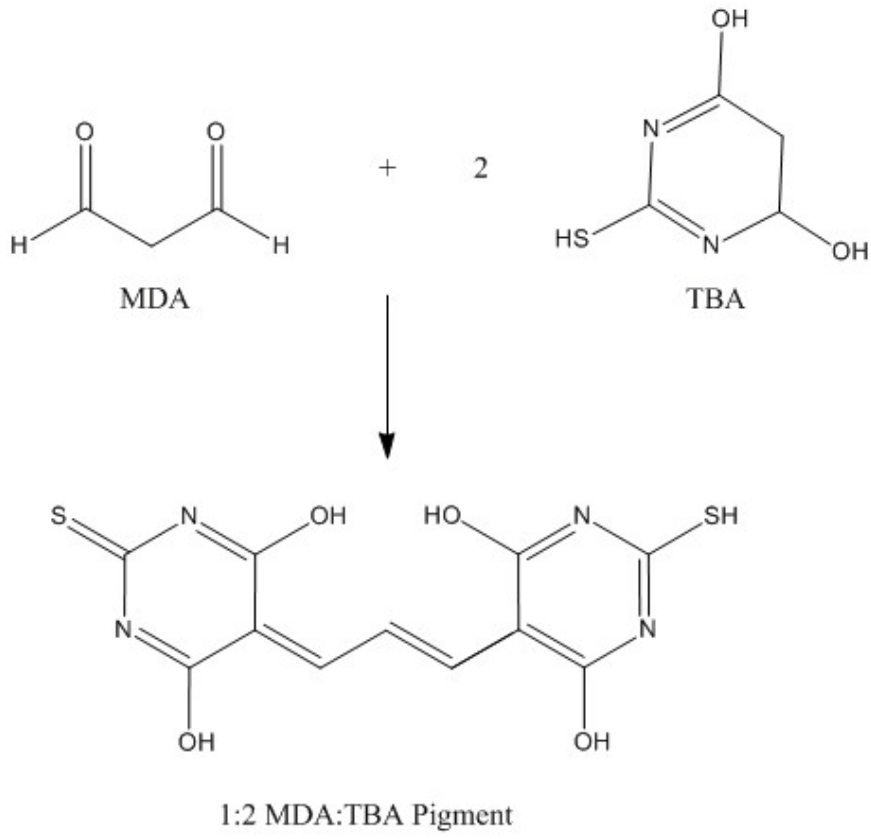

Figure 4.6: Derivatization scheme of MDA with 2 molecules of TBA. Adapted from [72].

Samples were analyzed or kept in the -80°C freezer until analysis within a week. Each day, samples were stored in the -20°C throughout the day during analysis.

4.4.2.4 Malondialdehyde analysis

MDA in a microdialysis sample was measured using capillary electrophoresis with fluorescence detection by the method developed by Cooley *et. al*.[74]. A 75 μm ID capillary was cut to 55 cm with a 2 mm window burned into the capillary 10 cm from the end (45 cm effective length). The capillary was then inserted into the Beckman Coulter MDQ CE System® cartridge (Beckman Coulter, Inc., Brea, CA, USA). A laser Module (488nm) was then connected to the Beckman Coulter MDQ CE system with a 560 nm band pass emission filter. Data analysis was performed by the 32-Karat Software (Beckman Coulter, Inc., Brea, CA, USA).

Immediately prior to standard or sample analysis, the sample was removed from the -80 or -20 °C freezer (samples stored throughout day), and allowed to thaw for a few minutes at room temperature and then stirred by vortex for about 5 seconds. The derivatized standard or sample was then ready for injection onto the capillary, which utilized a background electrolyte (BGE) of 200 mM boric acid solution with 4.5 mM brij 35 with the pH adjusted to 8.4 using sodium hydroxide (NaOH). At the beginning of each week, the capillary was conditioned with a full capillary rinse of 5 minutes methanol, 2 minutes 1 M hydrochloric acid (HCl), 2 minutes nanopure water, 20 minutes NaOH, 2 minutes nanopure water, and 20 minutes BGE at 20 psi each. The capillary rinse between injections was 2 minutes methanol, 2 minutes sodium hydroxide, and 3 minutes of BGE at 20 psi each. After the initial capillary rinse, the capillary was injected with buffer several times before the capillary is fully functional. Hydrodynamic

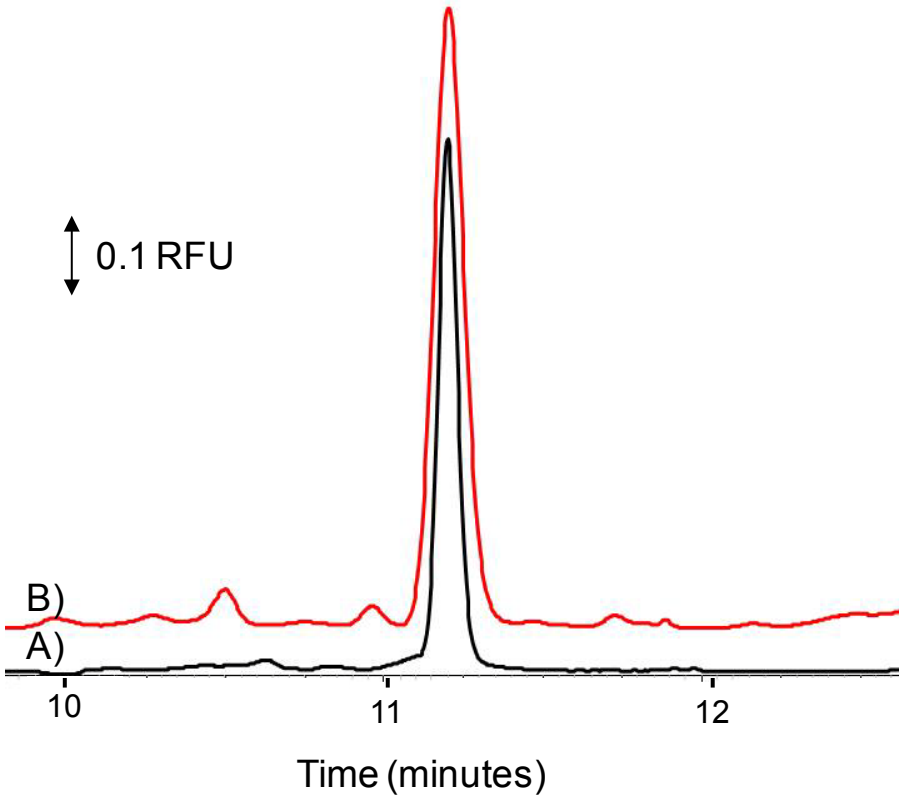

Figure 4.7: Electropherograms of A) 75 nM standard of MDA and B) brain dialysate sample

injection was employed for 5 seconds at 0.5 psi. The separation voltage was set at normal polarity at 10 kV. Example electropherograms for a 75 nM standard of MDA and a brain dialysate sample are shown in Figure 4.7.

4.5 Results and discussion

4.5.1 Chemotherapy administration: short-term studies

4.5.1.1 Doxorubicin pharmacology and neuromechanisms

Doxorubicin (DOX) is a common chemotherapy agent typically given in combination with other compounds for the treatment of a variety of cancers. Doxorubicin exerts its anticancer effects through three mechanisms[5]. The first mechanism is through intercalation into DNA bases allowing cross-links to form, thus preventing DNA replication. The second mechanism is through the formation of a topoismerase II-DNA complex, preventing the DNA from unwinding [4]. The third mechanism produces large amounts of ROS/RNS by the production of superoxide ($O_2^{\cdot -}$), resulting in extreme damage to the DNA, as shown in Figure 4.8.

Superoxide is produced through the reduction of the quinone moiety to the reactive semiquinone moiety in the doxorubicin structure. This reduction can take place by several oxidoreductase enzymes such as NADH dehydrogenase, cytosolic xanthine oxidase or NADPH-cytochrome P450 reductase[5, 75]. After the semiquinone is produced by one of these enzymes, generation of more free radicals is possible with the further reaction of the semiquinone with molecular oxygen to create superoxide (O_2^-), promoting protein oxidation, lipid

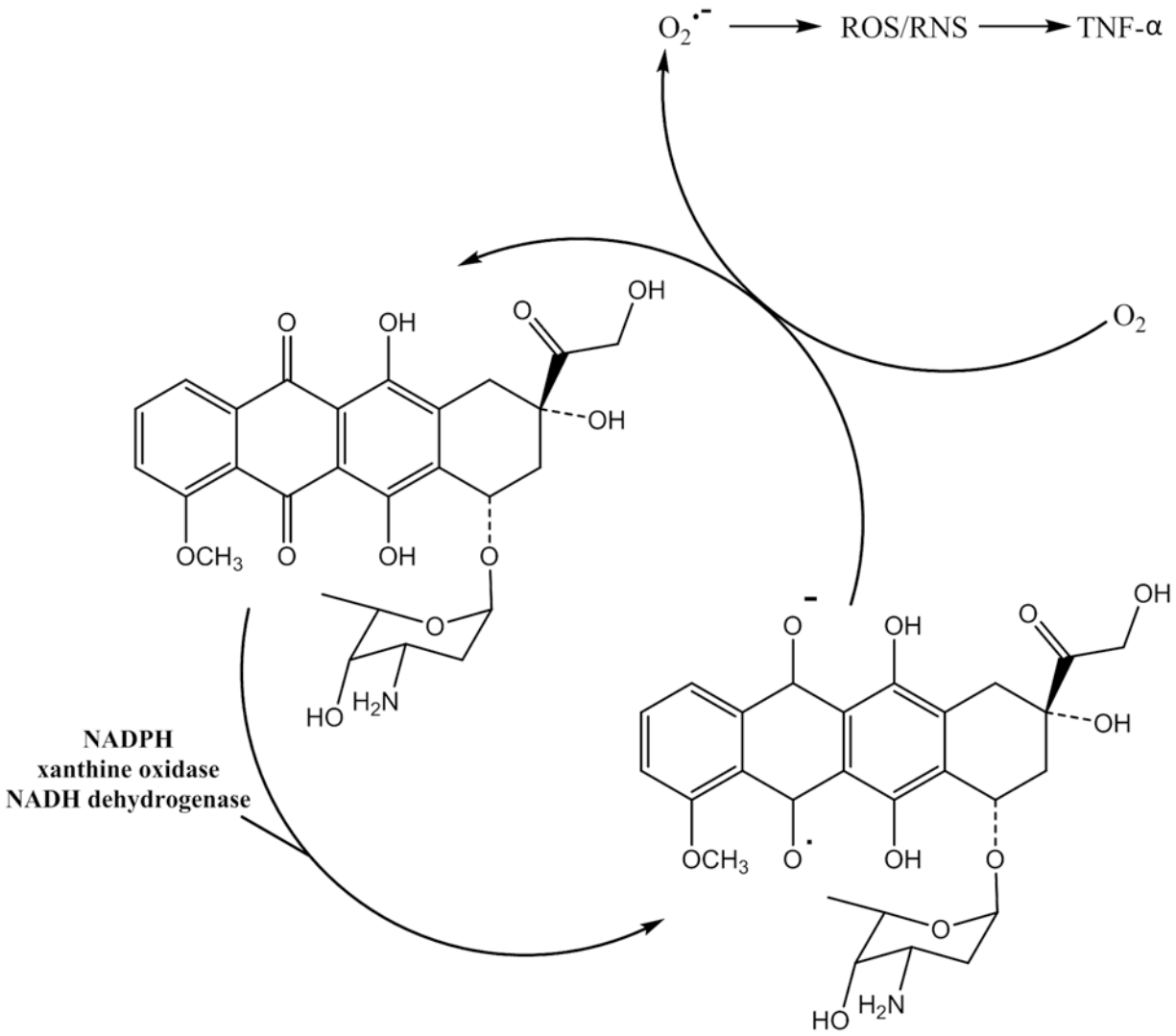

Figure 4.8: Doxorubicin redox cycling inducing ROS/RNS and TNF-α. Adapted from reference [5]

peroxidation, DNA/RNA oxidation, and increasing ROS/RNS generation. This then induces peripheral TNF-α production, which is capable of crossing into areas of the brain protected by the BBB, such as the hippocampus[5]. The brain can also produce TNF-α which activates nitric oxide synthase, also increasing ROS/RNS in the brain[5]. Due to the generation of large amounts of ROS/RNS in the brain, administration of different antioxidants (such as L-glutamine and glutathione) has been shown to decrease the toxic side effects of doxorubicin[76-77]. The generation of ROS/RNS systemically and in the brain is one of the possible mechanisms responsible for chemobrain in patients, thus causing a change in MDA, glutamate, and GABA.

4.5.1.2 Doxorubicin brain perfusion

The primary purpose of this study was to determine the direct effects of doxorubicin on the neurotransmitters glutamate and GABA and on oxidative stress, using MDA as a biomarker. As seen in Figure 4.9, glutamate and GABA responses were extremely different in magnitude from one experiment to another. The first rat exhibited an increase in glutamate after the perfusion of doxorubicin through the microdialysis probe. The second and third rat also had increases in glutamate; however the response was less than the preceding rat, as determined by ANOVA tukey test, even though the same pharmaceutical grade solution of doxorubicin was used. These differences in response might be from a change in the delivery or uptake of doxorubicin in the rat. A higher delivery or uptake would cause a higher response in the neurotransmitters.

Interestingly, the vast differences in responses were observed in the GABA response to doxorubicin as well (Figure 4.9). Unexpectedly, the GABA increase was much higher than

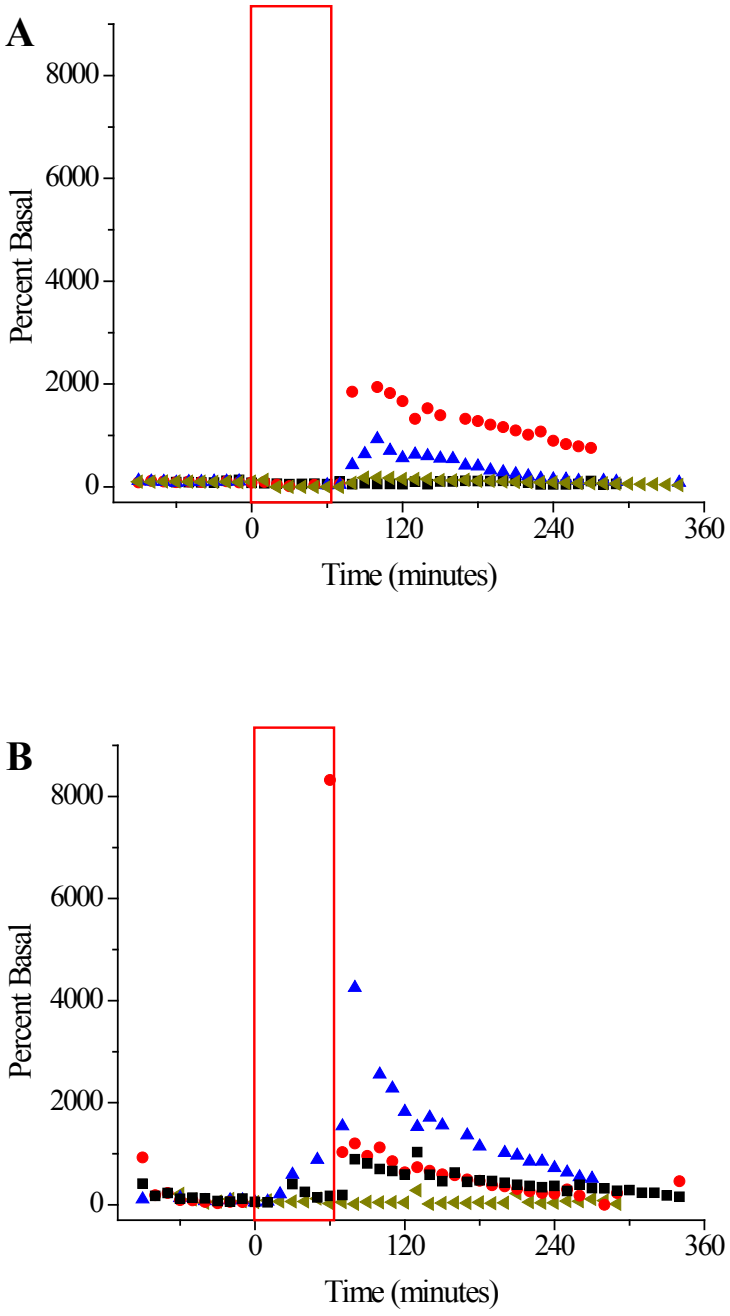

Figure 4.9: Doxorubicin (3.4 mM) perfusion in the hippocampus of anesthetized rats. Comparison of A) glutamate and B) GABA recovered from microdialysate samples as a function of percent basal. Time zero represents the start of a 60 minute perfusion of doxorubicin (indicated by box). Rat 1 (●), rat 2 (▲), rat 3 (■), and control (◀).

glutamate. This increase in GABA may be due to a couple of factors. The first possibility was that GABA was actually performing as an excitatory neurotransmitter, and particularly in the CA3 portion of the hippocampus[78]. Another possible reason for the higher GABA increase is due to the inverse relationship of GABA with norepinephrine[79-80]. Either way, these studies confirm that doxorubicin exhibits a profound response in glutamate and GABA with direct perfusion.

In this study, as seen in many other studies performed in our laboratory, doxorubicin was problematic in the microdialysis experiment and in the analysis system. Doxorubicin is very hydrophobic, and thus interacts with the microdialysis membrane, which affects the recovery and delivery of the doxorubicin. This was shown in a previous experiment performed by Whitaker *et al.*[81]. In addition to the complications with doxorubicin in the microdialysis experiment, doxorubicin also interfered with the analysis of MDA, since doxorubicin is also fluorescent. Solid phase extraction by Ziptips was utilized to remove doxorubicin from the sample, since it is hydrophobic. This reduced the interference of doxorubicin in the sample, but not sufficiently for the analysis of MDA.

Moreover, doxorubicin has repeatedly demonstrated a large variability in results obtained in liver studies performed in our laboratory with doxorubicin perfused through the microdialysis probe [74, 82]. In the study by Price *et al.*, a comparison of awake studies to anesthetized rats was performed. The anesthetized rats had more variability[75]. This inconsistency was attributed to different metabolic mechanisms of carbohydrates (in particular glucose) due to the animal being anesthetized during the experiment. Since these experiments were performed under anesthesia, changes in metabolic rates could have definitely influenced the results.

Glucose undergoes glycolysis to form pyruvate in peripheral tissues, which is then converted to alanine with the consumption of glutamate. If this process was occurring in the brain, an increase in alanine with a decrease in glutamate would be expected. This could possibly be the reason for the decrease in response of glutamate observed in the different rats. However, in order to be certain, more analytes (e.g. glucose) would need to be monitored in the future.

Another variation in the effects of doxorubicin microdialysis observed by Cooley *et al.* was due to the placement of the microdialysis probe in relation to a major vessel in the liver[83]. For instance, if the probe was implanted in close proximity to a blood vessel, a higher delivery of doxorubicin occurred. This is because the perfusion rate of an analyte is dependent upon the transport mechanisms surrounding the probe, correlating to more tissue damage around the probe. Since the brain is not a highly perfused organ like the liver, an increase in the transport was most likely not from the proximity to a blood vessel. However, if the different rats had different expressions of a transport protein (e.g. multidrug resistance protein), this could change the transport of doxorubicin from the probe, thus changing the delivery rate of doxorubicin, and changing the damage to tissue surrounding the probe. The latter could be determined by the histology of the tissue around the probe.

4.5.1.3 Doxorubicin intravenous infusion

The next study was to administer intravenous infusions of doxorubicin to determine if any changes occurred in glutamate, GABA, and MDA levels in the hippocampus of male Wistar rats. Typically, chemotherapy compounds are administered via intravenous infusion in human cancer patients. These studies were designed to simulate the administration technique performed in

humans while monitoring the changes in the specified analytes in Wistar rats. In this experiment, no change was observed in glutamate, GABA, or in MDA (Figures 4.10 and 4.11). This confirmed that insufficient doxorubicin crossed the BBB to cause changes, since no immediate damage was observed.

As discussed in the beginning of this section, doxorubicin is known to undergo redox cycling, which induces a cascade of peripheral events. Numerous experiments have shown a decrease in glutamine and glutathione in both peripheral and brain tissue due to the redox cycling. Since the brain cannot quickly regenerate large amounts of antioxidants, this makes the brain more susceptible to damage at lower levels of ROS/RNS (oxidative damage). The time it takes for these events to happen peripherally and cause an effect on the brain is not known. Currently, no other literature has reported glutamate or GABA level changes upon doxorubicin administration; however, levels of glutathione and glutamine have been reported to decrease upon peripheral administration along with a down regulation of glutamate transporters upon the administration of paclitaxel[84-85]. Since glutamine is a precursor to glutamate, and glutamine levels have been shown to decrease in the brain, most likely glutamate levels would decrease. Increased levels of glutamate would be expected only if the cells undergo apoptosis, thus dumping intracellular glutamate into the ECF, which could be another possible mechanism. Glutamate would also increase if long-term potentiation occurred, which again would cause glutamate to be leaked into the ECF.

Oxidative stress markers are of primary interest upon doxorubicin administration, since antioxidants such as glutathione have been shown to alleviate some of the adverse symptoms of doxorubicin. In the literature, several others reported an increase in oxidative damage in

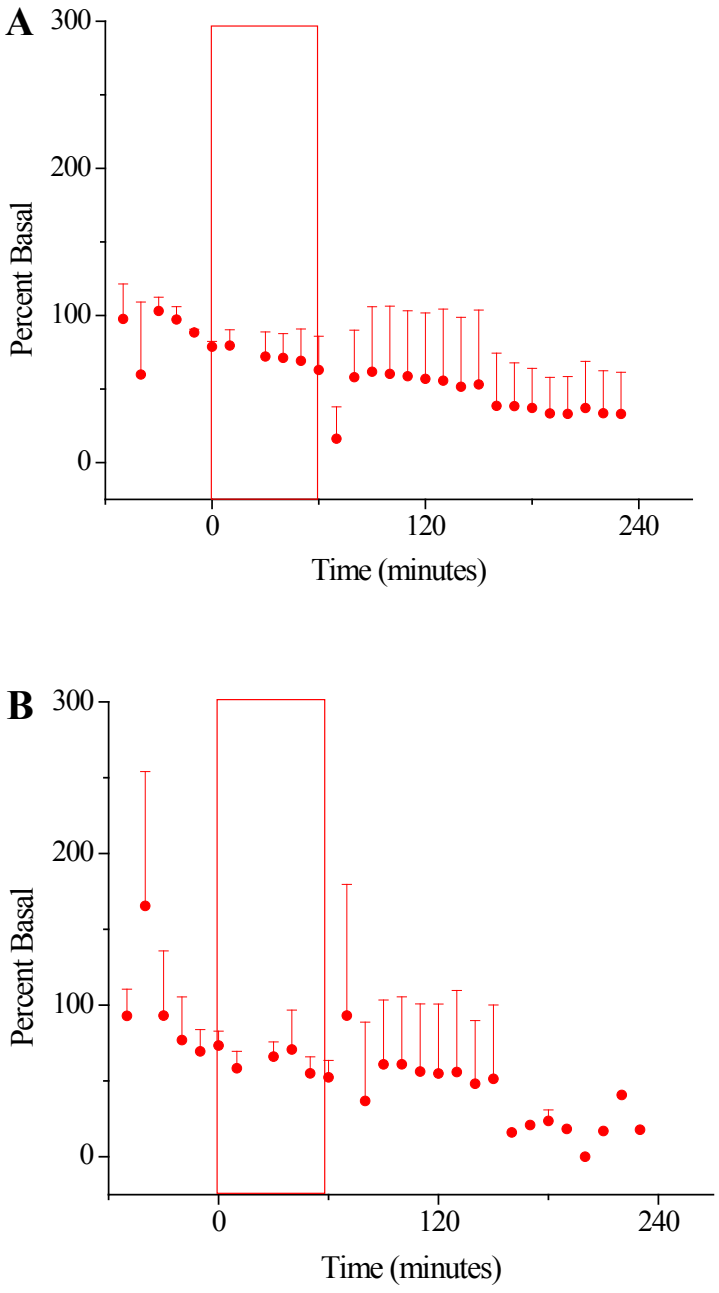

Figure 4.10: Doxorubicin (3.4 mM) intravenous infusion in anesthetized rats. Comparison of A) glutamate and B) GABA recovered from hippocampus microdialysate samples as a function of percent basal (n=2). Time zero represents start of doxorubicin infusion for 60 minutes (indicated by box).

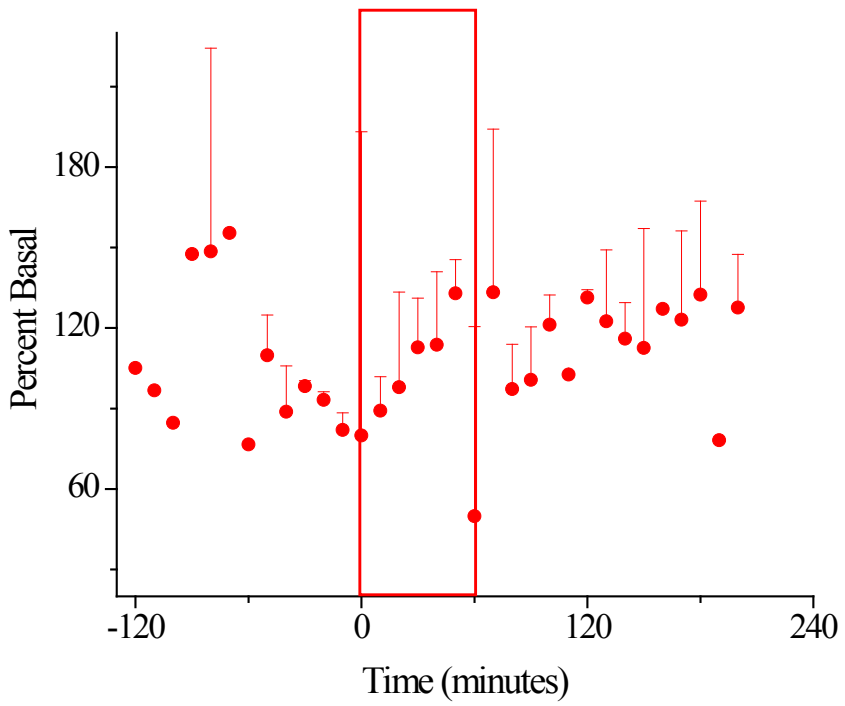

Figure 4.11: Doxorubicin (3.4 mM) intravenous infusion in anesthetized rats. Time plot of MDA recovered from hippocampus microdialysate samples as a function of percent basal (n=2). Time zero represents start of doxorubicin infusion for 60 minutes (indicated by box).

the brain, determined by various markers such as 4-hydroxynonenal (HNE) and MDA (both markers of lipid peroxidation by ROS/RNS) in rats or mice[5-6, 24, 86-91]. Most of these measurements of oxidative damage are taken at least 24 hours after the administration of doxorubicin and at discrete time points. A study by Merzoug *et al.* reports a slight increase in MDA in the brain one hour after intraperitoneal administration of doxorubicin in male Wistar rats[90]. This increase in MDA concentration was determined by the absorption of the TBA solution at 532 nm. The absorption reported in this study as being MDA could actually be from other aldehydes and pyrimidines, since they possess similar absorption bands[92]. Using a separation method developed by Cooley *et al.*[74], (which separates all other analytes from MDA) a slight increase in MDA was observed in brain microdialysate samples during intravenous administration of doxorubicin; however, it was not significant (Figure 4.11). Many other studies, including the one by Merzoug *et al.*, show a more significant increase in MDA days after doxorubicin administration[90].

In a study performed by Joshi *et al.*, lipid peroxidation determined by HNE and protein oxidation occurred in mice 72 hours after systemic administration of doxorubicin[88]. This study indicates that oxidative damage does occur, but the level of oxidative damage between drug administration and 72 hours is not known, nor are the effects of doxorubicin on neurotransmitters. The oxidative damage was determined by mixing the homogenate solution with the appropriate antibody and then incubated with a nitrocellulose membrane. The membrane was then exposed to sigma fast tablets [5-bromo-4-chloro-3-indolyl phosphate//nitro blue tetrazolium substrate (BCIP/NBT)], dried and then scanned in Adobe Photoshop, and quantified in Scion Image. In a previous study of Joshi *et al.* using systemic administration, a significant decrease was

found in glutathione, with a concomitant increase in expression was observed in multidrug-resistant protein-1, glutathione-S-transferase, glutathione peroxidase, and glutathione reductase. In a further study, Joshi showed that the administration of γ-glutamyl cysteine ethyl ester (GCEE) prior to systemic doxorubicin administration in mice decreased lipid peroxidation and protein oxidation in the brain[88].

4.5.1.4 Cyclophosphamide pharmacology and neuromechanisms

Cyclophosphamide, in conjunction with doxorubicin, is one of the most commonly used chemotherapy regimens for the treatment of cancer[93]. Cyclophosphamide acts by different mechanisms than doxorubicin, which increases the death and/or decreases cancer cell multiplication, thus increasing the survival of cancer patients. The utilization of adjuvant therapy can also lead to its own toxic products and also possibly increase BBB permeability of drugs that would not normally cross the BBB. Since cyclophosphamide is a pro-drug, it must first be converted into its main active pharmacological alkylating metabolite of phosphoramide mustard (Figure 4.12). This activation also causes the toxic side product acrolein to be formed[26]. Phosphoramide mustard exerts its anticancer effects by forming adducts with the oxygen and nitrogen present in DNA bases, thus preventing cell replication[4]. DNA damage caused by cyclophosphamide can be repaired by the removal of the alkylated bases, which could cause changes in DNA repair pathways, influencing the toxicity of this alkylating agent. Another form of toxicity from cyclophosphamide is due to an increase in ROS/RNS generated from acrolein, thus leading to oxidative stress[94-95]. An increase in lipid peroxidation products, such as MDA, has been demonstrated in many tissues (such as brain, liver, lung, and

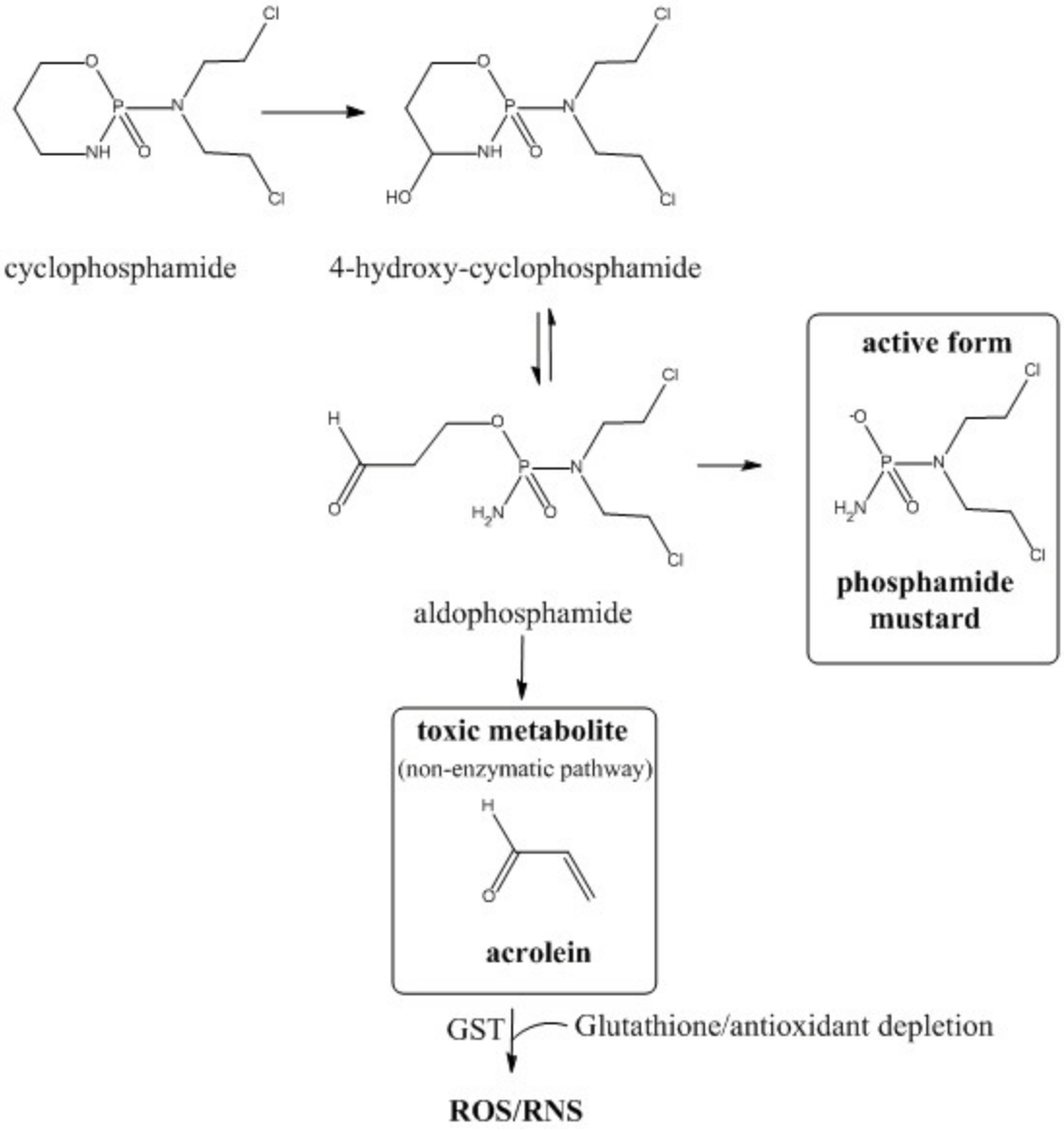

Figure 4.12: Cyclophosphamide bioactivation producing ROS/RNS. Adapted from [102] with permission.

kidney) after administration of cyclophosphamide[94, 96-101]. Pre-administration of antioxidants (such as melatonin and glutamine) alleviate some of the toxicity from cyclophosphamide, as determined by MDA analysis[96, 99].

4.5.1.5 Cyclophosphamide brain perfusion

In these experiments glutamate, GABA, and MDA were continuously monitored by microdialysis during the direct perfusion of cyclophosphamide into the brain. As can be seen in Figure 4.13A, a significant increase in glutamate was observed with direct perfusion of cyclophosphamide compared to the controls for time points during the administration (between 0 and 60 minutes), determined by t-test with unequal variance. During the direct administration of cyclophosphamide, GABA levels barely increased above the limits of detection (LOD: S/N = 3). Statistical analysis does show a significant increase in GABA with the same statistical analysis as glutamate (Figure 4.13B). Further analysis of GABA on a system with the concentration of GABA above the limits of quantitation (LOQ: S/N =10) would provide more certainty to any increases observed. The same was true for MDA levels, hence the variability seen in Figure 4.14. Interestingly, the increase in glutamate and GABA was essentially identical in both rats, with the first sample after compound perfusion spiking immediately to the highest level and then remaining elevated until the end of administration. With the return of glutamate and GABA levels to basal immediately after the perfusion of cyclophosphamide, this indicates that the increase was not permanent. However, even a temporary increase in glutamate and GABA levels could have caused damage to several important processes in the brain. As shown by many articles, increased levels of glutamate can cause long-term potentiation, which can cause a

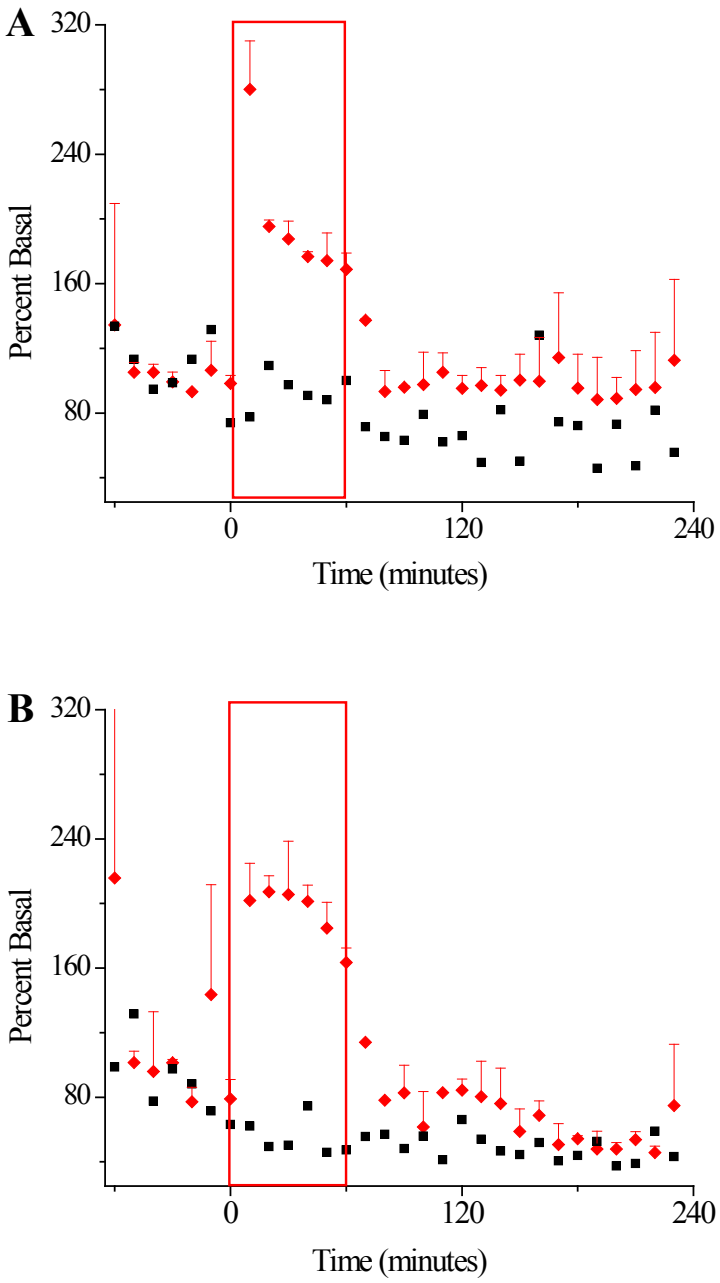

Figure 4.13: Percent deviation for amino acid neurotransmitters versus time profiles for constant perfusion of cyclophosphamide (25 mM) in the hippocampus of anesthetized rats. Comparison of A) glutamate and B) GABA recovered from microdialysate samples as a function of percent basal. Time zero represents start of cyclophosphamide (◆) (n=2) or sham (■) (n=1) perfusion for 60 minutes (indicated by box).

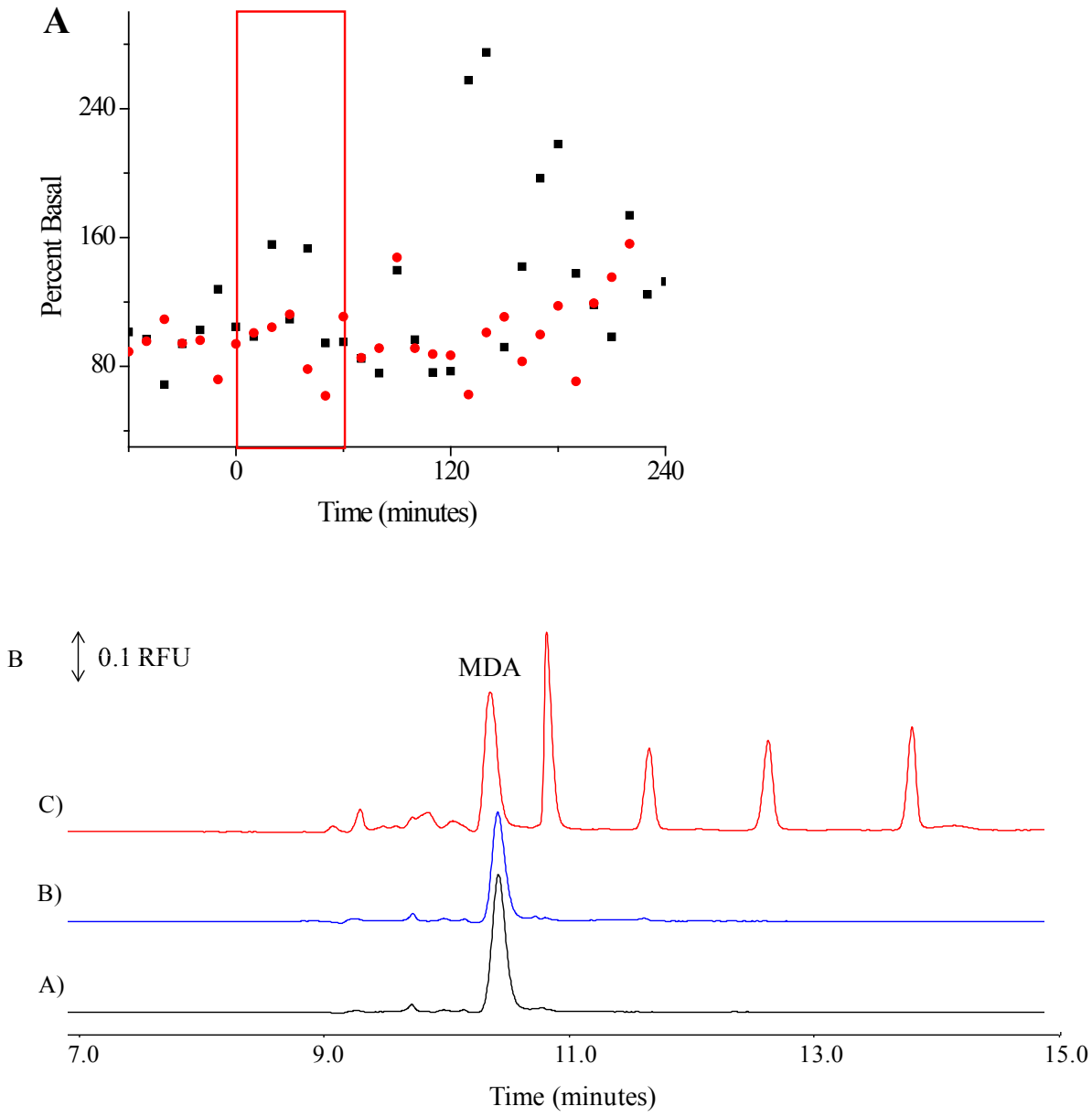

Figure 4.14: Graph A. Percent deviation for MDA versus time profile for constant perfusion of cyclophosphamide (25 mM) in the hippocampus of anesthetized rats. Time zero represents start of cyclophosphamide (◆) (n=1) or sham (■) (n=1) perfusion for 60 minutes (indicated by box). Graph B. A) Typical chromatogram of MDA in a 25 nM standard, B) microdialysate sample from 30 minutes before the perfusion of cyclophosphamide (25 mM) in the hippocampus of anesthetized rats, and C) 30 minutes after the start of the perfusion (Graph B).

decline in cognitive function[51, 103].

While cyclophosphamide does not undergo redox cycling like doxorubicin, it has been well documented to increase oxidative stress by the production of acrolein. This byproduct then increases ROS/RNS, thus increasing other lipid peroxidation markers such as MDA. Due to this, an increase in MDA was expected. The timing of the increase was not known, since other experiments generally report time points at least 24 hours after compound administration. Due to antioxidants reducing the levels of oxidative damage, changes were also expected in glutamate and GABA as well. To the best of our knowledge, these changes have not been monitored previously.

Several studies report a significant change in MDA after the administration of cyclophosphamide (typically via IV/IP injections). However, these studies usually use non-specific detection methods and tissue homogenation, which also include the intracellular levels [96, 100, 104]. In addition, most studies take the first time point at least 24 hours after cyclophosphamide administration. From our results, MDA was found to not increase significantly during the time of this study.

As expected, the increase in glutamate was not nearly as substantial as those seen in doxorubicin administration. Glutamate increased approximately 2-fold with cyclophosphamide perfusion, while doxorubicin administration saw an increase of 12-fold demonstrating that doxorubicin exhibited a higher increase and damage than cyclophosphamide (Figure 4.15). Due to doxorubicin's redox cycling producing large amounts of ROS/RNS, this smaller and shorter increase in glutamate due to cyclophosphamide was to be expected. Thus, cyclophosphamide did not elicit the large change in glutamate as seen with doxorubicin with direct perfusion.

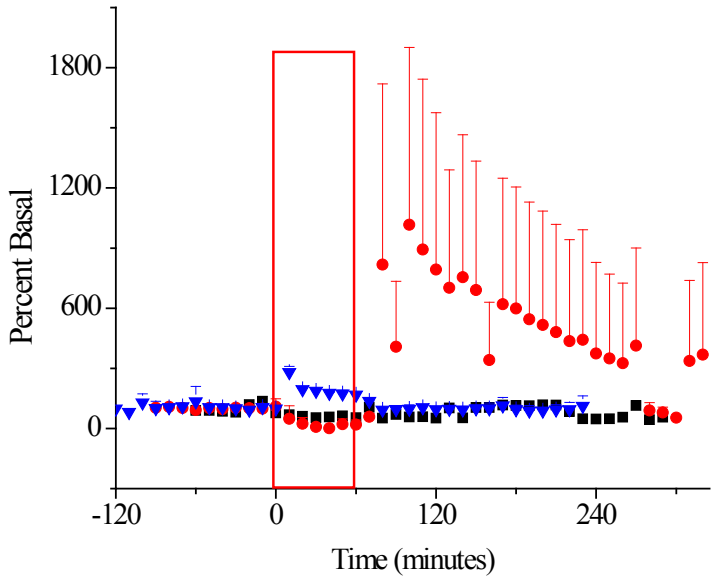

Figure 4.15: Comparison of glutamate recovered from hippocampus microdialysate samples of anesthetized rats given different chemotherapy agents. Time zero represents start of doxorubicin (●) (n=3), cyclophosphamide (▼) (n=2) and sham (■) (n=1) perfusion for 60 minutes (indicated by box).

Upon direct perfusion of cyclophosphamide to the brain, glutamate and GABA levels increased, while MDA did not. This demonstrates that cyclophosphamide does not appear to be as toxic upon direct perfusion to the brain as doxorubicin.

4.5.1.6 Cyclophosphamide intravenous infusion

Once the direct effects of cyclophosphamide had been determined, the next goal was to monitor the effects of cyclophosphamide during intravenous infusion. Upon the intravenous administration of cyclophosphamide, no detectable changes were observed in glutamate, GABA, or MDA concentrations in brain microdialysate. The glutamate levels from anesthetized rats in brain microdialysate samples upon intravenous infusion are shown in Figure 4.16. The parent drug, cyclophosphamide, is not expected to cross the BBB since it is highly hydrophilic[105]. Since some of the metabolites (not shown in Figure 4.12) can easily cross the lipid bilayer membranes of many cells through passive diffusion, these are potentially able to cross the BBB[105]. Metabolite permeability of the BBB may be responsible for the confusion, seizures, or coma patients endure when high concentrations of cyclophosphamide are utilized in chemotherapy regimens[50]. From studies performed previously in our laboratory, chemically induced seizures increase the levels of glutamate and GABA significantly[80]. Thus, one would expect these neurotransmitters to change if a seizure was induced. The timing or extent of the changes upon cyclophosphamide administration has not been determined. Peak concentration of cyclophosphamide metabolites in urine generally occur approximately 10-14 hours after administration; therefore the changes in neurotransmitters might take longer than a few hours to develop[106]. Due to this, cyclophosphamide was studied in the long-term microdialysis

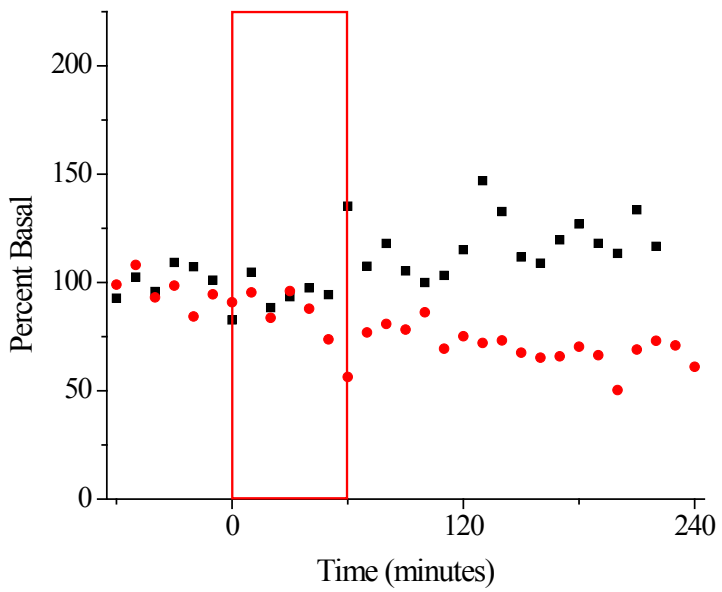

Figure 4.16: Cyclophosphamide intravenous infusion in anesthetized rats: 25 mM (■) (n=1) and 175 mM (●) (n=1). Glutamate recovered from hippocampus microdialysate samples as a function of percent basal. Time zero represents start of cyclophosphamide infusion for 60 minutes (indicated by box).

experiments to determine if changes in these analytes were observed at a later time, as discussed in Chapter 5.

4.5.1.7 5-Fluorouracil pharmacology and neuromechanisms

5-Fluorouracil (5FU) is commonly given in combination with other chemotherapeutic agents, such as cyclophosphamide and doxorubicin. It is one of the most common chemotherapy adjuvant therapies administered in cancer patients, especially in breast and colon cancer patients. The different modes of action of each agent help defeat cancer and increase life expectancy. With its uracil (pyrimidine) analog structure (Figure 4.17), the mechanism of action of 5FU is through its incorporation into DNA and RNA, along with the inhibition of thymidylate synthase (TS) [107]. The only difference between 5FU and uracil is the fluorine atom at the C-5 position in place of the hydrogen allowing 5FU to enter cells using the same transport mechanism as uracil.

Once in the cell, 5FU is converted to its three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and flurouridine triphosphate (FUTP). FdUMP and FdUTP cause DNA damage and cell death by FdUMP binding to the thymidylate synthase enzyme which blocks thymidine synthesis (Figure 4.17). This is necessary for DNA repair by the nucleotide excision repair enzyme uracil-DNA-glycosylase (UDG). RNA damage essentially occurs throughout all the different transcription levels with the metabolite FUTP. The incorporation of these 5FU metabolites causes DNA and RNA excision and repair mechanisms to malfunction, leading to cell death unless thymidine kinase can convert thymidine to thymidylate. This can make 5FU ineffective in the treatment of

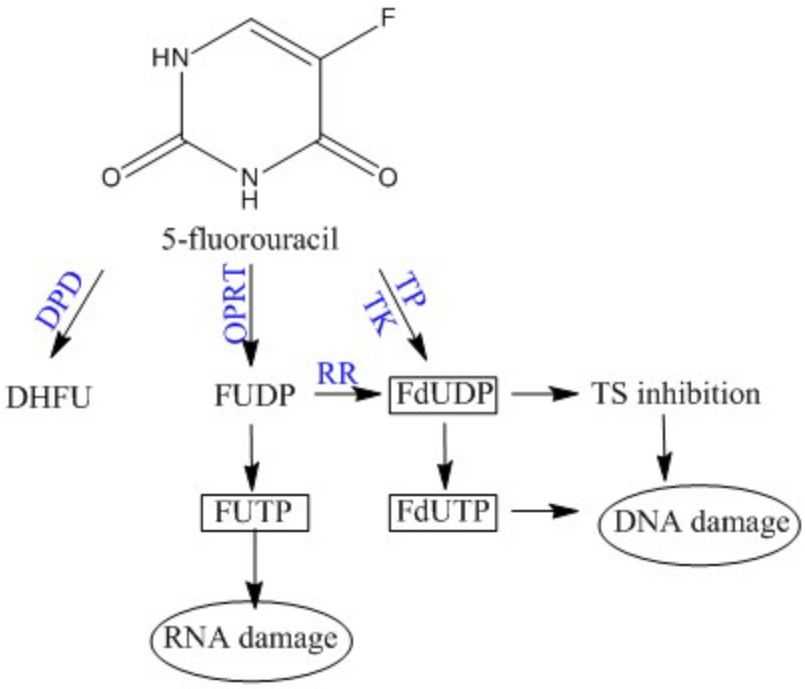

Figure 4.17: 5-Fluorouracil metabolism. Adapted from [107].

cancer. Another scenario that makes 5FU ineffective for cancer treatment is if there is a deficiency in the enzyme dihydrodpyrimidine dehydrogenase (DPD). DPD is primarily responsible for conversion of 5FU to dihydrofluorouracil (DHFU), and a deficiency in this enzyme commonly leads to neurotoxicity or even death[50]. Most neurotoxicity and/or cognition decline associated with 5FU are often reported with adjuvant therapy or with the co-administration of leucovorin (a calcium folinate).

4.5.1.8 5-Fluorouracil brain perfusion

As with the other brain perfusion experiments, 5FU was directly perfused through the microdialysis probe while glutamate, GABA, and MDA, were monitored continuously throughout the experiment. Upon administration of 5FU through the microdialysis probe, no significant differences were seen in any of the neurotransmitter or ROS levels when compared to the controls (Figures 4.18, and 4.19). As shown in Figure 4.18A, glutamate does not appear to change upon direct perfusion of 5FU through the microdialysis probe. This is consistent with other results observed with 5FU, especially when administered by itself[12, 50]. Due to 5FU's ability to cross the BBB[108], direct perfusion of 5FU through the microdialysis probe gives an indication of the effects of the drug directly on the brain. As seen in this study, 5FU seems to cause minimal neurotoxicity, if any.

In studies that do observe a change in the common effects experienced upon 5FU administration, 5FU was typically administered with other compounds, such as leucovorin or a part of adjuvant treatment with doxorubicin and/or cyclophosphamide. The effects rarely observed following 5FU administration consist of dysarthria, ataxia, dizziness, nystagmus,

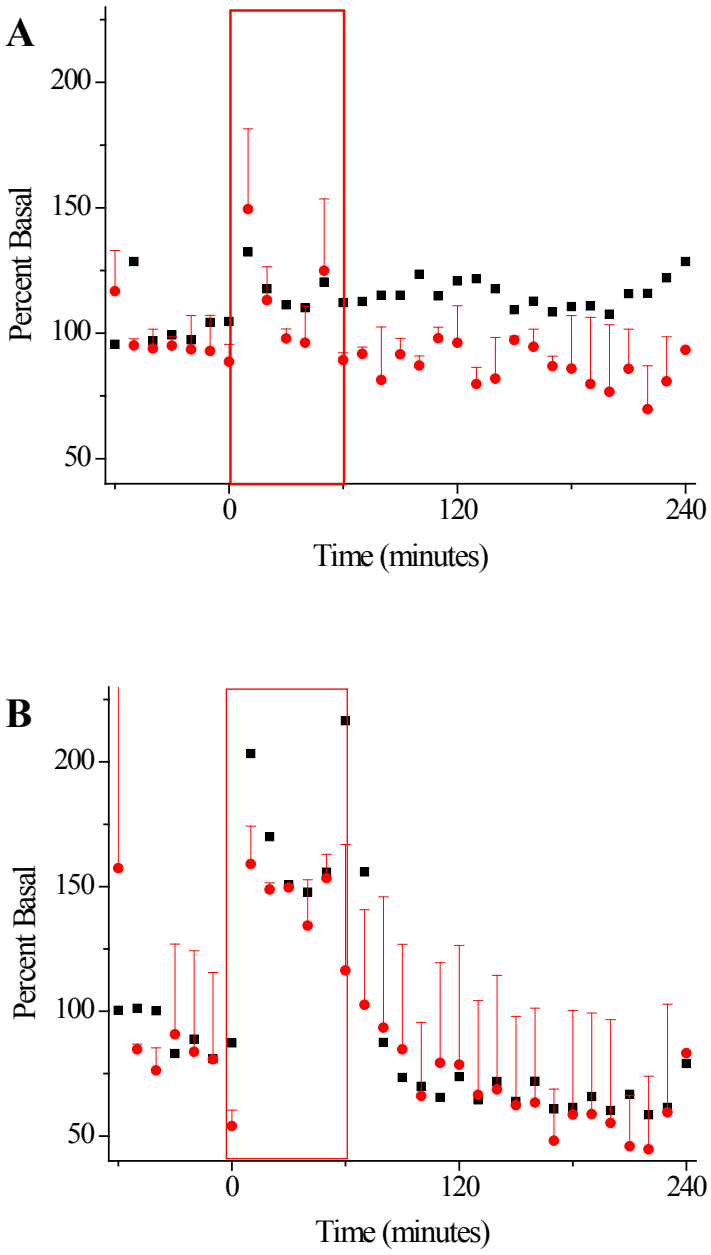

Figure 4.18: Percent deviation for amino acid neurotransmitters versus time for the constant perfusion of 5FU (25 mM) in the hippocampus of anesthetized rats. Comparison of A) glutamate and B) GABA recovered from microdialysate samples as a function of percent basal. Time zero represents start of 5FU (◆) (n=2) or sham (■) (n=1) perfusion for 60 minutes (indicated by box).

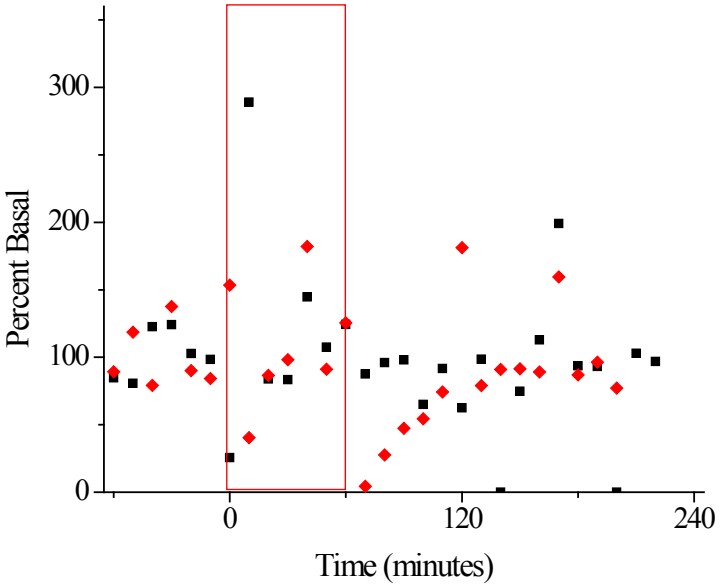

Figure 4.19: Percent deviation for MDA versus time profile for constant perfusion of 5FU (25 mM) in the hippocampus of anesthetized rats. Time zero represents start of 5FU (◆) (n=1) or sham (■) (n=1) perfusion for 60 minutes (indicated by box).

delayed damage to myelin, weight loss, and neurogenesis[109]. Most of these effects are acute and return to normal within weeks to months after cessation of 5FU administration[12]. In a study by Janelisons *et. al.* 5FU resulted in the second highest weight loss, with doxorubicin administration resulting in the highest[108]. The weight loss is most likely due to 5FU targeting proliferating cells, including the ones in the intestinal wall, causing a decrease in nutrient absorption. In another study by Mustafa *et al.* the effects of 5FU on proliferating cells were believed to reduce cognitive ability by inhibiting neurogenesis. Brain-derived neurotrophic factor (BDNF) and doublecortin (DCX), both of which are necessary for neurogenesis, decreased in the dentate gyrus of the hippocampus after five IV administrations of 5FU with leucovorin (which potentiates the action of 5FU) over twelve days in male Lister-hooded rats[46]. In the same study, Mustafa *et al.* also found a decline in spatial working memory using object location recognition. In a mouse study using novel object recognition, no difference was observed in mice receiving weekly injections of 5FU. However, significant weight loss was recorded a few days after every injection of 5FU[38]. Even though most studies report no changes in the brain and cognitive function, some studies do show that biological changes can occur with this chemotherapy drug. Further emphasizing that the extent of these changes and many of the aspects causing these changes are not understood. One important aspect responsible for these differences may be the administration schedule of the chemotherapeutics. Typical chemotherapy compound administration is given to rats on a weekly basis, which makes comparing studies difficult. This is most common treatment interval in animal studies, especially for those simulating adjuvant therapies given to patients for breast cancer. This will be discussed more in chapter five.

As with all other compounds, different administration schedules (slow infusion versus long infusion) can cause different responses. Since 5FU passively diffuses through the BBB[108], levels of 5FU in the cerebral spinal fluid (CSF) and the brain vary depending upon the administration techniques used[110]. Different doses of 5FU exhibit different responses based on concentration, although overall neurotoxicity is quite low with the administration of 5FU. The exception is if the person or animal has a deficiency in the enzyme dihyrodpyrimidine dehydrogenase (DPD). To the best of our knowledge, this is not the case for Wistar rats[50]. The differences seen could also be a result of the different species used or due to the different dosing regimen or reflecting the differences in the human population, where some show cognitive decline and others do not[38].

Due to these compounds not exhibiting any response in the brain perfusion studies, intravenous studies were not performed. The other two compounds, doxorubicin and cyclophosphamide, elicited a change in response with perfusion of the compounds through the microdialysis probe, but not from intravenous infusion. Therefore, it was highly unlikely that 5FU would cause changes with intravenous infusions and not brain perfusions.

4.5.1.9 Carboplatin

Carboplatin is a second-generation platinum chemotherapy drug occasionally used in breast cancer treatment. Carboplatin exerts its anticancer effects by reactions with adjacent guanines on a DNA strand, forming a cross-link between them and preventing DNA replication[111]. The first generation platinum drug was cisplatin, which has several toxic side effects such as neurotoxicity, nephrotoxicity, and many others[111]. Carboplatin does not seem

to have the high levels of toxic side effects as its parent drug. Carboplatin's main toxicity is bone-marrow suppression with minimal nephrotoxicity and neurotoxicity[111-112]. This difference in toxicity might occur due to the structure difference or the cross-links that are formed between the bases on the DNA strands. The structure of carboplatin removes two of the chlorides present on cisplatin and replaces them with a cyclobutyldicarboxylate, as shown in Figure 4.20. This then changes the mechanism of activation, including the cross-links formed on the DNA. Bloomart and collaborators reported cisplatin formed more cross-links intrastrand, while carboplatin formed more cross-links interstrand[111]. Whenever possible, carboplatin is used for breast cancer treatment, due in part to its lower toxicity issues.

In addition, another collaborator (Dr. Michael Johnson) in the Chemistry Department at the University of Kansas monitored the release of DA when different concentrations of carboplatin were administered to male Wistar rats. After four weekly administrations of carboplatin, a decrease in dopamine release was determined with increasing carboplatin concentrations, indicating a correlation between decreasing dopamine release and cognitive function[113]. Thus, it was used in these studies to determine any changes in glutamate, GABA and MDA.

4.5.1.10 Carboplatin brain perfusion

As with the other studies performed for these experiments, carboplatin was perfused directly to the brain. Brain perfusion of carboplatin did not cause any appreciable changes in glutamate or GABA (not shown) (Figure 4.21). This was expected, since carboplatin is reported to cause minimal neurotoxicity. MDA was not analyzed due to interference from carboplatin.

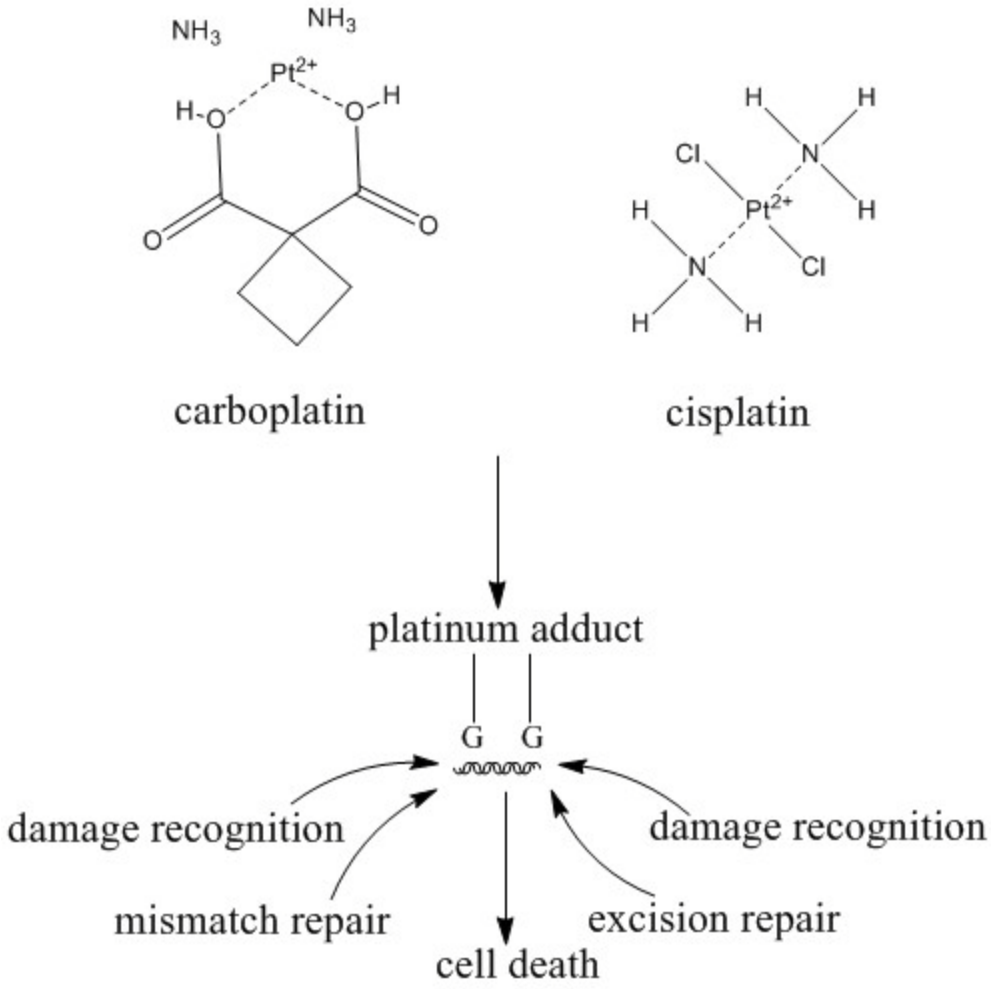

Figure 4.20: Platinum metabolism. Adapted from [116] with permission given by PharmGKB and Stanford University.

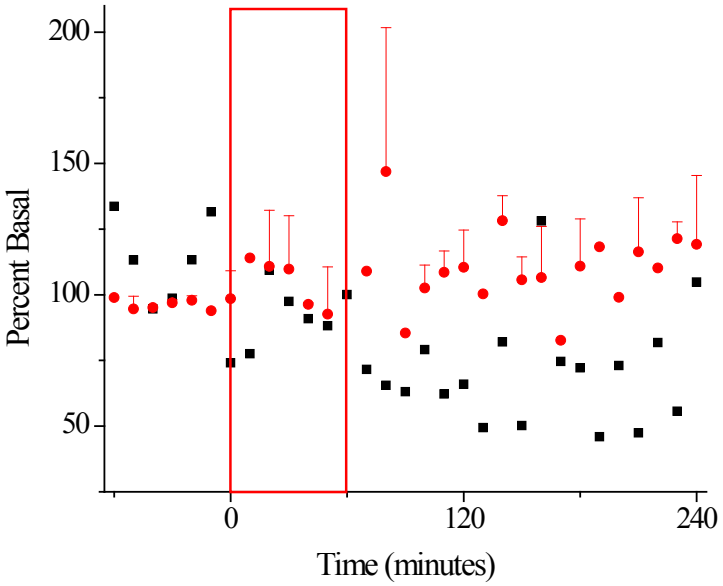

Figure 4.21: Percent deviation for glutamate versus time for the constant perfusion of carboplatin (25 mM) in the hippocampus of anesthetized rats. Glutamate recovered from hippocampus microdialysate samples as a function of percent basal. Time zero represents start of carboplatin (◆) (n=2) or sham (■) (n=1) perfusion for 60 minutes (indicated by box).

Neurotoxicity occurs in 50% of the patients treated with cisplatin, whereas only 5% occurs with carboplatin[114]. This minimal neurotoxicity correlated well with another study using female Wistar rats[114-115]. Carboplatin studies in female Wistar rats, the second-highest ranking of peripheral nerve accumulation of the compounds was observed among the platinum compounds[115]. This is consistent with more peripheral toxicity than neurotoxicity. Due to the loss of nerve function, some people also experience tinnitus and the loss of position sense, which was confirmed in a Sprague-Dawley rat study performed by Okur *et al.*[117]. In this study, high doses of carboplatin increased the production of nitric oxide in the cochlea of female Sprague-Dawley rats, which is associated with loss of positioning, due to the loss of nerve sensation throughout the body. In this same study pre-treatment with N-aceytlcysteine (NAC) prior to carboplatin administration seemed to decrease the production of nitric oxide, which would then decrease the formation of RNS/ROS. Again, these changes were primarily seen in the peripheral tissues and not in the brain, therefore it was not expected to see significant changes in the analytes monitored, in these acute experiments.

Since direct perfusion of carboplatin did not cause any changes in neurotransmitters, it was not studied with intravenous administration. It seemed highly unlikely that a change would be seen with systemic administration, since a change was not seen with direct administration to the brain. In addition, with limited toxicity reported in literature, short term changes were not likely to be seen in these studies. This compound was studied in the long-term experiments, which will be discussed in chapter five.

4.6 Conclusions

With direct perfusion of doxorubicin and cyclophosphamide, an increase was observed in glutamate and GABA, while 5FU and carboplatin did not have any appreciable changes with the perfusion of these compounds directly to the brain. Due to doxorubicin's redox cycling, the highest increase in glutamate and GABA was seen with this compound, with the return to basal levels taking several hours. While cyclophosphamide increased glutamate and GABA levels immediately upon perfusion, both glutamate and GABA levels immediately returned to basal levels after the dosing period. These results indicate the changes that would be observed in glutamate and GABA with direct compound administration. Interestingly, this corresponds well with the reports from human patients, with cyclophosphamide and doxorubicin patients generally experiencing more cognitive issues than other treatment regimens, such as 5FU and carboplatin. GABA and MDA were also monitored in these experiments; however, a change was not observed or was not detected due to interferences. Intravenous administrations of cyclophosphamide and doxorubicin were also performed, but no significant changes were observed during the time frame of these experiments. More experiments with these compounds were also tested in the next chapter to determine the long-term effects of intravenous administration of these compounds.

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~Chapter 5~

Monitoring chemobrain in long-term experiments by microdialysis

5.1 Introduction

Microdialysis is a viable technique to monitor multiple analytes in a variety of tissues in a single entire experiment using one animal. It has been used extensively for short term experiments, while only a few studies have been repeated that utilize microdialysis sampling for longer experiments (one month)[1-2]. Typical microdialysis experiments are performed over a few days to a couple of weeks. This sampling technique has been used to investigate nervous system diseases (e.g. dopamine-related disorders), neurobiological events (e.g. glutamate and GABA), and neurobiological mechanisms [3]. The use of microdialysis sampling to monitor neurochemical processes in long-term studies could provide valuable information about these disease processes. Two microdialysis studies have been published where various neurotransmitters have been monitored for up to a month; however the success rate is not reported, and each report only shows the results for a single rat[1-2]. The goal of this chapter was to develop robust, reliable and sensitive procedures for long-term microdialysis studies and apply it to chemobrain studies.

The ability to monitor various neurochemical processes continuously over an extended period of time could make it possible to elucidate the point at which neurotransmission and oxidative stress occurs in chemobrain, and to what extent these changes occur. In the previous chapter, short-term microdialysis experiments were performed with several chemotherapy drugs. Appreciable changes were seen following direct perfusion of doxorubicin and cyclophosphamide; however no changes were seen in intravenous studies. This indicated that these chemotherapy agents do not cause any short-term changes in the brains of anesthetized

animals. In the experiments described in this chapter, the chemotherapy agents will be tested in long-term studies, where one animal will be monitored throughout the entire time for a maximum of 10 weeks. Since the animals are monitored continuously before, during and after chemotherapy treatment, this will provide more information regarding the long-term of chemotherapeutics on the brain than obtained from longitudinal studies in human patients or other animal studies.

Chemobrain typically occurs in human patients' weeks to months after the initial treatment, and in some cases years later[4-5]. This same varied time course has also been observed in animal studies. The variable results could be from the fact that neurochemical processes with conventional sampling methods, such as decapitation are only monitored at discrete time points; therefore, if the neurochemical process only changes for a short period of time or if one animal has a higher basal level than another animal, this would provide mixed results.

5.1.1 Chemotherapy treatment

In order to compare the effects of chemotherapy administration between human patients and animal studies, the timing of the administration of the chemotherapy compounds need to simulate to the patient and the animal as much as possible across species. In order to do this, the administration schedule, dose, and concentration should be as close as possible.

5.1.1.1 *Human patient studies*

5.1.1.1.1 Administration agents

The treatment of breast cancer requires or involves many different chemotherapy agents and regimens, which adds to the difficulty of determining which compound or combination is

causing chemobrain, especially in human patients. Doxorubicin (adriamycin), cyclophosphamide, 5FU, and epirubicin are common drugs used for the treatment of breast cancer. These agents are typically used in combination chemotherapy. Breast cancer patients at the University of Kansas Medical Center (KUMC) typically receive treatment with one of the combinations shown in Table 5.1. Another aspect in human patients to keep in mind is the most important objective of these chemotherapy regimens is patient survival. Now that the survival rates are increasing substantially and the life-span after treatment is increasing as well, more focus is being given to the quality of life after cancer treatment, as discussed in chapter 3.

5.1.1.1.2 Administration schedule

The administration schedule in human patients for most chemotherapy agents is once every three weeks. This is the schedule that is used for the adjuvant chemotherapy agents AC±T, FAC, and FEC. If a more intense administration schedule is necessary, AC±T may be administered once every two weeks instead of every three weeks. An adjuvant therapy that deviates from this schedule is CEF. In this treatment regime, cyclophosphamide is administered every day for 14 days, with epirubicin and 5FU given on days 1 and 8. Since the most common administration cycle is once every three weeks, this was the administration schedule that was simulated in these rat experiments.

5.1.1.2 *Animal research studies*

5.1.1.2.1 Administration agents

The goal of these animal studies was to administer the same chemotherapy agents given in human patients and to determine the effects of these agents on different

Regimen	Cycles		Frequency
AC	4	Adriamycin Cyclophosphamide	 every 3 weeks dose dense every 2 weeks
FAC	6	5 Fluorouracil Adriamycin Cyclophosphamide	 every 3 weeks
FEC	6	5FU Epirubicin Cyclophosphamide	 every 3 weeks
CEF	6	Cyclophosphamide Epirubicin 5 Fluorouracil	D1-D14 D1 & D8 D1 & D8
AC----T	4	Adriamycin Cyclophosphamide	every 3 weeks dose dense every 2 weeks
T	4	Docetaxel (Taxotere) or Paclitaxel	every 3 weeks dose dense every 2 weeks
TAC	6	Docetaxel (Taxotere) Adriamycin Cyclophosphamide	 every 3 weeks
CMF	6 or 12	Cyclophosphamide Methotrexate 5 Fluorouracil	D1 & D8 D1 & D8 D1 & D8 every 4 weeks

Table 5.1: Common chemotherapy regimens for breast cancer patients at KUMC.

neurochemical pathways in rats. One of the advantages of animal studies is the ability to administer these compounds individually or in combination, which is not ethically possible in human cancer patients. In addition to the different administration possibilities in animal studies, different concentrations of the same compound(s) can be given as well. This allows the effects of dose on neurochemistry to be determined. In human patients this is not the common practice, as discussed in the previous chapter, and a comparison between a "standard"-dose group and a "high-dose" group typically consists of patients that have been administered different agents. In these human studies, it is difficult to determine if the decrease in cognitive function is due to the higher concentrations or from the different compounds used, or a combination of both. Animal studies can help elucidate this information. Since doxorubicin and cyclophosphamide are the primary agents used in human chemotherapy treatments, these compounds were initially tested in the long-term animal experiments.

5.1.1.2.2 Administration schedule

In addition to using the most common chemotherapy agents, administering these agents on the same schedule is important as well. To replicate the dosing schedule of human patients in rats, the typical dosing schedule is weekly administrations. This shortened administration interval is common because a rat's life expectancy is much shorter than humans, with a typical lifespan of 3 years for rats compared to 78 years for humans. Shorter administration intervals could be utilized; however, toxicity usually occurs in animals with chemotherapy compounds due to the systemic damage of these agents. For example, when doxorubicin is given systematically, widespread oxidative stress occurs throughout the body, leading to death if administration is repeated to soon[6]. For the most part, weekly administration intervals are commonly used in the majority of animal studies and will be used in these studies.

5.1.1.3 *Neurochemical information collection*

Neurochemical information can be collected in numerous ways in animal studies, as discussed in chapter 1, which again emphasizes the benefits of animal research in addition to human studies. Numerous animal studies have been performed to monitor the effects of chemotherapy administration, although most of these animal studies sample at discrete time points since most techniques utilize tissue homogenation. These studies provide valuable information; however this method requires numerous animals per time point in order to obtain statistically significant differences and exhibits increased variation due to interanimal variability. Moreover, these studies do not collect any samples between the selected time points, loosing valuable temporal information. When the analytes of interest are neurotransmitters, tissue homogenation techniques measure the concentration not only in the extracellular fluid (ECF), but also from the intracellular space as well (i.e. total brain content). Since the neurotransmitters in the ECF are usually the ones that induce other mechanisms/processes, these are the ones of primary interest.

A sampling technique that monitors the analytes in the ECF and is commonly used in other studies is microdialysis. This sampling method allows the neurochemical and oxidative stress events of the chemotherapy treatment to be monitored throughout the dosing regimen in each animal, which will decrease the interanimal variability, simulating the longitudinal studies in the human cognition studies. To date, only one other chemotherapy study has utilized microdialysis sampling to monitor the neurotransmitters (dopamine, norepinephrine, and serotonin) upon the administration of methotrexate intracerebroventricularly in male Wistar rats[7]. In addition to neurotransmitters, oxidative stress biomarkers can also be monitored with microdialysis[8]. Multiple neurotransmitters and oxidative stress biomarkers have been directly

correlated to cognitive and psychological function of many studies[3, 9-10]. Therefore applying this sampling technique to monitor these biochemical processes during chemotherapy could potentially elucidate important information regarding etiology to the decrease in cognitive function in humans.

The goal of these experiments was to implement microdialysis sampling over two months to study the effects of chemotherapy on the brain. In this study, the selected analytes were sampled continuously throughout the entire experiment utilizing microdialysis sampling. The longest continuous microdialysis experiment repeated to date has been one month, and it appears this was only accomplished in a single Sprague-Dawley rat[1]. This strain of rat is less aggressive than Wistar rats. Another long-term study monitoring dopamine with microdialysis sampling went 23 days in a male Wistar rat[2]. The success rate of these long-term studies was not really discussed in either of these articles. Therefore, the goal of this project was to determine if long-term studies could be applied to the study of chemotherapy agents.

5.2 Specific aims of research

The ultimate goal of the research described in this chapter was to optimize the microdialysis experiments to monitor different neurochemical processes over months, instead of days. With microdialysis sampling, an animal can be monitored before, during and after compound administration, which allows the same animal to serve as its own control in the long-term studies. Short-term animal experiments were monitored to determine the immediate effects of different chemotherapeutic compounds. The ability to monitor the administration of different chemotherapeutic compounds in long-term experiments would provide valuable information to elucidate the long-term mechanisms responsible for chemobrain that were not found in short-term studies.

For the long-term microdialysis studies many issues had to be addressed. Since the majority of microdialysis studies only last less than one week, numerous aspects of the study had to be optimized in order to increase the success rate of the long-term experiments. The desired protocol in these studies was to monitor the rats for a week prior to drug administration, the duration of the chemotherapeutic administration, and six weeks post administration (i.e. a total of 10 weeks). This would be the longest microdialysis experiment to ever be recorded. The methods implemented to accomplish these long-term studies will be discussed in detail. In addition to keeping the animals viable during the long-term experiments, several analytes were monitored, the neurotransmitters such as glutamate, GABA, and the catecholamines, and MDA as a marker for oxidative stress.

5.3 Chemicals and solutions

All of the chemicals were obtained as described in the previous chapters.. Ropivacaine hydrochloride injection (AAP pharmaceuticals, LLC.; Schamburg, IL) was purchased from Patterson Logistics (Webster veterinary; Kansas City, MO, USA)

5.4 Microdialysis methods

5.4.1 Surgical procedures

All animal experiments were performed in accordance with IACUC animal protocols. All experiments utilized male Wistar rats (Charles-River, Portage, ME) weighing 250-500 grams, unless otherwise specified. Animals were kept on a 12 hour light/dark schedule at all times, and had free access to food and water. The body temperature of the rat was maintained at 37°C by an automated heating pad (CMA Microdialysis AB, Sweden). All solutions were filtered using a 0.22 micron nylon syringe filter (Pittsburgh, PA, USA). Surgical tools, drapes,

sutures, cannulas, and other items were either sterilized by ethylene oxide, autoclave, or opened from a package.

Animals used in the long-term experiments were pre-anesthetized with isoflurane and maintained by isoflurane throughout the duration of the surgery. All animal surgeries for the long-term studies were performed aseptically. Incision sites were prepared by shaving as much hair around the immediate area as possible and by performing alternate scrubs of betadine and alcohol (70%) at least three times. After femoral cannulation implantation and/or brain surgery, the animals were allowed to recover in the Raturn® system (BAS, West Lafayette, IN), unless stated otherwise. All rats received hydration (25 mg kg^{-1} of saline) by subcutaneous injection before surgery. Prior to brain surgery, the local analgesics lidocaine and ropivicaine were applied to three places: ears (via cream) subcutaneously and topically directly on the skull. Special permission from the IACUC was granted to not administer any systemic analgesic to avoid any inhibition of enzymatic pathways that would prevent changes in the neurotransmitters or oxidative damage in these long-term experiments upon chemotherapeutic administration. If prolonged enzyme inhibition results from the administration of analgesics, then these agents could provide a protective effect to any biological mechanisms that would otherwise result in changes in biological function.

5.4.1.1 *Brain probe implantation*

This procedure was performed as described in Section 4.4.1.1.

5.4.1.2 *Intraperitoneal injections*

In the initial experiments animals were administered the appropriate adjuvant therapy or saline via i.p. injections. Several restraint techniques were tested for this administration route for

the long-term experiments. At first the rats were administered the compounds while still connected to the Raturn® system with the brain probe connected, in order to minimize handling and sampling lapses. However, this technique was not successful since these animals were not used to being handled in this manner and the access to the Raturn® cage was very narrow. In the Raturn® system, animal restraint is limited and ineffective, and the animal can move more than desired during chemotherapeutic administration. The movement caused more damage to the cannula and probe than ideal, and the rat would usually displace the harness straps in the process. Further experimentation found that it was beneficial and caused less stress to the animal to remove it from the Raturn® and give it the i.p. injection. Towels were used to cover the animals to minimize the stress, as instructed by the ACU veterinarian. During the administration, the rats would still cause some damage to the brain cannula since the towel would slip and move reducing the ability to restrain the rats effectively, especially the rats given the chemotherapeutic compounds. In addition, over time the rat harness tethered to the Raturn® would also cause irritation behind the front legs and/or in the middle of the chest. This irritation would cause the rats to loose their hair and by week two they would generally have the area red and/or raw. Any handling with any redness increased the agitation of the rat with any handling. Due to these conditions, the animals were implanted with a femoral cannula for the remaining long-term experiments for dosing to minimize the effects from handling and to lower the stress levels of everyone involved. The animals were also acclimated to human handling at least two weeks before surgery to minimize the stress of handling during the study.

5.4.1.3 *Jugular vein implantation*

This procedure was performed as described in section 4.4.1.2. In addition to the technique described previously, the animals underwent the following procedures. First the neck

was shaved as closely as possible between the shoulder blades and on the ventral side around the pectoral muscle (or around the jugular vein if it was visible). This neck area then underwent alternating scrubs with betadine and 70% alcohol, and a nick was made superior to the shoulder blades. A sterile piece of gauze was placed on the nick before the rat was positioned on its dorsal side. The ventral area was then shaved and scrubbed with betadine and 70% alcohol.

5.4.1.4 *Femoral vein implantation*

The femoral vein cannulation was performed in a similar manner as the jugular cannula implantation. A nick was made on the dorsal side of the rat between the shoulder blades immediately after the alternating scrubs. The animal was then placed on its back and an incision, approximately 1 cm long, was made in the midline of the area adjoining the hind leg and the abdomen. The blood vessels were then located and adventitious tissue was cleared from the blood vessels. MicroRenathane® (MRE-033) tubing (Braintree Scientific, Braintree, MA, USA) or a BAS femoral cannula tubing (BAS, West Lafayette, IN, USA) was externalized by tunneling a trochar subcutaneously to the back of the neck. A suture was superficially threaded in the abdomen tissue to hold it out of the way, if necessary. The femoral vein, the large blue vessel, was then carefully isolated from the connective ligament and femoral artery, the larger red vessel, by the insertion of forceps. The adventitious tissue was then completely removed from the isolated vein with forceps or a cotton swab with saline on it, with a flat metal spatula under the vein. Removal of the adventitious tissue was extremely important in order to make a small cut in the vein with the spring scissors (Fine Science Tools, Foster City, CA, USA). Before cutting, two ligation sutures (3-0 Dexon) were put around the isolated vessel approximately 1 cm apart. After the nick was made in the vein near the lateral ligation, tubing was inserted as quickly as possible into the vein. Sometimes a guide, which was a metal dental pick, was needed

to get the tubing into the vessel, especially if any delays of tubing insertion occurred. The tubing was inserted approximately 4-5 cm into the femoral vein, whenever possible, to the vena cava. Insertion of the tubing to the proper location was a prominent issue in Wistar rats, as discussed in section 5.5.5.1.

5.4.1.5 *Vein cannula infusions*

The jugular vein was cannulated before the implantation of the brain cannula using the procedures described above. Immediately after the surgeries, the brain probe would be perfused with aCSF, as described above, and the jugular cannula would be infused at 1 μL min^{-1} with saline, unless stated otherwise.

5.4.1.6 *Long-term microdialysis system*

A Raturn® system (BASi, West Lafayette, IN, USA) was utilized for the long-term experiments. This system allowed for continuous monitoring of the animals by both recording the activity of each animal and by collecting microdialysis samples for analysis on multiple analytical systems. In order to do this, the system was comprised of the following components: a BASi Culex® four station mobile cart, a BASi Culex® metabolic and stackable cages, a BASi® syringe pumps (BASi, West Lafayette, IN, USA), a Hamilton syringe 5 mL (Reno, NV, USA), and a BASi Honey Comb Fraction Collectors. The BAS syringe pumps were set at 0.20 μL min^{-1} (with a 5 mL syringe this equals 1 μL min^{-1}), which was then connected to approximately 75 cm of FEP Teflon microdialysis tubing (BAS, West Lafayette, IN, USA) to the inlet of the CMA microdialysis brain probe. The outlet of the brain probe was connected to approximately 80 cm of FEP Teflon microdialysis tubing and then connected to the BASi Fraction Collector for sample collection in 250 μL polypropylene microcentrifuge tubes

(Pittsburgh, PA, USA). For long term experiments, sample collection was set for 30 or 60 minute intervals. Samples were put in the -80°C freezer until analysis.

5.4.2 Analytical methods

5.4.2.1 *Amino acid neurotransmitter analysis*

This procedure was performed as described in section 4.4.2.2.

5.4.2.2 *Catecholamine neurotransmitters and metabolites*

Prior to sample collection, a solution of 0.1M perchloric acid with 0.1% cysteine was put in the 250 µL polypropylene microcentrifuge tubes in a 1:10 v/v ratio (e.g. 1 µL of 0.1 M perchloric acid with 0.1% cysteine was added for every 10 µL of microdialysis sample). A portion of the microdialysis sample (10 µL) was put in a separate vial along with 1 µL of 550 nM 3,4-dihydroxybenzylamine-HBr (DHBA) immediately before analysis. This sample (11 µL) was mixed by vortex and then immediately injected (10 µL) onto the following system: Shimadzu LC-20AD prominence LC pumps, a Rheodyne 7725i stainless steel sample injector connected to an Agilent Zorbax SB-C18 Micro Bore Rapid Resolution column (1.0 x 150 mm, 3.5 micron, PJ Cobert Associates Inc., St. Louis, MO, USA), and a BAS Amperometric Detector LC-4C (BASi, West Lafayette, IN, USA). The electrochemical cell was a 3mm glossy carbon electrode embedded in a PEEK block (BASi, West Lafayette, IN, USA), at a potential of +750 mV vs Ag/AgCl reference electrode (BASi, West Lafayette, IN, USA). The flow rate was set at 0.1 ml min^{-1} with the mobile phase consisting of 140 mM sodium phosphate monobasic anhydrous, 0.75 mM 1-octanesulfonic acid, and 2 mM ethylenediamine tetraacetic acid, disodium salt dehydrate (EDTA) with the pH adjusted to 3.5 with 85% o-phosphoric acid with

9% methanol added prior to final dilution of mobile phase. The data was collected with Chrom & Spec software version 1.5 (Ampersand International Inc. Beachwood, OH, USA).

5.4.2.3 *Malondialdehyde analysis*

This procedure was performed the same as described in Section 4.4.2.4.

5.5 Results and discussion

5.5.1 Column comparison for catecholamine analysis and their metabolites

The type of chromatography column needed for the analysis of catecholamines and their metabolites was investigated. A smaller i.d. column than what was typically used in our laboratory with a C18 stationary phase, Zorbax (Agilent) with 3.5 µm particle size, was utilized, based on previous methods used in our laboratory. The initial column tested for catecholamine analysis consisted of a column with an i.d. of 2.1 mm and length of 75 mm with 3.5 µm particle size. Using this column, norepinephrine was not well separated from the void, as shown in Figure 5.1. For simplicity, a column with a different i.d. and length was tested, while all of the other parameters were kept consistent. First a Zorbax column with a smaller i.d. (1 mm) was used, with a length of 50 mm. By decreasing the i.d. of the column, a reduction in band broadening was expected to obtain well-resolved peaks in a short amount of time. As shown in Figure 5.2, the norepinephrine peak co-eluted in the void peak. To increase the capacity factor, a Zorbax column with a length of 150 mm was tested, while keeping the i.d. at 1 mm. This column separated the norepinephrine peak from the void peak (Figure 5.3). The separation of norepinephrine was excellent in both the standard and rat samples and was used in all further studies.

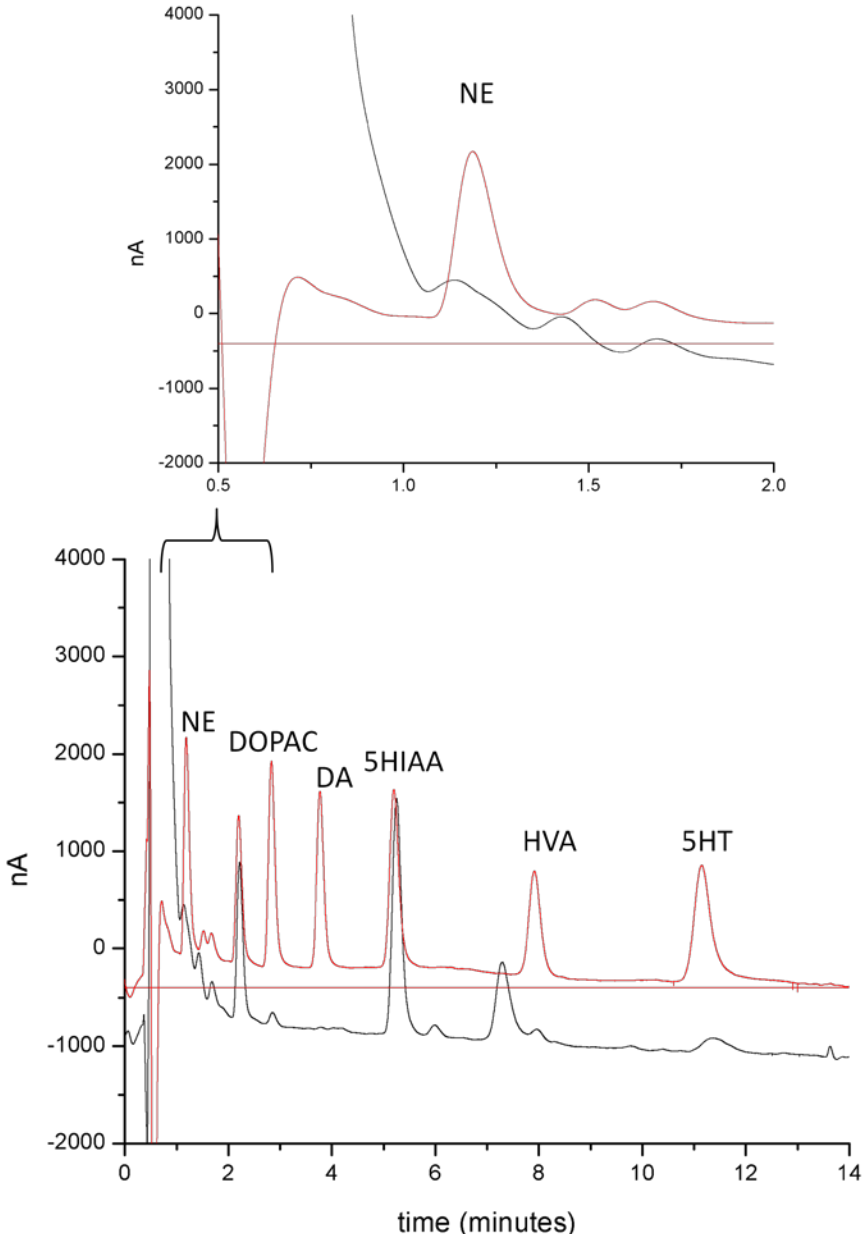

Figure 5.1: Chromatogram comparison of A) 50 nM standard mix (with NE, DOPAC, DA, 5HIAA, HVA, and 5HT) (red) and B) rat sample (black) separated on a Zorbax C18 column (3.5 µM SBC18 2.1 x 75 mm). Graph A) consists of a narrow window to view the NE peak, while B) represents the entire chromatogram.

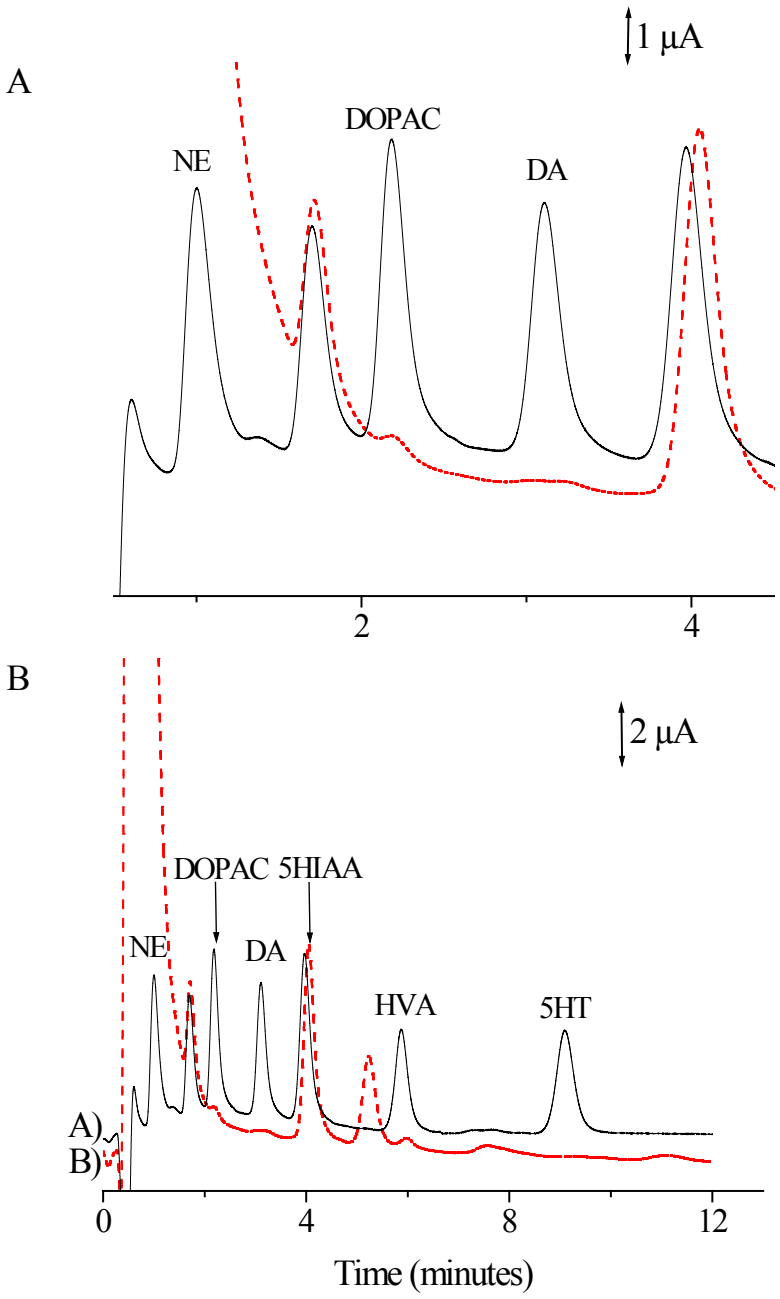

Figure 5.2: Chromatogram comparison of A) 50 nM standard mix (with NE, DOPAC, DA, 5HIAA, HVA, and 5HT), and B) rat sample separated on a Zorbax C18 column (3.5 µM SBC18 1 x 50 mm). Graph A) consists of a narrow window to view the NE peak, while B) represents the entire chromatogram.

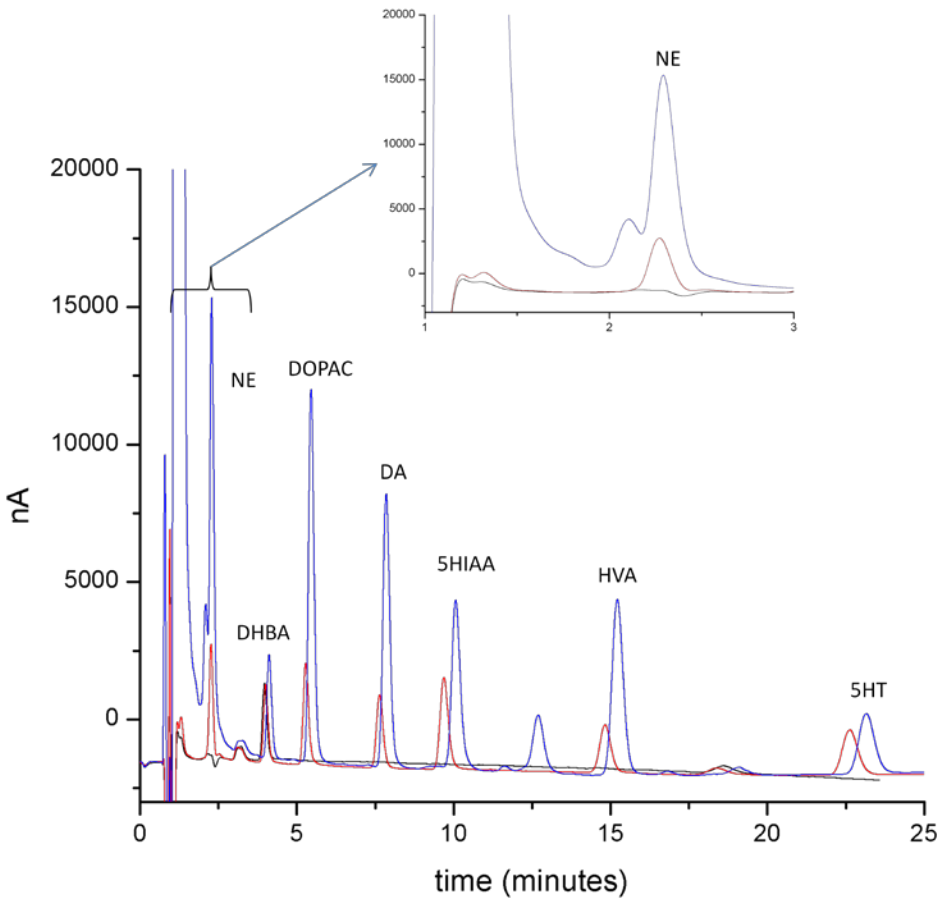

Figure 5.3: Chromatogram comparison of aCSF sample (black), 50 nM standard mix (with NE, DOPAC, DA, 5HIAA, HVA, and 5HT) (blue) and rat sample (red) with a Zorbax C18 column (3.5 µM SBC18, 1 x 150 mm). Graph A) consists of a narrow window to view the NE peak, while B) represents the entire chromatogram.

5.5.2 Catecholamine stability comparison

To assure sample stability of the catcholamines and their metabolites, different concentrations of perchloric acid were added to the microdialysate sample tested with and without cysteine. As seen in Figure 5.4, dopamine was more stable with the addition of 0.1 M perchloric acid and cysteine than with 0.1 M perchloric acid by itself or with 1 M perchloric acid. Sample one represents the level of dopamine at time zero. Sample two reflects the sample immediately stored in the -80° C freezer and then analyzed a week later (i.e. 7 days), while samples three and four were kept in the fraction collector for at least 12 hours and then put in the -80° freezer for several days. As shown in Figure 5.4, the best stability was observed for samples that contained 0.1 M perchloric acid and 0.1% cysteine. This solution was added to the vials in all further studies to decrease sample degradation.

5.5.3 Animal acclimation

Based on the experience of a few long-term experiments, animal acclimation to human handling was determined to be extremely beneficial prior to any surgical technique. Throughout these long-term experiments, the animals required multiple procedures that necessitated handling the rats throughout these 10 week studies. Any rat handling was done in a careful manner to prevent damage to the brain cannula (and also the intravenous cannula) to allow successful sampling of the various analytes throughout the duration of the experiment. Animal handling was necessary throughout the duration of the experiment for events such as weight measurements, and cage changes, as required by IACUC regulations. Additional handling was necessary to fix issues, such as low dialysate volumes, microdialysate tubing disconnected from the probe, and from harness movement, which increased with the new protocol of keeping the harness not as tight as the typical protocol for short-term experiments.

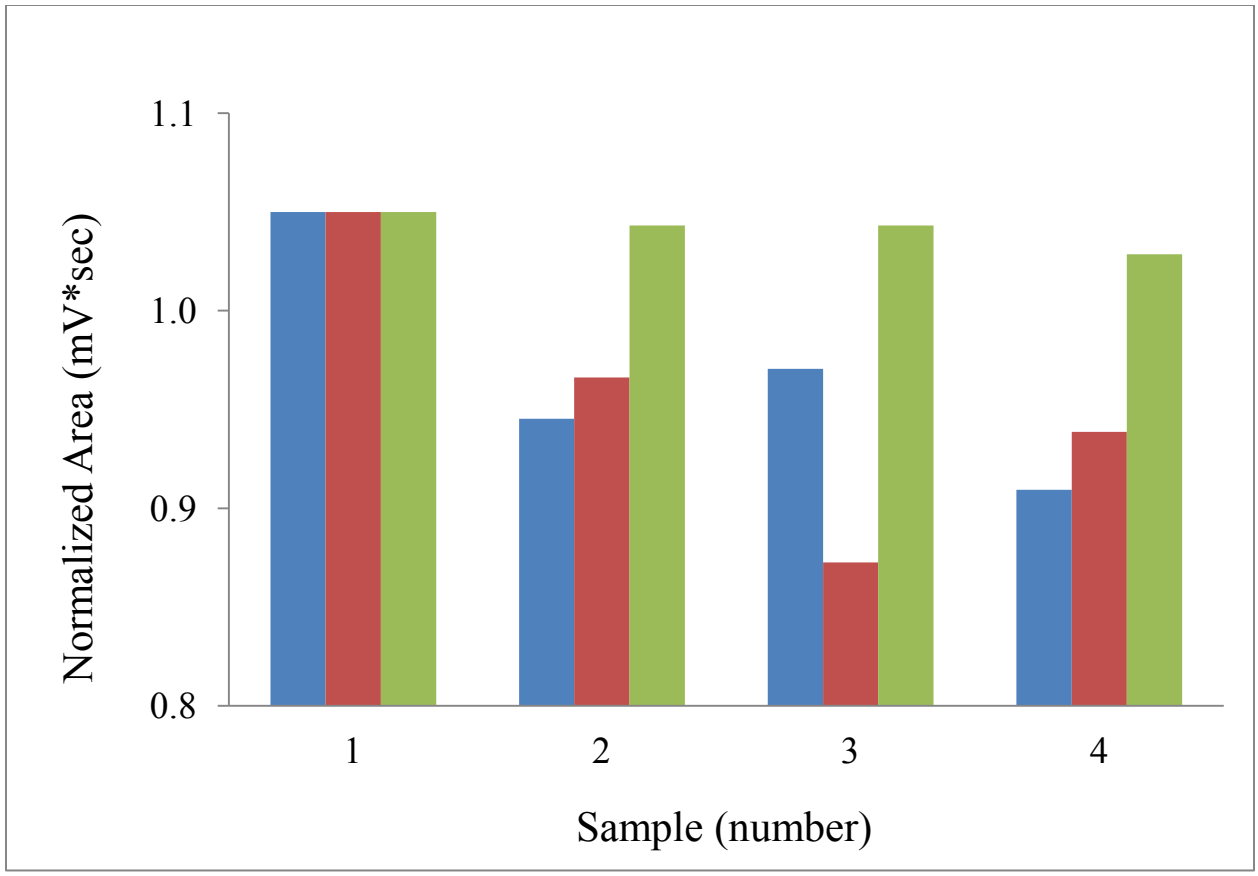

Figure 5.4: Stability comparison of dopamine with 0.1 M PCA (■), 1 M PCA (■), and 0.1 M PCA with 0.1% cysteine (■). Sample 1) immediately analyzed, 2) immediately stored in -80°C freezer, 3&4) stored in fraction collector for a minimum of 12 hours and then stored in -80°C freezer until analysis. All samples were n=1.

With this much handling, animal acclimation to handling prior to any surgery was determined to be essential to minimize the nervousness of the rat, thereby reducing the aggression, which increased the success rate of the experiments. Positive re-enforcement was also utilized since it was found to increase the success rate of the experiments. This was accomplished by giving the rats' crackers or treats in order to increase the positive interaction between the animal and its handler. Treats were used prior to the experiment and throughout the experiment as a continual reward system, as recommended by the attending ACU veterinarian.

5.5.4 Chemotherapy administration: long-term studies i.p. administrations

5.5.4.1 *Animal i.p. administration*

In the first set of experiments, i.p. administration was tested to determine the execution and application to performing long-term microdialysis experiments. The results of i.p. administration led to the control rat lasting over a month prior to experiment termination, while the rat receiving chemotherapy treatment only lasted three weeks. Rats administered chemotherapy had a shorter time-profile. This is because the brain cannula was damaged during the struggle to administer the chemotherapy to the rat. This increase in resistance to being handled was more pronounced in the rats administered chemotherapy than the control rats, which led to more damage to the brain cannula of the rats receiving chemotherapy compounds. To minimize rat handling and the loss of data points, the rats were originally administered the compounds by i.p. injection while in the Raturn®; however, this method seemed to be problematic. If the rat struggled, the rat would move the harness to an inappropriate position. This would then cause the rat more discomfort leading to more agitation. To increase the success rate and reduce the stress levels of the rat, the next logical approach at the time consisted of unhooking the animal from the Raturn® to administer the compounds. This was done for

both the control and chemotherapy administered rats. The removal of the animal from the Raturn® did increase the success rate initially, especially in the animals receiving chemotherapy. However, over time, the rat's receiving chemotherapy still became extremely agitated and were more difficult to restrain during administration than the control rats. This was probably due to the burning sensation caused by the chemotherapy drugs, especially doxorubicin. Any administration mixture that contained doxorubicin would always lead to a more excited state or even aggressive behavior. If this occurred, isoflurane would be used to get the animal tethered quickly and correctly in the Raturn® to minimize the loss of data points.

To minimize animal handling, microdialysis studies typically utilize cannulas or probes for the administration of the desired compounds. This is especially important when monitoring neurotransmitters, since handling can cause a response in itself. In these long-term studies, i.p. administration was tested first to reduce complications from long-term cannula failure. Short-term studies generally only last a few days or a couple weeks and cannula patency is not a huge concern. In chemotherapy administration studies, the compounds are typically administered in weekly intervals over four weeks. The cannula patency in microdialysis studies must last an additional week for basal level determinations. This means the cannula has to remain patent for five weeks during these studies. A typical cannula is only patent for three or four weeks. Therefore i.p. administration was attempted first to hopefully increase the success rate of the experiments. Even though i.p. administration did not work with these compounds, i.p. administration might be more applicable to other chemotherapy compounds, such as carboplatin, that have a lower burning/stinging sensation upon administration. However, due to doxorubicin's well-known ROS generation and cyclophosphamide's induced change in glutamate in the short term studies, these compounds were the main focus of these long-term

experiments. Furthermore, more cognitive complaints are received for these compounds (doxorubicin and cyclophosphamide) than for the other compounds (5-FU and carboplatin) with the KU Medical Center patients receiving treatment for breast cancer.

5.5.4.2 *Analysis of microdialysis samples*

Glutamate increased by 8-fold in the hippocampus for the rat receiving doxorubicin (3 mg kg^{-1}) and cyclophosphamide (30 mg kg^{-1}) by i.p. administration; however a increase of four-fold was also seen in the control rat (saline), as shown in Figure 5.5. The results were the same for both weekly, week one and week two, administrations of doxorubicin and cyclophosphamide. The increase in glutamate observed in the rats administered chemotherapeutics could have been from the handling or anesthesia, and not the actual adjuvant therapy itself. In addition, the increase was only seen in a few samples, since the samples were collected in 60 minute intervals. These samples were also initially analyzed for catecholamines (e.g. dopamine, norepinephrine and their metabolites). During the analysis of these compounds, issues arose in the detection system. Sample analysis then got backlogged very quickly, and unfortunately the samples became unusable due to oxidation of the catecholamines. To prevent this from happening in the future, two systems were set up, one for each analytical system (amino acids and catecholamines). This prevented a back log of samples from occurring if one system quit working, especially for the catecholamine measurement. In future experiments, 0.1 M perchloric acid with cysteine was also added to the vials to improve stability of the catecholamine sample.

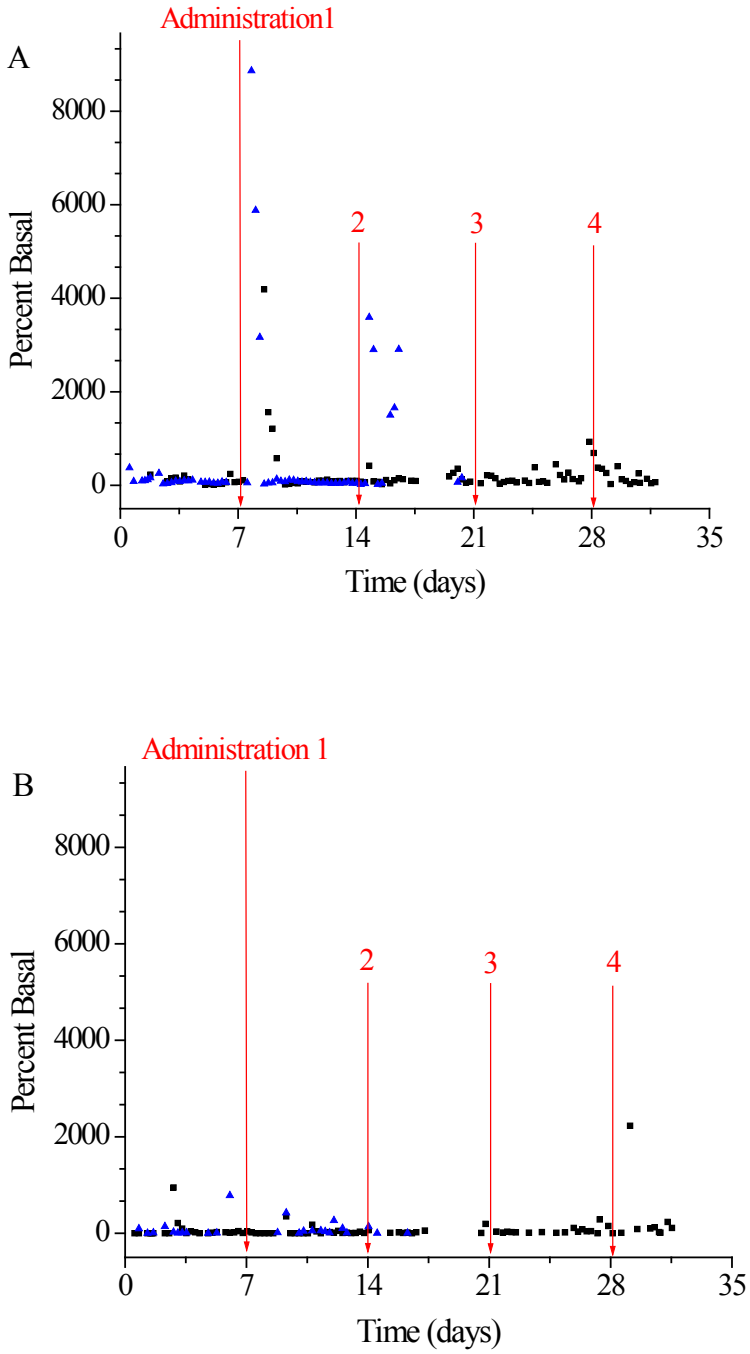

Figure 5.5: Percent deviation for amino acid neurotransmitters versus time profiles for i.p. administration of doxorubicin (3 mg kg^{-1}) and cyclophosphamide (30 mg kg^{-1}) in the hippocampus of awake Wistar rats. Comparison of A) glutamate and B) GABA recovered from microdialysate samples as a function of percent basal. Each red arrow represents the i.p. administration of chemotherapy (▲) or vehicle (■) by a bolus dose.

5.5.5 Chemotherapy administration: long-term studies i.v. administration

5.5.5.1 *Wistar rat femoral cannulation*

The goal of these experiments was to determine the applicability of intravenous (i.v.) cannulation to administer chemotherapy to the rats without exciting the animals. Wistar rats are commonly used for chemotherapy studies and were utilized in the initial studies (i.p. administration) here as well. Unfortunately, the implementation of a cannula in the femoral vein of the Wistar rat was much more difficult than expected. Upon insertion, the cannula would typically get stuck at the iliac branch, which is around two to three centimeters in the femoral vein, which is common in this strain of rat. The typical cannula implantation would have the cannula in the vein approximately four to five centimeters. After several attempts it was determined that Wistar rats were much more different to cannulate than other rats typically used in the lab (e.g. Sprague-Dawley). After several failed attempts (e.g. vein tore apart), histology was performed on a Wistar rat it was found that the end of the cannula was located in the iliac branch (Figure 5.6). Ideally the cannula should be extended well into the inferior vena cava, above the iliac branch, to minimize issues with the patency while maintaining the position of the cannula. With the cannula in the iliac branch instead of the vena cava, there is an increased chance of blood clots, since there was not any blood flow in this vessel. Cannula removal by the rat was also more likely, since the cannula was only in two centimeters inside the vein instead of the typical four to five centimeters. To solve this problem, different cannula tubing was tested. Initially, MRE tubing was utilized, which has an i.d. of 0.25” and o.d. of 0.37”. Other cannula tubing styles were also tested, such as the femoral-short catheter, the jugular catheter, and a portal vein catheter all manufactured by BAS, with the idea that these might have a smaller

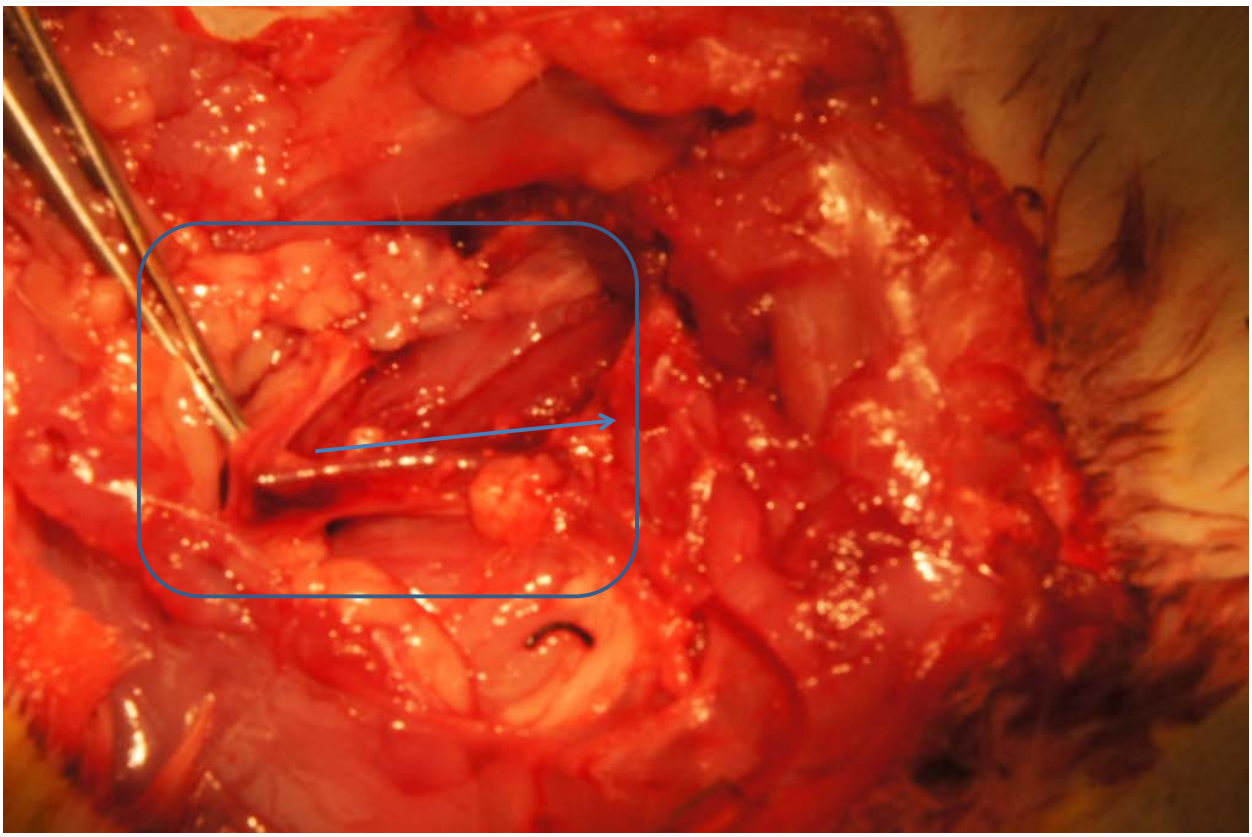

Figure 5.6: Femoral cannula stuck at the iliac branch. Blue arrow represents the cannula in the femoral vein with the arrow pointing to the insertion site of the cannula. The tip at the start of the arrow is at the iliac branch, which prevented further insertion of the cannula.

o.d.,and thus have a more successful implantation rate. The dimensions of the femoral and jugular catheters from BAS were the same, and the success rate of advancing these cannulas into the vein was not any more successful than the MRE-033 tubing. Due to the tip of the portal vein catheter being smaller, the catheter did advance further into the vein; however, since this cannula was not designed for the femoral vein, it did not work well for this application, especially for awake experiments. Therefore, none of these cannulas were utilized in the experiments with the Wistar rats, since the same success rate was obtained with MRE-033 as with these other cannulas.

5.5.5.2 *Wistar rat jugular cannulation*

Due to the issues with femoral cannulation in the Wistar rat, jugular cannulation was explored to determine if this administration technique would have a higher success rate. The success rate of the jugular cannulation in male Wistar rats was significantly higher than the femoral cannulation. The first two rats implanted with a jugular cannula were successful. Unfortunately, this same success rate was not observed throughout the duration of the study. One rat pulled the cannula out prior to compound administration, illustrating the concern with cannula patency throughout these studies. The second rat still had a patent cannula, and was administered carboplatin. This rat lasted three weeks, even with the harness on the incision site. Even without an incision site, the harness would typically start rubbing and causing tenderness around this time. Therefore this rat became more excited and agitated than the rats with the femoral cannula implanted. One possibility of reducing this irritation was to utilize a collar from BAS to connect the rat to the tether of the Raturn®. However, with these BAS collars, the animals prefer to move in one direction, and since animal activity can be monitored on the Raturn®, a harness was preferred. Another major disadvantage of the BAS collar was that the

collar had to be cut from the rat with scissors since a brain cannula was implanted. The idea of sharp objects around the animals head was not ideal, thus the BAS collars were only attempted a few times, since the collar had to either fit over the cannula or it had to be removed by scissors. Given that these experiments were carried out over several weeks, replacement collars were necessary far too often, as the rats usually doubled in size over the length of the study (i.e. 225 g to 450+g).

To test another possible administration route, in a quick experiment in one rat, doxorubicin was administered intravenously through the tail vein to see if this was a viable option. The first administration went well, particularly with the added handling prior to the experiment. However, repeated administration was not possible with this compound, due to the poor condition of the animal at the site of injection. The toxic reaction at the insertion site to the base of the tail along the entire blood vessel prevented the second weekly administration. A recovery period was given to allow the area to heal before another administration was attempted. Even without an additional administration, a few days later the animal was euthanized due to the animal's decline in health, especially at the site of injection. This demonstrates the extreme toxicity of doxorubicin, even after only one administration, especially in Wistar rats. Because of this, the ability to monitor Wistar rats dosed with doxorubicin was more difficult than anticipated, since neither cannulation or direct injections were successful in these experiments.

While there are several literature reports of administering doxorubicin intraperitoneally to male Wistar rats, most of these studies euthanize the rats 72 hours later[11-12]. There are other studies that repeated administration of doxorubicin weekly (with cyclophosphamide) for four weeks in Sprague-Dawley rats[13] or in B6C3 mice[14]. Due to the extreme complications

experienced with the Wistar rats, especially with regards to doxorubicin administration, Sprague-Dawley rats were tried next.

Sprague-Dawley rats were also tried, since during the individual administration of doxorubicin or carboplatin, an increase of glutamate in male Wistar rats within the three week period was not observed (Figure 5.7). Even though the glutamate level seems slightly higher in the doxorubicin treated rat, these changes do not correlate with the dose. An increase in glutamate was also observed at the end of the doxorubicin experiment, although the statistical significance of this result cannot be determined without several more successful experiments with the administration of this compound in long-term experiments.

5.5.5.3 *Sprague-Dawley rat femoral cannulation*

The objective of these experiments was to determine the length of time this strain of rat could last as a part of a microdialysis experiment while monitoring neurotransmitters and oxidative damage throughout the administration of chemotherapeutic agent. Glutamate was monitored in the hippocampus throughout the experiment following weekly administrations of doxorubicin (3 mg kg^{-1}) and cyclophosphamide (30 mg kg^{-1}) to male Sprague-Dawley rats (Figure 5.8). The length of this experiment was highly significant in that it lasted six weeks, which is longer than any microdialysis experiment of other rats administered chemotherapy, especially for doxorubicin. In addition, this rat was only euthanized due to the fact that no appreciable change in glutamate was seen following administration. There are a few experiments utilizing Sprague-Dawley rats in chemotherapy treatment that have been reported; however most of these papers report no changes as well[15]. Most chemotherapy experiments utilize Wistar rats [16-25], and probably do so because Wistar rats are reported to have a similar vasculature system and cognitive function as observed in humans upon administration of these

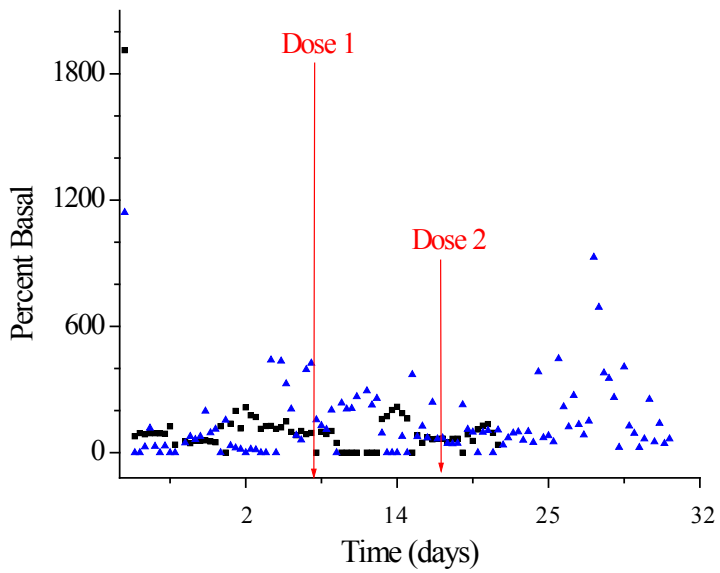

Figure 5.7: Percent deviation for glutamate versus time profiles for i.v. administration of doxorubicin (4.4 mg kg^{-1}) or carboplatin (20 mg kg^{-1}) in the hippocampus of awake Wistar rats. Each red arrow represents the i.v. administration of doxorubicin (▲) (only at arrow one) or carboplatin (■).

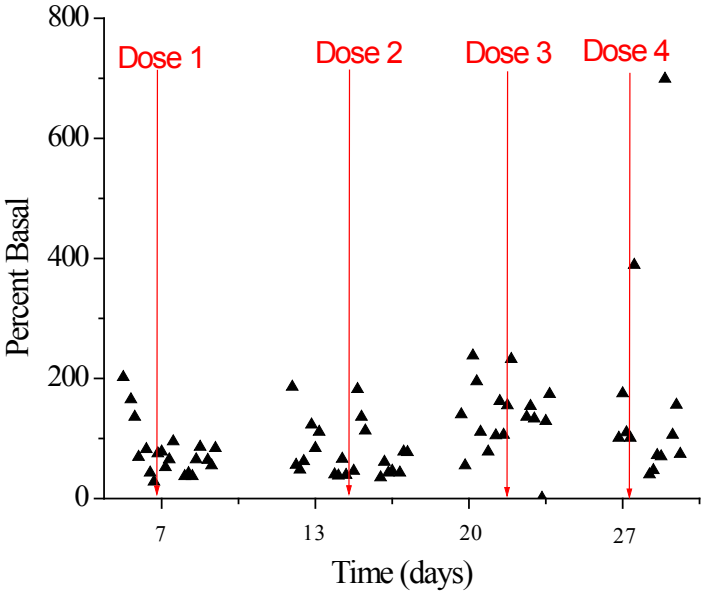

Figure 5.8: Percent deviation for glutamate versus time profiles for i.v. administration of doxorubicin (3 mg kg^{-1}) and cyclophosphamide (30 mg kg^{-1}) in the hippocampus of an awake Sprague-Dawley rat. Each red arrow represents the i.v. administration of the chemotherapy compounds.

compounds[26-27]. Even though the Sprague-Dawley rat was a more successful model for long-term microdialysis studies, further application of monitoring chemotherapy drugs will only be useful in species that more closely represents the changes observed in humans. Since no change was observed in any of the monitored analytes after six weeks, this rat was euthanized to explore other possibilities.

5.5.5.4 *Carboplatin*

In this experiment, an older Sprague-Dawley rat (446 g) was utilized to investigate changes in neurotransmitters upon the administration of carboplatin. This rat only lasted 10 days possibly due to his size, which was suspected as an issue throughout the previous studies. The elderly rat behavior consisted of completely taking the CMA microdialysis probe out of the guide cannula. Once the animal learned how to remove the microdialysis probe, it was hard to break the animal of this habit. This behavior occurred even though animal acclimation to handling was utilized in these experiments as well. Animal acclimation was definitely beneficial; however, upon any manipulation (such as surgery and compound administration), the rats definitely became more aggressive as they got bigger and older. The purpose of using these older rats was to see if age made a difference on neurotransmission and oxidative stress markers. However, during the course of the study, no appreciable changes were seen in glutamate (Figure 5.9), or in any of the other analytes monitored. No additional rats were tested for this reason.

5.6 Sample analysis considerations

One of the complicating issues with the length of these experiments was that the number of samples increased exponentially, especially with shorter microdialysis sampling intervals. The ability for one person to keep the animal going for up to 10 weeks while analyzing the numerous

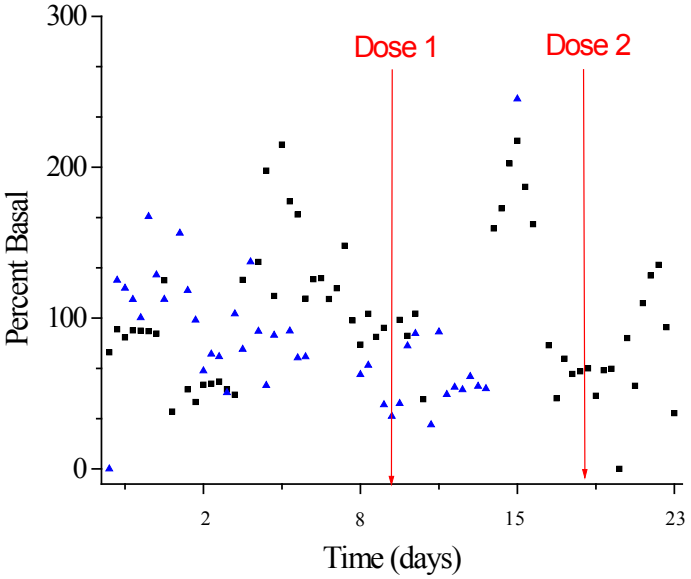

Figure 5.9: Comparison of glutamate recovered from hippocampus microdialysate samples of awake rats given multiple i.v. administrations of carboplatin (20 mg kg^{-1}). Each red arrow represents the i.v. administration of carboplatin in a Sprague-Dawley rat (▲) and a Wistar rat (■).

samples produced was inundating. Initially, all the samples were analyzed on three systems: two systems for the neurotransmitter analysis (one for the amino acids, and the other for the catecholamines) and one system for MDA analysis. As the sampling interval was decreased, the time required for analysis of the resulting sample increased exponentially.

During these experiments, thousands of samples were generated for each rat, especially if the study lasted for several weeks. To give an illustration of the time it took to perform sample analysis for these experiments, the sample analysis on one system, assuming a 15 minute analysis time, was used to monitor the neurotransmitters with a microdialysis sampling interval of 60 minutes, which was used in the initial studies. With this microdialysis sampling interval, the analysis time for the samples produced, one analytical system was approximately five weeks, assuming 80 hour weeks (24 samples/day x 70 days x 15 minute analysis / 60 min/hour / 80 hours/week = 5 weeks). A typical microdialysis sampling interval is 15 minutes (or less). The time to finish the analysis for one analyte with this microdialysis sampling interval just increased to 21 weeks (96 samples/day x 70 days x 15 minute analysis / 60 min/hour / 80 hours/week = 21 weeks). While sampling in 15 minutes intervals would be ideal to get a more accurate profile of the changes upon chemotherapy administration, this was not practical as this sampling interval would take 5 months, which is 21 weeks, to perform the analysis on one system. In addition, these calculations do not include any downtime of the instruments, for things such as maintenance or the amount of time to prepare the solutions or any standard analysis. This calculation also does not include the time it takes to prepare the vials for sample collection, especially regarding the required addition of the 0.1 M perchloric acid with cysteine for the catecholamine analysis, and for the maintenance required for the animals. For any future experiments, multiple analysts would be necessary in order to carry these experiments out with

any decent success of maintaining the animals while keeping up on the analysis of the microdialysate samples.

5.7 Conclusions

The longest microdialysis experiments performed lasted six weeks, in both a Wistar rat and a Sprague-Dawley rat, which is the longest continuous microdialysis experiment that has been performed to our knowledge. Unforeseen issues were experienced in the utilization of Wistar rats, including problems with femoral cannulation. Another issue with Wistar rats was the extreme aggression, which caused more experimental failure due to the inability to restrain these rats effectively, especially during doxorubicin administration. Due to these issues, Sprague-Dawley rats were utilized in the remaining studies. These studies were more successful in obtaining long-term experiments, especially if younger rats were used. This rat strain could have even lasted longer than six weeks, since the animal was still going strong. However, due to the lack of any detectable increase in any of the analytes monitored following the administration of doxorubicin, the animal was euthanized. In both strains, animal acclimation was found to be beneficial in these long-term experiments. Once rats got bigger/older, an increase in aggression and complications were definitely observed in both strains.

A higher success rate may definitely be possible with other compound administrations and/or in Sprague-Dawley rats for long-term microdialysis experiments. An improvement in success might also be possible in a younger/smaller rat. The disadvantage of utilizing a smaller rat is if an intravenous cannula is used, then this changes the cannula position over time, which decreases the cannula patency. This is especially true in the Wistar rat, since the cannula is only advanced two centimeters.

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~Chapter 6~

Conclusions and Future Work

6.1 Dissertation Overview

Microdialysis was successfully implemented to continuously monitor enzyme activity and neurochemical activity after compound administration. The first two microdialysis sampling methods were developed to screen the effects of novel drugs on enzyme activity. Microdialysis sampling was then used to monitor the effects of chemotherapy in short- and long-term experiments. The ability of microdialysis sampling to monitor biochemical events/reactions in a non-clinical species while generating an uninterrupted time-profile offers a huge advancement over conventional sampling methods for drug development studies. Application of microdialysis sampling in these studies provided a novel approach to study these biochemical mechanisms with good temporal and spatial resolution with a complete time-profile.

Other studies in these areas typically use tissue biopsy, which increases the inter-animal variability. This variation or disruption in sampling may cause a misinterpretation of the results, leading to inaccurate conclusions. In addition, during tissue harvest the method used for euthanasia can affect the results of the study. Moreover, contamination limits collection of small tissue regions, such as the submucosa of the colon (e.g. 60 μm). It is also important in the collection of biopsies that the sample be processed immediately to minimize further enzymatic reactions. Sample preparation generally constitutes 80% of the analysis time for biopsy samples, decreasing sample throughput. This is not the case for microdialysis experiments since the dialysate samples are aqueous and free of any proteins minimizing sampling clean-up if a low MWCO membrane is used. This generally allows the samples to be collected and analyzed

without any sample preparation, increasing sample throughput. Another advantage is the ability to split the sample to analyze on multiple analytical systems.

The limitations of microdialysis sampling are achieving a good temporal and spatial resolution due to the many factors that change the extraction efficiency. Many factors affect the EE of an analyte, including the perfusate flow rate, the surrounding tissue, and the membrane characteristics. The requirement of a continuous flow of the perfusate requires the balancing act of obtaining enough sample volume and analyte for the analytical system, while collecting microdialysis samples with good temporal and spatial resolution. Furthermore, if the analyte concentration is desired, than the extraction efficiency of the probe must be performed in the tissue of interest, since EE varies. Depending upon the microdialysate sample collection parameters chosen, this sampling technique may generate a continuous time-profile; however it can lead to the generation of a large number of samples, especially in long-term experiments. The ability to run the samples on multiple instruments to obtain a complete profile of each rat can lead to the elucidation of several biochemical pathways in disease states; however for a single graduate student to keep up with sample analysis was extremely daunting, especially while maintaining the rat as well. For example, the analysis on one analytical system took five weeks (assuming 80 hour weeks) for one long-term experiment. However, considering microdialysis sampling hardly requires any sample preparation time before analysis, performing these experiments with conventional methods, such as tissue collection, would not only require 80% longer for analysis due to sample preparation, but also sacrifice thousands of rats.

The application of microdialysis to elucidate multiple PK/PD components in drug development expedites throughput and minimizes animal usage. The efficient development of therapeutic drugs in a timely manner will improve human health and will minimize treatment

lapses, which can increase successful treatment. Before novel drugs are sold on the market, all drugs must be thoroughly tested in animal studies. The utility of microdialysis allows the PK/PD of the drugs to be studied in the tissue of interest. The implementation of microdialysis sampling in these studies made it possible to determine the mechanisms of drug actions that was not determined with other sampling methods.

6.2 Summary by section

6.2.1 Monitoring 11β-HSD enzyme activity by microdialysis

These studies were completed by developing multiple analytical techniques to monitor the processes of 11β-HSD1 activity *in vitro* and *in vivo*. Several studies were performed *in vitro* with rat microsomes and *in vivo* with Sprague-Dawley rats to determine the mechanisms of 11β-HSD1 in this species, primarily monitoring only cortisone and cortisol initially in the tissue of interest. After several delivery and recovery experiments monitoring several different metabolites, a different species (i.e. human) was investigated once the microsomes were determined to be viable *in vitro*. In human studies, the generation of cortisol correlated to the disappearance in cortisone, which was not the case in any of the rat studies. An extensive study of a common inhibitor of 11β-HSD1, carbenoxolone, was tested with different concentrations of substrate, cortisone. The *in vitro* results obtained with the addition of carbenoxolone in the presence of cortisone, correlated well with published literature demonstrating that microdialysis is a good sampling method to monitor enzyme inhibition.

6.2.2 Monitoring GC-C enzyme activity by microdialysis in the submucosa of the colon

This was the first study, to the best of our knowledge, to monitor the enzyme GC-C in the submucosa of the colon. The submucosa tissue layer was extremely difficult to study due to the

thinness of the tissue layer (approximately 60 μm). Previous studies have attempted to collect the submucosa by tissue biopsy; however contamination from the surrounding tissue (e.g. mucosa) makes this technique not feasible, and any tissue present from any other tissue of the colon significantly alters the results. A microdialysis implantation technique was developed that minimizes tissue manipulation and correlates the probe placement to the levels of cGMP. This technique was optimized to monitor the secretion of cGMP by GC-C activation.

Several microdialysis probe implantation techniques were utilized. The critical development from this project was the implantation of the lumen probe in the ascending colon to determine that the split response of cGMP was from the location of the microdialysis probe. The location of the microdialysis probe was critical since a small movement of the probe changed the concentration of cGMP by an order of magnitude. At first histology was performed by Dr. Thompson at LMH; however any movement of the microdialysis probe or tearing of the tissue that occurred after the experiment made the identification of the probe location within milli- or micrometer accuracy difficult. This then lead to the implantation of the lumen and submucosa microdialysis probe to compare cGMP levels between the two regions. This comparison of the cGMP level determined the split response was from the probe location and not a true split response in cGMP levels with the administration of the same concentration of STcore. This study was the first successful implantation of a microdialysis probe in the submucosa layer of the colon with the ability to confirm the microdialysis location and was necessary for determining the cGMP levels.

6.2.3 Monitoring chemobrain by microdialysis

The final application of microdialysis sampling in this dissertation was to optimize microdialysis sampling in long-term experiments to elucidate the mechanisms responsible for

cognitive dysfunction that is exhibited after the administration of chemotherapy. The main goal of the work in this chapter was to monitor the effects of chemotherapy drugs on neurotransmitters and oxidative stress over a long period of time (i.e. 6 weeks). Now that the optimization of long-term sampling has been performed, further studies can be planned to continuously monitor analytes such as neurotransmitter or oxidative stress biomarkers (e.g. MDA) in chronic diseases.

Chemotherapeutics are well-known to have multiple side-effects, and this was definitely observed in the animals treated with these agents. Even though many articles have been published monitoring a number of biochemical processes in animals after chemotherapy administration, the exact mechanism and extent of change in each analyte varies widely. This is most likely from the samples being collected in discrete intervals. Considering the time-of-onset of cognitive dysfunction in human varies, this will most likely be the case in other species as well, including rats. Most studies monitoring biochemical mechanisms *in vivo* perform tissue biopsy. This is extremely disadvantageous if the on-set is different for each rat and if each rat has different basal levels than the last. With the use of microdialysis sampling, the same rat can be compared before, during, and after compound administration reducing animal variability.

Several preliminary studies were performed to determine the direct effects of chemotherapy compounds. The long-term effects of these compounds were performed in the longest microdialysis experiment, to the best of our knowledge. These experiments determined that a detectable change in neurotransmitters, especially in the short-term direct perfusion experiments, occurred during the administration of doxorubicin and cyclophosphamide. Considering these are the most common compounds causing cognitive dysfunction during treatment in human patients, this was not surprising.

Further long-term experiments were performed; however the behavioral side effects of these toxic compounds definitely made monitoring these animals for a couple of months continuously with microdialysis sampling extremely difficult. This is apparent by the ability to obtain data from the control animals for a much longer period in each experimental design than any of the animals undergoing chemotherapy administration. The ability to monitor the control rats continuously for over a month demonstrated the advantage of the unique tissue-targeted microdialysis sampling method over conventional tissue harvesting techniques. While a long-term experiment was not successful in monitoring the neurochemical change caused by chemotherapy, the control experiments demonstrate that it is possible to perform long-term experiments, especially with less toxic compounds.

6.3 Future directions

6.3.1 Monitoring 11β-HSD enzyme activity by microdialysis

Future work of this study is to elucidate a similar metabolite in human and rat *in vitro* studies. If a primary metabolite of a specific inhibitor using human microsomes or in a preliminary clinical trial is similar to a metabolite generated in a non-clinical species, further studies could be performed in a tissue-specific fashion with microdialysis sampling. Interestingly, this experiment was recently performed by Amgen using an enzymatic bath with activity termination by acetonitrile[1]. Once the main metabolite was determined in a two human clinical trial, the specific inhibitor was administered to dogs and rats. The metabolite profile generated from each species was then compared. In their study, the compound produced the same plasma metabolite in all three species. In order to determine the PD of the drug, microdialysis sampling could be utilized to determine if the novel inhibitor is effective in the adipose tissue, the tissue of interest, instead of a systemic profile of the effects of the drug.

6.3.2 Monitoring GC-C enzyme activity by microdialysis in the submucosa of the colon

The next step for this project is to test the novel GC-C agonist, linaclotide, with microdialysis sampling in the submucosa of the colon. This will make it possible to measure the concentrations of cGMP in the desired location. An increase in cGMP following drug administration could demonstrate the ability of cGMP to reduce pain by acting as a gastrointestinal analgesic[2]. In addition, probenicid, an inhibitor of cGMP efflux pumps, could be administered with linaclotide to elucidate the export mechanism of cGMP from the lumen to the submucosa region[2]. This data would be revolutionary if the cGMP levels decrease with the administration of probenicid.

6.3.3 Monitoring chemobrain by microdialysis

The next step for the short-term chemobrain experiments is to perform extended studies in awake animals over a few days. This will determine the effects of the chemotherapy agents with an awake animal. Microdialysis sampling and implantation could be performed after the animal has received several chemotherapy administrations to determine the long-term effects. The disadvantage of this technique is that the basal levels would not be determined.

The main advantages of long-term experiments is the ability to monitor one animal continuously over an extended period of time making it it's own control. A microdialysis study in one animal can generate the same data that would require 1008 animals for tissue biopsy. For example, an animal that has microdialysis sampling collected in one hour intervals for six weeks would require 1008 animals in tissue collection studies (6 weeks x 7 days/week x 24/day = 1008). Microdialysis sampling determines the complete time-profile before, during, and after administration. Now that the long-term microdialysis sampling is optimized other chemotherapy agents can be tested. .

6.4 References

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