1.1. **Biomarkers**

Biomarkers are a critical component of the drug discovery process and are important in disease diagnosis and treatment. They are so critical, in fact, that the National Institutes of Health formed an Initiative on Biomarkers and Surrogate End Points to define key terms and issues more precisely. According to the initiative committee, a biomarker is described as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Biomarkers serve as a bridge between the observed clinical responses of patients and the changes that occur on a molecular level. These can be but are not limited to levels of a metabolite or protein – also included are data such as body weight, organ weight, genotype and urine volume. An ideal and robust biomarker should be noninvasive to sample, selective, sensitive, measured easily, relevant to the condition being studied and serve as an early warning for toxicity or disease. Additionally, interanimal and intraanimal
variability should be low and it should change on a timescale congruent with drug action or disease onset.

The measurement of biomarkers serves three primary purposes in drug development\(^2\): to quantify the dose to which the animal or human was exposed (biomarkers of exposure), to measure the pathological effect of the drug (biomarkers of response) and to identify animals or humans that are predisposed to the effects of the drug (biomarkers of susceptibility). One of the main bottlenecks in the drug discovery process is the identification of drug candidates that cause toxicity. Consequently, there is considerable interest from pharmaceutical firms in developing technologies that increase efficiency in this phase of development\(^3\)–\(^5\). Many companies have been formed in recent years that solely exist to identify sets of biomarkers most relevant to various drug candidates or disease conditions in hopes of identifying a promising drug earlier, saving a vast amount of resources in the process.

Biomarkers are also utilized to study disease onset and treatment. The primary purpose of biomarker identification in these studies is to discover compounds that can diagnose a disease earlier than currently established biomarkers or clinical endpoints\(^3\)–\(^6\). For these diagnostic biomarkers, although a level of the biomarker is obtained analytically, the value is then categorized as positive or negative. The place where the line is drawn between positive and negative is extremely important – while the level should not be low enough that a significant number of false positives results, it should not be high enough that a large number of false negatives are obtained and thus the disease remains untreated\(^5\).
Traditionally, scientists have searched for a single all-encompassing biomarker to use in the study of a pathological condition. The use of single biomarkers can be problematic. A single biomarker likely does not reflect all the changes induced by the drug or disease, so the information gleaned from single biomarker assays is incomplete. For example, many treatments are likely to lower levels of prostate specific antigen (PSA), but these effects may be unrelated to the success of the treatment of prostate cancer. Other pathways unrelated to PSA are tapped by cancer treatment. Biomarker qualification is also complicated by animal-to-animal variability and differences in response between species.

Consequently, there is a push in the scientific community to move to profiling experiments, so that many biomarkers of a condition can be analyzed simultaneously. It is hypothesized that an increased number of biomarkers increases the probability of accurately identifying a promising drug candidate or diagnosing a disease. The “-omics” technologies (genomics, proteomics, and metabonomics) have opened the door to allow for the simultaneous study of multiple biomarkers. A considerable amount of research is currently being conducted for gene, protein or metabolite combinations that collectively serve as more selective and sensitive biomarkers. While promising, this approach is not without limitations. With an increased number of variables comes an increased probability that chance (and thus misleading) correlations will be identified. Even though “-omics” techniques allow for many biomarkers to be characterized, it is important to ensure that these additional analytes add value to the study and thus are worth any extra effort or expense. Finally,
profiling techniques do not require that analytes be identified – what matters is their statistical significance relative to the drug dose or disease onset. This can be advantageous in that it is more efficient, but can also be disadvantageous. Although it is not necessarily required that the identity of a biomarker be known or that its exact role in the changes induced by a drug or disease are fully characterized, this information does help in the discernment of biomarkers that are most relevant to the study and thus most likely to give accurate information\textsuperscript{3,7}.

1.2. Metabonomics

1.2.1. History and Context

Although the term “metabonomics” was coined within the last ten years, scientists have been profiling metabolites in biofluids and tissues for decades. Some of the earliest studies were in 1965, involving the investigation of energy metabolites in cancerous tumors\textsuperscript{8} and livers\textsuperscript{9}. These studies were achieved by individual assays of the metabolites, requiring a great deal of work per sample. Studies progressed to the use of gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID) to analyze organic acids, steroids and other volatiles in urine\textsuperscript{10-14} and breath\textsuperscript{12}. Some of the first applications of nuclear magnetic resonance (NMR) spectroscopy to the study of small endogenous metabolite profiles in biofluids were in the late 1970s and early 1980s by Dallas Rabenstein\textsuperscript{15,16}, who examined erythrocyte metabolism. This was followed by work in the mid-1980s by J. K. Nicholson and P. J. Sadler\textsuperscript{17}. Over the years, a variety of applications have
emerged for metabonomics studies, centered on the identification of biomarkers (particularly of liver toxicity) and improved detection and diagnosis of disease. Metabolic profiling of urine by NMR has been used clinically to detect inborn errors of metabolism in urine\textsuperscript{18,19}. Dietary studies in humans have also found their application in metabonomics\textsuperscript{20}. Metabolic disturbances following conditions such as tissue injury\textsuperscript{21}, liver toxicity\textsuperscript{22-26}, liver disease\textsuperscript{27}, liver cancer\textsuperscript{28} and kidney toxicity\textsuperscript{29} have all been explored. Disease diagnosis and biomarkers of Type 2 diabetes\textsuperscript{30}, neurodegenerative disorders\textsuperscript{31,32}, meningitis\textsuperscript{33}, schizophrenia\textsuperscript{34} and ovarian cancer\textsuperscript{35} have been characterized. Metabonomics has captured the interest of pharmaceutical companies interested in expediting the drug development process. In 2001, a consortium of six major pharmaceutical companies was formed to study the feasibility of metabonomics methodology in the characterization of toxicity of drug candidates and to build metabolic profile databases of biofluid samples\textsuperscript{36}.

Metabonomics is based on the principal of a stressor, be it genetic, disease, pharmaceutical or environmental, causing changes in the levels of endogenous metabolites. Cells work to maintain homeostasis, so the application of a metabolic stressor results in changes of intracellular and extracellular metabolite levels to compensate for the stressor\textsuperscript{37}. The study of endogenous metabolite profiles has many different terms associated with it. The term “metabonomics” was coined in 1999. It is defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification\textsuperscript{38}.” The metabolome is defined as the complete set of endogenous small
molecules produced by an organism. Metabolomics is defined as the identification and quantification of the entire metabolome, to the greatest extent possible. In contrast, metabonomics is not concerned with characterizing the entire metabolome, although it is often used interchangeably in literature with metabolomics. It trades off the complete characterization of the metabolome with the ability to monitor many samples over a time course. Historically, metabolomics has primarily centered on the study of plant or bacterial metabolomes by GC/MS and metabonomics has focused on the study of animal metabolism by NMR analysis of biofluids.

Metabonomics is part of systems biology, which is a holistic technique that aims to characterize an organism’s response to a stimulus at the genetic, protein and metabolite level (Figure 1.1). The measurement of metabolite levels provides an important insight into both gene expression and protein levels or activity, as the endpoint of these changes is ultimately in the levels of small molecules. It should be noted that a stimulus does not necessarily affect change “top down” – that is, starting at the genetic level. In fact, many stimuli affect change directly at the metabolite level that is never observed at the genetic or protein level. Genes and proteins also respond to stimuli at different timescales than metabolites. Thus, metabolite levels may reflect a more immediate change from the stressor.

In fact, genomic, transcriptomic and proteomic studies face limitations that metabonomics studies do not. Both can be expensive – transcriptomic studies are at least 100 times more expensive than the same number of metabonomic studies, and proteomic studies are at least 50 times more expensive. Gene and protein
Figure 1.1. The study of systems biology, showing the layers of complexity of biological response of a cell to an external stimulus.
expression among species is not as highly conserved as metabolic pathways.\textsuperscript{45} Transcriptomic studies can be incomplete, as not all genes are transcribed into mRNA. Therefore, changes in some genes may not be detected by transcriptomic analyses (called “silent mutations”) that are detected by metabonomics.\textsuperscript{41,46} Additionally, metabonomics studies are typically much simpler to perform.\textsuperscript{45,46}

1.2.2. Sampling Approaches

Sampling is one of the most important considerations for a metabonomics experiment. The mode of sampling must balance the needs of the experiment with throughput and the effects sampling will have on the subject (animal or human). For example, while tissue-specific metabolic information may be desirable, the throughput of these experiments is notably diminished from the sampling of biofluids, particularly urine. There are many options available to the researcher for metabonomics studies. Virtually any biofluid can be studied, as well as tissue biopsies and extracellular fluid.

1.2.2.1. Blood

Plasma is a popular choice for metabonomics studies, but is considerably more challenging methodologically than urine, both for sampling and detection. Plasma sampling is somewhat invasive, but gives a good picture of the organism’s overall metabolic status. Particularly in the sampling of small animals such as rats or mice, the volume of blood drawn at a single time is limited to a few hundred microliters over a set time period. Sampling may also perturb the person’s or animal’s metabolism through stress experienced from the sampling itself, depending
on the method used to draw the blood. Thus, a high temporal resolution of less than 1-2 hours, which may be desirable for some studies, is extremely difficult to achieve with plasma sampling. Analysis of plasma by NMR (and MS) is complicated by the proteins present in the sample. Some choose to precipitate the proteins to clean up the sample, but this does not completely eliminate the problem. Additionally, extraction procedures may alter the observed metabolic profile of the biofluid. Protein precipitation is not always necessary for NMR experiments, although it is in LC/MS experiments. Specialized NMR pulse sequences are also used to suppress the signal from macromolecules in solutions such as plasma by capitalizing on the differences in diffusion and spin relaxation time. A final consideration is the stability of plasma samples. The stability of plasma samples can cause problems in analysis, mostly because of the enzymes present in the sample. Although the samples can be frozen during storage to a point where enzyme activity is minimal, after thawing for analysis, sample composition can begin to change.

1.2.2.2. Urine

Urine sampling is the most common sampling method for metabonomics studies. Studies with urine samples tend to focus on metabolic changes in the liver, kidney and intestinal microflora, but this biofluid is still considered to reflect the overall metabolic status of the organism. It is the least invasive biofluid to collect, as it does not involve any needles. Animal studies can be performed in metabolism cages, which contain a collection chamber underneath the mesh floor of the cage, allowing the animal to be disturbed as little as possible. In human studies, urine
sampling has good compliance with test subjects and does not require the assistance of a medical professional. Time resolution in urine sampling is limited by the length of time between voids, which is difficult to control or keep constant. Most studies collect samples continuously for a set length of time, with a typical time resolution of ~8 hours. For some metabolic stressors studied, this may not be sufficient. Urine can range in pH from 5 to 8, but the pH can increase with bacterial contamination\textsuperscript{51}. Urine samples are more stable than plasma, as no enzymatic degradation occurs as long as no bacteria are present. Thus, samples require an antibacterial agent (usually sodium azide) to prevent bacterial growth. Bacterial growth can cause changes in the metabolic profile, particularly in the levels of acetate, formate and ethanol\textsuperscript{52}.

\textit{1.2.2.3. Other Biofluids}

Many other biofluids have been studied by metabonomics, including cerebrospinal fluid\textsuperscript{33,34,53-55}, seminal fluid\textsuperscript{56,57}, saliva\textsuperscript{58} and amniotic fluid\textsuperscript{59,60}. Metabonomics analysis of cerebrospinal fluid has been used to study important conditions such as dementia\textsuperscript{53}, meningitis\textsuperscript{33}, schizophrenia\textsuperscript{34}, aneurysms\textsuperscript{54} and spinal problems such as herniated disks\textsuperscript{55}. Seminal fluid studies have focused on characterization of male contraceptives\textsuperscript{56} and prostate cancer\textsuperscript{57}.

Many of these fluids give information on metabolism in a more localized area than urine or plasma, but are more difficult and invasive to sample. Thus, samples from human subjects are limited. Samples from laboratory rats (a commonly used animal in metabonomics studies) are limited in volume (a few microliters, in some cases) and for some fluids are so invasive to sample that only one time point per rat
can typically be obtained. Additionally, time resolution may be inadequate because of the limited volumes and invasiveness of sampling, depending on the condition being studied. As a result of these limitations, studies of these biofluids are less popular, and many metabonomics studies on tissue metabolism make use of tissue biopsy samples instead.

1.2.2.4. Tissue Biopsy

The metabonomics community has a great interest in obtaining tissue-specific metabolic information. To date, the primary sampling method for this is through tissue biopsy. Tissues biopsied have included kidney, liver, brain and prostate\(^61\). Although interest has centered on these tissues, it is possible to profile virtually any tissue. Once surgically excised, the biopsy either undergoes an extraction procedure prior to analysis or is directly analyzed by a specialized NMR magic angle spinning (MAS) probe. (This will be discussed further in Section 1.3.2.) Both approaches have been utilized in metabonomics research. Tissue extract NMR has been applied to the study of traumatic brain injury\(^21\) and Batten disease, a genetic neurodegenerative disorder\(^62\). MAS-NMR has been applied to the study of prostate tumors\(^63\), neurodegenerative disorders\(^32\), liver toxicity\(^26,64,65\) and renal toxicity\(^65\).

Although tissue biopsy does give the more specific metabolic information, there are limitations with this sampling approach. First, only one time point can be taken per animal. This substantially increases the number of animals resulting in greater animal-to-animal variability. It could also limit the time resolution obtained for experiments by requiring a compromise to be made between number of animals
used and time resolution required. Tissue extraction creates selectivity in the experiment, as not all metabolites may be present in the extract sample depending on the eluent used. Third, macromolecule interference during analysis is also a problem, much like plasma samples, as discussed in Section 1.2.2.1.

1.2.2.5. Microdialysis

1.2.2.5.1. History and Basic Concepts

Microdialysis has been in use for decades as a sampling technique to probe metabolic changes in tissue extracellular fluid. Probes with a semipermeable membrane are surgically implanted into the tissue of interest. The probe is perfused with a solution isotonic to the surrounding tissue, so that there is no net fluid flow across the membrane. The perfusion of solution through the membrane creates a concentration gradient between the lumen of the membrane and the surrounding extracellular fluid. Consequently, molecules below the molecular weight cutoff (MWCO) of the membrane diffuse into the membrane lumen in accordance with their concentration gradient. Molecules above the MWCO cannot diffuse across the membrane and are thus not sampled, making dialysate samples clean and fairly stable (with regard to enzymatic sample degradation) relative to biofluids such as plasma.

Microdialysis sampling offers several unique advantages for in vivo experiments. One of the primary advantages lies in the ability to continuously obtain samples from the same tissue. Many in vivo studies of tissues require the researcher to sacrifice multiple animals per time point studied to harvest the tissue. Using microdialysis reduces the number of animals required for experimentation
substantially and reduces the animal-to-animal variability observed in experimental results. The clean samples tend to make analysis much easier, as sample cleanup is minimal and any interferences from macromolecules (such as matrix effects in MS) are virtually eliminated. Also, only free molecules are sampled – those that are protein-bound are not observed in the sample. For pharmacokinetic studies, this can be advantageous because only the active drug is analyzed in the samples. A careful balance must be achieved in these experiments between sample volume, perfusion rate and time resolution67. The analytical method employed will require a minimum sample volume, which will place constraints on the achievable time resolution. Time resolution can be improved by increasing the perfusion rate, but this lowers the recovery of the compound to perhaps undetectable levels.

Much of the work of microdialysis has centered on the brain and sampling of neurotransmitters. In fact, the first microdialysis experiments were performed in the brain in 1974 by Ungerstedt68. Since this time, the brain still remains the most studied tissue by a substantial margin. However, peripheral tissues have also been studied. To date, a wide variety of tissue types in mammalian organisms have been sampled by microdialysis, including the muscle, adipose tissue, heart, lung, liver, kidney, pancreas, eye, placenta and gastrointestinal tract69. Neurochemical and pharmacokinetic studies are particularly popular for microdialysis experiments, but many other applications where tissue-specific information is desirable or site-specific delivery of a compound is needed have been explored.
1.2.5.2. **Probe Designs**

Microdialysis probes require different geometries to achieve successful implantation and sampling in tissues\(^\text{67,70,71}\). Spatial resolution must be adequate to sample from the region of interest. While organs such as the liver are fairly homogenous, others are heterogeneous, such as the brain. Membrane length and probe implantation technique must be adjusted accordingly. Other considerations for probe design include anticipated movement of the rat – awake animals require tubing long enough to tunnel under the skin and probe implantation must be secure enough to avoid shifting of the membrane from rat movement. Additionally, the chemical properties of the analyte must be considered. Hydrophobic analytes can experience problems with sticking to probe components, particularly the dialysis membrane. This can lower analyte recovery and introduce variability into the probe performance.

The differing geometries of tissues, their functions and their location in an organism place differing requirements on microdialysis probe geometries. A microdialysis probe is composed of the dialysis membrane, which is often actual membrane used for kidney dialysis patients, tubing and an adapter that allows the probe to be attached to a syringe for perfusion. The membrane is the most important component of the probe. Its composition is chosen based on the analytes of interest in the experiment, as membrane polymers exhibit differing surface chemistries. Hydrophobic analytes in particular can be difficult to sample quantitatively by microdialysis, because they tend to stick to the membrane and other probe components. Commonly used membrane polymers include polyacrylonitrile (PAN),
polycarbonate, polyarylethersulfone, regenerated cellulose and cellulose acetate. Studies have been performed to compare membrane recovery properties – *in vitro* results show distinct differences but these differences become much less significant for *in vivo* studies\textsuperscript{72}. Most of these membranes have MWCOs ranging from 10-40 kDa. In recent years, a push has been made to increase this MWCO to allow for localized sampling of proteins to 100-300 kDa\textsuperscript{73,74}. The use of these probes is still being explored.

Some tissues, such as the brain, are difficult to access and undesirable to expose. In locations such as this, the concentric cannula style probe is used (Figure 1.2A). This probe design has concentric inlet and outlet tubing on the same side of the probe, with the membrane on the opposite side. The perfusate flows through the inner tubing, into the membrane lumen and then is pushed back through the outer tubing for sample collection. These probes can be implanted through a single point. Also, these probes tend to be rigid, as they are encased in a stainless steel cannula, which is advantageous for brain microdialysis experiments but is problematic for such applications as blood microdialysis in vessels. The rigidity can cause tissue damage or puncture vessel walls if the tissue moves when the animal moves or can rupture the membrane, so this probe style is limited to studies in the brain, where the probe can be secured to the skull. As an alternative to the rigid cannula, our laboratory designed a flexible cannula-style probe (Figure 1.2C)\textsuperscript{75} that can be used in a variety of peripheral tissues and is particularly useful for vessel implantation.
Figure 1.2. Probe designs for microdialysis sampling. A shows a commercial concentric rigid cannula probe suitable for brain microdialysis, B shows linear probes used in the study of peripheral tissues and C shows a flexible cannula probe used for microdialysis in blood vessels.
Microdialysis in peripheral tissues can be performed with linear probes (Figure 1.2B), in which the inlet and outlet tubing are on opposite sides of the membrane. Flow is unidirectional through the probe. The first microdialysis studies were performed with linear probes implanted transcerebrally\(^6^8\). Since this study, linear probes have expanded the scope of microdialysis studies to numerous peripheral tissues, including liver\(^7^6-7^8\), muscle\(^7^9-8^2\), stomach\(^8^3,8^4\), skin\(^8^0,8^5-8^8\), heart\(^7^7,8^9\) and tumors\(^7^9,9^0\). These probes cause the least amount of damage to the tissue during implantation of all the microdialysis probe geometries. Also, because they are used in more homogenous tissues and high spatial resolution is not necessary, the probes can typically use longer membrane lengths to achieve greater recovery\(^7^0\).

One final probe design that is not commonly used is the shunt probe, which was designed by our laboratory to sample dialysate from the bile duct\(^9^1\). Conventional studies of bile involve cannulation of the duct and removal of bile over the course of the experiment. Rats, the most common animal for this study, have low amounts of bile, so direct sampling depletes the biofluid substantially, changing the system being studied. The shunt probe is implanted in the bile duct. The design of the probe essentially allows for a linear probe to be implanted in the duct while bile flow is maintained.

1.2.2.5.3. **Probe Calibration**

Transport across the microdialysis membrane is bidirectional – probes can be used to both recover compounds from the tissue and deliver compounds to a localized area (around the implant site). During sampling, microdialysis does not reach a
steady state in the tissues due to the continuous flow of the perfusate. Consequently, only a fraction of the compound will be delivered or recovered, which is called the extraction efficiency (EE). EE can be changed by the perfusion rate, membrane and analyte chemical properties, temperature, probe geometry, active membrane length and physiological properties such as blood flow. The general equation for EE is as follows:

\[
EE = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}} - C_{\text{tissue}}} = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}} - C_{\text{tissue}}}
\]

In this equation, \( C_{\text{perfusate}} \) represents the analyte concentration entering the probe, \( C_{\text{dialysate}} \) represents the analyte concentration leaving the probe and \( C_{\text{tissue}} \) represents the analyte concentration already present in the tissue.

In order to quantify the analyte in dialysate samples, it is necessary to determine the probe EE for each experiment in vivo and often, over the course of the experiment. There are multiple methods available to calibrate probes, which can be chosen to best suit the needs of the experiment. The no net flux (NNF) method involves perfusing the probe with different analyte concentrations, and the analyte concentration is measured in the dialysate. By plotting the concentration in the perfusate against the difference in concentration between the perfusate and dialysate, two pieces of information can be obtained. First, the x intercept shows the concentration of the compound in the extracellular fluid and second, the slope of the line gives the recovery for the analyte. Retrodialysis is based on the principal that the percentage of analyte lost during delivery is equivalent to the percentage of...
analyte recovered from the extracellular fluid. For this calibration, the analyte is perfused through the probe prior to experimentation and the % delivery of the analyte is determined, which can then be used to correct for analyte concentrations observed during the actual experiment. For the studies presented in this dissertation, exact quantitation of each analyte is not possible due to the large number of analytes present and lack of prior identification. Also, the data presented examines changes in metabolite levels, rather than determining exact metabolite concentrations, so absolute quantitation is not necessary. Therefore, the delivery or internal standard method\textsuperscript{71,97} will be used to monitor changes in probe performance. In this method, a marker compound is chosen with similar chemistry to the analyte. The % delivery of this standard compound is used to gauge general changes in probe extraction efficiency over the course of the experiment.

1.2.2.5.4. Tissue Response to Probe Implantation

Membrane choice is important when considering the tissue reaction to the probe implant. Studies have shown both PAN and polycarbonate experience the same degree of biofouling over an eight day period\textsuperscript{98}. For long-term awake animal experiments, cellulose acetate is inappropriate because it dissolves over a 2-3 week period in the tissue\textsuperscript{99}. Cuprophan is a better choice for these studies, as the membrane integrity is maintained for at least 12 weeks \textit{in vivo}\textsuperscript{99}.

Immune reactions are unavoidable with microdialysis probe implantations, particularly those implanted for extended periods of time. These reactions have been well-characterized in the literature and tend to be consistent between tissue
types. After implantation, a small amount of hemorrhaging occurs around the implantation site from damage incurred, but good contact is still maintained between the tissue and membrane. Inflammation is the initial immune response of the tissue to the foreign body, occurring three to four hours after implantation. Blood flow increases at the implantation site and neutrophils infiltrate, surrounding the probe. Eight hours after implantation, neutrophil infiltration continues to increase and some necrosis is evident. After 1-3 days, macrophages infiltrate and begin to remove the necrotic tissue and any bacteria present. For extended implants (1 week or more), the tissue eventually begins to generate a capsule of connective tissue around the probe to isolate it from the rest of the tissue. Angiogenesis also occurs with the fibrous capsule.

1.2.2.5.5. Application to Metabonomics Studies

Very few microdialysis experiments have been studied by metabonomics. These have primarily been proof of concept studies examining neurotoxicity induced by tetradotoxin and liver toxicity induced by potassium and paracetamol. Our laboratory is the first to explore and refine the analytical methodology required for these tissue-targeted metabonomics studies.

1.3. Analytical Methods for Metabonomics

Metabonomics is extremely demanding on the analytical method. The desire is for nonselective detection in a very complex sample with a wide range of analyte concentrations. Analytical methods must achieve a balance between the degree of
nonselectivity and detection limits. Increasing selectivity improves detection limits, but narrows the scope of the compounds detected. Most metabonomics methods aim for nonselectivity while sacrificing detection limits – consequently, most of the compounds profiled are energy compounds, which are typically highest in abundance. Because of the large number of samples, the method should require little sample preparation and be high throughput with respect to analysis time. Analytically, the method should be robust and have a wide dynamic range. The complexity of the composition of a biofluid sample is illustrated in Figure 1.3, which depicts NMR detection of rat urine.

As discussed in the following sections, different analytical techniques bring different advantages to the metabolic profiling of biofluid samples. Although using multiple analytical techniques would give a broader picture of the metabolites in the sample, typically only one is employed to achieve higher throughput. Unlike metabolomics, metabonomics is more concerned with obtaining a snapshot of the detectable metabolites in a sample and observing how these change in response to a metabolic stressor.

1.3.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

Proton NMR (¹H-NMR) spectroscopy is by far the most popular analytical method for metabonomics studies. It is based on the property of nuclear spin. Some nuclei, including ¹H, ²H, ¹³C and ³¹P, have nuclear spin and are thus NMR active. The spinning nucleus generates a magnetic moment, which (in the simplest case) aligns in one of two directions when placed in an external magnetic field (B₀). This
Figure 1.3. $^1$H-NMR spectrum of rat urine in 10% D$_2$O (A). B and C show close-ups of sections of the original spectrum in A.
results in two populations of nuclei in different energy states – one state aligned parallel to $B_0$ (higher energy) and one aligned antiparallel to $B_0$ (lower energy). In the NMR experiment, a pulse of energy is applied at the resonant frequency of the nuclei in the form of radio waves, causing nuclei to change spin states after absorption of the energy. (The resonant frequency of the nucleus is its Larmor frequency, which is the rate that the nucleus precesses about the $B_0$ axis.) The difference in spin state population is what generates the NMR signal. This is relatively small in NMR compared to other types of spectroscopy, causing NMR’s known problems with sensitivity. The change in spin state generates a signal at a specific frequency that is related to the chemical environment around the proton of interest. Neighboring protons can also cause splitting patterns in the proton resonance signal. These two factors combined make it possible to learn a great deal about the structure of the compound of interest. NMR detection is inherently quantitative, as all protons will generate the same signal/nucleus, so only one known compound is required to obtain quantitative information about all the analytes detected, providing that the spectrum is acquired with suitable care.

$^1$H-NMR is one of the most nonselective analytical techniques available and is uniquely suited to measurements of endogenous metabolites, which generally contain protons. The proton has nearly the highest sensitivity, generating the most signal/nucleus of all NMR-active nuclei, except for tritium. No sample derivatization or $a$ priori decisions are required for analyte detection. Additionally, the sample is not destroyed during analysis. However, $^1$H-NMR suffers from poor sensitivity, and
is generally able to detect approximately a 10 μM concentration (depending on experimental conditions and amount of signal averaging). Some limitations in sensitivity can be overcome using cryoprobes, which are specialized NMR probes designed to reduce thermal or Johnson noise through the cooling of probe components. Although these improve signal-to-noise and reduce the required acquisition time, they are very expensive to purchase and maintain. Resonance overlap can also cause problems, reducing the number of analytes detected and causing quantitation problems. Moving to higher field magnets improves signal dispersion, and as a result generates many more resonances. In a single transient from NMR analysis on a urine sample on a high-field magnet (800 MHz), 5000 lines can be resolved\textsuperscript{52}.

Sample preparation is generally simple\textsuperscript{52}. All samples should be centrifuged to pellet any particulates. Urine samples are generally diluted by D\textsubscript{2}O to a 10% deuterium concentration. The D\textsubscript{2}O provides a lock signal for the NMR experiment, and an internal standard such as trimethylsilyl propionic acid (TSP) is often included as a chemical shift and quantitation standard. Sometimes buffer is used as well in the dilution to control the pH and thus reduce resonance shifting. Plasma sample preparation is also fairly straightforward. Although buffer is generally not added, samples are diluted in saline and then D\textsubscript{2}O is added for a lock signal. Some researchers choose to further clean up the sample by precipitating the protein, but this step is generally eliminated because of pulse sequences like the Carr-Purcell-Meiboom-Gill (CPMG) sequence that can suppress the macromolecule signal.
One of the main obstacles to be overcome in the NMR experiment is the large water signal from the aqueous samples, which causes resonance overlap, baseline distortion, radiation damping and dynamic range problems\textsuperscript{52,104,105}. In aqueous samples, water occurs at a concentration of 55 M. This is much greater than the analytes of interest, which are in the micromolar to millimolar range\textsuperscript{48,106}. The water signal can be reduced by removing the aqueous solvent through speed vacuuming or lyophilization and reconstituting in a deuterated solution, but even with this, some signal will remain. Water suppression pulse sequences are generally utilized to reduce the intensity of the water resonance. A variety of pulse sequences are available that achieve suppression from different mechanisms, including saturation of the resonance, pulsed field gradient resonance destruction and resonance zero net excitation\textsuperscript{105,106}. Saturation of the water resonance is one of the simplest ways to reduce water signal. It is accomplished by applying a long, low intensity pulse at the frequency of the water signal, equalizing or saturating the spin populations so that there is no net spin population to detect. This approach can cause saturation of exchangeable protons and can deplete signal around the HOD resonance\textsuperscript{105}. The second approach uses pulsed field gradients or soft pulses. These sequences, such as WATERGATE and WET, cause zero net inversion and dephasing of the water resonance, destroying its signal while solute signal is maintained. We routinely use WET on samples reconstituted in a deuterated solution. Also, the experiments are typically shorter in length and show few effects on the solute resonances\textsuperscript{104}. Finally, water signal can be suppressed by manipulation of the water resonance to place it out
of the axis of detection (z axis) while placing solute signal in the xy plane for
detection. These sequences have a lower efficiency of suppression of the water
signal$^{104}$.

$^1$H-NMR in metabonomics studies has faced challenges in the analysis of low
volume biofluid and microdialysis samples. Two primary choices are available to
reduce the volume requirement for these analyses – specialized NMR tubes or
specialized NMR probes. Typical 5 mm NMR tubes require a few hundred
microliters of sample. This volume requirement can be reduced by using smaller
NMR tubes, which can go down to 1 mm or by using Shigemi tubes, which reduce
sample volume requirements by only requiring enough sample to fill the active region
of the probe. Specialized NMR probes, such as nanoprobe or capillary probes$^{107-109}$
can also be used. These probes have a flow cell volume of a few microliters, with an
observe volume of 1-2 $\mu$L or less. These probes can be advantageous for the analysis
of these samples, but do require access to an NMR magnet either dedicated to
analysis with the capillary probe or that allows for routine probe changes.

Solid samples are also challenging for NMR analysis, because of restricted
analyte motion in the sample matrix and heterogeneity within the sample, causing
resonance broadening$^{106}$. Most solid tissue samples undergo tissue extraction
procedures prior to NMR analysis, but these procedures are time-consuming and
introduce selectivity, thus reducing the scope of metabolites that can be profiled. An
increasingly popular alternative to tissue extraction is magic angle spinning (MAS)
NMR$^{26,63-65,110-113}$, which directly analyzes small molecules in solid tissue samples.
The tissue sample is inserted into a small rotor, which is spun in the core of the magnet (~4-6 KHz spin rate) at the magic angle of 54.78°, so that molecular motion is similar to solution phase. Spinning at this angle reduces the line broadening observed in these solid samples, improving resolution and signal.

1.3.2. Mass Spectrometry (MS)

Next to NMR, most metabonomics studies are performed by LC/MS. Most metabolomic studies, which focus on quantitation of the entire metabolome, have been performed on the GC/MS, showing good sensitivity and resolution. However, GC/MS experiments have been limited in metabonomic studies\textsuperscript{114,115}, which focus on the analysis of multiple samples to determine an organism’s response to a metabolic stimulus. The throughput of GC/MS in these studies is low due to the need for derivatization of polar nonvolatile analytes such as amino acids and sugars, which comprise a large portion of the metabolome\textsuperscript{52,114}.

Therefore, LC/MS has been much more popular for metabonomics studies. This work was pioneered by the lab of Robert Plumb at Waters Corporation, with initial studies focusing on the detection of metabolites in urine samples using LC-ESI-MS\textsuperscript{62,116}. Sample preparation is much less involved than with GC/MS methodology – most samples can be either directly injected or undergo a short protein precipitation procedure. Throughput is lower than NMR, as experiment times can be upwards of 1 hour. The MS detection itself has improved detection limits compared with NMR (provided the molecule ionizes efficiently) and has a good dynamic range. The use of smaller diameter LC columns (1-2 mm) has improved resolution and
lowered sample volume requirements. Others have explored increasing the resolution through the use of ultra-performance LC (UPLC)\textsuperscript{117}, or the use of columns with smaller particle sizes. However, differences in ionization efficiencies between compounds introduce selectivity into this detection mode. Also, changes in ionization efficiency can occur during the actual MS experiment, when coeluting compounds affect the ionization of the other compounds\textsuperscript{118}. Analysis is generally performed using a gradient LC system with a reversed phase column and ionization is accomplished by electrospray in positive and negative mode. Those developing analytical methodology focus on increasing peak capacity. A peak capacity of 1500 has been achieved, but at the expense of throughput – this separation took approximately 20 hours\textsuperscript{119}. Improved resolution has also been explored through new column stationary phases, including monolithic\textsuperscript{120} and hydrophilic interaction chromatography (HILIC) phases\textsuperscript{121}.

While MS detection with separation does allow for lower detection limits, several new issues are introduced by this analytical technique. First, the technique itself is more selective – different classes of compounds will be detected depending on the column, mobile phase, ionization technique, mass analyzer and other conditions. Second, quantitation is difficult in mass spectrometry studies. One of the primary ideas behind metabonomics is no \textit{a priori} identification of metabolites of interest. However, the best quantitation in mass spectrometry studies comes using deuterated standards of analytes. Not only is this difficult to do in metabonomics studies, it is impractical based on the number of metabolites detected in a single
experiment. Additionally, drift in retention times can make comparison between runs difficult\textsuperscript{122}.

A small number of studies have also been performed by CE/MS\textsuperscript{123-127}. Some applications include characterization of enzyme activity\textsuperscript{123}. These dramatically reduce sample volume requirements, which could potentially improve time resolution or allow for the concentration of samples. CE/MS also has the potential to give high resolution separations. Separate run conditions need to be employed for anionic and cationic analytes. In the initial study, 1692 metabolites were detected in 20 hours, but only 150 were identified\textsuperscript{124}. This raises a couple of issues: first, it can be difficult to identify compounds and second, quantitation must be performed by running standards, making it difficult to not identify compounds of interest before analysis. CE/MS studies to date have primarily focused on more metabolomic applications in plants and bacteria, but some metabonomics studies on urine samples have been performed\textsuperscript{126}.

\subsection*{1.3.3. Other Techniques}

UV detection is not ideal for metabonomics studies, for two primary reasons\textsuperscript{37}. First, the data generated gives little information about the molecule generating it, and second, the linewidth of the signal generated is large, lowering peak resolution. Nevertheless, a few studies have been performed with this detection mode, including CE/UV\textsuperscript{128} and LC/UV\textsuperscript{129} analyses.

Fourier transform-infrared spectroscopy (FT-IR) has been utilized in limited studies to characterize biofluid samples\textsuperscript{130}. Its main advantages lie in the speed of
sample acquisition (several seconds) and its nonselectivity. However, it has found limited use in metabonomics studies because of the difficulty in signal assignment and less overall information present in a single spectrum. It is possible to distinguish different functional groups present in the sample, but individual metabolites typically cannot be distinguished.

Electrochemical (EC) analytical techniques have been applied to metabonomics studies, including studies with coulometric arrays\(^1\) and microchip technology\(^2\). This offers excellent detection limits and good dynamic range. However, detection is limited to redox-active compounds. Compound classes such as hormones, antioxidants, neurotransmitters and oxidative stress markers can be profiled using this technique\(^1\).

Combining detection modes in metabonomics is being explored, in an attempt to gain the advantages from two orthogonal analytical techniques and increase the number of metabolites detected. NMR and MS have been combined, primarily by splitting flow from the LC to each detection scheme\(^1\). An LC-EC-Array-MS parallel detection method has also been described\(^1\).

### 1.4. Statistics for Metabonomics

#### 1.4.1 Multivariate Statistics Overview

The complexity of the data generated in metabonomics analyses, be it from mass spectrometry or NMR spectroscopy, makes it very difficult to evaluate visually and very cumbersome to analyze in a univariate manner. It is also desirable to
monitor patterns of biomarkers, or how multiple biomarkers change simultaneously with respect to each other. Consequently, pattern recognition techniques are used on metabonomics data sets through multivariate statistical techniques. Generally, two types of pattern recognition methods exist: unsupervised and supervised. Unsupervised methods, including principal components analysis (PCA), examine patterns and clustering in the metabolite data without \textit{a priori} identification of groupings or trends. These are typically used to simply describe trends occurring in the data. Supervised methods, such as discriminant analysis, are used to classify the data into groups. In these methods, “training sets” of data are used to define the boundaries of groups (i.e., control and dosed) and then the model generated is used to classify unknown metabolic profiles into their respective groups with a certain degree of confidence. There are many statistical techniques available to the researcher. This discussion will focus on the two most relevant to this research, principal components analysis and partial least squares regression.

These statistical techniques are well-suited to metabonomics data analysis for several reasons. First, they are adaptable to many different types of data – MS, NMR, or data from any other analytical method can be translated into the matrices needed for these analyses\textsuperscript{135}. Also, the quality of these analyses tends to improve with an increasing number of variables, because the new or latent variables are a weighted average of the original variables\textsuperscript{135}.

Metabonomics data sets are incredibly complex, with a large number of variables. What the variables represent is dependent on the analytical method
employed. (For simplicity and because it is most pertinent to this research, the following discussion will be limited to NMR data. Variables from an NMR data set represent integral regions in the NMR spectra.) Data matrices for these variables contain two types of data – noise (or undesirable variation) and systematic variation directly related to the metabolic perturbation\textsuperscript{135}. It is the goal of the researcher to minimize the noise so that systematic variation can be studied without interference, which can be done through a variety of mechanisms, including scaling\textsuperscript{136}. This can be done in many ways and is discussed further in Chapter 2.

1.4.2. **Principal Components Analysis**

Principal components analysis (PCA) is by far the most prevalent statistical tool in metabonomics studies\textsuperscript{135,137}. It is fairly easy and efficient to obtain results and gives an accurate view of the data set. It can be used to point the way for further statistical tests or important variables to probe further, or can be used to describe the data on its own. PCA allows for visualization of trends in the data, both groupings and time trends and can identify data outliers\textsuperscript{136}.

PCA reduces complex sets of data (with a high number of correlated variables) to a model with fewer variables but containing the same amount of variance\textsuperscript{137-139}. This is called data reduction, making the data set more manageable and easier to study. Mechanistically, PCA accomplishes data reduction through the generation of new variables via an eigenanalysis of the original data set. The eigenanalysis is actually performed on a matrix of correlation coefficients between each variable. A set of new variables, called principal components, are generated by
creating linear combinations of the original variables (for example, NMR integral regions). Each principal component is orthogonal to the others\textsuperscript{135}. The set of new variables describes the same amount of variance in the original data set, with the first principal component explaining the most amount of variance and each subsequent principal component explaining successively less variance. PCA generates two matrices of information – a scores matrix and a loadings matrix. The loadings matrix describes the new variables/principal components by giving the coefficients used in the linear combination of original variables to calculate the new variable’s value. The scores matrix contains a data point for each sample in the original data matrix, which is calculated from the linear combination described in the loadings matrix. A PCA model can contain the same numbers of principal components as there are variables in the original data set, but this is typically not necessary. Most trends in the data can be visualized in the first few principal components. The PCA model is examined by generating scores plots, or by plotting the new principal components against each other. Clustering in the data will illustrate which samples have similarities (and differences). Time trends will also emerge in these plots. By observing which axis/principal component the trend is on, loadings for that principal component can be used to determine which of the original variables were important in defining that trend.

1.4.3. Partial Least Squares Regression

Partial least squares regression (PLS) is used to correlate complex metabonomics data sets (called predictors) to another set of data (called
Responses could correspond to quantitative variables, such as time or drug dosage or to qualitative variables, such as groupings. These data sets are represented as separate matrices in the analysis. A model is created that maximizes the covariance between the matrices by developing latent variables to describe the data set. The scores and loadings for the model can give information about the strength of metabolite correlation to the response matrix. PLS models are typically tested through cross-validation. In this test, part of the data set is removed and the model is recalculated. This is compared to the original model, and the difference between the models provides a gauge of the model robustness.

1.5. Tissue-Targeted Metabonomics Application: Oxidative Stress

Metabonomics has seen many applications over the years, as reviewed in Section 1.2.1. This research is primarily concerned with the application of metabonomics to study oxidative stress. Oxidative stress is a potentially devastating occurrence in the human body. It plays an important role in the pathogenesis of many debilitating or fatal conditions, including myocardial infarction (heart attack), stroke, atherosclerosis, Parkinson’s disease and Alzheimer’s disease. The societal impact of oxidative stress damage is widespread. In the United States alone, 8.1 million people suffered a heart attack and 5.8 million people suffered a stroke in 2005. Coronary heart disease is the leading cause of death in the United States, with stroke the third leading cause of death. Combined yearly healthcare costs associated with coronary heart disease and stroke are over 500 billion dollars.
Oxidative stress can occur from a variety of sources. Conditions such as stroke or heart attack cause oxidative damage to tissue resulting from ischemia/reperfusion, or the cessation and subsequent restoration of blood flow. Oxidative stress can also be induced chemically, such as through redox cycling of the quinone moiety on doxorubicin, a common chemotherapy agent\textsuperscript{145}. The resulting oxidative damage is often irreversible and could cause organ failure. Oxidative stress will result from any situation in which the body is unable to mediate free radical levels.

The agents causing oxidative damage, reactive oxygen species (ROS) and reactive nitrogen species (RNS), are highly reactive compounds, including free radicals and peroxides, produced in aerobic organisms\textsuperscript{146}. The ROS include superoxide (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), peroxynitrite (ONOO\(^-\)) and the hydroxyl radical (\(^*\)OH). Organisms constantly generate a low level of ROS under basal metabolic conditions. In the liver, the concentration of \(H_2O_2\) ranges from 10\(^{-7}\) to 10\(^{-9}\) M, and the concentration of \(O_2^-\) is approximately 10\(^{-11}\) M\textsuperscript{147}. ROS play a role in a few normal cellular processes, including catalysis by ribonucleotide reductase and phagocyte immune response\textsuperscript{148}. Free radicals mostly originate from the mitochondria (during cellular respiration)\textsuperscript{146} and the endoplasmic reticulum\textsuperscript{148}. There is a balance in the body between basal free radical production and levels of antioxidants to mediate the radicals. Many of these free radicals are dangerous, as they are highly reactive, with the capability to destroy virtually any cellular component if left unchecked due to insufficient antioxidant levels\textsuperscript{146,149}. 
On a molecular level, oxygen free radicals can be generated through a variety of pathways (Figure 1.4), in addition to cellular respiration in the mitochondria. First, enzymatic formation is common. Xanthine oxidase, mitochondrial cytochrome oxidase, cyclooxygenase, lipoxygenase and NADPH oxidases all can cause the transfer of a single electron to molecular oxygen\textsuperscript{148,150}. The autooxidation of compounds such as the catecholamines can also result in ROS production\textsuperscript{148,150}. Finally, reperfusion causes an insurgence of neutrophils to the site of ischemia which release proteolytic enzymes that generate free radicals\textsuperscript{149}. Reduction of molecular oxygen by one electron forms the superoxide anion, while two electron reduction of molecular oxygen results in the formation of hydrogen peroxide\textsuperscript{148}. Hydrogen peroxide is also formed through a dismutation reaction of superoxide, typically catalyzed by superoxide dismutase, although the reaction can slowly occur spontaneously\textsuperscript{148}.

\[
\text{O}_2^{\bullet+} + \text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

(1)

Superoxide can go on to form other products such as the highly reactive hydroxyl radical and peroxides. The Haber-Weiss reaction forms hydroxyl radicals and molecular oxygen from superoxide and hydrogen peroxide substrates\textsuperscript{149}. This is a two step reaction. The first step is the reaction of superoxide with Fe\textsuperscript{3+}, mediated by superoxide dismutase, forming Fe\textsuperscript{2+} and molecular oxygen\textsuperscript{148}. The Fenton reaction then forms the hydroxyl radical from hydrogen peroxide, as catalyzed by Fe\textsuperscript{2+} generated from the first reaction\textsuperscript{149,151}.

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \bullet\text{OH} + \text{OH}^-
\]

(2)
Oxidative Damage
Lipid peroxidation
DNA strand breakage
Enzyme inactivation

Antioxidants:
- Thiols
- Ascorbate
- Vitamin E
- β-carotene

O2

Fe2+ (O2•− + Fe3+ → Fe2+ + O2)

H2O2

GSH

Glutathione

Peroxidase

Glutathione Reductase

NADPH, H+

NADP+

Antioxidants:
- Thiols
- Ascorbate
- Vitamin E
- β-carotene

Oxidative Damage
Apoptosis
Necrosis

ONOO−

NO

O2•−

O2

Figure 1.4. Pathways of formation of oxygen free radicals. Adapted from reference 151.
The hydroxyl radical is highly toxic, as it is one of the most reactive species known\textsuperscript{148}. It can cause lipid peroxidation, inactivate enzymes and induce DNA strand breakage\textsuperscript{151}. Although not as toxic as the hydroxyl radical, superoxide itself can also instigate oxidative damage directly through reaction with FeS enzyme centers, resulting in enzyme inactivation. However, it is more likely that it contributes to oxidative stress indirectly through its reduction of transition metal ions and conversion to hydrogen peroxide\textsuperscript{148}. Superoxide can also react nonenzymatically with nitric oxide, producing peroxynitrite (ONOO\textsuperscript{-})\textsuperscript{148,149}, another causative agent of oxidative damage. This ROS is also highly reactive and has been shown to increase superoxide formation by inhibition of superoxide dismutase and interference with the electron transport chain\textsuperscript{148}.

The effects of oxidative damage are felt by every cellular component. Oxidative damage at DNA bases primarily occurs through oxidation of the guanine base. This causes an 8-hydroxyguanine lesion, leading to G to T transversions in DNA replication. These lesions are repaired by the body through base excision repair, which removes the damaged base, preventing further mutagenic damage from occurring. The extent of DNA damage from ROS is often measured by the levels of excreted base excision repair products in plasma or urine, such as the marker 8-hydroxy-2’-deoxyguanosine (8-OHdG)\textsuperscript{152}. Oxidative damage can also occur on lipids, where ROS react with polyunsaturated fatty acids, forming hydroperoxides, conjugated dienes and aldehydes. The hydroxyl or peroxynitrite radical produces an alkyl radical that produces a peroxyl radical (ROO\textsuperscript{*}) after reaction with molecular
oxygen. This instigates a cycle of lipid peroxidation, as the peroxyl radical can gain a hydrogen from the neighboring fatty acid, which creates a hydroperoxide and another peroxyl radical\textsuperscript{150}. Common markers of lipid peroxidation include malondialdehyde and other dialdehydes, as well as 4-hydroxynonenal (4-HNE)\textsuperscript{153}. Lipid peroxidation also produces formaldehyde, acetaldehyde, acetone and propionaldehyde from $\beta$-cleavage of lipid peroxides\textsuperscript{153}.

Free radicals are neutralized \textit{in vivo} by a variety of sources. Some proteins can indirectly mediate oxidative damage by chelation of metal ions\textsuperscript{148}. The enzymes catalase and glutathione peroxidase both inactivate hydrogen peroxide, converting it to water\textsuperscript{151}. The hydroxyl radical is not trapped \textit{in vivo}, but repair mechanisms are in place to mediate the damage caused, such as base excision repair discussed previously. Free radicals are also mediated nonenzymatically, through compounds such as thiols, ascorbate, uric acid, vitamin E and $\beta$-carotene\textsuperscript{147,150}. Thiols are the small molecule antioxidant defense system in the body – the most predominant being glutathione (GSH), a tripeptide consisting of glycine, glutamate and cysteine. Other endogenous thiols include cysteine, homocysteine and cysteinylglycine. The sulfhydryl group on the cysteine interacts with ROS, producing a thyl radical (GS$^\ast$) through one electron oxidation. The thyl radical reacts with GS$^-$ and superoxide to form glutathione disulfide (GSSG) and H$_2$O$_2$. Thiols act as a redox buffer by maintaining a balance between reduced and oxidized forms – the relative amounts of these can give some indication as to the oxidative stress status of the tissue.
Because oxidative stress is so closely linked to mitochondrial activity, there is a natural transitive relationship between oxidative stress and energy metabolism. Problems in the electron transport chain may result in ROS formation, which can then create a cycle of increasing ROS production. During ischemia, energy is generated anaerobically from the conversion of glucose to lactate. Energy stores are also utilized rapidly, including creatine phosphate, glycogen, and adenosine compounds. Without an energy supply, ions leak across the cell membrane, causing depolarization. Adenosine monophosphate (AMP) is metabolized to hypoxanthine, xanthine and uric acid by xanthine oxidase, producing the superoxide radical. A decrease in pH is observed from anaerobic metabolism, resulting in activation of lysosomal enzymes. The combined effects of these metabolic changes result in dysfunction for the organ, such as contractile problems in the heart. Although ischemia causes extreme changes in metabolism, studies suggest that most of the damage to the tissue occurs during reperfusion, when most of the ROS are formed. This is known as the oxygen paradox – although oxygen is essential for the survival of organisms, a resurgence of it after ischemia can injure the tissue through the formation of reactive oxygen species.

Oxidative stress can also be induced by the administration of doxorubicin (also known as adriamycin), a common chemotherapy drug used to treat a variety of cancers. Doxorubicin exerts its anticancer effects through intercalation into and cross-linking with DNA bases, causing problems with strand separation and interfering with the activity of helicase, topoisomerase II and DNA and RNA
polymerase$^{157,158}$. It also induces apoptosis$^{158-160}$. The doxorubicin dose that can be
given to a patient is limited due to its cardiotoxic side effects observed above a
threshold dose, leading to irreversible changes to the heart and congestive heart
failure$^{161}$.

The mechanism for this cardiotoxicity is unclear, but has been shown to be
linked to free radical production, mitochondrial dysfunction, excess calcium levels,
activity changes in membrane-bound enzymes and abnormal fatty acid oxidation$^{162-}$
$^{165}$. The evidence for free radicals causing cardiotoxicity is strong$^{145}$ because studies
have demonstrated the mediation of cardiomyopathy through administration of
antioxidants$^{166}$. Heart tissue is uniquely susceptible to doxorubicin toxicity, due to its
high rate of oxidative metabolism, lowered levels of antioxidants, and the affinity of
doxorubicin to cardiolipin (a phospholipid in cardiac mitochondrial membranes)$^{165}$. It is thought that cardiac cells are more susceptible to damage from doxorubicin
because of a different enzyme in the mitochondrial membrane that reduces
doxorubicin and causes it to displace CoQ$_{10}$, a critical element in the electron
transport chain$^{145}$. Although doxorubicin is notorious for its cardiotoxic side effects,
its toxicity is observed in other tissues as well, such as the liver$^{167-169}$. One of the
primary mechanisms by which doxorubicin generates its toxic side effects in the liver
is through lipid peroxidation ultimately ending in membrane disruption, as discussed
previously$^{145,167,169,170}$. Doxorubicin has been shown to increase levels of superoxide
dismutase, catalase and glutathione peroxidase in liver tissue, while decreasing levels
of cytochrome P450 and glutathione$^{168,169,171}$.  

41
Doxorubicin initiates oxidative damage in tissues through redox cycling. It contains a tetracyclic group, with a daunosamine sugar linked by a glycosidic bond. The tetracyclic moiety contains adjacent quinone and hydroquinone structures that allow for reduction to the semiquinone. The semiquinone in turn will give up the electron to molecular oxygen, creating the superoxide free radical that can go on to cause cellular damage or undergo conversion to the more highly reactive hydroxyl radical, causing damage to lipids, DNA and proteins. (This redox cycling is illustrated in Figure 1.5.) Doxorubicin can be reduced by enzymes, such as NADH dehydrogenase, xanthine oxidase or NADPH-cytochrome P450 reductase. Doxorubicin can also be reduced through complexation with metal ions, typically iron. In this reaction, Fe$^{3+}$ accepts an electron from doxorubicin, forming an Fe$^{2+}$-doxorubicin complex, which goes on to reduce oxygen to ROS. Thus, doxorubicin can be a chemical inducer of oxidative stress, allowing oxidative stress to be induced much more easily and reproducibly than with ischemic mechanisms.
Figure 1.5. Doxorubicin redox cycling. Adapted from reference 145.
1.6. **Focus of This Research**

This research focuses on the development of tissue-targeted metabonomics, an analytical approach to examining tissue-specific metabolism. There is a need in the metabonomics community for additional technologies that can provide insight into tissue metabolism, particularly if the technology is noninvasive and allows for continuous sampling from the same animal. Microdialysis sampling is used to collect the small endogenous metabolites in the interstitial fluid of the tissue of interest. (In this study, focus was primarily directed to the liver.) These samples were then analyzed by proton NMR and subjected to multivariate data analysis to find patterns in the metabolic data.

Initially, focus was placed on the development of the analytical methodology. Several issues had to be addressed before application of this technique to biological problems. First, the analysis of small volume dialysate samples by NMR had to be optimized. Variance in the data from experimental conditions had to be identified, characterized and then removed from the metabolic data by normalization. These factors included probe extraction efficiency, variation from the NMR analysis and animal-to-animal variability. Data pretreatment steps were examined for their impact on the results of the statistical analyses.

Next, inherent biological variation in the metabolic information was characterized. Processes such as animal-to-animal variability, circadian rhythms, animal activity and anesthesia effects can all cause changes in the basal metabolism
of the animal. It is important to be able to identify these changes so that they can be separated from metabolic changes induced by an experimental stressor.

Finally, the developed tissue-targeted metabonomics method was applied to the study of hepatic oxidative stress, as induced by the chemotherapy agent doxorubicin. This was a proof of concept study used to determine if the technique could successfully detect and follow metabolic changes over time. These studies were performed in conjunction with the quantitation of a biomarker of oxidative stress, glutathione, to determine if data analysis was more successful using a previously established marker to guide interpretation.

1.7. References


2.1. Introduction

Tissue-targeted metabonomics provides an alternative to traditional metabonomics sampling approaches using urine and plasma, which can be obtained fairly noninvasively and in sufficient quantity and concentration for metabonomics analytical techniques. Urine and plasma sampling, although popular, is not without limitations. Time resolution can be limited in these studies, as discussed in Chapter 1. The potential for changes in sample composition due to bacterial growth (as in urine) or enzymatic degradation (as in plasma) can also cause complications in sample collection and data interpretation.

Most importantly for this research, sampling biofluids like urine and plasma does not provide spatial information about metabolic changes. Many metabonomics studies have made use of tissue biopsies to obtain localized metabolic information. The tissues studied are diverse and have been derived from both animals and humans for basic research and clinical applications. They include the liver\textsuperscript{1-4}, kidney\textsuperscript{4}, brain\textsuperscript{1,5-7}, heart\textsuperscript{8}, prostate\textsuperscript{9}, tumors\textsuperscript{9-11} and human gut biopsies\textsuperscript{12}. One limitation of this
sampling approach is the termination of the experiment after a biopsy is taken, utilizing large numbers of animals to characterize metabolic differences and reducing experimental precision due to animal-to-animal variability. Samples must either be analyzed using a specialized NMR probe (high resolution magic angle spinning probe) or the tissue must be homogenized and then undergo an extraction procedure. A viable alternative to tissue biopsy is microdialysis sampling of extracellular fluid\textsuperscript{13-16}.

Microdialysis offers an inherent advantage for site-specific metabonomics sampling. Careful selection of the molecular weight cutoff of the membrane can selectively exclude macromolecules. Thus, samples are much cleaner and do not require additional cleanup steps that reduce throughput and increase method error. Microdialysis samples are typically small in volume (flow rates of \(\sim 1 \mu\text{L/min}\), with typical sampling times ranging from a few minutes to an hour). While not typically a problem for metabolic profiling with mass spectrometry, this does require special consideration when conducting NMR analysis, such as using tube inserts or low volume probes.

For microdialysis studies, additional sample preparation is required prior to NMR analysis. Dialysate samples are very low in volume and many components border on the detection limits of the NMR instrument. Thus, any dilution becomes problematic. One solution to this problem involves taking dialysate samples to dryness and reconstituting in a deuterated solution to provide a lock signal and avoid any dilution effects.
There are two primary options for low volume NMR analysis: capillary tube inserts and capillary probes or probes with reduced detection volumes. Capillary tube inserts are made to sit inside a typical 5 mm NMR tube with the aid of a plastic adapter. The capillary is submerged in sample-matching solvent (usually D$_2$O) and then the sample is centered in the active region of the magnet for analysis. Although tube inserts do not provide an efficient filling factor, they allow the analysis of small volume samples without specialized NMR probes. The low volume detection cells of specialized capillary probes and nanoprobes can accommodate smaller sample volumes and typically provide better mass sensitivity than capillary inserts, but they can be more costly and require flexibility in the NMR setup so that the probe can be changed.

2.2. Specific Aims

The purpose of this research was to develop and validate analytical methodology to enable metabolic profiling by $^1$H-NMR of microdialysis samples. Because this is a new approach to metabonomics studies, no literature exists detailing appropriate methodology and characterizing the important analytical considerations for these experiments. The analytical method involved three primary components that were characterized in this study: microdialysis sampling, $^1$H-NMR detection and multivariate statistical analysis by principal components analysis (PCA). Microdialysis sampling considerations included probe calibration, as well as sample preparation procedures that minimized sample composition changes but allowed for
concentration of the sample and reconstitution in a deuterated buffer. \(^1\)H-NMR analysis considerations primarily involved characterization of the small volume detection scheme. Considerations for the multivariate statistical analysis involved the integration of the NMR spectra, normalization of the integral data and optimization of the principal components analysis parameters. Time trend analysis was also performed on basal dialysate samples by both principal components analysis and partial least squares regression.

2.3. **Materials and Methods**

2.3.1. **Reagents and Solutions**

All deuterated solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Salts for Ringer’s solution and other reagents were purchased from Sigma Chemical Corporation (St. Louis, MO). Ringer’s solution composition was 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl\(_2\), and 1.2 mM MgCl\(_2\). Solutions were prepared in nanopure water (18.2 M\(\Omega\) resistivity, Labconco, Kansas City, KS) and filtered through a 0.22 \(\mu\)m pore filter prior to use.

2.3.2. **Microdialysis Methods**

2.3.2.1. **Microdialysis Probe Preparation and Calibration**

All probes were prepared in-house, using polyacrylonitrile (PAN) membrane (Hospal, Lakewood, CO) of dimensions 350 \(\mu\)m O.D. by 250 \(\mu\)m I.D. and polyimide tubing (MicroLumen, Tampa, FL) of dimensions 170 \(\mu\)m O.D. by 120 \(\mu\)m I.D. All probes used Tygon tubing attached at the inlet as a syringe adapter. Vascular probes
were prepared with a 10 mm membrane length. Liver probes were linear and also had a 10 mm membrane length. The outlet of the probe had a 7 cm piece of fused silica capillary (360 μm O.D. by 200 μm I.D.) attached to the polyimide to use as a needle guide. Heart probes were linear and were approximately 2 mm in membrane length. A 1 mm long Tygon tubing “stop” was secured directly behind the active window of the membrane to aid in implantation of the probe. The outlet of the probe had a 27 gauge stainless steel needle (bent to 45°) attached to aid in implantation. Both the liver and heart probe are pictured in Figure 2.1. All probes were perfused with Ringer’s solution at a rate of 1 μL/min and were flushed for 30 minutes prior to the start of sampling. Samples were collected over 60 minute intervals (producing 60 μL sample volumes) unless explicitly noted.

Probes were calibrated continuously through the experiment by delivery of 7 μM antipyrine in Ringer’s solution\textsuperscript{17,18}. Antipyrine is an established marker of microdialysis membrane extraction efficiency\textsuperscript{18} and will account for global changes in probe performance. The concentration of antipyrine was chosen by the maximum concentration that could be used without detection by NMR, to avoid interferences with endogenous metabolite resonances. Antipyrine was detected using LC/UV at 254 nm (Shimadzu, Columbia, MD). The isocratic LC system was equipped with a Shimadzu LC-10AD pump and SPD-10AV UV-Vis detector, which was integrated to EZStart software (version 7.3, Shimadzu Scientific Instruments, Inc., Kyoto, Japan) with an SCL-10AVP system controller. A Rheodyne 7725i injector fitted with a 25 μL PEEK loop was also used. Samples were injected onto a Phenomenex Synergi
Figure 2.1. Microdialysis probe design. A shows a linear microdialysis probe used for liver microdialysis, with an active membrane length of 10 mm. B shows a linear microdialysis probe used for heart microdialysis, with an active membrane length of 2 mm and a Tygon stop to aid in implantation.
Polar-RP column of 4 μm particle size, of dimensions 2 mm x 150 mm. The mobile phase was acetonitrile-sodium phosphate (pH 2.5, 25 mM) (17:83, v/v) and was set to a flow rate of 0.3 mL/min.

2.3.2.2. Animal Setup

All experiments utilized male Sprague-Dawley rats (Sasco, Wilmington, MA), weight range 350-450 g. Rats were pre-anesthetized by isoflurane inhalation and then fully anesthetized with a ketamine (67.5 mg/kg)/xylazine (3.4 mg/kg)/acepromazine (0.67 mg/kg) cocktail, administered subcutaneously. For nonsurvival surgeries, rats were maintained under anesthesia for the duration of all experiments, using ketamine booster doses administered as needed intramuscularly at one quarter of the initial dose. Rats undergoing survival procedures were only given the initial anesthesia cocktail, which maintained anesthesia for the duration of probe implantation. Rat body temperature was maintained at 37°C using an electric heating pad.

For survival surgeries, all procedures were performed aseptically. Rats were given 3 mL saline subcutaneously after the surgery to facilitate recovery and were housed in a Raturn© system (BASi, West Lafayette, IN). No buprenorphine analgesic was given to avoid problematic perturbations in basal tissue metabolism. All animal experiments were performed under approval of the local Institutional Animal Care and Use (IACUC) committee.

2.3.2.3. Jugular Probe Implantation

The jugular vein was exposed through a small midline incision in the neck skin tissue. The vein was isolated by separating the vessel from fine connective
tissue using blunt dissection and cotton swabs. A flat metal spatula was placed under the vein to externalize it, and a small incision made in the vessel using a pair of fine spring scissors. A vascular probe with an introducer was inserted into the vein toward the heart. Silk sutures were used to secure the probe in the vessel, and the introducer was then removed. (Surgery was performed by Shannon S. Vandaveer.)

2.3.2.4. Heart Probe Implantation

Pictures to illustrate the surgical procedure are shown in Figure 2.2. A tracheotomy was performed by externalizing the trachea through an incision on the midline of the neck. The trachea was isolated using a spatula and any fluid around the trachea was removed with cotton swabs (Figure 2.2A). A small nick was made between the rings of the trachea and an intubation tube was immediately inserted and secured with sutures. The rat was attached to a Digi-Med Sinus Rhythm Analyzer (Louisville, KY) to monitor heart rate and sinus rhythm. Immediately prior to opening the chest cavity, the intubation tube was connected to a respirator that supplied artificial ventilation for the rat. A constant-volume respirator using room air (Model 683 Rodent Respirator, Harvard Apparatus, Holliston, MA) was used. To expose the heart, a thoractomy was performed on the left side of the animal by making an incision between and parallel to the fifth and sixth ribs approximately 1.5 to 2 cm in length (Figure 2.2B), and the pericardium was opened, allowing the microdialysis probe to be inserted into the myocardium. Specifically, a bent 27 gauge needle attached to the probe was used to implant the probe into the apex of the beating heart muscle, near the left descending coronary artery along the longitudinal
Figure 2.2. Progression of microdialysis probe implantation in the heart. A shows the externalization and ventilation of the trachea. B shows the rat being prepared for the opening of the chest cavity. The rat has been connected to electrodes on three of its legs to monitor the heart rhythm, and an incision has been made in preparation for the thoractomy. C shows the chest cavity open immediately prior to implantation. The lungs have been retracted. The cotton swab in the left hand is used to expose the beating heart for implantation and the forceps in the right hand are holding the bent needle used as an implantation guide for the probe.
axis of the heart (Figure 2.2C). The lungs were then fully expanded by blocking the exhalation tube on the rodent respirator for several seconds. (Some of the surgeries were performed by Sara L. Thomas.)

2.3.2.5. Brain Probe Implantation

The animal was positioned in a stereotaxic apparatus with the incisor bar set to level the head between the lambda and bregma fissure lines of the skull. A 1 cm midline incision was made in the scalp. The incised skin was gently separated from the skull and then spread open. The exposed scalp was cleaned and dried. A guide cannula was implanted into the striatum (stereotaxic coordinates from bregma: anterior 1.0 mm, lateral 2.7 mm, vertical -3.6 mm). Two jeweler’s screws were partially screwed into the skull on either side of the cannula to act as an anchor for the dental cement, which was used to fix the cannula to the skull. The incision was stitched around the dental cement. After implantation, the guide cannula was replaced with the microdialysis probe, which had a 4 mm active polycarbonate membrane (CMA Microdialysis AB, Sweden). (Surgery was performed by Shannon S. Vandaveer.)

2.3.2.6. Liver Probe Implantation

Pictures to illustrate the surgical procedure are shown in Figure 2.3. A 2 cm incision was made along the midline of the abdomen directly below the diaphragm and xyphoid process, and the abdominal cavity was opened. The incision was spread with Bowman retractors and tissue forceps were used to expose the median liver lobe from beneath the ribs (Figure 2.3B). Two microdialysis probes were implanted using
Figure 2.3. Progression of microdialysis probe implantation in the liver (survival surgery).  A shows the rat prepared prior to surgery. The abdomen has been shaved and cleaned and placed on a heating pad. Immediately after this, the rat was covered with a sterile drape. B shows the midline incision made in the abdomen, with the median liver exposed using tissue forceps. C shows the fused silica in the liver tissue used as the probe implantation guide. D shows the muscle layer being stitched up, with the probe tubing externalized through the incision. E shows the trocar used to tunnel the probe tubing from the abdominal incision out the back of the neck, and F is the closing of the abdominal incision. G shows the rat emerging from anesthesia in the Raturn® bowl. The jacket is utilized to attach the rat to the harness, which signals rat activity and turns the bowl appropriately.
the fused silica capillary as a guide needle (Figure 2.3C). The probes were implanted 1 cm apart, which has been shown by previous studies to be a sufficient distance to prevent cross-talk between the probes\textsuperscript{19}. The tissue entrance and exit points for each probe were secured with a small amount of tissue glue applied with a plastic transfer pipet. After probe implantation, the incision was closed with sutures, with the probe inlets and outlets externalized through the incision (Figure 2.3D). For survival surgeries, the probe tubing was tunneled underneath the skin and externalized through a small incision in the back of the neck (Figure 2.3E). The muscle layer incision was closed with sutures and the skin was closed with staples (Figure 2.3F).

2.3.2.7. Microdialysis Sample Preparation

All dialysate samples (60 \( \mu \)L original volume) were split into two fractions – one 50 \( \mu \)L fraction for NMR analysis and one 10 \( \mu \)L fraction for LC/UV analysis of antipyrine. Fractions for NMR analysis were speedvacuumed to dryness at room temperature after collection and stored at -20\(^{\circ}\)C in the dark until analysis. Immediately prior to NMR analysis, the sample was thawed to ambient temperature and reconstituted in 25 \( \mu \)L of 25 mM deuterated acetate solution in D\(_2\)O, pD = 7.8, with 5 mM trimethylsilyl propionic acid (TSP-d\(_4\)) as an internal standard. Samples were vortexed for 30 seconds and then transferred to a linear capillary tube insert (Wilmad-Labglass, Buena, NJ) using a 10 \( \mu \)L syringe with a 10 cm fused silica needle. The insert was submerged using a PTFE adapter in 600 \( \mu \)L D\(_2\)O in a 5 mm NMR tube. A picture of the capillary tube setup is shown in Figure 2.4. The capillary was filled with 25 \( \mu \)L of a fluorescein solution to aid in visualization.
Figure 2.4. Capillary tube insert in a 5 mm NMR tube.
2.3.3. \textit{NMR Spectroscopy}

$^1$H-NMR analysis was performed on a Varian INOVA 600 MHz spectrometer equipped with VnmrJ software (Version 2.1 Revision B, Varian Instruments Inc., Palo Alto, CA) at 25°C. Samples were analyzed on a 5 mm Varian triple-axis gradient probe. All samples were analyzed with the WET water suppression pulse sequence. A tip angle of 90° was utilized, with an acquisition time of 1.8 sec and 25200 points. The pulse sequence is shown in Figure 2.5. Spectra were coadded for 3000 transients for a total acquisition time of 95 minutes, zero-filled to 65536 points, and a line broadening of 1 Hz was applied. All spectra were referenced to TSP-d$_4$ at 0.00 ppm. Resonances were assigned by comparison to standard spectra measured in the sample solvent solution and literature chemical shift values.

2.3.4. \textit{Data Analysis}

NMR spectra were exported to ACD/Labs software (Version 10.02, Advanced Chemistry Development, Inc., Toronto, Ontario) and spectral processing (zero-filling, line-broadening, baseline correction, and referencing) was performed as described above. Spectra were manually integrated over 40 defined integral regions between 0.8 and 4.5 ppm, and integral tables were exported as a spreadsheet for data manipulation. (For sample stability analyses, spectra were manually integrated over 57 defined integral regions.) Integral values were normalized to the TSP-d$_4$ integral and concentration, as well as the extraction efficiency of the corresponding microdialysis probe, as measured by the % delivery of antipyrine. Analysis by principal components, partial least squares regression (PLS) and univariate methods
Figure 2.5. WET pulse sequence parameters.
were performed with Minitab® Statistical Software (Release 14.20, Minitab, Inc., State College, PA). Principal components analysis was performed on a correlation matrix (data matrix was centered and standardized), with five principal components calculated. Partial least squares regression between time and metabolic profiles was also performed on a correlation matrix, with a maximum of five components calculated. The PLS model was cross-validated through jackknifing.

2.4. Results and Discussion

2.4.1. Microdialysis Sampling

2.4.1.1. Comparison of $^1$H-NMR Spectra of Plasma and Plasma Dialysate

To illustrate the differences between conventional metabonomics data and tissue-targeted metabonomics data, a comparison of $^1$H-NMR spectra of rat plasma and plasma dialysate taken from the same rat is shown in Figure 2.6. D$_2$O was added to the plasma to a final concentration of 10% to provide a lock signal, while the plasma dialysate was prepared as described in the methods (Section 2.3.2.7). The plasma, as expected, showed underlying broad signals throughout the spectrum due to proteins and lipids. Specifically, much of the macromolecule signal in plasma comes from albumin, immunoglobulins, N-acetylated sugars on glycoproteins, lipoproteins, triglycerides and chylomicrons$^{20}$. Very few small molecule resonances are detectable above this background. The major visible sharper resonances in the region from 3.1 to 3.9 ppm correspond to glucose and the sharp singlet at 3.0 ppm is due to creatine.
Figure 2.6. Comparison of the $^1$H-NMR spectra of (A) plasma and (B) plasma dialysate.
Other small molecule resonances may be present, but are not sufficiently above the background for positive identification.

In contrast, the plasma dialysate NMR sample was entirely devoid of any macromolecule signal. Glucose and creatine resonances are present at the frequencies indicated above. Lactate, alanine, lysine, valine, leucine, isoleucine, citrate, tyrosine, and many other small molecules are detectable. The largest differences between the spectra are noted in the more aliphatic regions (0.5-2.5 ppm). This region contains resonances from many amino acids, which are key substrates in or have strong influences on metabolic pathways such as glycolysis, the citric acid cycle, the urea cycle, glucose-alanine cycle, and many others. Thus, this is a critical area of the spectrum to characterize in tissue metabolic profiling, which has been achieved through $^1$H-NMR analysis of microdialysis samples. In the dialysate NMR spectra, spectral resolution is high, indicating that the use of inserts for NMR analysis of small volume dialysate samples does not compromise the quality of the NMR spectrum.

2.4.1.2. Probe Calibration by Delivery of Antipyrine

Quantitation of all the metabolites in any metabolic profile of a dialysate sample is extremely difficult. To truly quantitate levels of a metabolite in the extracellular fluid of the tissue surrounding the probe, it is ideal to calibrate the probe $in vivo$ prior to the start of the experiment with the same compound. Due to the nature of metabonomics studies, in which analytes are not identified prior to sample analysis, probe calibration for each individual metabolite is precluded because all the metabolites cannot be identified prior to analysis. In addition, it would be logistically
difficult to calibrate a probe for a large number of metabolites in a single experiment. However, exact quantitation is not necessarily the goal of these studies – rather, it is the relative change from baseline levels. This makes probe calibration by delivery a good option for metabolomics studies using microdialysis sampling. In this method, the delivery of a standard compound (antipyrine is commonly used) through the microdialysis probe to the tissue has been shown to be equivalent to the recovery of the same compound from the tissue to the probe lumen. Thus, the use of a single marker compound to account for changes in extraction efficiency of many endogenous small molecules is a simple and efficient way to account for variability in the data due to changes in probe recovery. Antipyrine was detected in dialysate samples using the LC/UV method detailed in section 2.3.2.1. Figure 2.7 shows the separation of antipyrine from endogenous components in basal liver dialysate. In general, probe extraction efficiency remained constant over the course of the experiment, but did show variance from rat to rat. Section 2.4.4.1.2 discusses the use of probe extraction efficiency to remove extraneous variance from the metabolomics data set.

2.4.2. Sample Preparation Effects and Stability

2.4.2.1. Speedvacuuming Effects

Dialysate samples are primarily collected into an isotonic Ringer’s solution. Although it is possible to effectively suppress the water resonance in solutions that are predominately (i.e., 90%) protic water, any analyte resonances within this region of the spectrum are also suppressed. More significantly, the residual water resonance
Figure 2.7. LC/UV chromatogram of antipyrine in liver dialysate. A shows the chromatogram at full scale and B depicts the same chromatogram at a lower scale to magnify lower intensity peaks. Antipyrine elutes at approximately 7.1 minutes.
may distort the spectral baseline, affecting analyte quantitation. For dilute solutions, the residual water signal may dominate the spectrum, limiting the dynamic range of the measurement. Therefore, we chose to reduce the water resonance by drying the dialysate sample and reconstituting in a deuterated solution. This approach is also advantageous because the sample could be concentrated, improving sample throughput by reducing the time required for signal-averaging. However, it is possible that speedvacuuming could alter sample composition.

For this reason, the effect of speedvacuuming on the composition of heart and plasma dialysate samples was determined. Samples were split into two fractions, with one frozen at -20°C and the other speedvacuumed to dryness before storage at -20°C. All sample pairs were analyzed by NMR on the same day. Immediately prior to NMR analysis, frozen samples were diluted to a final TSP-d₄ concentration of 5 mM and deuterated acetate concentration of 25 mM. Speedvacuumed samples were reconstituted in 25 mM deuterated acetate solution in D₂O with 5 mM TSP-d₄ to the same final volume as the speedvacuumed samples.

Visual inspection of the spectra revealed few differences between the sample preparation methods. For heart and plasma dialysate, the carbohydrate regions (< 3 ppm) and aromatic regions (> 5 ppm) showed no detectable differences in both resonance intensity and resonances present. For both dialysates, small differences were noted in the 3 – 4.5 ppm region, primarily containing glucose resonances.

The normalized integrals from each sample were averaged for the frozen samples and for the speedvacuumed samples. A student’s t-test was performed on the
two means for each integral region. Only one out of 40 integral regions showed significant differences between the speedvacuumed and frozen heart dialysate samples at 95% confidence: 3.22-3.29 ppm. For the plasma dialysate samples, one region was significant at 95% confidence: 3.75-3.77 ppm. Both of these regions are dominated by glucose resonances, but do overlap other metabolite resonances.

Principal components analysis (Figure 2.8) showed a clear segregation between the plasma dialysate and heart dialysate samples (with one set of plasma dialysate samples appearing as an outlier). No segregation was observed between dialysate samples that were directly frozen and those that were speedvacuumed before freezing. Although segregation was observed between heart and plasma dialysate as expected (with one outlying sample), no segregation between sample preparation methods was observed within clusters of dialysate types. Therefore, the scores plot in Figure 2.8 suggests that speedvacuuming did not significantly alter the sample composition in either plasma or heart dialysate. However, it should be noted that sample composition is not exactly the same, as corresponding frozen and speedvacuumed sample points do not overlap in the score plot. Measurement error is also reflected in the scores plot in Figure 2.8, accounting for some of the lack of overlap of sample pairs. Thus, speedvacuuming alters dialysate composition slightly, but not in a large and systematic manner as would be indicated by clustering in the scores plot. The univariate analysis and spectral examination detailed previously confirmed these conclusions. Although it is apparent that speedvacuuming does alter dialysate composition slightly due to loss of volatile components (primarily acetate)
Figure 2.8. Score plot comparing sample preparation methods for dialysate samples. ♦ represents samples speedvacuumed to dryness and reconstituted in deuterated solution immediately prior to NMR analysis. ▼ represents samples frozen prior to NMR analysis and then diluted to 10% D₂O. Each point is labeled with its individual sample number, and lines are drawn to connect sample pairs visually.
or possible degradation of unidentified unstable compounds, the changes it causes are confined to a small region of the NMR spectrum. Speedvacuuming dialysate samples also improves sample stability and spectral quality by improving water suppression. Although reconstitution in D$_2$O can cause loss of resonances from exchangeable protons, in general for small organic molecules there is less information lost by this route than by suppression of the solvent resonance in 90% H$_2$O.

2.4.2.2. Recovery by Dissolution

Biofluid composition is complex, especially plasma, whose components have varying degrees of solubility in aqueous solution. Additionally, many of the dialysate samples collected for this project were speedvacuumed to dryness and reconstituted in half the original volume, potentially exacerbating any solubility issues.

The recovery of plasma dialysate components by reconstitution in Ringer’s solution was examined. Plasma dialysate was collected and speedvacuumed to dryness. The plasma was reconstituted in deuterated acetate solution in D$_2$O (pD=7.8) and analyzed by NMR. Any solution remaining in the sample vial was removed and the same volume of methanol-d$_4$ was added to solubilize any residual metabolites that were not dissolved when the plasma dialysate was initially reconstituted. The NMR spectrum measured for this residual metabolite solution was compared to a control methanol-d$_4$ spectrum and to the original plasma dialysate spectrum. Only solvent resonances were detected in the residual metabolite solution, indicating that no detectable levels of metabolites were left undissolved by the
reconstitution buffer. No solubility concerns were noted with the dialysate sample preparation procedure.

2.4.2.3. pH Effects on Dissolution

In NMR experiments, solution pH is an important concern, as many resonances have a pH-dependent chemical shift. In metabonomics NMR experiments in particular, the choice of an appropriate pH for the sample as well as control of the pH with a buffer is important. Metabonomics NMR spectra tend to be crowded with hundreds of resonances, so even small changes in the chemical shift can change the appearance of the spectrum. When applying bucketing procedures for statistical analysis, the width of the buckets is typically so small (0.04 ppm) even a small change in pH can easily shift resonances between buckets. Therefore, the reproducibility of NMR spectra generated from the same sample at a normal in vivo pH and low pH was examined.

The purpose of this experiment was to examine the effect of pH on the NMR spectra of plasma dialysate. Two separate plasma dialysate samples (speedvacuumed to dryness after collection and stored at -20°C until analysis) were reconstituted in 25 mM deuterated acetate in D₂O, one with a pD of 7.8 and the other with a pD of 3.4. Spectra were examined for the shifting or disappearance of resonances. No major changes were noted when comparing the spectra. Most changes occurred in glutamate resonances, with shifts of 0.02 ppm and 0.04 ppm occurring at the resonances at 2.43 ppm and 2.09 ppm, respectively. Overall, the pD had a minimal effect on the NMR spectrum, with an average change in ppm of 0.009 over
approximately 4 pH units. Therefore, for dialysate samples reconstituted in buffer, small changes in pH should minimally change the NMR spectrum and therefore integration parameters.

2.4.2.4. Speedvacuumed Sample Stability

The storage conditions of the speedvacuumed samples were important in that the need for long NMR acquisition times to allow for signal averaging combined with the limitations on instrument access limit the number of samples that can be analyzed in a single day, requiring that samples from the same set be stored and analyzed on different days. It was therefore necessary to establish the stability window of the dialysate samples.

The appropriate storage conditions of the speedvacuumed samples were assessed. Plasma dialysate samples were split into equal volume fractions and all samples were speedvacuumed to dryness. Half of the speedvacuumed samples were stored at -20°C and the other half were stored at 4°C. All samples were reconstituted in equal volumes of 25 mM deuterated acetate solution in D₂O with 5 mM TSP-d₄ immediately prior to NMR analysis. Samples from each storage condition were analyzed on days 0 (immediately after speedvacuuming to determine the initial sample composition), 1, 2 and 7.

Results showed a clear demonstration of sample instability for some components at 4°C and improved sample stability at -20°C. Figure 2.9 shows resonances from 3.0-4.5 ppm, where resonances from glucose tend to dominate the spectrum. Figure 2.10 shows resonances from 0.5-3.0 ppm, containing the aliphatic
Figure 2.9. Comparison of NMR spectra acquired from speedvacuumed and reconstituted plasma dialysate samples stored at two different temperatures. A shows the spectrum of the original plasma dialysate acquired directly after sampling and speedvacuuming. B shows the spectrum acquired from the plasma dialysate sample stored in the refrigerator (4°C) for 7 days prior to analysis. C shows the spectrum acquired from the plasma dialysate sample stored in the freezer (-20°C) for 7 days prior to analysis.
Figure 2.10. Comparison of NMR spectra acquired from speedvacuumed and reconstituted plasma dialysate samples stored at two different temperatures. A shows the spectrum of the original plasma dialysate acquired directly after sampling and speedvacuuming. B shows the spectrum acquired from the plasma dialysate sample stored in the refrigerator (4°C) for 7 days prior to analysis. C shows the spectrum acquired from the plasma dialysate sample stored in the freezer (-20°C) for 7 days prior to analysis.
resonances of many amino acids and other metabolites. Aliphatic resonances showed substantial changes seven days after storage at 4°C. Although some resonances (such as alanine) showed a decline in relative intensity, many new resonances appeared. Minor changes in the frozen samples were noted, although these were smaller relative to changes observed in the refrigerated samples. Specifically, six out of 57 integral regions were considered significantly different at 95% confidence when comparing the frozen samples analyzed on the 7th day to the original sample integral values. In contrast, 18 of 57 integral regions were considered significantly different at 95% confidence when comparing the refrigerated samples analyzed on the 7th day to the original sample integral values. Most of the changes in sample composition for both frozen and refrigerated samples were observed in the 3.0 – 4.0 ppm region, which primarily contains glucose resonances. Upon inspection of the NMR spectra, the levels of glucose stayed constant. The differences observed are due to unidentified resonances growing into the spectra as time elapsed. Also, when comparing the integral values for frozen and refrigerated samples analyzed on the 7th day, 23 integral regions were considered significantly different at 95% confidence. The significant integral regions were also primarily found in the 3.0 – 4.0 ppm region. The exact integral regions showing significant differences are listed in Table 2.1.

In summary, these comparative studies showed instability in sample composition at 4°C, but improved stability at -20°C. It should be noted that changes in sample composition were observed in the frozen samples. Although minor, they could still alter the integral values obtained in subsequent experiments. In this study,
<table>
<thead>
<tr>
<th>Integral regions</th>
<th>Original/Frozen</th>
<th>Original/Refrigerated</th>
<th>Frozen/Refrigerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.06-1.09</td>
<td>1.06-1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.09-1.18</td>
<td>1.09-1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.18-1.23</td>
<td>1.18-1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.91-1.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.39-2.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.74-3.00</td>
<td>2.74-3.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.06-3.17</td>
<td>3.06-3.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.17-3.22</td>
<td>3.17-3.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.22-3.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.36-3.39</td>
<td>3.36-3.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.41-3.43</td>
<td>3.41-3.43</td>
<td>3.41-3.43</td>
<td></td>
</tr>
<tr>
<td>3.44-3.46</td>
<td>3.44-3.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.50-3.52</td>
<td>3.50-3.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.52-3.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.54-3.56</td>
<td>3.54-3.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.56-3.58</td>
<td>3.56-3.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.70-3.73</td>
<td>3.70-3.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.72-3.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.77-3.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.80-3.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.85-3.87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.87-3.90</td>
<td>3.93-3.94</td>
<td>3.93-3.94</td>
<td></td>
</tr>
<tr>
<td>3.94-4.09</td>
<td>3.94-4.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.09-4.15</td>
<td>4.09-4.15</td>
<td>4.09-4.15</td>
<td></td>
</tr>
<tr>
<td>6.80-8.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Integral regions significantly different between the original, frozen and refrigerated sample groups, as determined by the student’s t test at a 95% confidence level. Not all the possible integral regions are shown in the table – only those shown to cause significant differences between the sample groups.
both frozen and refrigerated samples were analyzed 1, 2 and 7 days after analysis of the original sample. The day 1 and 2 spectra acquired from frozen samples were very similar in composition to the original sample, suggesting that it is only after a period of a few days that sample composition changes significantly in the frozen samples. Thus, speedvacuumed samples were frozen until NMR analysis, and samples from the same experiment were analyzed within one day of each other to avoid introducing variance from sample stability.

2.4.3. **NMR Analysis**

The analysis of small volume samples can be challenging for NMR experiments. Conventional NMR analysis uses samples of about 600 μL for normal 5 mm NMR tubes and about 220 μL for specialized 5 mm Shigemi tubes. (Shigemi NMR tubes reduce the required sample volume by utilizing glass plugs in the tube whose magnetic susceptibility is matched to the sample solvent. The sample volume needs only to fill the active window of the probe.) Smaller diameter tubes, typically 3 mm in diameter, can also be used. Conventional 3 mm tubes require about 230 μL for analysis and 80 μL for specialized Shigemi tubes. Even the smaller diameter tubes require sample volumes that would yield poor time resolution (a few hours) for microdialysis sampling (using typical μL/min flow rates). One final alternative utilizes microcell assemblies that insert smaller tubes inside conventional 5 mm NMR tubes, such as those sold by Wilmad-Labglass, Inc (Figure 2.4). These inserts come in a variety of geometries, including cylindrical (8 μL/cm volume), spherical bulb (18 μL total volume) and cylindrical bulb (110 μL total volume). Cylindrical inserts were
chosen because sample insertion and shimming were easier with this insert probe geometry.

Specialized NMR probes are an alternative to reduce the sample volume requirements of the NMR experiment. A microcoil probe (CapNMR probe produced by Protasis/MRM) used in these experiments was composed of a length of capillary with a bubble cell. The observe volume of this bubble cell was 1.5 μL and total probe volume was approximately 10 μL. Thus, sample volume requirements are reduced significantly when compared to traditional NMR analysis. A further advantage of microcoil NMR analysis is increased mass sensitivity, meaning that given the same mass of analyte detected, the microcoil probe will give a larger response than a traditional 5 mm NMR probe\textsuperscript{21}. Another study of microdialysis sampling for metabonomics studies used a Varian nanoprobe cell\textsuperscript{15}, which required 20 μL sample volumes.

A comparative experiment was conducted to characterize the analysis of microdialysis samples by NMR using both specialized tube inserts and a specialized microcell probe. Plasma dialysate samples were collected and split into two equal volume fractions. The samples were speedvacuumed to dryness and reconstituted in the same volume of deuterated acetate solution. For each sample pair, one sample was placed in a cylindrical tube insert in a conventional 5 mm NMR tube (Figure 2.4). The sample was analyzed as usual on a triple-axis gradient probe. The other sample was injected into the capillary microcell probe until the flow cell was completely filled with sample. Samples were acquired using WET water suppression
with a 90° tip angle calibrated for each probe. Spectra were processed as described in the methods and visually compared.

The results of the comparative study are shown in Figure 2.11. No significant differences between sample analysis methods were noted from 3.0-4.5 ppm. However, differences were noted in other regions of the spectra. First, in Figure 2.11, resonances from the capillary microcell probe are broader than those analyzed using tube inserts. This is not necessarily observed in all resonances – for example, 7.75 ppm shows sharp resonances for both analytical approaches. The shims were similar between the two analytical approaches, as judged by the linewidth of the internal standard, TSP-d4. So, the broad resonances are likely not due to poorer shimming of the probe, but perhaps solute interactions with the surface of the capillary. Both A and B in Figure 2.11 also show differences in resonances detected, both in the appearance of new resonances in the samples analyzed by the microprobe and in differences in relative intensity of various resonances. New resonances are noted at 2.20 and 7.75 ppm. Changes in relative resonance intensity were noticed in the region of 6.75-7.35 ppm, as well as the doublet next to lactate (1.30 ppm), alanine (1.45 ppm), and the mixture of resonances at 2.10 ppm.

The results from this comparative study indicate that the use of tube inserts for NMR analysis of microdialysis samples is a superior alternative to the use of capillary microprobes for this specific application. Interactions of the sample with the capillary wall resulted in broadening of some of the resonances, therefore poorer spectral quality was obtained. Although the capillary microprobes offer superior
Figure 2.11. Comparison of plasma dialysate analysis by (1) a cylindrical tube insert with a conventional triple-axis gradient probe and (2) a capillary microprobe. Results are representative of the experimental results. A shows resonances from 0.75-3.0 ppm and B shows resonances from 6.0-9.0 ppm.
detection volumes relative to the NMR tube inserts, the disadvantages preclude their use in these studies. Not only is the spectral quality problematic, the flow probe format of the microprobe also reduces the flexibility in the NMR setup. In contrast, samples in tube inserts can be analyzed using nearly any probe, including cryoprobes. Although tube inserts do not provide as efficient a filling factor as the capillary probes, they allow the analysis of small volume samples without specialized NMR probes and generate spectral data of superior quality in this application.

2.4.4. Data Analysis

2.4.4.1. Data Pretreatment

Once the NMR spectrum is acquired, steps must be taken to translate the spectral domain data into a form more appropriate for statistical analysis. Interpretation of large metabonomics data sets can be cumbersome when considered in a univariate manner. Multivariate statistical methods such as principal components analysis (PCA) have been a tremendous tool for metabonomics researchers for data evaluation. However, the quality of data input for PCA determines the quality of the results obtained. The purpose of this study was to evaluate the effects of data pretreatment on PCA of tissue-targeted metabonomics data, including integration methodology, data normalization and data standardization. This pretreatment can strongly affect the statistical analysis and the interpretation of the results. Thus, data pretreatment steps must be chosen that will give results that accurately reflect trends in the data, rather than bringing irrelevant artifacts to light.
2.4.4.1.1. Data Integration

First, the method of integration was considered. Many metabonomics studies use bucketing, an automated approach to integration in which the NMR spectrum is divided somewhat arbitrarily into integral regions of equal ppm width\textsuperscript{22}. This is by far the most popular approach to metabonomics integration, as the automation is attractive to many researchers. Another approach is to use manual integration, whereby the researcher examines the spectra and determines the divisions between integral regions. The regions will likely not be of equal width, but the end points of the regions will occur at natural breaking points in the spectra. The manual integration divisions are kept consistent between spectra.

In this comparison, a sample set of basal heart and liver dialysate was integrated in two ways. First, the spectra were bucketed from 4.5-0.8 ppm after spectral processing in ACD/Labs software with settings of 0.04 ppm bucket width and 10\% looseness. (The \% looseness setting determines the flexibility that the program is allowed in dividing spectra, so as to avoid splitting a resonance into two integral regions as much as possible.) Second, the same set of spectra was manually integrated from 4.5-0.8 ppm. Both sets of integrals were normalized to TSP-d\textsubscript{4} intensity and to the average \% delivery of the microdialysis probe. PCA was performed on a correlation matrix, and the results are shown in Figure 2.12. The resulting scores plots were similar, with clusters for the liver dialysate samples observed in roughly the same coordinates in each plot. The heart dialysate shows about the same amount of spread with each integration mode. Although slight
Figure 2.12. Comparison of integration methods on basal heart and liver dialysate data. A shows spectral data integrated by bucketing and B shows spectral data integrated manually. ■ represents basal liver dialysate data and ○ represents basal heart dialysate data.
differences in data coordinates were noted, none of the changes would alter the data interpretation. Thus, no significant differences were noted between integration modes.

The occupation of many integral regions is one means by which a molecule may dominate the PCA (in this case, glucose). Thus, the decisions that the researcher makes when dividing the NMR spectrum into integral regions may determine which molecules become important in the PCA. Most metabonomics experiments utilize bucketing, perhaps the most impartial method of dividing the NMR spectrum into integral regions. However, divisions can occur across resonances and it may not be advantageous for all data sets, particularly for tissue-targeted metabonomics data, where glucose is high in intensity and occupies a large space in the NMR spectrum. Our studies have shown that the integration method does not significantly alter the interpretation of the data set. Subsequent analyses will be performed with manual integration, allowing for more control over how the integrals represent the data set and decreasing complications from shifting of resonances between integral regions.

2.4.4.1.2. Data Normalization

Data normalization was also examined. Metabonomics data analysis by PCA is based on variance in the data set. The assumption is that most of the variance in the data is from metabolic changes induced by the metabolic stressor. However, this is not necessarily the case. Variance can be introduced into these experiments by a variety of routes, including error in the actual analysis. In tissue-targeted metabonomics studies, variations in the probe extraction efficiency can also cause
changes in metabolite concentration unrelated to the metabolic stressor. With this in mind, it is important to identify and account for sources of variance unrelated to the metabolic stressor so that changes related to the metabolic stressor can be more easily and confidently identified.

Three principal component analyses were performed: on the raw data set, the data set normalized to TSP-d$_4$, and the data set normalized to both TSP-d$_4$ and the average extraction efficiency for the microdialysis probe (as gauged by the % delivery of antipyrine). Manual integrals from the basal heart and liver dialysate data set were again utilized. Figure 2.13 shows the three scores plots generated. Without normalization the data shows scatter, with no clustering or segregation between heart and liver dialysate. Data normalized to TSP-d$_4$ intensity showed segregation between dialysate types, but with no real clustering. Finally, data normalized to both TSP-d$_4$ and extraction efficiency of the microdialysis probes showed a tight cluster of liver dialysate samples that were segregated from the more scattered heart dialysate samples. Although the liver dialysate data clustered tightly when normalized to both TSP-d$_4$ and % delivery, the heart dialysate data showed less variance when normalized only to TSP-d$_4$.

From the progression of effects of normalization observed in Figure 2.13, it is clear that the variation in the data due to these two factors is greater than the variation between liver and heart dialysate composition. Therefore, the initial PCA was dominated by experimental (not metabolic) variance and underlying patterns could not be resolved. For the liver dialysate data, the more variation corrected for prior to
Figure 2.13. Comparison of data normalization methods on basal heart and liver dialysate data. A shows unnormalized spectral data, B shows spectral data normalized to the internal standard, TSP, and C shows spectra data normalized to both the internal standard and to the average extraction efficiency of the microdialysis probe. ■ represents basal liver dialysate data and ○ represents basal heart dialysate data.
the PCA, the tighter the clustering. This was not observed with the heart dialysate data, as more scatter was observed after normalization to probe delivery. This is likely due to two key differences in heart and liver microdialysis probe performance, as shown in Figure 2.14. First, due to spatial constraints in probe implantation in the heart apex, the membrane length is 8 mm shorter than the liver microdialysis probe. Thus, the extraction efficiency is approximately 30% less in the heart than in the liver. Second, the heart is a continuously moving organ, unlike the liver. This leads to greater variation in probe extraction efficiency. When this is considered with the smaller overall extraction efficiency of the heart microdialysis probes, it is likely that corrections for probe performance does not effectively remove variation in the heart dialysate data set. These results are consistent with what would be expected both experimentally and biologically. Subsequent analyses of liver dialysate data will include normalization to TSP-d₄ and the probe extraction efficiency, while heart dialysate will only include normalization to TSP-d₄.

2.4.4.1.3. Data Standardization

The disparity between metabolite levels in an NMR spectrum from tissue-targeted metabonomics data seems to be exacerbated relative to typical metabonomics data because most of the molecules of interest in the NMR spectrum are present at concentrations at least ten times lower than the most abundant molecule, glucose. However, the patterns in their changes are as important, if likely not more important, than that of glucose. This is an important consideration for data pretreatment, particularly in the choice of a correlation or covariance matrix for principal
Figure 2.14. The % delivery of antipyrine in heart (n=4) and liver (n=5) microdialysis experiments.
components analysis. PCA can be performed using either a correlation matrix or a covariance matrix. Covariance matrix data has been unstandardized, while correlation matrix data has been standardized to a zero mean and unit variance. Higher intensity resonances would have a greater influence on the principal components analysis using a covariance matrix, while use of a correlation matrix would place equal emphasis on all variables, regardless of intensity. Thus, the effect of data standardization was examined.

Figure 2.15 is a comparison of loadings values from a PCA performed on the same data set using a correlation and covariance matrix. The loadings values from the PCA on a covariance matrix show a greater range of values, with most variables contributing to the analysis minimally. When these loadings values are aligned with the corresponding resonances in the NMR spectrum as in Figure 2.16, a correlation can be noted between the highest intensity resonances and the largest loading values. In contrast, the loadings values for PCA performed on a correlation matrix show much less of a range than the covariance matrix loadings, but now each variable shows roughly equal importance to the PCA. Note from the corresponding NMR resonances that no correlation can be found between loadings values and resonance intensity as in the covariance matrix PCA.

The loadings plot in Figure 2.15 demonstrates clearly the importance of standardization in the data set when the dynamic range of the data is high. If no standardization occurs, the high intensity metabolites dominate the PCA. However, contributions from individual variables become more equal when standardization is
Figure 2.15. Comparison of loadings values for the same data set with a principal components analysis performed using a correlation matrix and covariance matrix. NMR spectra positioned above the graph approximate the resonances that correspond to each integral region. ■ represents loadings calculated from a correlation matrix. ○ represents loadings from a covariance matrix.
applied (that is, when a correlation matrix is used). There is still some variation in importance to the PCA, but overall, the concentration of the compound does not determine its importance in the analysis. A potential problem with the use of a correlation matrix is the lack of discrimination between variables. When everything becomes important, in essence, everything loses its importance. It becomes difficult to identify just a few resonances that might be important biomarkers of the conditions being studied. However, this concern is not enough to prevent the use of correlation matrices in these studies. Thus, all PCA analyses were performed using correlation matrices to equalize variable contribution to the statistical analysis.

2.4.5. Analysis of Tissue-Targeted Metabonomics Data: Perspectives and Time Trends

Metabonomics data sets can be analyzed on three primary levels: multivariate, univariate and by spectral examination. Most metabonomics studies solely use multivariate statistics to interpret trends in the data, since it is an efficient way to examine the data. However, this may provide an incomplete picture of trends in the data. It is also important to characterize any trends in basal metabolism or from experimental parameters, such as from anesthesia, diurnal rhythms, food/fasting cycles and animal activity, which will be more fully discussed in Chapter 3. For tissue-targeted metabonomics in particular, the description of time trends is key to data interpretation.

These data analysis strategies were applied to a data set comparing basal liver dialysate from both anesthetized and awake rats. For the anesthetized rats, sampling
began 30 minutes after probe implantation and continued every hour for 12 hours. For the awake rats, sampling began 24 hours after probe implantation to allow the animals sufficient time to recover from the anesthesia and continued every hour for 96 hours. Comparisons were made between tissue metabolic states (awake, anesthetized, and an awake rat that developed an infection post-surgery). Additionally, time trends were analyzed by both PCA and PLS regression.

First, general groupings in the data were examined. Principal components analysis of the data, shown in Figure 2.16, shows three distinct groupings of data points along principal components 1 and 3. These three groupings correspond to previously noted metabolic states of the animal: awake healthy, anesthetized and awake infected. Principal component 1 separates the healthy awake samples from the anesthetized and awake infected samples. Principal component 3 separates the awake infected samples from the anesthetized samples. The loadings indicate that principal component 1 is a contrast axis between time after probe implantation and all integral regions except those corresponding to lactate, meaning that the time variable has a negative loading while all loadings for the integral regions are positive in magnitude. Loadings for principal component 3 show lactate, valine, and resonances in the 3.51-3.63 ppm region (including some glucose resonances) and the 2.46-2.60 ppm region as important in segregating the samples from anesthetized and awake infected animals.

The data was also analyzed in a univariate manner by plotting time after probe implantation against the normalized integrals from each integral region of the NMR
Figure 2.16. Comparison of liver metabolic states by principal components analysis. ○ represents samples of basal liver dialysate from an awake rat with sepsis (n=1). ■ represents samples of basal liver dialysate taken from healthy awake rats (n=3). ♦ represents samples of basal liver dialysate taken from anesthetized healthy rats (n=4).
spectrum (40 in total). This allowed both time trends and segregation of groups within a single variable to be visualized. Examination of the plots showed discrepancies in regions containing glucose resonances. As shown in Figure 2.17, although A and B both contain glucose resonances, the trends observed in the samples from awake infected rats differ remarkably between these regions, while the trends observed in the samples from awake healthy and anesthetized rats remain the same. The 3.51-3.63 ppm region was also shown to be important in principal component 3 for segregating the samples from anesthetized rats from the samples from awake infected rats. Spectral examination of the data confirmed the findings from the PCA and univariate data analysis.

PCA (described above) showed a time trend along principal component 2, as illustrated in Figure 2.18. This time trend was unidirectional and confined to the samples from anesthetized animals. The samples from awake (both healthy and infected) animals did not show a definitive time trend although a trend was observed in the univariate time plots in Figure 2.17. Loadings for principal component 2 (not shown) indicate glucose, creatine and lysine as being most important in defining the time trends for samples from anesthetized animals.

PLS regression showed a slightly different time trend. PLS was performed separately on correlation matrices for samples from anesthetized and awake animals, correlating the NMR integral data with sampling time. The anesthetized sample set showed a good correlation between the predictors (NMR integrals) and responses (sampling time), with an $R^2$ of 0.916 for 2 components and an $R^2$ predicted of 0.874.
Figure 2.17. Comparison of liver glucose levels over time. All rat samples are plotted. ○ represents samples of basal liver dialysate from an awake rat with sepsis. ■ represents samples of basal liver dialysate taken from healthy awake rats (n=3). ♦ represents samples of basal liver dialysate taken from anesthetized healthy rats (n=4). A shows changes in the 3.51-3.52 ppm region and B shows changes in the 3.36-3.46 ppm region.
Figure 2.18. Time trends observed in basal liver dialysate using principal components analysis. ○ represents samples of basal liver dialysate from an awake rat with sepsis. ■ represents samples of basal liver dialysate taken from healthy awake rats. ♦ represents samples of basal liver dialysate taken from anesthetized healthy rats. The arrow shows the direction of the time trend observed in the anesthetized rats.
for 2 components, as determined through cross-validation. The standardized coefficients generated, which are similar to PCA loadings, are shown in Figure 2.19A. Again, there is a contrast between glucose and creatine, but glucose is now also contrasted with lactate and citrate. These resonances were not significant in the PCA loadings, likely because the samples from awake animals were also included in the analysis. Figure 2.19B shows the coefficients for the PLS regression correlation between the NMR integral data for the awake samples and the sampling time. Here, the correlation is not as strong as observed for the samples from anesthetized rats, with an $R^2$ of 0.488 and an $R^2$ predicted of 0.152, as determined by cross-validation. A similar trend was also observed in the PCA analysis. However, a loose correlation was still defined by the analysis largely by a contrast between the lysine, isoleucine, and valine and citrate, creatine, alanine and $\beta$-hydroxybutyrate resonances.

This research examines a three-tiered approach for the analysis of a metabonomics data set. Principal components analysis provided a good overall description of the trends in the data set. Groupings in the data were identified on the scores plot with principal components 1 and 3, while time trends were identified on the scores plot with principal components 1 and 2. While these trends were confirmed by examining the data from a univariate and spectral perspective, no significant trends were overlooked by the PCA. Analysis on the univariate and spectral levels, however, is still important to more fully describe trends identified in the multivariate analysis. The multivariate analysis can streamline the more time consuming univariate and spectral analysis steps by focusing attention on the most
Figure 2.19. Standardized coefficients for PLS regression analysis. A shows the coefficients from PLS analysis of basal liver dialysate from anesthetized rats. Model statistics showed an $R^2$ of 0.916 and an $R^2$ predicted of 0.874. B shows the coefficients from PLS analysis of basal liver dialysate from awake rats. Model statistics showed an $R^2$ of 0.488 and an $R^2$ predicted of 0.152. The approximate correspondence of the integral regions to chemical shift is shown by the chemical shift scale above each graph.
relevant integral regions, both increasing throughput and ensuring an in-depth analysis of the data set.

Because a time course of microdialysis samples are collected from a single animal, the data generated by tissue-targeted metabonomics naturally lends itself to analysis of time trends as opposed to the typical group classification. Time trends can be observed with PCA and then more accurately described using PLS regression. PCA was useful in identifying data sets with strong time trends, but was unable to identify weaker time trends. The principal components loadings correctly identified most of the key resonances responsible for defining strong time trends, as they corresponded to the coefficients calculated from PLS regression. However, compared with PCA, PLS identified more resonances as significant. Additionally, PLS regression could identify and describe both strong and weak time trends, as illustrated by the samples from anesthetized and awake animals.

2.5. Conclusions

Tissue-targeted metabonomics shares many of the same goals as “traditional” metabonomics studies, the primary goal being the identification of new biomarkers without the prejudice of the more common single biomarker assays. Theoretically, by expanding the scope of metabolites studied, the probability of uncovering new biomarkers increases. However, tissue-targeted metabonomics differs from traditional metabonomics in several ways. First, of course, is the site specificity of the sampling approach. Second, microdialysis sampling does not require biopsy of
the tissue of interest and the tissue remains intact. Third, because many samples can be obtained from the same animal over the time course of the experiment, tissue-targeted metabonomics better lends itself to the examination of time trends in metabolic data, rather than simpler group classification experiments. This is not to imply that traditional metabonomics cannot study time trends (or that tissue-targeted metabonomics cannot study group classification experiments) – in fact, many do\textsuperscript{24,25}. However, the vast majority of metabonomics studies using urine or plasma sampling report group classifications as the end result of data interpretation. Finally, an additional benefit of the time trend approach of tissue-targeted metabonomics is the use of an animal as its own control, as is also done in some conventional metabonomics studies. By characterizing the basal metabolic patterns of an animal prior to perturbing its metabolism, corrections for animal-to-animal variation can be applied, potentially improving data interpretation.

This tension between high and low abundance metabolites is a significant barrier to metabolic profiling experiments in general, both for detection schemes and data analysis. The instrumental dynamic range required to effectively characterize the complete metabolome is immense. Thus, it should be considered that while the data generated by metabonomics analysis is extensive, it is not comprehensive. Many metabolites of key significance to the metabolic perturbation being studied but lower than the detections limits of the instrument may be excluded. A related problem is the difficulty of detecting metabolites present in low abundance due to overlap with the more intense resonances of the major components, an inherent consequence of
performs the analysis on the intact mixture without a separation. This problem can be partially alleviated by using a higher field magnet since dispersion increases linearly with magnetic field. Still, there are practical limits such as the instrumentation available to the investigator, and even at the highest field available many important low abundance metabolites will still be missed due to resonance overlap. Dynamic range is also a problem in metabonomics data analysis. A balance must be established between the dominance of high abundance metabolites in the multivariate analysis and the inflation of regions of noise when trying to increase the contribution of low abundance metabolites in the multivariate analysis.

In addition to dynamic range, the complexity of metabonomics data analyses can create challenges for the technique. Balancing a thorough investigation of the data with time constraints can be difficult. Using PCA to identify the pertinent trends in the data, while efficient, can easily overlook important metabolic trends, particularly from lower abundance metabolites, which tend to become overshadowed in principal components analysis even with unit variance scaling. The studies presented in this research suggest that principal components analysis gives an accurate overview of the data and can be used to focus deeper data analysis. Partial least squares regression is also a powerful tool, particularly in describing time trends in the data. For both of these multivariate statistical techniques, it is important to probe the data further using univariate analysis and spectral inspection.

In summary, tissue-targeted metabonomics focuses on biomarker discovery for localized metabolic perturbations, as observed through time trends in the
metabolic profiles. These time trends point to potential biomarkers for the condition, leading to more targeted single biomarker diagnostic assays. Perhaps more importantly, the improved time resolution with reduced animal-to-animal variability enables the researcher to observe the development or recession of a physiological condition, as well as to identify the key metabolic pathways involved throughout the onset or retraction of a metabolic perturbation. These perturbations may be identifiable well before any changes are observed in urine or plasma. Thus, the use of tissue-targeted metabonomics may lead to tests that enable the detection of site-specific diseases or drug toxicity much faster than conventional assays.

2.6. References


3.1. Introduction

Tissues have differing energy requirements and utilize different pathways for energy generation, according to their specific function and the levels of enzymes present in the tissue\(^1\). Thus, the differing functions an organism’s tissues serve are reflected in their metabolic profiles. The brain requires a steady supply of glucose (15% of the body’s total energy consumption) and oxygen (20% of the body’s total oxygen consumption) to generate enough energy to maintain membrane potentials necessary for transmitting nerve impulses\(^1-3\). Even within the brain, a notably heterogeneous organ, the different regions have different metabolic needs\(^1\). Although glucose is the primary fuel, the brain can utilize ketone bodies for energy generation under fasting conditions. The muscle requires energy for contraction of the tissue, allowing it to do work. It can utilize a variety of energy substrates, including fatty acids (used while resting), glucose (used during exertion) and ketone bodies. During exertion, energy is produced by the dephosphorylation of muscle creatine phosphate stores and glucose is generated from the muscle’s glycogen stores. The glucose is
then metabolized. Glycolytic flux is greater than flux through the citric acid cycle, which causes accumulation of lactate and alanine in the tissue. Fatty acids are used during resting while the muscle’s glycogen stores are depleted. Muscle tissue will also breakdown the protein in the tissue for energy under starvation conditions. The heart, with its constant pumping and activity, maintains a more constant energy usage than skeletal muscle and is strictly aerobic in its metabolism. Like the brain, it has few energy stores and requires a steady supply of oxygen and energy substrates, primarily fatty acids but also glucose, lactate and ketone bodies. The liver is perhaps the most metabolically diverse organ in the body. It uses glucose, fatty acids and amino acids to supply its own energy demands. What makes it distinct is that it also assists other organs and tissues in their metabolic needs through the synthesis of fatty acids, glucose, glycerol, amino acids and ketone bodies, the metabolism/detoxification of exogenous compounds, the regulation of blood glucose levels and the elimination of nitrogenous compounds through the urea cycle.

Not only does metabolism vary between tissues, but it also varies over time within the same tissue. It has been well established that tissue metabolism can change over time due to a variety of factors. Biological rhythms are cycles that are influenced by environmental cues, primarily light/dark cycles. Circadian rhythms are biological rhythms on a roughly 24 hour period. These particular rhythms occur without entrainment by any external cues, suggesting that there is some internal pacemaker responsible for establishing these rhythms. (Diurnal rhythms are those cycles that are controlled by external stimuli.) Rhythms or cycles in metabolism are
under the control of the suprachiasmatic nucleus (SCN), the “pacemaker” of the body. The SCN has been shown to regulate melatonin production by the pineal gland, but the exact mechanism of control is poorly understood. Rhythms are observed in a variety of processes and metabolite levels that all relate directly or indirectly to tissue metabolism (and possibly directly or indirectly to each other) – glucose uptake, plasma glucose levels, blood pressure, heart rate, hormone secretion, activity levels, sleep/wake cycles, body temperature, melatonin levels, cortisol levels and eating.

In the liver, circadian rhythms in carbohydrate metabolism are well established. But, these have not been studied in a comprehensive manner – rather, the information is pieced together from various studies. Enzymes important to metabolic pathways such as the citric acid cycle have been shown to exhibit circadian rhythmicity in their levels. In addition, “clock genes” have been identified in nonneural tissue, including the liver, suggesting that at least some metabolic rhythms are intrinsic to the liver tissue itself, outside of the influence of the SCN. Rhythmicity in liver metabolism has been studied for cholesterol metabolism, amino acid metabolism and glucose metabolism. Both hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, a participant in cholesterol synthesis, and cholesterol 7α-monooxygenase, a participant in bile acid synthesis from cholesterol have an established circadian rhythm. Amino acid metabolism circadian rhythms are less clear – rhythms in actual metabolites appear to be secondary to eating cycles, but some enzyme cycles have been shown to be independent of eating cycles. These
enzymes include tyrosine transaminase, alanine transaminase, tryptophan 2,3-dioxygenase, lactate dehydrogenase, L-serine dehydratase and arginase. There are strong correlations between light/dark cycles and many key enzymes involved in glycolysis. Some glucose metabolism cycles, specifically the citric acid cycle, are less established. Isocitrate dehydrogenase and malate dehydrogenase (malic enzyme) have been studied, but it is unclear if the rhythms observed are circadian or secondary to feeding cycles. Additionally, it has been shown that glycogen levels (as well as gluconeogenic and glycogen metabolic enzymes) in the liver vary over the course of the day, with maximum levels occurring during the active/dark period.

Most of the studies on basal changes in liver metabolism have been performed examining enzyme levels and individual metabolite levels. This is true of metabolic studies in a variety of tissues, not just the liver. Very few, if any, studies have been performed with metabolic profiling experiments. To date, many metabonomics studies have been published that characterize basal metabolic trends in animals and humans, as well as the factors contributing to these changes. Most of these studies have focused on whole body metabolic trends, with urine the predominant sample analyzed. Basal metabolism is affected by a myriad of factors, some genetic, some environmental. Not surprisingly, distinct metabolic differences occur between species, as illustrated in Figure 3.1, which compares urine samples from rat, mouse and human subjects. Differences in the metabolic profiles of different genetic strains of rats and mice have been clearly demonstrated, as have differences between genders of the same strain. The hormonal fluctuations experienced by female
Figure 3.1. Analysis of urine from three different species. The samples analyzed are aliquots from pooled urine samples. □ represents human urine (n=7), ○ represents rat urine (n=9) and Δ represents mouse urine (n=10).
rats causes metabolic perturbations over the course of the estrus cycle\textsuperscript{17}, which is why most metabonomics studies are limited to male subjects. The age of the animal is also an important factor, particularly in the early development stage\textsuperscript{18}. The animal shows large changes in body weight over the first few months of life and its gut microflora is still developing. Animal diet, including the food supplied, as well as the time of food consumption and amount of water consumption can all dramatically affect urinary metabolic profiles\textsuperscript{18-20}. Related to this is the composition of the gut microflora in the intestines, which can vary due to a variety of genetic and environmental factors\textsuperscript{21}. Environmental factors such as room temperature (and fluctuations in) and noise (affecting the animal’s sleep quality and stress levels) also change animal metabolism. Finally, general diurnal rhythms in urinary metabolic profiles have been observed, with distinctions observed between samples collected in the morning and at night\textsuperscript{14,16,17}.

3.2. **Specific Aims**

Nearly all of the metabonomic studies of basal metabolism have been performed in biofluids, and these studies are almost exclusively in urine. Urine can give some information on liver function, but its composition is influenced by a variety of organs, as reviewed in Section 1.2.2.2. Information is lacking on basal trends in tissue metabolism, as observed through the metabolic profiling of tissue biopsies or dialysate samples of the tissue extracellular fluid.
Thus, the focus of the study was to apply tissue-targeted metabonomics to the study of basal metabolism in tissue. These studies will lend new insight to both the metabonomics community and the biological community interested in circadian rhythms in tissue metabolism. Experiments were performed to examine and describe circadian rhythms in tissue metabolism, as well as to establish the expected animal-to-animal variability in metabolism. Additionally, the effects of anesthesia on basal tissue metabolism were studied, as this is a component of the tissue-targeted metabonomics experiment. The focus of this study will be liver metabolism, although some perspective will also be given on myocardial tissue metabolism.

3.3. **Materials and Methods**

All materials and methods are described in Section 2.3. Additions to the methods are described below.

3.3.2.2. **Animal Setup**

Complete consistency between experiments was attempted to avoid introducing variance from circadian rhythms and environmental factors. All surgeries were performed at the same time of day and sampling commenced at the same time as well. Rats were fed the same rodent chow (LabDiet 5001 Rodent Diet) and maintained on a 12 hour light/dark cycle (6:00 AM/PM).
3.4. Results and Discussion

3.4.1. Comparison of Dialysate Metabolic Profiles from Various Tissues

The primary purpose of this study was to characterize differences in basal tissue metabolism. Each tissue will have its own distinctive metabolic profile under basal conditions, due to variance in metabolism. Tissues preferentially use different substrates for energy generation and other major metabolic processes\textsuperscript{21}, as reviewed in the chapter introduction (Section 3.1). In the heart, energy is generated through β-oxidation of fatty acids and glucose metabolism via the citric acid cycle. Brain metabolism is dominated by glucose metabolism through glycolysis and the citric acid cycle. Finally, liver metabolism is dominated by glycolysis, gluconeogenesis, β-oxidation of fatty acids and fatty acid synthesis. The levels of extracellular metabolites will reflect these differences in dominant metabolic pathways.

Figures 3.2, 3.3 and 3.4 show differences in the NMR spectra reflecting the metabolic profiles of basal dialysate sampled from heart, brain and liver tissues in anesthetized rats. In general, liver dialysate tended to be more concentrated than brain and heart dialysate. This is partially due to the fact that the liver can accommodate a larger active probe membrane length (10 mm) than brain or heart tissues (2-4 mm), increasing the extraction efficiency of the membrane by a factor of 3-5. This can be accounted for in data analysis by normalizing to probe delivery, as described previously. Although the brain is as large as or larger than the liver, the heterogeneity of the tissue makes it necessary to reduce the probe membrane size to sample from a single region (in this case, the striatum). The heart is smaller in size
Figure 3.2. Comparison of $^1$H-NMR spectra (0.5-3.0 ppm) reflecting the metabolic profiles of different tissue dialysates. **A**: heart dialysate; **B**: brain dialysate; **C**: liver dialysate.
Figure 3.3. Comparison of $^1$H-NMR spectra (3.0-4.5 ppm) reflecting the metabolic profiles of different tissue dialysates. A: heart dialysate; B: brain dialysate; C: liver dialysate.
**Figure 3.4.** Comparison of $^1$H-NMR spectra (6.0-9.0 ppm) reflecting the metabolic profiles of different tissue dialysates. **A:** heart dialysate; **B:** brain dialysate; **C:** liver dialysate.
than both the brain and liver, and implantation is complicated both by the movement of the tissue during implantation and the need to implant in the muscle wall of the heart and not in the atrial or ventricular chambers, thus necessitating a smaller membrane length. It is also possible that the liver extracellular fluid is simply more concentrated than the brain or heart extracellular fluid. Finally, the liver and heart are both highly perfused organs, increasing the extraction efficiency of the membrane in these tissues.

In the aliphatic region of the NMR spectra (Figure 3.2), the heart and liver dialysate show some differences but are generally more similar to each other than each individually are to brain dialysate. Brain dialysate shows a noticeable absence or depletion of alanine, β-hydroxybutyrate, valine, leucine, isoleucine and citrate. Heart and liver dialysate show more similarities, probably due to the fact that both are so well perfused and therefore reflect to a certain extent metabolite levels in the plasma. The region of 2.2-2.5 ppm seems to most clearly show differences in tissue metabolism in this region of the spectrum. For example, glutamine is observed in heart dialysate, but is not identifiable in brain and liver dialysate. In the glucose region of the NMR spectra (Figure 3.3), less differences are noted. There were relative differences in the levels of lactate and glucose, but both were present in significant concentrations. Creatine levels were fairly constant across the dialysate types. Because glucose is such a dominant metabolite in all three dialysate types and has multiple resonances, any resonances overlapping glucose will not be detected. Thus, information is lost about lower level metabolites occurring in this region of the
spectrum, which could potentially include metabolites like taurine and choline. In this region of the spectra, key differences were noted in the region of 3.55-3.7 ppm. The brain dialysate shows a significant level of myo-inositol and glycine that is not seen in the liver and heart dialysate. The aromatic region of the NMR spectra (6.0-9.0 ppm), shown in Figure 3.4, contains resonances from low abundance metabolites. Heart and liver dialysate shows a signal from tyrosine that is not seen in brain dialysate. In general, liver dialysate has more abundant aromatic metabolites than brain or heart dialysate.

Principal components analysis (PCA) was performed to compare the metabolic profiles obtained under basal metabolic conditions from heart, brain and liver in anesthetized rats. The resulting scores plot is shown in Figure 3.5. In this analysis, the integral data was only normalized to the internal standard, TSP-d4. In chapter 2, it was demonstrated that normalizing to the average % delivery of the probe adds variance to the heart dialysate metabolic profiles, rather than removing it. Additionally, no % delivery information was available for the brain dialysate samples. Therefore, there is some variance in the data set from probe delivery that could not be accounted for in this analysis. In the scores plot, groupings are observed between dialysate types, although they do not show tight clustering. Principal component 1 distinguishes clearly between brain and heart dialysate, with some delineation of heart and liver dialysate present. However, it is only in combination with principal component 3 that heart and liver dialysate are able to be distinguished. The loadings for principal component 1 indicate that nearly every integral region is
Figure 3.5. Comparative PCA study of metabolic profiles representing basal metabolism in liver (■) (n=4), heart (▲) (n=3) and brain (●) (n=1) tissues of anesthetized rats. * indicates a sample of basal heart dialysate that is an outlier.
important in defining the separation for this axis, with the exception of creatine and empty regions of the spectrum at 2.17-2.36 ppm and 1.09-1.18 ppm. The loadings for principal component 3 are more defined/focused, with glycine, β-hydroxybutyrate, valine, glutamine, alanine, lysine and citrate resonances most responsible for the trends observed along this axis.

The data presented demonstrates that tissues exhibit unique metabolic profiles that typically correspond with the known preferred energy generation pathways for the tissue, with some exceptions. For example, in the brain, no ketone bodies were present in the extracellular fluid (ECF), even though the rat was anesthetized and therefore fasting during sampling. Also, no citrate was present in the ECF, even though the brain utilizes the citric acid cycle as a primary means of energy generation. Very few studies have been performed correlating the ECF composition with energy state of the cell and with its internal composition of energy metabolites. One study has demonstrated that lactate, adenosine, inosine and hypoxanthine levels in dialysate samples from porcine hearts correlate fairly well to levels of the compounds extracted from biopsy samples taken from the same tissue, both under normal and ischemic conditions\textsuperscript{25}. However, literature characterizing other endogenous metabolites or tissues remains scarce. These results give a new perspective to basal tissue metabolism studies.

3.4.2. Time Trends in Metabolism: Heart and Liver Tissues

There are differences in metabolism between tissues, but perhaps more importantly to this research, basal tissue metabolism in each tissue changes over time
for a variety of reasons, including experimental factors such as anesthesia and circadian rhythms. These individual issues will be explored in detail in subsequent sections. For these experiments, basal tissue metabolism was characterized for heart and liver tissues.

Heart dialysate was sampled from anesthetized rats. Samples were collected every 60 minutes for up to 10 hours. PCA and partial least squares regression (PLS) was performed on NMR integrals normalized to TSP-d$_4$ (the NMR internal standard) only. (Prior studies, detailed in Chapter 2, have shown that normalization to the % delivery of the microdialysis probe adds variance to data sets obtained from heart dialysate.) The scores plot from the PCA is shown in Figure 3.6. A time trend is observed along the principal component 2 axis, with early samples beginning at lower scores on the axis and increasing (to varying degrees) over time. PCA identified creatine, alanine, isoleucine and glucose as the most important in defining the time trend in the second component. Specifically, alanine, creatine and isoleucine increase over time as glucose decreases. Creatine time trends are shown in Figure 3.7, and are representative of the time trends observed in alanine and isoleucine. Glucose time trends are shown in Figure 3.8. Increases in creatine are likely due to dephosphorylation of the store of creatine phosphate in cardiac tissue. With the rat in a fasting state due to anesthesia and glucose levels falling, the cardiac tissue must turn to other means to supply energy. Creatine phosphate is stored in tissue for this very purpose. Increases in alanine are likely due to perturbations in the glucose-alanine cycle$^{26}$, in which pyruvate is transaminated with glutamate to yield alanine and $\alpha$-
Figure 3.6. PCA of basal heart dialysate metabolic profiles (n=8). The arrow indicates the direction of the time trend. Data points are labeled by hour of sampling. Symbols are as follows: ■, hour 1; ○, hour 2; ▲, hour 3; ◊, hour 4; ►, hour 5; □, hour 6; ●, hour 7; Δ, hour 8; ♦, hour 9; ▼, hour 10.
Figure 3.7. Creatine levels in basal heart dialysate over time (n=8). Individual rats are differentiated by different symbols to show the time trend for each rat.
Figure 3.8. Glucose levels in basal heart dialysate over time (n=8). Individual rats are differentiated by different symbols to show the time trend for each rat.
ketoglutarate. The large amount of glucose near the beginning of the experiment likely causes a flux in the glycolytic pathway, resulting in a buildup of pyruvate that is available for transamination and subsequent transport to the liver. Isoleucine was an unexpected significant metabolite. Although it can participate in metabolism in the heart tissue, it is typically not thought of as a key metabolite in heart metabolism. Isoleucine levels may also be affected by transamination pathways, which are also affected by pyruvate levels as well as α-ketobutyrate levels. Partial least squares analysis was also performed on this data set, correlating the NMR integrals (normalized only to TSP) to time. A 5 component model was calculated, with an $R^2$ of 0.86 and $R^2$ predicted of 0.76 ($R^2$ predicted values are calculated by cross validating the model and are an indication of the model robustness). Interestingly, the PLS model identified a few metabolites as important in defining the time trends in the data set that PCA did not identify. Glucose was identified by both analyses as key to the time trend in the data, but PLS identified myo-inositol, leucine and valine as important as well, although not of the same importance as glucose. Leucine and valine are likely changing in a similar transamination reaction like isoleucine, as they are all branched-chain amino acids and participate in similar metabolic pathways. Myo-inositol is produced from glucose-6-phosphate, indicating that this may also be indicative of glycolytic flux in the cardiac tissue.

The basal metabolism of liver tissue in anesthetized animals was also studied. Samples were collected every 60 minutes for up to 12 hours. PCA and PLS were performed on NMR integrals normalized to TSP-d$_4$, the average % delivery of the
microdialysis probe and the integral value for the initial time point prior to PCA and PLS analysis. The PCA scores plot is shown in Figure 3.9. It is shown on the same scale as Figure 3.6 to allow for comparison between the time trends in heart and liver tissue. The scores plot shows that the first principal component is responsible for describing the time trends in liver dialysate from anesthetized rats. The loadings for the first principal component indicate that lysine, alanine, valine, creatine, glucose and myo-inositol are significant. Figure 3.10 shows the changes in lysine, which is representative of the changes in alanine, valine and creatine. These metabolites increase over time. Figure 3.11 shows changes in glucose, which are similar to changes in myo-inositol. Both decrease over time. Note that many of the same metabolites are affected in both the heart and liver tissue. Creatine increases, indicating the liver uses its stores of creatine phosphate as the animal fasts and glucose levels drop. Alanine increases as glucose decreases, again indicating flux through glycolysis, resulting in transamination of pyruvate. Valine and myo-inositol, identified as key in the PLS model for heart metabolism, are also important in liver metabolism, according to the PCA. Lysine appears to be unique to basal liver metabolism time trends in anesthetized rats. It reacts with α-ketoglutarate and a reductase enzyme to form saccharopine. A PLS model was also calculated to correlate basal liver metabolism in anesthetized rats to time. The model was quite strong, with an $R^2$ value of 0.99 and $R^2$ predicted value of 0.98. In this case, PLS identified the same metabolites as significant as the PCA model did.
Figure 3.9. PCA of basal liver dialysate metabolic profiles (n=5). Data points are labeled to show time trends. Symbols are as follows: ■, hour 1; ○, hour 3; ▲, hour 5; ◊, hour 7; ▶, hour 7; □, hour 11.
Figure 3.10. Lysine levels in basal liver dialysate from anesthetized rats over time (n=5). Individual rats are differentiated by different symbols to show the time trend for each rat.
Figure 3.11. Glucose levels in basal liver dialysate from anesthetized rats over time (n=5). Individual rat experiments are differentiated by different symbols to show the time trend for each rat.
Changes in basal liver metabolism in awake rats over time were also characterized. Samples were collected from awake rats every 60 minutes up to 72 hours. PCA and PLS models were calculated from NMR integrals normalized to TSP-d₄, the average % delivery of the microdialysis probe and the integral value for the initial time point. For the PCA model, no correlations were noted to time in the scores plots (5 principal component axes were examined). The PLS model showed a weak correlation to time ($R^2 = 0.74$), but the model itself was poor ($R^2$ predicted = 0.27). No significant time trends were identified in this data set.

Overall, it appears that changes in basal metabolism in both the heart and liver are highly linked to changes in carbohydrate metabolism - specifically, glucose. Both tissues show evidence of flux through glycolysis, as many of the amino acids that rise in level are typically produced as a result of transamination with pyruvate or other glycolytic intermediates. The citric acid cycle is the rate-limiting step in carbohydrate metabolism, so it stands to reason that an abundance of glucose, as observed at the beginning of the sampling period, would rapidly progress through glycolysis and accumulate while waiting to enter the citric acid cycle. In addition to glucose metabolism, creatine phosphate stores in both tissues are depleted over the course of the experiment, which is expected when rats are under low energy conditions like fasting, such as in these experiments.

3.4.3. Anesthesia Effects

Microdialysis experiments require the use of anesthesia during probe implantation. This can cause significant perturbations to the animal’s local and
general metabolism. Anesthesia affected liver metabolism to a much greater extent than circadian rhythms or changes in animal activity. The anesthetics used in these experiments were isoflurane, ketamine, xylazine and acepromazine. Isoflurane is an inhalation anesthetic used to sedate the rat briefly during administration of the anesthesia cocktail, which included ketamine, xylazine and acepromazine. Ketamine is a dissociative injectable anesthetic, that acts to change the reaction of the central nervous system to sensory stimuli\textsuperscript{27}. Ketamine is known to cause tremoring and muscle rigidity, so it is commonly administered in a cocktail with one or two other agents\textsuperscript{28}, typically xylazine and acepromazine (also known as acetylpromazine maleate). Xylazine is both a sedative and an analgesic that causes muscle relaxation, and acepromazine is primarily used for its sedative properties\textsuperscript{29}.

Comparison experiments to determine the effects of anesthesia on basal liver metabolism were performed by sampling liver dialysate from anesthetized and awake rats. The liver was chosen as a model tissue for two primary reasons. First, the surgery was simpler and more amenable to awake studies than heart microdialysis. Second, the liver was planned for use in subsequent experiments detailed in Chapter 4, so it was important to characterize these perturbations to interpret the data in the subsequent studies.

Probe implantation was performed in the same manner for both awake and anesthetized animals. For the anesthetized animal experiments, sampling began about one hour after probe implantation and continued for approximately 12 hours thereafter. Anesthetized animal experiments are limited to about 12 hours, primarily
due to limitations in the time the rat can be maintained under anesthesia and to avoid the onset of infection. It should be noted that anesthetized animal experiments can be conducted for longer than 12 hours if the probe implantation is under sterile conditions and if the rat can be maintained under anesthesia. However, for these experiments, 12 hours of sampling was sufficient. For the awake animal experiments, sampling began about 24 hours after probe implantation, when the rat was fully recovered from the implantation and the anesthesia had left its system. Sampling continued for 72 hours. Experiments were also performed during which sampling commenced immediately after probe implantation to study the metabolic effects of anesthesia leaving the rat.

A comparison of the metabolic profiles in awake and anesthetized rats is shown in the scores plot in Figure 3.12. A separation between metabolic profiles of awake and anesthetized animals is observed in the plot, demonstrating that there are metabolic differences caused by the anesthesia. This separation is defined solely by principal component 1. Also, the size of the clusters shows a marked difference between awake and anesthetized samples. It is evident that anesthesia introduces large amounts of variance into the data set and that awake animals experience relatively much less metabolic variance over time. This additional variance in the anesthetized animals is defined by both the first and second principal component axes.

Figure 3.13 shows the time trends observed in the scores plot in Figure 3.12. Figure 3.12 shows data from multiple rats for each group. Figure 3.13 is the same
Figure 3.12. Comparison of basal liver metabolites in awake (n=3) and anesthetized rats (n=4). ○ represents samples from awake animals and □ represents samples from anesthetized animals.
Figure 3.13. Time trajectory plots comparing the basal liver metabolism of awake and anesthetized rats. ○ represents averaged samples from awake animals (n=3) and □ represents averaged samples from anesthetized animals (n=4). A shows a comparison of time trajectories from awake and anesthetized animals and B shows an expanded view of the time trajectory in awake animals shown in A.
data set, but data points from individual rats were averaged at each time point to more easily track time trends in the data. Arrows in the plot show the directions of the metabolic time trends. In Figure 3.13A, note that anesthetized animals experience a large unidirectional time trend that is not observed in the awake animals. This is indicative of a large shift in metabolism that does not return to the original levels of metabolites over the time of the experiment. Figure 3.13B shows an expanded view of the metabolic time trends for the awake animals. No patterns are observed, whether unidirectional or cyclical. The PCA loadings for principal component 1 identify all integral regions as significant in defining the separation between awake and anesthetized animals, except for both regions containing lactate. The time trend along principal component 2 for the anesthetized rats is defined by glucose and creatine.

These experiments illustrate an important point for these experiments: the variance introduced by anesthesia is much greater than the variance introduced by animal activity, feeding/fasting cycles and diurnal variations in metabolism for tissue-targeted metabonomics experiments, as all of these potential sources of variance are included in the awake animal data set. This introduction of large amounts of variance unrelated to the metabolic stressor of the metabonomics experiment can be detrimental to the success of the experiment and data interpretation. Thus, although some microdialysis experiments may be better suited to the use of anesthetized animals, tissue-targeted metabonomics studies in liver tissue should avoid the use of anesthesia during sampling from the animal.
One of the main reasons for the dramatic time trends observed in the anesthetized animal samples is the effect of anesthesia on glucose levels. Glucose was identified in the principal component loadings for the analysis shown in Figures 3.12 and 3.13 as important both in distinguishing between awake and anesthetized animals and in establishing the time trend observed in anesthetized animals. Both ketamine/xylazine and isoflurane anesthetics have been shown to cause hyperglycemia in rodents. Xylazine in particular is an $\alpha_2$-adrenergic agonist and causes a massive release of glucose stores from the liver upon initial administration.28 Isoflurane also causes surges in glucose levels independently, but the mechanism for this effect is less clear.28 Figure 3.14 shows the dramatic trends in glucose observed in rats under anesthesia, those emerging from anesthesia and those completely removed from anesthesia. Rats under anesthesia initially have large amounts of glucose in the liver extracellular fluid, which fall dramatically over the course of several hours, almost by a factor of ten. Glucose levels begin to level around 20 hours as it clears from the extracellular fluid and as the anesthesia wears off. To avoid introducing glucose as a large source of variance unrelated to the experimental metabolic stressor, Figure 3.14 suggests that sampling should begin at least 20 hours post-probe anesthesia. It should be noted that in experiments with anesthesia, rats are fasting, as they are unable to eat. It is difficult to determine what, if any, effect fasting has on the levels of glucose in the experiment.
Figure 3.14. Timecourse of glucose in the liver extracellular fluid, as affected by the anesthetic agents ketamine, xylazine and isoflurane. ■ represents samples from anesthetized animals, ★ represents samples from animals emerging from anesthesia and ● represents samples from awake animals.
3.4.4. Animal-to-Animal Variability

In any *in vivo* experiment, some variance in the data set will originate from animal-to-animal variability in metabolism. Each animal will exhibit its own unique metabolic profile, due to small variations in metabolic rate (related to age and body weight) and other undetermined innate factors. In all experiments, every effort was taken to maintain consistency in the animals studied, through the use of male Sprague-Dawley rats from the same commercial source, within the weight range 350-450 g. Because rat weight tracks well with age, the rats used should be similar in age. The data analyses shown in this section is the same data as shown in Section 3.4.2, but relabeled to look at the data from a different perspective.

Animal-to-animal variability in heart metabolism is illustrated in the PCA scores plot in Figure 3.15. The first principal component separates the different animals, giving information on what causes the animal-to-animal variability in heart microdialysis experiments. The loadings for the first principal component show valine, leucine and glutamine as most important in differentiating between individual rats. A univariate plot of valine time trends in each rat is shown in Figure 3.16. There are no definable time trends for valine in these experiments – rather, it is the differences in levels of valine between rats that makes it so significant. The other significant integral regions, including leucine and glutamine, show similar trends. In this data set, some variance may still be present from the % delivery of the microdialysis probe, as the data was not normalized to this. (This was determined to be inappropriate for heart dialysate data, as shown in Section 2.4.4.1.2.) This could
Figure 3.15. PCA of basal heart dialysate metabolic profiles (n=8). Individual rats are differentiated by different symbols to show the time trend for each rat.
Figure 3.16. Valine levels in basal heart dialysate from anesthetized rats over time (n=8). Individual rats are differentiated by different symbols to show the time trend for each rat.
appear as animal-to-animal variability in the scores plot. It should also be noted that while there is variance between animal metabolism overall along the first principal component axis, there is also variance among the animals with regard to the way the metabolism changes over time. Along the second principal component axis in Figure 3.15, there is a large variation between the “length” of the time trends. This indicates that some animals experience a substantial change in basal metabolism over the course of sampling, while others experience much less change. In fact, the first principal component axis sorts rats based on the magnitude of the metabolic change experienced over time, with rats experiencing greater change at higher scores values.

Figure 3.17 shows the scores plot illustrating animal-to-animal variability in liver metabolism in anesthetized rats. All variance between rats should be primarily due to differences between animals, as other identified sources of variance have been corrected for in the raw data set prior to analysis. The fourth principal component axis best defines metabolic differences between the rats. In general, the anesthetized liver experiments showed much less animal-to-animal variation in metabolism than the heart microdialysis experiments, as observed by both multivariate and univariate analysis of the data. β-hydroxybutyrate and lactate were most significant in differentiating between anesthetized animals, as they show the most variation from animal to animal. Liver metabolism in awake rats contains animal-to-animal variation, but it is more difficult to define. Figure 3.18 shows the scores plot for the data set. Unlike the previous scores plot, there is no clearly defined axis describing animal-to-animal variation in the data set, although it is present.
Figure 3.17. PCA of basal liver dialysate metabolic profiles in anesthetized rats (n=5). Individual rats are differentiated by different symbols to show the time trend for each rat.
Figure 3.18. PCA of basal liver dialysate metabolic profiles in awake rats (n=4). Individual rats are differentiated by different symbols to show the time trend for each rat.
Animal-to-animal variation is an important consideration for metabonomics experiments. Studies are needed to clearly establish expected variance from innate differences in metabolism between animals, so that metabolic changes induced by a metabolic stressor can be confidently identified. In tissue targeted metabonomics, which is primarily focused on the identification and analysis of time trends in tissue metabolism, it is important to establish both differences between animal metabolism at single time points and differences between basal tissue metabolism over the time course of the experiment. In these studies, liver and heart tissues showed different metabolites as significant in varying between animals. Heart tissue showed the most variance from amino acids (leucine, valine and glutamine), while liver tissue showed the most variance from energy substrates or intermediates (lactate and β-hydroxybutyrate).

3.4.5. Circadian Rhythms

The presence of circadian rhythms was investigated in samples taken from the liver of awake rats. Circadian rhythms should not be significant in anesthetized animal experiments, due to their sampling period being 12 hours or less. PCA and PLS have already shown a poor correlation of liver metabolism in awake rats to time, as detailed in Section 3.4.2. However, it is possible that cyclical trends in the data could be missed, so the data was examined in a univariate manner. Only one rat exhibited cyclical trends in some metabolites, including leucine, isoleucine, valine, alanine and citrulline. Figure 3.19 shows levels of valine in the liver of three different awake rats over the course of three days of sampling. Roughly 24 hour
Figure 3.19. Timecourse of valine in the extracellular fluid of liver tissue of awake rats (n=3). The gray bars represent the dark period of the day, 6:00 PM – 6:00 AM.
cycles of valine levels are observed in one rat. The other rats show little to no cycling in metabolite levels. This plot is representative of trends observed in leucine, isoleucine, alanine and citrulline. All other metabolites showed very little cycling in levels over a 24 hour period. In addition to metabolite levels, cycling in animal activity was noted in all three rats, as expected. An example of animal activity is given in Figure 3.20. The rat with the greatest difference between activity in the light and dark period was the one with the cycling observed in metabolite levels. The other two rats showed activity cycling consistent with the time of day, but the differences in activity levels between the light and dark periods were less distinct.

These experiments have demonstrated that circadian rhythms do not significantly impact the composition of the liver extracellular fluid, at least in a large and consistent manner. While one rat showed cycling in a few metabolite levels, this was not observed in the other two rats studied. The fact that these effects are limited to a few metabolites and only in a small number of rats is positive for tissue-targeted metabonomics data interpretation. Little variance is added to the data set by circadian rhythms, making interpretation of other more interesting metabolic effects easier. The relatively small magnitude of circadian rhythms on liver extracellular fluid composition is confirmed in Figure 3.12, showing that metabolic changes from anesthesia are much greater than any circadian rhythms observed. This is the first known study of circadian rhythms in the composition of liver extracellular fluid. Numerous microdialysis studies of circadian rhythms in neurotransmitters have been
Figure 3.20. Animal activity histogram for one rat. The y axis represents the number of times that the optical sensor in the Ratum® system was activated over an hour period. The gray bars represent the dark period of the day, 6:00 PM – 6:00 AM.
performed in the brain tissue. This is an important study for tissue-targeted metabonomics experiments, helping in interpretation of the data acquired.

3.4.6. Tissue Immunological Response

Histology studies were performed on all tissues. Hearts and livers were harvested immediately after the end of the experiment, and the tissue was fixed in 10% buffered formalin and stored at 4°C for at least a week. The tissue was embedded in paraffin, sliced and stained in hematoxylin and eosin. Tissue responded as described previously\textsuperscript{30}. Probes implanted in anesthetized animals were in the tissue for 12-13 hours. Small amounts of damage from implantation were noted, but the primary tissue immune reaction was the beginning of neutrophil implantation into the implantation site. Awake animals had the probe implanted for much longer periods of time, typically around 96 hours. Consequently, the immune reaction was much greater. Neutrophil infiltration was much greater, some macrophage recruitment was present and a fibrotic capsule had begun to form around the probe. Unfortunately, the effects of probe implantation on metabolic profiles were unable to be determined. This is a common quandary in microdialysis experiments, as samples of extracellular fluid cannot be taken without the microdialysis probe.

Histology did show that one awake rat developed an infection over the course of the experiments. (Anesthetized rat experiments are not concerned with infection, as sampling is completed before infection begins to develop.) These results were reported in Chapter 2, Section 2.4.5 and Figures 2.16 and 2.17. It is important to comment here that infection causes changes in the metabolic profiles of liver tissue
(and presumably any other tissue). Thus, it is important to maintain sterile field during probe implantation, closely monitor rats for signs of infection during sampling and perform tissue histological analysis after sampling to determine if an infection developed. Conversely, this finding could provide an interesting future application for tissue-targeted metabonomics studies as a potentially more sensitive detector of sepsis.

3.5. Conclusions

These results demonstrate the potential of tissue-targeted metabonomics for studies requiring metabolic information from specific tissues. The ability to collect multiple samples over time from the same animal offers a unique advantage for the use of microdialysis sampling in metabonomics studies. As with all in vivo studies, metabolite levels are prone to change from factors unrelated to the metabolic stressor of the experiment, due to metabolite level variability between animals, circadian rhythms and animal activity. It is particularly important to characterize these trends in metabonomics studies because of the large number of interconnected endogenous metabolites monitored in a single analysis. The union of microdialysis sampling with metabonomics studies also presents anesthesia as an additional factor to be considered in characterizing metabolic perturbations unrelated to the metabolic stressor.

Tissue-targeted metabonomics data sets experience variance from a variety of sources. Tissues have different metabolic profiles, according to their specific
functions and energy needs. Within the same tissue, variance occurs primarily from anesthesia (if sampling from anesthetized animals) and animal-to-animal variability. Circadian rhythms do not appear to contribute significantly to variance in basal metabolism.

3.6. References


4.1. Introduction

Tissue-targeted metabonomics has the potential to give a new perspective on metabolic changes in specific tissues that experience oxidative stress. Oxidative stress was reviewed in the Introduction, Section 1.5. Some metabonomics studies have been previously conducted to examine the effects of oxidative stress on brain\textsuperscript{1,2}, liver\textsuperscript{3-6}, kidney\textsuperscript{7,8}, vascular smooth muscle\textsuperscript{9,10} and neonate urine\textsuperscript{11}. These studies have examined oxidative stress in tissues originating from various sources, including traumatic brain injury, hepatotoxicity, kidney transplantation, schizophrenia, diet and fatty liver disease. The studies use a mixture of biofluid and tissue extract analysis, often comparing the metabolic profiles to concurrent proteomic or genomic data. In these studies, no clear metabolic patterns resulting from oxidative stress were identified. A few endogenous metabolites changed with the timecourse of oxidative damage, including ophthalmic acid, allantoin, taurine, polyunsaturated fatty acids and ascorbate, although no consistent metabolic changes were noted among the studies. To date, no metabonomics studies have been performed examining two common
causes of oxidative stress, ischemia/reperfusion and chemically induced oxidative stress by doxorubicin. Additionally, no metabonomics studies have been performed characterizing oxidative stress in specific tissues with microdialysis sampling – all tissue-specific work has been done through biopsies, so animal-to-animal variability is high in these experiments.

4.2. Specific Aims

With this in mind, the purpose of this research was to apply tissue-targeted metabonomics to the study of oxidative stress, as induced locally by doxorubicin. Prior to in vivo studies, in vitro studies were performed to determine the solution stability of doxorubicin and its behavior in a microdialysis system. Myocardial oxidative stress, as induced by doxorubicin and ischemia/reperfusion, was characterized in the anesthetized rat. However, the research primarily focused on hepatic oxidative stress, as induced by doxorubicin. This was examined in both awake and anesthetized animals. Additionally, a marker of oxidative stress, the antioxidant glutathione, was monitored in liver dialysate samples and correlated to changes in metabolic profiles. The purpose behind the glutathione analyses was to better identify metabolic changes specific to the oxidative stress induced by doxorubicin by providing some metric of oxidative stress to which to correlate the metabolic profiles.
4.3. **Materials and Methods**

See Section 2.3. Deviations or additions to the methods described are noted in the following sections.

4.3.1. **Reagents and Solutions**

Doxorubicin hydrochloride, ≥ 98% purity, was purchased from Sigma Chemical Corporation (St. Louis, MO), as were the glutathione and glutathione disulfide standards.

4.3.2. **Microdialysis Methods**

4.3.2.1. **In Vitro Experiments**

Doxorubicin *in vitro* experiments were performed to characterize the behavior of doxorubicin in the microdialysis setup. A 37°C bath was prepared using a 118 mL polypropylene specimen jar filled with 80 mL of Ringer’s solution. (A polypropylene container was used in place of the typical glass container, due to doxorubicin’s propensity to stick to glass surfaces.) The specimen jar was kept at constant temperature using a Thermolyne Dri-Bath and was stirred slowly throughout the experiment by placing the bath on a magnetic stirrer plate. The specimen jar was isolated using a lid, with holes drilled in the lid for the microdialysis probes. The probe membrane was submerged in the middle of the bath, and the probe was secured in position. The bath was allowed to equilibrate to temperature for 30 minutes and the probes were allowed to equilibrate while being perfused at 1 μL/min for an additional 30 minutes. Delivery experiments were typically performed first. In these experiments, the bath contained Ringer’s solution and the probe was perfused with 10
μM doxorubicin and 10 μM antipyrine in Ringer’s solution. (Concentrations were varied in some experiments.) In the recovery experiments, the bath contained 10 μM doxorubicin and 10 μM antipyrine in Ringer’s solution and the microdialysis probe was perfused with Ringer’s solution. Samples were collected every 15 minutes for LC/UV analysis, as described in Section 2.3.2.1 and 4.3.3.

4.3.2.2. Isoflurane Anesthesia

A subset of rats was anesthetized using isoflurane inhalation anesthesia to determine the effects of anesthesia on the timecourse of glutathione and glutathione disulfide in liver dialysate. The setup is shown in Figure 4.1. The isoflurane was delivered to the rat using an isoflurane vaporizer (VetEquip, Pleasanton, CA). The vaporizer mixed isoflurane vapor with an aerobic gas mixture (95% oxygen/5% carbon dioxide). The airflow was maintained at 1.5 L/min. Rats were initially anesthetized at 5% isoflurane. The level was lowered to approximately 2% to maintain anesthesia, but was adjusted as necessary to maintain appropriate levels of sedation. Exhaled isoflurane was trapped using a VaporGuard activated charcoal absorption filter.

4.3.2.3. Doxorubicin Dosage

Doxorubicin was dosed in two different ways. First, preliminary studies for doxorubicin were performed in heart microdialysis experiments with anesthetized rats. In these studies, doxorubicin was dosed at 15 mg/kg intraperitoneally. Second, doxorubicin was dosed locally through the microdialysis probe in the liver microdialysis experiments. Two probes were implanted in the median lobe of the
Figure 4.1. Isoflurane inhalation anesthesia setup, as utilized during a liver microdialysis experiment. **A** labels the vaporizer and **B** designates the charcoal filter. The syringe pump and fraction collector on the right show the microdialysis sampling equipment.
liver (Figure 4.2). One probe (posterior, Probe A) was used to monitor basal changes in the liver extracellular fluid composition and the second probe (anterior, Probe B) was used to both dose doxorubicin and monitor changes in liver metabolism due to the doxorubicin dose. The probes were spaced 1 cm apart, a distance previously established to prevent crosstalk between the probes\textsuperscript{12}. Probes were perfused at 1 μL/min with 7 μM antipyrine in Ringer’s solution. After collection of basal dialysate from both probes, the perfusate for Probe B was switched to 2 mg/mL doxorubicin dissolved in the original perfusate solution. Probe B was perfused for 1 hour with the doxorubicin solution, after which the perfusate was returned to the original composition. In anesthetized rat experiments, basal dialysate was collected for three hours, with samples collected every 60 minutes. After the doxorubicin dose, samples were collected every 60 minutes for eight hours. In awake rat experiments, basal dialysate samples were collected every 60 minutes for 24 hours. After the doxorubicin dose, sampling continued for 47 hours.

4.3.3. LC/UV Doxorubicin Analysis

Doxorubicin was detected in liver dialysate samples using LC/UV, with the system described in Section 2.3.2.1. The isocratic method utilized the mobile phase 25% acetonitrile/75% 25 mM sodium phosphate buffer, pH = 2.5. The flow rate was 0.3 mL/min, and the detection wavelength was 254 nm.

4.3.4. CE/UV Glutathione Analysis

Glutathione (GSH) and glutathione disulfide (GSSG) were quantified in liver dialysate using a CE/UV method similar to that previously described\textsuperscript{13}. A homebuilt
Figure 4.2. Dual probe approach to doxorubicin studies in the liver. A shows the basic implantation geometry, with B showing the localized dosing of doxorubicin through Probe B.
CE/UV system was used, with a Spellman CZE1000R high voltage power supply and SpectraPhysics UV1000 detector. A 50 μm inner diameter (360 μm outer diameter) fused silica capillary was utilized, cut to 60 cm in total length. A 1 cm detection window was burned in the capillary coating, making the capillary 45 cm in effective length. The background electrolyte (BGE) was 0.1 M ammonium chloride with 0.5 mM tetradecltrimethylammonium bromide (TTAB), pH = 8.4. TTAB, a cationic surfactant, was used to reverse the electroosmotic flow of the separation. Samples were injected onto the capillary electrokinetically by applying a potential of -10 kV for 30 seconds, followed by an electrokinetic injection of 0.1 M NaOH for 60 seconds at -10 kV for pH-mediated stacking. The separation potential was -10 kV, and the detection wavelength was 214 nm. Data was acquired using Chrom&Spec software (version 1.52L, Ampersand International, Beachwood, OH). A sample electropherogram is shown in Figure 4.3. At the beginning of each day, the capillary was fully conditioned using the following rinsing procedure (times represent the length of time a pressure of 10 psi was applied): 10 minutes, methanol; 2 minutes, water; 10 minutes, 1 M HCl; 2 minutes, water; 20 minutes, 0.1 M NaOH; 2 minutes, water; 10 minutes, BGE. In between injections, the capillary was rinsed with hydrodynamic injections of 0.1 M NaOH and BGE, 4 minutes each.

4.3.5. LC/EC Glutathione Analysis

Glutathione (GSH) and glutathione disulfide (GSSG) were quantified in liver dialysate using a dual electrode LC/EC method similar to those previously described in literature\textsuperscript{14-17}. The LC/EC system was composed of a single Shimadzu LC-10AD
Figure 4.3. CE/UV electropherogram of basal liver dialysate. The arrow indicates the peak representing GSSG.
pump and a Rheodyne 7725i injector, fitted with a 25 μL PEEK injection loop (10 μL injection volume). The inlet tubing for the pump was composed of PEEK, to prevent the reoxygenation of the mobile phase after sparging. A Phenomenex Synergi Hydro-RP column, 2 mm x 150 mm, 4 μm particle size was used. The mobile phase consisted of 98% 0.12 M monochloroacetic acid 7 mM sodium octylsulfonic acid, pH = 3.0/2% methanol, at a flow rate of 0.35 mL/min. A dual gold-mercury electrode system was used as the detection scheme. The electrode consisted of two 3 mm gold electrodes in a series configuration. The gold electrodes were amalgamated and placed in the electrode block with a thin-layer gasket 0.005” thick. A Ag/AgCl reference electrode was utilized. Two BAS LC-4C amperometric detectors were utilized and interfaced to Chrom&Spec software (v. 1.52L, Ampersand International, Inc., Beechwood, OH). The upstream electrode was set to -1.0 V (range = 1 μA) and the downstream electrode was set to 0.15 V (range = 50 nA). The upstream electrode was used for disulfide reduction, and the downstream electrode was used for thiol detection. In all microdialysis experiments for the detection of glutathione, samples were collected into an equal volume of acid (0.12 M monochloroacetic acid pH = 3.0) to stabilize the thiol/disulfide ratio. A representative chromatogram of a liver dialysate sample is shown in Figure 4.4.

4.3.6. NMR Spectroscopy

See Section 2.3.3.

4.3.7. Data Analysis

See Section 2.3.4.
Figure 4.4. LC/EC chromatogram of liver dialysate. 1 corresponds to GSH and 2 corresponds to GSSG.
4.4. Results and Discussion

Tissue-targeted metabonomics lends itself to the study of conditions that induce spatially-specific metabolic changes that would otherwise go undetected by conventional metabonomics sampling approaches. One such condition is oxidative stress, which can be induced by a variety of mechanisms. Here, metabolic changes induced by ischemia/reperfusion or doxorubicin in heart and liver tissue are presented, along with concurrent studies of oxidative stress using established biomarkers, glutathione and its disulfide. However, initially, the behavior of doxorubicin in the analytical setup was characterized.

4.4.1. Doxorubicin Method Development

Doxorubicin presented some significant analytical challenges for both microdialysis and LC experiments, due to its hydrophobicity and propensity to photodegradation. Doxorubicin was detected by LC/UV in liver dialysate, as detailed in Section 4.3.3. Carryover for the method was about 1%. Because doxorubicin is fairly hydrophobic, sticking to the glass syringe or tubing components created a significant carryover problem. This was corrected by rinsing the syringe with three volumes of isopropanol followed by three volumes of Ringer’s solution (the sample matrix). The injection loop (25 μL volume) was also rinsed with four volumes of isopropanol followed by four volumes of Ringer’s solution. The linear range for doxorubicin was 1-75 μM, and precision was 2-3%. A series of in vitro experiments were performed to characterize doxorubicin behavior with probe components (tubing and membrane), as well as with the glass syringe used for perfusion. Additionally,
the degradation of doxorubicin over time and by light was studied. The setup for these experiments is described in Section 4.3.2.1.

4.4.1.1. Doxorubicin Adsorption in Microdialysis Experiments

The adsorption of doxorubicin to microdialysis probe components was examined. Microdialysis probes are composed of Tygon tubing (used as an adapter between the probe and syringe), polyimide tubing and the membrane, which can be composed of many different polymers. (Membrane selection will be examined in Section 4.4.1.3.) For linear microdialysis probes, either polyimide tubing or fused silica is used. In this laboratory, polyimide is the tubing of choice, due to its improved flexibility, making surgical probe implantation and tubing tunneling under the skin easier. However, polyimide may be more prone to interactions with hydrophobic compounds. Thus, it is important to characterize *in vitro* the effects of the equipment setup and microdialysis probe composition on doxorubicin levels in the perfusate.

Figure 4.5 shows the adsorption of doxorubicin to the microdialysis probe setup. In this experiment, the doxorubicin solution was analyzed in three ways: the perfusate solution prior to perfusing through the microdialysis probe, the solution perfused through a length of polyimide tubing and the solution perfused through a microdialysis probe (1 cm PAN membrane active window). The level of doxorubicin was constant for both the solution from the polyimide tubing and from the microdialysis probe, showing a constant doxorubicin delivery over time. A small but significant loss of doxorubicin was observed after perfusing the solution through the
Figure 4.5. Examination of doxorubicin adsorbance to the probe components. The dashed line indicates the initial level of doxorubicin in the perfusate solution (10 μM doxorubicin in Ringer’s solution). ▲ indicates the level of doxorubicin after perfusing the solution through a length of polyimide tubing. ◊ indicates the level of doxorubicin after perfusing the solution through a complete microdialysis probe with a 10 mm PAN membrane.
polyimide tubing alone, indicating adsorption in some component of the in vitro experiment setup. This could be due to one of three components that the perfusate solution is exposed to: the glass syringe, Tygon tubing or polyimide tubing. Figure 4.6 shows a repeat of the experiment performed in Figure 4.5, but each of the probe components were examined individually. The loss of doxorubicin from the syringe, Tygon tubing and polyimide tubing was the same. Therefore, it can be concluded that the primary source of adsorption of doxorubicin in its perfusion through a microdialysis probe originates with the glass syringe. This is the first component of the perfusion setup that the doxorubicin solution contacts. While it is important to note these sources of adsorption, this should not pose a problem for in vivo microdialysis experiments. The levels of doxorubicin in the solution from the syringe are constant over time, indicating that adsorption will not cause unwanted fluctuations in doxorubicin levels, which could potentially introduce variance into the metabolic profile data.

4.4.1.2. Doxorubicin Solution Stability

The stability of doxorubicin solutions was a concern, as it is known to undergo degradation, particularly at lower concentrations. It has been demonstrated that doxorubicin is most stable at 2 mg/mL (~3 mM) concentration for storage purposes\textsuperscript{18}. However, the doxorubicin concentration in at least some of the dialysate samples was expected to be in the low micromolar range. Therefore, it was important to establish the window of stability during which the dialysate samples could be analyzed for doxorubicin levels by LC/UV. A set of doxorubicin standards (10, 5, 1
Figure 4.6. Examination of doxorubicin adsorbance to individual microdialysis probe components. The perfusate solution was 10 μM doxorubicin in Ringer’s solution. ■ indicates the level of doxorubicin in the perfusate, which was stored in the dark in a polypropylene vial through the experiment. Δ indicates the level of doxorubicin after perfusing the solution through a length of Tygon tubing. ◊ indicates the level of doxorubicin after perfusing the solution through a length of polyimide tubing. ● indicates the level of doxorubicin sampled directly from the glass syringe used to perfuse solution through the tubing.
and 0.5 μM) were prepared in Ringer’s solution and analyzed by LC/UV in triplicate. (Standards were stored in the dark at room temperature between injections on the same day.) The remaining standard solutions were then aliquoted into 10 μL portions in polypropylene vials, protected from light and stored at 4°C. The doxorubicin stock solution (2 mg/mL in Ringer’s solution) was also stored with the standard solutions. After 2 days, fresh standards were prepared from the stock solution and analyzed, along with the two-day-old standards. Figure 4.7 shows the results of the study. The standards prepared from the stock solution on the second day match the calibration curve generated on the initial day of analysis, confirming that the doxorubicin stock solution is stable at the high concentration and under the storage conditions described. A large drop in response was observed for the older standard solutions. The lower doxorubicin standard concentrations (1 and 0.5 μM) did not give a detectable signal. The results of this study suggest that dialysate samples should be analyzed without delay after collection to avoid any quantitation problems due to degradation.

Another concern with the microdialysis setup is the stability of the doxorubicin solution in the glass syringe over the course of the experiment. Depending on the doxorubicin dosing scheme, doxorubicin could remain in the syringe for several hours at room temperature while being perfused through the microdialysis probe. Photodegradation effects could be avoided by covering the syringe with foil, but other degradation mechanisms could cause significant changes in doxorubicin levels over time in the syringe. To characterize this potential problem, a solution of 10 μM doxorubicin and 10 μM antipyrine in Ringer’s solution was
Figure 4.7. Stability of doxorubicin standards in Ringer’s solution. ■ indicates standard solutions prepared on day 0, the first day of analysis. ○ indicates standard solutions prepared two days after the initial analysis from the original stock solution. ▲ indicates standard solutions prepared on day 0 and analyzed two days later.
prepared, and two glass 1 mL syringes (Hamilton Company, Reno, NV) were filled with the solution. The syringes were covered in foil and placed in the syringe pump. The flow was set to 1 μL/min. Samples were collected hourly over the course of the day and analyzed by LC/UV immediately. The perfusate solution (stored in the dark in a polypropylene vial) was also analyzed over the course of the day. The syringes (and perfusate solution) were then allowed to sit overnight at room temperature, still wrapped in foil. The following day, the delivery experiment was repeated, and injections were made of solution from the syringe to show the long-term stability of the solution in the syringe. Figure 4.8 shows the stability of both antipyrine and doxorubicin over time. Antipyrine showed good stability with no adsorption of the analyte to the syringe, which was expected. Doxorubicin levels in the solution from the syringes were significantly lower at 95% confidence than levels in the initial solution due to adsorption of the analyte to the glass syringe. However, doxorubicin in all experiment groups showed fairly good stability, particularly over the course of the initial 10 hours of analysis. The RSD of doxorubicin levels in all experiment groups was approximately 5%. Therefore, although doxorubicin showed adsorption to the walls of the glass syringe, this did not impact the stability of doxorubicin levels coming from the syringe. This is an important point for the dosage of doxorubicin by delivery through the microdialysis probe.

Doxorubicin has been shown to degrade in the presence of light\textsuperscript{18,19}. Photodegradation of a solution of 10 μM doxorubicin was examined. The solution was split into two polypropylene vials. One vial was stored at room temperature in
Figure 4.8. Stability of doxorubicin (A) and antipyrine (B) in a glass syringe over time. ■ indicates solute levels in the solution stored in a polypropylene vial in the dark, and □ indicates solute levels in the solution stored in a glass syringe in the dark.
the dark, while the other vial was stored at room temperature at normal indoor lighting conditions on the laboratory bench. Samples were analyzed periodically from both solutions. The results of the experiment are shown in Figure 4.9. The results from this experiment are difficult to interpret. The solution stored in the dark showed a low response from doxorubicin at three consecutive timepoints (150-250 minutes) that returned to initial levels for the last two time points. The origin of these low data points is unknown. Samples taken from the solution stored in light showed a steady decline in doxorubicin levels over the course of the sampling period (~6 hours). It is unclear whether this is a significant decline, due to the variation observed from samples analyzed from the dark solution. Regardless, some photodegradation was expected, and vials were covered in foil to lessen its effects in doxorubicin quantitation.

4.4.1.3. Doxorubicin In Vitro Behavior

Microdialysis probes were calibrated with doxorubicin in vitro to characterize the behavior of the probe and approximate its behavior in vivo. Each probe was characterized by both a delivery and a recovery experiment, as described in Section 4.3.2.1. Probe extraction efficiency can be calculated by characterizing either probe delivery or recovery – theoretically, either technique should yield the same probe extraction efficiency. This has been demonstrated for linear probes previously by this laboratory. In general, delivery experiments were performed first, followed by the recovery experiment. Figure 4.10 shows the result of microdialysis probe calibrations by doxorubicin, with the recovery and delivery experiments performed in different
Figure 4.9. Photostability of 10 μM doxorubicin in Ringer’s solution. The dashed line indicates the initial level of doxorubicin in the perfusate solution (10 μM doxorubicin in Ringer’s solution). ■ indicates the solution stored in the dark, and ○ indicates solution stored on the benchtop, exposed to normal indoor lighting conditions.
Figure 4.10. Method for calibration of microdialysis probes (1 cm PAN membrane) with doxorubicin. ■ indicates the delivery of doxorubicin, as performed first in the calibration experiment. □ indicates the recovery of doxorubicin, as performed second in the calibration experiment. ● indicates the delivery of doxorubicin, as performed second in the calibration experiment. ○ indicates the recovery of doxorubicin, as performed first in the calibration experiment.
orders. For both experiments, the delivery of doxorubicin was level and consistent throughout the experiment. The delivery was unaffected by the order of the calibration experiment. The recovery of doxorubicin showed significant differences between experiments. Additionally, the observed probe extraction efficiency began low for each recovery experiment and took about 30 minutes for extraction efficiency values to stabilize. The order of the experiment significantly changed the extraction efficiency values observed for the recovery portion of the experiment, by 20-25%. The reason for this large discrepancy is unclear, but is likely connected to doxorubicin’s hydrophobicity. Doxorubicin readily adsorbs to PAN membrane. The membrane is a deep red color after perfusion with doxorubicin. Immersion of the probe in a doxorubicin solution rapidly increases the amount of doxorubicin adsorbed to the membrane, while this seems to be a slower process in the delivery experiments. In these experiments, the doxorubicin solution is only perfused through the probe, while the probe itself is submerged in Ringer’s solution.

When such a large (and reproducible) discrepancy is observed in data, it is advisable to verify the analytical methodology and technique of the scientist performing the experiment. With this in mind, the in vitro experiments described above were repeated by two scientists. Probes were constructed by each scientist, and each scientist independently calibrated one probe fabricated by the other (two probes total). All experiments were performed on the same day. The probes were perfused with 10 μM doxorubicin and 10 μM antipyrine in Ringer’s solution. Delivery experiments were performed first, followed by recovery experiments. Figure 4.11
Figure 4.11. Comparison of microdialysis probe (1 cm PAN membrane) calibration technique, as performed by two different scientists. Calibrated for doxorubicin (A) and antipyrine (B) with two different probes – one made by each scientist. ■ indicates delivery values as determined by scientist #1. □ indicates recovery values as determined by scientist #1. ● indicates delivery values as determined by scientist #2. ○ indicates recovery values as determined by scientist #2.
shows the results of the experiment. Probe calibration by antipyrine shows consistent results, with matching values for probe delivery and recovery over time. The antipyrine results indicate the precision of both the probe fabrication and analytical methodology, so any discrepancies observed in the doxorubicin data were due to the behavior of doxorubicin itself. The doxorubicin results show good reproducibility for the delivery of doxorubicin, reaching a similar steady-state level, both between scientists and probes. These values were also close to those obtained to antipyrine. However, there is a large difference between the delivery and recovery values for both probes, as determined by both scientists. The recovery shows variation at the initial time points, with some convergence in recovery values observed at later time points. Although the recovery of doxorubicin in vitro was problematic, the delivery was really of primary concern. This is the mechanism by which the drug will be delivered to the tissue. It is consistent and reproducible, indicating that the drug dose will be consistent as well.

The effect of the probe membrane material on the in vitro behavior of doxorubicin was examined. Many membrane polymers are available, but polyacrylonitrile (PAN) and cuprophan membranes were characterized in this investigation because of their accessibility and successful use in previous microdialysis experiments. PAN membranes are advantageous for microdialysis experiments because the membrane is robust, i.e., does not kink or break easily. This simplifies both fabrication and implantation. PAN is a hydrophilic membrane with a 20 kDa molecular weight cutoff (MWCO) and is highly negatively charged on the
membrane surface\textsuperscript{20}. Cuprophan (regenerated cellulose) is also a hydrophilic membrane, but the membrane surface is neutral\textsuperscript{20}. It has a 12 kDa MWCO and is much more brittle than PAN membrane. The delivery and recovery for doxorubicin and antipyrine (as described for previous experiments) was determined for microdialysis probes made of 1 cm PAN or 1 cm cuprophan membrane, both prepared in-house. Figure 4.12 shows the results of the experiment. Antipyrine behaved similarly with both membrane types. (One probe with a PAN membrane showed lowered recovery values, but this was the only probe out of numerous \textit{in vitro} experiments that exhibited this behavior for antipyrine.) Doxorubicin showed significant differences between the delivery values obtained from the PAN and cuprophan membranes. Both cuprophan and PAN showed much lower recovery values for doxorubicin. Thus, this discrepancy between delivery and recovery values of doxorubicin was not remedied using the cuprophan membrane.

Recovery and delivery values can change with analyte concentration. The effects of doxorubicin concentration on probe delivery and recovery were characterized (antipyrine was used as a control). Probes were calibrated by both delivery and recovery of antipyrine and doxorubicin at 10, 50 and 100 \(\mu\text{M}\) concentrations for both analytes in Ringer’s solution. Both PAN and cuprophan membranes were compared. The results are shown in Figure 4.13. (In the 100 \(\mu\text{M}\) experiment, the cuprophan membrane was torn, and recovery results could not be obtained.) Overall, no concentration dependence was noted for the recovery or
Figure 4.12. The effects of membrane composition on the probe extraction efficiency for doxorubicin (A) and antipyrine (B). ■ indicates delivery values for the probe made with PAN membrane. □ indicates recovery values for the probe made with PAN membrane. ● indicates delivery values for the probe made with cuprophan membrane. ○ indicates recovery values for the probe made with cuprophan membrane.
Figure 4.13. The effect of solute concentration on probe extraction efficiency for both PAN (A, C) and cuprophan (B, D) membranes. Doxorubicin is measured in A and B, and antipyrine is measured in C and D. Square symbols show the probe extraction efficiency for a 10 μM solution, circle symbols show the probe extraction efficiency for a 50 μM solution, and triangle symbols show the probe extraction efficiency for a 100 μM solution. Closed symbols show probe delivery and open symbols show probe recovery.
delivery of either antipyrine or doxorubicin. Probes behaved as expected for each membrane type and analyte, as determined by previous experiments.

In summary, doxorubicin shows deviations from “ideal” behavior for microdialysis experiments, including adsorption to glass and discrepancies between the delivery and recovery of the analyte by the microdialysis probe. In these studies, these discrepancies were characterized to aid in the interpretation of data in the in vivo experiments. These experiments demonstrated an important property of doxorubicin that was key in data interpretation for metabonomics experiments. First, the delivery of doxorubicin was very consistent over time, matching the delivery observed for antipyrine (the internal standard). In most of the experiments, doxorubicin was dosed through the microdialysis probe. These results show that the doxorubicin dose will be consistent between animals and likely will not add to variation in the experiments.

4.4.2. Myocardial Oxidative Stress

One of the most susceptible tissue targets of oxidative stress is the heart. Whether the oxidative stress is physiologically induced by ischemia/reperfusion or is chemically induced by the administration of a drug such as doxorubicin, metabolism in the tissue is disrupted, resulting in detrimental and perhaps permanent effects to the tissue, including contractile dysfunction and congestive heart failure.

Preliminary studies were performed to characterize metabolic changes induced in heart tissue by both physiologically induced and chemically induced oxidative stress. All experiments were performed on anesthetized animals, as the
induction of myocardial ischemia is nearly impossible to perform in awake animals, for both surgical and ethical reasons. In these experiments, basal heart dialysate was collected from rats for two hours (samples collected every 30 minutes) prior to the induction of oxidative stress. Oxidative stress was induced physiologically by temporary ligation of the left anterior descending coronary artery or induced chemically by the administration of a systemic dose of doxorubicin (15 mg/kg intraperitoneally).

The metabolic consequences of both methods of inducing oxidative stress were compared in the principal components analysis (PCA) scores plot in Figure 4.14. The analysis indicates that physiological and chemical oxidative stress may have different metabolic signatures in heart tissue. The right-facing arrow in the scores plot indicates the metabolic trend observed as a result of ischemia/reperfusion, while the downward-facing arrow shows the direction of the metabolic trend induced by doxorubicin administration. In this scores plot, the first principal component distinguishes between control experiment samples and ischemia/reperfusion experiment samples. The second principal component distinguishes between control experiment samples and doxorubicin experiment samples. The loadings for the first principal component show lysine, alanine, glutamine, lactate, citrate, β-hydroxybutyrate and glucose as important. Interestingly, creatine is not identified in this analysis as important, even though ischemia/reperfusion is known to significantly alter the levels of creatine through dephosphorylation of creatine phosphate. In Figure 4.15, the simultaneous metabolic changes induced by ischemia/reperfusion in
myocardial ischemia/reperfusion and indicates samples from rats dosed with doxorubicin. The arrows designate the direction of metabolic trends resulting from ischemia/reperfusion or doxorubicin dose. Integral data was normalized to the initial time point prior to PCA.

**Figure 4.14.** Principal components analysis of heart dialysate metabolic profiles. △ indicates samples from control rats, ■ indicates samples from rats undergoing myocardial ischemia/reperfusion and ● indicates samples from rats dosed with doxorubicin. The arrows designate the direction of metabolic trends resulting from ischemia/reperfusion or doxorubicin dose. Integral data was normalized to the initial time point prior to PCA.
Figure 4.15. Comparison of NMR spectra of heart dialysate from a rat experiencing myocardial ischemia/reperfusion. A depicts basal heart dialysate, B depicts ischemic heart dialysate and C depicts reperfused heart dialysate. Three key metabolites in the spectra are labeled: 1, lactate; 2, creatine; 3, glucose.
three key energy metabolites are depicted. Glucose falls rapidly and lactate increases after the induction of ischemia. Creatine remains level, with a small increase during the reperfusion period. These results are in agreement with the PCA loadings. Doxorubicin affects different metabolites; the loadings for the second principal component indicate that glucose, valine, citrulline and creatine levels are altered in response to doxorubicin administration.

The results described above are preliminary, with the doxorubicin-dosed group only containing n=1. However, early indications suggest a differential metabolic response. Some of the metabolites changing in response to myocardial ischemia/reperfusion are in accordance with previously established trends in literature, with glucose and lactate changing as a result of the heart muscle’s switch to anaerobic metabolism under restricted blood flow. The other metabolites (lysine, alanine, glutamine, citrate and β-hydroxybutyrate) are involved in energy metabolism, but through many different metabolic pathways. Doxorubicin may tap into different pathways than ischemia/reperfusion, with different metabolites earmarked as important. No anaerobic metabolism was induced, but alterations in energy stores were noted, as were changes in nitrogen metabolism. This is the first time that myocardial oxidative stress has been examined using metabonomics, particularly using a sampling technique to focus on the actual tissue metabolism. Additionally, this is the first metabonomics comparison of oxidative stress events induced by different mechanisms.
In spite of these preliminary results, it was decided to move the oxidative stress investigations to the liver. Experimentally, microdialysis sampling of heart tissues imposes restrictions on the metabonomics studies that can be overcome by moving to liver studies. Heart microdialysis probes are limited in the active membrane length, thus limiting probe extraction efficiency. By lowering the concentration of metabolites in the dialysate sample, several possible negative effects on the method could result. First, the number of detected metabolites will likely decrease because the concentration in the original dialysate sample will be lowered due to poorer extraction efficiency. Second, the throughput of the method could be lowered due to increased NMR experiment time to compensate for the more dilute samples by signal averaging longer. Third, the time resolution of the method could be worsened by sampling for longer periods of time to concentrate the sample to compensate for the lower extraction efficiency of the heart microdialysis probes. Thus, the decision was made to move to liver microdialysis experiments. For these experiments, the active membrane length can be longer, thus increasing probe extraction efficiency. Additionally, the microdialysis experiments are more amenable to awake animal studies.

4.4.3. *Hepatic Oxidative Stress*

In these experiments, doxorubicin was used to chemically induce oxidative stress only in the liver tissue immediately surrounding one of the implanted microdialysis probes. One of the advantages of microdialysis sampling is that drugs can be delivered to the surrounding tissue while the endogenous metabolites are
collected from the same tissue being dosed. By adding a second probe in the liver lobe unaffected by the doxorubicin dose as a control (referred to as Probe A), natural changes in liver metabolism could be identified and separated from metabolic changes induced by doxorubicin (Figure 4.2).

Doxorubicin can be dosed to examine toxicity from two perspectives – acute and chronic. The cardiotoxic side effects of doxorubicin are typically the result of chronic toxicity, induced by a constant, lower dose of the drug. However, in these studies, the acute toxicity of doxorubicin was probed for several reasons. First, the liver was studied rather than the heart, making induction of cardiotoxicity less interesting. Second, acute toxicity studies are more efficient and easier to study by microdialysis because the timescale is hours to days rather than weeks to induce the metabolic change. Finally and most importantly, it would be difficult to separate the initial toxic effects of doxorubicin from changes caused by tissue repair and adaptation in chronic toxicity studies\textsuperscript{22}. It should be acknowledged that the toxic side effects of doxorubicin may differ depending on the dose or time given\textsuperscript{23-25}. For example, apoptosis may be observed at lower concentrations of doxorubicin, while free radical-mediated toxicity is dominant at higher concentrations\textsuperscript{23}. Doxorubicin has been shown to have varying degrees of toxicity when it is administered at different times of day\textsuperscript{26}. In these studies, the doxorubicin dose was given at the same time of day for each rat.
4.4.3.1. Doxorubicin Dosage Studies

The doxorubicin dosing scheme in liver microdialysis experiments was described in Section 4.3.2.3. Doxorubicin was analyzed in dialysate from both probes using LC/UV, in order to both monitor the time course of doxorubicin and confirm that no doxorubicin was detected in the control probe. Figure 4.16 shows doxorubicin levels over time. There is an initial spike of the drug when it is perfused through the probe, and then it gradually decays over the next few hours. Thus, the dose of doxorubicin provides a single, intense metabolic insult to the tissue. Figure 4.16 also confirms that this metabolic insult is localized, as no doxorubicin was detected in the dialysate from the control probe A. This is further confirmed by histological analysis of the liver tissue, as shown by the representative picture in Figure 4.17. The tissue around probe B shows a large necrotic zone, which is lighter in color than the surrounding healthy tissue. This zone does not extend to the tissue around probe A, confirming that the dialysate sampled at probe A should be representative of the natural metabolic profile of healthy liver tissue.

The time course of damage from the doxorubicin dose was examined through a histology study. In this study, probes were implanted as described previously. Rats were maintained under anesthesia. Thirty minutes after probe implantation, probes were perfused with Ringer’s solution for three hours to mimic the basal dialysate sampling period. The perfusate was then switched to 2 mg/mL doxorubicin in Ringer’s solution for one hour. At the end of the dosing period, the rats were put down at various time points (0, 2, 5, 6 and 8 hours post-dose). Livers were harvested
Figure 4.16. Timecourse of doxorubicin dosage and clearance through the microdialysis probe, as measured by the dialysate concentration. ▲ represents samples from Probe A, the undosed/control probe and Δ represents samples from Probe B, through which doxorubicin was dosed.
Figure 4.17. Histology of liver with two microdialysis probes implanted (A and B). Doxorubicin was perfused through probe B. The light area around Probe B is a zone of necrotic tissue.
and prepared for histological analysis. Dr. Mike Thompson, pathologist at Lawrence Memorial Hospital, helped in the interpretation of the slides. The results of the histological study are shown in Table 4.1. The control probe (Probe A) showed a normal immune response to the probe, with small amounts of damage from probe implantation and some neutrophil recruitment at later time points. Very little tissue damage occurred around Probe B immediately after dosing – it became more evident 2 hours post-dose. Maximum damage appeared to occur 5 hours post-dose. Consistently, the amount of damage around Probe A from implantation and immune response was substantially lower than the damage induced by doxorubicin. The data from this study was also used to approximate a doxorubicin “diffusion coefficient” from the probe through the tissue. The maximum distance of damage from the probe was measured and divided by the time post-dose. The results are shown in Table 4.2. Diffusion coefficient approximations were fairly consistent among the time points, with an average coefficient of 11 nm/min through the liver tissue for doxorubicin.

Additionally, the pharmacokinetics of the doxorubicin dose was explored, as no literature has been published on the dosing of doxorubicin through a microdialysis probe. The doxorubicin dose (perfusate concentration of 2 mg/mL perfused through the microdialysis probe at 1 μL/min for 1 hour) was given in three experimental settings: *in vitro*, in the liver of anesthetized animals and in the liver of awake animals. All microdialysis probes utilized were of the same membrane length. The overall time course for each set of experiments is shown in Figure 4.18. The pharmacokinetics of doxorubicin in awake and anesthetized animals does not differ
<table>
<thead>
<tr>
<th>Time Post-Dose (Hour)</th>
<th>Necrotic Zone Area</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Probe A: 0 μm²</td>
<td>Probe A: Minimal change</td>
</tr>
<tr>
<td></td>
<td>Probe B: 0 μm²</td>
<td>Probe B: Minimal change</td>
</tr>
<tr>
<td>2</td>
<td>Probe A: 5.4 x 10³ μm²</td>
<td>Probe A: Some cell death from probe implantation</td>
</tr>
<tr>
<td></td>
<td>Probe B: 1.3 x 10⁷ μm²</td>
<td>Probe B: Liver toxicity developing; geographic necrosis evident</td>
</tr>
<tr>
<td>5</td>
<td>Probe A: 7.4 x 10⁵ μm²</td>
<td>Probe A: Some cell death from probe implantation</td>
</tr>
<tr>
<td></td>
<td>Probe B: 1.9 x 10⁷ μm²</td>
<td>Probe B: Zonal necrosis, but peripheral region still viable</td>
</tr>
<tr>
<td>6</td>
<td>Probe A: 1.3 x 10⁴ μm²</td>
<td>Probe A: Some cell death from probe implantation</td>
</tr>
<tr>
<td></td>
<td>Probe B: 2.8 x 10⁷ μm²</td>
<td>Probe B: Zonal necrosis, but peripheral region still viable</td>
</tr>
<tr>
<td>8</td>
<td>Probe A: 2.2 x 10⁴ μm²</td>
<td>Probe A: Some cell death from probe implantation</td>
</tr>
<tr>
<td></td>
<td>Probe B: 1.1 x 10⁷ μm²</td>
<td>Probe B: Zonal necrosis, but peripheral region still viable</td>
</tr>
</tbody>
</table>

**Table 4.1.** Progression of damage from doxorubicin, as gauged by histological analysis of liver tissue harvested at various time points post-dose.
<table>
<thead>
<tr>
<th>Time Post-Dose (Hour)</th>
<th>Maximum Distance (mm)</th>
<th>Diffusion Coefficient (nm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.2</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>14</td>
</tr>
</tbody>
</table>

*Average: 11*

**Table 4.2.** Doxorubicin diffusion coefficient approximation, as estimated from the histology study of the time course of doxorubicin damage.
Figure 4.18. Comparison of doxorubicin levels post-dose in liver dialysate samples from *in vitro* (■), anesthetized rat (Δ) and awake rat (●) experiments.
significantly, at the 95% confidence level. Additionally, it can be seen from the logarithmic scale that doxorubicin exhibits biphasic behavior both in vitro and in vivo — a distribution and elimination phase is identifiable in the plot. This biphasic behavior is likely from doxorubicin sticking to the probe membrane after dosing and subsequently releasing from the membrane over time. This hypothesis was tested in vitro by “dosing” both doxorubicin and antipyrine through the same microdialysis probe for one hour. Antipyrine does not stick to PAN membrane, so if the biphasic behavior of doxorubicin was due to membrane sticking, antipyrine levels should fall rapidly in the dialysate after dosing. Figure 4.19 shows the results from this experiment. This hypothesis was confirmed, as antipyrine did not linger in the dialysate after dosing. The pharmacokinetic parameters of the doxorubicin dose were calculated by fitting the doxorubicin concentration curves on a logarithmic scale to a biexponential decay, according to the following equation:

\[ C = Ae^{-at} + B^{-bt} \]  

(1)

Doxorubicin pharmacokinetic parameters were then calculated from the coefficients\(^{27}\), and the results are shown in Table 4.3. No statistically significant differences in distribution and elimination between awake and anesthetized animals were found. This was unexpected, as anesthesia has been shown to alter the pharmacokinetics of many other drugs. However, these drugs have been dosed systemically, usually either intravenously or intraperitoneally. Very few pharmacokinetics studies have been performed examining drug dosage through a microdialysis probe. The use of microdialysis probes as a dosing vehicle may lead to
Figure 4.19. Comparison of doxorubicin (●) and antipyrine (□) levels in dialysate samples from an in vitro microdialysis experiment.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>In Vitro (n=2)</th>
<th>Anesthetized (n=3)</th>
<th>Awake (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1/2 (distribution)</td>
<td>0.219 +/- 0.018</td>
<td>0.883 +/- 0.339</td>
<td>0.849 +/- 0.221</td>
</tr>
<tr>
<td>t1/2 (elimination)</td>
<td>3.07 +/- 0.05</td>
<td>17.6 +/- 13.2</td>
<td>11.4 +/- 9.0</td>
</tr>
<tr>
<td>Cmax (μM)</td>
<td>2540 +/- 370</td>
<td>1180 +/- 480</td>
<td>1300 +/- 1050 (693)</td>
</tr>
<tr>
<td>AUC</td>
<td>915 +/- 182</td>
<td>1280 +/- 450</td>
<td>1740 +/- 1700 (755)</td>
</tr>
<tr>
<td>Clearance (x 10^-10)</td>
<td>1.78 +/- 0.33</td>
<td>0.864 +/- 0.288</td>
<td>0.952 +/- 0.677 (1.34)</td>
</tr>
</tbody>
</table>

**Table 4.3.** Doxorubicin pharmacokinetic calculations. In the awake rat data, one rat appeared to be an outlier. The numbers in parentheses correspond to the average parameter value when the possible outlier point is removed.
experiments in which the results are less susceptible to anesthesia effects, perhaps because such a small portion of the animal is affected.

4.4.3.2. Dual Probe Experiments in Anesthetized Animals

Doxorubicin was first administered to anesthetized animals. Although strong metabolic trends were observed as a result of the anesthesia, as described above, it was initially not known whether the magnitude of the metabolic changes induced by doxorubicin would be greater than those induced by the anesthesia. Three hours of basal dialysate were collected from each probe prior to dosing doxorubicin (2 mg/mL perfusate concentration) for one hour through probe B. Samples were collected for 8 hours post-dose from both probes.

Figure 4.20 shows the scores plot of the first two principal components in the PCA analysis of data for anesthetized animals dosed with doxorubicin. The data segregates into dosed and undosed animals, with both data sets grouping in the top left of the plot. Each rat trends away from the starting point, with undosed rats trending downward along principal component 2 and dosed rats trending to the right along principal component 1. Samples from rats taken at hours 6 and 12 prior to doxorubicin dosing are marked with a star. In the dosed animals, there is no segregation between probe A and B, as would be expected if doxorubicin only perturbed the metabolism of the tissue around probe B. The timecourse data for individual rats from the PCA is shown in Figure 4.21. (This is the same PCA as Figure 4.20, but the plots are broken up to highlight individual rats’ metabolic trends.) There is good consistency for both the dosed and control groups of data. It should be
Figure 4.20. Doxorubicin liver metabolic effects in anesthetized rats. ■ represents samples from Probe A of undosed rats, □ represents samples from Probe B of undosed rats, ▲ represents samples from Probe A of dosed rats and Δ represents samples from Probe B of dosed rats (dosed probes). Samples points with an * are samples acquired before doxorubicin was dosed.
Figure 4.21. Doxorubicin liver metabolic effects in anesthetized rats. This is the same PCA as Figure 4.18, but displayed by individual rat. ■ represents samples from Probe A of undosed rats, □ represents samples from Probe B of undosed rats, ▲ represents samples from Probe A of dosed rats and Δ represents samples from Probe B of dosed rats (dosed probes). A, B, and C show individual anesthetized rats dosed with doxorubicin and D shows time trends from undosed anesthetized rats (n=4).
noted that the trends observed in dosed animals are unidirectional, meaning that the changes observed to the tissue from perfusing doxorubicin through the probe are permanent and irreversible (at least as observed over a 12 hour period).

From the scores plot in Figure 4.20, it appears that doxorubicin is causing a metabolic change in the dosed rats, but it is possibly a global effect rather than localized at the dosage site. This is because the metabolic profiles from both Probe A and B change in the same way in response to the doxorubicin dose. Histological analysis did confirm that no necrosis was observed around Probe A and LC-UV analysis did not detect doxorubicin in probe A. Principal component 1 is the component responsible both for separating control and dosed samples, as well as defining the time trend observed in dosed animals. The loadings for principal component 1 show the following metabolites as important: lysine, alanine, valine, leucine, isoleucine, creatine and β-hydroxybutyrate. Metabolic implications of this will be discussed further in Section 4.4.3.5.

4.4.3.3. Glutathione: Biomarker of Oxidative Stress

Metabonomics in general is an exploratory analytical technique, with one of the primary goals being to identify new biomarkers of pathophysiological conditions. However, biomarkers have been previously established for many conditions, although their reliability and relevance to the condition being studied may be questionable (thus necessitating these exploratory techniques for additional and/or better biomarkers). It would be prudent to perform concurrent studies of established biomarkers of the condition of interest with exploratory metabonomics studies. This
could both aid in interpretation of the metabonomics data and help to validate potential newly identified biomarkers.

Oxidative stress in particular has many previously established biomarkers, both small molecules and enzymes. Oxidative stress can be gauged by products of oxidative damage, including malondialdehyde, a product of lipid peroxidation, and spin trapping of the actual free radicals. It can also be gauged by the presence of repair products or alterations in antioxidant levels. Glutathione is one of the most prevalent antioxidants in the body, serving a variety of purposes. It is well established that both the levels of glutathione and the ratio of the thiol to its disulfide are markers of oxidative stress. Doxorubicin when administered systemically causes a decrease in liver glutathione concentration\textsuperscript{28}. Glutathione also detoxifies doxorubicin through conjugation, as catalyzed by glutathione-S-transferase\textsuperscript{28,29}. The conjugate is excreted into the bile.

Glutathione (GSH) and its disulfide (GSSG) were chosen as the biomarkers of oxidative stress to monitor in conjunction with liver dialysate metabolic profiles. GSH and GSSG were chosen for a couple of reasons. First, they are present in the extracellular fluid in the high nanomolar to low micromolar range, making quantitation amenable to a variety of analytical techniques. Second, the response of glutathione to oxidative stress is rapid. Biomarkers such as 8-hydroxy-2’-deoxyguanosine from the base excision repair process can have a significant lag time in response to an oxidative stress event. For these experiments, it would be difficult
to correlate more immediate metabolic changes to biomarker fluctuations occurring several hours later.

These studies were initially performed in anesthetized animals to establish patterns that could be correlated to the metabolic profile changes described in the previous section. Initially, glutathione was quantitated using an established method in our laboratory, CE/UV. However, this could only monitor total glutathione levels. An LC/EC method was then developed to quantitate GSH and GSSG separately in liver dialysate samples. This method was used to compare trends in rats anesthetized with ketamine and isoflurane inhalation, as well as to examine trends in awake and anesthetized rats dosed with doxorubicin.

4.4.3.3.1. CE/UV: “Total” Glutathione

Total glutathione in liver dialysate samples was quantified by a CE/UV method previously established in our laboratory, as described in Section 4.3.4. This method was unable to detect both GSH and GSSG, due to on-capillary oxidation effects previously noted in our laboratory. As a result, all of the GSH was oxidized to GSSG, resulting in a measure of total glutathione through the concentration of GSSG. (The GSH and GSSG did not comigrate in the separation – any GSH present in the sample would be detected. However, none was detected in the samples.) The detection limit for GSSG was 500 nM, with a precision of 3% and linear range of 0.75 – 50 μM.

The experiments dosing doxorubicin to anesthetized animals through the microdialysis probe were repeated, but at a higher time resolution than the
metabonomics experiments. In these studies, two probes were implanted in the liver, and doxorubicin was dosed through Probe B after 3 hours of sampling basal dialysate. Samples were collected every 15 minutes, rather than every hour as in the metabonomics experiments. Three sets of experiments were performed – rats not dosed with doxorubicin, rats dosed with doxorubicin after 3 hours of basal dialysate collection and rats dosed with doxorubicin after 6 hours of basal dialysate collection. For all rats dosed with doxorubicin, Probe B was perfused with 2 mg/mL doxorubicin in Ringer’s solution for 60 minutes.

Figure 4.22 shows the results of the CE/UV analysis of the total glutathione (as measured by GSSG) in liver dialysate samples. The basal dialysate showed a high level of glutathione at the beginning of the experiment that fell and eventually leveled around 5 hours. Ideally, doxorubicin would be dosed after basal glutathione levels reached a steady state. However, the metabonomics experiments performed previously dosed doxorubicin 3 hours after the start of sampling. Therefore, doxorubicin was dosed at two time points in the experiment – at hour 3, to mimic the metabonomics experiments and at hour 6, after the total glutathione levels had steadied. In both dosing regimes, doxorubicin had no effect on total glutathione levels. There was no significant difference between glutathione levels in Probe A and B, as well as between experiment sets of dosed and control animals.

4.4.3.3.2. **LC/EC: Reduced and Oxidized Glutathione**

The CE/UV results suggested that total glutathione was not a good marker of oxidative stress in liver dialysate. It was hypothesized that measuring reduced and
Figure 4.22. Basal levels of total glutathione in liver dialysate over time. ■ represents samples from Probe A (control probe) and ○ represents samples from Probe B (dosed probe). A shows glutathione levels in basal liver dialysate (n=3). B shows glutathione levels in liver dialysate from rats dosed with doxorubicin at hour 3, as represented by a dashed line (n=3). C shows glutathione levels in liver dialysate from rats dosed with doxorubicin at hour 6, as represented by a dashed line (n=2).
oxidized glutathione separately would be a better gauge of the oxidative stress of the tissue, as the ratio of the reduced to oxidized form has been shown to indicate the oxidative stress level of the tissue.

One common method of thiol analysis is LC/EC with dual gold-mercury amalgam electrodes (Figure 4.23). In this method, the two electrodes are placed in series configuration relative to the flow from the LC column. The upstream electrode is used as a reducing, or “generator”, electrode. The potential applied to this electrode is sufficient to reduce any disulfide bonds to thiols. As thiols (either in the sample or from reduction at the first electrode) pass over the downstream or working electrode, the slightly positive potential is sufficient to form a thiol-mercury complex, creating a detectable signal. Thus, every compound is detected in the system as a thiol, but they can be quantified separately because they are separated chromatographically. One advantage of using gold-mercury electrodes over glassy carbon or plain gold for thiol detection is that a much lower potential can be used, resulting in lower background current, better selectivity and improved detection limits\(^\text{16}\). This setup also allows for the easier identification of thiols in the sample – simply turning off the upstream electrode will remove signal from the disulfides in the chromatogram.

An LC/EC method was developed to quantify GSH and GSSG in liver dialysate. The final method is described in Section 4.3.5. One important consideration for this type of analysis is the presence of dissolved oxygen in the mobile phase. The reducing electrode is set at such a strongly negative potential that
Figure 4.23. Dual-electrode LC/EC analysis of thiols with gold-mercury electrodes.
current is generated from dissolved oxygen in the mobile phase. This can create high background currents, as well as erratic baselines in the chromatogram. Consequently, the mobile phase was continuously sparged with helium and the mobile phase inlet tubing was composed of PEEK, rather than PTFE. PEEK is only slightly permeable to oxygen, and the use of PEEK tubing resulted in much lower and stable background currents for both electrodes (upstream: -2 μA, downstream: -1.5 nA). An oxygen scrubber was also placed in the helium gas flow. The precision of the method was 1-2%. The limit of detection (LOD) of GSH was 100 nM, with a limit of quantitation (LOQ) of 250 nM. The LOD of GSSG was 500 nM, with a LOQ of 900 nM. The dynamic range for GSH was 250 nM – 50 μM, and the dynamic range for GSSG was 900 nM – 90 μM. Sample stability is an important concern in thiol analysis, as thiols are easily oxidized, particularly in Ringer’s solution. Acidification of the sample stabilizes the thiol/disulfide ratio. As a result, all samples were diluted by 50% in the mobile phase buffer (0.12 M monochloroacetic acid, pH = 3.0) without the ion-pairing agent. The samples were shown to be stable for two days at -20°C and through one freeze-thaw cycle.

4.4.3.3. Anesthetized Rats

The effects of anesthesia on basal levels of GSH and GSSG were characterized, as shown in Figure 4.24. (In all the glutathione plots, the levels were normalized to the % delivery of the microdialysis probe.) GSH levels were high at the start of the experiment, but eventually dropped and leveled around 6 hours after sampling began (about 7 hours post-implantation). The initial high levels of GSH
**Figure 4.24.** Comparison of levels of GSH (A) and GSSG (B) in rats anesthetized with ketamine (n=3). ■ represents samples taken from Probe A, and ○ represents samples taken from Probe B.
may have been due to tissue trauma from probe implantation that eventually cleared as the wound healed. Basal GSSG levels were fairly stable but level over the course of sampling.

The effect of anesthesia on the basal levels of GSH and GSSG was examined by comparing results from rats anesthetized with ketamine and rats anesthetized with isoflurane inhalation. Figure 4.25 shows a comparison of GSH levels, and Figure 4.26 shows a comparison of GSSG levels. For both analytes, similar time trends were noted in GSH and GSSG levels between rats anesthetized by ketamine or isoflurane. A student’s $t$ test was performed to determine if any differences were statistically significant. None were found at 95% confidence.

4.4.3.3.4. Awake Rats

Comparison studies were then performed between glutathione levels in anesthetized and awake rats. The results are shown in Figure 4.27. Both GSH and GSSG are at low levels and stable at the start of sampling (24 hours post-probe implantation). GSSG was below the LOQ of the method in awake animals, with occasional quantifiable levels. GSH levels were low in awake animals, but still within the dynamic range of the method. It should be noted that neither GSH nor GSSG levels changed significantly over the course of three days of sampling, showing that immunological changes from probe implantation do not alter glutathione levels in the extracellular fluid of liver tissue.

Doxorubicin was dosed in awake animals at sampling hour 25 and the levels of both GSH and GSSG were monitored. The results are shown in Figure 4.28. No
Figure 4.25. Comparison of GSH levels from rats anesthetized with isoflurane and ketamine. ■ represents samples from Probe A of rats anesthetized with ketamine; □ represents samples from Probe A of rats anesthetized with isoflurane; ● represents samples from Probe B of rats anesthetized with ketamine; ○ represents samples from Probe B of rats anesthetized with isoflurane. A compares GSH levels in Probe A and B compares GSH levels in Probe B. C shows an average of GSH levels from each experiment group.
Figure 4.26. Comparison of GSSG levels from rats anesthetized with isoflurane and ketamine. ■ represents samples from Probe A of rats anesthetized with ketamine; □ represents samples from Probe A of rats anesthetized with isoflurane; ● represents samples from Probe B of rats anesthetized with ketamine; ○ represents samples from Probe B of rats anesthetized with isoflurane. A compares GSSG levels in Probe A and B compares GSSG levels in Probe B. C shows an average of GSSG levels from each experiment group.
Figure 4.27. Comparison of GSH (A) and GSSG (B) levels from rats anesthetized with isoﬂurane (n=3) and ketamine (n=3), as well as awake rats. The axis represents time past probe implantation ■ represents samples from Probe A of rats anesthetized with ketamine; □ represents samples from Probe A of rats anesthetized with isoﬂurane; ● represents samples from Probe B of rats anesthetized with ketamine; ○ represents samples from Probe B of rats anesthetized with isoﬂurane; ▲ represents samples from Probe A of awake rats; △ represents samples from Probe B of awake rats.
Figure 4.28. Comparison of GSH (A) and GSSG (B) levels from awake rats dosed and undosed with doxorubicin (at t=0). ▲ represents samples from Probe A of awake rats undosed with doxorubicin; Δ represents samples from Probe B of awake rats undosed with doxorubicin; ♦ represents samples from Probe A of awake rats dosed with doxorubicin; ◊ represents samples from Probe B of awake rats dosed with doxorubicin.
significant and consistent changes were noted as a result of the doxorubicin dose, either a decrease in GSH levels or increase in GSSG levels that would signify oxidative stress conditions. In these studies, it appears that GSH and GSSG are not good markers of the hepatic oxidative stress induced by doxorubicin, as they do not change in response to the dose. The dose to the tissue is large, sufficient to induce a large amount of oxidative stress in the liver tissue surrounding the probe. Therefore, glutathione levels could not be used to correlate to changes observed in metabolic profiles. Metabonomics studies were still performed in awake animals, as described below, but no statistical comparisons were made between glutathione levels and metabolite levels.

4.4.3.4. Dual Probe Experiments in Awake Animals

Doxorubicin was dosed in awake rats, removing the metabolic effects of anesthesia from the experiment so that the metabolic impact of the doxorubicin dose could be more clearly discerned. As in the anesthetized animal experiments, doxorubicin was dosed through probe B, with probe A used as a marker for natural changes in liver metabolism. In the awake animal experiments, sampling began 24 hours after probe implantation. Basal dialysate was collected from both probes for 24 hours. Doxorubicin was then dosed through probe B for one hour (2 mg/mL perfusate concentration), and dialysate was collected for an additional 48 hours post-dose.

The patterns observed in awake rats as a result of the doxorubicin dose are shown in Figure 4.29. As in Figure 4.20, both the undosed and dosed rats show a
Figure 4.29. Doxorubicin liver metabolic effects in awake rats. ● represents samples from Probe A of undosed rats, ○ represents samples from Probe B of undosed rats, ♦ represents samples from Probe A of dosed rats and ◊ represents samples from Probe B of dosed rats (dosed probes).
starting point in the top left corner of the scores plot. The undosed rat samples continue down the principal component 2 axis, as in Figure 4.20. However, in Figure 4.29, a separation was observed between probe A and B in the dosed rats. The probe A samples tracked along the principal component 2 axis, along with the samples from the undosed animals. In contrast, the probe B samples tracked along the principal component 1 axis, separating from the control animals and samples taken at probe A in the dosed animals at the time point that doxorubicin was dosed (and subsequent time points post-dose). Figure 4.30 shows the individual time courses of rats dosed with doxorubicin. Variance in response to the dose can be visualized from the plots.

These results are consistent with a localized response to the doxorubicin dose, in that the liver tissue metabolism around probe B was altered but the healthy tissue around probe A maintained metabolic trends consistent with normal healthy tissue. This is in stark contrast to the anesthetized animal results in Figure 4.20, where more global changes from doxorubicin were observed. This suggests that anesthesia alters the metabolic changes resulting from doxorubicin administration to the liver, causing it to have a more global effect. Loadings from principal component 1 show lysine, alanine, leucine, valine, glutamine, creatine and citrulline as significantly changing in response to the doxorubicin dose. Citrulline and glutamine changes were not observed in anesthetized animal experiments. Figure 4.31 shows a representative plot for how each metabolite changed individually over time, with lysine used as an example. All metabolites showed a similar time trend, increasing after the doxorubicin dose and then falling back to near-basal levels. Metabolites were fairly
Figure 4.30. Doxorubicin liver metabolic effects in awake rats. Plots A, B and C show the metabolic time course from individual rats dosed with doxorubicin. ♦ represents samples from Probe A of dosed rats and ◊ represents samples from Probe B of dosed rats (dosed probes).
Figure 4.31. Time course of lysine in liver dialysate. A shows the metabolite time course in undosed rats. ● represents samples from Probe A of undosed rats and ○ represents samples from Probe B of undosed rats. The dashed line indicates the dosage of doxorubicin through Probe B. ♦ represents samples from Probe A of dosed rats and ◊ represents samples from Probe B of dosed rats (dosed probes). B shows the metabolite time course in dosed rats.
stable in the control/undosed rats, as well as in the undosed microdialysis probes of the dosed rats. However, the administration of a doxorubicin dose induced an increase in the levels of the endogenous energy metabolite lysine (in this example). It should be noted that no significant differences were noted in the rate of clearance of doxorubicin between awake and anesthetized animals.

4.4.3.5. *Metabolic Effects of Doxorubicin*

The damage induced by doxorubicin is often measured by established biomarkers of oxidative stress, such as malondialdehyde to measure the extent of lipid peroxidation\textsuperscript{32}. From the studies of these single biomarkers, it is apparent that large changes occur in cellular small molecule levels in response to doxorubicin. Therefore, it was anticipated that changes in the small molecules detected in the liver extracellular fluid (primarily energy compounds) would be responsive to the doxorubicin dose. Doxorubicin has not been previously studied by metabonomics, although the metabolic consequences of the drug have been broached primarily by investigations on the activity of enzymes pertinent to cell respiration.

Doxorubicin has been demonstrated to cause a depletion of energy stores in the cell in a dose-dependent manner\textsuperscript{28,33}, in the forms of both ATP and phosphocreatine. In fact, it has been demonstrated that both ATP and phosphocreatine levels fall dramatically within one hour of doxorubicin dosage, and this depletion of energy stores can be sustained over a period of several days\textsuperscript{33}. This depletion occurs by several mechanisms, including the inhibition of creatine kinase and transporters of ATP from the mitochondria to the cytosol, preventing the transfer
of the high energy phosphate from ATP to creatine for longer term storage in the cellular energy reserves. The accumulation of creatine in response to the doxorubicin dose shows evidence that doxorubicin interferes in the energy stores of liver tissue. The creatine is not phosphorylated for energy storage, causing it to accumulate in the cell. This is reflected by the increase in creatine in the extracellular fluid levels, which was observed in both the anesthetized and awake animals.

Another noteworthy metabolic effect of doxorubicin is the change in levels of amino acids in the liver extracellular fluid, including lysine, alanine, glutamine, valine, leucine and isoleucine. This is likely due to doxorubicin-induced alterations in amino acid metabolism, specifically changes in aminotransferase levels and activity in the liver, including alanine aminotransferase and aspartate aminotransferase\textsuperscript{34}. The liver is the major site of amino acid catabolism in the body, because it is the first organ to receive amino acids after feeding via the portal vein. It is also the only organ that can eliminate nitrogen from the amino acids via the urea cycle. In general, amino acids are metabolized in the liver by transaminases, which remove the amino group to make a keto acid that can go on to participate in cellular respiration in either the liver itself or other tissues, such as muscle. (For example, the transamination of alanine produces pyruvate.) This is an important link between carbohydrate and amino acid metabolic pathways. The process is generally regulated by substrate supply and glucagon and cortisol levels. However, because doxorubicin alters the transamination pathways in the liver, significant changes in the levels of the amino acids listed above are measured. All of the amino acids listed above showed
an increase in level within one hour of the doxorubicin dose, and levels gradually returned to normal as the doxorubicin was eliminated from the liver.

Doxorubicin may further alter amino acid metabolism by interfering in urea cycle metabolic pathways, as judged by alterations in the levels of citrulline, glutamine and creatine. The urea cycle is used to eliminate nitrogen by the production of urea through a reaction cycle involving ornithine, citrulline and arginine. Glutamine feeds into the urea cycle by its ultimate conversion to the urea cycle intermediate argininosuccinate via conversion to glutamate. Creatine production is also metabolically linked to the urea cycle through the conversion of arginine to creatine.

Increases in β-hydroxybutyrate levels in the liver extracellular fluid were also observed in response to the doxorubicin dose. Previous studies have shown alterations in lipid metabolism in the liver in response to doxorubicin.32 Specifically in this study, increased lipid peroxidation products such as acetone were observed in the urine, indicating increased β-oxidation of fatty acids or an accumulation of acetyl-CoA by decreased metabolism of carbohydrates. Both acetone and β-hydroxybutyrate are ketone bodies. Because doxorubicin has been shown to increase acetone levels in previous studies, it seems logical that doxorubicin might also result in an increase in β-hydroxybutyrate levels by a similar mechanism to acetone. (The detection of acetone in these samples is precluded due to the speedvacuuming of the samples prior to NMR analysis, thus removing volatile components like acetone.)
Figures 4.20 and 4.29 demonstrate that doxorubicin induces different metabolic effects in the liver tissue of awake and anesthetized rats. Anesthetized rats experienced more of a global metabolic effect in the liver lobe, while awake animals experienced more of a localized metabolic effect. The reason for this is unclear at this time, although several possibilities can be considered. First, it is established in literature that doxorubicin-induced oxidative stress is dependent on tissue oxygen levels\textsuperscript{35}. Depleted oxygen levels will decrease the observed toxicity of the drug. Related to this, regions of the liver may experience differences in oxygen levels as a result of their proximity to the portal vein\textsuperscript{36,37}. Although no differences were observed between probes under basal metabolic conditions, it is possible that changes in blood flow and heart rate due to anesthesia could alter the liver’s metabolic response to the doxorubicin dose. Second, changes in liver enzyme levels and activity induced by the anesthesia could alter the liver’s response to the doxorubicin dose. It should be noted that the metabolites that changed in response to doxorubicin were similar in anesthetized and awake animals, indicating that anesthesia likely did not change the mechanism of doxorubicin hepatotoxicity dramatically. However, two new metabolites (citrulline and glutamine) were identified as significantly changing in response to the doxorubicin dose when awake animals were used. This would suggest that anesthesia can influence the identification of key metabolites in tissue-targeted metabonomics studies, perhaps through alterations in metabolic pathways caused by the anesthesia itself.
4.5. Conclusions

The purpose of the studies presented in this chapter was to illustrate the advantages of using tissue-targeted metabonomics to study a pathophysiological condition targeting the metabolism of a specific tissue, such as oxidative stress. Studies (of both ischemia/reperfusion and doxorubicin) were initially performed in the heart, but were ultimately moved to the liver, as the heart is less amenable to metabonomics studies. The biomarker of oxidative stress, glutathione, was characterized in both awake and anesthetized animals. Surprisingly, this biomarker did not respond to doxorubicin-induced oxidative stress (although evidence for oxidative stress induction by doxorubicin was shown in histological analysis of the liver). In future studies, however, the concurrent analysis of biomarkers with metabolic profiles could help to both interpret the metabolic changes induced by the metabolic stressor and to validate new biomarkers identified by the metabonomics analysis.

The primary metabolic stressor of this experiment, doxorubicin, was used to monitor perturbations in patterns of small molecules in the extracellular fluid of the liver in response to oxidative stress conditions. This chapter presents the first metabonomics study of doxorubicin-induced oxidative stress. Data indicated that multiple metabolic pathways were affected by the doxorubicin dose, including depletion of energy stores and alterations in amino acid and fatty acid metabolism. Thus, the utility of combining microdialysis sampling with NMR analysis is illustrated by these results: many metabolic pathways can be monitored
simultaneously to characterize conditions best observed through the metabolism of specific tissues rather than biofluids.

4.6. References


2(E)-nonenal derived mercapturic acids and 1H NMR metabonomics potential
biomarkers of chemically induced oxidative stress in the kidney? *Toxicol. 230*, 244-255.

PKCδ. *Circ. Res. 94*, e87-e96.

10. Mayr, M., Chung, Y.-L., Mayr, U., Yin, X., Ly, L., Troy, H., Fredericks, S.,


preconcentration of glutathione and glutathione disulfide using pH-mediated
base stacking for the analysis of microdialysis samples by capillary

chromatographic behavior of biological thiols and the corresponding


liver samples using liquid chromatography/electrochemistry. *J. Liq.
Chromatogr. 8*, 691-706.


243


5.1. Dissertation Overview

Tissue-targeted metabonomics was successfully developed as an analytical tool to probe localized metabolic changes over time in an organism. This is a novel approach to metabolic profiling experiments, offering a superior alternative to tissue biopsy sampling studies. Multiple samples can be taken from the same tissue site over time, making this approach uniquely suited to studying time trends. Most metabonomics studies simply focus on group classification, but following time trends opens the possibility of evaluating metabolic perturbations in a unique light. Additionally, this analytical approach allows for simultaneous time trend studies of multiple tissues, as microdialysis probes can be implanted in several locations in the body during a single experiment. Dialysate samples also are much cleaner than typical plasma or tissue samples. This improves sample stability by removing enzymatic degradation, improves throughput by reducing the sample preparation requirements and simplifies NMR and LC analysis.
The primary limitation of tissue-targeted metabonomics analytical methodology is throughput. NMR analysis time limits the number of samples that can be analyzed per rat, with nearly 2 hours per sample required on the magnet used in these experiments, a 600 MHz spectrometer. Dialysate samples are dilute, requiring more signal averaging than biofluid samples like urine. Also, dialysate samples are small in volume. This also makes NMR analysis challenging. Because samples are already dilute, it is not feasible to dilute the samples to the appropriate volume. In these studies, tube inserts were used rather than a specialized capillary probe. Although this approach gives more flexibility in the magnet that can be used (no special probe is required), the fill factor is low and more signal averaging is required.

In addition (and related) to throughput, tissue-targeted metabonomics also faces limitations because of the n value required to make confident conclusions about the biological processes underlying the changes observed in the metabolic profiles. Animal-to-animal variability is a challenge in any in vivo experiment, and although some of this variability can be removed through data normalization, it is impossible to completely remove it. One of the best ways to separate animal-to-animal variability from an actual response to a metabolic perturbation is to increase the number of animals studied. An adequate number of samples must be analyzed to sufficiently characterize the response to the metabolic perturbation. However, the number of replicates must be balanced with the analysis time required.
While tissue-targeted metabonomics offers some interesting possibilities for the study of metabolic changes in vivo, this technology is still developing and has obstacles to be overcome. Throughput of the method must be increased, allowing for more samples to be analyzed and more animals to be studied. This in turn would improve the quality of the data set analyzed and allow more significant conclusions to be made about biological changes observed.

Metabonomics studies have presented an interesting challenge for analytical chemists. There is a switch in focus from the traditional pursuit of selectivity to the new focus on detector nonselectivity. With this comes a new set of methodological problems to tackle, mostly centering on the data set complexity. Tissue-targeted metabonomics fills a void in the metabonomics community by offering the possibility of continuous sampling of tissue-specific metabolism while still maintaining the profiling/nonselective detection scheme. This dissertation presents in-depth studies of the analytical methods required to perform these investigations and shows the potential for monitoring a tissue-specific metabolic perturbation that develops and changes over a period of time.

5.2. Summary by Section

5.2.1. Tissue-Targeted Metabonomics: Analytical Considerations

The analytical methodology for tissue-targeted metabonomics studies was optimized. Microdialysis sampling and subsequent sample preparation was examined. It was determined that speedvacuuming the samples to remove water and
reconstituting in a deuterated solution improved water suppression in the NMR experiment, allowed for sample concentration and dramatically improved sample stability. As with most choices made in analytical methods, speedvacuuming is not without a sacrifice. Volatile solutes are lost, and sample composition changes slightly. However, these changes appear to be consistent among samples. Much larger changes in sample composition result from directly freezing the dialysate samples. Thus, the improvements achieved with speedvacuuming were deemed to outweigh the cost of slight changes in sample composition.

Much of the important details in the analytical methodology for tissue-targeted metabonomics lies in the data treatment and analysis. The number of variables is quite large and complex statistical techniques are utilized. As with all statistics, the quality of the data set analyzed will determine the quality of the conclusions that can be reached after applying statistics. The goal of the data treatment is to minimize the noise in the data set from variation unrelated to the metabolic perturbation. It was determined for liver microdialysis experiments that the NMR analysis and probe extraction efficiency are primary contributors to the noise in the data. However, significant noise sources may vary depending on the experiment, and it is important to establish these prior to experimentation.

5.2.2. *Tissue-Targeted Metabonomics: Biological Considerations*

With such complex data sets, it is important to determine the changes expected in the metabolic profiles of tissue extracellular fluid under basal conditions. Many factors contribute to metabolic changes under normal metabolism, some from
the animal itself and some as artifacts of the experimental conditions. These studies showed that anesthesia in particular causes significant alterations in basal metabolism. While this is not unexpected, it is important to characterize because its use is unavoidable in microdialysis experiments. While some microdialysis experiments maintain the rat under anesthesia for the duration of sampling, the results suggest that tissue-targeted metabonomics experiments should be confined to awake animals, at least in the study of liver metabolism.

Another important characteristic of in vivo experiments is natural cycles in metabolism due to cycles such as circadian rhythms, feeding, activity and estrus cycles (this can be avoided by using males). In these studies, cycles were observed in animal activity. However, these were not reflected in the metabolic profiles of liver dialysate. It was concluded that at the time scale being observed and the tissue extracellular fluid being sampled for these experiments, endogenous rhythms in metabolism did not consistently or significantly contribute to variance in the metabolic profiles. This dissertation presents studies of endogenous rhythms in metabolic profiles of tissue extracellular fluid.

5.2.3. Tissue-Targeted Metabonomics Application: Oxidative Stress

There are many potential applications for tissue-targeted metabonomics methodology. This dissertation presents the study of hepatic oxidative stress, as induced locally by the perfusion of doxorubicin through the microdialysis probe. The microdialysis setup in these experiments provided a unique opportunity to differentiate basal changes in metabolic conditions from those induced by
doxorubicin by the implantation of two probes in the liver lobe. This approach could be utilized in other microdialysis experiments, particularly those performed in homogenous tissues.

This study was the first time doxorubicin was investigated by metabonomics. Studies have been performed previously characterizing the effects of doxorubicin on liver tissue homogenate. However, these studies have centered on enzyme levels and activity with very little or no attention paid to the levels of endogenous metabolites. This dissertation shows that doxorubicin has been directly associated with changes in the levels of creatine, lysine, alanine, glutamine, valine, leucine, isoleucine, citrulline and β-hydroxybutyrate in the rat liver. The simultaneous changes in these metabolites are also presented, providing another dimension to the study of doxorubicin effects in the liver over time. The results obtained in this study do not contradict the existing literature on the changes in enzyme levels and activity from a doxorubicin systemic dose (no previous studies have been performed on the direct perfusion of doxorubicin through the liver). It should be emphasized that these results are preliminary and are used to illustrate the possibilities that tissue-targeted metabonomics methodology offers. The data presented represents observations from the principal components analysis performed on the dialysate metabolic profiles. Experiments with additional animals would be needed to make any biological claims on the effects of doxorubicin in the liver with confidence.

It was hypothesized that monitoring an established marker of oxidative stress would aid in the identification and interpretation of relevant changes in the metabolic
profile of liver extracellular fluid. Thus, glutathione (GSH) and glutathione disulfide (GSSG), as established biomarkers of oxidative stress, were chosen to perform this study. Unfortunately, GSH and GSSG was not an ideal oxidative stress marker in this particular experimental setup, as no dose-dependent response was noted. Although this approach was not successful here, it could still be applied in other studies.

5.3. Future Directions

5.3.1. Tissue-Targeted Metabonomics: Analytical Considerations

As stated previously, the primary limitation to tissue-targeted metabonomics methodology is throughput. The poor sensitivity of NMR combined with dilute, small volume dialysate samples requires long analysis times in the NMR experiment. Future work on analytical methodology for tissue-targeted metabonomics should focus on increasing throughput. One way to do this is by improving detection limits and/or sensitivity. The NMR analysis parameters could be explored that aim to decrease the number of spectra required for coaddition, but this may prove difficult. Studies could also be performed using capillary probes, which could allow for greater concentration of the sample and thus lower analysis time. Another type of probe that could improve detection limits is a cryoprobe. A cryoprobe, with electrical components such as the rf coil and preamplifier cooled with liquid helium, yields improved sensitivity and detection limits through the reduction of thermal noise. Larger modifications to the method to improve detection limits could include moving to a higher field magnet. Because signal to noise (S/N) scales with $B_0^{3/2}$, increasing
the field strength of the magnet will increase S/N. For example, if a 900 MHz magnet was utilized with the same number of coadded spectra, the S/N would increase by almost a factor of two. This could be used to either increase the S/N obtained in the same amount of time as the acquisition parameters utilized in this research or be used to increase throughput by achieving the same S/N in a shorter period of time.

Detection limits could also be improved by using a different analytical method. Metabonomics studies using LC/MS$^{1,2}$ and CE/MS$^3$ have grown in popularity in recent years, as discussed in Chapter 1. The exploration of these techniques as detection schemes for dialysate samples could prove advantageous for tissue-targeted metabonomics studies. Both mass spectrometry techniques have improved detection limits when compared to NMR detection, albeit with a new set of analytical challenges. The metabolites detected by these techniques may differ from NMR due to some molecules not ionizing efficiently. Also, mass spectrometry is generally more amenable to automation than NMR detection. While run times for these techniques may still be somewhat high (an hour or more), they will likely be lower than the NMR analysis time required for dialysate samples. Additionally, throughput would be improved by opening the possibility for automation, an option not usually found on NMR spectrometers for the analysis of small volume dialysate samples. However, it should be noted that the high salt content in dialysate samples may present an obstacle for MS studies by creating ionization problems.

Sensitivity is not the only limitation on the NMR analysis of dialysate samples. Dynamic range is also an important constraint on the method.
of metabolite resonances places limitations on the number and concentration of metabolites that can be detected. Due to overlap, even if sensitivity is improved by any of the mechanisms discussed above, metabolites at lower concentrations may still not be detected. Metabolites at higher concentrations, typically energy metabolites, will prevent the detection of the lower intensity resonances they overlap at any sensitivity level. This can be improved slightly by moving to higher field magnets to increase signal dispersion. Two dimensional NMR experiments can also improve signal dispersion but are not practical for the routine analysis of dialysate samples.

The limitations on sensitivity and dynamic range faced by tissue-targeted metabonomics using $^1$H-NMR analysis may be challenging to improve upon. With this in mind, the use of class-specific analytical methods should be explored and compared to tissue-targeted metabonomics methods. Class-specific methods, such as the profiling of endogenous thiols by gold-mercury electrodes to characterize oxidative stress, retain some of the nonselectivity of metabonomics detection schemes, thus still allowing for biomarker discovery. However, these analyses are more focused than conventional metabonomics approaches, so sensitivity and dynamic range will likely be improved. These methodologies may represent the best compromise between specific, highly sensitive assays of single biomarkers and the very nonselective but poorly sensitive metabonomics analytical approaches.

5.3.2. Tissue-Targeted Metabonomics: Biological Considerations

The studies presented in this research focused on metabolic processes observed in extracellular fluid in liver tissue. Each tissue has its own distinct
metabolic processes, which could be affected differently by endogenous rhythms. Therefore, it is important that each new tissue studied be fully characterized for basal metabolic rhythms before studying the effects of a metabolic stressor.

The effects of different anesthetic agents should also be examined for their effects on basal metabolic profiles. The agents studied in this dissertation were those readily available, typically used and approved for use in the laboratory. However, other anesthetic agents are available that could affect the metabolic profiles in different ways. The optimum anesthetic agent used for tissue-targeted metabonomics studies may also be tissue-dependent.

5.3.3. Tissue-Targeted Metabonomics Application: Oxidative Stress

The results of these studies suggest that tissue-targeted metabonomics with NMR detection is most suited to the study of metabolic conditions that impact the levels of energy metabolites in the extracellular fluid of the tissue of interest. These compounds tend to be higher in concentration and are thus detected by the NMR analysis. There are many potential applications of tissue-targeted metabonomics methodology.

One potential application is the study of diabetes, metabolic syndrome and obesity. All of these conditions, which can be interconnected, alter energy metabolism in the body\textsuperscript{4,5}. These diseases are increasing in prevalence in the United States and many questions remain regarding the development and treatment of these conditions. Multiple tissues are involved, including the muscle, brain, liver, pancreas and kidney. Each tissue is uniquely impacted by changes in levels of or response to
metabolic regulatory hormones such as insulin. Additionally, energy metabolites are involved in intertissue communication regarding the metabolic status of the organism\textsuperscript{4}. In this way, tissues impact other tissues’ response to metabolic disorders via metabolite levels in the extracellular matrix. Time trends in energy metabolism can also be used as predictors for the development of these metabolic disorders. Alterations in circadian rhythms of adipocyte metabolism have been linked to the development of metabolic syndrome\textsuperscript{5}. These factors combined make these metabolic conditions an ideal application for tissue-targeted metabonomics.

Another potential application of tissue-targeted metabonomics is the study of sepsis\textsuperscript{6}. In many cases, it is difficult to detect the presence of this infection until it is fairly well established. Previous studies in our research have shown that infection causes alterations in the metabolic profiles of liver tissue extracellular fluid (Section 2.4.5). Additionally, other studies have also demonstrated the alteration in energy metabolism observed during sepsis, but these have only focused on one or two analytes\textsuperscript{7}. Tissue-targeted metabonomics has the unique potential to identify new markers of sepsis so that it can be detected and therefore treated more quickly.

5.4. References


spectrometry and multivariate statistical analysis shows promise for the
detection of drug metabolites in biological fluids. *Rapid Commun. Mass
Spectrom.* **17**, 2632-2638.

(2003). Quantitative metabolome analysis using capillary electrophoresis

communication involved in energy homeostasis: potential therapeutic targets

rhythms and the regulation of metabolic tissue function and energy


A. (2000). Evolution of lactate/pyruvate and arterial ketone body ratios in the
early course of catecholamine-treated septic shock. *Crit. Care Med.* **28**, 114-
119.