Development of Analytical Methods Coupled to Microdialysis Sampling for Studying Biomarkers of Oxidative Stress

By

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Abstract

This research describes the development of analytical methods based upon separation techniques coupled to microdialysis sampling for the detection of potential biomarkers of oxidative stress. The research focused on two biomarkers, glutathione and the hydroxyl radical (‘OH). Microdialysis sampling was utilized for the continuous monitoring of these biomarkers in rat liver and heart. Oxidative stress was induced, causing the generation of reactive oxygen species (ROS). It was hypothesized that ROS generation would increase in biological systems due to induced oxidative stress.

In one investigation, a capillary electrophoresis (CE) method with ultra-violet (UV) detection was developed employing pH-mediated stacking, an on-column preconcentration technique, to detect two forms of glutathione, reduced glutathione (GSH) and oxidized glutathione (GSSG), simultaneously in microdialysates. The detection limits necessary for measuring GSH and GSSG in microdialysates with UV detection could not be achieved without the use of pH-mediated stacking. The method was utilized to monitor glutathione in microdialysates from rat liver that was subjected to chemically induced oxidative stress using adriamycin. GSSG concentration was found to increase from the basal concentration as a result of oxidative stress, suggesting increased antioxidant activities during oxidative stress.

For the indirect detection of ‘OH in microdialysates, a trapping agent, 4-hydroxybenzoic acid (4-HBA), was employed. Indirect determination involved the
trapping of ‘OH by 4-HBA and then detection of the radical adduct, 3,4-dihydroxybenzoic acid (3,4-DHBA), by the developed CE-UV method. In vitro studies of ‘OH generation systems (e.g., UV photolysis of H₂O₂, the Fenton reaction, and hypoxanthine/xanthine oxidase systems with and without superoxide dismutase) demonstrated the capability of 4-HBA to trap ‘OH and the ability of the CE-UV method to detect 3,4-DHBA in the reaction products.

Finally, an in vivo investigation was performed using CE-UV and high performance liquid chromatography (HPLC) method with electrochemical (EC) detection to detect 3,4-DHBA in microdialysates from rat heart where 4-HBA was delivered through the microdialysis probe to trap ‘OH. Increased generation of 3,4-DHBA was observed in microdialysates of rat heart subjected to physically induced oxidative stress (ischemia-reperfusion), suggesting increased generation of ROS during oxidative stress.
Dedication

To My Mother (1952 - 2007) & Father
Acknowledgement

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Table of Contents

Chapter 1: Introduction to Oxidative Stress, Microdialysis, CE, and HPLC

1.1 Oxidative Stress ................................................................. 1
  1.1.1 Biomarkers of Oxidative Stress ................................. 1
  1.1.2 Sources of Reactive Oxygen Species ......................... 3
  1.1.3 Ischemia-Reperfusion ................................................. 6
  1.1.4 Antioxidant Defenses ................................................. 8
    1.1.4.1 Enzymatic Antioxidants .................................... 10
    1.1.4.2 Non-Enzymatic Antioxidants ............................... 11
1.2 Microdialysis ................................................................. 14
  1.2.1 Principles ............................................................... 15
  1.2.2 Probe Design ........................................................... 15
  1.2.3 Extraction Efficiency ................................................. 17
  1.2.4 Tissue Damage and Response ...................................... 19
1.3 General Analytical Needs with Microdialysis for Measuring Oxidative Stress ................................................. 20
1.4 Capillary Electrophoresis .................................................. 22
  1.4.1 Basic Concepts .......................................................... 23
  1.4.2 Detection Modes ......................................................... 32
    1.4.2.1 UV Detection .................................................... 32
    1.4.2.2 Fluorescence Detection ....................................... 34
    1.4.2.3 Electrochemical Detection .................................. 35
    1.4.2.4 Mass Spectrometric Detection ............................... 37
  1.4.3 On-Column Preconcentration Techniques ....................... 37
    1.4.3.1 Field-Amplified Sample Stacking .......................... 37
    1.4.3.2 pH-Mediated Stacking ....................................... 38
    1.4.3.3 Transient Isotachophoresis ................................ 39
    1.4.3.4 Transient Pseudo Isotachophoresis ........................ 42
1.5 High Performance Liquid Chromatography ............................... 42
  1.5.1 Basic Concepts .......................................................... 42
  1.5.2 Detection Modes ........................................................ 46
1.6 Scope of the Dissertation .................................................. 47
1.7 References ........................................................................ 49

Chapter 2: Development of a CE-UV Method Coupled to Microdialysis for the Simultaneous Determination of GSH and GSSG

2.1 Introduction ...................................................................... 56
2.2 Analytical Methods for the Determination of GSH and GSSG in
| Chapter 3: 4-Hydroxybenzoic Acid as a Trapping Agent for Hydroxyl Radicals – An *In Vitro* Evaluation by CE-UV Coupled to Microdialysis |
| 3.1 Introduction ................................................................. 101 |
| 3.2 Analytical Methods for the Detection of Hydroxyl Radicals in Biological Samples ................................................................. 103 |
| 3.3 Specific Aims ................................................................. 110 |
| 3.4 Experimental ................................................................. 110 |
| 3.4.1 Materials ................................................................. 110 |
| 3.4.2 Sample Preparation ................................................................. 112 |
| 3.4.3 CE System ................................................................. 113 |
| 3.4.4 Microdialysis Sampling ................................................................. 114 |
| 3.4.4.1 Apparatus ................................................................. 114 |
| 3.4.4.2 Probe Fabrication ................................................................. 115 |
| 3.4.5 *In Vitro* Hydroxyl Radical Generation Systems ................................................................. 115 |
| 3.4.5.1 UV Photolysis of H₂O₂ ................................................................. 116 |
| 3.4.5.2 Fenton Reaction ................................................................. 116 |
| 3.4.5.3 Hypoxanthine/Xanthine Oxidase System ................................................................. 116 |
| 3.4.5.4 Hypoxanthine/Xanthine Oxidase System in the Presence of Superoxide Dismutase ................................................................. 118 |
| 3.4.6 Microsomal Metabolism of 4-HBA ................................................................. 119 |
3.5 Results and Discussion ................................................................. 120
  3.5.1 CE-UV Method ................................................................. 120
  3.5.2 Method Validation ............................................................ 124
  3.5.3 Stability of 3,4-DHBA and 4-HBA ....................................... 126
  3.5.4 Hydroxyl Radical Generation Systems ............................... 126
    3.5.4.1 UV Photolysis of $H_2O_2$ ........................................... 126
    3.5.4.2 Fenton Reaction ....................................................... 128
    3.5.4.3 Hypoxanthine/Xanthine Oxidase Systems ..................... 128
  3.5.5 Microsomal Metabolism of 4-HBA ..................................... 135
3.6 Conclusion ................................................................................. 138
3.7 References ................................................................................. 139

### Chapter 4: Monitoring Hydroxyl Radicals as 3,4-Dihydroxybenzoic Acid in Microdialysis Samples

4.1 Introduction .............................................................................. 143
4.2 Specific Aims ........................................................................... 144
4.3 Experimental ........................................................................... 144
  4.3.1 Materials ............................................................................ 144
  4.3.2 Sample Preparation ............................................................ 145
  4.3.3 CE-UV System ................................................................. 145
  4.3.4 Microdialysis Sampling ....................................................... 145
    4.3.4.1 Apparatus ................................................................. 145
    4.3.4.2 Probe Fabrication ....................................................... 146
    4.3.4.3 Surgical Procedure ..................................................... 146
4.4 Results and Discussion ........................................................... 149
  4.4.1 Detection of 3,4-DHBA in Heart Microdialysates by CE-UV .... 149
  4.4.2 Detection of 3,4-DHBA in Heart Microdialysates by HPLC-EC-UV ...................................................................................... 159
4.5 Conclusion ................................................................................. 176
4.6 References ................................................................................. 179

### Chapter 5: Summary and Future Work

5.1 Overview of the Dissertation .................................................... 181
5.2 Summary .................................................................................. 182
  5.2.1 Detection of Glutathione by CE-UV in Rat Liver Microdialysates . 182
  5.2.2 Indirect Determination of ‘OH by CE-UV .............................. 183
  5.2.3 Monitoring of ‘OH in Rat Heart Microdialysates .................... 183
5.3 Future Work ............................................................................. 184
  5.3.1 Detection of GSH and GSSG by CE-UV and CLC-EC ............ 184
  5.3.2 CE-EC for the Indirect Determination of ‘OH ....................... 185
5.3.3 HPLC-EC for Oxidative Stress Investigations .......................... 186
5.3.4 Monitoring of GSH, GSSG, and \( \cdot \mathrm{OH} \) ........................................ 187
5.4 References ...................................................................................... 188
# List of Figures

<p>| Figure 1.1 | An illustration of pathways for the generation of ROS during ischemia-reperfusion | 7 |
| Figure 1.2 | Hypoxanthine/xanthine oxidase system | 9 |
| Figure 1.3 | Structure of glutathione and glutathione disulfide | 12 |
| Figure 1.4 | Glutathione redox cycle | 13 |
| Figure 1.5 | Illustration of the principle of microdialysis | 16 |
| Figure 1.6 | Basic components of a CE-UV system | 24 |
| Figure 1.7 | EOF in normal polarity CE | 27 |
| Figure 1.8 | Reverse EOF in CE | 29 |
| Figure 1.9 | Electrochemical cell with a decoupler for CE with EC detection | 36 |
| Figure 1.10 | pH-mediated base stacking | 40 |
| Figure 1.11 | Schematic of a HPLC system | 43 |
| Figure 2.1 | Glutamyl cycle | 57 |
| Figure 2.2 | Transport and metabolism of glutathione | 60 |
| Figure 2.3 | Reduction of adriamycin and generation of superoxide | 62 |
| Figure 2.4 | GSH and GSSG detection without pH-mediated stacking | 75 |
| Figure 2.5 | Optimization of injection length with ammonium buffer system | 78 |
| Figure 2.6 | Stability study of GSH | 81 |
| Figure 2.7 | Representative electropherogram of liver microdialysate | 83 |
| Figure 2.8 | Electropherograms of unspiked and spiked liver microdialysates | 84 |
| Figure 2.9 | Plots of in vivo NNF experiments | 85 |
| Figure 2.10 | Time profile of extracellular GSH and GSSG in liver microdialysates | 86 |
| Figure 2.11 | Change in extracellular GSSG in liver microdialysates of rats subjected to ADR induced oxidative stress | 90 |
| Figure 2.12 | Extracellular GSSG in liver microdialysates: Control experiments | 90 |
| Figure 2.13 | GSH detection as GSSG | 93 |
| Figure 3.1 | Reactions of DMPO, PBN, and POBN with ‘OH | 105 |
| Figure 3.2 | Hydroxylation of 2-HBA | 106 |
| Figure 3.3 | Hydroxylation of 4-HBA | 108 |
| Figure 3.4 | Microdialysis sampling process employed for the radical trapping experiment | 111 |
| Figure 3.5 | In vitro microdialysis setup for the HX/XO system | 117 |
| Figure 3.6 | Detection of 3,4-DHBA by CE-UV | 122 |
| Figure 3.7 | Separation of 3,4-DHBA, 4-HBA, and potential interferents | 123 |
| Figure 3.8 | Analysis of 3,4-DHBA from 2.5 µL sample in vial | 125 |</p>
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Derivatizing agents: Chromophores</td>
<td>64</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Derivatizing agents: Fluorophores</td>
<td>64</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>HPLC methods for the analysis of glutathione in microdialysates</td>
<td>66</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>CE methods for the analysis of glutathione in microdialysates</td>
<td>66</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Separation efficiency as a function of injection ratio</td>
<td>79</td>
</tr>
<tr>
<td>Table 2.6</td>
<td>GSH and GSSG in liver microdialysates of rats</td>
<td>87</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Effect of injection length</td>
<td>121</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Peak height of 3,4-DHBA vs. volume of sample in vial</td>
<td>125</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction to Oxidative Stress, Microdialysis, CE, and HPLC

1.1 Oxidative Stress

1.1.1 Biomarkers of Oxidative Stress

Biological markers (i.e., biomarkers) are measurable cellular, biochemical, or molecular changes which occur in biological samples, such as fluids, cells, or tissues [1]. More recently, the National Institutes of Health (NIH) has proposed a broader definition of biomarkers: “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention” [2]. Monitoring biomarkers in biological systems is a powerful approach for predicting and understanding the cause, diagnosis, progression, and prevention of diseases, or pathological conditions [1].

Oxidative stress in living systems refers to the imbalance between the formation of reactive oxygen species (ROS) and antioxidant defenses [3]. This imbalance occurs when there is an increase in production of ROS and/or a decrease in antioxidant activities. The most common ROS in biological systems are superoxide radicals (O$_2^-$), hydroxyl radicals (·OH), hydrogen peroxide (H$_2$O$_2$), and peroxyl radicals (ROO$^\cdot$) [4]. These ROS can react with biomolecules including lipids, deoxyribonucleic acid (DNA), and proteins. As a result of oxidation reactions (such as lipid peroxidation or DNA and protein oxidation), disintegration and modification of biomolecules may take place. The typical end products of lipid peroxidation are
malondialdehyde (MDA) and 4-hydroxynonenal whose presence may be an indicator of oxidative stress [4]. DNA damage is a result of oxidation of DNA bases by ‘OH, usually forming 8-oxoguanine (8oxoG) or 8-hydroxy-2′-deoxyguanosine (8OHdG) [5]. 8oxoG is an oxidized guanine base product, and 8OHdG is the modified nucleoside, where a 2-deoxyribose sugar is attached to the oxidized guanine base. Protein modifications include fragmentation of the protein, oxidation of amino acid side chains, and protein carbonyl generation [6, 7]. On the other hand, antioxidant defense mechanisms act in a preventive manner through the scavenging of ROS before they attack biomolecules. However, some ROS may escape in healthy systems and cause damage to biomolecules, as mentioned above.

Oxidative stress is involved in the development and progression of cardiovascular diseases [8]. According to the American Heart Association’s published data, cardiovascular disease is the number one killer in the United States [9, 10]. In 2003, total deaths from cardiovascular disease were 0.911 million, whereas the number of deaths from cancer were 0.555 million. Other pathophysiological conditions, such as Alzheimer’s disease, Parkinson’s disease, and aging, are also believed to be related to an increased amount of ROS in living systems [11, 12]. Hence, monitoring the concentrations of ROS, oxidative damage products of biomolecules, and antioxidants will help to determine the extent of oxidative injury, follow the progression of these diseases, and take preventive measures against them. In this regard, ‘OH, MDA, 8OHdG, and glutathione (an endogenous antioxidant, GSH) have been widely used as potential biomarkers of oxidative stress [3, 13].
1.1.2 Sources of Reactive Oxygen Species

Superoxide (O$_2^-$) is the precursor of ROS, such as ´OH and H$_2$O$_2$, in biological systems. It is formed during normal metabolic processes as a result of a one electron reduction of molecular oxygen (O$_2$) (Eq. 1.1). It is estimated that 1 to 2% of the O$_2$ consumed by an organism escapes as O$_2^-$ [14, 15].

\[
O_2 + e^- \rightarrow O_2^-(1.1)
\]

Mitochondria are the main source of O$_2^-$ generation. The electron leakage of O$_2$ in the mitochondrial electron transport chain (METC) leads to the formation of O$_2^-$. The METC consists of six electron carriers which are associated in the inner mitochondrial membrane. These are NADH-coenzyme Q reductase (complex I), succinate-coenzyme Q reductase (complex II), coenzyme Q (CoQ or ubiquinone), coenzyme Q-cytochrome c reductase (complex III), cytochrome c, and cytochrome c oxidase (complex IV), with $E_0^-$ ranging from -0.320 to +0.380 V [16]. Through these protein complexes, a series of redox reactions passes electrons from NADH to O$_2$ to produce H$_2$O (Eq. 1.2). The free energy released from the reactions involving the electron transfer is utilized for the ATP synthesis. Although the majority of electrons are used for the reduction of O$_2$ to form H$_2$O, some of the electrons in the chain may leak out to O$_2$ to generate O$_2^-$ ($E_0^-' = -0.160$ V) [17].
Another source of \( \text{O}_2^- \) generation is phagocytosis. In this process, leukocytes, such as neutrophils and macrophages, become activated, and as a result, \( \text{O}_2 \) consumption is increased [11, 18]. NADPH oxidase from leukocytes, which is a membrane-bound enzyme, catalyzes the one electron reduction of \( \text{O}_2 \) to form \( \text{O}_2^- \) (Eq. 1.3). This may be converted to other ROS, as discussed later in this section. Leukocytes use these reactive species as a part of the defense mechanisms against microorganism.

\[
\text{NADPH} + \text{O}_2 \xrightarrow{\text{Oxidase}} \text{NADP}^+ + \text{O}_2^- + \text{H}^+ \quad (1.3)
\]

Superoxide can also be formed by cytochrome P-450, a ubiquitous enzyme in living systems [19]. Cytochrome P-450, which is located in the endoplasmic reticulum membrane, is responsible for the metabolism of toxic substances. During metabolism, it reduces \( \text{O}_2 \) to \( \text{O}_2^- \), where NADPH acts as an electron donor [20]. The other common sources of \( \text{O}_2^- \) generation are the oxidation of various endogenous and exogenous compounds in the cytoplasm [18]. These compounds include flavins, hemoglobin, thiols, hydroquinone, and \( \text{Fe}^{2+} \) complexes [18].
In biological systems, $\text{O}_2^-$ generation is always accompanied by the production of $\text{H}_2\text{O}_2$ through enzymatic pathways. Superoxide dismutase (SOD), a metalloprotein enzyme, catalyzes the conversion of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and $\text{O}_2$ (Eqs. 1.4 and 1.5). SOD is present in the mitochondria, cytosol, and extracellular spaces [14]. Additional enzymes, that convert $\text{O}_2$ to $\text{H}_2\text{O}_2$ by a two electron reduction, include acyl-CoA oxidase, xanthine oxidase (XO), glutathione oxidase, and monoamine oxidase [18].

\[
\begin{align*}
\text{O}_2^- + \text{SOD}[\text{Cu}^{2+}\text{ state} ] & \rightarrow \text{O}_2 + \text{SOD}[\text{Cu}^+\text{ state} ] \\
\text{O}_2 + \text{SOD}[\text{Cu}^+\text{ state} ] + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{SOD}[\text{Cu}^{2+}\text{ state} ]
\end{align*}
\]

$\text{H}_2\text{O}_2$ becomes biologically significant when it is converted to $\cdot\text{OH}$, which is the most unstable ROS with a half life of $\sim 10^{-9}$ s [21]. Metal complexes, such as metalloproteins, iron citrate, or iron-nucleotides, are responsible for generating $\cdot\text{OH}$ from $\text{H}_2\text{O}_2$ according to the following reactions (Eqs. 1.6 and 1.7).

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH} \quad \text{(Fenton reaction)} \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \rightarrow \text{HO}^- + \cdot\text{OH} + \text{O}_2 \quad \text{(Haber-Weiss reaction)}
\end{align*}
\]
‘OH can also be generated from the decomposition of peroxinitrous acid at the physiological pH of 7.4 (Eq. 1.8). Peroxinitrous acid is the protonated form of peroxinitrite (ONOO-) with a pKₐ of 6.8. ONOO- is the reaction product of O₂⁻ and nitric oxide (NO). NO is a free radical, and it is produced biologically by nitric oxidase and activated phagocytic cells [22].

\[
\text{HOONO} \rightarrow \cdot \text{OH} + \text{NO}_2
\]  

(1.8)

1.1.3 Ischemia-Reperfusion

Ischemia-reperfusion results in oxidative stress in biological systems. Ischemia is the restriction of blood flow to the tissue or a portion of the tissue, and reperfusion is the restoration of blood flow after a short period of ischemia. The most common ischemic conditions are heart attack and stroke. During ischemia and reperfusion, generation of ROS is elevated due to the increased electron leakage from the altered METC, increased XO activity, and activation of polymorphonuclear leukocytes [23]. Through these mechanisms, first O₂⁻ is formed, and then other ROS can be formed from O₂⁻, as described in Section 1.1.2. Figure 1.1 shows the pathways for ROS generation during ischemia-reperfusion and antioxidant activities [24, 25].

The supply of NADH into the METC system is disrupted due to the lack of O₂ during ischemia. This situation leaves the mitochondrial carrier in the reduced state. As a result, more electron leakage to residual O₂ entrapped in the inner mitochondrial membrane may result in the generation of O₂⁻[26].
Figure 1.1. An illustration of pathways for the generation of ROS during ischemia-reperfusion.
During ischemia, adenosine triphosphate (ATP) is degraded to adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, inosine, and finally to hypoxanthine (HX) [4, 23]. Also, in the event of ischemia, xanthine dehydrogenase (XDH) is transformed to xanthine oxidase due to proteolysis by a Ca\(^{2+}\) dependent protease [27]. Thus, accumulation of HX and XO occurs. In the presence of O\(_2\), XO catalyzes the conversion of HX to xanthine, and it can further catalyze the conversion of xanthine to uric acid (UA). During reperfusion, when O\(_2\) is re-introduced after ischemia, the activities of HX and XO are increased. As a result, xanthine, UA, H\(_2\)O\(_2\), and O\(_2^-\) are formed (Figure 1.2). This HX/XO system has been hypothesized to be the most important mechanism of oxidative injury during reperfusion [23, 28, 29].

Additional production of ROS is due to the activation of phagocytic cells during ischemia-reperfusion. Activation involves circulating neutrophils or macrophages into the affected tissue areas to aid in the repair [30, 31]. These cells may or may not be the major source of ROS since it is dependent on how soon they arrive in the affected tissues [26].

1.1.4 Antioxidant Defenses

The antioxidant defense mechanisms in biological systems are both enzymatic and non-enzymatic. Antioxidants scavenge ROS or prevent their generation. Oxygen free radicals are very reactive with reaction rate constants in the range of \(10^4\) to \(10^9\) M\(^{-1}\)s\(^{-1}\) [32]. Hence, the antioxidants need to have similar rate constants to effectively
Figure 1.2. Hypoxanthine/xanthine oxidase system.
reduce the formation of ROS. The common enzymatic antioxidants are SOD, catalase, and glutathione peroxidase (GSHPx), and the non-enzymatic antioxidants include GSH, vitamin C, vitamin E, and ubiquinone (coenzyme Q) [32]. Some of the enzymatic and non-enzymatic antioxidants are discussed in the following two sections.

1.1.4.1 Enzymatic Antioxidants

As mentioned in Section 1.1.2, SOD catalyzes the conversion of \( O_2^- \) to \( H_2O_2 \). SOD is a Cu-Zn or Mn containing enzyme, and \( O_2^- \) is its substrate [33]. Cu-Zn SOD, a dimer, is found mainly in cytosol, and Mn SOD, a tetramer, is found in the mitochondria. Both have similar rates of reaction, approximately \( 2 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) [4, 33].

Catalase is a heme containing protein with four subunits. It decomposes \( H_2O_2 \) into \( H_2O \) and \( O_2 \) at a rate of \( 10^8 \text{ M}^{-1}\text{s}^{-1} \) [32]. Catalase is mostly located in organelles called peroxisomes. Large amounts of catalase are found in the liver while small amounts are located in the brain and heart [32, 33].

Finally, GSHPx (a selenoenzyme) catalyzes the degradation of peroxides, such as \( H_2O_2 \) and organic peroxides (ROOH), using GSH as a cofactor. The decomposition rates of \( H_2O_2 \) and ROOH by GSHPx are \( 1.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \) and 1 to 3 \( \times 10^7 \text{ M}^{-1}\text{s}^{-1} \), respectively [32]. This tetrameric enzyme is located in the mitochondria, in peroxisomes, in the endoplasmic reticulum, and also in the cytoplasm [32]. GSHPx
acts effectively at low concentrations of $H_2O_2$ ($\sim 10^{-6}$ M), while catalase functions effectively at high $H_2O_2$ concentrations.

1.1.4.2 Non-Enzymatic Antioxidants

GSH is a tripeptide of glutamic acid, cysteine, and glycine (Figure 1.3). This endogenous low molecular-weight thiol compound is found intracellularly in the concentration range of 1 to 10 mM [34]. It is also present in the extracellular space in micromolar concentrations. In the liver, the intracellular concentration of GSH is at its the highest ($\sim 10$ mM) [35, 36]. GSH plays an important role in scavenging ROS. This sulfhydryl (-SH) antioxidant reacts with $O_2^-$ and $OH$ directly by radical transfer reactions (Eqs. 1.9 and 1.10). In the presence of GSHPx, GSH converts $H_2O_2$ to $H_2O$ with the generation of glutathione disulfide (GSSG), a dimer of GSH, where two molecules of GSH are attached through a disulfide linkage (-S-S-), as shown in Figure 1.3. GSSG is converted to GSH by glutathione reductase (GSHR) with the loss of NADPH (Figure 1.4). This interconversion of GSH and GSSG is termed the GSH redox cycle. The relative concentrations of GSH and GSSG in biological systems may be used to determine the extent of oxidative stress since GSSG is formed as a result of antioxidant activity of GSH. A higher ratio of GSSG to GSH (GSSG/GSH) would indicate an increased level of oxidative stress [37, 38].
Figure 1.3. Structure of glutathione (A) and glutathione disulfide (B).
Figure 1.4. Glutathione redox cycle.
Besides GSH, other small molecule antioxidants, such as vitamin E and vitamin C, play a significant role by removing ROS from biological systems [33]. The -OH group in the vitamin E structure is responsible for its antioxidant activity. Vitamin C (ascorbic acid) is converted to dehydroascorbate while also reducing ROS. Diets, that include vegetable oils and fruits, are rich in vitamin E and vitamin C which may aid in removing ROS from biological systems. Ubiquinone is an endogenous antioxidant, and it is present in biological membranes and lipoproteins [39].

1.2 Microdialysis

Microdialysis is a useful in vivo sampling tool for the continuous monitoring of analytes of interest in biological samples [40]. It is used to sample from the extracellular fluid (ECF) of living systems. Both endogenous and exogenous substances can be monitored by this technique. Microdialysis has become a routine sampling method in neuroscience, pharmacokinetic, and drug deposition studies [41, 42]. Initially, this technique was applied for the investigation of neurotransmitters release in the brain [40]. Subsequently, it has been successfully applied for the determination of analytes in tissues and fluids, such as liver, heart, muscle, skin, bile, kidney, tumor, and blood [41].
1.2.1 Principles

Microdialysis sampling is performed by implanting a probe with a semipermeable membrane at the site of interest. The microdialysis probe is perfused with a solution of similar ionic strength and pH as the ECF. The semipermeable membrane of the probe is a short length hollow fiber typically made from polycarbonate, regenerated cellulose, or polyacrylonitrile (PAN). These membranes usually have a molecular weight cut-off (MWCO) between 6 to 42 kDa [43]. Mass transport occurs through the membrane due to the concentration gradient between the ECF and perfusate. The analytes below the MWCO of the membrane diffuse in or out of the probe due to the concentration gradient. Larger molecules above the MWCO of the membrane, such as proteins, are unable to diffuse through the probe membrane. Figure 1.5 illustrates the principle of microdialysis. Microdialysis sampling is termed as a recovery experiment when the analyte concentration in the ECF is higher than that in perfusate. Conversely, in a delivery experiment, the analyte concentration in the ECF is less than in the perfusate.

1.2.2 Probe Design

Probe design or geometry is dependent upon the sampling site in which the probe is implanted [43]. Different types of microdialysis probes have been designed, such as the rigid cannula probe, flexible probe, linear probe, and shunt or by-pass probe [43]. The rigid cannula, flexible, and shunt probes are used for sampling in the brain, blood vessel, and bile duct, respectively. The linear probe is designed for
Figure 1.5. Illustration of the principle of microdialysis.
sampling at peripheral sites such as the skin, muscle, liver, gastrointestinal (GI) tract, and heart.

1.2.3 Extraction Efficiency

Microdialysis sampling is a dynamic sampling method, where the perfusate is continuously passed through the probe [44, 45]. Microdialysate is collected from a non-equilibrium system. The concentration of the analyte of interest in microdialysate is less than the actual concentration of that analyte in the ECF in case of recovery experiment. Hence, the extraction efficiency (EE) is used to calculate the actual concentration of an analyte in the ECF. The EE of a microdialysis probe is the change in concentration of the perfusate relative to the concentration gradient from outside to inside the probe and is defined as:

\[
EE = \frac{(C_p - C_d)}{(C_p - C_s)}
\]  

(1.11)

where \(C_s\) is the known concentration of the analyte of interest in the external medium (e.g., ECF), \(C_p\) is the initial concentration of the analyte in the perfusate, and \(C_d\) is the concentration of the analyte in the microdialysate [46].

Extraction efficiency is dependent upon several parameters: perfusion rate, temperature, concentration, molecular weight, molecular shape, analyte ionic state, diffusion coefficient of the analyte, chemical interactions with the membrane, and
blood flow [40, 44, 47, 48]. Among those parameters, it has been shown that perfusion rate and temperature have a major influence on recovery [44].

Microdialysis probe calibration is performed by determining the EE of a particular probe under experimental conditions. Both in vivo and in vitro probe calibrations are performed. The most commonly used calibration methods are an in vitro or vivo delivery experiment, in vitro recovery experiment, in vitro or in vivo no net flux (NNF) experiment, and retrodialysis (an internal standard delivery method).

Delivery experiments involve the use of an analyte of known concentration (C_p) in the perfusate. Diffusion of the analyte occurs from the perfusate into the external medium. After determining C_d, EE_D can be calculated using Eq. 1.12.

\[
EE_D = \frac{(C_p - C_d)/C_p}{(where \ C_s = 0 \ for \ delivery \ experiment)}
\]

For the recovery experiment, the perfusate does not contain the analyte of interest (C_p = 0), and its concentration in the external medium is known. In this case, the diffusion of the analyte occurs from the external medium to the perfusate, and EE_R is calculated from Eq. 1.13.

\[
EE_R = \frac{C_d}{C_s} \quad (where \ C_p = 0 \ for \ recovery \ experiment)
\]
NNF experiments involve the determination of the mass transport of analyte through the probe as a function of analyte concentration in the perfusate. If the concentration of the analyte is higher in the perfusate than in the external medium, then diffusion of analyte occurs from the perfusate into the external medium. The analyte diffuses into the perfusate when the concentration is lower in the perfusate than in the external medium. There is no net flux of the analyte when the concentration of the analyte in the perfusate is equal to the concentration in the external medium. $E_{NNF}$ can be determined from the slope of $(C_d - C_p)$ vs $C_p$ plot. The value of $C_p$ at $(C_d - C_p) = 0$ is equal to the concentration of analyte in the external medium [46]. In the case of calibration by retrodialysis, an exogenous internal standard is used in the perfusate [49, 50]. This is a delivery experiment, where it is assumed that the recovery of the analyte is equal to the delivery of the internal standard. This method requires an internal standard that behaves similarly to the analyte of interest. No significant differences have been observed between an in vitro calibration by recovery, delivery, or NNF experiments for various analytes [46]. Also, no differences were determined between an in vivo delivery and in vivo NNF experiment performed in muscle tissue [46].

1.2.4 Tissue Damage and Response

A microdialysis probe is implanted into the target tissue of an experimental animal by an invasive surgical procedure. As a result, tissue damage is unavoidable during probe implantation. However, damage may be minimized using a probe of
smaller dimensions [45, 51]. Also, the type of probe used determines the extent of
damage in the tissue. For example, implantation of a linear probe causes less cell
damage than a cannula probe [51]. During probe implantation, a slight hemorrhage
occurs at the point of probe entry and in some cases, at the tissue adjacent to the
probe membrane [51]. It is known that the severity of surgical invasion is dependent
upon the tissue site. Probe implantation into the liver is less invasive to the animal
than in the heart or brain [51].

As a result of probe implantation, inflammatory response was observed at the
site of probe implantation [51, 52]. Responses were found to be time dependent, as
concluded from reported histopathological examinations of tissue slices of liver [51].
Immediately after surgery, no disruption of tissue was observed. However, ~ 6 hours
after surgery, a moderate infiltration of polymorphonuclear leukocytes (PMN) was
observed adjacent to the implanted probe [51, 53]. After 40 hours of implantation,
PMN and macrophages surrounded the probe. However, the delivery experiment
showed no change in the delivery of the test compound for ~ 24 hours after
implantation [53].

1.3 General Analytical Needs with Microdialysis for Measuring Oxidative Stress

Microdialysis has been used to monitor potential biomarkers of oxidative
stress (8-OHdG, MDA, GSH, and ‘OH, as mentioned in Section 1.1.1) in the liver,
brain, and heart of experimental animals [25, 54-57]. Generally, evaluation of
oxidative stress is performed by determining these biomarkers in biological samples
In order to measure the concentration of analytes of interest in microdialysis samples, a suitable analytical technique is required.

The commonly used separation technique, high performance liquid chromatography (HPLC), has been widely used to study potential biomarkers of oxidative stress in conjunction with microdialysis sampling [54, 55, 57-64]. These biomarkers included 8-OHdG [58, 59], MDA [60, 61], GSH [55, 62], and OH [57, 63, 64]. HPLC requires sample volumes in the range of 10 to 25 µL for a single analysis.

There are only a few reports on the application of capillary electrophoresis (CE) with microdialysis to study oxidative stress [65, 66]. CE is a powerful separation technique used to study a wide range of analytes, such as amino acids, nucleic acids, neurotransmitters, and pharmaceuticals [67]. Microdialysis generates microliter volumes of sample, and CE is an ideal technique to couple to microdialysis, as it consumes only a small amount of sample (1 to 10 nL). It is possible to make an injection into the CE system from ~1.5 µL sample volume. Therefore, multiple sample injections can be made from the same sample volume. Another advantage of coupling CE to microdialysis is increased temporal resolution. The temporal resolution is dependent upon the sampling interval, e.g., the smaller the sampling interval the higher the temporal resolution. A high temporal resolution will help to determine how early changes in the concentration of biomarkers occur within a small time frame during oxidative events, such as ischemia and reperfusion. As a result, it would provide information about the extent of oxidative stress over time.
It is necessary to analyze multiple biomarkers in order to get a better understanding of what is happening in vivo as a result of oxidative stress. Therefore, the analysis of multiple biomarkers within the same sample volume (e.g., 10 µL of sample) is required in order to establish a correlation between them. In this regard, CE will be very useful because of small sample volume consumption. Different types of biomarkers give different information regarding oxidative stress. For example, determining 8-OHdG and MDA concentrations provides information on the extent of oxidative damage in DNA and lipids, respectively. Measurement of an endogenous antioxidant (e.g., GSH) and ROS (e.g., ‘OH), gives information regarding antioxidant activity and ROS generation in biological systems, respectively.

1.4 Capillary Electrophoresis

CE is a separation technique which is used to analyze charged species. Separation is achieved in a capillary filled with a background electrolyte (BGE) by applying an electric field. Hjerten introduced the capillary form of electrophoresis in 1967 [68]. Further developments of this technique were reported by Virtanen [69], Mikkers et al. [70], and Jorgenson et al. [71]. In 1984, the introduction of micellar electrokinetic capillary chromatography (MEKC) by Terabe et al. enabled the analysis of neutral analytes by CE [72]. Applications of CE are expanding day by day. A variety of analytes have been separated successfully using CE including nucleic acids, proteins, pharmaceuticals, chiral molecules, inorganic ions, and organic ions [67]. Some advantages of CE over conventional separation techniques, such as
HPLC, include high separation efficiency, increased resolution, and smaller sample volume requirements.

1.4.1 Basic Concepts

The basic components of a CE system are shown in Figure 1.6. These are a polyimide coated fused silica capillary, a high voltage power supply, two BGE reservoirs, a detector, and a data acquisition system. As shown in Figure 1.6, both capillary ends and electrodes (anode and cathode) are immersed in BGE vials. An on-column (capillary) ultra-violet (UV) absorbance detector is shown in Figure 1.6.

In order to perform a CE separation, the sample is injected from the inlet end into a capillary filled with BGE. The most commonly used sample introduction techniques are hydrostatic, hydrodynamic, and electrokinetic injection [73]. Following sample introduction, a high voltage is applied across the capillary. Charged species start migrating to the electrode of opposite charge under the influence of an electric field. Ions are separated due to differences in migration that arise from the differences in charge to size ratio of the charged species.

In an applied electric field, the accelerating force \( F_e \) of an ion can be written as in Eq. 1.14:

\[
F_e = qE \tag{1.14}
\]

where \( q \) is the charge on the ion and \( E \) is the field strength (i.e., applied voltage (V) per unit length (L), \( E = V/L \)) [74, 75]. In the solution, the drag or retarding force \( F_d \)
Figure 1.6. Basic components of a CE-UV system.
of the ion is the product of the frictional coefficient (f) and migration or electrophoretic velocity ($v_{ep}$) (Eq. 1.15).

$$F_d = f v_{ep} \quad (1.15)$$

The direction of the two forces is opposite. Within a short time ($10^{-11}$ second) [74, 75], both forces are counterbalanced and ionic species begin to migrate with a steady state velocity, as described in Eqs. 1.16 - 1.18:

$$F_e = F_d \quad (1.16)$$

$$v_{ep} = qE/f \quad (1.17)$$

$$\mu_{ep} = v_{ep}/E = q/f \quad (1.18)$$

where $\mu_{ep}$ is the electrophoretic mobility. From the Stokes equation, it is known that $f = 6\pi\eta R$ (where $\eta$ is the viscosity of the medium, and $R$ is the hydrodynamic radius of the ion). Therefore, the electrophoretic mobility can be equated as follows.

$$\mu_{ep} = q/6\pi\eta R \quad (1.19)$$

From the above equations, it is evident that the larger the radius or size of the ions (the lower the charge to size ratio), the lower the electrophoretic mobility.
In CE, the simultaneous analysis of cations, anions, and neutrals is possible in a single run due to the presence of the electroosmotic flow (EOF). The function of EOF in CE is similar to that of a pump in HPLC. EOF provides the bulk flow in the capillary and originates from the ionizable silanol groups (SiO\(^{-}\)) on the inner capillary wall at a pH above 3 (the pI of the fused silica is \(\sim 1.5\)) [67, 76]. The negatively charged silanol groups form an electrical double layer (rigid layer and diffuse layer) with the cations of the BGE (Figure 1.7). Upon application of an electric field across the capillary, cations in the diffuse layer migrate toward the cathode, carrying the bulk flow of solution with them. A key characteristic of EOF is that it produces a flat flow profile. As a result, reduced analyte dispersion occurs, leading to high peak efficiencies. This flow pattern in CE is different from HPLC, where a parabolic flow profile is generated by an external pump. The EOF can be defined by the following equations (Eqs. 1.20 - 1.22):

\[
\nu_{\text{eof}} = \frac{\varepsilon \zeta}{4 \pi \eta} E \quad (1.20)
\]
\[
\mu_{\text{eof}} = \frac{\varepsilon \zeta}{4 \pi \eta} \quad (1.21)
\]
\[
\nu_{\text{eof}} = \mu_{\text{eof}} E \quad (1.22)
\]

where \(\nu_{\text{eof}}\) = electroosmotic velocity, \(\mu_{\text{eof}}\) = electroosmotic mobility, \(\varepsilon\) = dielectric constant, and \(\zeta\) = zeta potential. Viscosity is temperature dependent, and the zeta potential increases with increasing surface charge. Any changes in EOF are due to variations in \(\varepsilon, \eta, \zeta,\) and \(E\) [77]. In the presence of EOF, the apparent migration or
Figure 1.7. EOF in normal polarity CE.
electrophoretic velocity \( (v_{\text{app}}) \) of a charged analyte is the sum of the electrophoretic and electroosmotic velocity, as shown in the following equations:

\[
\mu_{\text{app}} = \mu_{\text{ep}} + \mu_{\text{eof}} \quad (1.23)
\]

\[
v_{\text{app}} = (\mu_{\text{ep}} + \mu_{\text{eof}})E \quad (1.24)
\]

where \( \mu_{\text{app}} \) is the apparent mobility of an ion. However, neutral molecules migrate with the EOF since they do not have their own mobilities.

In normal CE mode, cations migrate towards the cathode (detection end) and anions to the anode (sample introduction end) (Figure 1.6). In this mode, the EOF is also in the direction of the cathode. In reverse CE mode, the EOF is reversed by treating the capillary wall with a cationic surfactant (e.g., tetradecyltrimethylammonium bromide (TTAB)) (Figure 1.8), and the separation is performed by applying a voltage of negative polarity. As a result, anions and the EOF move in the same direction (toward the anode, the detection end). The migration order in normal and reverse CE modes are cations \(<\) neutrals \(<\) anions and anions \(<\) neutrals \(<\) cations, respectively.

Due to the flat flow profile produced in CE, high separation efficiencies are obtained. The separation efficiency can be expressed in terms of theoretical plates \((N)\), and is summarized by Eqs. 1.25, 1.26, and 1.27:
Figure 1.8. Reverse EOF in CE.
\[ N = 16 \left( \frac{1}{W} \right)^2 \]  

where \( N \) is the number of theoretical plates, \( W \) is the peak width, \( W_{1/2} \) is the peak width at half of the peak height, and \( D \) is the diffusion coefficient of the analyte [77]. The \( N \) value in CE generally lies in the range of 100,000 to 200,000. On the other hand, for HPLC, this value is 5,000 to 20,000 [78]. Resolution (\( R_s \)) in CE can be calculated from the following equations:

\[ R_s = \frac{\left( t_{R2} - t_{R1} \right)}{\sqrt{W_2 + W_1}} \]  

(1.28)

\[ R_s = \frac{1}{4} \left( \mu_{ep2} - \mu_{ep1} \right) \left\{ \frac{V}{2D(\mu_{avg} - \mu_{eof})} \right\}^{1/2} \]  

(1.29)

where \( t_{R2} \) = migration time of peak 2, \( t_{R1} \) = migration time of peak 1, \( W_2 \) = width of peak 2, \( W_1 \) = width of peak 1, \( \mu_{ep2} \) = electrophoretic mobility of analyte 2, \( \mu_{ep1} \) = electrophoretic mobility of analyte 1, and \( \mu_{avg} \) = average electrophoretic mobility of analytes 1 and 2 [77, 78]. A value equal to or greater than 1.5 is necessary for baseline resolution between two peaks [78].

Peak efficiency and resolution can be affected by peak or band broadening in CE. There are three parameters which are found to be responsible for peak broadening. These are the length of the sample plug, longitudinal diffusion (which occurs during electromigration), and adsorption of the analyte on the capillary wall.
Large volume sample injections are not compatible with CE, as this leads to band broadening. In fact, small sample volumes (1-2% of the capillary volume) should be injected for improved peak shape [73]. Adsorption of an analyte can occur as a result of analyte-wall interactions, and this leads to peak broadening. Injection of a high ionic strength sample, such as microdialysates, also leads to peak broadening [73]. A high ionic strength sample zone exhibits a higher conductivity and a lower field relative to the rest of the capillary. As a result, analytes migrate very slowly in the sample zone, resulting in band broadening or destacking. To minimize the destacking effect, the ratio of BGE ionic strength to sample ionic strength has to be optimized. It is recommended that the sample ionic strength should be at least 100-fold lower than the BGE ionic strength [73].

Another common phenomenon in CE, which may lead to band broadening, is Ohmic or Joule heating. This is a direct result of the applied separation voltage across the capillary. The heat generated inside the capillary causes temperature changes due to ineffective heat dissipation and temperature gradients across the capillary [76, 77, 80]. The temperature gradient (ΔT) across the capillary is equated as:

\[ ΔT = 0.24 \frac{Wr^2}{4K} \] (1.30)

where \( W \) = power, \( r \) = capillary radius, and \( K \) = thermal conductivity. The detrimental effect of Joule heating can be minimized by performing the separation in a capillary of smaller inner diameter (ID) at an optimum separation voltage determined from an Ohm’s plot (a plot of applied voltage and current).
The mode of CE discussed in this section is commonly known as capillary zone electrophoresis (CZE). Another mode of CE is micellar electrokinetic capillary chromatography (MEKC or MECC). It was first reported by S. Terabe et al. [72]. In MEKC, a surfactant (a pseudo stationary phase) is added to the BGE. The surfactant (e.g., sodium dodecyl sulfate, SDS), which has a nonpolar moiety in the interior and a charged polar group at the surface, forms micelles above its critical micelle concentration (CMC). Separation is the result of partition of the analyte between the BGE and micelle. The mobility of the analyte is dependent on its electrophoretic mobility (based on the mass to charge ratio), the partition coefficient between the BGE and the micelle, the micelle’s mobility, and EOF [67]. Both neutral and charged species with similar mobilities can be separated with MEKC.

1.4.2 Detection Modes

Detection modes in CE have been reviewed extensively [81-85]. The choice of a detector for a particular analysis is dependent on the type or nature of the analytes, their selectivity, and the detection limit required. The commonly used detection techniques in CE are discussed in the following sections.

1.4.2.1 UV Detection

UV detection is widely used, due to its ease of operation and incorporation with CE. In CE, UV detection is employed on-column by removing a small length (~2 mm) of the polyimide coating and directing a light beam through it. Analytes, that
possess chromophores, may be detected by UV. However, the sensitivity of CE with UV detection (CE-UV) is low. This can be attributed to the short path length of light (25 to 75 µm, the ID of the capillary). The typical concentration detection limit in CE-UV is in the range of 1x10^{-3} to 1x10^{-8} M [86]. According to Beer’s law, the absorbance is dependent on the molar absorptivity, the concentration of the analyte, and the path length of light. Hence, the common strategies for sensitivity enhancement for UV detection are path length extension and large volume sample loading. Also, both sensitivity and selectivity, which are dependent upon the wavelength of absorption, may be increased by derivatization of the analyte with a UV labeling agent of higher molar absorptivity.

Different approaches have been taken to increase the path length of the light in a capillary. A bubble cell was made into the capillary at the detection window in order to increase the path length [87]. A “Z” bend capillary was used in another approach [88]. In a different approach, the path length was increased by measuring the absorbance perpendicular along the length of a capillary [89]. Unfortunately, all of these approaches resulted in band broadening and were difficult to apply, although sensitivity did improve slightly with the increased path length. In a different design, multiple reflections were made into the capillary to increase the path length. To accomplish this, the capillary wall was coated with metallic silver to form silver mirrors in both sides of capillary [90]. In this case, a laser beam was used as the UV source.
A large volume sample injection into the capillary is an effective way to improve the sensitivity in CE-UV. Employing on-column preconcentration techniques in CE, which are discussed in Section 1.4.3, make injection of a large sample volume (higher mass of analyte) possible [56, 91, 92]. However, the amount of sample to be injected needs to be optimized without any loss of separation efficiency and/or resolution.

1.4.2.2 Fluorescence Detection

Fluorescence is a selective detection technique. This detection scheme has low detection limits, and it has been applied for the analysis of biological samples [86, 93]. Not all analytes have native fluorescence, and in most cases, analytes of interest are derivatized with a fluorescent tag (fluorophore). Derivatization can be accomplished by various approaches, such as pre-column, on-line, on-column, and post-column derivatization [94]. On-line fluorescence detection is performed using either a tungsten or xenon arc lamp as an excitation source. In laser-induced fluorescence (LIF) detection, the high intensity monochromatic laser line is used instead of a conventional excitation source. CE with LIF detection (CE-LIF) provides concentration detection limits of $10^{-10}$ to $10^{-16}$ M [86]. The use of CE-LIF has been reviewed by several authors [95, 96].
1.4.2.3 Electrochemical Detection

Electrochemical (EC) detection is suited to the analysis of electroactive compounds. EC methodologies in CE have been reviewed by different authors [83-85, 97]. There are three modes of EC detection in CE which are conductimetric, potentiometric, and amperometric detection. The conductimetric and potentiometric detectors measure any changes in conductivity and potential, respectively, between two electrodes in an electrolyte. These are universal detectors. However, they have several disadvantages including poor detection limits and relatively high sample volume requirements [98]. Amperometric detection is the most commonly used EC detection system in CE. This mode of detection is highly selective and sensitive [83, 86]. Typical detection limits for EC detection are in the range of $1 \times 10^{-5}$ - $1 \times 10^{-11}$ M. Many electroactive analytes of biological importance can be detected in CE without any need for derivatization. Amperometric detection may be performed in three different modes: such as end-column, off-column, and on-column [99]. The major problem with using amperometric detection is the isolation of the separation voltage from the detection circuit. The isolation of the separation current is performed using a decoupler (e.g., Nafion® and cellulose acetate decoupler) in the separation capillary (Figure 1.9) [97, 100].
Figure 1.9. Electrochemical cell with a decoupler for CE with EC detection. RE = reference electrode and WE = working electrode.
1.4.2.4 Mass Spectrometric Detection

Mass spectrometric (MS) detection offers structural information. Coupling of CE with MS detection (CE-MS) is a growing area of research. Electrospray ionization (ESI) is widely used ionization technique in CE-MS [73]. CE-MS is a very informative technique for the analysis of complex samples, where analyte identification based on the migration time is problematic. Detection limits of $1 \times 10^{-5}$ - $1 \times 10^{-11}$ M have been achieved with CE-MS [86].

1.4.3 On-Column Preconcentration Techniques

Preconcentration of analytes is necessary in order to achieve lower limits of detection (LOD), especially when UV detection is employed in CE. The main goal of all on-column preconcentration techniques for CE is to maintain or create a low conductivity sample zone of high velocity. In this case, a large sample volume (higher mass of analyte) injection may be possible without any loss of efficiency. The commonly used on-column preconcentration techniques are discussed in the following sections.

1.4.3.1 Field-Amplified Sample Stacking

The field-amplified sample stacking (FASS) technique is based upon the amplification of the electric field in a sample zone. In CE with FASS, the low ionic strength sample is injected electrokinetically into the capillary, which is filled with a high ionic strength BGE. The low ionic strength sample is characterized either as
having a high electric field strength, low conductivity, or a high velocity zone. To lower the ionic strength of the sample, it may be diluted with low ionic strength BGE or water. After sample injection, separation is accomplished with the high ionic strength BGE using the optimum separation voltage. Analytes migrate faster in the low ionic strength sample zone to the interface between the sample zone and the high conductivity BGE. At the interface, analytes slow down to form a narrow band, and as a result, peak height and efficiency are increased [90, 101].

1.4.3.2 pH-Mediated Stacking

Lunte et al. developed pH-mediated stacking methods in order to analyze high ionic strength samples, such as microdialysis samples [91, 92]. It is an alternative to the FASS technique, as the high ionic strength sample can be injected directly without prior dilution or any sample pretreatment. In this technique, a high ionic strength sample zone is neutralized on-column to a low ionic strength sample zone by either a strong acid or base. There are two types of pH-mediated stacking: acid stacking for the analysis of cations and base stacking for the analysis of anions. Various kinds of anions and cations have been investigated successfully using pH-mediated stacking [56, 91, 102]. A sensitivity enhancement of 66-fold has been reported for several anions using CE-UV with pH-mediated stacking [91]. Separation efficiency was also found to increase with an increase in ionic strength of the sample matrix using CE-UV with pH-mediated stacking [56, 91].
In acid stacking, the BGE is made from the salt of a weak acid, such as sodium acetate ($\text{CH}_3\text{COONa}$). Here, sample injection is followed by an acid injection, such as hydrochloric acid (HCl). Both the sample and HCl injection are made electrokinetically. Anions in the sample zone (e.g., chloride ion) are displaced by BGE anions. Hydrogen ions ($\text{H}^+$) migrate through the sample zone and neutralize BGE anions (e.g., acetate ions), creating a low conductivity zone. In the low conductivity sample zone, analytes move faster and stack at the interface of the sample zone and BGE [91, 92].

Base stacking is performed in CE with reverse EOF. In this technique, the BGE is made from the salt of a weak base, such as ammonium chloride ($\text{NH}_4\text{Cl}$). Sample injection is followed by base (NaOH) injection. The ammonium ions ($\text{NH}_4^+$) displace the cations (e.g., $\text{Na}^+$) in the sample zone, and $\text{OH}^-$ neutralizes $\text{NH}_4^+$ in the sample zone. This creates a zone of low conductivity. Figure 1.10 illustrates the principle of pH-mediated base stacking [56, 91].

1.4.3.3 Transient Isotachophoresis

In isotachophoresis (ITP), two buffer systems (i.e., a heterogeneous buffer system) are used [103-106]. One electrolyte is known as the leading electrolyte (LE), and the other is called the terminating electrolyte (TE). The LE has a higher mobility than the TE, and the mobility of the analyte of interest is between these two electrolytes. The sample is injected between the LE and TE, where field strength is higher in the TE zone and lower in the LE zone. When a separation voltage is applied,
Figure 1.10. pH-mediated base stacking. (1) Capillary filled with BGE consisting of the salt of a weak base (NH$_4^+$). (2) Electrokinetic injection (EKI) of sample; migration of cations and anions under the electric field. (3) EKI of NaOH; titration of cation (NH$_4^+$) by OH$^-$, creation of a narrow zone of analyte. (4) Replacement of the NaOH vial with the BGE vial; migration of stacked analytes towards the detection window. Adapted and modified from reference [56].
analytes are separated within the sample zone into narrow bands according to their mobilities. Analytes lagging into the TE zone will accelerate, and analytes will decelerate if they migrate into the LE zone. This phenomenon also happens between sample bands within the sample zone. At steady state, the leading, analyte, and terminating zones move with the same velocity (as isotachophoresis means). The success of ITP is dependent upon the proper choice of the two electrolytes.

In transient isotachophoresis (tITP), initially an on-column preconcentration is performed and achieved using the ITP principle, and subsequently the separation proceeds as in the CZE mode of separation. It is called tITP because the migration mode transforms into ITP from CZE in a single operation [104, 105].

tITP can be achieved using single capillary and double capillaries (connected on-line) [103]. tITP can be induced in a single capillary by manipulating the sample composition, called self-induced tITP. In this instance, one component (natural or added) in the sample acts as the leading ion, and the co-ion in the BGE acts as the terminating ion [103]. In the double capillaries system, initially ITP is performed in one capillary, and subsequently, zone electrophoresis is carried out in the other capillary. The major disadvantage of using ITP in CZE is the difficulty in acquiring a suitable leading or terminating ion for the analysis of a particular analyte of interest [103].
1.4.3.4 Transient Pseudo Isotachophoresis

To overcome the limitation of tITP, transient pseudo isotachophoresis (tpITP; also called acetonitrile stacking) was proposed [107]. The sample matrix in this technique consists of acetonitrile and a salt mixture. A large volume of sample is injected into the capillary filled with BGE. After sample injection, the sample vial is replaced with the BGE vial to perform the separation. The salt ion (e.g., chloride ion) in the sample zone acts as the leading ion, and acetonitrile acts as the terminating ion (pseudo terminating ion). The presence of acetonitrile in the sample zone produces a low conductivity zone (high field strength). As a result, analyze velocities are increased, and stacking is achieved. In fact, the ITP effect is very brief, and the separation begins as soon as acetonitrile enters the BGE zone [107].

1.5 High Performance Liquid Chromatography

HPLC is the most widely used separation technique in bioanalytical applications. It is the major separation tool because of its robustness. A wide variety of substances, including amino acids, proteins, nucleic acids, and drugs, can be separated by HPLC [108, 109].

1.5.1 Basic Concepts

The basic components of a HPLC system are a pump, an injector, a column, a detector, and a data acquisition system (Figure 1.11) [78]. In chromatography, substances in the sample are separated from each other due to their distribution
Figure 1.11. Schematic of a HPLC system.
between the mobile phase and the stationary phase to varying degrees. The sample is carried by the mobile phase through the solid stationary phase, and a continuous flow of the mobile phase is achieved using a high pressure pump. The typical mobile phase flow rate is 0.1 to 1 mL/min. The mobile phase can be uniform in composition (isocratic) or change in composition (gradient). Isocratic separations are widely used because of their simplicity. A gradient separation is advantageous over isocratic separations since it can separate different types of analytes (polar and nonpolar substances) in a complex mixture in a single run. The column is packed with a porous particle (i.e., silica-based particles) with diameters in the range of 3 to 10 µm. The length and diameter of the columns are in the range of 10 - 30 cm and 1 - 4.6 mm, respectively.

Based on the relative polarities of the mobile and stationary phases, chromatography is referred to as either normal-phase chromatography (NPC) or reversed-phase chromatography (RPC). In RPC, the stationary phase is nonpolar (hydrocarbon, C8 or C18; supported on silica), and the mobile phase is relatively polar (water, methanol, or acetonitrile). On the other hand, in NPC, the stationary phase is polar (a polar functional group, e.g., cyano, diol, or amino), and the mobile phase is relatively nonpolar (hexane or ethylether).

The effectiveness of a chromatographic column is dependent in part on the retention factor or capacity factor. The capacity factor (k´) describes the migration rate of solutes, and can be defined as:
\[ k' = \frac{(t_R - t_M)}{t_M} \]  \hspace{1cm} (1.31)

where \( k' \) is the distribution constant for a species, \( t_R \) is the migration time of the species, and \( t_M \) is the migration time of unretained species. The retention factor for the species should be in the range of 2 - 10 for an ideal separation. \( k' \) is a useful parameter in chromatography since it is independent of the nature of the mobile and stationary phase [78].

Another important parameter of a column is its column efficiency. Column efficiency can be described in terms of plate height (H, cm), and is defined by the van Deemter equation:

\[ H = A + \frac{B}{u} + Cu \]  \hspace{1cm} (1.32)

where \( u \) is the linear velocity (cm/sec) of mobile phase, and A, B, and C correspond to the multiple flow paths, longitudinal diffusion, and the mass transfer between phases, respectively [78, 110]. As seen in Eq. 1.32, H varies with the flow rate or velocity of the mobile phase, as it moves through the column. The term A occurs when a molecule takes multiple pathways in a column during elution. Since the length of each path varies, the residence time of molecules in the column is variable. This leads to band broadening. Longitudinal diffusion (the term B) is produced if molecules diffuse from the band with and against the direction of the mobile phase flow in the column. It is inversely proportional to the velocity of the mobile phase.
The term C arises when the rates of mass transfer of molecules between the stationary and mobile phases are slower than the movement of mobile phase. The mass transfer effect is directly proportional to the flow rate of the mobile phase. H and N (column efficiency in terms of plate number, \( N = L/H \)) can be calculated from the following equations:

\[
H = \frac{LW^2}{16t_R^2} \quad (1.33)
\]
\[
N = 16\left(\frac{t_R}{W}\right)^2 \quad (1.34)
\]

where \( L \) is the length of column, and \( W \) is the peak width. \( N \) for a HPLC column varies from a few hundred to several thousand plates [78, 111].

1.5.2 Detection Modes

Detection schemes for HPLC are similar to CE, as described in detail in Section 1.4.2. In a HPLC system, detection is performed in a flow cell, where the eluant flows from the column to the detection cell through a plumbing system. UV detection is commonly used in HPLC because most analytes possess UV absorbing chromophores [112]. Fluorescence detection is more sensitive and selective than UV detection [112]. Since fewer analytes have natural florescence, derivatization steps are typically performed in order to make compounds fluoresce. EC detection (e.g., amperometric, conductivity, and coulometric detection) is also used in HPLC [112]. However, analytes have to be electroactive for this scheme of detection. Like
fluorescence detection, it is a sensitive detection technique, and selectivity can be achieved by performing detection at lower electrode potentials. The mass detection limits of UV, fluorescence, and EC detection are in the range of 100 pg - 1 ng, 1 - 10 pg, and 10 pg - 1 ng, respectively [78].

1.6 Scope of the Dissertation

The goal of this research was to develop analytical methods based on separation techniques coupled to microdialysis sampling in order to determine biomarkers of oxidative stress in vivo. Particularly, the research focused on two biomarkers, GSH and ‘OH. GSH was used as a measure of antioxidant activity in vivo, and ‘OH was used to determine free radical generation in vivo. For the in vivo studies, Sprague Dawley rats were used as experimental animals.

Chapter 2 describes the development of a CE method for the simultaneous determination of GSH and GSSG in microdialysis samples. A UV detection scheme was used, and in order to improve the detection limit necessary for the quantification of GSH and GSSG in microdialysis samples, a pH-mediated stacking technique was employed. The developed method was then applied for the determination of GSH and GSSG in liver microdialysates of anesthetized rats.

Chapter 3 presents the in vitro evaluation of 4-hydroxybenzoic acid (4-HBA) as a trapping agent for the indirect determination of ‘OH. First, a CE-UV method coupled to microdialysis was developed to detect 3,4-dihydroxybenzoic acid (3,4-DHBA). Indirect determination involved the trapping of ‘OH with 4-HBA to form
3,4-DHBA and then quantifying the hydroxylated adduct with the CE-UV method. Applicability of the method was demonstrated through the detection of 3,4-DHBA in 
‘OH generation systems in vitro with 4-HBA.

Chapter 4 focuses on the application of the CE-UV method to determine ‘OH in vivo as 3,4-DHBA in heart microdialysates of anesthetized rats. 3,4-DHBA was monitored in microdialysates before and after the induced ischemia-reperfusion to examine the effect of oxidative stress.

Finally, Chapter 5 describes the summary of the research performed. Also, it outlines future efforts resulting from the research presented in this dissertation.
1.7 References


66. Reyes, M.G., Capillary electrophoresis with electrochemical detection for the determination of glutathione in biological samples. 1997, University of Kansas: Lawrence, KS.


Chapter 2: Development of a CE-UV Method Coupled to Microdialysis for the Simultaneous Determination of GSH and GSSG

2.1 Introduction

Glutathione is a thiol found intracellularly at high concentrations (1-10 mM), and is also present in small amounts (~ μM) in the extracellular fluid (ECF) [1]. It exists as reduced glutathione (GSH) and in an oxidized form as glutathione disulfide (GSSG), as shown in Figure 1.2 of Chapter 1. More than 95% of the glutathione pool in the cell remains in its reduced form [2]. Both forms of glutathione are found mainly in the cytosol. In eukaryotic cells, ~ 90% of GSH is found in the cytosol, ~ 10% in the mitochondria, and a very small percentage in the endoplasmic reticulum [3]. GSH has great reducing power because of the -SH group (thiol or sulfhydryl group) in its structure. Both GSH and GSSG are anions at physiological pH since pK values of the -COOH (Glu), -NH₃⁺ (Glu), -SH (Cys), and -COOH (Gly) functional groups are 2.12, 8.66, 9.12, and 3.53, respectively [4].

Glutathione plays several important roles in biological systems. Functions of GSH can be categorized as follows: its antioxidant activity, as shown in Eqs. 2.1 to 2.3, detoxification of xenobiotics (Eq. 2.4), regulation of the immune system, storage and transport of cysteine (by the γ-glutamyl cycle, as shown in Figure 2.1 and described in following paragraph), and regulation of proteins (Eq. 2.5) [3, 5]. Reaction of GSH with reactive oxygen species (ROS) results in the formation of GSSG. Decreases in GSH and increases in GSSG concentrations have been suggested
Figure 2.1. Glutamyl cycle. (1) Transport of amino acid (AA) into the cell through AA transporters. (2) Conversion of γ-glutamyl-amino acid (γ-Glu-AA) to glutamate (Glu) and AA through formation of 5-oxoproline. (3) Formation of γ-glutamylcysteine (γ-Glu-CysH) from Glu and cysteine (CySH) by γ-glutamylcysteine synthetase (GCS). (4) Generation of GSH, γ-glutamylcysteinylglycine (γ-Glu-CyS-Gly) from γ-Glu-CySH and glycine (Gly) by GSH synthetase. (5) Transport of GSH into extracellular space by GSH transporters. (6) Generation of γ-Glu-AA and cysteinylglycine (CyS-Gly) from GSH and AA by γ-glutamyltransferase (GGT). (7) Conversion of CyS-Gly to CySH and Gly by dipeptidase (DP). Adapted and modified from references [1, 3, 6].
to be linked to a variety of diseases, such as cancer, Alzheimer’s disease, heart
disease, stroke, diabetes, and AIDS [1, 7-9].

\[
\begin{align*}
2\text{OH} + 2\text{GSH} &\rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad (2.1) \\
\text{H}_2\text{O}_2 + 2\text{GSH} &\rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad (2.2) \\
\text{R-O-O-H} + 2\text{GSH} &\rightarrow \text{GSSG} + \text{R-OH} + \text{H}_2\text{O} \quad (2.3) \\
\text{GSH} + \text{Xenobiotics} &\rightarrow \text{GSX} \quad (2.4) \\
\text{Protein-SSG} + \text{GSH} &\rightarrow \text{P-SH} + \text{GSSG} \quad (2.5)
\end{align*}
\]

All biological functions of GSH, as mentioned above, deplete cellular GSH.
The loss of intracellular GSH is balanced by the conversion of GSSG to GSH through
the redox cycle of glutathione, as shown in Figure 1.4 of Chapter 1 and also by the
biosynthesis of GSH from its precursor amino acids [3]. This biosynthesis involves
two steps [3]. In the first step, glutamate reacts with cysteine in the presence of ATP
to form \(\gamma\)-glutamylcysteine, catalyzed by \(\gamma\)-glutamylcysteine synthetase (Eq. 2.6). The
second step is catalyzed by glutathione synthetase, where \(\gamma\)-glutamylcysteine reacts
with glycine in the presence of ATP to produce \(\gamma\)-glutamylcysteinylglycine (Eq. 2.7).
Among the precursors, it is the availability of cysteine that determines the rate of
GSH synthesis [3]. Cysteine is supplied through the diet, protein breakdown,
methionine metabolism, and the \(\gamma\)-glutamyl cycle. The \(\gamma\)-glutamyl cycle is a series of
six enzyme-catalyzed reactions which are responsible for the synthesis and
metabolism of GSH (Figure 2.1) [3, 10]. Dietary supplements (e.g., precursors of GSH) and natural foods rich in precursors of GSH may increase the biosynthesis of GSH [11]. Meister reported that GSH deficiency might be prevented by the administration of GSH-ester [12]. The GSH-ester can be transported into the cell readily and hydrolyzed to generate GSH inside the cell. A study showed that GSH-ester can protect reperfusion injury by supplying GSH intracellularly and extracellularly [13].

\[
\text{Glu} + \text{CySH} + \text{ATP} \xrightarrow{\gamma-\text{Glu-CySH synthetase}} \gamma-\text{Glu-CySH} + \text{ADP} + P \quad (2.6)
\]

\[
\gamma-\text{Glu-CySH} + \text{Gly} + \text{ATP} \xrightarrow{\text{GSH synthetase}} \gamma-\text{Glu-Cys-Gly} + \text{ADP} + P \quad (2.7)
\]

Intracellular formation of GSSG, as illustrated in Eqs. 2.1 to 2.5, could be a useful quantitative index to assess the extent of ROS formation in biological systems [14] during oxidative stress since it alters the ratio of GSH to GSSG in the cell. Increased production of intracellular GSSG is accompanied by the export of GSSG to the extracellular space through active transport [3, 15, 16]. Like GSSG, efflux of GSH also occurs as a normal cellular function [17], and this translocation can occur by diffusion or active processes [18, 19]. Efflux of GSH and GSSG is unidirectional. The transport and metabolism of glutathione are summarized in Figure 2.2. Increased release of GSH into the extracellular space was observed as a result of induced
Figure 2.2. Transport and metabolism of glutathione. (1) & (1’) Transport of GSH & GSSG into extracellular space. (2) Conversion of GSH to GSSG in extracellular space. (3) & (3’) Degradation of GSH and GSSG to amino acids and transport of amino acids into the cell. Adapted and modified from reference [19].
ischemia-reperfusion [20-22]. Hence, the change of GSH and GSSG concentrations in the extracellular fluid before and after oxidative stress may be used as an indicator of oxidative stress in vivo.

In oxidative stress investigations, adriamycin (ADR) has been utilized to chemically induce oxidative stress in rats [23]. It is hypothesized that the presence of ADR in biological systems would increase the generation of ROS. The increased generation of ROS in biological systems could be explained by the chemical structure of ADR (Figure 2.3). The quinone moiety in ADR may accept an electron to generate the semiquinone free radical, and subsequently, the electron on the semiquinone can be donated to O$_2$ to form O$_2^-$. NADPH, cytochrome P450 reductase, or xanthine oxidase (XO) could catalyze the reduction of the quinone to the semiquinone free radical [25, 26]. The hypothesized site for the generation of O$_2^-$ is in the mitochondria [25]. From O$_2^-$, other ROS, such as H$_2$O$_2$ and `OH, can be generated, as discussed in Section 1.1.2 of Chapter 1. In these circumstances, antioxidants (e.g., GSH) would scavenge ROS as part of natural defense mechanisms in biological systems. A significant decrease in the GSH/GSSG ratio (decrease in GSH and increase in GSSG) has been observed in brain homogenates of rats dosed with ADR [23].
Figure 2.3. Reduction of adriamycin and generation of superoxide.
2.2 Analytical Methods for the Determination of GSH and GSSG in Microdialysis Samples

Several methods have been used to determine glutathione in biological samples. Various authors have reviewed analytical methods for glutathione and other thiols in different sample matrices [27-32]. Approaches to analyzing biological samples for GSH and GSSG can be classified as either non-separation or separation methods. The commonly used non-separation methods include spectrophotometric and spectrofluorometric assays. The separation-based methods use high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Every method; however, has its advantages and disadvantages. The selection of a method depends on the required limit of detection (LOD), selectivity, sample matrix, and expected concentration levels in the samples.

Both spectrophotometric and spectrofluorometric determinations are derivatization-based methods. GSH is derivatized with a suitable chromophore or fluorophore to improve the sensitivity. For GSSG determination, GSSG is reduced to GSH and then is quantified as GSH. Examples of some chromophores and fluorophores employed are shown in the Tables 2.1 and 2.2, respectively. The chromophores or fluorophores used to derivatize glutathione in biological samples are either -SH or -NH$_2$ group specific. Therefore, spectrophotometric and spectrofluorometric methods are not selective since interferences may come from the other biological thiols (cysteine and homocysteine) or disulfides (cystine and homocystine) in the samples.
### Table 2.1. Derivatizing agents: Chromophores [27, 33]

<table>
<thead>
<tr>
<th>Chromophores</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-ethylmaleimide (NEM)</td>
<td>−SH</td>
</tr>
<tr>
<td>Monoiodoacetic acid (MIAA)</td>
<td>−SH</td>
</tr>
<tr>
<td>N-(2,4-dinitrophenylaminoethyl)-maleimide</td>
<td>−SH</td>
</tr>
<tr>
<td>Eosin-5-maleimide (EMA)</td>
<td>−SH</td>
</tr>
<tr>
<td>Pyrenyl-maleimide</td>
<td>−SH</td>
</tr>
<tr>
<td>5′,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Elman’s reagent)</td>
<td>−SH</td>
</tr>
<tr>
<td>2,4-Dinitrofluorobenzene (DNFB)</td>
<td>−SH &amp; −NH₂</td>
</tr>
<tr>
<td>2-Chloro-1-methylquinolininium-tetrafluoroborate</td>
<td>−SH</td>
</tr>
</tbody>
</table>

### Table 2.2. Derivatizing agents: Fluorophores [27, 34, 35]

<table>
<thead>
<tr>
<th>Fluorophores</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Phthalaldehyde (OPA)</td>
<td>−SH &amp; −NH₂</td>
</tr>
<tr>
<td>Naphthalene-2-3-dicarboxyaldehyde</td>
<td>−SH</td>
</tr>
<tr>
<td>Monobromobimane (BrB)</td>
<td>−SH</td>
</tr>
<tr>
<td>4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F)</td>
<td>−SH</td>
</tr>
<tr>
<td>4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F)</td>
<td>−SH</td>
</tr>
<tr>
<td>4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F)</td>
<td>−SH</td>
</tr>
<tr>
<td>Fluorescein-5-maleimide (FM)</td>
<td>−SH</td>
</tr>
<tr>
<td>N-(1-pyrenyl)-maleimide (NPM)</td>
<td>−SH</td>
</tr>
<tr>
<td>2-(4-N-maleimidophenyl)-6-methoxybenzofuran</td>
<td>−SH</td>
</tr>
<tr>
<td>3-Iodoacetylaminobenzanthrone (IAB)</td>
<td>−SH</td>
</tr>
<tr>
<td>N-hydroxyuccinimidyl-α-(9-acridine)-acetate (HSAA)</td>
<td>−NH₂</td>
</tr>
<tr>
<td>N-hydroxysuccinimidyl-α-(9-phenanthrene)-acetate (HSMPA)</td>
<td>−NH₂</td>
</tr>
<tr>
<td>4-Chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl)</td>
<td>−NH₂</td>
</tr>
</tbody>
</table>

Biological samples are complex in nature, and separation-based methods are more suitable to analyze complex samples because interferences can be minimized by performing a separation. HPLC has been used successfully to determine GSH in biological samples, such as tissue, plasma, urine, and microdialysates. The detection
schemes used with HPLC are ultraviolet-visible (UV-Vis), fluorescence, electrochemical (EC), and mass spectrometric (MS) detection [27, 28]. Both UV-Vis and fluorescence detection techniques required a derivatization step to increase sensitivity. EC detection has the advantage over other detection techniques since it does not need any derivatization procedure.

Like HPLC, CE has been used successfully to determine GSH in biological samples of different matrices (e.g., tissue, plasma, cell, and microdialysates) [32]. Modes of detection employed with CE are similar to those used with HPLC. The application of CE for the determination of GSH in samples of biological origin is a growing area of research. However, very few papers have been reported to determine GSH in microdialysates. Microdialysates, collected through in vivo microdialysis sampling from targeted tissue sites of awake or anesthetized animals, can give tissue specific chemical information. Microdialysis sampling produces only a few microliters of sample with good temporal resolution. To analyze this small volume of sample, CE is an ideal technique since it consumes a small amount of sample (~ nL). Comparatively, HPLC needs sample volumes in the range of 10 to 25 µL for a single injection. Hence, coupling of microdialysis sampling with CE will result in increased temporal resolution relative to HPLC. The use of separation methods to determine glutathione in microdialysis samples is summarized in Tables 2.3 and 2.4.

Both HPLC and CE have been used to detect GSSG in biological samples [31]. EC, UV-Vis, and fluorescence detection schemes have been employed with both separation techniques. EC detection uses a dual electrode system (in series
Table 2.3. HPLC methods for the analysis of glutathione in microdialysates

<table>
<thead>
<tr>
<th>Sample Matrices</th>
<th>Detection</th>
<th>Concentration (µM)</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat striatum</td>
<td>EC, Functionalized carbon nano-tube</td>
<td>2.75 µM (GSH)</td>
<td>[36]</td>
</tr>
<tr>
<td>Rat cerebral cortex</td>
<td>EC, Au electrode</td>
<td>0.41 µM (GSH); 1.82 µM (GSSG)</td>
<td>[37]</td>
</tr>
<tr>
<td>Rat brain</td>
<td>EC, Pd/IrO\textsubscript{2} modified glassy carbon electrode</td>
<td>420 µM (GSH)</td>
<td>[38]</td>
</tr>
<tr>
<td>Rat striatum</td>
<td>EC, Pt particle modified electrode</td>
<td>2.6 µM (GSH)</td>
<td>[39]</td>
</tr>
<tr>
<td>Rat liver</td>
<td>EC, Au(Hg) electrode</td>
<td>1.03 µM (GSH)</td>
<td>[40]</td>
</tr>
<tr>
<td>Rat striatum</td>
<td>EC, Chemically modified electrode</td>
<td>2.70 µM (GSH)</td>
<td>[41]</td>
</tr>
<tr>
<td>Rat liver</td>
<td>EC, Au(Hg) electrode</td>
<td>25.2 µM (GSH)</td>
<td>[42]</td>
</tr>
<tr>
<td>Tibialis anterior muscle</td>
<td>Fluorescence, N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM)</td>
<td>2.18 µM (GSH); 0.14 µM (GSSG)</td>
<td>[43]</td>
</tr>
<tr>
<td>Rat liver</td>
<td>EC, Au(Hg) electrode</td>
<td>~ 4 µM (GSH)</td>
<td>[45]</td>
</tr>
</tbody>
</table>

Table 2.4. CE methods for the analysis of glutathione in microdialysates

<table>
<thead>
<tr>
<th>Sample Matrices</th>
<th>Detection</th>
<th>Concentration (µM)</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>EC, Au(Hg) electrode</td>
<td>4.16 to 76.5 µM (GSH)</td>
<td>[20]</td>
</tr>
<tr>
<td>Rat brain cortex</td>
<td>Fluorescence, methanolic monobromobimane</td>
<td>2.10 µM (GSH)</td>
<td>[22]</td>
</tr>
<tr>
<td>Melanoma tissue</td>
<td>Fluorescence, DACM</td>
<td>204 µM (GSH)</td>
<td>[44]</td>
</tr>
<tr>
<td>Rat subcutaneous tissue</td>
<td>EC, Au(Hg) electrode</td>
<td>~ 4 µM (GSH)</td>
<td>[45]</td>
</tr>
</tbody>
</table>
configuration) to detect GSSG as GSH. In this approach, GSSG is reduced to GSH at a gold/mercury (Au-Hg) electrode, the upstream electrode (-1.0 V vs. Ag/AgCl), and GSH is detected at an other Au-Hg electrode, the downstream electrode (+ 0.15 V vs. Ag/AgCl) (Eqs. 2.8 and 2.9). The detection of GSH is indirect, where Hg is oxidized in the presence GSH [48, 49]. GSH and GSSG can be monitored independently since both are separated chromatographically.

\[
\text{GSSG} + 2\text{H}^+ + 2e^- \rightarrow 2\text{GSH} \quad (2.8)
\]

\[
2\text{GSH} + \text{Hg} \rightarrow \text{Hg(SG)}_2 + 2\text{H}^+ + 2e^- \quad (2.9)
\]

Simultaneous determination of GSH and GSSG is a growing area of interest because of the biological significance of the ratio of GSH to GSSG. Separation based methods have been used to determine of GSH and GSSG simultaneously in biological samples [9, 35, 50-62]. However, there is no report for the simultaneous detection of GSH and GSSG in microdialysis samples using CE with any detection scheme. Simultaneous detection of GSH and GSSG in urine samples was first reported by S. Lunte using CE with EC detection (mixed-valance ruthenium cyanide-modified carbon fiber microelectrode) [51]. Besides EC detection [51, 52, 55, 56, 58-60], UV-Vis and fluorescence detection schemes have also been utilized with CE [9, 35, 50, 53, 54, 57].
2.3 Specific Aims

In this study, *in vivo* microdialysis sampling was used to collect biological samples. To analyze microdialysis samples, a CE-UV method was developed. The coupling of microdialysis sampling with CE-UV allowed continuous monitoring of the target analytes, GSH and GSSG, in extracellular fluid. Ultra-violet (UV) detection was used since it is easy to operate and couple to the CE system. An on-column (capillary) preconcentration technique, pH-mediated stacking, was employed in order to enhance the sensitivity of the CE-UV method. The CE-UV method was then utilized to monitor changes in concentrations of GSH and GSSG in liver microdialysates of anesthetized Sprague Dawley rats due to oxidative stress induced by adriamycin.

2.4 Experimental

2.4.1 Materials

Reduced glutathione (~98%) and oxidized glutathione (~98%) were purchased from Sigma-Aldrich Company (St. Louis, MO). Inorganic salts, such as sodium chloride (NaCl), potassium chloride (KCl), and calcium chloride (CaCl₂), used for making Ringer’s solution were certified ACS grade and were obtained from Fisher Scientific Company (Fair Lawn, NJ). Ammonium chloride (certified ACS grade, Fisher Scientific Company) and tetradecyltrimethylammonium bromide (TTAB) (99%, Sigma-Aldrich Company) were used to prepare the buffer solutions. Other chemicals such as sodium hydroxide (NaOH, certified ACS grade), hydrochloric acid
(HCl, 37%), and methanol (MeOH, HPLC grade) were obtained from Fisher Scientific Company. Nanopure water (18.2 MΩ, Water Pro PS, Labconco, Kansas City, MO) was used for making stock standard and buffer solutions. Anesthetics (isoflurane, xylazine, and ketamine) were employed for animal studies and were supplied by the Animal Care Unit, the University of Kansas.

2.4.2 Sample Preparation

A 100 mM ammonium hydroxide buffer (NH₄⁺/NH₃, prepared from 100 mM NH₄Cl, pH adjusted with NaOH) with 0.5 mM TTAB at pH 8.4 was used as the CE background electrolyte (BGE). Ringer’s solution consisted of 155 mM NaCl, 5.5 mM KCl, and 2.3 mM CaCl₂·2H₂O. Both the GSH (10 mM) and the GSSG (5 mM) stock solutions were prepared in BGE. GSH stock solution (10 mL) was prepared daily, and GSSG stock solution (10 mL) was prepared weekly and kept in the refrigerator. Working standard solutions of GSH and GSSG were prepared daily from their stock solutions by multiple dilutions with Ringer’s solution. All buffer and Ringer’s solutions were bubbled with argon gas for 20 min to remove dissolved oxygen. Oxygen was eliminated from the solutions in order to minimize the oxidation of GSH to GSSG. All solutions were filtered through a 0.22 μm pore size syringe filter (Millipore Millex™GP, Fisher Scientific) prior to analysis by CE-UV.
2.4.3 CE-UV System

A home-built CE system with a SpectraPhysics UV1000 (a ThermoSeparation product) was used for this study. Polyimide coated fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a 50 μm inner diameter (ID) and 360 μm outer diameter (OD) was cut to a total length of 60 cm long with 45 cm effective length. A small UV window (~1.5 mm) was made in the capillary by burning the polyimide coating carefully, and the capillary window was aligned with the UV light path of the detector. For conditioning, new capillaries were flushed hydrodynamically at 15 psi with MeOH (10 min), H₂O (5 min), 1 M HCl (5 min), H₂O (5 min), 0.1 M NaOH (10 min), H₂O (5 min), and then BGE (10 min).

CE-UV analysis was performed in reversed electroosmotic flow (EOF) mode. EOF was reversed using a cationic surfactant (i.e., TTAB) in the BGE and performing separation at a voltage of negative polarity. Both GSH and GSSG were detected on-column by the UV detector at a fixed wavelength (214 nm). A –10 kV voltage (resulting current of ~ 45 μA) was applied between the anode and cathode using a high voltage power supply unit (CZE1000R, Spellman High Voltage Electronics, Hauppauge, NY, USA) to drive the electrophoresis.

All injections were made electrokinetically by applying –10 kV (i.e., in the negative polarity mode). The microdialysis samples were injected into the capillary without any pretreatment or dilution. CE-UV analyses were performed at ambient temperature. After each run, the capillary was rinsed with 0.1 M NaOH for 2 min and then BGE for 2 min to regenerate the capillary inner surface. Data were collected by a
computer using a data acquisition computer card and the LabView 5.1 data acquisition software (National Instrument, Austin, TX).

2.4.4 pH-Mediated Base Stacking

To perform base stacking, first the sample was injected into the capillary filled with BGE. Then, the sample vial was replaced with the NaOH (0.1 M) vial to inject strong base into the capillary. After sample and base injections, the separation was performed using BGE. All injections were performed electrokinetically at – 10 kV. The length of sample and base injections were optimized as discussed in Section 2.5.1.

2.4.5 Microdialysis Sampling

Sprague Dawley rats (male and female) of 350 – 400 gram body weight were used in these studies. Microdialysis sampling was performed in the rat for the in vivo studies. Microdialysis sampling apparatus, procedures for making linear probes, probe calibration, and surgery are described in the following sections.

2.4.5.1 Apparatus

The microdialysis sampling apparatus consists of two parts: a microinjection pump and the microdialysis probe. A CMA/100 microinjection pump (CMA Microdialysis AB, Stockholm, Sweden) was used to pump Ringer’s solution (the perfusate) through the microdialysis probe. Linear probes were used in these studies.
The dimensions of the linear probe are mentioned in Section 2.4.5.2. After implanting the semipermeable membrane into the tissue site (as discussed in Section 2.4.5.3), one end of the linear probe was connected to 1 mL syringe (Hamilton gas tight # 1001, Hamilton, Reno, Nevada) which was placed in the microinjection pump. The other end of the probe was placed in the sample collection vial. The perfusate was delivered through the linear probe by the microinjection pump, and microdialysates were collected in sample vials for further analysis.

2.4.5.2 Probe Fabrication

Linear probes were made from polyimide tubing with 127 µm OD and 100 µm ID (MicroLumen Inc., Tampa, FL) and Spectra/Por® in vivo microdialysis hollow fibers of regenerated cellulose (Spectrum, Rancho Domnguez, CA) with 216 µm OD and 200 µm ID. The two pieces of polyimide tubing (6 inch long each) were inserted into a 3 cm long piece of the hollow fiber membrane to give an active length of 10 mm. The membrane had a molecular weight cut-off of 18 kDa. The hollow fiber ends were glued to polyimide tubing using UV glue (UVEXS, Ultraviolet Exposure Systems, Sunny Vale, CA). A small piece of polyimide coated fused silica capillary (360 µm OD and 200 µm ID) was glued at one end of the linear probe using UV glue. This was used as an implantation guide during the surgery. The other end of the probe was glued with a small piece of TYGON® microbore tubing (1524 µm OD and 508 µm ID) (Norton Performance Plastics, Akron, OH). Through this tubing, the probe was connected to the needle (22s gauge, 2 inch long, and point style 3) of the syringe.
2.4.5.3 Surgical Procedure

Prior to the surgery, Sprague Dawley rats were pre-anesthetized using isoflurane and then fully anesthetized by intra-muscular injection of a ketamine (100 mg/kg dose) and xylazine (10 mg/kg dose) mixture. Additional doses of ketamine (1/4 of original dose) were injected as needed to keep the rats anesthetized throughout the entire experiment. The abdominal area was shaved and disinfected by swabbing with ethyl alcohol. The anesthetized rats were kept on a heated pad to maintain body temperature during the surgery and sampling. The liver was exposed after making an abdominal incision. The liver lobe was drawn outside using cotton swab sticks. The fused silica capillary end of the linear probe was threaded through the rat liver lobe to place the semipermeable membrane portion of the probe in the middle of the liver lobe. Then, it was carefully glued with the tissue by a tissue adhesive (3M Vetbond\textsuperscript{TR}, 3M, USA) to keep in place. After completion of implantation, the liver was replaced, and the cut skin and muscle were glued with the tissue adhesive. The fused silica capillary was cut off from the polyimide tubing before connecting the probe to the microdialysis apparatus. The implanted probe was perfused with Ringer’s solution at a flow rate of 1 \(\mu\text{L/min}\) in all experiments. Prior to the collection of microdialysates at 10 min intervals (unless otherwise mentioned), the implanted probe was flushed with Ringer’s solution for 30 min.

For the induction of oxidative stress chemically, microdialysates were collected for 2 hours using Ringer’s solution as the perfusate. Then, ADR (2 mg/mL in Ringer’s solution) was delivered as the perfusate for 1 hour. After this, the
perfusate was switched back to Ringer’s solution. Microdialysates were collected for 2 hours after delivery of ADR to the liver.

2.4.5.4 Probe Calibration by In Vivo No Net Flux Experiments

In vivo no net flux (NNF) experiments were performed by determining the mass transport of GSH and GSSG through the implanted probe as a function of their concentrations in the perfusate. After implantation of the probe in the liver, the probe was flushed with Ringer’s solution at a flow rate of 1 µL/min for 2 hours to attain a steady state. Once steady state was established, four known concentrations of each analyte were perfused through the probe. Each concentration was perfused for 1 hour, and after that, samples were collected at 10 min intervals until a stable concentration was obtained. Between concentrations, the Ringer’s solution was perfused through the probe for 1 hour to obtain a steady state concentration. It took 16 to 18 hours to finish the whole in vivo NNF experiment. To determine the in vivo recovery value, the change of concentration ($C_d - C_p$) was plotted against analyte concentration in the perfusates ($C_p$), and the slope of the regression line was the in vivo NNF recovery of the analyte.

2.5 Result and Discussion

2.5.1 Optimization of Sample Injection

Figure 2.4 shows the effect of different injection times on sensitivity when the sample is a mixture of a 10 µm GSH and GSSG in Ringer’s solution. The BGE
Figure 2.4. GSH and GSSG detection without pH-mediated stacking. (A) 3 s electrokinetic injection (EKI) of sample (mixture of 10 μM GSH and 10 μM GSSG in Ringer’s solution). (B) 30 s EKI of sample. Conditions: 50 μm ID capillary x 60 (45) cm, 100 mM NH₄Cl BGE with 0.5 mM TTAB at pH 8.4, EKI at –10 kV, separation at –10 kV, and detection at 214 nm.
employed was 100 mM NH₄Cl with 0.5 mM TTAB buffer of pH 8.4, and separation was performed at – 10 kV. Using TTAB and reversed polarity, EOF and electromigration of GSH and GSSG were achieved in the same direction toward the anode (detection end). From Figure 2.4, it is evident that both sensitivity and resolution are very low for both GSH and GSSG with 3 s and 30 s injections (electrokinetic injections at – 10 kV from the cathode). Normal injection without stacking did not provide sufficient concentration detection limits for the quantification of GSH and GSSG in biological samples even when injecting large volume samples (e.g., 30 s sample injection). The poor sensitivity and separation efficiency are the result of the small path length of light (50 µm) in the capillary and the destacking phenomena of high ionic strength samples. Sample destacking occurs when the sample zone has a higher conductivity than that of the BGE. High ionic strength samples, such as those in Ringer’s solution, have high conductivity, and charged analytes migrate slowly in the sample zone. Analytes migrate faster when they enter the BGE zone from the sample zone, thus resulting in sample destacking or band broadening during CE separation. The extent of destacking is higher with the longer sample injection times and higher ionic strength samples [63-65].

In this study, an on-column preconcentration technique, pH-mediated base stacking, was employed to enhance sensitivity as well as resolution. Optimization of the injection ratio (ratio of sample to base injection time) is critical to achieve maximum sensitivity without deteriorating the separation. The amount of NaOH to be injected into the capillary is dependent on the amount of sample injected. Hence, the
first step of CE with pH-mediated stacking is to determine the optimum sample and base injection times. Figure 2.5 and Table 2.5 show that for a 30 s sample injection (EKI at \(-10\) kV), S/N increases, and peak tailing and fronting decrease with an increase of base (0.1 M NaOH) injection time. Both GSH and GSSG peaks are baseline resolved when base injection length is 60 s. Base injection times longer than 60 s did not improve the separation. As a result, the optimum injection protocol was found to be a 30 s injection of sample followed by a 60 s injection of 0.1 M NaOH (30 s/60 s). This injection scheme produced an increased resolution of GSH and GSSG with greatly improved sensitivities. Analysis of 10 µM GSH and 10 µM GSSG standard solutions using the optimized injection protocol showed a 26-fold increase in sensitivity for both GSH and GSSG in comparison to the normal injection (30 s EKI without stacking).

In pH-mediated stacking, the CE capillary serves two purposes, titration or neutralization and separation. In the titration step, the EKI of OH\(^-\) results in the titration of NH\(_4^+\) (i.e., BGE cation). Thus, NH\(_4^+\) is converted to NH\(_3\). This creates a low conductivity sample zone, where analytes move faster and stack at the interface of the sample and BGE zones. The peak tailing is the result of incomplete titration of the sample zone as an insufficient amount of base relative to sample is injected. In the case of peak fronting, the titrated zone does not reach the front of the sample zone, and as a result, analytes move faster to create a broad zone or an unstacked zone [64, 65].
Figure 2.5. Optimization of injection length with ammonium buffer system. (A) 30 s EKI of sample (mixture of 10 µM GSH and 10 µM GSSG in Ringer’s solution)/0 s EKI of NaOH. (B) 30 s EKI of sample /30 s EKI of NaOH. (C) 30 s EKI of sample/40 s EKI of NaOH. (D) 30 s EKI of sample /50 s EKI of NaOH. (E) 30 s EKI of sample /60 s EKI of NaOH. (F) 30 s EKI of sample /70 s EKI of NaOH. Conditions: same as in Figure 2.4.
Table 2.5. Separation efficiency as a function of injection ratio*

<table>
<thead>
<tr>
<th>Injection Ratio</th>
<th>S/N</th>
<th>Efficiency (x 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GSH</td>
</tr>
<tr>
<td>30 s/0 s</td>
<td></td>
<td>A broad unresolved peak (Figure 2.5A)</td>
</tr>
<tr>
<td>30 s/30 s</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td>30 s/40 s</td>
<td>30</td>
<td>62</td>
</tr>
<tr>
<td>30 s/50 s</td>
<td>30</td>
<td>66</td>
</tr>
<tr>
<td>30 s/60 s</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>30 s/70 s</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

*Conditions: mixture of 10 µM GSH and 10 µM GSSG, 50 µm ID capillary x 60 (45) cm, 100 mM NH_4Cl BGE with 0.5 mM TTAB at pH 8.4, EKI at –10 kV, separation at –10 kV, and detection at 214 nm.

It has been shown that too long of a base injection (more than optimum amount) results in the deterioration of the separation efficiency and resolution [65]. This is because the most of the capillary length is used for stacking, and only a small portion of the capillary is left for the separation. Also, it has been demonstrated that the optimum base injection time is a function of capillary length, ionic strengths of both the sample and BGE, and electrophoretic mobility of the analyte [63, 65].

2.5.2 Method Validation

The performance of the CE-UV method was evaluated in terms of reproducibility of migration time and sensitivity by comparing migration time and sensitivity between analyses of 10 µM GSSG standard solutions in the same capillary. The reproducibility of migration time (7.17 ± 0.08 min) and sensitivity (0.257 ± 0.008 (mAU/µM) were acceptable since RSDs were below 5% (1.14% and 3.08%,
respectively and n = 12) for the analysis within the same day. The resolution of GSH and GSSG was found to be $2.53 \pm 0.17$ (n = 12). The peak height (mAU) versus concentration (µM) exhibited linearity over the range of 0.75 µM to 40 µM with an equation of $y = 0.060x + 0.192$ ($R^2 = 0.978$ and n = 6) for GSH and the range of 0.25 µM to 50 µM with an equation of $y = 0.111x + 0.431$ for GSSG ($R^2 = 0.950$ and n = 7) for GSSG. LOD for the analysis of GSH and GSSG were found to be 0.75 µM (S/N = 6) and 0.25 µM (S/N = 6), respectively.

2.5.3 Stability of GSH

To perform a stability study of GSH, 2 mL of 10 µM GSH standard solution in a 4 mL amber glass sample vial (closed with cap) was left at room temperature, and an aliquot of 10 µL was taken for analysis every hour. It was observed that the peak height of GSH decreased and that of GSSG (oxidation product of GSH) increased. Within 1 hour, the peak height of GSH decreased by ~4% and after 2 hours decreased by ~7% (Figure 2.6). A decrease of ~20% in peak height was observed within 7 hours. The stability study shows that the oxidation of GSH to GSSG occurs under normal storage conditions.

It has been reported that acidified sample reduces the oxidation of GSH to GSSG [21, 66]. However, the analysis of acidified GSH standards by the CE-UV method resulted in reduced (32% decrease) sensitivity. Therefore, acidification of the sample was found not to be suitable for the analysis.
Figure 2.6. Stability study of GSH. (A) 0 hour. (B) 1 hour. (C) 2 hours. (D) 7 hours.

Conditions: 10 µM GSH in Ringer’s solution, 30 s/60 s base stacking, and all other conditions same as in Figure 2.4.
2.5.4 Monitoring GSH and GSSG In Vivo

A representative electropherogram of liver microdialysates of anesthetized Sprague Dawley rats is shown in Figure 2.7. GSH and GSSG peaks in the electropherograms were confirmed by spiking (Figure 2.8). To perform spiking experiments, 5 µL of microdialysate was spiked with 1 µL of 100 µM GSH and 1 µL of 100 µM GSSG standard solutions. The concentrations of GSH and GSSG were quantified from GSH and GSSG calibration curves (GSH: $y = 0.130x + 0.012$, $R^2 = 0.995$, $n = 3$ & GSSG: $y = 0.252x + 0.127$, $R^2 = 0.997$, $n = 5$). The recoveries of GSH and GSSG determined by in vivo NNF experiments (Figure 2.9) were 49.5 % and 47.0 %, respectively, and these values were used to calculate basal concentrations. In Figure 2.9, the points of NNF correspond to the extracellular concentrations of GSH (1.13 µM) and GSSG (6.06 µM) in rat livers.

It was found that immediately after probe implantation GSH and GSSG concentrations were high in the microdialysis samples. Figure 2.10 shows the time profile of extracellular GSH and GSSG in liver microdialysates of a rat. As shown in this figure, concentrations are higher in the first two microdialysis samples. This may be due to the cell damage resulting from probe implantation [20]. As a result of cell damage, the intracellular species go into the extracellular fluids. Over time, the levels of GSH and GSSG decreased to the basal levels. As shown in Figure 2.10, the GSH and GSSG concentrations reached basal levels 1 to 2 hours after implantation.
Figure 2.7. Representative electropherogram of liver microdialysate. Conditions: 30 s/60 s base stacking and all other conditions same as in Figure 2.4.
Figure 2.8. Electropherograms of unspiked and spiked liver microdialysates. (A) liver microdialysates; black line. (B) spiked liver microdialysates (5 µL microdialysate spiked with 1 µL of 100 µM GSH and 1 µL of 100 µM GSSG standard solutions); red line. Conditions: 30 s/60 s base stacking and all other conditions same as in Figure 2.4.
Figure 2.9. Plots of *in vivo* NNF experiments. (A) GSH (male rat 7 in Table 2.6). (B) GSSG (male rat 6 in Table 2.6). $C_p = C_d$ = concentration of GSH or GSSG in the perfusate and $C_d$ = concentration of GSH or GSSG in liver microdialysates.
Figure 2.10. Time profile of extracellular GSH and GSSG in liver microdialysates. A female rat and sampled at 15 min intervals.
The basal concentrations of GSH and GSSG in liver microdialysates of several anesthetized Sprague Dawley rats (male and female) are shown in Table 2.6. Concentrations were found to vary from rat to rat. Average concentrations of GSH and GSSG in liver microdialysates of male rats were found to be $4.14 \pm 2.55$ µM ($n = 8$) and $5.10 \pm 3.59$ µM ($n = 8$), respectively. In the female rats, the concentration of GSSG was determined to be $0.635 \pm 0.209$ µM ($n = 3$) which was a lower concentration than that determined in male rats. GSH concentrations in microdialysates of all female rats and 1 male rat were not quantified because their concentration levels were below the LOD. However, GSSG concentrations were detected in microdialysates of all rats.

<table>
<thead>
<tr>
<th>Rat</th>
<th>GSH (µM)</th>
<th>GSSG (µM)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 1</td>
<td>7.37 ± 2.04</td>
<td>9.06 ± 0.24</td>
<td>0.81</td>
</tr>
<tr>
<td>Male 2</td>
<td>2.62 ± 1.37</td>
<td>9.78 ± 1.65</td>
<td>0.27</td>
</tr>
<tr>
<td>Male 3</td>
<td>5.14 ± 0.24</td>
<td>8.54 ± 3.35</td>
<td>0.60</td>
</tr>
<tr>
<td>Male 4</td>
<td>3.59 ± 0.25</td>
<td>1.86 ± 0.06</td>
<td>1.93</td>
</tr>
<tr>
<td>Male 5</td>
<td>3.93 ± 0.10</td>
<td>1.53 ± 0.13</td>
<td>2.57</td>
</tr>
<tr>
<td>Male 6</td>
<td>7.66 ± 2.21</td>
<td>5.70 ± 1.95</td>
<td>1.34</td>
</tr>
<tr>
<td>Male 7</td>
<td>2.79 ± 0.11</td>
<td>2.14 ± 0.05</td>
<td>1.30</td>
</tr>
<tr>
<td>Male 8</td>
<td>*</td>
<td>2.22 ± 0.29</td>
<td>-</td>
</tr>
<tr>
<td>Female 1</td>
<td>*</td>
<td>0.867 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>Female 2</td>
<td>*</td>
<td>0.462 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>Female 3</td>
<td>*</td>
<td>0.577 ± 0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

*Below the LOD
- Not calculated
The reported GSH concentration in liver microdialysates of anesthetized male Sprague Dawley rats in literature is in the concentration range of 1.03 to 76.5 µM, and there is no reported value for female rats. In a study using CE with EC detection, the basal concentration of GSH was found to be $4.7 \pm 1.6$ µM ($n = 6$) [21]. In another study using HPLC with EC detection, the extracellular concentration of GSH was found to be in the range of 4.16 to 76.5 µM ($25.12 \pm 27.46$, $n = 10$) [20]. The other reported GSH concentration, also determined by HPLC with EC detection, was $1.03 \pm 0.51$ (n = 8) [40]. No literature values for GSSG concentration in microdialysis samples were found.

The ratio of GSH to GSSG ([GSH]/[GSSG]) in the cytoplasm of cells has been shown to vary from 30:1 to 100:1 [67]. In this study, the ratio of extracellular GSH and GSSG (in liver microdialysates) varied from 0.27 to 2.57. Currently, there is no literature value to compare with this result. It is known that intracellular GSSG is converted to GSH by glutathione reductase, and both GSH and GSSG are transported out of the cell (as illustrated in Figure 2.2) as part of normal physiological functions [2]. As a result, the cell maintains the intracellular concentration of GSSG at a low level preventing the accumulation of GSSG within the cell [68]. Hence, small changes in the ratio of intracellular GSH and GSSG may be reflected in their extracellular concentration levels. Once GSH and GSSG become extracellular, they may enter into the glutathione synthesis cycle ($\gamma$-glutamyl cycle, as illustrated in Figure 2.1). Therefore, the differences in efflux and rate of disappearance by the $\gamma$-glutamyl cycle may lead to the variation in extracellular GSH and GSSG levels in

88
different rats. Besides these in vivo factors, a change in the recoveries of GSH and GSSG might contribute to the differences.

In this present study, the influence of oxidative stress on extracellular GSH and GSSG in rat livers was also investigated. ADR was utilized to chemically induce oxidative stress. The change in extracellular GSSG in liver microdialysates of rats subjected to ADR induced oxidative stress is presented in Figure 2.11. As shown in Figure 2.11, GSSG increased 6-fold from basal levels. However, the CE-UV method could not detect GSH in microdialysates. Data from the control experiments was plotted and analyzed in a similar manner as the data from the experiments with induced oxidative stress (Figure 2.12). From the comparison of Figures 2.11 and 2.12, it was concluded that the increase of GSSG might be the result of induced oxidative stress. As mentioned in Section 2.1, ROS can be generated in ADR treated rats. Therefore, this may result in increased antioxidant activities in biological systems. Increased antioxidant activities could possibly result in changes in the levels of GSH, GSSG, or the GSH to GSSG ratio [14]. An increase in GSSG in ADR treated rat livers (Figure 2.11) could be attributed to this fact.

The decrease in GSSG after 60 min (Figure 2.11) was observed when ADR delivery (and thus induction of oxidative stress) was stopped. Also, it suggests that GSSG may diffuse away from the probe area or enter into the systems that transport and metabolize glutathione, as shown in Figures 2.1 and 2.2. There is no report on the use of ADR and microdialysis for the continuous monitoring of GSH and GSSG in
Figure 2.11. Change in extracellular GSSG in liver microdialysates of rats subjected to ADR induced oxidative stress (n = 3). Male rats and ADR delivery beginning at 0 min for 1 hour.

Figure 2.12. Extracellular GSSG in liver microdialysates: Control experiments (n = 3). Male rats, Ringer’s solution as the perfusate, and no ADR delivery.
microdialysates. Therefore, the data found in this study could not be confirmed. However, from the literature, it has been demonstrated that ADR lowers GSH in liver tissue of mouse [69]. A significant decrease in GSH/GSSG ratio (decrease in GSH and increase in GSSG) has been observed in brain homogenates of rats dosed with ADR [23]. Also, it has been shown that GSH levels in microdialysates increase from basal levels as a result of physically induced oxidative stress in rat livers [20].

It was mentioned earlier that increased production of GSSG is accompanied by the release of GSSG into the extracellular space [15, 16]. Efflux of GSH also occurs as part of normal cellular activities, and GSH efflux increases during oxidative stress [20]. Furthermore, intracellular components may become extracellular if there is damage in cellular membranes caused by ROS [20]. Thus, the concentrations of GSH and GSSG could change in extracellular space as a result of oxidative stress.

As shown in Figure 2.11, GSH was not detected in liver microdialysates of rats subjected to chemically induced oxidative stress, and this may be attributed to the poor stability of GSH. Besides GSSG, the other possible oxidization product could be glutathionesulfonic acid (GSO₃H). To check for the possibility of GSO₃H formation from GSH, a GSO₃H standard was analyzed by a CE-UV method. A LOD of 2.25 µM was obtained. GSO₃H with a migration time of ~ 6.5 min was found to be well separated from GSH and GSSG. However, no GSO₃H was detected in GSH standards or in liver microdialysates.

It has been reported that storing the sample at acidic pH reduces the oxidation of GSH to GSSG [21, 66], and conversely, basic pH facilitates the oxidation of GSH
to GSSG. In this study, CE analysis conditions were at a basic pH. The pH of the BGE was 8.4, and NaOH (0.1 M) was injected after the sample injection for stacking. Therefore, to determine if there is an effect of the CE operating conditions on GSH stability, a GSH standard was analyzed by both the CE-UV method and a HPLC method using dual electrode detection. The HPLC analysis was performed by K. Price in the Craig Lunte Lab using her analytical system. The dual electrode detection for the HPLC analysis involved the use of two Au-Hg electrodes in series configuration, as described in Section 2.2 (Eqs. 2.8 and 2.9) [49]. GSSG was reduced at the upstream electrode (– 1.0 V vs. Ag/AgCl) and detected as GSH at the downstream electrode (+ 0.15 V vs. Ag/AgCl). GSH and GSSG were monitored independently since both were separated chromatographically. The mobile phase consisted of 0.12 M monochloroacetic acid with 7 mM sodium octyl sulfate at pH 2.7 and MeOH (97.25: 2.75). When a 10 µM standard of GSH was analyzed by CE-UV, only GSSG was detected (Figure 2.13). This observation suggested that GSH was oxidized to GSSG, and detected as GSSG. However, HPLC-EC only detected GSH, and no GSSG was detected. The comparative study by two analytical systems suggests that during the analysis by CE, GSH might be converted to GSSG in the capillary.

Further investigations illustrated a discrepancy in the stability of GSH (Figure 2.6A and Figure 2.13A). It was found that the extent of oxidation of GSH to GSSG might be varied. In the first case, slow or less oxidation was occurring, and in the latter case, oxidation was occurring more quickly. This could be the result of small
Figure 2.13. GSH detection as GSSG. (A) 10 µM GSH. (B) 10 µM GSSG. Conditions: 30 s/60 s base stacking and all other conditions same as in Figure 2.4.
changes in the experimental and environmental parameters to which oxidation might be dependent upon. These parameters include the amount of dissolved oxygen and trace metal content in the solution, temperature (ambient temperature or temperature within the capillary), basic pH of BGE, and ionic strength of NaOH. However, all of these parameters excluding the temperature should be the same for both cases. Temperature could vary since the analysis was performed in a home built CE system at ambient temperature. It is unlikely; however, that small changes in temperature would result in accelerated oxidation of GSH in the capillary. Another parameter which is different between the two stability studies was the capillary. Two different batches of fused silica capillary were used, and it is not known whether the manufacturing procedure or metal content varied between the two. Therefore, taking this into account, it might be possible that the discrepancy in the stability of GSH was a direct result of these two variables (temperature and fused silica capillary).

2.6 Conclusion

A CE-UV method with pH-mediated base stacking was developed to detect GSH and GSSG simultaneously in high ionic strength sample matrices. This method provided a simple and effective way for the on-column preconcentration and detection of analytes in a single run. Sensitivity to GSH and GSSG detection was increased by a 26-fold relative to the normal sample injection without stacking. The limits of detection for GSH and GSSG in high ionic strength sample matrices were 0.75 µM and 0.25 µM, respectively.
The method was successfully used to determine basal concentrations of GSH and GSSG in liver microdialysates of anesthetized Sprague Dawley rats. In the study of chemically induced oxidative stress, extracellular GSSG was found to increase 6-fold from its basal level, indicating increased antioxidant activities as a result of oxidative stress. However, this method was not able to detect GSH, and this may be attributed to the poor stability of GSH in Ringer’s solution, as well as the CE operating conditions.
2.7 References

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47. Wang, S., et al., Direct determination of reduced glutathione in biological fluids by Ce(IV)–quinine chemiluminescence *Talanta 2006*, 70, 518-521.


Chapter 3: 4-Hydroxybenzoic Acid as a Trapping Agent for Hydroxyl Radicals – An *In Vitro* Evaluation by CE-UV Coupled to Microdialysis

3.1 Introduction

Various pathophysiological conditions, such as myocardial infarction, stroke, and Parkinson’s disease are believed to be the result of increased levels of reactive oxygen species (ROS) in biological systems [1, 2]. Biologically relevant ROS include superoxide (O$_2^-$) and the hydroxyl (‘OH) and peroxyl (ROO$^.$) radical [3]. The hydroxyl radical is the most reactive among them, with a half-life of $1 \times 10^{-9}$ s [4]. It is formed within living systems through normal metabolic processes, and its generation is increased during oxidative stress, as described in Chapter 1. ‘OH reacts with proteins, lipids, deoxyribonucleic acid (DNA), and carbohydrates with rate constants in the range of $1 \times 10^7$ to $10^9$ M$^{-1}$s$^{-1}$, as a result causing damage to these biomolecules [3]. Hence, measurement of ‘OH in biological samples is important to better predict the oxidative damage of biomolecules [5-7]. In this regard, analytical methods with appropriate sampling and detection techniques are necessary for the continuous *in vivo* monitoring of ‘OH with increased temporal resolution.

Some commonly used sampling techniques which are used to obtain biological samples include tissue extraction, tissue homogenization, blood sampling, urine collection, and microdialysis. Plasma and urine are widely used as biological samples because they are easy to sample from living systems. However, these samples represent the overall biochemical information, and do not give any site or organ specific information. Homogenized tissue or tissue extract can give site specific
information but it is not suitable for the continuous monitoring of the analyte of interest in real time. On the other hand, microdialysates collected through in vivo microdialysis sampling from the targeted tissue sites of awake or anesthetized animals give tissue specific chemical information [8-10]. Microdialysis is a powerful sampling technique, and it has been used in the investigation of potential biomarkers of oxidative stress [6, 11-13].

Using microdialysis sampling, a radical trapping agent can be delivered (as the perfusate) into biological systems to trap ‘OH forming radical adducts. Subsequently, the radical adducts can be recovered. This approach enables the indirect detection of ‘OH in vivo through quantification of the radical adduct by a suitable analytical technique. Capillary electrophoresis (CE) is a suitable technique to couple to microdialysis sampling since it consumes only a few nanoliters of sample. The use of CE provides increased temporal resolution in comparison to high performance liquid chromatography (HPLC). Improved temporal resolution is needed for oxidative stress investigations in order to determine how ‘OH concentration changes can occur in a small period of time during ischemia-reperfusion. A highly resolved time profile for ‘OH formation in vivo will be useful to develop antioxidant therapies that reduce cellular damage since treatment of reperfusion injury is time dependent [14]. In Section 3.2, different analytical methods used for the detection of ‘OH in biological samples are discussed.
3.2 Analytical Methods for the Detection of Hydroxyl Radicals in Biological Samples

The different analytical methods employed to detect ‘OH in biological samples have been reviewed previously by Cheng et al. and Charbonnet [4, 13, 15-17]. The methods can be classified as either direct or indirect detection. The direct approach involves the detection of ‘OH, as it produced, and the indirect approach involves the detection of stabilized reaction products from ‘OH and a trapping agent.

Electron paramagnetic (spin) resonance (EPR or ESR) can be used for the direct detection of ‘OH, but this is not suitable or realistic for in vivo analysis because of the short life of ‘OH in biological systems [4]. Indirect detection is a more realistic approach, and EPR has been used in this regard. EPR needs radical adducts that are paramagnetic and stable enough during the time of analyses [16]. The main disadvantages of EPR are its low sensitivity, the complexity in data interpretation, and artifact problems [18]. The detection limits obtained with EPR lie in the micromolar range [18]. Because of limitations associated with EPR, separation techniques (e.g., HPLC and CE) coupled with a sensitive detection scheme give an alternative approach for the indirect detection of ‘OH in biological samples [4].

There are several trapping agents (spin traps) available that form adducts with free radicals of biological importance (e.g., ‘OH) [4, 16, 17]. The most commonly used trapping agents (diamagnetic compounds) are 2-hydroxybenzoic acid (2-HBA), 4-hydroxybenzoic acid (4-HBA), 5,5-dimethyl-1-pyrroline-n-oxide (DMPO), α-phenyl-n-tert-butyl nitroline (PBN), and α-(4-pyridyl-1-oxide)-n-tert-butyl nitroline (4-
Articles reporting the use of microdialysis sampling, separations, and trapping agents are of particular interest and are discussed below.

2-HBA (salicylic acid) is the most widely used trapping agent. The o-, p-directed hydroxylated products of 2-HBA, 2,4-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) (Figure 3.2), have been determined by HPLC with electrochemical (EC) detection (amperometric detection) [19-21]. Since two product are formed (quantitatively important), the ‘OH signal is split between 2,3-DHBA and 2,5-DHBA [22]. This may lead to decreased detection sensitivity. Between these two products, only 2,3-DHBA may be monitored for the accurate reflection of ‘OH in vivo since there are reports that 2,5-DHBA could be formed through enzymatic pathways by the action of cytochrome P450 [23].

Both systematic dose and delivery through microdialysis have been used to administer 2-HBA to experimental animals (rats) [19, 24]. The basal concentration of ‘OH (as 2,3-DHBA or 2,5-DHBA) was reported to be 35 nM in heart microdialysates of anesthetized rats [19]. Increased generation of ‘OH was observed in the heart and brain of rats as a result of ischemia-reperfusion [25-28]. Myocardial ischemia was induced by occlusion of the left anterior descending (LAD) coronary artery [25-27], and cerebral ischemia was performed by clamping both carotid arteries and the cerebral artery [29-31]. Reperfusion was achieved by removing the occluder or clamps.

More recently, 4-HBA has gained much attention as a radical trap, although it is a constituent of urine, produced from tyrosine by the intestinal flora [6]. Normal rat
Figure 3.1. Reactions of DMPO, PBN, and POBN with \( \cdot \text{OH} \).
Figure 3.2. Hydroxylation of 2-HBA.
blood contains very low concentrations of 4-HBA (~ 100 nM) and 3,4-DHBA (~ 10 nM) [6]. Unlike 2-HBA, hydroxylation of 4-HBA produces one specific product (quantitatively important), the \( p \)-directed hydroxylated product, 3,4-dihydroxybenzoic acid (3,4-DHBA) (Figure 3.3) [6, 18, 29, 30, 32]. Besides this, there is no report on the oxidative metabolism of 4-HBA by cytochrome P450. The reaction rate of \( \cdot \text{OH} \) and 4-HBA for the formation of 3,4-DHBA is \( 8.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) [33]. Montgomery et al. published the first report on the \textit{in vivo} application of 4-HBA for the continuous monitoring of \( \cdot \text{OH} \) in microdialysis samples [6, 34, 35]. HPLC-EC (coulometric and amperometric detection) and gas chromatography-mass spectrometry (GC-MS) have been used to monitor 3,4-DHBA and 4-HBA [6, 34, 35]. The basal concentration of \( \cdot \text{OH} \) (as 3,4-DHBA) was reported to be in the range of 56-77 nM in the brain cortex [31]. Like 2-HBA, 4-HBA was administered by systematic dose and also by delivery through a microdialysis probe [6, 29-31]. Very low production of 3,4-DHBA was observed following systemic administration of 4-HBA [6]. Delivery of 4-HBA through a microdialysis probe was determined to be the preferred method over systemic delivery since delivery of 4-HBA was maintained at a constant level over the time course of the experiment (before and after induced ischemia-reperfusion) [29, 30]. 4-HBA has been mainly used in the rat brain [6, 29-32, 34-38]. An increased level of 3,4-DHBA was observed after cerebral ischemia-reperfusion [29-31]. Although 4-HBA has been used successfully to monitor 3,4-DHBA \textit{in vivo} by HPLC-EC, there is no report for an \textit{in vitro} study to determine the specificity of 4-HBA hydroxylation. For example, the hydroxylated product, 3,4-DHBA, could be formed
Figure 3.3. Hydroxylation of 4-HBA.
in vivo from 4-HBA through its oxidative metabolism (e.g., catalyzed by cytochrome P450 in biological systems (not only by the direct addition of \( \cdot OH \) to 4-HBA).

There are few reports on the use of nitroene spin traps (DMPO and POBN) with microdialysis and HPLC. A previous report with microdialysis has used spin trapping (DMPO) EPR detection [39]. Most recently, Allyson Charbonnet demonstrated the use of DMPO and POBN to trap \( \cdot OH \) in brain microdialysates using HPLC-EC coupled to microdialysis, but this approach was not able to detect radical adducts of \( \cdot OH \) and trapping agents (DMPO and POBN) in microdialysates [13]. The other nitroene spin trap, PBN, has been used as a neuroprotective agent, while 4-HBA was delivered systematically. Microdialysis sampling was subsequently used to monitor \( \cdot OH \) production (as 3,4-DHBA) by HPLC-EC [31, 36]. PBN has been attributed to scavenge \( \cdot OH \) during traumatic brain injury in rats, as reflected in the decrease of 3,4-DHBA formation during ischemia-reperfusion [31, 36].

The application of CE to determine \( \cdot OH \) in biological samples is not common. Presently, there is no report for the indirect determination of \( \cdot OH \) in biological samples using CE. As mentioned in Section 3.1, this technique is suitable for the analysis of small sample volumes, such as microdialysis samples. Development of a sensitive CE method along with microdialysis sampling for the indirect determination of \( \cdot OH \) in biological samples will be beneficial in oxidative stress investigations.
3.3 Specific Aims

The purpose of this work was to evaluate 4-HBA as a trapping agent for the indirect determination of \( \cdot \text{OH} \) (as 3,4-DHBA). To perform this study, first an analytical method using microdialysis and CE was developed for the determination of 3,4-DHBA. The indirect determination involved trapping of \( \cdot \text{OH} \) with 4-HBA and quantifying 3,4-DHBA (the adduct of \( \cdot \text{OH} \) and 4-HBA) using the developed CE method. Ultra-violet (UV) detection was employed for this investigation. To demonstrate the ability of 4-HBA to trap \( \cdot \text{OH} \) and the applicability of the CE-UV method to detect 3,4-DHBA, \( \cdot \text{OH} \) generation systems \textit{in vitro}, such as UV irradiation of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), the Fenton reaction and hypoxanthine/xanthine oxidase (HX/XO) system, were studied. Also, microsomal metabolism of 4-HBA was investigated \textit{in vitro} to determine if microsomal enzymes catalyzed the oxidation of 4-HBA to 3,4-DHBA. Figure 3.4 shows the microdialysis sampling process for the delivery of the trapping agent and recovery of 3,4-DHBA through a microdialysis probe.

3.4 Experimental

3.4.1 Materials

3,4-dihydroxybenzoic acid (3,4-DHBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), 4-hydroxybenzoic acid (4-HBA), hydroquinone (HQ), resorcinol, uric acid (UA), xanthine, hypoxanthine (HX), sodium tetraborate decahydrate (\( \text{Na}_2\text{B}_4\text{O}_7.10\text{H}_2\text{O} \), borax), tetradecyltrimethylammonium bromide (TTAB), \( \beta- \)
Figure 3.4. Microdialysis sampling process employed for the radical trapping experiment. (1) Delivery of 4-HBA through the microdialysis probe as the perfusate. (2) Diffusion of 4-HBA into the extracellular space around the probe. (3) Formation of 3,4-DHBA (trapping of OH with 4-HBA). (4) Diffusion of 3,4-DHBA across the semipermeable membrane from the tissue site into the probe. (5) Collection of microdialysate containing 3,4-DHBA.
nicotinamide adenine dinucleotide phosphate sodium salt (β-NADP), D-glucose 6-phosphate sodium salt, 3,4-dihydroxyphenylacetic acid (DOPAC), and perchloric acid (HClO₄, 70%) were purchased from Sigma (Saint Louis, MO). Enzymes such as xanthine oxidase (XO, 18 mg/mL protein, 10.64 Units/mL), superoxide dismutase (SOD, 2 mg/mL protein, 9600 Units/mL), and glucose 6-phosphate dehydrogenase (0.260 mg/mL protein, 213 Units/mL) were obtained from Sigma (Saint Louis, MO). Microsomes from male rat liver (20 mg/mL protein content) were purchased from Sigma (Saint Louis, MO). Inorganic salts, such as sodium chloride (NaCl), calcium chloride (CaCl₂.2H₂O), potassium chloride (KCl), magnesium chloride (MgCl₂.6H₂O), ferric chloride (FeCl₃), ferrous chloride (FeCl₂), sodium dihydrogenphosphate monohydrate (NaH₂PO₄.H₂O), and disodium hydrogen phosphate (Na₂HPO₄) were purchased from Fisher Scientific (Fair Lawn, NJ). Also, sodium hydroxide (NaOH), hydrochloric acid (37%, HCl), methanol (MeOH), and hydrogen peroxide (H₂O₂, 30%) were obtained from Fisher Scientific (Fair Lawn, NJ). Anesthetics (isoflurane, xylazine, acepromazine, and ketamine) were supplied by the Animal Care Unit, the University of Kansas.

3.4.2 Sample Preparation

Nanopure water (18.2 MΩ, Water Pro PS, Labconco, Kansas City, MO) was used to prepare all solutions. Stock solutions (10 mM) of 3,4-DHBA, 2,4-DHBA, 4-HBA, HX, HQ, and resorcinol were prepared in 0.1 M HClO₄. Xanthine and UA stock solutions (10 mM) were prepared in basic solution. All stock solutions were
stored in the refrigerator. Working standard solutions were made from stock solutions by serial dilution with Ringer’s solution and were prepared fresh daily. Ringer’s solution was prepared in the lab and consisted of 155 mM NaCl, 5.5 mM KCl, and 2.3 mM CaCl₂·2H₂O. Both FeCl₃ (5 mM) and FeCl₂ (5 mM) were made in nanopure water. The background electrolyte (BGE) consisted of borax and TTAB. Before use all solutions were filtered through 0.22 µm pore size syringe filters (Millipore Millex™ GP, Fisher Scientific).

3.4.3 CE System

A home-built CE system was used in this study. UV detection was performed with a SpectraPhysics UV1000 (Thermoseparation, San Jose, CA) at wavelengths of 254 nm and 295 nm. For EC (amperometric) detection, a BAS LC-4C (Bioanalytical Systems, Inc., West Lafayette, IN) was used. On-column (capillary) decouplers and carbon fiber (33 µm diameter) electrodes were fabricated according to the procedures described in the literature [40, 41]. The separation was performed in a polyimide coated fused silica capillary of 50 µm inner diameter (ID) and 360 µm outer diameter (OD) (Polymicro Technologies, Phoenix, AZ). The injection end of the capillary was housed in a Plexiglass safety box fitted with an interlock. New capillaries were conditioned by flushing hydrodynamically at 10 psi with MeOH (10 min), H₂O (5 min), 1 M HCl (5 min), H₂O (5 min), 0.1 M NaOH (10 min), H₂O (5 min), and BGE (10 min). Separation voltage in the negative polarity mode was applied across the capillary using a high voltage power supply unit (CZE1000R, Spellman High Voltage
Electronics, Hauppauge, NY) to drive electrophoresis. To reverse the EOF, TTAB (a cationic surfactant) was added to the BGE at a concentration of 0.5 mM. As a result, electromigration of anions and EOF were achieved in the same direction, toward the detector and anodic end. Sample injection was performed electrokinetically, and made from a 10 µL sample volume, unless otherwise noted. All experiments were performed at room temperature. Data was collected using Chrom&Spec™ data acquisition software (Ampersand International, Inc., Beachwood, OH).

3.4.4 Microdialysis Sampling

3.4.4.1 Apparatus

A CMA/100 microinjection pump (CMA/Microdialysis AB, Stockholm, Sweden) with a 1 mL syringe (Hamilton gas tight # 1001, Hamilton, Reno, NV) was used to deliver perfusate through the microdialysis probe. Linear probes of 2 mm active length (as described in Section 3.4.4.2) were used for microdialysis studies. All *in vitro* microdialysis experiments were performed in a 1 mL glass vial. The vial was placed in a dry heat block, and a temperature of 37 °C was maintained with gentle stirring. The perfusate was delivered through the probe at a flow rate of 1 µL/min by the microinjection pump. The collected microdialysates were analyzed without sample pretreatment or dilution.
3.4.4.2 Probe Fabrication

Linear microdialysis probes of 2 mm active length (12.2 cm long) were made in-house from polyacrylonitrile (PAN) semipermeable membrane (Hospal, Meyzieu, Germany) and polyimide tubing (MicroLumen Inc. Tampa, FL). The ID and OD of the PAN membrane were 350 and 250 µm, respectively, and its molecular weight cut-off was 40 kDa. The ID and OD of the polyimide tubing were 122 and 163 µm, respectively. Probes were manufactured as follows. Two pieces of polyimide tubing (6 inch long each) were inserted into a 1.5 cm long piece of the PAN membrane to give an active length of 2 mm. Then, two ends of the membrane were glued to the polyimide tubing using UV glue (UVEXS, Ultraviolet Exposure Systems, Sunny Vale, CA). One end of the linear probe was glued with a small piece of TYGON® tubing (0.050 inch ID and 0.090 inch OD) (Cole-Parmer Instrument Company, Vernon Hills, Illinois). Through this tubing, the probe was connected to a peek needle (51 mm length, 0.76 mm ID, and 1.52 mm OD, Hamilton Company, Reno, NV) of the syringe placed in the microinjection pump.

3.4.5 In Vitro Hydroxyl Radical Generation Systems

Three in vitro hydroxyl radical generation systems (UV photolysis of H$_2$O$_2$, the Fenton reaction, and HX/XO system) were investigated to demonstrate the capability of 4-HBA to trap ‘OH. To reduce any non-specific production of 3,4-DHBA, glassware was cleaned with nitric acid (50%), and a non-metal syringe needle (i.e., peek needle) was used in the microdialysis studies.
3.4.5.1 UV Photolysis of H$_2$O$_2$

UV photolysis of H$_2$O$_2$ provides a simple way to generate ‘OH in vitro. This system has been used to trap ‘OH by 2-HBA and DMPO [13, 22, 42]. To perform this experiment, 80 µL of Ringer’s solution, 10 µL of H$_2$O$_2$ (30%), and 10 µL of 4-HBA (1 mM) were added to a 5 mL beaker. Then, UV irradiation was performed by placing a UV lamp directly on top of the beaker for different durations (6, 12, and 18 min). Samples (before and after irradiation) were analyzed by CE-UV for 3,4-DHBA.

3.4.5.2 Fenton Reaction

The Fenton reaction has been utilized previously to evaluate the trapping ability of 2-HBA and 4-HBA [6, 43, 44]. To carry out the Fenton reaction in the presence of 4-HBA, 840 µL of Ringer’s solution, 100 µL of H$_2$O$_2$ (30%), and 20 µL of 4-HBA (10 mM) were placed in a 1 mL glass vial. Then, 40 µL of FeCl$_2$ (5 mM) was added to generate ‘OH. The samples (before and after addition of FeCl$_2$) were analyzed by CE-UV for the radical adduct (3,4-DHBA).

3.4.5.3 Hypoxanthine/Xanthine Oxidase System

The HX/XO system has been used previously to generate ‘OH in vitro [17, 18, 22, 45]. This experiment was performed in an in vitro microdialysis setup (Figure 3.5), and a buffered Ringer’s solution (a solution of 145 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$.6H$_2$O, 1.2 mM CaCl$_2$.2H$_2$O, 0.45 mM NaH$_2$PO$_4$.H$_2$O, and 2.33 mM Na$_2$HPO$_4$ at pH 7.4) was used to prepare the reaction bath. A mixture of 20 µL of HX
Figure 3.5. *In vitro* microdialysis setup for the HX/XO system.

(a) Reaction mixture of HX, FeCl$_3$, 4-HBA, and XO
(b) Reaction mixture of HX, FeCl$_3$, 4-HBA, SOD, and XO
(10 mM), 20 µL of 4-HBA (10 mM), 20 µL of FeCl₃ (5 mM), and 902.4 µL of buffered Ringer’s solution at pH 7.4 was placed in a 1 mL glass vial. A linear microdialysis probe was placed into the vial, and the probe was perfused with buffered Ringer’s solution at a flow rate of 1 µL/min using the microdialysis apparatus. Six microdialysates (before the addition of XO in the mixture) were collected at 10 min intervals to obtain the baseline concentration of 3,4-DHBA. Once a baseline concentration was obtained, a volume of 37.6 µL of XO was added, and microdialysates (after the addition of XO) were collected for 60 min at 10 min intervals and analyzed for 3,4-DHBA.

For the deoxygenated HX/XO system, prior to experiments, the aforementioned reaction mixture was flushed with argon gas for 45 min to remove dissolved oxygen. Also, the vial contents were maintained under argon throughout the course of the experiment.

3.4.5.4 Hypoxanthine/Xanthine Oxidase System in the Presence of Superoxide Dismutase

This experiment was performed in a manner similar to the HX/XO system except that 10 µL of SOD and a reduced content of buffered Ringer’s solution (892.4 µL) were placed in a glass vial (1 mL). A linear microdialysis probe was placed into the vial, and buffered Ringer’s solution was used as the perfusate (1 µL/min). Microdialysates were collected at 10 min intervals. First, six microdialysates were collected to obtain the baseline concentration of 3,4-DHBA, and then a 37.6 µL
volume of XO was added to the vial. After the addition of XO to the vial contents, six microdialysates were collected and analyzed to determine any change in the concentration levels of 3,4-DHBA in response to the activity of XO in the presence of SOD.

3.4.6 Microsomal Metabolism of 4-HBA

An *in vitro* microdialysis set up was employed for this experiment. All experiments were performed in a 1 mL glass vial. Incubations were carried out according to the application notes provided with microsomal fractions and procedure used by Ingelman-Sundberg *et al.* [23, 46]. The reaction mixture (1 mL total volume) consisted of 1.3 mM β-NADP, 3.3 mM glucose 6-phosphate, 0.4 Unit/mL glucose 6-phosphate dehydrogenase, and 200 μM 4-HBA in buffered Ringer’s solution at pH 7.4 and maintained at 37 °C. A linear microdialysis probe was immersed into the reaction mixture, and buffered Ringer’s solution was perfused through the probe at 1 μL/min. First, six microdialysates were collected at 10 min intervals and analyzed for 3,4-DHBA. Then, 10 μL of rat liver microsomal fractions (i.e., a source of cytochrome P450 enzymes, 0.2 mg/mL protein) was added to the vial. Six microdialysates collected after the addition of the enzyme were analyzed to determine any change in the concentration levels of 3,4-DHBA in response to the enzymatic activity of microsomal fractions.
3.5 Results and Discussion

3.5.1 CE-UV Method

For the separation of 3,4-DHBA and 4-HBA by CE, a number of buffer systems were investigated, such as ammonium, methylamine, and borate. A borate buffer system (B(OH)$_3$/B(OH)$_4$, $pK_a$ 9.24) was chosen as the BGE since this system had been shown to be effective for the separation of polyhydroxy isomers which have similar electrophoretic mobilities [47, 48]. Borate selectively forms a borate-analyte complex with vicinal hydroxyl groups, and thus it alters the charge to size ratio of the compound. This results in different electrophoretic mobilities among the charged analytes. In this experiment, borax (Na$_2$B$_4$O$_7$.10H$_2$O) with TTAB was used as the BGE.

pH dependence of the peak height of 3,4-DHBA (100 µM) was investigated at different pH values (8.4, 9.0, 9.30, and 9.6) for the BGE concentration of 100 mM borax with 0.5 mM TTAB. At a pH of 8.4, the peak height of 3,4-DHBA was at its greatest. In this buffer system, 4-HBA and 2,4-DHBA co-migrated. However, 3,4-DHBA was baseline resolved from both of these analytes. A BGE composition of 75 mM borax with 0.5 mM TTAB at pH 8.4 produced similar results. In this experiment, the highest possible separation voltage was applied to drive the electrophoresis because application of a higher separation voltage resulted in a reduced analysis time for the analytes. The highest voltage that can be applied with this buffer composition and capillary dimensions (60 cm x 50 µm) was – 8 kV, as determined from an Ohm’s plot. At – 8 kV separation voltage, the buffer system of 75 mM borax with 0.5 mM
TTAB at pH 8.4 generated a current of ~ 26 µA whereas 100 mM borax with 0.5 mM TTAB at pH 8.4 generated ~ 41 µA. Because of the reduced current generation with lower ionic strength borax, this was chosen as the BGE for the CE-UV analysis.

Injection length has an effect on both the sensitivity and resolution of analytes. The effect of injection length was investigated by varying injection times, from 30 to 60 s (Table 3.1). A longer injection time gave higher sensitivity but lower resolution between 3,4-DHBA and 4-HBA. At higher injection voltages, a longer sample plug or zone is injected into the capillary thereby increasing the peak height. As shown in the Table 3.1, a 60 s electrokinetic injection (EKI) at –8 kV gave an increased sensitivity for 3,4-DHBA and good baseline resolution between 3,4-DHBA and 4-HBA.

Table 3.1. Effect of injection length

<table>
<thead>
<tr>
<th>Injection length*</th>
<th>Sensitivity (mAU/µM)</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 s</td>
<td>0.565</td>
<td>7.10</td>
</tr>
<tr>
<td>45 s</td>
<td>0.926</td>
<td>4.70</td>
</tr>
<tr>
<td>60 s</td>
<td>0.994</td>
<td>3.40</td>
</tr>
</tbody>
</table>

*Conditions: 50 µm ID capillary x 60(45) cm, 75 mM borax BGE with 0.5 mM TTAB at pH 8.4, EKI at –8 kV, separation at –8 kV, and UV detection at 295 nm.

The electropherogram for the detection of 100 µM 3,4-DHBA in Ringer’s solution is shown in Figure 3.6. 3,4-DHBA was found to be well separated from 4-HBA, 2,4-DHBA, and other potential interfering compounds, such as HQ, resorcinol, HX, xanthine, and UA (Figure 3.7). HQ and resorcinol are minor products of the
Figure 3.6. Detection of 3,4-DHBA by CE-UV. Conditions: 50 µm ID capillary x 60(45) cm, 75 mM borax BGE with 0.5 mM TTAB at pH 8.4, 60 s EKI at –8 kV, separation at –8 kV, and UV detection at 295 nm.
Figure 3.7. Separation of 3,4-DHBA, 4-HBA, and potential interferents. (A) Separation of 100 µM 3,4-DHBA, 4-HBA, resorcinol, and HQ in Ringer’s solution. (B) Separation of 100 µM 3,4-DHBA, 4-HBA, 2,4-DHBA in Ringer’s solution, UA, xanthine, and HX. Conditions: 30 s EKI at –8 kV, UV detection at 254 nm, and all other conditions same as in Figure 3.6.
hydroxylation reactions of 4-HBA [33, 49]. Xanthine and UA are the products of the HX/XO system which is described later in Section 3.5.4.3.

Figure 3.8 and Table 3.2 show the effect of sample volume in the injection vial on the peak height of 3,4-DHBA. In this experiment, sample injections were made from different volumes of samples (1.25, 2.5, 5, and 10 µL), and the peak heights were subsequently compared. Injections made from different sample volumes resulted in similar peak heights for 1 µM 3,4-DHBA. For a perfusion rate of 1 µL/min in microdialysis sampling, 1.25 min temporal resolution can be achieved with the developed CE method.

3.5.2 Method Validation

The analytical performance of the developed CE-UV method was evaluated at a wavelength of 295 nm and an injection time of 60 s (EKI at –8 kV). A limit of detection (LOD) of 74 nM (S/N = 3) for 3,4-DHBA was achieved. Peak efficiency and peak width at half-height were (15.38 ± 0.22) x 10^6 (n = 3) and 0.3 ± 0.0 s (n = 3), respectively. Linearity with a regression equation of \( y = 2.2234x - 0.0289 \) (\( R^2 = 0.999 \)) was obtained in the concentration range of 125 nM to 10 µM. Intra-day reproducibility with respect to migration time and sensitivity was investigated by making consecutive injections of a 100 µM 3,4-DHBA standard solution. The reproducibility of migration time (min) and sensitivity (mAU/µM) was 8.33 ± 0.06 (n = 3) and 0.994 ± 0.002 (n = 3), respectively. The resolution between 3,4-DHBA and 4-HBA was calculated to be 3.40 ± 0.07 (n = 3).
Figure 3.8. Analysis of 3,4-DHBA from 2.5 µL sample in vial. Sample: 1 µM 3,4-DHBA in Ringer’s solution. Conditions: same as in Figure 3.6.

Table 3.2. Peak height of 3,4-DHBA vs. volume of sample in vial

<table>
<thead>
<tr>
<th>Volume of sample in vial* (µL)</th>
<th>Peak height (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>2.13 ± 0.18</td>
</tr>
<tr>
<td>2.5</td>
<td>2.10 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>2.06 ± 0.11</td>
</tr>
<tr>
<td>10</td>
<td>2.08 ± 0.12</td>
</tr>
</tbody>
</table>

* Sample: 1 µM 3,4-DHBA in Ringer’s solution. Conditions: same as in Figure 3.6 and n = 3.
3.5.3 Stability of 3,4-DHBA and 4-HBA

To perform stability studies, 100 µM 3,4-DHBA and 4-HBA standard solutions (2 mL each) in a 4 mL amber glass vials were left on the lab bench at room temperature for 8 hours. Samples were analyzed by the developed CE-UV method every hour by taking an aliquot of 10 µL each time. Both analytes were found to be stable during the experiment time (8 hours) since peak heights of 3,4-DHBA and 4-HBA did not change significantly (RSD in the range of 4.19 - 6.09%, n = 9). Also, a stability study over 3 days was performed. From this experiment, both 3,4-DHBA and 4-HBA were found to be stable, as peak heights of both analytes did not change significantly (RSD below 5%).

3.5.4 Hydroxyl Radical Generation Systems

3.5.4.1 UV Photolysis of H₂O₂

The trapping ability of 4-HBA was evaluated by the detection of the adduct of \(^{\cdot}\)OH and 4-HBA by the developed CE-UV method. The control experiment consisted of analyzing a reaction mixture of H₂O₂ and 4-HBA before exposure to UV radiation. Figure 3.9A shows that there is no 3,4-DHBA peak in the electropherogram before exposure to UV light, while the 3,4-DHBA peak (confirmed by spiking with a 3,4-DHBA standard) appears upon exposure to UV light (Figure 3.9B). 3,4-DHBA was formed through the hydroxylation of 4-HBA after UV irradiation as predicted, and 3,4-DHBA was readily detected by CE-UV. The concentration of 3,4-DHBA increased over the UV irradiation times (6, 12, and 18 min), indicating increased
Figure 3.9. UV photolysis of \( \text{H}_2\text{O}_2 \): Trapping of \(^\text{•}\text{OH}\) with 4-HBA. (A) Electropherogram of control (mixture of 4-HBA and \( \text{H}_2\text{O}_2 \)). (B) Electropherogram of reaction products after 12 min UV irradiation. (C) Effect of incubation time on 3,4-DHBA formation (n = 3). Conditions: same as in Figure 3.6.
production of \( \cdot \text{OH} \) resulting from longer exposure to UV radiation (Figure 3.9C). This UV photolysis study suggests that 4-HBA can trap \( \cdot \text{OH} \), and 3,4-DHBA can be detected by CE-UV. Also, the peak heights of the unknown products (peaks 1 and 2 of Figure 3.9B) increased with longer irradiation times.

### 3.5.4.2 Fenton Reaction

Upon addition of \( \text{FeCl}_2 \) into the mixture of \( \text{H}_2\text{O}_2 \) and 4-HBA, 3,4-DHBA appeared (Figure 3.10). As shown in Figure 3.10B, the Fenton reaction produced 3,4-DHBA (~2.69 \( \mu \text{M} \)) and other products, unknown peaks 1 and 2. The identity of 3,4-DHBA peak was confirmed by spiking with a known concentration of 3,4-DHBA. \( \cdot \text{OH} \) was formed, according to Fenton chemistry, between \( \text{H}_2\text{O}_2 \) and \( \text{Fe}^{2+} \), and 3,4-DHBA formation was due to the attachment of \( \cdot \text{OH} \) to the 3 position of 4-HBA. As in the photolysis experiment, it can be concluded that 4-HBA can trap \( \cdot \text{OH} \), and 3,4-DHBA can be readily detected by CE-UV. The time course for the formation of 3,4-DHBA and others products is shown in Figure 3.10C. 3,4-DHBA and unknown products (peaks 1 and 2) are plotted together with 4-HBA, assuming all products have a similar molar extinction coefficient. It is evident from Figure 3.10C that the amount of products increases with the disappearance of 4-HBA over incubation time.

### 3.5.4.3 Hypoxanthine/Xanthine Oxidase Systems

As mentioned in Chapter 1, the HX/XO system is one of the proposed pathways for the generation of superoxide (\( \text{O}_2^- \)) in living systems during ischemia-
Figure 3.10. Fenton reaction: Trapping of ‘OH with 4-HBA. (A) Electropherogram of control (mixture of 4-HBA and H₂O₂). (B) Electropherogram of reaction products 10 min after addition of Fe²⁺ (mixture of 4-HBA, H₂O₂, and FeCl₂). (C) Time course of products’ formation and 4-HBA disappearance (n = 3). Conditions: same as in Figure 3.6.
reperfusion [3, 14, 50-53]. It involves enzymatic processes where HX is a substrate of the XO enzyme. In this system, XO catalyzes the conversion of HX to xanthine, and it can further catalyze the conversion of xanthine to UA. During these oxidation processes, the escape of one electron to O₂ produces O₂⁻. The reaction products of this system are xanthine, UA, H₂O₂, and O₂⁻ (Figure 1.2 of Chapter 1). In the presence of Fe³⁺, O₂⁻ can react with Fe³⁺ to form Fe²⁺ which then can react with H₂O₂ to produce ·OH, according to Fenton chemistry (Eqs. 3.1 and 3.2). Hence, the amount of ·OH formed is dependent upon the availability of O₂⁻. ·OH generation in the HX/XO system in the presence of SOD, a metalloprotein enzyme, is expected to be reduced since SOD catalyzes the conversion of O₂⁻ to O₂ and H₂O₂. This is called the dismutase reaction (Eqs. 3.3 to 3.5).

\[
\begin{align*}
\text{Fe}^{3+} + O_2^- & \longrightarrow \text{Fe}^{2+} + O_2 \quad \text{(Eq. 3.1)} \\
\text{Fe}^{2+} + H_2O_2 & \longrightarrow \text{Fe}^{3+} + \cdot OH + \cdot OH \quad \text{(Fenton reaction)} \quad \text{(Eq. 3.2)} \\
O_2^- + H_2O_2 & \longrightarrow \cdot OH + \cdot OH + O_2 \quad \text{(Haber-Weiss reaction)} \quad \text{(Eq. 3.3)} \\
2O_2^- + 2H^+ & \xrightarrow{\text{SOD}} O_2 + H_2O_2 \quad \text{(Eq. 3.4)} \\
4\text{-HBA} + \cdot OH & \longrightarrow 3,4\text{-DHBA} \quad \text{(Trapping reaction)} \quad \text{(Eq. 3.5)}
\end{align*}
\]
Figures 3.11 and 3.12 show electropherograms obtained from the HX/XO systems without and with SOD, respectively. In the case of both systems after the addition of XO, UA and xanthine were produced as a result of XO activity. The time course of 3,4-DHBA formation in HX/XO systems is shown in Figure 3.13. Initially, some 3,4-DHBA was seen in the reaction mixture containing 4-HBA in both systems (Figures 3.11 and 3.12). Upon addition of XO in the reaction mixture (without SOD) at 0 min, there was an increase (~1.78 times from the baseline value) in the concentration of 3,4-DHBA. But in the presence of SOD, the formation of 3,4-DHBA was reduced significantly, as shown in Figure 3.13. This indicates that SOD was involved in the dismutase reaction (Eq. 3.4), and therefore a reduced amount of ‘OH was formed. Hence, it can be concluded that the formation of 3,4-DHBA was due to the specific addition of ‘OH (generated due to XO activity) to the 3 position of 4-HBA.

Oxygen plays a significant role in the HX/XO system since it is a substrate of XO. The products formed by the HX/XO system are dependent upon the availability of oxygen in the reaction mixture, as shown in Figure 3.14. In the case of the deoxygenated HX/XO system, UA was not observed after the addition of XO. As mentioned above, xanthine is oxidized to UA, and this oxidation is catalyzed by XO in the presence of oxygen. Therefore, conversion of xanthine to UA was not observed because of the lack of oxygen. Also, it is evident from Figures 3.14A and 3.14B that the conversion of HX to xanthine or UA and the generation of 3,4-DHBA were reduced in comparison to the normal HX/XO system (with no deoxygenation).
Figure 3.11. HX/XO system without the presence of SOD. (A) Electropherogram of a mixture of 4-HBA, Fe$^{3+}$, and HX (before the addition of XO). (B) Electropherogram of the reaction products 10 min after the addition of XO in a mixture of 4-HBA, Fe$^{3+}$, and HX. Conditions: same as in Figure 3.6.

Figure 3.12. HX/XO system in the presence of SOD. (A) Electropherogram of a mixture of 4-HBA, Fe$^{3+}$, HX, and SOD (before the addition of XO). (B) Electropherogram of the reaction products 10 min after the addition of XO in a mixture of 4-HBA, Fe$^{3+}$, HX, and SOD. Conditions: same as in Figure 3.6.
Figure 3.13. Time course of 3,4-DHBA formation in HX/XO systems without and with SOD (n = 3). Conditions: XO added at 0 min and all other conditions same as in Figure 3.6.
Figure 3.14. Comparison of normal and deoxygenated HX/XO systems. (A) Electropherogram of the reaction products 10 min after the addition of XO (normal HX/XO system). (B) Electropherogram of the reaction products 10 min after the addition of XO (deoxygenated HX/XO system). Conditions: same as in Figure 3.6.
3.5.5 Microsomal Metabolism of 4-HBA

An *in vitro* investigation was performed to determine if cytochrome P-450 enzymes catalyzed the oxidation of 4-HBA to 3,4-DHBA. Liver microsomal fractions from male rats were used as a source of cytochrome P-450 enzymes. Cytochrome P-450 enzymes are a family of heme containing proteins found in tissues, particularly in the liver [54, 55]. These enzymes are associated with the oxidative metabolism of xenobiotics in biological systems [56, 57]. The types of reactions catalyzed by cytochrome P450 include the oxidation of alcohols and aldehydes, carbon hydroxylation, and aromatic hydroxylation (Figure 3.15) [56, 58].

The time course for the formation of 3,4-DHBA in the microsomal metabolism study is shown in Figure 3.16. As shown in Figure 3.16, upon addition of microsomal enzymes in the reaction system at 0 min, 3,4-DHBA generation increased to 0.450 µM (at 10 min) from a baseline value of ~ 0 µM. This significant increase in 3,4-DHBA concentration suggested that 4-HBA was metabolized to 3,4-DHBA by cytochrome P-450 enzymes. However, no literature exists on the *in vitro* microsomal metabolism study of 4-HBA to compare with this result. But a lower metabolic production of 3,4-DHBA *in vivo* has been reported when 4-HBA has been used as a trapping agent in the perfusate for the determination of ‘OH as 3,4-DHBA in the rat brain [6]. Non-specific production of 3,4-DHBA from 4-HBA through oxidative metabolism is not desirable when 4-HBA is used to trap ‘OH *in vivo*. This is because of the fact that assaying 3,4-DHBA would not reflect the actual concentration of ‘OH in biological systems.
Figure 3.15. Hydroxylation of a substrate by cytochrome P450. Heme iron represents the active site of cytochrome P450. (1) Binding of the substrate (RH). (2) Reduction of the Fe$^{3+}$ to the Fe$^{2+}$ state. (3) Binding of O$_2$ (formation of a ferric-superoxide species). (4) Transfer of an electron and addition of protons (formation of a ferric-proxy species). (5) Release of H$_2$O and formation of a Fe$^{5+}$=O (perferryl) species. (6) Abstraction of hydrogen atom from the substrate to produce a substrate radical. (7) Addition of –OH to substrate radical (R'). (8) Release of the hydroxylated product (ROH) and return of iron to Fe$^{3+}$ state. Adapted and modified from reference [58].
Figure 3.16. Microsomal metabolism of 4-HBA: Time course of 3,4-DHBA formation (n = 3). Conditions: microsomes added at 0 min, 30 s. EKI at –8 kV, and all other conditions same as in Figure 3.6.
3.6 Conclusion

A CE-UV method coupled to microdialysis was developed using 4-HBA as a trapping agent for the determination of ‘OH (as 3,4-DHBA, the adduct of ‘OH and 4-HBA). The detection limit determined for 3,4-DHBA was 74 nM.

The CE-UV method was utilized in ‘OH generation systems in vitro (UV photolysis of \( \text{H}_2\text{O}_2 \), the Fenton reaction, and HX/XO systems) in order to evaluate 4-HBA as a trapping agent of ‘OH. It was found that 4-HBA was capable of trapping ‘OH, and the CE-UV method developed was able to detect 3,4-DHBA in the reaction products. Particularly, the experiments involving HX, XO, and SOD suggested that the formation of 3,4-DHBA was due to the specific addition of ‘OH to 3 position of 4-HBA.

The hydroxylation of 4-HBA by microsomal enzymes was also observed in the in vitro study. This suggested that 3,4-DHBA could be generated in vivo through the oxidative metabolism of 4-HBA (where 4-HBA would be used in the in vivo analysis as a trapping agent). Hence, an in vivo study was needed to verify this.
3.7 References


Chapter 4: Monitoring Hydroxyl Radicals as 3,4-Dihydroxybenzoic Acid in Microdialysis Samples

4.1 Introduction

As presented in Chapter 3, a capillary electrophoresis (CE) method was developed for the indirect determination of hydroxyl radicals (‘OH) in microdialysis samples using 4-hydroxybenzoic acid (4-HBA) as a trapping agent. The results of the \textit{in vitro} studies showed the effectiveness of the CE-UV method to detect 3,4-dihydroxybenzoic acid (3,4-DHBA) in ‘OH generation systems. The \textit{in vitro} microsomal incubation study of 4-HBA suggested that 3,4-DHBA could be produced \textit{in vivo} through the oxidative metabolism of 4-HBA. Non-specific generation of 3,4-DHBA is not desirable since it makes data interpretation difficult. Hence, it is necessary to evaluate further the potential of 4-HBA as a trapping agent in experiments \textit{in vivo}. The \textit{in vivo} investigations will help to determine the usefulness of CE-UV for the detection of 3,4-DHBA in biological samples and also assess the extent of non-specific production of 3,4-DHBA, if any.

4-HBA has previously been utilized \textit{in vivo} as a trapping agent of ‘OH where only the change in the concentration of 3,4-DHBA from its basal concentration (as a result of induced oxidative stress) was monitored to assess the generation of ‘OH. As mentioned in Chapter 3, there is no report on the use of CE coupled to microdialysis to investigate oxidative stress by monitoring ‘OH \textit{in vivo}. High performance liquid chromatography (HPLC) has been commonly used with microdialysis for the indirect determination of ‘OH (as 3,4-DHBA) in the rat brain using 4-HBA as the trapping
agent [1]. A concentration detection limit of ~ 1.5 nM with 15 µL injection volume has been readily achieved using HPLC with electrochemical (EC) detection [2]. The reported temporal resolution for the in vivo analysis using HPLC coupled with microdialysis lies in the range of 10 to 30 min [2-6].

4.2 Specific Aims

This chapter focuses on the application of the CE-UV method developed (as presented in Chapter 3) for the indirect determination of ‘OH in biological samples. A microdialysis sampling technique was used to deliver 4-HBA continuously into the heart of anesthetized Sprague Dawley rats. 3,4-DHBA (the adduct of ‘OH and 4-HBA) was monitored in heart microdialysates before and after the induced ischemia-reperfusion in order to assess any changes in 3,4-DHBA.

4.3 Experimental

4.3.1 Materials

3,4-DHBA and 4-HBA were purchased from Sigma (Saint Louis, MO). Sodium chloride (NaCl), calcium chloride (CaCl₂.2H₂O), potassium chloride (KCl), disodium ethylenediamine tetra-acetate (Na₂EDTA), sodium phosphate monobasic (NaH₂PO₄.2H₂O), and methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ). Tetradecyltrimethylammonium bromide (TTAB), sodium tetraborate decahydrate (Na₂B₄O₇.10H₂O, borax), and perchloric acid (HClO₄, 70%) were purchased from Sigma (Saint Louis, MO). All anesthetics, such as isoflurane,
xylazine, acepromazine, and ketamine, were supplied by the Animal Care Unit, the University of Kansas.

4.3.2 Sample Preparation

All solutions were prepared with nanopure water (18.2 MΩ, Water Pro PS, Labconco, Kansas City, MO). Stock solutions of 4-HBA (10 mM) and 3,4-DHBA (10 mM) were prepared in 0.1M HClO₄ and stored in the refrigerator. Working standard solutions of 4-HBA and 3,4-DHBA were made fresh daily by dilution of stock solutions with Ringer’s solution. Ringer’s solution was made in the lab and consisted of 155 mM NaCl, 5.5 mM KCl, and 2.3 mM CaCl₂·2H₂O.

4.3.3 CE-UV System

A home-built CE-UV system, which was described in Section of 3.4.3 of Chapter 3, was used for this study. The background electrolyte (BGE) consisted of 75 mM borax with 0.5 mM TTAB at pH 8.4. Before use, BGE and Ringer’s solution were filtered through 0.22 µm pore size syringe filters (Millipore Millex™ GP, Fisher Scientific).

4.3.4 Microdialysis Sampling

4.3.4.1 Apparatus

A microinjection pump (CMA/100, CMA Microdialysis AB, Stockholm, Sweden) with a 1 mL syringe (Hamilton gas tight # 1001, Hamilton, Reno, NV) was
used to deliver perfusate through the linear microdialysis probe. For the in vivo analysis, the probe was implanted in the hearts of anesthetized male Sprague Dawley rats, as described in Section 4.3.4.3. The perfusate (Ringer’s solution or 100 µM of 4-HBA in Ringer’s solution) was delivered through the implanted probe at a flow rate of 1 µL/min. The collected microdialysates were analyzed without any sample pretreatment or dilution.

4.3.4.2 Probe Fabrication

Linear microdialysis probes (2 mm active length) were made in-house from polyacrylonitrile (PAN) membrane (Hospal, Meyzieu, Germany) and polyimide tubing (MicroLumen Inc. Tampa, FL). Fabrication of probes was described in Section 3.4.4.2 of Chapter 3. In addition, a 27 gauge needle (0.5 inch long) was glued at one end of the probe using UV glue. This was used as an implantation guide during the surgery.

4.3.4.3 Surgical Procedure

Male Sprague Dawley (350 – 400 gm) rats were pre-anesthetized by inhalation of isoflurane, and then a mixture of ketamine (67.5 mg/kg), xylazine (3.4 mg/kg), and acepromazine (0.67 mg/kg) was injected subcutaneously for full anesthetia. To maintain anesthesia throughout the entire experiment, booster doses of ketamine ($1/4^{th}$ of the initial dose) were given every hour. The anesthetized rats were maintained at a temperature of 37 °C by keeping them on heated mats connected to a
CMA/150 temperature controller. Body temperature was monitored by means of a rectal temperature probe attached to the temperature controller. The electrocardiogram (ECG, Figure 4.1) of the rat was monitored continuously by a PowerLab system (ADInstruments, Colorado Spring, CO).

During surgery, initially the throat of the anesthetized rat was shaved and cut longitudinally. Then, the trachea was isolated and intubated with a plastic tube for artificial respiration. Artificial respiration was provided by a constant-volume respirator (Rodent Respirator, Model 683, Harvard Apparatus, Holliston, MA). The thoracic cavity was shaved and cleaned for the implantation of the probe. After opening the chest by a left thoracotomy, a small portion (~ 2 cm) of rib positioned directly above the heart was removed to expose the heart. To keep the rib cage open, a rib spreader was placed in the incision. The needle end of the linear probe was threaded through the apex of the heart (i.e., adjacent to the left anterior descending (LAD) artery) to place the semipermeable membrane. The needle was cut off from the polyimide tubing before connecting the probe to the microdialysis apparatus. The probe was flushed for 3 hours with the trapping agent (100 µM 4-HBA in Ringer’s solution) prior to collection of samples. Microdialysis samples were collected in 10 min intervals.

For inducing occlusion (physically induced ischemia), initially a suture was threaded under the LAD artery, and a snare was formed by passing both ends of the suture through a small plastic tube [7-9]. Then, tension was created by clamping the
Figure 4.1. PQRST wave of ECG.
snare against the surface of the heart. Reperfusion was obtained after ischemia (occlusion phase) by releasing the tension (Figure 4.2). This procedure is a model for myocardial ischemia-reperfusion [8, 10, 11]. Myocardial ischemia caused typical changes in the ECG. Representative ECGs of a rat heart at different phases (before and during occlusion and during reperfusion) are shown in Figure 4.3. The ST segment elevations of the PQRST waves in ECG during occlusion were an indication of ischemia, as illustrated in Figure 4.3B [12].

Occlusion was performed for either 20 or 30 mins after collecting microdialysates for 1 hour (as basal microdialysates). After the occlusion phase (during the reperfusion phase) microdialysates were collected for 2 hours. At the end of the experiment, the experimental animals were sacrificed. The collected microdialysates were analyzed by either by CE-UV or HPLC-EC-UV.

4.4 Results and Discussion

4.4.1 Detection of 3,4-DHBA in Heart Microdialysates by CE-UV

Initially, a control experiment was conducted using Ringer’s solution as the perfusate. The electropherograms obtained from the CE-UV analysis of basal heart microdialysates are shown in Figure 4.4. In both electropherograms, a peak was observed at the migration time of 3,4-DHBA which was further confirmed by spiking with a standard solution of 3,4-DHBA. However, 3,4-DHBA was not expected to be present while perfusing with Ringer’s solution. This indicates that an endogenous compound is co-migrating with 3,4-DHBA.
Figure 4.2. Schematic of a microdialysis probe and an occluder implanted into the rat heart. Adapted and modified from reference [13].
Figure 4.3. Changes in the ECG of a rat heart caused by occlusion. (A) Before occlusion. (B) During occlusion. (C) During reperfusion. Axis: X axis = Time (0.8 s window) and Y axis = ECG (−0.2 to +0.2 mV).
Figure 4.4. Analysis of heart microdialysates by CE-UV. (A) Microdialysate obtained using Ringer’s solution as the perfusate. (B) Microdialysate obtained using 4-HBA (100 µM in Ringer’s solution) as the perfusate. Conditions: 50 µm ID capillary x 60(45) cm, 75 mM borax BGE with 0.5 mM TTAB at pH 8.4, 60 s EKI at –8 kV, separation at –8 kV, and UV detection at 295 nm.
To help identify the endogenous compound, a DOPAC standard solution was analyzed by the developed CE-UV method. It was chosen initially because of its similarity in structure with 3,4-DHBA (Figure 4.5). DOPAC is a metabolite of dopamine, and is found at high concentrations in the extracellular fluid (~ 1 µM in brain) [14]. An electropherogram of a mixture of 3,4-DHBA and DOPAC standards is presented in Figure 4.5. It shows that these two compounds behave the same electrophoretically. Hence, the endogenous compound, whose migration time is the same as 3,4-DHBA, could be either DOPAC or another endogenous compound (which has the same electrophoretic mobility as 3,4-DHBA).

Initially, attempts were made to separate 3,4-DHBA and DOPAC. In order to improve their separation, micellar electrokinetic chromatography (MEKC) was utilized. In MEKC, two buffer systems were investigated, 75 mM borax with 5 mM TTAB at pH 8.4 and 75 mM borax with 10 mM TTAB at pH 8.4. For MEKC, the surfactant employed must be above its critical micelle concentration (CMC). For TTAB (the surfactant), the CMC is ~3.5 mM [15]. MEKC with these two buffer systems did not resolve DOPAC from 3,4-DHBA. Furthermore, the sensitivity (mAU/µM) of the method for 3,4-DHBA decreased drastically with MEKC (as shown in Figure 4.6). No further TTAB concentrations (above 10 mM) were investigated since a change in TTAB concentration resulted in a decreased signal (mAU) for 3,4-DHBA. Hence, MEKC with UV detection using BGE compositions of borax and TTAB could not be used for the analysis.
Figure 4.5. Electropherogram of a mixture of 3,4-DHBA and DOPAC standards. Conditions: same as in Figure 4.4.
Figure 4.6. Detection of 3,4-DHBA showing the effect of increasing TTAB concentration. (A) 75 mM borax BGE with 0.5 mM TTAB at pH 8.4. (B) 75 mM borax BGE with 5 mM TTAB at pH 8.4. (C) 75 mM borax BGE with 10 mM TTAB at pH 8.4. Conditions: same as in Figure 4.4 except BGE composition.
To improve the separation of 3,4-DHBA and DOPAC, the BGE composition was optimized. Using a BGE composition of 25 mM borax with 0.5 mM TTAB at pH 9.3 in a CE-EC system, DOPAC and 3,4-DHBA were separated. EC detection was employed in CE since CE-UV analysis with this BGE composition was not useful because the necessary detection limit (10 – 20 nM) for 3,4-DHBA was not achieved. An electropherogram of a mixture of DOPAC and 3,4-DHBA standards with CE-EC is shown in Figure 4.7. A detection potential of 800 mV (vs. Ag/AgCl) was employed, which was determined from the hydrodynamic voltammogram (HDV) as shown in Figure 4.8. The detection limit determined for 3,4-DHBA was 250 nM.

A broad peak for 3,4-DHBA was obtained with CE-EC. As a result, the expected detection limit was not achieved. The peak width obtained was increased (to 30 s) when compared to CE-UV where the peak width was 1.8 s. As a result, a decreased signal was obtained. Here, it should be emphasized that UV and EC detection were performed on-column (capillary) and off-column, respectively (Figure 4.9). Off-column EC detection uses a decoupler to isolate the separation current from the EC detection circuit [16, 17]. As shown in Figure 4.9, off-column detection is performed in the electric field free region (a region of no separation voltage) where analytes do not have electrophoretic mobilities, and in which EOF does not exist. Analytes in the field free region are expected to experience longitudinal diffusion which results in peak broadening. In addition to this, a significant peak tailing (Figure 4.7) suggests that adsorption of analyte onto the wall was occurring. Therefore,
Figure 4.7. Separation of 3,4-DHBA, DOPAC, and 4-HBA by CE-EC. Conditions: 50 µM each analyte in Ringer’s solution, 50 µm ID capillary x 65 cm, 25 mM borax BGE with 0.5 mM TTAB at pH 9.3, 45 s. EKI at –12 kV, separation at –12 kV, and detection at 800 mV vs. Ag/AgCl.

Figure 4.8. Hydrodynamic voltammogram of 3,4-DHBA (n = 2). Conditions: same as in Figure 4.7 except detection potential varied throughout.
Figure 4.9. On-column UV detection and off-column EC detection in CE.
analyte-wall interactions in the field free region might be a significant factor in peak broadening.

To improve the sensitivity (nA/µM) of CE-EC for 3,4-DHBA, acetonitrile (ACN) stacking was employed. The mechanism of ACN stacking was discussed earlier in Section 1.4.3.4 of Chapter 1. An electropherogram obtained employing ACN stacking is shown in Figure 4.10. Using ACN stacking, sensitivity was increased 2-fold (Figures 4.7 and 4.10), and a detection limit of 125 nM was achieved. However, this concentration detection limit was not sufficient enough for the in vivo analysis which required a low nanomolar detection limit. Hence, further improvement of the CE-EC method was necessary before it can be applied for the in vivo determination of ‘OH as 3,4-DHBA. This will be discussed in Chapter 5. To continue with this investigation, HPLC was chosen as a secondary analytical method to measure 3,4-DHBA in heart microdialysates, and it is presented in Section 4.4.2.

4.4.2 Detection of 3,4-DHBA in Heart Microdialysates by HPLC-EC-UV

The HPLC system consisted of a pump (ESA 582 solvent delivery system, ESA, Chelmsford, MA), an amperometric detector (LC-4C, BAS, West Lafayette, IN), and a UV detector (SPD-10AV, Shimadzu, Columbia, MD) (Figure 4.11). Separations were performed on a C18 column (Phenomenex Synergi Hydro-RP 150 x 4.6 mm, 4 µm, 80 Å) using a flow rate of 0.3 mL/min. The mobile phase consisted of 85:15 (v/v) mixture of 10 mM NaH₂PO₄·2H₂O with 0.5 mM Na₂EDTA at pH 2.5 and MeOH. This mobile phase was modified from literature [18]. Simultaneous detection
Figure 4.10. Detection of 3,4-DHBA by CE-EC with ACN stacking. Conditions: 50 µM each analyte in Ringer’s solution and ACN (50:50 mixture), 50 µm ID capillary x 65 cm, 25 mM borax BGE with 0.5 mM TTAB at pH 9.3, 45 s. EKI at –12 kV, separation at –12 kV, and detection at 800 mV vs. Ag/AgCl.
Figure 4.11. HPLC system with tandem detection scheme.
of 3,4-DHBA and 4-HBA was achieved using a tandem detection system in which the analytes were first detected by EC (detection of 3,4-DHBA) and subsequently, monitored by UV (detection of 4-HBA) (Figure 4.11). The sample injection loop utilized was 20 µL, and an injection volume of 8 µL was employed. Data was recorded by Chrom&Spec™ (Ampersand International, Inc., Beachwood, OH) data acquisition software.

Chromatograms of 3,4-DHBA and 4-HBA standards are shown in Figure 4.12. A detection potential of 750 mV (vs. Ag/AgCl, glassy carbon electrode) for 3,4-DHBA was determined from its HDV (Figure 4.13). Amperometric detection was not used for 4-HBA since it oxidizes at higher potentials (~ 1100 mV). Therefore, UV detection (at 254 nm wavelength) was employed for its determination. As shown in Figure 4.12B, a small peak of 3,4-DHBA (23.62 ± 6.18 nM, n = 8) was observed in the chromatogram when 100 µM of 4-HBA standard solution was analyzed by HPLC. The linearity equation for 3,4-DHBA (2.5 to 100 nM) was $y = 0.0084x + 0.0196$ ($R^2 = 0.999; n = 4$) and for 4-HBA (12.5 to 100 µM) was $y = 0.7075x + 0.2113$ ($R^2 = 1; n = 4$).

For the in vivo study, first a control experiment was performed using Ringer’s solution as the perfusate. 3,4-DHBA was not detected in heart microdialysates, as shown in Figure 4.14A. However, 3,4-DHBA was detected in heart microdialysates when 4-HBA (100 µM 4-HBA in Ringer’s solution) was used as the perfusate (Figure 4.14B). Using 4-HBA as the perfusate, the concentration of 3,4-DHBA determined in microdialysates was $12.69 \pm 5.07$ nM (n = 8). The in vivo delivery of 4-HBA was
Figure 4.12. Detection of 3,4-DHBA and 4-HBA by HPLC with a tandem detection scheme (EC-UV). (A) Injection of 3,4-DHBA (100 nM in Ringer’s solution). (B) Injection of 4-HBA (100 µM in Ringer’s solution). Conditions: mobile phase - 10 mM NaH₂PO₄.2H₂O with 0.5 mM Na₂EDTA at pH 2.5 and MeOH (85:15, v/v), column - C18 (Phenomenex Synergi Hydro-RP 150 x 4.6 mm, 4 µm, 80 Å), injection volume - 8 µL, flow rate - 0.3 mL/min, EC detection at 750 mV (vs Ag/AgCl, glassy carbon electrode), and UV detection at 254 nm.
Figure 4.13. Hydrodynamic voltammogram of 3,4-DHBA (n = 2). Conditions: all chromatographic conditions same as in Figure 4.12.
Figure 4.14. Representative chromatograms of heart microdialysates by HPLC-EC. (A) Ringer’s solution as the perfusate. (B) 100 µM of 4-HBA in Ringer’s solution as the perfusate. Conditions: same as in Figure 4.12.
As mentioned above, initially a small amount of 3,4-DHBA (23.62 ± 6.18 nM, n = 8) was present in the perfusate (100 µM 4-HBA in Ringer’s solution). Hence, the concentration of 3,4-DHBA present in microdialysates (C_d = 12.69 ± 5.07 nM, n = 8) is less than that in the perfusate (C_p = 23.62 ± 6.18 nM, n = 8). This observation suggests that there was less (or no significant) amount of 3,4-DHBA formation \textit{in vivo} due to the non-specific hydroxylation (e.g., from oxidative metabolism by enzymes) of 4-HBA. This result was supported by the reported data [2].

It was found from the \textit{in vitro} experiment (as discussed in Chapter 3) that 3,4-DHBA could be formed as a result of microsomal metabolism of 4-HBA. The discrepancies between \textit{in vitro} and \textit{in vivo} data cannot be readily explained. Furthermore, it should be noted that unknown pathways may be active \textit{in vivo} that further metabolize 3,4-DHBA, as it is produced in normal biological systems.

Representative chromatograms of microdialysates obtained from a rat, which has been subjected to a physically induced ischemia-reperfusion, are shown in Figure 4.15. Figure 4.16 shows the time course of 3,4-DHBA formation (presented as “3,4-DHBA/4-HBA (\% change) vs. time”) for all \textit{in vivo} experiments with occlusion, and Figure 4.17 shows the average of this data. It should be noted that 3,4-DHBA production may be dependent upon the availability of 4-HBA at the sampling site [18, 19], and the availability of 4-HBA in the sampling site is dependent on the delivery of 4-HBA. The \textit{in vivo} delivery of 4-HBA varied from rat to rat (22.23 ± 7.25 %, n = 8) (Figure 4.18). Therefore, expressing the time course of the experiments as “3,4-
Figure 4.15. Effect of ischemia-reperfusion on 3,4-DHBA formation. (A) Before occlusion. (B) During occlusion. (C) During reperfusion. Conditions: same as in Figure 4.12.
Figure 4.16. 3,4-DHBA/4-HBA (% change) vs time: Individual rats subjected to occlusion. *For rat 2, data points at 30 min = 956% and 40 min = 1596%. Occlusion at 0 min for all rats and reperfusion at 30 min for rats 1, 3, 4 and 5 and at 20 min for rat 2. No data points for rat 2 after 50 min and for rat 5 after 100 min because of their death.
Figure 4.17. 3,4-DHBA/4-HBA (% change) vs. time: Rats subjected to occlusion (n = 3). p values were calculated by comparison with the baseline (data points before occlusion). Rats 2 and 5 were not included because of their early death.
Figure 4.18. Delivery of 4-HBA to rat hearts subjected to occlusion. (n = 3, rats 1, 3, and 4).
DHBA/4-HBA vs. time” would account for the changes in delivery of 4-HBA during the experiments [18, 19]. As shown in Figures 4.15 - 4.17, increased formation of 3,4-DHBA was observed during occlusion and reperfusion phases in comparison to basal levels.

As mentioned in Chapter 1, it has been hypothesized that generation of \(^{\cdot}\)OH will increase during reperfusion due to the increased xanthine oxidase (XO) activity and increased electron leakage from the altered mitochondrial electron transport chain (METC) [20-23]. A 1.3 to 2.5 times increase in \(^{\cdot}\)OH concentrations (relative to basal levels) has been observed as a result of induced ischemia-reperfusion when the trapping method has been used in the stroke model of rats [2-4, 6, 18, 19, 24].

During ischemia, \(^{\cdot}\)OH concentrations (relative to the basal levels) may decrease, and this decrease may be due to the lack of oxygen supply resulting from complete occlusion [4, 25]. Gido et al. and Christensen et al. found a decrease in \(^{\cdot}\)OH concentration (determined as 3,4-DHBA and 2,5-DHBA, respectively) during ischemia relative to its basal concentration [25]. However, an increase in the concentration of \(^{\cdot}\)OH (determined as 2,3-DHBA, 2,5-DHBA or 3,4-DHBA) has also been observed during ischemia [8, 11, 19, 26, 27].

In this present study, a small increase in 3,4-DHBA (~ 140% change, Figure 4.17) during the occlusion phase (ischemia) from the baseline was observed. Generation of \(^{\cdot}\)OH during the ischemic phase may be occurring when residual oxygen is present [23] or when a complete occlusion is not achieved. Reactive oxygen species (ROS) formation is possible even when tissues contain only 5% of normal oxygen...
levels [27]. Increased concentrations of xanthine and XO in biological systems occur at the event of ischemia [20, 21]. Increased XO activity is expected during ischemic phase if there is residual oxygen in the biological system, and as a result ‘OH generation may increase.

As shown Figure 4.17, there is a continued rise of 3,4-DHBA (up to ~ 190% change) during reperfusion phase. A continued increase of 3,4-DHBA during the reperfusion phase implies that reperfusion is detrimental to biological systems. Observations from the in vivo experiments showed that the rats subjected to occlusion produced variable amounts of 3,4-DHBA. During reperfusion, variability in the amount of 3,4-DHBA or sustained generation of ‘OH cannot be explained readily. However, 3,4-DHBA formation might be dependent on various factors. These may be the extent of induced ischemia, the extent of reperfusion injury, and the probability of reaction between generated ‘OH and 4-HBA. Also, variability could occur as a result of the surgery itself, e.g., variation in occluder and the probe placement in the heart (Figure 4.2). As mentioned in Section 4.3.4.3, the LAD was targeted for occlusion, and the probe was implanted at the apex of the heart to obtain microdialysates. In addition to all of the above factors, variations in rat age and % recovery of 3,4-DHBA should also be considered.

To determine if the % change from the baseline (Figure 4.17) is significant, p values were calculated. The p values, as shown in Figure 4.17, suggest that the increase in 3,4-DHBA (% change) from the baseline is very significant, and this change may be the result of induced ischemia-reperfusion. However, in a comparison
with control experiments (experiment with no occlusion), a different conclusion may be reached. Data from the control experiments was plotted and analyzed in a similar manner as the data from the experiments with occlusion. The results of the control experiments are shown in Figure 4.19. The p values of both experiments (control and occlusion) were compared (Figure 4.17 and 4.19), and it can be concluded that the % changes at 10, 20, 30, 40, 50, 60, 70, and 90 min in Figure 4.17 are significant. The control experiments also suggest that an increased amount of 3,4-DHBA starts to form after 70 min (Figure 4.19) (5.5 hours since the start the experiment). After this time, unknown pathways \textit{in vivo} become active to generate ROS. One of the possible pathways might be inflammatory cell (neutrophil) migration. Hence, a suitable (maximum) time frame to perform the study involving heart occlusion is 5.5 hours or less. Figure 4.20 shows the combined plots of control and occlusion experiments within this time frame. From this figure, it can be concluded that increased generation of 3,4-DHBA may be the result of induced ischemia-reperfusion.

Increased generation of 3,4-DHBA may be an indication of significant production of ‘OH in biological systems due to higher levels of XO activity and greater electron leakage from the altered METC [20-23]. However, other possible sources of ‘OH generation cannot be excluded. For example, ‘OH can also be generated from the decomposition of peroxinitrous acid (as mentioned in Section 1.1.2 of Chapter 1) [28], enzymatic hydroxylation of 4-HBA, activation of neutrophils, and other unknown pathways.
Figure 4.19. 3,4-DHBA/4-HBA (% change) vs. time: Control experiments (n = 3). p values were calculated by comparing with data points before 10 min (-50 to 0 min).
Figure 4.20. 3,4-DHBA/4-HBA (% change) vs. time: Control and occlusion experiments (n = 3). Occlusion at 0 min and reperfusion at 30 min for the experiments with occlusion.
In order to verify the damages in the rat heart as a result of occlusion, histology was performed on the heart tissues (at the implanted probe area) and analyzed at the Lawrence Memorial Hospital (LMH), Lawrence, Kansas. Histology (hemotoxylin and eosin (H & E) staining) showed morphological changes in the heart tissues of rats subjected to occlusion, suggesting tissue damage due to an ischemic event (Figure 4.21). The ischemic changes to the myocardium include coagulation necrosis with cells showing edema, increased eosinophilic cytoplasm, loss of nuclei, and loss of or decreased muscle striations. Also, neutrophil migration was observed at the ischemic site.

4.5 Conclusion

The present study has demonstrated the combination of ‘OH trapping with 4-HBA and microdialysis for the real time monitoring of ‘OH (as 3,4-DHBA) in heart microdialysates of rats. The CE-UV method was found to be ineffective for the detection of 3,4-DHBA in vivo since the radical adduct (3,4-DHBA) and an endogenous compound (possibly DOPAC) were found to co-migrate. 3,4-DHBA and DOPAC were separated by modifying the buffer system in CE-EC. However, the CE-EC method was not employed further, as the detection limit obtained for 3,4-DHBA was not capable of monitoring low nanomolar concentration of 3,4-DHBA. Hence, further optimization is needed to improve (lower) the detection limit so that this method can be applied to in vivo oxidative stress investigations.
Figure 4.21. Histology (H & E staining) of rat hearts showing morphological changes due to occlusion (100x magnification). (A) Control rat heart. (B) Rat heart subjected to occlusion (ischemia-reperfusion). Black arrows: necrosis and edema. White arrows: inflammatory cells migration.
3,4-DHBA was successfully detected in heart microdialysates by HPLC with EC detection. No significant amount of 3,4-DHBA formation \textit{in vivo} (due to the oxidative metabolism) was observed following the delivery of 4-HBA (as the perfusate) into the rat heart. Increased production of 3,4-DHBA \textit{in vivo} as a result of physically induced ischemia-reperfusion was observed. It is hoped that HPLC-EC method coupled with microdialysis and 4-HBA will be beneficial for the measurement of \textsuperscript{·}OH as 3,4-DHBA in oxidative stress events, such as heart attack and stroke.
4.6 References

Chapter 5: Summary and Future Work

5.1 Overview of the Dissertation

In this dissertation, the development of analytical methods coupled to microdialysis for the determination of potential biomarkers of oxidative stress are presented. The application of the methods developed to monitor changes in the levels of biomarkers in biological systems as a result of induced oxidative stress are illustrated. Oxidative stress results in increased generation of reactive oxygen species (ROS) [1]. Therefore, increased antioxidant activities are expected in biological systems that experience oxidative stress.

The research focused on two biomarkers: glutathione and the hydroxyl radical (‘OH). Glutathione is an endogenous antioxidant that exists as two forms, reduced glutathione (GSH) and oxidized glutathione (GSSG). GSH plays an important role in scavenging ROS, and as a result, GSSG is formed. ‘OH is the most reactive ROS in biological systems, and it damages biomolecules. Methods based on separation techniques were developed for GSH, GSSG, and ‘OH. Microdialysis sampling enabled the continuous monitoring of GSH, GSSG, and ‘OH at tissue sites (liver and heart) of experimental animals, anesthetized Sprague Dawley rats. GSH and GSSG were measured to obtain information regarding antioxidant activity in biological systems, and ‘OH was monitored to assess the extent of ROS generation.
5.2 Summary

5.2.1 Detection of Glutathione by CE-UV in Rat Liver Microdialysates

A CE-UV method with pH-mediated base stacking was developed to detect GSH and GSSG simultaneously in microdialysis samples. This method provides a simple and effective way for on-column (capillary) preconcentration and detection of analytes in a single run. The sensitivity of GSH and GSSG detection was increased 26-fold relative to normal sample injection without stacking. The limits of detection (LOD) for GSH and GSSG in high ionic strength sample matrices were 0.75 µM and 0.25 µM, respectively.

Using the developed method, basal concentrations of GSH and GSSG in liver microdialysates of anesthetized Sprague Dawley male rats were found to be 4.14 ± 2.55 µM (n = 8) and 5.10 ± 3.59 µM (n = 8), respectively. Also, the CE-UV method was utilized to monitor changes in GSSG due to chemically (adriamycin) induced oxidative stress. It was hypothesized that the generation of ROS would increase as a result of oxidative stress. In the oxidative stress investigation, GSSG levels increased 6-fold from its basal levels, suggesting increased antioxidant activities during oxidative stress. However, the method was not able to detect GSH in microdialysates, and therefore, the GSH/GSSG ratio was not determined. The method’s inability to detect GSH may be attributed to GSH’s poor stability in Ringer’s solution, as well as the CE operating conditions.
5.2.2 Indirect Determination of ‘OH by CE-UV

A CE-UV method coupled with microdialysis sampling for the indirect determination of ‘OH as 3,4-dihydroxybenzoic acid (3,4-DHBA) was developed. 3,4-DHBA is the adduct of ‘OH and 4-hydroxybenzoic acid (4-HBA), the trapping agent. A detection limit of 74 nM for 3,4-DHBA was achieved. 4-HBA was able to trap ‘OH, and the CE-UV method was capable of detecting 3,4-DHBA in the reaction products of in vitro ‘OH generation systems (UV photolysis of H$_2$O$_2$, the Fenton reaction, and hypoxanthine/xanthine oxidase (HX/XO) systems without and with superoxide dismutase (SOD). Furthermore, experiments involving HX/XO systems with and without SOD implied that formation of 3,4-DHBA was the result of specific addition of ‘OH to 4-HBA. However, the microsomal incubation study with 4-HBA suggested that 3,4-DHBA could be formed through oxidative metabolism of 4-HBA.

5.2.3 Monitoring of ‘OH in Rat Heart Microdialysates

The CE-UV method developed to monitor ‘OH was found to be ineffective for the detection of 3,4-DHBA in heart microdialysates since 3,4-DHBA and an endogenous compound were found to co-migrate. However, 3,4-DHBA was successfully detected in heart microdialysates by a HPLC-EC method. Microdialysis sampling enabled the continuous delivery of 4-HBA (as the perfusate) into and the recovery of 3,4-DHBA from rat hearts. A tandem detection scheme in HPLC (HPLC-EC-UV) was employed. Therefore, the simultaneous detection of 3,4-DHBA (EC detection) and 4-HBA (UV detection) in heart microdialysates was achieved.
Increased amounts of 3,4-DHBA as a result of physically induced ischemia-reperfusion was observed, suggesting increased generation of 'OH during oxidative stress.

5.3 Future Work

5.3.1 Detection of GSH and GSSG by CE-UV and CLC-EC

Future work should focus on the stability of GSH. This may be achieved either by the derivatization of GSH or stabilization of GSH in an acidic sample matrix. It should be noted that an acidic sample matrix or use of an acidic background electrolyte (BGE) is not practical for CE with pH-mediated base stacking, because the BGE for base stacking consists of the salt of a weak base (e.g., NH$_4^+$).

In the derivatization approach, GSH will be derivatized on the -SH group with a suitable chromophore or derivatizing agent, and then detection of the adduct will performed by CE-UV. A list of common derivatizing agents specific to -SH and -NH$_2$ were presented in Table 2.1 of Chapter 2. Employing a derivatization procedure may stop (or reduce) the oxidation of GSH to GSSG. Under these conditions, previously developed CE-UV method (as presented in Chapter 2) would have to be re-optimized for the simultaneous detection of GSH and GSSG.

Alternatively, capillary high performance liquid chromatography (CLC) with dual electrode detection may be applied. An acidic sample matrix and an acidic mobile phase will be utilized during method development. Conventional HPLC with dual electrode detection (i.e., amperometric) has been used to monitor GSH and
GSSG simultaneously in biological samples [2, 3]. The dual electrode detection approach uses two gold/mercury (Au-Hg) electrodes in series configuration. The upstream and downstream electrodes are set at -1.0 V (vs. Ag/AgCl) and +0.15 V (vs. Ag/AgCl), respectively. GSH and GSSG can be monitored independently since both are separated chromatographically. CLC uses a smaller inner diameter (ID) column (0.2 - 0.8 mm) [4], and conventional HPLC uses a column ID of 4 - 6 mm. An injection volume of 1 µL or less can be analyzed by CLC [5]. The capability of analyzing small sample volumes will be an advantage when CLC is coupled with microdialysis. The use of dual electrochemical (EC) detection in CLC may enable the detection of low micromolar concentrations of GSH and GSSG in microdialysates. This could not be achieved by CE-UV without an on-column preconcentration technique (pH-mediated base stacking).

Hopefully, these new methods will be able to simultaneously detect GSH and GSSG in microdialysates of anesthetized Sprague Dawley rats (male and female), enabling the ratio of GSH/GSSG to be used as an indicator of oxidative stress.

5.3.2 CE-EC for the Indirect Determination of ‘OH

Further improvement of the CE-EC method for the determination of ‘OH (as 3,4-DHBA) is needed before it can be applied for *in vivo* analysis. Future work should focus on the enhancement of peak efficiency. As presented in Chapter 3, an off-column EC detection scheme resulted in peak broadening of 3,4-DHBA. To overcome this effect, end-column EC detection with a glassy carbon disk working
electrode (diameter of 300 µm or less) without using a decoupler should be explored [6]. Capillaries with a smaller ID (e.g., 25 µm or less) will be used to reduce the generation of the separation current caused by the BGE. This is due to the fact that the higher separation current may interfere with the EC detection cell to a great extent. Also, the BGE ionic strength should be optimized so that also it produces less current within the capillary.

5.3.3 HPLC-EC for Oxidative Stress Investigations

The HPLC-EC method will be utilized to monitor the change in 3,4-DHBA in the brains of rats as a result of a physically induced oxidative stress. Oxidative stress in the brain will be induced by ligation of both carotid and vertebral arteries [7]. In addition to this model of induction of oxidative stress, adriamycin will also be utilized to chemically induce oxidative stress. As presented in Chapter 2, adriamycin was delivered (as the perfusate) to the rat liver through a microdialysis probe for the induction of oxidative stress locally.

It is hypothesized that increased generation of 3,4-DHBA will be observed as a result of oxidative stress. Therefore, further experimentation will also include investigating the effect of a radical scavenger on the formation of 3,4-DHBA. A radical scavenger (e.g., α-phenyl-n-tert-butyl nitronate, PBN) can be delivered (as the perfusate) continuously to the heart or brain through a microdialysis probe. The hypothesis is that the administration of a radical scavenger will help to reduce the
generation of 3,4-DHBA. This experiment will provide a better insight into antioxidant therapy at the event of an oxidative event.

Also, future work should focus on the development of a CLC method that will be an alternative to the conventional HPLC method. Therefore, an analytical method will be developed that will be capable of analyzing small sample volumes and which will be ideally suited to coupling to microdialysis sampling.

5.3.4 Monitoring of GSH, GSSG, and ‘OH

Once proper analytical methods for GSH, GSSG, and ‘OH are developed, the next step will be the analysis of GSH, GSSG, and ‘OH in the same sample volume (e.g., 10 µL). This will help to determine any relationship among the biomarkers’ concentrations in the same aliquot of sample.
5.4 References


