Development of chromatography and mass spectrometry methods for enzyme kinetics and inhibition in glycolysis and the methylerythritol phosphate (MEP) pathways

Allison Fabino Carr

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ABSTRACT

DEVELOPMENT OF CHROMATOGRAPHY AND MASS SPECTROMETRY METHODS FOR ENZYME KINETICS AND INHIBITION IN GLYCOLYSIS AND THE METHYLERITHRITOL PHOSPHATE (MEP) PATHWAYS

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Northern Illinois University, 2019
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This dissertation focuses on method development for enzymatic activity and inhibition assays for use in screening potential antibacterial compounds. Infection by antibiotic-resistant pathogens is on the rise globally and several enzymes, including those in the methylerythritol phosphate (MEP) pathway and fructose bisphosphate aldolase, are strong targets for novel pharmaceuticals because they are not present in humans. Several assays exist for the MEP pathway and aldolase, but there is always work to be done to improve sensitivity, increase throughput, and lower cost. This dissertation describes the development of several methods – hydrophilic interaction chromatography (HILIC), liquid chromatography-mass spectrometry (LC-MS), and high resolution-mass spectrometry (HR-MS) – to detect and quantitate enzymatically-relevant analytes. The HILIC method was used to identify nucleotide analytes from the MEP pathway and was applied to LC-MS with no post-column desalting. In addition, the procedure was transferred for the analysis of fructose bisphosphate (FBP), the substrate of the aldolase reaction, with no changes. Additionally, an HR-MS was developed and validated to detect and quantitate FBP with high specificity and precision. Furthermore, the validated HR-MS method was used to obtain kinetic data from rabbit muscle aldolase uninhibited and with known inhibitors adenosine.
monophosphate (AMP) and adenosine triphosphate (ATP). Results confirmed that the HR-MS method was able to detect and quantitate enzyme turnover in quenched aliquots of reaction mixture. Future work will focus on using HR-MS detection to continuously monitor reactions \textit{in situ} in order to save time, labor, and cost. Finally, the goal is to combine the HILIC method with the continuous HR-MS method to study kinetics and inhibition of a variety of target enzymes against libraries of potential inhibitors \textit{in vitro} and \textit{in vivo}.
DEVELOPMENT OF CHROMATOGRAPHY AND MASS SPECTROMETRY METHODS FOR ENZYME KINETICS AND INHIBITION IN GLYCOLYSIS AND THE METHYLERITYRITOL PHOSPHATE (MEP) PATHWAYS

BY

ALLISON FABINO CARR
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A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

Doctoral Director:
Victor Ryzhov
Many people contributed to this project over the years. First and foremost, I want to thank my doctoral advisor, Dr. Victor Ryzhov, for guiding and supporting me, while also encouraging me to solve problems independently. I also want to thank my committee members Drs. James Horn, Timothy Hagen, Lee Sunderlin, and Linda Yasui for their helpful feedback. I have been fortunate to work with a wonderful group of people both in this project and others and, in particular, I want to acknowledge Michael Lesslie, Elettra Piacentino, and Kevin Parker for their many hours of help and support.

On a personal note, I want to thank my family for their unwavering love and support over the years. To my mom, Barbara; brother, Michael; sister, Janean; mother-in-law, Margaret; and father-in-law, Bob: your encouragement, praise, and support mean the world to me and I love you all dearly. And finally, I want to thank my husband, Michael. Thank you for being an incredible partner and supporting me day-in and day-out throughout this process. I love you forever.
DEDICATION

In honor of Robert Fabino and Eugenia Szumski

who are always right there with me
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ix</td>
</tr>
</tbody>
</table>

Chapter

1. INTRODUCTION ........................................................................................................ 1

2. METHODS .................................................................................................................. 14

3. COMPARISON OF REVERSED-PHASE, ANION-EXCHANGE, AND HYDROPHILIC INTERACTION HPLC FOR THE ANALYSIS OF NUCLEOTIDES INVOLVED IN BIOLOGICAL ENZYMATIC PATHWAYS .......... 37

4. QUANTITATIVE HR-MS: METHOD DEVELOPMENT AND VALIDATION........... 64

5. KINETIC ANALYSIS OF RABBIT MUSCLE ALDOLASE IN VITRO BY HR-MS....88

6. CONCLUSIONS AND FUTURE WORK................................................................. 124

REFERENCES........................................................................................................... 128

APPENDICES............................................................................................................ 145

A. RAW DATA AND SUPPLEMENTAL INFORMATION FOR CHAPTER 4............ 145

B. RAW DATA AND SUPPLEMENTAL INFORMATION FOR CHAPTER 5.........147
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. A timeline produced by the US Centers for Disease Control and Prevention (CDC) highlighting the issue of antibiotic resistance by showing the year an antibiotic is introduced (right) compared to the first known instance of resistance (left).</td>
<td>3</td>
</tr>
<tr>
<td>1.2. Example reactions of frequently studied enzymes in the MEP pathway: IspD, IspE, and IspF.</td>
<td>6</td>
</tr>
<tr>
<td>1.3. Illustrated reaction schemes showing the basis of the Kinase-Glo assay, sold by Promega Corporation.</td>
<td>7</td>
</tr>
<tr>
<td>1.4. Reaction scheme of the EnzChek phosphate assay.</td>
<td>8</td>
</tr>
<tr>
<td>1.5. Reaction scheme of the reversible aldolase-catalyzed cleavage of fructose bisphosphate.</td>
<td>11</td>
</tr>
<tr>
<td>1.6. Illustrated procedure for the multi-step Elite Kit fluorometric assay for aldolase activity.</td>
<td>12</td>
</tr>
<tr>
<td>1.7. Reaction scheme for the coupled-enzyme colorimetric assay for aldolase activity.</td>
<td>13</td>
</tr>
<tr>
<td>2.1. A visual representation of the overlap between HILIC chromatography and ion-exchange (IEX), reversed-phase (RPLC), and normal-phase (NPLC).</td>
<td>16</td>
</tr>
<tr>
<td>2.2. Example of selected ion monitoring and selected reaction monitoring of a dipeptide using triple quadrupole mass spectrometry.</td>
<td>21</td>
</tr>
<tr>
<td>2.3. A table summarizing the confidence levels for identification of metabolites and the requisite data requirements.</td>
<td>26</td>
</tr>
<tr>
<td>2.4. An instrument schematic showing the cycling between high and low collision energies during an MS$^E$ experiment.</td>
<td>29</td>
</tr>
</tbody>
</table>
2.5. Example spectra showing the difference between high and low collision energy spectra for xanthuric acid. While the low collision energy spectra mainly shows only the intact ion, the high collision energy one also shows various fragments useful for identification and structural elucidation. 

2.6. The prototypical Michaelis-Menten curve (bottom left) and the Michaelis-Menten equation (left), as well as their double reciprocal transformations.

3.1. Representative HPLC chromatogram showing separation of five nucleotides by IP-RP HPLC. This chromatogram excludes the wash stage of the gradient.

3.2. Structure of the cyclofructan stationary phase used in the FRULIC-N and FructoShell HPLC columns. The crown-ether center is able to dock cations (such as ammonium, which was used in this study) and act as an ion-exchanger for retention of acidic analytes.

3.3. Analysis of five nucleotides on the XBridge Amide column under isocratic elution.

3.4. Repeatability analysis of a CDP, ADP, and ATP separation under isocratic conditions after a one-hour initial column equilibration.

3.5. Repeated analysis of CMP under the same chromatographic conditions as those in Figure 3.4, but with overnight column equilibration.

3.6. Representative HPLC chromatogram of five nucleotides analyzed on the FructoShell HILIC column under isocratic conditions at 0.425 mL/min.

3.7. LC-MS analysis of nucleotides by a 10 min isocratic elution scheme (0.8 mL/min) followed by SIM(-) detection of analyte m/z (values given in Table 3.5).

3.8. LC-MS/MS spectrum showing retention of fructose bisphosphate on the FructoShell HILIC column with selected reaction monitoring (SRM) detection.

3.9. Representative chromatogram of nucleotide separation of SAX. Varying degrees of coelution of CTP and ATP were present in all runs.

3.10. Isocratic elution of five nucleotides on a 15cm column with a buffer/THF mobile phase.

3.11. Sample gradients of THF-buffer to improve separation and peak shape of nucleotide analytes.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.12. Nucleotide analysis by a shortened gradient in UHPLC mode</td>
<td>60</td>
</tr>
<tr>
<td>4.1. A schematic showing the components of an ESI-QqTOF-MS. Obtained from</td>
<td>66</td>
</tr>
<tr>
<td>4.2. Structures and m/z values for the substrate fructose bisphosphate (FBP, right) and the internal standard ($^{13}$C$_6$-FBP, left)</td>
<td>72</td>
</tr>
<tr>
<td>4.3. FBP full scan</td>
<td>74</td>
</tr>
<tr>
<td>4.4. FBP SRM</td>
<td>75</td>
</tr>
<tr>
<td>4.5. IS full scan</td>
<td>76</td>
</tr>
<tr>
<td>4.6. IS SRM</td>
<td>77</td>
</tr>
<tr>
<td>4.7. Full scan of matrix without S and IS</td>
<td>78</td>
</tr>
<tr>
<td>4.8. Standard curve for average normalized signal vs. FBP concentration over the range of 0.1-500 μM</td>
<td>79</td>
</tr>
<tr>
<td>4.9. Linearity data for low concentration samples with error bars showing extreme variability in signal intensity among triplicate analyses</td>
<td>80</td>
</tr>
<tr>
<td>4.10. Low CE (7 eV)</td>
<td>84</td>
</tr>
<tr>
<td>4.11. High CE (30 eV)</td>
<td>85</td>
</tr>
<tr>
<td>4.12. A concentration vs. time kinetic plot obtained using this HR-MS method with a starting [FBP] of 10 μM, an enzyme concentration of 12.7 nM, and 10 μM [IS]</td>
<td>86</td>
</tr>
<tr>
<td>5.1. PDB entry 6ALD showing the rabbit muscle aldolase tetramer with FBP bound to each of the four active sites</td>
<td>89</td>
</tr>
<tr>
<td>5.2. Illustration of the fluorescent coupled-enzyme assay for aldolase</td>
<td>93</td>
</tr>
<tr>
<td>5.3. The enzyme-catalyzed conversion of resazurin to resorufin that is used as a fluorescent probe because of the co-conversion from NADH to NAD$^+$</td>
<td>94</td>
</tr>
<tr>
<td>5.4. A schematic showing the reaction set-up for the continuous enzyme assay</td>
<td>100</td>
</tr>
</tbody>
</table>
5.5. A sample reaction profile for the aldolase starting with 10 μM FBP and no added inhibitor.  

5.6. The first few time points representing the initial reaction from Figure 5.5, and the linear fit to yield the initial reaction rate of 2.7985 μM/min.  

5.7. Reaction profiles from two trials of reaction with 1 μM initial FBP concentration. Both the shapes of the curves and the calculated initial rates are markedly different from each other.  

5.8. Reaction profile from the 50 μM initial FBP concentration reaction. There is no sharp decrease in substrate concentration indicative the initial rate phase, so the first three data points were used to calculate initial rate.  

5.9. The calculated Michaelis-Menten curve obtained from the initial rates of reactions ranging from 0-50 μM FBP in the absence of inhibitor.  

5.10. The double reciprocal plot with a linear fit used to determine the Michaelis constant and maximum reaction rate.  

5.11. The Michaelis-Menten plot produced from reactions with 1 μM AMP.  

5.12. The Lineweaver-Burk plot with the linear fit.  

5.13. The extracted ion chromatogram showing signal for FBP (blue) and IS (orange) for the 25 μM initial [FBP] after 2 min incubation time.  

5.14. The Michaelis-Menten plot for aldolase with 1 μM ATP.  

5.15. The double reciprocal plot with linear fit for aldolase in the presence of ATP.  

5.16. An overlaid chart of the Michaelis-Menten curves with no inhibitor (blue), AMP (orange), and ATP (gray).  

5.17. Chromatogram trace of signal from FBP (green) and the IS (purple) for the continuous reaction with 5 μM initial [FBP].  

5.18. Overlay of quantitation data extracted from duplicate trials of 5 μM continuous reactions. The slight differences in curve shapes translate to a 43% difference in calculated reaction rates.
Figure 5.19. Extracted ion chromatograms from FBP (purple) and the IS (green) from a continuous reaction with 100 μM aldolase .......................................................... 121

5.20. Continuous reaction analysis starting with 10 μM FBP monitoring signal from FBP (blue) and the IS (orange). ................................................................. 121
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Interday average retention times and variabilities for ion-pair reversed-phase HPLC over four days</td>
<td>46</td>
</tr>
<tr>
<td>3.2. Intraday relative standard deviations in retention times for nucleotides analyzed by IP-RP HPLC</td>
<td>46</td>
</tr>
<tr>
<td>3.3. Intraday retention time variability from HILIC analyses given as %RSD values</td>
<td>51</td>
</tr>
<tr>
<td>3.4. Average retention times and variabilities for interday analysis of nucleotides by HILIC</td>
<td>52</td>
</tr>
<tr>
<td>3.5. The m/z values monitored in SIM(-) LC-MS analysis of nucleotides (Figure 3.7)</td>
<td>52</td>
</tr>
<tr>
<td>3.6. Interday average retention times and deviations for nucleotide analytes analyzed by strong anion exchange HPLC</td>
<td>55</td>
</tr>
<tr>
<td>3.7. Intraday variabilities in retention times for nucleotides analyzed by SAX</td>
<td>56</td>
</tr>
<tr>
<td>3.8. Summary of various chromatographic parameters for each nucleotide analyzed by IP-RP, SAX, and HILIC</td>
<td>57</td>
</tr>
<tr>
<td>3.9. Average intraday %RSD values for each analyte for each of the three separation methods tested</td>
<td>61</td>
</tr>
<tr>
<td>4.1. Summary of mass accuracy data</td>
<td>72</td>
</tr>
<tr>
<td>4.2. Summary of mass accuracy data after adjusting capillary distance</td>
<td>73</td>
</tr>
<tr>
<td>4.3. Summary of repeatability experimental results</td>
<td>81</td>
</tr>
<tr>
<td>4.4. Intraday intermediate precision data for days 1 and 2</td>
<td>82</td>
</tr>
<tr>
<td>4.5. Interday intermediate precision between days 1 and 2</td>
<td>82</td>
</tr>
</tbody>
</table>
4.6. Variability data from analyses between spray needles........................................83

5.1. Summary of kinetic parameters obtained for rabbit muscle aldolase without inhibitor ..............................................................................................................107

5.2. Summary of kinetic parameters for aldolase with 1 μM AMP ..................................109

5.3. The summary of kinetic parameters for aldolase with 1 μM ATP...............................111

5.4. Examples of $K_M$ values for rabbit muscle aldolase in the literature obtained using the colorimetric or fluorometric coupled-enzyme assay ........................................113

5.5. Summary of the kinetic parameters for all three aldolase conditions .......................114
CHAPTER 1

INTRODUCTION

A Brief History of Antibiotics

Antibiotics have been used by humanity since ancient times.¹ Perhaps the most well-known and widely-successful antibiotics in history is penicillin, which was serendipitously discovered in 1928 by Alexander Fleming. Years after the initial discovery, Ernst Chain and Howard Florey were able to purify and mass-produce penicillin, helping it to become “the wonder drug” that saved countless lives during World War II.² In the decades since the end of World War II, penicillin and its derivatives – including amoxicillin, ampicillin, and methicillin – have continued to be used. Additionally, other antibiotic classes against several targets have been developed such as cephalosporins, fluoroquinolones, sulfonamides, and tetracyclines. However, in recent decades antibiotic development has slowed significantly and only two new classes have made it to market since the mid-1960s.³

The Emerging Threat of Antibiotic-Resistant Pathogens

When Alexander Fleming discovered penicillin, he also predicted that there would come a time in the future when it would no longer be an effective treatment due to bacterial resistance.⁴
Bacterial antibiotic resistance is an unfortunate effect of natural selection and the theory of evolution. As bacteria reproduce extremely quickly, mutations that induce resistance can form and be passed on to future generations within a short timeframe from initial antibiotic treatment. Overuse in both humans and livestock, misuse, and the recent plateau in new antibiotic development have led both the US Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) to declare antibiotic resistant pathogens as a serious global threat. Examples of antibiotic resistant pathogens are seen in the figure below and include: MRSA/VRSA, MDR/XDR tuberculosis, and malaria.

New Targets for Antibacterial Drugs

Although the pathogens described above represent the biggest threats to humanity and work on pathogen-specific antibiotics is underway, the true future of antibiotics lies in creating broad-spectrum drugs designed to treat a wide array of infections. These new drugs should display several desirable characteristics including being effective at low doses (high potency) with few debilitating side effects (low toxicity). The best way to ensure a low occurrence of off-target effects is to choose a bacterial target (such as an enzyme or receptor) that is not present in humans. The targets described below are very promising because of their necessity for bacterial survival, and the idea that a compound could be designed that has a specificity for the target and limited toxicity of the host organism.
Figure 1.1: A timeline produced by the US Centers for Disease Control and Prevention (CDC) highlighting the issue of antibiotic resistance by showing the year an antibiotic is introduced (right) compared to the first known instance of resistance (left).
One long-studied antibacterial target that is not present in humans is the enzymes involved in cell wall biosynthesis. The primary product of this series of enzymes is peptidoglycan, and because human cells have membranes but not walls, it is not essential for the viability of human cells. On the other hand, bacterial cells have cell walls and peptidoglycan is vital to cell survival. This difference in humans and bacteria has been used to great effect with β-lactam antibiotics – e.g., penicillins and cephalosporins – which inhibit the group of proteins that cross-link peptidoglycan, aptly named penicillin-binding proteins (PBPs), into cell walls. However, bacteria have developed mechanisms to deactivate β-lactams (namely β-lactamase enzymes) and novel antibiotics are constantly needed to keep up with resistance. Another high priority target for antibiotic development is enzymes involved in energy metabolism. Energy production and metabolism are vital for cell survival and many of these enzymes are either not present in humans or display a different catalytic mechanism and/or active site structure than the human versions, including but not limited to: fructose bisphosphate aldolase, acetate kinase, phosphotransacetylase, formate acetyltransferase, and xylitol dehydrogenase. The specific targets discussed below each represent one of the classic antibacterial targets absent in humans: the methylerythritol phosphate pathway, which aids in isoprenoid synthesis for cell membranes and fructose bisphosphate aldolase, an essential glycolysis enzyme.

The Methylerythritol Phosphate (MEP) Pathway

The methyl erythritol phosphate (MEP) pathway is essential for the survival of bacterial gram-negative and some gram-positive species, making it an attractive target for novel antibiotics to treat drug-resistant infections. The MEP pathway involves a series of seven enzymes that
produce the isoprenoid precursors IPP and DMAPP. All MEP pathway enzymes can be inhibited but based on previous research on the MEP pathway, the first five enzymes in the pathway – DXS, DXR, IspD, IspE, and IspF – are the strongest targets for future drug development. The isoprenoids produced from the precursors are essential for cellular membranes, steroid production, and ubiquinone synthesis for use in cellular respiration.

Therefore, inhibitors of MEP pathway enzymes are bactericidal. Animals also biosynthesize IPP and DMAPP, but they do so via the mevalonate pathway, which does not have any enzymes in common with the MEP pathway. This is the main reason the MEP pathway is such a popular target for antibiotic development. The goal is to develop drugs that would be very effective against bacterial infections, with little effect against human cells.

![Figure 1.2: Example reactions of frequently studied enzymes in the MEP pathway: IspD, IspE, and IspF.](image-url)
As there is a lot of interest in the MEP pathway, many methods exist to evaluate inhibition of MEP pathway enzymes. These methods represent a variety of different platforms, sensitivities, and throughputs. Plate-based assays are commonly used, most of which are coupled-enzyme assays. One such example is the Kinase Glo assay, which quantifies kinase activity based on the decrease in ATP concentration.\textsuperscript{22} The Kinase Glo assay can be used to study IspE activity.\textsuperscript{23} The kinase reaction will convert ATP to ADP until the reaction reaches equilibrium, and any leftover ATP will be used in the enzymatic conversion of luciferin to oxyluciferin by the enzyme luciferase.\textsuperscript{22b, 24} The luciferase-catalyzed reaction releases a photon and the chemiluminescent signal can be read on a plate reader.\textsuperscript{24} Luminescent signal is inversely proportional to ATP concentration and the ATP concentration can be stoichiometrically related to product formation to yield enzymatic reaction rates.\textsuperscript{23} Kinase Glo assay kits are commercially sold and most of the experimental conditions are optimized.

Figure 1.3: Illustrated reaction schemes showing the basis of the Kinase-Glo assay, sold by Promega Corporation.\textsuperscript{25}
For the IspD-catalyzed reaction, phosphate ions released as product can be quantified by the EnzChek phosphate assay kit. The assay relies on coupled enzyme reactions, in which the inorganic phosphate released by the IspD reaction is used alongside 2-amino-6-mercapto-7-methylpurine riboside (MESG) as the substrates of purine nucleoside phosphorylase (PNP). One of the products formed by this reaction, 2-amino-6-mercapto-7-methylpurine, absorbs at 360 nm and by monitoring the absorbance over time on a plate reader, the absorbance can be directly related to the amount of IspD activity.

![Reaction scheme of the EnzChek phosphate assay](image)

Figure 1.4: Reaction scheme of the EnzChek phosphate assay.
Another example of a plate-based assay uses fluorescence detection to quantitate IspF activity.\(^{28}\) In this scheme, the CMP produced as a by-product of the IspF reaction is combined with ATP and nucleotide monophosphate kinase (NMK) to produce ADP. That ADP is used in the pyruvate kinase reaction to produce pyruvate. Pyruvate oxidase is then used to produce hydrogen peroxide, which is used as an electron donor to convert Amplex-Red to resorufin horseradish peroxidase. This oxidation reaction can be monitored by fluorescence with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. This is another indirect assay, with signal from resorufin being directly proportional to the amount of CMP produced by the IspF reaction.\(^{28}\) The plate-based format makes these assays high-throughput, and chemiluminescence and fluorescence are very sensitive forms of detection. Also available in plate format is an assay that follows the UV signal from the depletion of NADH. However, these assays are indirect, involves multiple enzymatic steps, sometimes requiring special detectors or expensive assay kits.

A more direct assay for IspE utilizes TLC separation with radiometric detection.\(^{28}\) For this assay, the reaction is run \textit{in vitro} with all of the typical reagents but using \(\gamma\)-\(^{32}\)P-labelled ATP. The IspE kinase reaction converts ATP to ADP, TLC separates compounds from the reaction mixture, and labelled ATP is detected with a phosphoimager. A decrease in ATP signal indicates that the IspE reaction is proceeding forward.\(^{28}\) This assay can be quantitative and be used to determine reaction rates. It is also very specific, with very low background signal and artifacts, extremely sensitive, and relatively quick. The main issue with this assay is expense: it requires a radio-labelled substrate, the cost of which can add up over a series of runs.

Other MEP pathway assays utilize more widely-used techniques, like high performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS). Anion
exchange HPLC is a common choice for phosphate-containing analytes. IspD-binding compound CDP-ribitol can be assayed using strong anion exchange HPLC in gradient elution mode and quantitated by measuring absorbance at 280 nm. Similarly, IspF inhibition can be assayed with an isocratic strong anion exchange method. In both cases, UV absorbance is used as a means of determining concentrations and reaction rates. However, UV detection has fairly low sensitivity and a more sensitive detection method such as mass spectrometry is needed for very dilute samples. Unfortunately, neither method is ESI-MS-applicable because of high non-volatile salt concentrations in the mobile phase: 0.05-0.5 M ammonium phosphate and 0.5 M sodium phosphate, respectively. Another HPLC technique that is commonly used is reversed-phase (RP) combined with an ion-pairing (IP) reagent in the mobile phase. Since reversed-phase columns are nonpolar and MEP pathway compounds contain polar phosphate groups, retention necessitates the use of between 5-500 mM ion-pairing reagent, often in a shallow gradient of increasing concentration of organic solvent. IspF reaction mixtures have been analyzed in this way using a short “rocket” column running at a high flow rate. Reaction products CMP and CDPME2P were well separated in a short gradient. In this case, 5 mM tetrabutyl ammonium bisulfate in phosphate buffer was used as the ion-pairing reagent; and that, in addition to the 0.1% SDS quenching reagent, made ESI-MS non-viable, thereby limiting the sensitivity of the method. Other IP reagents are more applicable for ESI-MS, such as tributylamine which can be interfaced with ESI-MS at low-millimolar concentrations.
Fructose Bisphosphate Aldolase

Another potentially interesting target for antibiotic development is fructose bisphosphate aldolase, or simply aldolase, which is part of two of the most critical enzymatic pathways in all of biology: glycolysis and gluconeogenesis. Nearly complete opposites of each other, glycolysis is the pathway that processes glucose to form free energy and pyruvate for the Krebs cycle, and gluconeogenesis synthesizes glucose using energy and pyruvate. Both are integral to cellular metabolism, energy production, and respiration so it is easy to see their value as “druggable” enzymatic pathways. Aldolase catalyzes the reversible cleavage of fructose bisphosphate (FBP) into dihydroxyacetone phosphate (DHAP) and glycerol 3-phosphate (G3P), an aldol cleavage/condensation step. Depending on the type of organism, aldolase comes in two forms: class I and class II. The mechanism of action for class I aldolases involves a Schiff-base intermediate to link a lysine residue in the active site to the carbonyl group on dihydroxyacetone phosphate. This type of aldolase is found in plants and animals. On the other hand, bacteria use class II aldolases, which have a totally different mechanism of action and little sequence homogeneity to the active site of class I aldolases. Class II aldolases are metalloenzymes, which use a Zn\(^{2+}\) to bind to and polarize the carbonyl group on DHAP. Due to the difference in active site structure and the extreme importance of the enzyme, aldolase is a very promising target for future antibiotic development.
Figure 1.5: Reaction scheme of the reversible aldolase-catalyzed cleavage of fructose bisphosphate.

The major assays that currently exist to study aldolase activity are colorimetric or fluorometric coupled-enzyme assays following the reduction of NADH (which absorbs light at 340 nm) to NAD$^+$ (which can be coupled to a fluorescence reaction). Therefore, colorimetric assays will measure the decrease in A$_{340}$ and fluorometric assays will measure the decrease in signal from the excitation-emission (usually in the 520-590 nm range). The colorimetric assay is more commonly used, but fluorescence provides a much higher sensitivity, albeit at higher cost because of the necessity of a fluorophore. Although these assays are extremely sensitive, they are plate-based and require expensive and material-heavy kits to be purchased, lowering the throughput of the assay. As many labs already contain the required instrumentation, HPLC and LC-MS assays offer cost savings and can run continuously with little user input. In addition, LC-MS can directly analyze compounds in the aldolase reaction, instead of requiring coupled enzyme reactions and stoichiometrically relating signal from NADH to aldolase products. This direct method also requires less incubation time and sample preparation, saving time, effort, cost, and materials while reducing matrix effect.
Figure 1.6: Illustrated procedure for the multi-step Elite Kit fluorometric assay for aldolase activity.\(^{39}\)

Figure 1.7: Reaction scheme for the coupled-enzyme colorimetric assay for aldolase activity.
Aim of This Work

The aim of this work is to develop LC, MS, and LC-MS methods able to quantitate enzyme activity and inhibition and offering significant improvements in throughput, sensitivity, cost, and material use compared to traditional plate-based, coupled-enzyme, and spectrophotometric assays. Additionally, the methods will also provide direct analysis of reaction biomarkers in vivo. This allows for confirmation that an inhibitor is acting on the enzyme of interest and not an off-target site. This information is pertinent when developing drugs because it ensures specificity. Ideally, the protocols developed here could be used for various enzymes and pathways with only minor changes. The long-term goal of this work is to use the data obtained to describe the potency and specificity of enzyme inhibitors for use as potential antimicrobial drugs, which are desperately needed in the age of antibiotic-resistant pathogens.
CHAPTER 2

METHODS

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography has been used for decades as a robust and high-throughput method to separate and quantitate analytes in the liquid phase. The basis of the separation power lies in the extrapolation of property (usually polarity) differences between compounds in a mixture and involves two phases: the stationary phase (an immobile solid phase, usually a packed column) and mobile phase (a solvent which carries the sample through the stationary phase). Similar to a liquid-liquid extraction, separation occurs based on partitioning of compounds between the two phases. Since the stationary and mobile phases have different polarities themselves, compounds will differentially partition toward one phase or another, and over time this will lead to their separation. Depending on the type of analyte, stationary/mobile phase properties, column length, temperature, mobile phase flow, etc. can all be fine-tuned. Therefore, HPLC is considered one of the most useful and widely applicable techniques in analytical chemistry.
Hydrophilic Interaction Liquid Chromatography (HILIC)

Since its development in 1990, hydrophilic interaction, or HILIC, chromatography has revolutionized the analysis of polar and ionizable compounds. HILIC combines a polar stationary phase (as in normal phase chromatography) that often contains ionizable groups (as in ion chromatography) with the water-acetonitrile mobile phase used in reversed-phase chromatography. The retention mechanism in HILIC chromatography is two-fold. As the name implies, the primary retention mechanism is based on the interactions between the hydrophilic stationary phase and analyte. This can be due to dipole-dipole, charge-dipole or charge-charge interactions. In addition to hydrophilic interactions, there is also partitioning of the analyte within the water-rich layer of the mobile phase around the stationary phase particles and the organic-rich layer. Different stationary phases provide varying degrees of hydrophilic interaction vs. partitioning mechanisms depending on their polarity and the number of ionizable groups present.

At present, a variety of HILIC stationary phases are commercially available, the most common of which is bare silica. At near-neutral pH, silica can act as a cation exchanger because of the formation of deprotonated silanol groups; however, the specificity is limited to more basic analytes and retention of acidic analytes is poor. Also widely used are neutral bonded phases, such as hydroxyl- and amido-bonded silica. Hydroxyl phases show almost no ion-exchange activity and retention of polar analytes is predominately due to partitioning. On the other hand, amide phases exhibit a high degree of hydrogen-bonding to analytes while also creating strong water-acetonitrile partitioning. Therefore, amide columns are applicable for a variety of neutral and charged analytes.
Zwitterionic (charged, but net neutral) stationary phases exhibit very high overall retention and are also effective at separating neutral and charged analytes. Despite the presence of charged groups on the stationary phase, ion-exchange is not the principal retention mechanism. Instead, the extremely high stationary phase hydrophilicity promotes analyte partitioning within the mobile phase. For anion-exchange of acidic analytes, there are also amino stationary phases which can be protonated. Furthermore, the above only represent the most commonly used HILIC stationary phases, and a variety of other commercial and proprietary phases exist for more narrow application.

With such a large number of HILIC stationary phases available, great care must be taken when selecting a stationary phase for a particular application or analyte. In addition, several mobile phase factors such as %ACN and pH also play vital roles in determining the ionization state of
both the stationary phase and analyte and the degree of partitioning. A generic mobile phase used for HILIC is a combination of a large fraction of acetonitrile and a small fraction of water (but no smaller than 5% (v/v) to ensure some partitioning). In practice, the solution is often buffered to control pH, as the analyte and/or stationary phase may contain pH-dependent ionizable groups. HILIC runs can either be isocratic or gradient, and column equilibration times are at least 20 column volumes. Because of the long equilibration times, isocratic elution is preferable for fast analysis. On the other hand, the multi-faceted retention mechanism increases molecular drag and causes peaks to tail in most HILIC methods. In this case, using a gradient or incorporating core-shell technology into particles can improve the peak shape.\textsuperscript{41b}

As the majority of the analytes of interest in this work are contain phosphate moieties, HILIC is the ideal separation choice for LC and LC-MS. Chapter 3 focuses on HPLC method development and the comparison of HILIC to other separation techniques for phosphate analytes: strong anion exchange (SAX) HPLC and ion-pair reversed-phase (IP-RP). In this chapter, the advantages and disadvantages of each of these three techniques are evaluated. In addition, method development and comparative analyses are shown for five nucleotide analytes in the MEP pathway.

**Drawbacks of HPLC Alone**

Liquid chromatography analysis is applicable to complex matrices and can separate (based on stationary phase) and detect (based on detector) analytes with various properties. Many researchers utilize LC, and since it is the most popular analytical technique worldwide, there are
lots of resources for method development across different applications. However, HPLC is not applicable to every analyte in every instance. The biggest disadvantage of HPLC is the limited number of detectors available commercially. In order of increasing specificity, the most typical detectors are refractive index (RI), evaporative light scattering (ELS), UV or UV-Vis, and fluorescence. Depending on the number of analytes and their respective absorption properties, these four detectors may not provide the requisite specificity or selectivity needed for quantitative analysis. In that case, HPLC can be interfaced with other detection techniques.

For the nucleotides and sugar-phosphates of interest in this work, the typical HPLC detectors are applied with mixed success. For complex matrices such as biological samples, RI and ELS are generally negated due to their low specificity. Many of the compounds of interest for the MEP pathway, namely nucleotides, have aromatic, UV-active DNA bases (e.g. cytosine and adenine) that absorb UV at 272 and 260 nm, respectively. However, MEP pathway compounds that do not contain these functional groups are excluded from detection because they lack any chromophore. This is also the case with fructose bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate of the aldolase-catalyzed reaction. Because the ultimate aim of this work is to develop enzymatic assays that can be applied to multiple pathways with little-to-no changes, UV is not the optimum detector because it only applies to a fraction of the compounds on interest. Fluorescence detection has the same issue; none of the analytes in the MEP pathway or the aldolase reaction naturally fluoresces.

Although UV and/or fluorescence detection may not work for many phosphate-containing compounds in their native state, significant effort has been done to derivatize them to possess a chromophore or fluorophore. For example, inositol phosphates, a group of biologically-relevant
sugar phosphates lacking a chromophore for UV detection, have been derivatized post-column with iron (III) nitrate for detection at 290 nm.43 Amino- and hydroxyl-groups on phospholipids can be derivatized with chromophores or fluorophores.44 One example is derivatization of amino-phospholipids with either 1-dimethylaminonaphthalene-5-sulfonyl (DNS) or succinimidyl 2-naphthoxyacetate groups to enable fluorescence detection.44 Also, the thiamine group in thiamine-containing nucleotides can be oxidized to form thiochrome fluorophore, significantly increasing sensitivity compared to UV detection.45 For these and other derivatization methods to be viable, the reaction needs to be relatively fast and high-yielding, leaving huge barriers to becoming a routinely-used detection scheme.44 Therefore, direct analyte analyses are highly preferred compared to derivatization.

Liquid Chromatography-Mass Spectrometry (LC-MS)

The interface of liquid chromatography and mass spectrometry is an extremely powerful tool for qualitative and quantitative analysis. In this setup, a mass spectrometer is used either in place of or in tandem with a typical UV detector. Mass spectrometers separate analytes based on their mass-to-charge ratio (m/z) and are much more sensitive than UV detectors. In addition, MS is a highly selective detector which can detect analytes that do not absorb in the UV range based on their mass-to-charge, eliminating the need for analyte modification. Finally, MS provides an extra degree of separation to LC, and is able to separate coeluting LC species as long as they have different m/z values. This is especially helpful because it allows for the use of isotopically-labelled internal standards, which would coelute in LC but be separated in MS.46
There are several MS detection protocols that are commonly paired with chromatography. The fastest and most basic detection scheme looks for certain ions in the mixture and refers them to LC peaks. When one or more ion of interest is selected for screening and the other ions are filtered out, it is called selected ion monitoring (SIM). To obtain an even greater level of identification and sensitivity than single-stage MS, a technique called tandem mass spectrometry (MS/MS) - which collects fragmentation data for a selected ion - can be performed. Fragmentation is often induced by collision-induced dissociation (CID), in which the analyte of interest is fragmented via collisions with a neutral gas such as He, N$_2$, or Ar. The user inputs an energy value added to the collision gas that determines the strength of collisions, which can lead to a variety of fragmentation patterns in the analyte. Tandem mass spectrometry is especially important in complex samples where more than one ion may have the same mass-to-charge ratio. These isobaric ions would not be separated in single-stage MS, but MS/MS fragmentation data can elucidate them using structure-based fragmentation patterns. For quantitation experiments, selected reaction monitoring (SRM) is extremely common. One MS/MS ion pair is used for detection purposes (the qualifier) and another is used for quantitation (the quantifier, usually the highest intensity peak). These ion pairs are assigned based on their relative abundance in the spectra and the quantitation limits of the system. When more than one pair of ions are being detected simultaneously, the process is called multiple reaction monitoring (MRM).
A variety of mass spectrometers can be used as part of an LC-MS system, but there are a few that are more popular than others. These include the single quadrupole (Q), triple quadrupole (QqQ), and hybrid instruments such as the quadrupole time-of-flight (QqTOF) or the linear ion-trap combined with the Orbitrap (LIT-Orbitrap). For fast separation of ions less than 1000 Da, single quadrupole instruments are used in conjunction with an LC system. The quadrupole mass filter is able to screen out unwanted m/z values, either by selecting a mass range to get a full spectrum (scanning mode) or a single m/z for selected ion monitoring (filtering mode). Q-MS detectors are effective for general use and screening experiments but are not able to provide MS/MS fragmentation data to distinguish isobaric ions. Therefore, for applications requiring
identification of very low concentrations of analytes in complex sample matrices, single quadrupole detectors may not provide sufficient sensitivity and/or selectivity.\textsuperscript{46}

By far the most popular system used in LC-MS is the triple quadrupole or QqQ. Designated the “workhorse instrument,” labs across all disciplines use triple quadrupole MS for validation and quantitation of small molecules. Like the name suggests, a triple quadrupole instrument is made up of three quadrupoles: Q1 and Q3 act as mass filters and q2 acts as a collision cell for MS/MS fragmentation. Like with single quadrupole MS, ions can be detected in selected ion monitoring mode by allowing passage through Q1 and q2, and operating Q3 in filtering mode for the DC and RF voltages corresponding to the m/z of interest. However, this does not give optimum performance or sensitivity. Instead, most QqQ methods will either be selected reaction monitoring or multiple reaction monitoring. In both cases, Q1 will be used to filter out all ions except a selected precursor ion, and from there the precursor will travel to q2 and undergo fragmentation by collision induced dissociation with a neutral gas. This filtration process plays an important role in increasing sensitivity. With a narrower range of masses travelling through the system, the scan time is decreased, and thus more scans can be collected per unit time. Finally, all fragment ions enter Q3 where all but a specified product ion are filtered out. Methods monitoring a single ion pair are SRM, and MRM for multiple pairs. Reaction monitoring schemes are extremely valuable for identifying analytes in complex sample matrices because selectivity is very high, and background signal is diminished or eliminated by mass filtering leading to higher signal to noise (S/N). Both of these contribute to the high sensitivity and low limits of quantitation (LOQ) that make the triple quadrupole such a popular instrument.\textsuperscript{46}
When high mass accuracy is needed alongside sensitivity and MS/MS, a high-resolution
mass spectrometer can be coupled to LC. One example is a quadrupole time-of-flight (QqTOF),
which is set up like the triple quadrupole except that Q3 is a TOF detector. The time-of-flight mass
spectrometer applies a voltage along the flight tube (usually about 3 m long) whereby ions of
different masses and charge states will separate and arrive at the detector at different times. The
newest innovation in mass spectrometry technology is the Orbitrap, an ion-trap instrument in
which the ions orbit around a central spindle-shaped electrode and are separated based on the
different rotational frequencies caused by their m/z values in relation to the electric field strength.
Orbitrap mass analyzers do not exist as standalone instruments and are combined usually with
either a linear ion trap (LIT) or a quadrupole.46

Despite being the most widely used analytical technique, LC-MS presents a number of
challenges for method developers and analysts to overcome. Most LC-MS systems use
electrospray ionization (ESI) to get analytes into the gas phase. While ESI is extremely popular
and effective, maintaining appropriate ionization efficiency requires some method development of
LC mobile phase conditions. Spray efficiency is inversely related to flow rate in ESI-MS, but to
accommodate high LC flow rates parameters such as desolvation gas flow, auxiliary gas flow, and
spray temperature can be raised with limited positive results. Most modern ESI systems limit LC
flow rate to 1 mL/min, but many chromatography columns meant for LC-MS will run at lower
flow rates such as 0.2-0.8 mL/min. Choosing columns with smaller internal diameters to reduce
flow rates or introducing splitters into the post-column tubing can help with this issue without the
need for extensive MS tuning.47
Another important consideration for ESI-MS is additives in the mobile phase. Electrospray relies on gas- and temperature-based desolvation to get ions into the gas phase and is very sensitive to non-volatile species. For example, buffer salts such as Tris, sulfates, and phosphates, as well as IA and IIA salts (Na or Mg salts for example) will coat the spray source with crystals and eventually completely obstruct flow into the mass spectrometer. Therefore, these salts should be avoided, or the mobile phase should be desalted prior to entering the electrospray source. Also, many mobile phases contain charged additives that can act as ion-pairing reagents or interfering ions. A common example is sodium dodecyl sulfate (SDS), a detergent commonly found in biological samples. SDS readily ionizes and presents as a very strong peak at m/z 265 in the negative ion mode. This could be problematic, especially if there is an analyte of interest with m/z at or near that value. Residual sodium (either from glassware or solutions) will be ionized (m/z 23) and can pair with negatively-charged analytes, leaving a net zero charge and losing MS visibility. The same is true of acidic additives like acetic acid or formic acid and their ability to bind to positively-charged analytes. On the other hand, mobile phase additives in small concentrations can be extremely useful if the analyte(s) of interest are not readily ionizable, which may not be seen in mass spectrometry otherwise.

High Resolution Mass Spectrometry (HR-MS) for Quantitation

Although MS is often combined with another separation technique (most often LC), it is very valuable as a standalone technique. Mass spectrometry is very sensitive, requiring very little material and significantly reducing experiment cost. It is also very fast, with total analysis times in seconds or minutes. Any mass spectrometer is capable of obtaining quantitative data, but some
instruments are better for sensitivity, structural elucidation, mass accuracy, etc. High resolution mass spectrometry (HR-MS) gives high mass accuracy, such that the exact masses of compounds are measured and those with the same nominal masses are separated.\textsuperscript{49} In general, high-resolution mass spectrometers measure m/z to 0.001 mass units and a mass accuracy of 1-2 ppm.\textsuperscript{49b, 50} From the exact masses, HR-MS can give chemical formulas for unknown analytes,\textsuperscript{51} and structural information when MS/MS fragmentation is obtained. Time-of-flight (TOF), Orbitrap, and Fourier transform-ion cyclotron resonance (FT-ICR) instruments are all high resolution – with resolving powers of 60000, 240000, and 1000000, respectively\textsuperscript{52} - and can be hybridized with quadrupoles in order to be MS/MS-capable.\textsuperscript{49b} A popular example is the QqTOF, which is commonly used to identify unknown metabolites in complicated matrices. Mass spectrometry is particularly useful for metabolomics research because a wide array of compounds can be detected and analyzed in a single scan. When in full-scan mode, while a researcher may be looking to analyze a specific compound, they can also identify and analyze other sample components.

For example, when analyzing a complicated matrix (e.g. blood plasma) for drugs, a researcher may come across a peak at m/z 207.145 in positive ion mode.\textsuperscript{53} Using the exact mass, the molecular formula is determined with confidence to be C\textsubscript{13}H\textsubscript{18}O\textsubscript{2}. Even with the molecular formula, more information is needed to confidently identify the compound among the C\textsubscript{13}H\textsubscript{18}O\textsubscript{2} isomers. This could be achieved through other methods such as NMR, or within the same run with tandem mass spectrometry (MS/MS). The ability to gain formula and fragmentation data in a single run is one of the major advantages for using HR-MS for metabolomics. Therefore, the analyst can isolate and fragment the unknown peak, and be able to confirm that it is ibuprofen by the loss of m/z 46.013 corresponding to formic acid.\textsuperscript{53} Additionally, several databases exist to compare
experimental data to standards to gain an extra level of confidence in identification. The summary of identification levels for metabolites is shown below in Figure 2.3.54

Figure 2.3:  A table summarizing the confidence levels for identification of metabolites and the requisite data requirements. Reproduced from: 54

In mass spectrometry, signal is not only proportional to concentration, but to ionization efficiency as well.48, 55 Therefore, internal standards play an extremely important role in quantitation. At minimum, a compound or compounds with comparable ionization efficiency to the analyte(s) of interest should be used. However, the best internal standards are ones that have identical ionization efficiency compared to the analyte, and in mass spectrometry, these are isotopically-labelled versions of analytes with at least a 3 Da mass difference from the unlabeled compound.56 With this technique, the mass spectrometer can easily distinguish between the compounds because of their different mass-to-charge ratios, even after accounting for peak overlap and/or naturally-occurring mass shifts from isotopes.46 Common labelling techniques selectively
label H or C atoms with heavy D or $^{13}$C.\textsuperscript{57} If lower cost is desired, compounds with similar structures to the analyte can also be used in place of isotopically-labelled compounds, although this introduces the potential for ionization efficiency differences.\textsuperscript{57a}

Quantitative mass spectrometry, particularly HR-MS, has wide-reaching applications in fields such as toxicology, environmental science, pharmacology, and others. The exact mass data obtained from HR-MS leads to much higher specificity of analytes vs. matrix components compared to low-resolution mass spectrometry.\textsuperscript{58} This means that quantitation can be done with confidence that signal from a particular m/z is only coming from the analyte of interest and not another compound in the sample. In addition, sensitivity of high-resolution mass spectrometers is very high and quantitation at nanomolar or sub-nanomolar levels is routine.\textsuperscript{59} In most instances, a separation technique such as GC, LC, or solid-phase extraction (SPE) is used prior to quantitative MS, especially when analyzing a complicated sample matrix. This allows for samples varying from bodily fluids, soil, hair, foodstuffs, etc. to be separated and identified based on both polarity and mass-to-charge, as well as eliminating interferences from the sample matrix.\textsuperscript{60} The majority of workflows use targeted screening, meaning the analytes of interest are at least somewhat known and the experimental conditions are tailored for detection and quantitation of those analytes. There are countless examples of this in the literature including but not limited to qualitative identification of rodenticides in whole blood\textsuperscript{61}, detection and quantitation of date-rape drug GHB in human hair\textsuperscript{62}, quantitative determination of the antifungal natamycin in wine\textsuperscript{63}, and detection of polycyclic aromatic hydrocarbons (PAHs) in smoked meats and edible oils\textsuperscript{64}.

In addition to targeted screening, many HR-MS instruments can provide qualitative and quantitative data for most ionizable compounds in a sample in full-scan mode. Because the exact
mass information can yield molecular formula and fragmentation can give clues about molecular structure, previously unknown compounds in a sample can be identified.\textsuperscript{60, 65} In many cases, spectra are compared to those in large databases for an extra level of confidence in identification.\textsuperscript{66} These non-targeted analyses are useful for identifying novel natural products in biological samples,\textsuperscript{67} novel metabolites in humans and other organisms,\textsuperscript{68} contaminants and xenobiotics in food,\textsuperscript{69} and many more.

**Mass Spectrometry Elevated Energy (MS\textsuperscript{E})/ Broadband Collision-Induced Dissociation (bbCID)**

As an alternative to MRM, mass spectrometry elevated energy or broadband collision-induced dissociation is a form of tandem mass spectrometry in which the instrument alternates between high and low collision energy, demonstrated in Figure 2.4. Under low collision energy conditions, a high-resolution mass spectrometer can display the exact mass and a chemical formula can be inferred. Under high collision energy conditions, fragmentation will occur and can be used to determine structural characteristics. Therefore, full-scan and MS/MS data is gathered in a single run. Another huge advantage of using MS\textsuperscript{E} analysis is that exact mass and fragmentation data are obtained for each ion in the selected mass range. This is different from SRM or MRM analyses (discussed above in the LC-MS section) in which precursor ions are selected while discarding extraneous ions. The higher informational content of MS\textsuperscript{E} scans is advantageous for complex or unknown samples.
Figure 2.4: An instrument schematic showing the cycling between high and low collision energies during an MS\textsuperscript{E} experiment. Reproduced from: \textsuperscript{70}

For example, after separation by LC, MS\textsuperscript{E} was used to analyze gelatin in commercial dairy products.\textsuperscript{71} By combining the high mass accuracy of the ESI-Q-TOF-MS with the wealth of indiscriminate fragmentation data obtained by MS\textsuperscript{E} scans, the method was able to differentiate gelatin produced from bovine and porcine in commercial cheese, yogurt, and ice cream. As gelatin is a protein, the unique peptide signatures following trypsin digestion were used as biomarkers for identification. Similarly, in another food science study, \textit{Lycium ruthenicum} fruits were analyzed by MS\textsuperscript{E} on the ESI-Q-TOF-MS to identify polyphenols in order to subsequently study their antioxidant activity.\textsuperscript{72} These workflows are also extremely applicable to the health science fields. In studies of chronic kidney disease, rats were dosed with renal-failure-inducing doses of adenine and their kidney tissues were analyzed by HR-MS to determine metabolic biomarkers of the disease.\textsuperscript{73} In this non-targeted metabolomics study, all ions were considered and those appearing
in adenine-dosed rats and not in control rats were considered potential renal failure metabolites. MS\textsuperscript{E} is particularly useful in this type of experiment because unknown ions could be identified by their intact mass and fragmentation in a single run by alternating between high and low collision energy. Using this workflow, 12 biomarkers for renal failure were identified in negative ion mode and assigned to a biochemical pathway, yielding a wealth of information about the biology of this disease. Additionally, a similar workflow investigated the therapeutic effect of \textit{Porta cocos} epidermis through the metabolic signature of treated and untreated rats.\textsuperscript{74} These and other examples of untargeted metabolomic studies using MS\textsuperscript{E} significantly improve ease and time while still collecting the same quality information as other HR-MS workflows.\textsuperscript{70}

![Figure 2.5: Example spectra reproduced from reference (\textsuperscript{70}) showing the difference between high and low collision energy spectra for xanthuric acid. While the low collision energy spectra mainly shows only the intact ion, the high collision energy one also shows various fragments useful for identification and structural elucidation.](image)
The studies addressed so far all comprise complicated sample matrices, but HR-MS, and MS\textsuperscript{E} in particular, can also be used for general identification purposes. Another study combined both MS/MS and MS\textsuperscript{E} to confirm the identities and elucidate the fragmentation patterns of illicit drugs, and their metabolites and degradation products.\textsuperscript{59} For many of the illicit drugs studied, low-resolution mass spectrometry data has already been obtained. However, there is some debate about the completeness of the data and the interpretation of the mass spectra. The high-resolution data was able to confirm the fragmentation patterns elucidated from low-resolution experiments, and MS\textsuperscript{E} improved the workflow speed by eliminating the need to isolate and fragment ions individually (like in MS/MS).

**Method Development and Validation**

Assay development and validation is often a long and arduous process, with many variables to consider. Several governing bodies, such as the US Food and Drug Administration (FDA) and International Conference on Harmonisation (ICH) have created guidelines for scientists in the pharmaceutical industry and academia to follow to promote uniformity in analytical assays.\textsuperscript{75} When developing an analytical assay, the principle and scope need to be determined first. Enzyme inhibition assays generally measure, either directly or indirectly, the formation of a product or depletion of a substrate. Depending on the type of assay, the procedure could be transferred for use with other enzymatic reactions as well. For example, the work presented here directly assays substrates and/or products by HPLC, MS, or LC-MS, techniques that are widely used and applicable to a variety of biologically relevant compounds. This is in contrast to many existing enzymatic assays, known as coupled enzyme assays, which measure the product of a secondary
enzyme reaction and stoichiometrically relate it back to the analyte of interest. Enzyme inhibition assays can also be continuous or discontinuous (end point) depending on whether samples are analyzed continuously or at discrete intervals along the reaction progression after a quenching step. Assays that employ separation techniques like LC or LC-MS are discontinuous assays.

Once a method type is chosen, there are factors related to the assay conditions that need to be optimized. Some are related to the operating parameters of the reaction, such as buffer salt, ionic strength, temperature, incubation time, and pH. Optimization at this stage ensures that the reaction is actually proceeding in the intended direction with reasonable speed and helps reduce artifacts. Other factors relate to the analysis method itself; for example, in LC-MS the stationary and mobile phases, separation method, LC-MS interface, and MS instrument method all need to be optimized to be compatible with analytes and be quantitative. The sample preparation protocol also requires optimization, both to properly end the reaction (quench) and to make the samples ready for analysis. Sample preparation is an extremely important aspect of LC, LC-MS, and MS analysis as some components of enzymatic reaction solutions are incompatible with those techniques. For example, when using HPLC, enzymes must be removed by either filtration or precipitation because they will clog HPLC columns; and certain reagents such as quench reagents, buffers, and salts will not electrospray and need to be removed before ESI-MS.

To be robust and quantitative, assays must contain the appropriate standards and controls. Various concentrations of the analyte(s) of interest must be prepared to obtain standard curves for quantitation. In addition, positive and negative controls are needed to elucidate artifacts from the reaction or sample matrix. A negative control is a sample in which no response is expected, such as one with no substrate or enzyme present. In a positive control, on the other hand, a response is
expected and tests if your assay is able to measure the reaction signal. When studying enzyme inhibition, a positive control in which no inhibitor is present is needed to show the typical way the enzymatic reaction will proceed. Each experimental run requires both negative and positive controls.

Before being used for quantitation of analytes in real samples, all methods must go through several validation steps. Method validation defines the conditions under which the assay is applicable, the accuracy and precision of measurements from the assay, and assay robustness and reproducibility. Of fundamental importance to method validation is to establish method selectivity, the ability to distinguish the analyte(s) from other components in the sample. Blank analyses of sample matrix should be evaluated for extraneous peaks that could interfere with analyte quantitation either by coelution or signal depletion. Additionally, the signal that is being quantitated must be confirmed to be from the analyte. Selectivity should be confirmed for each analyte to be quantitated down to the lower limit of quantitation. In LC, MS, and LC-MS unique m/z ratios and retention times are used for identity, and a signal-to-noise ratio of three constitutes the lower limit of quantitation (LLOQ).

Standard curves evaluate the linearity of a method by measuring the signal intensity vs. analyte concentration. The calibration curve should include a zero-concentration sample and 4-6 non-zero concentration samples, all prepared in sample matrix. The series of samples of increasing concentration are measured in triplicate and successful linearity is defined as an $R^2 > 0.99$. If the curve passes, it can be used for subsequent quantitation of the analyte in unknown samples. Calibration curves are also important in evaluating the linear dynamic range of a method, the range
over which signal will be proportional to sample concentration. Samples with concentrations outside the range of linearity should not be quantitated with the calibration curve.

Another important method benchmark is a high accuracy and precision in signal and concentration from the method. Precision is analyzed by performing replicate analyses of standard samples and evaluating the percent relative standard deviation (\%RSD) of retention times and peak areas. There are two subsets of precision: intra-batch, inter-batch (intermediate). Intra-batch precision, also called repeatability, is evaluated in by completing replicate analyses within a single analytical experiment; whereas inter-batch precision is evaluated over time and under varying experimental conditions (e.g.: analyst, equipment, reagent lot, etc.). In both cases, precise methods are those in which the deviation is less than 15%. On the other hand, accuracy compares the quantitative data obtained from a sample to the known concentration and measuring the deviation. Accuracy is established by doing replicate analyses of samples with known concentrations of analytes. The concentration of analyte calculated from the method should be within 15% error from the true value for the method to be considered accurate. Both precision and accuracy are extremely important and should be evaluated periodically throughout the use of the method.

\textit{In vitro} Compound Screening for Target Affinity

Modern organic synthesis techniques for drug discovery often lead to libraries of thousands of compounds that need to be tested against the intended target. Therefore, analytical techniques with high throughput and sensitivity are necessary. Assays studying enzyme-substrate or enzyme-inhibitor binding can be conducted \textit{in vitro} (in solution) and binding constants obtained. The most
common experimental procedure is to incubate the enzyme and substrate under biological conditions and study reaction aliquots at different time points, obtaining the reaction velocity. When these experiments are done for various concentrations of substrates and the reaction velocities are plotted versus substrate concentration, a Michaelis-Menten plot with a corresponding Michaelis constant ($K_m$) can be obtained for that enzyme-substrate interaction. When inhibitors are added to the reactions at varying concentrations, inhibition constants ($K_i$) are elucidated that describe the strength of binding between the enzyme and inhibitor. And by modifying the data to form Lineweaver-Burk plots, the inhibition can be described as competitive, non-competitive, or mixed. However, a strong binding affinity to the target enzyme does not guarantee that the compound will be a potent inhibitor. However, *in vitro* screening methods can significantly narrow the number of compounds that are tested further for efficacy by *in vivo* tests.

$$V_0 = V_{\text{max}} \left( \frac{[\text{Substrate}]}{[\text{Substrate}]+K_m} \right) \quad \frac{1}{V_0} = \frac{K_m}{V_{\text{max}}} \cdot \frac{1}{S} + \frac{1}{V_{\text{max}}}$$

Figure 2.6: The prototypical Michaelis-Menten curve (bottom left) and the Michaelis-Menten equation (left), as well as their double reciprocal transformations. Reproduced from: 81
Initial velocity determinations are made by measuring the rate of product formation over time before the reaction has reached equilibrium. Then those initial rates are plotted vs. their respective concentration of substrate to form a Michaelis-Menten curve, which eventually asymptotically approaches $V_{\text{max}}$. $V_{\text{max}}$ is the point at which all binding sites on the enzyme are occupied. The substrate concentration at which 50% of $V_{\text{max}}$ is reached is known as the Michaelis-Menten constant, $K_m$. The $K_m$ value is a measure of binding affinity, with lower $K_m$ values indicating stronger enzyme-substrate binding. The $K_m$ value can also be determined graphically as the x-intercept of a double reciprocal plot (also known as a Lineweaver-Burk plot).\textsuperscript{81}

Michaelis-Menten experiments can also be used to determine inhibition constants, or $K_i$ values. The presence of inhibitors the $K_m$ and/or $V_{\text{max}}$ value, which is very noticeable on a Lineweaver-Burk plot. Competitive inhibitors affect the $K_m$ of the enzyme-substrate interaction (making the binding weaker) and will shift the x-intercept of a double-reciprocal plot toward zero. On the other hand, uncompetitive inhibitors, which do not directly compete with the substrate for space in the enzyme binding site, will increase binding affinity and therefore shift the x-intercept away from zero. They will also decrease the $V_{\text{max}}$ value of the reaction. Finally, non-competitive inhibitors, which affect enzyme activity by binding to a non-binding site on the enzyme, do not affect the $K_m$ of the interaction (no change in x-intercept) but will lower the $V_{\text{max}}$. Therefore, by doing Michaelis-Menten experiments with inhibitors at varying concentrations it is possible to simultaneously the type of inhibitor (competitive, uncompetitive, or non-competitive) and the inhibition constant ($K_i$).\textsuperscript{81}
CHAPTER 3
COMPARISON OF REVERSED-PHASE, ANION-EXCHANGE, AND HYDROPHILIC INTERACTION HPLC FOR THE ANALYSIS OF NUCLEOTIDES INVOLVED IN BIOLOGICAL ENZYMATIC PATHWAYS

Introduction

Nucleotides are an important class of biological molecules that play a variety of roles such as acting as the building blocks for DNA and RNA\textsuperscript{81-82}, storing and releasing chemical energy\textsuperscript{83}, and playing integral roles in enzymatic reactions\textsuperscript{21a,84}. Therefore, there is a lot of interest in developing methods capable of quantitating nucleotides in a variety of sample matrices. High performance liquid chromatography (HPLC) is a very common and effective technique for analyzing biological samples and separating analytes based on their relative polarities\textsuperscript{85}. Nucleotides contain between one and three phosphate groups that are negatively charged at pH slightly above 2\textsuperscript{82a}. Consequently, HPLC method development focuses on interactions between the stationary phase of the column and the phosphate group(s) of the nucleotides. In addition to separation by HPLC, electrospray ionization-mass spectrometry (ESI-MS) is often required for definitive peak identification and more sensitive quantitation than spectrophotometric detection\textsuperscript{46, 86}. For this reason, another important focus of HPLC method development is MS compatibility.
Of particular interest is the role of nucleotides and other phosphate-containing compounds in enzymatic reactions. As the primary biological energy carrier, adenosine triphosphate (ATP) and its dephosphorylated counterpart, ADP, are heavily involved in biological systems including phosphorylation reactions. The primary focus of our work is to develop effective, high-throughput, and robust LC-MS methods to test the efficacy of potential antimicrobial compounds. Several of the pathways and enzymes of interest involve phosphate-containing compounds. One such pathway is the methylerthritol phosphate (MEP) pathway, which was introduced in Chapter 1, and that produces isoprenoid precursors in eukaryotic bacteria and some plants. As isoprenoids are essential to bacterial survival and the MEP pathway is not present in humans, it is an excellent target for antibiotic development. The main enzymes of interest are IspD, IspE, and IspF—all of which catalyze reactions including nucleotides and similar compounds (see Figure 1.2 in Chapter 1).

Also introduced in Chapter 1 was another enzyme of interest for antibiotic development: fructose bisphosphate aldolase, or simply aldolase. Aldolase catalyzes the reversible cleavage of fructose 1,6-bisphosphate (FBP) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P) in the glycolysis (forward reaction) and gluconeogenesis (reverse reaction) pathways (Figure 1.5 in Chapter 1). As these pathways are involved in the production and metabolism of glucose, they are essential for the survival of most organisms. Aldolases are divided into two categories based on mechanism of action: class I and class II. Class I aldolases are found in animals, including humans, and use a Schiff base intermediate on a lysine residue. This is distinct from class II which use Zn ions that bind to a carbonyl group in the active site. These aldolases are found in bacteria, and are a strong
target for antibacterial drugs since there is little sequence homology between the two classes. Assays to study \textit{in vitro} aldolase activity are typically coupled to glycerophosphate dehydrogenase and measure the conversion of NADH to NAD\(^+\) at 340 nm. LC-MS analysis eliminates the need for coupled enzyme assays, significantly reducing costs. The standard nucleotides studied here are secondary reactants and products of the IspE and IspF reactions of the MEP pathway. Due to the expense and instability of MEP pathway substrates, analysis of secondary reactants is a useful alternative for \textit{in vitro} studies. Our goal is to develop effective and high-throughput separations for real-time analysis of enzymatic reaction samples involving phosphate-containing analytes - such as the MEP pathway and aldolase - both \textit{in vitro} and \textit{in vivo}.

Strong anion exchange (SAX) HPLC caters to the strongly anionic nature of nucleotides by using a positively-charged stationary phase containing a quaternary amine moiety. The phosphate group(s) of the nucleotides form charge-charge interactions with the stationary phase to gain retention and are eventually eluted in one of two ways: displacement by a strong anion such as chloride or a pH switch to modify the chemistry of the nucleotide. For example, the sugar-nucleotide product of the antibacterial target IspD enzymatic reaction was analyzed from a reaction mixture using a 0-500 mM NaCl gradient. In general, optimum resolution is achieved when using a steep gradient. However, this approach rules out the possibility of a subsequent MS analysis as salt concentrations for elution are generally in the high-millimolar to molar range. Some groups have attempted to use post-column online desalting as a means of reducing the salt concentration prior to electrospray mass spectrometry and achieving sufficient MS signal.

A majority of LC-MS methods utilize reversed-phase HPLC which contains a non-polar stationary phase such as octadecyl (C18)-bonded silica. On the surface, this seems counterintuitive
for retention and resolution of phosphate-containing nucleotides. Effective separation of nucleotides necessitates the use of ion-pairing (IP) reagents to neutralize the charge and optimize retention. Tetrabutyl ammonium bisulfate was used by Meyers et al. as a mobile phase additive to separate nucleotides in the IspF reaction of the MEP pathway. Although this method provided a short and effective separation scheme using a “rocket” column, a short, high-load column with dimensions 53x7mm, and a high mobile phase flow rate, it did not use a volatile ion-pairing agent and cannot be coupled to MS. However, using low concentrations of volatile ion-pairing reagents such as triethylamine or dibutylamine can counter this effect enough to give acceptable MS signal. Odom et al. performed IP-RP LC-MS analysis with success using tributylamine. However, as is often the case with ion-pair RPLC, a relatively longer equilibration time is required with gradient elution and, in this case, it led to a very long runtime not suitable for near real-time analysis.

As described previously in Chapter 2, hydrophilic interaction HPLC (HILIC) is a subset of normal phase chromatography, combining separation power from the liquid-liquid partitioning between the polar, solvated stationary phase and the water-deficient mobile phase with hydrogen bonding and ion exchange on the surface of the stationary phase to achieve retention of polar analytes and seamless MS compatibility. A variety of HILIC stationary phases are available, each with unique properties and suited for different analytes. Because of the interplay between partitioning and ion-exchange in the HILIC retention mechanism and the wide range of columns offered, finding the optimum separation scheme can be challenging. Due to the aqueous layer of the mobile phase solvating on the surface of the relatively polar stationary phase, HILIC stationary phases often suffer from retention time drift and peak tailing affecting the robustness and
reproducibility of HILIC methods. Retention and separation of nucleotides can be achieved using amino or amide-bonded silica phases, or zwitterionic phases containing sulfonates and quaternary amines. In most cases, when retaining ionic analytes, a stationary phase oppositely-charged is used. However, the recently developed electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) can separate highly charged analytes by utilizing repulsion from a like-charged stationary phase. ERLIC has been used for several applications including the separation of peptides, nucleic acids and nucleotides, and carboxylates.

In recent years, mixed-mode chromatography techniques have been utilized for difficult separations. Mixed-mode methods add a secondary stationary phase modality to increase resolving power. For organic anions such as the phosphate-containing compounds of interest here, a combination of reversed-phase and anion-exchange or hydrophilic interaction and anion-exchange are common. For example, sugar phosphates, including structural isomers, have been effectively separated using a combination of weak anion exchange and reversed-phase under HILIC elution conditions. Similarly, nucleic acid and oligonucleotide separations were investigated on several ionic-hydrophobic stationary phases to determine the optimum support material; by fine-tuning the hydrophobicity of the stationary phase, several large oligonucleotides (80-90 chain length) were able to be separated.

In this chapter, separation of nucleotides is performed with ion-pair RPLC, anion exchange, and HILIC mode of liquid chromatography. The performance of stationary phases in these modes of chromatography and their ability to differentiate nucleotides is compared. The robustness of the three methods was also compared with inter and intraday retention drift. The utility of FructoShell-N HILIC column was further explored for in vitro analysis and quantitation of phosphates in
enzymatic pathway reactions. The efforts led to a robust, fast, and MS-compatible isocratic method for separation of nucleotides.

Materials and Methods

Instruments

All HPLC analysis was done at room temperature on an Agilent 1100 HPLC system with a multiwavelength detector (MWD) set at 260 nm. For each run, 20 μL of sample was injected into the HPLC. Retention time data from five identical runs were analyzed over the course of four days to investigate intra- and interday retention time drift. LC-MS analysis was conducted on a Shimadzu 8030 UHPLC (Nexera)-QqQ system.

Chemicals

5’- adenosine triphosphate (ATP) trisodium salt hydrate, 5’- guanosine monophosphate (GMP) disodium salt, 5’- cytidine monophosphate (CMP), and 5’- adenosine diphosphate (ADP) sodium salt were purchased from Sigma (St. Louis, MO). 5’- cytidine triphosphate (CTP) disodium salt, D-fructose 1,6-bisphosphate trisodium salt octahydrate (98%), acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (glacial), phosphoric acid (85%), formic acid, triethylamine (TEA), sodium chloride (NaCl), ammonium acetate (>99%), and sodium phosphate monobasic (anhydrous) were purchased from Fisher (Chicago, IL). All water used in experiments was filtered with an in-house Milli-Q 18MΩ.cm R-O system (EMD Millipore, Billerica, MA).
Methods

Ion-pair, reversed-phase (IP-RP) HPLC

The IP-RPLC method was developed based on previous reports by Klawitter et al.\textsuperscript{100} Analysis of 0.1 mg/mL GMP, CMP, ADP, ATP, and CTP in starting mobile phase was carried out using a Hydro-RP C18 column (150x4.6 mm, 5 μm, Phenomenex, Torrance, CA). The elution gradient used a mixture of two solvents: 0.1M triethylammonium acetate (TEAA) buffer at pH 5.5 (A) and methanol (B). After initial column equilibration at 92/8 (v/v) A/B, the mobile phase fraction was held at 8% B for 1 min, then linearly increased to 27% B over 12 min, increased to 100% B over 2 min, held at 100% B for 4 min, and decreased to the initial mobile phase 8% B over 1 min, followed by a 15 min re-equilibration period. The flow rate throughout the 20-min run was 700 μL/min.

Strong-anion exchange (SAX) HPLC

For this analysis, the 0.1 mg/mL nucleotide mixture was dissolved in DI water. Separations utilized a Phenosphere SAX column (150x3.2 mm, 5 μm, Phenomenex, Torrance, CA) and a mobile phase consisting of 2 mM sodium phosphate monobasic pH-adjusted to 2.5 with phosphoric acid (A) and the identical pH 2.5 with 1 M NaCl (B). Separation of the nucleotides was achieved in a 30-min run with a column flow rate of 350 μL/min. The gradient elution scheme began after initial equilibration at 0% B for 5 min, then up to 40% over 10 min, followed by an increase to 60% over 6 min, then up to 100% B over 3 min, 100% B for 4 min for a column wash, back down to 0% B over 2 min, followed by re-equilibration for 10 min.
Hydrophilic Interaction (HILIC) HPLC

The HILIC runs analyzed a mixture 0.1 mg/mL CTP, CMP, ADP, ATP, GMP in 55/40/5 acetonitrile/water/methanol on a FructoShell-N HILIC core-shell column (100x3.0 mm, 2.7 μm, AZYP, LLC, Arlington, TX) graciously provided free-of-charge by the company. The 20-min isocratic run used a mobile phase of 85/15 (v/v) of 55.5/39.5/5 Acetonitrile/water/methanol with 100 mM ammonium acetate pH 6.6 overall (A) and acetonitrile with 0.1% formic acid (v/v) (B) at 425 μL/min. For LC-MS analysis, the column flow rate was increased to 800 μL/min and nucleotide m/z values were monitored in negative mode using selected ion monitoring (SIM) over the 10-min run time. A sample of 2 mM FBP in starting mobile phase was analyzed under the same chromatographic conditions with MS/MS detection of dephosphorylated FBP (m/z 339.1>241.1; CE -35.0V) in selected reaction monitoring (SRM) mode.

Prior HILIC analyses included those done on zwitterionic (Nucleodur HILIC by Macherey-Nagel), amide (Xbridge amide by Waters), bare silica (Luna silica by Phenomenex), and amino (Selectosil NH2 by Phenomenex) columns. A mixture of the same five analytes listed above were analyzed on a Waters XBridge Amide column (2.1 x 30 mm, 3.5 μm particle size) with an isocratic elution scheme of 65/35 MeOH/100 mM ammonium formate buffer pH 4.01 at 0.2 mL/min. Additionally, the Nucleodur HILIC column (3.0 x 100 mm, 3 μm particle size) was used to separate CDP, ADP, and ATP in an isocratic run with 70/30 ACN/10mM ammonium bicarbonate buffer pH 7.0 at 0.5 mL/min. A quick retention test of ATP was conducted on a Luna Silica column (2.0 x 100mm, 3μm particle size), but the results were not favorable, and development did not continue.
Results

IP-RP

Reversed phase liquid chromatography (RPLC) is the de facto standard in HPLC methods due to its high sensitivity, robustness, and availability of a wide selection of stationary phases. However, most RPLC stationary phases are based on Octadecyl bonding which offer poor retention to polar analytes. In many cases, highly polar analytes, such as nucleotides, may experience pore expulsion where they are unable to access the surface area inside the pores and elute in void volume. Addition of ion-pairing agents is an effective remedy to retain these polar molecules. For separation of nucleotides, this particular method utilized a high concentration of 0.1 M triethylamine (TEA) to separate five nucleotides commonly found in biological reactions: CMP, CTP, GMP, ADP, and ATP. This method was able to distinctly separate all five analytes with no coelution and offered the best resolution of all the methods tested (Figure 3.1).

Figure 3.1: Representative HPLC chromatogram showing separation of five nucleotides by IP-RP HPLC. This chromatogram excludes the wash stage of the gradient.
At low concentrations, triethylamine is volatile enough for ESI-MS analysis, but prior experiments showed that low concentrations of TEA (~10 mM) did not give sufficient separation of nucleotides (data not shown). Interday variability in retention times was between 3-7% (Table 3.1) and is likely due to mobile phase degradation, mixing problems, or solvent evaporation. Intraday retention times also varied, with relative standard deviations reaching 4-8% on Day 3 compared to 1-3% on Day 2 and <1.5% on Day 4 (Table 3.2). Such drift is not ideal for increasingly relied-upon automated analyses in which the instrument software selects peaks for identification and analysis based on retention time and hence this method is not robust.

Table 3.1: Interday average retention times and variabilities for ion-pair reversed-phase HPLC over four days.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Average ( t_r ) (min)</th>
<th>sd</th>
<th>%rsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>6.94</td>
<td>0.34</td>
<td>4.85</td>
</tr>
<tr>
<td>CTP</td>
<td>3.08</td>
<td>0.10</td>
<td>3.09</td>
</tr>
<tr>
<td>GMP</td>
<td>7.70</td>
<td>0.34</td>
<td>4.38</td>
</tr>
<tr>
<td>ADP</td>
<td>4.54</td>
<td>0.31</td>
<td>6.75</td>
</tr>
<tr>
<td>ATP</td>
<td>3.80</td>
<td>0.17</td>
<td>4.50</td>
</tr>
</tbody>
</table>

Table 3.2: Intraday relative standard deviations in retention times for nucleotides analyzed by IP-RP HPLC.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Day 1 %RSD</th>
<th>Day 2 %RSD</th>
<th>Day 3 %RSD</th>
<th>Day 4 %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>2.14</td>
<td>1.88</td>
<td>5.98</td>
<td>0.91</td>
</tr>
<tr>
<td>CTP</td>
<td>1.01</td>
<td>1.02</td>
<td>4.29</td>
<td>0.56</td>
</tr>
<tr>
<td>GMP</td>
<td>2.56</td>
<td>1.47</td>
<td>5.82</td>
<td>0.89</td>
</tr>
<tr>
<td>ADP</td>
<td>2.11</td>
<td>2.58</td>
<td>7.74</td>
<td>1.19</td>
</tr>
<tr>
<td>ATP</td>
<td>2.04</td>
<td>0.89</td>
<td>8.36</td>
<td>1.16</td>
</tr>
</tbody>
</table>
HILIC

After preliminary screening of several commercially available HILIC columns such as bare silica, zwitterionic, amino, and amide, they provided poor peak symmetry and/or poor resolution (see example chromatogram in Supplemental Information). This led to choosing FructoShell-N HILIC column\textsuperscript{108}, a HILIC stationary phase containing a native cyclofructan 6 moiety (Figure 3.2) which allows for retention of ionic analytes by an ion-exchange mechanism made possible by docking a cation (such as K\textsuperscript{+}, Ba\textsuperscript{2+}, or NH\textsubscript{4}\textsuperscript{+}) into the crown ether core\textsuperscript{109}. Figure 3 shows that the native cyclofructan stationary phase bonded to 2.7 μm core-shell silica support particles and ammonium acetate buffer led to low peak asymmetry which improved sensitivity and resolution.

Figure 3.2: Structure of the cyclofructan stationary phase used in the FRULIC-N and FructoShell HPLC columns. The crown-ether center is able to dock cations (such as ammonium, which was used in this study) and act as an ion-exchanger for retention of acidic analytes.
Of the other HILIC columns tested, although the Xbridge Amide column offered a fast elution scheme, the short size (30mm length) did not allow for adequate separation of analytes (Figure 3.3). The column dimensions of the Nucleodur column are much more similar to those of the FrustoShell column, making the results a better fit for comparison. The best separation was achieved with a mobile phase of 70/30 ACN/10mM ammonium bicarbonate buffer pH 7.0 run isocratically at 0.5 ml/min (Figure 3.4). While the separation was very good with this column, there were issues with retention time stability. This issue was alleviated with overnight column equilibration (Figure 3.5), but this technique is cost-prohibitive and extremely time consuming. With buffers such as ammonium acetate instead of ammonium bicarbonate, tailing increased and severely impacted resolution. Finally, a bare silica column was briefly investigated, but as it is mainly applicable to basic analytes, the retention of the nucleotides was very low and peak tailing was prominent.

Figure 3.3: Analysis of five nucleotides on the XBridge Amide column under isocratic elution.
The FructoShell-N method yielded the lowest intraday peak drift values (Table 3.3) despite retention drift being a common issue with other HILIC phases. One contributing factor was the use of a premixed organic/aqueous mobile phase A to increase temperature stability and prevent gas bubbles from forming. Additionally, the method offered short run time with an isocratic elution scheme, meaning samples can be continuously analyzed with no column re-equilibration. Table 3.4 shows that interday peak drift was generally <5% (with the exception of GMP) and could likely be improved by preparing fresh mobile phase daily. Interday data show a small but consistent downward shift of all analyte peaks.
Figure 3.5: Repeated analysis of CMP under the same chromatographic conditions as those in Figure 3.4, but with overnight column equilibration.

Figure 3.6: Representative HPLC chromatogram of five nucleotides analyzed on the FructoShell HILIC column under isocratic conditions at 0.425 mL/min.
Table 3.3: Intraday retention time variability from HILIC analyses given as %RSD values

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Day 1 %RSD</th>
<th>Day 2 %RSD</th>
<th>Day 3 %RSD</th>
<th>Day 4 %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>0.13</td>
<td>0.31</td>
<td>0.82</td>
<td>0.17</td>
</tr>
<tr>
<td>CTP</td>
<td>0.62</td>
<td>0.49</td>
<td>2.49</td>
<td>0.06</td>
</tr>
<tr>
<td>GMP</td>
<td>0.26</td>
<td>0.27</td>
<td>0.43</td>
<td>0.13</td>
</tr>
<tr>
<td>ADP</td>
<td>0.64</td>
<td>0.47</td>
<td>3.15</td>
<td>0.23</td>
</tr>
<tr>
<td>ATP</td>
<td>0.72</td>
<td>0.51</td>
<td>2.68</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 3.4: Average retention times and variabilities for interday analysis of nucleotides by HILIC

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Average ( t_r ) (min)</th>
<th>sd</th>
<th>%rsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>9.79</td>
<td>0.46</td>
<td>4.73</td>
</tr>
<tr>
<td>CTP</td>
<td>19.91</td>
<td>0.63</td>
<td>3.18</td>
</tr>
<tr>
<td>GMP</td>
<td>8.84</td>
<td>0.47</td>
<td>5.29</td>
</tr>
<tr>
<td>ADP</td>
<td>14.28</td>
<td>0.62</td>
<td>4.36</td>
</tr>
<tr>
<td>ATP</td>
<td>16.63</td>
<td>0.59</td>
<td>3.55</td>
</tr>
</tbody>
</table>

To test the ESI-MS compatibility of the method, the analysis was performed on a Shimadzu 8030 UHPLC-QqQ LC-MS system. MS detection of nucleotides was conducted in negative ion mode with selected ion monitoring (Table 3.5). In an attempt to increase throughput, the column flow rate was increased from 425 µL/min to 800 µL/min. Results show that the increased flow rate decreased the run time from 20 min to 10 min while maintaining resolution between the five analytes (Figure 3.7). It should be noted that under current LC-MS conditions, some ATP undergoes in-source fragmentation forming ADP at the same retention time. This is a common issue in the analysis of nucleotides due to the lability of phosphate groups and the high voltages used in the MS source and ion guides. Further experiments will focus on optimizing conditions to prevent in-source dephosphorylation of the nucleotide analytes.
Table 3.5: The m/z values monitored in SIM(-) LC-MS analysis of nucleotides (Figure 3.7).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>[M-H]^- m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>322.1</td>
</tr>
<tr>
<td>CTP</td>
<td>482.1</td>
</tr>
<tr>
<td>GMP</td>
<td>362.1</td>
</tr>
<tr>
<td>ADP</td>
<td>426.1</td>
</tr>
<tr>
<td>ATP</td>
<td>506.1</td>
</tr>
</tbody>
</table>

Figure 3.7: LC-MS analysis of nucleotides by a 10 min isocratic elution scheme (0.8 mL/min) followed by SIM(-) detection of analyte m/z (values given in Table 3.5).

As a means of evaluating the broader applicability of this method to other phosphate-containing analytes, we tested the retention of fructose bisphosphate – the substrate of aldolase that contains two non-bridged phosphate groups – under the same chromatographic conditions as the above experiments. With none of the reactants and products of the aldolase reaction being UV-
active, chromatographic assays of enzyme activity and/or inhibition must rely on sensitive MS analysis. Figure 3.8 shows an isocratic run at 0.8 mL/min in which FBP retains on the FructoShell-N column and is analyzed by its fragmentation pattern (loss of phosphoric acid). This result is promising for the development of a sensitive and high-throughput aldolase analysis method as an alternative to the traditional spectrophotometric assay.

Figure 3.8: LC-MS/MS spectrum showing retention of fructose bisphosphate on the FructoShell HILIC column with selected reaction monitoring (SRM) detection.

SAX

Strong anion exchange is a popular choice for HPLC analysis of nucleotides without subsequent MS analysis. Our analysis used a Phenosphere SAX quaternary-amine stationary phase and a mobile phase buffered at very low pH of 2.5 to prevent irreversible retention and promote fast elution of
nucleotides when increasing salt concentration. Even using a steep NaCl gradient, we had difficulty obtaining acceptable resolution between ATP and CTP (Figure 3.9) with this particular method. However, the very low interday retention time drift, as evidenced in Table 3.6, allowed for resolution to remain $\geq 1.0$. Additionally, the intraday %RSD values for CTP and ATP retention times remained very low, with an average of <2% for both analytes (Table 3.7).

**Nucleotide Separation by Strong Anion Exchange HPLC**

![Chromatogram of nucleotide separation](image)

Figure 3.9: Representative chromatogram of nucleotide separation of SAX. Varying degrees of coelution of CTP and ATP were present in all runs.
Table 3.6: Interday average retention times and deviations for nucleotide analytes analyzed by strong anion exchange HPLC.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Average $t_r$ (min)</th>
<th>sd</th>
<th>%rstd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>3.23</td>
<td>0.03</td>
<td>0.80</td>
</tr>
<tr>
<td>CTP</td>
<td>14.57</td>
<td>0.19</td>
<td>1.34</td>
</tr>
<tr>
<td>GMP</td>
<td>4.31</td>
<td>0.07</td>
<td>1.57</td>
</tr>
<tr>
<td>ADP</td>
<td>11.29</td>
<td>0.14</td>
<td>1.23</td>
</tr>
<tr>
<td>ATP</td>
<td>13.54</td>
<td>0.18</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Table 3.7: Intraday variabilities in retention times for nucleotides analyzed by SAX.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Day 1 %RSD</th>
<th>Day 2 %RSD</th>
<th>Day 3 %RSD</th>
<th>Day 4 %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>1.11</td>
<td>0.81</td>
<td>1.52</td>
<td>1.10</td>
</tr>
<tr>
<td>CTP</td>
<td>2.65</td>
<td>0.21</td>
<td>3.57</td>
<td>0.71</td>
</tr>
<tr>
<td>GMP</td>
<td>3.02</td>
<td>1.96</td>
<td>2.86</td>
<td>2.22</td>
</tr>
<tr>
<td>ADP</td>
<td>0.49</td>
<td>0.28</td>
<td>0.89</td>
<td>0.71</td>
</tr>
<tr>
<td>ATP</td>
<td>1.04</td>
<td>0.22</td>
<td>0.67</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Discussion

Separation parameters for each analyte are listed in Table 3.8, with parameters listed for the developed FructoShell-N HILIC method and IP-RP and SAX method values for reference. Retention factors ($k$) demonstrate the degree of retention of an analyte relative to the void time of the column, and a range of 2-10 is considered optimal. While the experiments described here analyzed clean samples, unretained compounds from complicated sample matrices could potentially overshadow these peaks. For the nucleotides analyzed by FructoShell-N, the retention factors were often greater than 10. While this is not explicitly a problem, phenomena such as peak broadening tend to be exacerbated as retention increases. However, higher retention is clearly
better given the eventual goal of analyzing biological samples which will leave a lot of unretained components.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean $t_r$ (min)</th>
<th>Mean $k^b$</th>
<th>$\alpha^c$</th>
<th>Mean $N^d$</th>
<th>Mean $R_s^e$</th>
<th>Mean $S^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>6.92</td>
<td>1.78</td>
<td>2.18</td>
<td>37084</td>
<td>16.68</td>
<td>0.69</td>
</tr>
<tr>
<td>CTP</td>
<td>3.07</td>
<td>0.23</td>
<td>--</td>
<td>18773</td>
<td>--</td>
<td>0.71</td>
</tr>
<tr>
<td>GMP</td>
<td>7.68</td>
<td>2.09</td>
<td>1.17</td>
<td>28586</td>
<td>4.18</td>
<td>0.44</td>
</tr>
<tr>
<td>ADP</td>
<td>4.52</td>
<td>0.82</td>
<td>1.56</td>
<td>22081</td>
<td>5.98</td>
<td>0.77</td>
</tr>
<tr>
<td>ATP</td>
<td>3.80</td>
<td>0.52</td>
<td>2.24</td>
<td>9666</td>
<td>4.69</td>
<td>0.49</td>
</tr>
</tbody>
</table>

HILIC (n=19)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean $t_r$ (min)</th>
<th>Mean $k^b$</th>
<th>$\alpha^c$</th>
<th>Mean $N^d$</th>
<th>Mean $R_s^e$</th>
<th>Mean $S^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>9.77</td>
<td>7.72</td>
<td>1.12</td>
<td>4951</td>
<td>1.71</td>
<td>0.62</td>
</tr>
<tr>
<td>CTP</td>
<td>19.89</td>
<td>16.8</td>
<td>1.21</td>
<td>10465</td>
<td>4.21</td>
<td>0.62</td>
</tr>
<tr>
<td>GMP</td>
<td>8.82</td>
<td>6.87</td>
<td>--</td>
<td>6084</td>
<td>--</td>
<td>0.84</td>
</tr>
<tr>
<td>ADP</td>
<td>14.26</td>
<td>11.7</td>
<td>1.52</td>
<td>8294</td>
<td>7.17</td>
<td>0.48</td>
</tr>
<tr>
<td>ATP</td>
<td>16.61</td>
<td>13.8</td>
<td>1.18</td>
<td>9675</td>
<td>3.48</td>
<td>0.61</td>
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</tbody>
</table>

SAX (n=20)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean $t_r$ (min)</th>
<th>Mean $k^b$</th>
<th>$\alpha^c$</th>
<th>Mean $N^d$</th>
<th>Mean $R_s^e$</th>
<th>Mean $S^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>3.23</td>
<td>0.34</td>
<td>--</td>
<td>1704</td>
<td>--</td>
<td>0.51</td>
</tr>
<tr>
<td>CTP</td>
<td>14.54</td>
<td>5.03</td>
<td>1.09</td>
<td>2865</td>
<td>0.93</td>
<td>0.32</td>
</tr>
<tr>
<td>GMP</td>
<td>4.31</td>
<td>0.79</td>
<td>2.32</td>
<td>6458</td>
<td>5.03</td>
<td>0.92</td>
</tr>
<tr>
<td>ADP</td>
<td>11.27</td>
<td>3.68</td>
<td>4.67</td>
<td>66092</td>
<td>39.71</td>
<td>1.38</td>
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<tr>
<td>ATP</td>
<td>13.53</td>
<td>4.61</td>
<td>1.26</td>
<td>7016</td>
<td>3.50</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table 3.8: Summary of various chromatographic parameters for each nucleotide analyzed by IP-RP, SAX, and HILIC.

$^a$n = total number of runs for each method

$^b$Mean $k$ was obtained by subtracting column void time ($t_0$) from retention time ($t_r$) for each run then taking the mean

$^c$α for each analyte is the ratio of $k$ values for adjacent peaks and was not calculated for the first-eluting analyte from each method

$^d$Mean $N$ was calculated as $N = 16 \left( \frac{t}{w} \right)^2$ then averaged

$^e$Mean $R_s$ was calculated using the fundamental resolution equation for adjacent, subsequent peaks

$^f$Symmetry factors were calculated by the Agilent ChemStation software, and $S$ values for each run were averaged.
The reversed-phase separation that we used offered very high efficiency. Ion pairing is widely known to have this effect because it produces sharp peaks and modulates retention. Our goal was to model this efficiency with the HILIC method, and when using the FructoShell-N column, the separation efficiency ranged between about 5000-10000 plates. As mentioned previously, peak shape (particularly broadening and tailing) is a common problem with HILIC separations. The core-shell particles utilized in the FructoShell-N column treat this problem by decreasing the eddy dispersion significantly while also reducing the longitudinal diffusion and resistance to mass transfer, thereby providing a more homogenous packed bed that provides a higher performing column. Additionally, the FructoShell-N column is UHPLC capable, and increasing the column flow rate can be used as a means of decreasing run times (as evidenced in Figure 3.7), and increasing resolution by improving column efficiency\textsuperscript{110}. Some initial studies have been done to improve separation by substituting acetonitrile as the organic solvent for tetrahydrofuran. As THF is aprotic and less polar than acetonitrile, the partition coefficient between the aqueous and organic layers are greater in this case, theoretically leading to a more effective separation. The examples of THF/ammonium acetate isocratic and gradient elution schemes, and high flow rate runs are given in the Supplemental information (Figures 3.10-3.12). Important to note is that the column used in these studies was 15cm long, so comparisons are made only to data from the 15cm column. Compared to the isocratic THF/ammonium acetate mobile phase (3.10), the gradient elution schemes (3.11) provide an increase in separation efficiency by improving peak shape of late-eluting triphosphates. The impact is even greater when the column is run in UHPLC mode (3.12) and, as mentioned previously, running at a higher flow rate cuts the run time approximately in half.
Figure 3.10: Isocratic elution of five nucleotides on a 15cm column with a buffer/THF mobile phase.

Figure 3.11: Sample gradients of THF/buffer to improve separation and peak shape of nucleotide analytes.
Figure 3.12: Nucleotide analysis by a shortened gradient in UHPLC mode.

Table 3.9: Average intraday %RSD values for each analyte for each of the three separation methods tested.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>SAX average %RSD</th>
<th>HILIC average %RSD</th>
<th>IP-RP average %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>1.14</td>
<td>0.36</td>
<td>2.73</td>
</tr>
<tr>
<td>CTP</td>
<td>1.79</td>
<td>0.92</td>
<td>1.72</td>
</tr>
<tr>
<td>GMP</td>
<td>2.52</td>
<td>0.27</td>
<td>2.69</td>
</tr>
<tr>
<td>ADP</td>
<td>0.59</td>
<td>1.12</td>
<td>3.41</td>
</tr>
<tr>
<td>ATP</td>
<td>0.55</td>
<td>1.00</td>
<td>3.11</td>
</tr>
</tbody>
</table>

The data in Table 3.9 show that average intraday %RSD for each analyte was generally <1% for FructoShell-N HILIC. The small drift between runs has important implications for the development of fully-automated analysis schemes being used in many applications. These results are in stark contrast to the those obtained from a repeatability analysis of three nucleotides – ADP, ATP, and CDP – on the zwitterionic Nucleodur HILIC column, seen in Figure 3.4, in which visible peak drift was observed between six replicate runs and subsequently required overnight
equilibration (Figure 3.5). Finally, FructoShell-N HILIC is ESI-MS compatible without the time-intensive and expensive desalting efforts required when using the high concentrations of salts and ion-pairing reagents displayed in these reference methods. It is important to note, however, that the methods presented here are only a small sampling of the variety of HPLC methods applicable to organic phosphate analysis. Ion-pair reversed-phase methods are extremely common and are even sometimes combined with MS when a volatile ion-pair reagent is used in low concentrations, and the steep gradients often used in ion-exchange methods promote high-throughput.\textsuperscript{30-31, 89} For example, inhibition of IspD by fosmidomycin was previously quantitated using an LC-MS/MS method in which 10 mM tributylamine is used as an ion-pairing reagent with an almost 30-min. stepwise gradient from 10-60% ACN followed by a 100% ACN column wash on the Hydro-RP C18 column.\textsuperscript{32} By using a low concentration of a volatile ion-pairing reagent combined with a RP column with polar endcapping, matrix effect was largely avoided in this case.

In addition, changes in phase chemistry and column size can make significant changes toward improving the quality and efficiency of a separation. When the analytes are UV-Vis-active, an IP-RP or SAX method with good resolution may be the most appropriate analysis technique. The ultimate goal of this work is to develop an LC-MS method that can be applied across multiple enzymatic pathways with polar, acidic analytes with little or no intermediate method development required. Because HILIC does not necessitate the use of high concentrations of salts or ion-pairing reagents for separation, we chose to focus our method development on that. Based on our initial method development results from a few of the many HILIC phases offered, we present a strong case here that FructoShell-N is a promising stationary phase for further research in this type of applications.
Conclusions

For decades, HPLC analysis of nucleotides and other phosphates was done by either anion exchange or ion-pair reversed-phase. However, great care must be taken when interfacing separation techniques with mass spectrometry, as these methods often require non-volatile mobile phase additives. Hydrophilic interaction liquid chromatography (HILIC) can alleviate the issues with AX and RP, but not all HILIC stationary phases are suited for separating strongly ionic nucleotides. Based on the data presented here and previous evaluations of several HILIC phases, the FructoShell HILIC column successfully separated the nucleotide analytes from the MEP pathway via isocratic elution without dramatic peak tailing or co-elution and was able to retain fructose bisphosphate from the aldolase reaction with no method modifications. In addition, FructoShell HILIC gave comparable retention time stability to the reference SAX and IP-RP methods, and was shown to be ESI-MS compatible without desalting. Finally, increasing the flow rate to reduce run time did not compromise resolution, making this method a good choice for high-throughput UHPLC or LC-MS analysis of phosphate-containing compounds in biological systems such as the MEP pathway, aldolase, and many more.
CHAPTER 4

QUANTITATIVE HR-MS: METHOD DEVELOPMENT AND VALIDATION

Introduction

The aldolase enzyme is present in all organisms and this reaction plays a vital role in the transition between the preparatory and pay-off phases of the glycolysis pathway.\textsuperscript{81} Glycolysis converts monosaccharides (primarily glucose) into the biological energy carrier ATP and precursors for the Krebs cycle or cellular respiration (pyruvate and NADH). The aldolase enzyme is also involved in gluconeogenesis, essentially the opposite of glycolysis in which glucose is synthesized to maintain appropriate blood glucose levels.\textsuperscript{111} Because of the widespread importance of the pathway in sustaining life, glycolysis enzymes are of scientific interest for mechanistic, kinetic, and inhibitory studies. The aim of this part of the study is to develop and qualify an ESI-MS method, without prior HPLC separation, to be used for \textit{in vitro} kinetic analysis of the fructose bisphosphate (FBP) aldolase-catalyzed enzymatic reaction (shown in Figure 1.5 in Chapter 1).

As seen in the Figure 1.5, all of the compounds involved in the aldolase reaction are phosphates or bisphosphates. Phosphate-containing analytes are great candidates for MS detection because the phosphate group is anionic at almost any pH.\textsuperscript{112} Therefore, these compounds can be analyzed by ESI in negative ion mode without adjusting the pH of the solution.\textsuperscript{113} This does not mean, however, that method development is not necessary. Phosphate groups can readily cleave if ESI and ion transfer conditions are harsh, including a process known as in-source fragmentation.\textsuperscript{114}
It is important to optimize intact ion signal while keeping in-source fragmentation to a minimum, and MS parameters can be tuned to achieve the best balance.

The MS method developed here utilizes a high-resolution ESI-QqTOF system, a schematic of which is shown in Figure 4.1. HR-MS demonstrates very high sensitivity and mass accuracy, helping to avoid analyte interference and decreasing the amount of sample needed. As mentioned in Chapter 2, the QqTOF system can distinguish ions with mass differences of 0.001 m/z with a resolving power of up to 60,000. In the post-run analysis software, the exact mass data can be matched to the corresponding chemical formula and compounds of the same nominal mass are ranked according to mass error. An added benefit of the QqTOF is the capability of MS/MS which when combined with the chemical formula allows the user to confidently identify analytes based on both formula and structure.

Figure 4.1: A schematic showing the components of an ESI-QqTOF-MS. Obtained from: 
MS quantitation is based on comparative signal between the analyte and an internal
standard.\textsuperscript{46} In this case, the analyte of interest is reaction substrate fructose bisphosphate (FBP)
and isotopically-labelled analog, in this case (\textsuperscript{13}C)\textsubscript{6}-FBP, is the internal standard. The method
development and validation process followed the goals of specificity, high sensitivity, mass
accuracy, linearity, and precision. Guidelines for evaluation of these parameters are provided by
ICH, a procedure which is nearly universally used for analytical methods.\textsuperscript{75a} The MS methods
developed here were designed to meet the guidelines provided by the ICH, and only validation
data for the optimized method is presented.

As part of the method validation process, mass accuracy for the QqTOF-MS was studied
by calculating mass error between the theoretical and experimental m/z values for both the
substrate and internal standard. Identities were also confirmed by MS in both selected reaction
monitoring (SRM) and alternating collision energy (MS\textsuperscript{E}) modes. Once the analytes were
thoroughly identified, a “blank” \textit{in vitro} reaction sample was analyzed to confirm method
specificity for these analytes.

In order to be quantitative, an assay must exhibit linearity over a useful range of standard
concentrations. Typically, calibration curves must include at minimum: triplicate analyses of five
different standard concentrations. In the case of mass spectrometry, for which concentration is not
easily deciphered by signal intensity alone, FBP signal is normalized against the signal from a
constant concentration of internal standard. The standard curve can also elucidate the linear
dynamic range. To do this, standards over several orders of magnitude of concentration are needed.
The linear dynamic range of an assay is the number of orders of magnitude during which linearity
holds.
Precision was evaluated as repeatability and two forms of intermediate precision (intra- and inter-day). Both are important for constant and long-term data gathering. Repeatability analyzes at least six replicates of a single sample and reports deviation in both signal and m/z. Intermediate precision is broken down in several ways: variability on a single day of separate sample preparations and replicate analysis (intraday), and comparison of the intraday analyses from more than one day (interday). In this case, data was also gathered for samples analyzed using a different ESI spray needle – the variability of which can offer insight into method robustness and transferability.

All of these parameters give insight into the effectiveness of HR-MS for quantitation. However, the validation data shown here analyzed clean mixtures containing only substrate and internal standard in reaction solvents. Therefore, because the eventual goal is to apply this method for quantitation of in vitro reaction kinetics, an example reaction is run using the optimized MS conditions as a “proof of concept,” to confirm that real, useful quantitative data can be gathered and applied to enzymatic kinetic reaction models.

Materials and Methods

Chemicals

D-fructose 1,6-bisphosphate trisodium salt octahydrate (98%), ammonium acetate, sodium chloride, and acetonitrile (HPLC grade) were purchased from Fisher (Chicago, IL). Aldolase from rabbit muscle was purchased from Sigma-Aldrich (St. Louis, MO). \((^{13}\text{C})_6\)-D-fructose bisphosphate sodium salt hydrate (98%) was purchased from Cambridge Isotope Laboratories Inc. (Andover,
All water used in experiments was filtered with an in-house Milli-Q 18MΩ.cm R-O system (EMD Millipore, Billerica, MA).

Instrument Parameters

All samples were analyzed on a Bruker MaXis Plus ESI-QqTOF instrument in negative ion mode from m/z 100-800. The ESI source parameters were as follows: 210 C dry gas temperature, 8.0 L/min dry gas (N₂) flow, 1.6 bar nebulizer gas (N₂) pressure, 3.5 kV capillary voltage, and 700V end plate offset. The QqTOF-MS was tuned as follows: funnel RF 300 Vpp, multipole RF 200 Vpp, collision RF 1000.0 Vpp, ion cooler RF 800 Vpp, isCID (in-source collision-induced dissociation) 0.0 eV, ion energy 4.0 eV, collision energy 7.0 eV, pre-pulse storage 5.0 μs, transfer time 100.0 μs. This MS method was used for sample analyses unless otherwise specified.

Mass Accuracy

To evaluate mass accuracy of FBP and the IS, a sample of 0.1 mg/mL of each in 50/50 acetonitrile/10mM ammonium acetate buffer was analyzed using the instrument method above. In addition, fragmentation experiments were conducted in selected ion monitoring (SRM) mode for each analyte by isolating the ion and applying 16 eV of energy to q2.

Standard curves

Fructose 1,6-bisphosphate (FBP) at various concentrations between 0.1-500 μM in 50/50 (v/v) reaction buffer/acetonitrile with a constant concentration of 10 μM (13C₆)-fructose 1,6-
bisphosphate (the internal standard). The reaction buffer used was that of class I aldolase: 10 mM ammonium acetate at pH 7.6 with 2 mM NaCl. A standard curve for FBP was created by plotting average normalized signal (arbitrary counts) from triplicate sample analyses vs. concentration (μM).

Specificity

To simulate a “blank” reaction sample, a solution of 12.7 nM rabbit muscle aldolase (tetramer) in 50/50 buffer/acetonitrile was prepared. The full scan spectrum from this sample was evaluated for interfering ions at m/z 338.99 or 345.01 +/- 0.01, the m/z values of FBP and (13C)6 -FBP, respectively.

Repeatability and Intermediate Precision

Repeatability was studied by comparing the m/z values and signal intensities from six replicate injections of 0.1 mg/mL FBP and 13C-FBP in 50/50 buffer/ACN. The mean, standard deviation, and relative standard deviation (%RSD) were calculated and reported.

Intermediate precision was evaluated over two days. On each day, three solutions of 0.1 mg/mL FBP and 13C-FBP in 50/50 buffer/ACN were each analyzed twice and the means, standard deviations, and %RSDs were reported (intraday precision). The data from the two days were compared to yield interday precision values. An additional experiment on the second day used an alternative spray needle than the one used for previous experiments. The samples prepared on Day 2 were also used for this experiment and the results were compared to the intraday data from Day 2 (robustness).
The instrument method used for MS$^E$ was different from the one used for other experiments. In this case, the ESI source parameters were: 220 °C dry gas temperature, 8.0 L/min dry gas (N$_2$) flow, 1.6 bar nebulizer gas (N$_2$) pressure, 3.0 kV capillary voltage, and 500V end plate offset. The QqTOF tune parameters were: funnel RF 150 Vpp, multipole RF 50 Vpp, collision RF stepped between 650.0 and 2000.0 Vpp, and ion transfer time ramped between 41.1 and 64.0 μs, isCID 0.0 eV, ion energy 4.0 eV, collision energy 7.0 eV, pre-pulse storage 1.0 μs, transfer time 100.0 μs. The collision energy was alternated between 7.0 eV (low energy) and 30.0 eV (high energy) for MS$^E$. The experiment was conducted with a solution of 0.1 mg/mL FBP and $^{13}$C-FBP in 50/50 buffer/ACN.

Results

Mass Accuracy

Full scan and SRM scans for both FBP and $^{13}$C-FBP are presented below (Figures 4.3-4.6). The data show that the m/z values of 338.9922 for FBP and 341.0112 for $^{13}$C-FBP. Table 4.1 shows the comparison between the theoretical calculated exact masses and the measured ones. For both ions and their fragments, mass accuracy is very high, with the differences in m/z values being between 1 and 4 mDa and 6-11 ppm. These ions were confirmed as those corresponding to the negative ion of FBP and $^{13}$C-FBP, [M-H]$^-$, and SRM fragments corresponding to the loss of phosphoric acid, [M-H$_3$PO$_4$-H]$^-$. Replicate trials show slight variability in the third and fourth decimal places, so henceforth, FBP will be identified as m/z 338.99 +/- 0.01 and $^{13}$C-FBP as 345.01
+/- 0.01. This range of error also incorporates the theoretical mass values. It is important to note, however, that these ranges are subject to change over time or with instrument modification. Table 4.2 shows mass accuracy data produced after the spray needle distance was adjusted and demonstrated a roughly three-fold increase in mass error for each compound and fragment compared to Table 4.1. Therefore, mass accuracy will be continuously monitored, and the instrument will be re-calibrated as necessary to keep mass error as low as possible.

Figure 4.2: Structures and m/z values for the substrate fructose bisphosphate (FBP, right) and the internal standard \( ^{13}C_6\text{-FBP}, \text{left} \).

\[
\begin{align*}
6^{13}C\text{-FBP} & \quad [M-H]^+ = 345.01 \\
\text{FBP} & \quad [M-H]^+ = 338.99
\end{align*}
\]
Table 4.1: Summary of mass accuracy data.

<table>
<thead>
<tr>
<th>Name</th>
<th>Calculated exact mass (Da)</th>
<th>Measured exact mass (Da)</th>
<th>Δ (Da)</th>
<th>Δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose bisphosphate [M-H]⁻</td>
<td>338.988772</td>
<td>338.9922</td>
<td>0.0033</td>
<td>10.11</td>
</tr>
<tr>
<td>Fructose bisphosphate fragment* [M-H₃PO₄-H]⁻</td>
<td>241.011333</td>
<td>241.0134</td>
<td>0.0021</td>
<td>8.58</td>
</tr>
<tr>
<td>(¹³C)₆-fructose bisphosphate [M-H]⁻</td>
<td>345.008356</td>
<td>345.0116</td>
<td>0.0032</td>
<td>9.40</td>
</tr>
<tr>
<td>(¹³C)₆-fructose bisphosphate fragment** [M-H₃PO₄-H]⁻</td>
<td>247.031460</td>
<td>247.0330</td>
<td>0.0015</td>
<td>6.23</td>
</tr>
</tbody>
</table>

*The mass difference corresponds to phosphoric acid (exact mass: 97.976898 Da, delta 0.001802)

** The mass difference corresponds to phosphoric acid (exact mass: 97.976898 Da, delta 0.001702)

Table 4.2: Summary of mass accuracy data after adjusting capillary distance

<table>
<thead>
<tr>
<th>Name</th>
<th>Calculated exact mass (Da)</th>
<th>Measured exact mass (Da)</th>
<th>Δ (Da)</th>
<th>Δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose bisphosphate [M-H]⁻</td>
<td>338.988772</td>
<td>339.0009</td>
<td>0.0121</td>
<td>35.78</td>
</tr>
<tr>
<td>Fructose bisphosphate fragment* [M-H₃PO₄-H]⁻</td>
<td>241.011333</td>
<td>241.0205</td>
<td>0.0092</td>
<td>38.04</td>
</tr>
<tr>
<td>(¹³C)₆-fructose bisphosphate [M-H]⁻</td>
<td>345.008356</td>
<td>345.0212</td>
<td>0.0128</td>
<td>37.23</td>
</tr>
<tr>
<td>(¹³C)₆-fructose bisphosphate fragment** [M-H₃PO₄-H]⁻</td>
<td>247.031460</td>
<td>247.0404</td>
<td>0.0089</td>
<td>36.19</td>
</tr>
</tbody>
</table>

*The mass difference corresponds to phosphoric acid (exact mass: 97.976898 Da, delta 0.003502)

** The mass difference corresponds to phosphoric acid (exact mass: 97.976898 Da, delta 0.003902)
Figure 4.3: FBP full scan
Figure 4.4: FBP SRM
Figure 4.5: IS full scan
Specificity

One of the major benefits of HR-MS is the extremely high specificity. As opposed to a low-resolution method which can only measure exact mass to within about 0.01-0.1, HR-MS measures exact mass to roughly 0.001 m/z. Because of this, even if interfering peaks are present at the same nominal mass as the analyte, they will not have the same exact mass. For this method, the matrix was relatively simple but specificity can also be evaluated by HR-MS for complicated matrices such as whole blood. Analyzing a complex matrix increases the chances of isobaric ions in MS,
even in high-resolution mode. The QqTOF-MS, which is capable of obtaining fragmentation data, allows those isobaric ions to be structurally elucidated from each other.

Specificity for the ESI-QqTOF method was evaluated by studying a sample of 12.7 nM rabbit muscle aldolase in class I aldolase buffer. This is essentially an aldolase reaction sample without substrate (FBP) or internal standard ($^{13}$C-FBP). Figure 4.7 shows a full scan spectrum of this blank sample and demonstrates that there is negligible signal in the range of m/z 338-346. Additionally, none of the noise peaks in this range match the exact mass of fructose bisphosphate (m/z 338.99) or ($^{13}$C)$_6$-fructose bisphosphate (m/z 345.01), conclusively confirming the specificity of the method.
For this study, linearity was investigated over three orders of magnitude of fructose bisphosphate concentration (0.1-500 μM) and under the buffer conditions for both Class I aldolase. Figure 4.8 shows that over the full series of micromolar concentrations, triplicate analyses yielded a correlation coefficient (R²) of 0.9982. Error bars are displayed for each average normalized signal as +/- 1 SD value (raw data shown in Figure 4.S1).
Based on the y-intercept for the calibration, it is evident that linearity breaks down at very low FBP concentrations (< 1µM). Standard curve data from the low concentration values are shown below in Figure 4.9. Error bars on these values correspond to %RSD in normalized signal - ~90%, 33%, and 15%, respectively. These low concentrations, especially 0.1 µM, are approaching the limit of detection for this method and thus signal varies widely scan-to-scan. For example, of the three replicate analyses of 0.1 µM FBP, two gave average m/z 338.99 signal of 50 counts or less and one could not detect the ion at all. In the 0.5 µM and 1 µM samples, variability decreases as the analyte concentration moves away from the limit of detection, but signal is still low enough (<100 counts for 0.5 and <1000 for 1) for small variances in sample signal to have a huge impact. Despite the high variability in FBP signal among these samples, their removal from the data set did not affect the correlation coefficient and therefore they cannot be considered outliers. Future analyses at low concentrations will require more replicates to counter the random signal variability.
Figure 4.9: Linearity data for low concentration samples with error bars showing extreme variability in signal intensity among triplicate analyses.

The high linear dynamic range when using this buffer demonstrates the importance of keeping the concentrations of non-volatile buffer salts to a minimum for ESI-MS. Overall, linearity experiments show promising results for analysis of *in vitro* aldolase reactions. It is important to note however that the samples analyzed here did not contain enzyme and were not meant to evaluate enzyme activity. Because the buffer salt concentrations used here were intentionally kept significantly lower than those used for plate-based assays, additional experiments will need to be conducted to ensure that the enzyme has catalytic activity. If any additional method development is required, this would likely affect the linearity.

**Precision**
Results of the six replicate repeatability trials are summarized in Table 4.3. The m/z of both the substrate and internal standard extremely stable between runs, with standard deviations of 0.0002 m/z, which translates to %RSD values around 4e-5 %. The signal intensities exhibit some variability run-to-run, but it is relatively low at <5%. The low run-to-run variability in signal intensities is very promising, meaning that aliquots from a single enzymatic reaction can be analyzed using one calibration which will save significant analysis time and materials.

Table 4.3: Summary of repeatability experimental results.

<table>
<thead>
<tr>
<th></th>
<th>FBP m/z</th>
<th>FBP Signal</th>
<th>13C-FBP m/z</th>
<th>13C-FBP Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>338.9980</td>
<td>503364</td>
<td>345.0183</td>
<td>113592</td>
</tr>
<tr>
<td>SD</td>
<td>0.0002</td>
<td>16739</td>
<td>0.0002</td>
<td>3612</td>
</tr>
<tr>
<td>%RSD</td>
<td>4.44e-5</td>
<td>3.33</td>
<td>4.36e-5</td>
<td>3.18</td>
</tr>
</tbody>
</table>

The first studies of intermediate precision are based on variability on a single day between different sample preparations (intraday intermediate precision, Table 4.4). As with the repeatability experiment, there is almost no variation in m/z values between replicates and sample preparations. On each of the two days, signal intensity %RSD is between 2-13%. While this is relatively low, especially for day 2, intraday precision can easily be improved by re-running the quantitation calibration solution between subsequent reaction experiments. At minimum, recalibration should occur daily and when changing equipment because interday precision (Table 4.5) and precision between ESI spray needles (Table 4.6) are very poor. However, this is only the case for signal intensity, which has %RSD ranging from 20-45%. On the other hand, although m/z %RSD is one order of magnitude larger interday and between needles, it is still very low at 1-2e-4 %.
Table 4.4: Intraday intermediate precision data for days 1 and 2.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBP m/z</td>
<td>338.9964 5.00e-5 1.47e-5</td>
<td>338.9976 0.0004 1.28e-4</td>
</tr>
<tr>
<td>FBP signal</td>
<td>516753 63084 12.21</td>
<td>384734 11094 2.88</td>
</tr>
<tr>
<td>$^{13}$C-FBP m/z</td>
<td>345.0166 5.77e-5 1.67e-5</td>
<td>345.0179 0.0004 1.26e-4</td>
</tr>
<tr>
<td>$^{13}$C-FBP signal</td>
<td>111584 10112 9.06</td>
<td>58403 1453 2.49</td>
</tr>
</tbody>
</table>

Table 4.5: Interday intermediate precision between days 1 and 2.

<table>
<thead>
<tr>
<th></th>
<th>FBP m/z</th>
<th>FBP signal</th>
<th>$^{13}$C-FBP m/z</th>
<th>$^{13}$C-FBP signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>338.9970</td>
<td>450744</td>
<td>345.0172</td>
<td>84993</td>
</tr>
<tr>
<td>SD</td>
<td>0.0009</td>
<td>93351</td>
<td>0.0009</td>
<td>37604</td>
</tr>
<tr>
<td>%RSD</td>
<td>2.61e-4</td>
<td>20.71</td>
<td>2.60e-4</td>
<td>44.24</td>
</tr>
</tbody>
</table>

Table 4.6: Variability data from analyses between spray needles.

<table>
<thead>
<tr>
<th></th>
<th>FBP m/z</th>
<th>FBP signal</th>
<th>$^{13}$C-FBP m/z</th>
<th>$^{13}$C-FBP signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>338.9972</td>
<td>315138</td>
<td>345.0175</td>
<td>47965</td>
</tr>
<tr>
<td>SD</td>
<td>0.0005</td>
<td>98425</td>
<td>0.0005</td>
<td>14762</td>
</tr>
<tr>
<td>%RSD</td>
<td>1.60e-4</td>
<td>31.23</td>
<td>1.50e-4</td>
<td>30.78</td>
</tr>
</tbody>
</table>

**MS$^E$**

As an alternative to individual SRM experiments, full-scan fragmentation data was obtained through MS$^E$. Figures 4.10 and 4.11 below show the comparative scans between the low collision energy (CE) state (7 eV) and the high CE (30 eV) state, respectively. In the low CE scan, signal for the FBP and $^{13}$C-FBP fragments are very low: about 2.5-2.6% of the signal for the intact molecules. These 2.5-2.6% are likely due to the in-source fragmentation experienced by the ions as they undergo the desolvation process by ESI and mass filtering before reaching the mass analyzer. In-source fragmentation is especially relevant for phosphate-containing compounds, as
the phosphate group is very labile and can dissociate as a highly stable phosphoric acid molecule. When 30 eV of collision energy is used to disrupt stability of the ions, fragmentation is significantly increased: 16.5-17% relative to the intact molecules.

In the MS<sup>E</sup> workflow, collision energy is constantly alternated between the high and low state. Therefore, the data collected here represent data obtained from two distinct fragmentation conditions within a single run for all ions in the range of 100-800 m/z. This is unique from the classic multiple reaction monitoring (MRM) experiment which requires that ion pairs of interest be selected and others be excluded from detection. In this case, with only two analytes of interest with known fragmentation patterns, the MS<sup>E</sup> and MRM experiments are equally useful. However, for untargeted studies or those studying a variety of compounds, MS<sup>E</sup> is a much simpler way to obtain intact and fragmentation data in a single run. The downside is that the spectra can become very difficult to interpret as more and more compounds are fragmented. By controlling the high CE value, different fragmentation pathways can be accessed. For this study, 30 eV only resulted in 16-17% analyte fragmentation by neutral loss of phosphoric acid. This allows identification of unique fragments without decimating ion signal with destabilizing collision energies. As the eventual goal of this study is to use HR-MS for both in vitro and in vivo enzymatic reaction kinetics, MS<sup>E</sup> can be a very useful tool for identifying unknowns or quantitating various compounds in the matrix.
Figure 4.10: Low CE (7 eV)
The goal of this method is to analyze enzymatic reaction samples and obtain kinetic plots. To this end, data from the reaction with 10 μM substrate are presented below. This data was obtained using a discontinuous assay, the details of which are further described in the next chapter. In short, aliquots are removed at various time points and quenched with acetonitrile and internal standard before MS analysis. To save time and materials, quantitation is achieved with the use of a single-point response factor as opposed to a full standard curve.
Figure 4.12: A concentration vs. time kinetic plot obtained using this HR-MS method with a starting [FBP] of 10 μM, an enzyme concentration of 12.7 nM, and 10 μM [IS].

The plot shown above (Figure 4.12) with data obtained from the developed and validated HR-MS method has the correct shape for an enzymatic reaction profile: a quick decrease in substrate concentration as the transition from substrate to product happens, and equilibrium eventually being reached and substrate concentration levelling out. This is evidence that rabbit muscle aldolase has catalytic activity under the low-ionic strength conditions developed for this method, and that the substrate turnover follows the typical profile seen for other aldolase assays. Additionally, the concentration values (in the Appendix as Table 4.S2) calculated from the single-point response factor make sense based on the initial FBP concentration of 10 μM. These results demonstrate the efficacy of this method for use as an aldolase assay.
Conclusions

The data presented throughout this chapter show the development, validation, and use of a high-resolution ESI-QqTOF-MS method for quantitation of fructose bisphosphate, the substrate of the forward aldolase-catalyzed reaction. After MS parameters were optimized, the identities of the substrate and internal standard were confirmed by exact mass to within 0.01 m/z and fragmentation by both selected reaction monitoring (SRM) and mass spectrometry-elevated energy (MS²). Neither the m/z for FBP nor the isotopically-labelled internal standard appeared in the spectrum of the blank matrix sample containing buffer and aldolase enzyme, eliminating the chance of isobaric ions in the matrix affecting quantitation. Linearity was established for FBP concentration over four orders of magnitude, although increased signal variance was seen when studying extremely low concentrations (< 1 μM). Based on precision experiments, quantitation calibration should take place at least once daily to avoid errors in quantitation caused by signal %RSD and as a best practice a new response factor will be determined prior to every reaction.

Method validation yielded very promising results, and a sample reaction was run to evaluate catalytic activity of the enzyme under method conditions and the usefulness of the method for FBP quantitation in the matrix. The experiment was able to confirm substrate turnover and the quantitative ability of the QqTOF-MS. We believe that these results verify the high separative and quantitative power of HR-MS, even without prior separation and/or clean-up by HPLC or SPE. Future work will utilize the method developed and validated here to obtain kinetic parameters and inhibition constants for the aldolase reaction in the hopes of providing a faster, less expensive, and higher-throughput assay.
CHAPTER 5

KINETIC ANALYSIS OF RABBIT MUSCLE ALDOLASE IN VITRO BY HR-MS

Introduction

As discussed previously, fructose bisphosphate aldolase is a strong target for drugs to treat infectious diseases and human diseases such as cancer.\textsuperscript{120} To summarize, aldolase catalyzes the reversible conversion of fructose bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate in glycolysis and gluconeogenesis. Aldolases are divided into two categories (Class I and Class II) based on their mechanism of action, with human aldolase falling into class I and bacterial in class II. Therefore, most infectious disease treatments aim to target class II aldolases exclusively. A notable exception is the malarial parasite \textit{Plasmodium falciparum} which is known to have class I aldolase.\textsuperscript{38} Therefore, class I aldolase is a potential target for future antimalarial drugs. Additionally, many human cancer cells upregulate glycolysis enzymes to promote tumor growth, making class I aldolase inhibitors a possible target for oncology drugs.\textsuperscript{120c, 121} The aldolase tetramer from rabbit muscle is shown below in Figure 5.1.
Class I aldolases are characterized by the formation of a Schiff base in the active site by the carbonyl group on the substrate – either DHAP or FBP depending on the direction of the reaction – and the amino group of a local lysine residue. This mechanism of action is quite different from that of class II aldolase, which requires a divalent cation such as Zn\textsuperscript{2+} in the active site to interact with the carbonyl group on the substrate. Therefore, inhibitor design is highly dependent on which type of aldolase is targeted. Phosphoglycolamide and phosphoglycolohydroxamic acid are structural analogues of DHAP that act as inhibitors of class I aldolases.\textsuperscript{122} Both compounds were shown to competitively inhibit rabbit muscle aldolase as well as the class II aldolase from
Geobacillus stearothermophilus\textsuperscript{122} and Giardia lamblia\textsuperscript{92}. Additionally, hexitol 1,6-bisphosphate, which is similar in structure to fructose bisphosphate, also inhibits both class I and class II aldolases.\textsuperscript{34, 37, 123}

Many compounds can act as aldolase inhibitors including metals\textsuperscript{124}, chelators\textsuperscript{125}, synthetic organic compounds\textsuperscript{34, 37, 126}, and biological compounds\textsuperscript{127}. For example, common physiological compounds such as inorganic phosphate and adenosine nucleotides (AMP, ADP, and ATP) all act as inhibitors of Trypanosoma brucei, rabbit muscle, and Staphylococcus aureus class I aldolases.\textsuperscript{127a} The mechanism and degree to which these compounds act as inhibitors varies. ATP is a strong competitive aldolase inhibitor for all three species, with \textit{K}_i values roughly between 0.3-1.3 mM. In general, as the net charge of the nucleotide decreases, its inhibition strength decreases as well. Also, aldolase from \textit{S. aureus} was generally less susceptible to inhibition by phosphates and nucleotides. Other previous work\textsuperscript{37} has shown that hydroxynapthaldehyde phosphate derivatives napthyl-2,6-bisphosphate (NA-P\textsubscript{2}) and 1,6-dihydroxy-2-napthaldehyde phosphate (HNA-P) potently inhibit Schiff base formation in rabbit muscle aldolase. With fructose bisphosphate as the substrate, NA-P\textsubscript{2} competitively inhibited the aldolase reaction with a \textit{K}_i of 0.28 +/- 0.03 \textmu M, and HNA-P slowly binds to the aldolase active site and acts as a reversible inhibitor with an estimated \textit{K}_i of 24 +/- 5 nM. NA-P\textsubscript{2} was also shown to inhibit aldolase from \textit{Trypanosoma brucei} – the causative agent of sleeping sickness. Further study showed that a similar hydroxynapthaldehyde phosphate derivative called TBK1 could selectively inhibit parasitic class I aldolases with little effect of rabbit muscle and human liver aldolases.\textsuperscript{34} After incubation with 100 \textmu M TBK1 for 15 min, rabbit muscle and human liver aldolases retained 100\% of their activities whereas \textit{Plasmodium falciparum} (malaria) and \textit{Leishmania mexicana} (leishmaniasis)
only retained 30% and 15% of activity, respectively. TBK1 appears to be primarily selective toward *T. brucei* aldolase as it has <0.5% residual activity under these experimental conditions. The ability of an inhibitor to selectively affect parasitic class I aldolases over mammalian aldolases has important implications for safe and effective treatments for infectious diseases.

As discussed in Chapter 1, assays for fructose bisphosphate activity are usually plate-based, multi-enzyme, and either colorimetric or fluorometric. Both the colorimetric and fluorometric assays are indirect and discontinuous and colorimetric being utilized more often. The coupled reaction scheme for colorimetric analysis of aldolase products is shown below in Chapter 1 in Figure 1.7. As the aldolase-catalyzed reaction converts fructose bisphosphate (FBP) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P), any G3P is isomerized to DHAP by triose phosphate isomerase, and the DHAP aids in the conversion of NADH to NAD\(^+\) by G3P dehydrogenase. This assay spectrophotometrically monitors the absorbance of NADH in the UV at 340 nm. Therefore, as the aldolase reaction proceeds in the forward direction, the observed signal from NADH absorbance would decrease over time.

The colorimetric assay is generally done in a continuous manner by directly analyzing the reaction solution in a cuvette on a spectrophotometer during the initial reaction time. After the signal from NADH absorbance is stoichiometrically converted to fructose bisphosphate concentration, the plot of [FBP] vs. time will give the initial reaction velocity (\(V_0\)). In order to obtain a Michaelis-Menten curve, at least 7-10 different concentrations of FBP must be analyzed, and often in duplicate or triplicate. The total reaction volume is often around 1.0 mL and contains enzyme concentrations in the high nanomolar to low micromolar range and a variety of micromolar to millimolar concentrations of substrate. Therefore, the material usage can really add up over time.
and, despite only dedicating a few minutes per sample, the assay throughput is severely hindered. Large volumes or high concentrations are necessary for spectrophotometric determination because of the low selectivity and sensitivity of the UV-Vis detector. Absorbance at 340 nm will detect NADH as well as other components in the matrix that have an absorption maximum at or near that wavelength. Because of this, matrix controls are crucial for getting accurate results, especially when analyzing complicated matrices such as cell cultures—which will likely contain NADH from other reactions. Additional complications from stray light, temperature or pH changes can also negatively affect the precision and accuracy of spectrophotometric results. Finally, sub-micromolar concentrations of NADH challenge the sensitivity of the UV-Vis detector and can negatively affect measurements. However, when high enough concentrations are used and matrix interferences are accounted for, UV-Vis spectrophotometry provides reliable results on an inexpensive and accessible detector. For these reasons, the colorimetric coupled-enzyme assay is by far the most popular for kinetic analysis of the aldolase-catalyzed reaction.

To combat the poor sensitivity and selectivity of UV-Vis, fluorescence-based assays are also available for aldolase. As seen in the illustrated schematic (Figure 5.2), this workflow is also a coupled-enzyme assay, in this case added an extra step to activate a fluorescent probe through NADH oxidation. A common fluorescent probe for this purpose is the reduction of resazurin—which is a faint pink color in solution—to resorufin, a red fluorescent compound detected at 585 nm after excitation at 535 nm, the enzyme-catalyzed reaction for which is shown below in Figure 5.3. Fluorescence detection is much more sensitive than UV-Vis with detection limits often in the picomolar range and in general fluorescence is less prone to matrix interference than UV-Vis spectrophotometry. Therefore, the fluorescence assay requires very small sample volumes (< 100
μL) and is done on microplates with detection on a plate reader. Microplates come in a variety of sizes - including 96, 384, and 1536 wells – which allows for all reaction replicates and controls to be done in one experiment on a single plate. The time and materials savings from using a single, small volume plate for all experiments makes this assay extremely high-throughput, a powerful advantage for inhibitor-screening applications. On the other hand, this assay can be cost-prohibitive because assay materials are often sold as expensive kits. In some cases, a 100-sample reaction kit can cost $300-500\textsuperscript{128} and even without using a kit the fluorescent probe reagent is very expensive.

Figure 5.2: Illustration of the fluorescent coupled-enzyme assay for aldolase. Reproduced from: \textsuperscript{39}
Figure 5.3: The enzyme-catalyzed conversion of resazurin to resorufin that is used as a fluorescent probe because of the co-conversion from NADH to NAD$^+$. 

Mass spectrometry is a powerful separation tool both on its own and combined with another separation technique such as LC or GC. While the majority of workflows utilize LC to account for complicated sample matrices and provide a greater degree of separation, it is not always necessary for \textit{in vitro} kinetic studies. Excluding an LC gradient and instead relying on just MS significantly improves analysis time, with MS spectra being collected over the course of seconds or a few minutes. Additionally, mass spectrometry directly analyzes reaction components based on their mass-to-charge ratio and does not require expensive labels for detection. Mass spectrometry also provides a lot of variety in terms of the types of ionization sources and mass analyzers available.
Electrospray ionization (ESI) is most often used for biological samples in the liquid phase, but matrix-assisted laser desorption ionization (MALDI) can be used for samples on a solid surface. It is important to note, however, that each ionization technique has its own specific sample requirements for optimum detection. For example, ESI necessitates volatile buffer salts (or pre-injection desalting if salt cannot be avoided) in order to prevent ion suppression, and MALDI requires the sample to be combined with the matrix for desorption. Therefore, reaction protocols are not always transferrable from spectroscopic or chromatographic assays to MS, and some method optimization is usually required. Mass analyzers with high sensitivity, high linear dynamic range, high mass accuracy, and MS/MS capability are preferred. Some examples include: QqTOF, Orbitrap hybrids, and FT-ICR hybrids. All of these fall into the category of high-resolution mass spectrometry (HR-MS), but low-resolution instruments such as ion traps are still used for these applications.

Several examples of assays for enzyme kinetics by MS exist and are categorized as either continuous or discontinuous. In continuous assays, the reaction takes place in the syringe and a small amount of the solution is constantly introduced into the mass spectrometer. A single run tracks the substrate and product intensity vs. time and concentrations are determined by comparison to signal from an internal standard. In discontinuous assays, on the other hand, the reaction is run on the benchtop and small aliquots are removed, quenched, and analyzed by MS at various time points. The majority of published MS assays for enzyme kinetics are discontinuous.

Kinetics and inhibition of hexokinase, the first enzyme in the glycolysis pathway, were previously determined by an MS-only method. Similar to aldolase, hexokinase is usually studied by coupled-enzyme spectrophotometric assays, and interferences by auxiliary enzymes and
organic inhibitors cause discrepancies in measured kinetic parameters. This MS method can directly assay reaction substrates and products by their mass-to-charge ratios, and quantitation takes place through the use of an internal standard. In this case, 2-deoxy-glucose-6-phosphate was used as an internal standard for the product glucose 6-phosphate, which is a less expensive but somewhat less accurate alternative to using isotopically-labelled glucose 6-phosphate. In addition, the kinetic parameters for the reaction were determined using a low-resolution ion trap mass spectrometer, losing mass accuracy and resolving power but improving total assay cost. $K_M$ and $V_{\text{max}}$ values were determined by analyzing reaction aliquots (quenched by methanol addition) over time. The workflow for inhibitor library screening was different than the initial experiments: hexokinase was immobilized on a gel, incubated with substrates and inhibitors for a few minutes, and washed prior to ESI-MS analysis on a high-resolution FT-ICR mass spectrometer. Mass spectra were taken before and after inhibitor incubation and were compared to show the presence or absence of reaction biomarkers. In this inhibition study, a variety of potential inhibitors were screened quickly and those showing inhibitory activity were studied further to obtain $K_i$ values. HR-MS was chosen for this study because the analyte ions could be identified with high accuracy. Using this fast and accurate workflow, 10/12 compounds in a mock library were identified as potential hexokinase inhibitors based on differences in mass spectra.\textsuperscript{130}

The case of phosphoglucomutase, the enzyme that catalyzes the reversible isomeric conversion of glucose 1-phosphate (G1P) to glucose 6-phosphate (G6P), presents a unique difficulty for direct MS detection because the substrate and product ions have the same exact mass. As a way to circumvent this issue, ion-molecule reactions (IMR) using the mass spectrometer as a reaction vessel provide an indirect method of identification. In this case, the neutral compound
trimethylborate (TMB), which is known to react with the phosphate groups of phosphorylated compounds, was introduced into the ICR cell of the ESI-FT-ICR instrument. Based on the position of the phosphate group, G1P and G6P yielded unique product ion distributions. Each product was assigned a diagnostic ion and the relative intensities of those ions were used for quantitation. As the enzymatic reaction proceeded, aliquots were removed and quenched with three volumes of acetonitrile, making this a discontinuous assay. Due to the reaction buffer containing Mg\(^{2+}\) the samples were desalted a strong-cation exchange resin prior to MS, helping to reduce ion suppression in negative ion mode. Performing reactions with varying concentrations of substrate and in both directions allowed this workflow to be used to determine \(K_M\) values for two different substrates of phosphoglucomutase, \(V_{\text{max}}\) values for both directions of the G1P to G6P conversion, and the equilibrium constant (\(K_d\)) for the enzyme-substrate interaction.\(^{131}\)

As part of their work on MEP pathway enzymes and inhibitors, Narayanasamy \textit{et al.} confirmed IspF activity by TOF-MS.\(^{28}\) For this workflow, the \textit{in vitro} reaction was run under biological conditions with MOPS buffer and 5 mM MgCl\(_2\) and quenched with EDTA after 30 min. As the reaction conditions were not suitable for ESI, the solution was completely dried and redissolved in 1:1 MeOH: H\(_2\)O prior to MS analysis. The TOF-MS monitored the conversion of the substrate CDP-ME2P to MEcPP based on m/z in the negative ion mode. The assay was not quantitative in this case, but instead was used to confirm enzyme activity as a supplement to radiometric and spectrophotometric methods.

The aim of this study is to use HR-MS to obtain kinetic data for class I fructose bisphosphate aldolase from rabbit muscle without the use of HPLC. Methods for both continuous and discontinuous reaction monitoring are described and results from both workflows are
compared. Additionally, known aldolase inhibitors adenosine monophosphate (AMP) and adenosine triphosphate (ATP) were evaluated as part of the proof-of-concept.\textsuperscript{127a}

Materials and Methods

**Chemicals**

D-fructose 1,6-bisphosphate trisodium salt octahydrate (98%), ammonium acetate, sodium chloride, and acetonitrile (HPLC grade) were purchased from Fisher (Chicago, IL). Aldolase from rabbit muscle, adenosine 5’-monophosphate (AMP, >99%), and adenosine 5’-triphosphate (ATP, disodium salt hydrate, 99%) were purchased from Sigma (St. Louis, MO). (\textsuperscript{13}C)\textsubscript{6}-D-fructose bisphosphate sodium salt hydrate (98%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). All water used in experiments was filtered with an in-house Milli-Q 18MΩ.cm R-O system (EMD Millipore, Billerica, MA).

**Instrument Parameters**

Samples were analyzed on Bruker MaXis Plus ESI-QqTOF in the negative ion mode between 100-800 m/z. Sample was delivered by KD Scientific model 100 syringe pump (KD Scientific Inc., Holliston, MA) at a rate of 200 μL/hr. For the discontinuous assay, the ESI source parameters were as follows: 210 °C dry gas temperature, 8.0 L/min dry gas (N\textsubscript{2}) flow, 1.6 bar nebulizer gas (N\textsubscript{2}) pressure, 4.0 kV capillary voltage, and 500V end plate offset. The QqTOF-MS was tuned as follows: funnel RF 300 V pp, multipole RF 200 V pp, collision RF 1000.0 V pp, ion cooler RF 800
Vpp, isCID 0.0 eV, ion energy 4.0 eV, collision energy 7.0 eV, pre-pulse storage 5.0 μs, transfer time 50.0 μs. Some tune parameters were modified for the continuous assay: 150 °C dry gas temperature, 2.5 bar nebulizer gas pressure, 3.5 kV capillary voltage, and 1200 V end plate offset for the source.

**Response Factor for Quantitation**

Prior to analyzing samples from reactions, a solution of equal concentration of substrate and internal standard was run. From this solution a response factor (F) was calculated using the below equation, and the response factor was used for quantitation of reaction samples. This equation was then rearranged in order to calculate the concentration of FBP for each sample.

\[
R = \frac{I_{IS} \times [A]}{[IS] \times I_A}
\]

\[
[A] = \frac{[IS]I_AR}{I_{IS}}
\]

\(I_{IS}\) and \(I_A\) are intensities of internal standard and analyte, respectively

\([IS]\) and \([A]\) are concentrations of internal standard and analyte, respectively

Equation 5.1: (top) the equation used to calculate response factor and (bottom) the rearranged equation used to calculate FBP concentration

**Discontinuous Assay**

In vitro aldolase reaction: 12.7 nM aldolase tetramer from rabbit muscle was incubated at room temperature in reaction buffer (10 mM ammonium acetate pH 7.5 with 2 mM NaCl) and the
enzymatic reaction was initiated by addition of varying concentrations (2-50 μM) FBP, and the final reaction volume was 1.0 mL. At various time points, 100 μL aliquots of reaction solution were removed and 125 μL acetonitrile was added to quench the reaction. Samples were stored in the freezer until the time of analysis, at which point 6-^{13}C FBP was added to a final concentration of 10 μM and reaction buffer was added to a final sample volume of 250 μL. Each aliquot was analyzed by ESI-MS in negative ion mode and the concentration of FBP was calculated from the response factor. Reaction progress was plotted as concentration of FBP vs. time, and initial velocity ($V_i$) was calculated from the reaction curve. A Michaelis-Menten plot was generated based on the range of initial concentrations of FBP. From that curve and its double-reciprocal plot, kinetic parameters $K_M$, $V_{max}$, $k_{cat}$, and turnover number were calculated.

\[
k_{cat} = \frac{v_{max}}{[E]}
\]

Equation 5.2: The equation for catalytic rate ($k_{cat}$)

\[
\text{turnover number} = \frac{k_{cat}}{K_m}
\]

Equation 5.3: The equation for turnover number used for comparison of different inhibitors
In syringe pump 1, 12.7 nM aldolase tetramer and varying concentrations of FBP were combined in reaction buffer and delivered to ESI source at 200 μL/hr. Syringe pump 2 delivered 10 μM $^{13}$C-FBP (the IS) at the same flow rate. The schematic for the reaction set-up is shown in Figure 5.4. The lines from the two syringe pumps were connected by a mixing tee prior to entering the ESI source. From the time the enzymatic reaction was started by addition of FBP, the MS monitored the signal from m/z 338.99 (FBP) and 345.01 (IS). The reaction was allowed to proceed for roughly 10 min while extracted ion chromatograms (EIC) monitored the signal of substrate and internal standard over time. The intensities of these two ions and the calculated response factor were used to determine the concentration of FBP over time, and from that curve the initial velocity was obtained. A plot of $V_i$ vs. initial [FBP] yielded $V_{\text{max}}$ and $K_M$ values.
Results

**Discontinuous Assay with No Inhibitor**

Below is a sample reaction profile from the 10 μM initial substrate reaction (Figure 5.5) and the calculation of initial rate ($V_i$) based on the first few data points of the reaction (Figure 5.6). Additional reaction profiles and initial rate graphs are presented in the Appendix C. The shape of the reaction profile is prototypical of a forward reaction monitoring decreasing substrate concentration: a sharp decrease in the beginning of the reaction time followed by a levelling of concentration as the reaction reaches equilibrium. The data points in the sharp decrease (0-2 min) were used to calculate the initial rate of 2.7985 μM/min. The linearity is very high over this stretch of time with a correlation coefficient of 0.9759. This process was repeated for various concentrations between 2-50 μM FBP, with only the first 5-7 min of the reaction analyzed in order to save time and materials. Because the reaction reaches equilibrium faster at higher substrate concentrations, the number and identity of time points used for initial rate calculations and the strength of linear fit varies slightly.

While most reactions proceeded in a typical manner, there were some issues with reaction monitoring and quantitation at low and high substrate concentrations, albeit for different reasons. When investigating precision for this HR-MS method in the last chapter, high variability in signal intensity and quantitation was reported at concentrations at or below 1 μM FBP. Figure 5.7 demonstrates the extreme discrepancy in reaction curves obtained from duplicate reactions with 1
Figure 5.5: A sample reaction profile for the aldolase starting with 10 μM FBP and no added inhibitor.

Figure 5.6: The first few time points representing the initial reaction from Figure 5.5, and the linear fit to yield the initial reaction rate of 2.7985 μM/min.
μM initial FBP concentration. The calculated initial reaction rate from Trial 2 is more than double that of Trial 1, and because of continued variability in quantitation from these low concentrations, 2 μM was the lowest condition tested. On the other hand, as shown in Figure 5.8, reaction profile from 50 μM initial FBP concentrations does not show the usual exponential decrease in substrate concentration. It is widely known that as initial substrate concentration increases, initial reaction rate also increases. Therefore, the reaction reaches equilibrium very quickly after the addition of the substrate to trigger the start of reaction; and when the assay is run in a discontinuous manner with only a finite number of time points, those data points may not fully represent the initial reaction phase. In those cases, the first few time points (0-0.5 min) were used indiscriminately for $V_i$ calculation. The linear correlation is much poorer than those of lower concentrations, but the data obtained by this approach appear to fit the Michaelis-Menten model nonetheless.

The Michaelis-Menten and Lineweaver-Burk plots obtained by analyzing seven concentrations of FBP in the absence of inhibitor are shown below in Figures 5.9 and 5.10. Both plots confirm that the Michaelis-Menten model is appropriate for determining reaction kinetics for rabbit muscle aldolase. In other words, the Michaelis-Menten plot is hyperbolic and the double-reciprocal (Lineweaver-Burk) plot is linear, showing a lack of allosteric binding that would cause a sigmoid-shaped plot and the inability to determine kinetic data with this model. Kinetic parameters extracted from this data are listed in Table 5.1.
Figure 5.7: Reaction profiles from two trials of reaction with 1 μM initial FBP concentration. Both the shapes of the curves and the calculated initial rates are markedly different from each other.

Figure 5.8: Reaction profile from the 50 μM initial FBP concentration reaction. There is no sharp decrease in substrate concentration indicative the initial rate phase, so the first three data points were used to calculate initial rate.

Initial Rates:
Trial 1: 0.6392 μM/min
Trial 2: 1.4006 μM/min
Figure 5.9: The calculated Michaelis-Menten curve obtained from the initial rates of reactions ranging from 0-50 μM FBP in the absence of inhibitor.

Figure 5.10: The double reciprocal plot with a linear fit used to determine the Michaelis constant and maximum reaction rate.
Table 5.1: Summary of kinetic parameters obtained for rabbit muscle aldolase without inhibitor.

<table>
<thead>
<tr>
<th>Kinetic Parameters for Rm aldolase without Inhibitor</th>
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<td>$K_M$ (μM)</td>
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<td>$V_{max}$ (μM/s)</td>
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<td>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</td>
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1 μM AMP as an Inhibitor

Experiments with the same initial concentrations of FBP were repeated with the addition of 1 μM AMP, a known inhibitor of rabbit muscle aldolase. Figures 5.11 and 5.12 show the new Michaelis-Menten and Lineweaver-Burk plots, respectively. The hyperbolic shape of the Michaelis-Menten curve is less obvious with AMP, but there is still no evidence of allosteric binding. Table 5.2 lists the new kinetic parameters for the inhibited enzyme. As with the uninhibited reactions, the individual reaction profiles and initial rate calculations are listed in the Appendix. It is important to note that in this case only the first 5-7 minutes of the reactions were measured for FBP concentration in an effort to save time and materials.
Figure 5.11: The Michaelis-Menten plot produced from reactions with 1 μM AMP.

Figure 5.12: The Lineweaver-Burk plot with the linear fit.
Reactions were again repeated, this time with another known nucleotide inhibitor, ATP. In this case, there were issues with quantitation of the 25 μM sample because the signal for FBP and IS are approximately equal and role of scan-to-scan signal variability was exacerbated in the averaging process (Figure 5.13). Because of this, data from a 100 μM reaction was added to keep the number of data points constant throughout all three analyses. In spite of issues with 100 μM reaction quantitation problems under uninhibited conditions, with ATP present the reaction is slowed enough that the initial rate can be elucidated. Figures 5.14 and 5.15 show the kinetic plots obtained from experiments with ATP, and the calculated kinetic parameters are listed in Table 5.3. Again, there is no evidence of allosteric binding with ATP, but the Lineweaver-Burk plot presents some error in linear fit primarily due to mid-range FBP concentration data points. This contributes to the overall method error, although the correlation coefficient is still relatively high at 0.9585.

<table>
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<th>Kinetic Parameters for Rm aldolase with 1 μM AMP Inhibitor</th>
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Figure 5.13: The extracted ion chromatogram showing signal for FBP (blue) and IS (orange) for the 25 μM initial [FBP] after 2 min incubation time.

Figure 5.14: The Michaelis-Menten plot for aldolase with 1 μM ATP.
Figure 5.15: The double reciprocal plot with linear fit for aldolase in the presence of ATP.

Table 5.3: The summary of kinetic parameters for aldolase with 1 μM ATP.

| Kinetic Parameters for *Rm* aldolase with 1 μM ATP Inhibitor |
|---------------------------------|-----------------|
| Km (μM)                         | 145.6           |
| Vmax (μM/s)                     | 7.71e-2         |
| kcat (s⁻¹)                      | 1.52            |
| kcat/Km (M⁻¹s⁻¹)                | 1.04e4          |
Discussion

Kinetic Parameters for Rabbit Muscle Aldolase by a Discontinuous Assay

Based on the data presented above, analysis of reaction aliquots with QqTOF-MS is a viable discontinuous assay for studying aldolase activity in vitro. The method was able to effectively monitor aldolase reactions and obtain kinetic parameters using the Michaelis-Menten model. Table 5.4 samples $K_M$ values determined for rabbit muscle aldolase in the absence of inhibitor using the typical colorimetric or fluorometric coupled-enzyme assays. The literature values range from approximately 2-20x lower than the one obtained using this HR-MS method. The most likely explanation for the decrease in catalytic activity is the low ionic strength in solution for this method compared to those in the literature, and previous studies have confirmed that aldolase activity is inversely proportional to ionic strength ($I$) at $I < 0.1$M. In this case, the ionic strength was intentionally kept lower than the typical assay conditions in order to be better suited for ESI-MS. Specifically, the buffer was changed from the usual TEA-HCl to ammonium acetate for more efficient electrospray and the concentration was reduced to 10 mM from the typical 50-100 mM. In addition, the 2 mM NaCl used for this study represents a dramatic reduction from the 50 mM often used and the chelator EDTA was eliminated from the assay protocol altogether. In spite of these changes to the reaction conditions, aldolase still remained active and the $K_M$ was generally on the same order of magnitude as the literature values.
Table 5.4: Examples of $K_M$ values for rabbit muscle aldolase in the literature obtained using the colorimetric or fluorometric coupled-enzyme assay.

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<td>Colorimetric coupled enzyme assay 0.1 M glycyl-glycine buffer pH 7.4 with 0.2 M KOAc</td>
<td>134</td>
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<td>12.8</td>
<td>Colorimetric coupled enzyme assay 50 mM TEA-HCl pH 7.6; I = 0.1 M</td>
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<tr>
<td>13</td>
<td>Colorimetric coupled enzyme assay 100 mM TEA-HCl pH 7.6 with 50 mM NaCl; I = 0.15M</td>
<td>37</td>
</tr>
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<td>9.5</td>
<td>Colorimetric coupled enzyme assay, 30 °C 50 mM TEA-HCl pH 7.4</td>
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<tr>
<td>5.1</td>
<td>Colorimetric coupled enzyme assay</td>
<td>136</td>
</tr>
<tr>
<td>15</td>
<td>Colorimetric coupled enzyme assay 0.1 M glycyl-glycine buffer pH 7.4 with 0.2 M KOAc</td>
<td>91a</td>
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</table>

In the presence of inhibitors AMP and ATP, the data show a decrease in reaction rate and other kinetic parameters – demonstrated visually in the overlay of the three Michaelis-Menten plots in Figure 5.16 and listed in Table 5.5. As AMP and ATP are known to inhibit rabbit muscle aldolase, this is not a surprising result but does allow for confirmation that our HR-MS method is able to detect enzyme inhibition. Figure 5.16 shows that the overall $V_{\text{max}}$ of the reaction is lowered when incubated with either 1 μM of AMP or ATP. Based on comparison of the turnover numbers ($k_{\text{cat}}/K_M$), rabbit muscle aldolase activity is roughly 2.7x lower in the presence of AMP and about 10.4x lower with ATP. AMP appears to be a non-competitive inhibitor of rabbit muscle aldolase based on the difference $V_{\text{max}}$ value, but very similar $K_M$ value, 32.7 μM, compared to 38.3 μM for the uninhibited reaction. This is in opposition to a study in the literature which identifies it as a competitive inhibitor.\textsuperscript{127a} For an inhibitor to be competitive based on the Michaelis-Menten model,
the $V_{\text{max}}$ in the presence of an inhibitor would be approximately the same than that of the uninhibited one and the $K_M$ values would vary. In the same study, ATP is also listed as a competitive inhibitor. However, our data show the inhibition as mixed, with both $K_M$ and $V_{\text{max}}$ being different from the parameters for the uninhibited reaction. Future work will include more replicates of these experiments and more reactions with varying concentrations of inhibitors to be more certain about the exact degree and mechanism of inhibition.

Figure 5.16: An overlaid chart of the Michaelis-Menten curves with no inhibitor (blue), AMP (orange), and ATP (gray).
As mentioned previously, each of the reactions were only run for 5-7 minutes to save time and materials. This dramatically increased speed, but full reaction curves were not obtained. Additionally, the analysis time per sample was very low: 1-2 min of data collection followed by several minutes of instrument rinse. This is comparable to that of the spectrophotometric assay, and for HR-MS no incubation time was required from reaction to quenching to analysis (as is often the case with coupled enzyme assays). The total time required to run and analyze the one reaction was about 1 hr using this method, and a single Michaelis-Menten curve could be obtained in 7-8 hrs. At this point, the run time of this assay significantly hinders the throughput compared to a plate-based assay in which all reactions and replicates can be performed on a single microplate. However, unlike colorimetric and fluorometric assays, this method is a direct analysis of fructose bisphosphate and does not require auxiliary compounds for detection, significantly simplifying the sample matrix and subsequently the analysis process.

Not only is the method direct, but it is very specific. Only the [M-H]⁺ ions for FBP and the IS +/- 0.01 m/z are taken into account, decreasing the overall matrix interference. For example,
several samples contained a peak at m/z 339.20 – very close to that of FBP at m/z 338.99. Without the specificity provided by the exact mass measurements, this peak may have been incorrectly applied to the quantitation of FBP and could have severely impacted the accuracy of kinetic data. Moreover, the specificity and sensitivity of HR-MS is much higher compared to colorimetric assays, which are especially susceptible to background interference. In addition, any matrix interferences to quantitation are taken into account in HR-MS during the quantitation calibration process with the isotopically-labelled internal standard. In other words, since FBP and its isotopically-labelled counterpart have identical ionization efficiencies and are affected equally by adduction, in-source fragmentation and other forms of ion suppression, recalculation of the sample response factor prior to each reaction analysis increases precision and accuracy by taking small run-to-run changes in ionization and signal into account.

Important to note is that the specificity and sensitivity provided by MS is achieved without analyte tags or derivatization, although quantitation requires an isotopically-labelled IS. This provides a significant improvement over assays requiring fluorescent or colorimetric tags and/or probes in other assays. However, as aldolase will also turn over \((^{13}\text{C})_6\text{-FBP}\), it is vital that the IS is added after reaction quenching and immediately before sample analysis to keep signal consistent throughout the run and ensure accurate quantitation.

Despite the many advantages provided by using HR-MS over traditional assays for aldolase activity, method development for this application is still in its early stages and is not without issues. One issue that was noted in this study is with the quantitation of FBP at concentrations greater than or equal to 50 μM. As a possible solution, reactions with an initial FBP concentration above 50 μM were tried with a response factor solution of 50 μM each of FBP and the IS, but that did
not make a difference and the issue seems to be unrelated to the quantitation calibration. It is more likely that the reaction rate is very high at high concentrations of FBP such that the few time points analyzed below 30 sec reaction time are not enough to accurately calculate the initial rate.

Another drawback of the assay in its discontinuous form is the amount of benchtop labor required for MS analysis. The multitude of pipetting steps needed for preparation of stock solutions, reactions solutions, and quenched aliquots each comes with random error and to the total error of the assay. However, the same can be said of traditional aldolase assays, with the exception of those that utilize automatic pipetting systems. In addition to labor, the discontinuous method also requires consumables (sample tubes, pipet tips, etc.), the waste and cost of which can add up over time. However, there is room for improvement in terms of material use. As this study represents the early stages of method confirmation, the 1.0 mL total reaction volume and 250 μL sample volume were purposefully kept higher than necessary. In reality, at 200 μL/hr and 1-2 min per sample, the required sample volume is extremely low. Theoretically, sample volumes could be cut 5-10-fold, although this would require a much smaller sample syringe than the 500 μL one used here and possibly a special low-volume syringe pump.

The largest cost barrier for this method is obviously the high-resolution instrumentation, which is generally in the hundreds of thousands of dollars. But as more and more labs acquire HR-MS instrumentation, their use can be expanded to include enzyme kinetics. Besides the internal standard (~$250-500/50mg), the operational cost of this assay is relatively inexpensive. This is a significant improvement for fluorescent assays in particular because fluorescent tags are often very costly.
The biggest issue with using ESI-MS is the stringent sample requirements, specifically with respect to non-volatile salts. As discussed above, the low enzyme turnover rate compared to literature values is likely a symptom of the low ionic strength of the reaction buffer. For additional experiments in which the ionic strength is raised and/or a more complicated sample matrix (e.g. cell lysate for *in vivo* studies) is analyzed, a sample clean-up technique such as filtration or solid phase extraction (SPE) will be needed. SPE is a relatively fast, simple, inexpensive, and effective process with a variety of resins available commercially depending on the application. In this case, widely available desalting or anion-exchange cartridges would be optimal. SPE clean-up is usually achieved in a few minutes, saving significant time compared to HPLC. Therefore, a wider variety of samples could be analyzed with optimum aldolase activity without compromising specificity and sensitivity and while keeping operational cost relatively low.

**Continuous Assay**

Attempts to elucidate kinetic parameters for aldolase via a continuous assay were much less successful than the discontinuous assay. Figure 5.17 below shows a full 45 min reaction with 5 μM initial FBP concentration and extracted ion chromatograms for FBP (green) and the IS (purple) monitored continuously. This chromatogram indicates a forward-proceeding reaction based on the decrease in FBP signal in the first several minutes. After roughly 5 minutes of reaction time, the signal for FBP stays roughly the same for the rest of the run time. Based on this, subsequent reactions were only analyzed for about 10 min to save time. As described in the assay protocol, the internal standard solution is pumped into the MS using a separate syringe from the enzyme and substrate mixture. This is due to the fact that the enzyme does not discriminate between native
FBP and isotopically-labelled FBP. It is clear from the stability of signal in purple trace that the enzyme is not affecting the internal standard during their brief mixing period before entering the MS. This ensures that quantitation is true to the actually concentration of the IS and quantitation error is not due to modifications in IS concentration caused by the enzyme.

Figure 5.17: Chromatogram trace of signal from FBP (green) and the IS (purple) for the continuous reaction with 5 μM initial [FBP].

Although the chromatogram shows that the enzyme-substrate reaction is occurring, precision for this assay is very low. Figure 5.18 shows data extracted from duplicate analyses of 5 μM FBP reactions on the same day. In general, both trials show the decrease in FBP concentration over the first several minutes of the reaction. However, when initial rates are calculated, the two rates of 1.8161 and 2.6090 μM/min, respectively, demonstrate a 43% difference. This significantly hinders assay robustness and throughput as it is likely that many trials of each reaction will need to be done to get a precise and accurate result.
Figure 5.18: Overlay of quantitation data extracted from duplicate trials of 5 μM continuous reactions. The slight differences in curve shapes translate to a 43% difference in calculated reaction rates.

In addition, not all continuously-monitored reactions demonstrate the decrease in FBP signal over time. One example is Figure 5.19, which shows the chromatogram for the reaction with 100 μM initial FBP concentration. In this case, there is no characteristic drop in FBP signal and it appears that the reaction has already reached equilibrium by the time data collection started. In another more bizarre example (Figure 5.20), the FBP signal (blue trace) from the 10 μM initial concentration reaction appears to stay constant up until 25 min reaction time before steadily increasing over the final 20 min of the run. This is particularly odd because the IS signal (orange) stays fairly constant throughout the whole time and does not increase along with FBP in the last 20 min.
Both Figures 5.19 and 5.20 represent instances in which no useful kinetic data could be obtained from runs with the continuous HR-MS assay. Alongside the issues mentioned above with run-to-run precision, this invalidates the usefulness of this assay in its current form. In order for this assay to be successful in the future, several aspects of the protocol will need to be optimized. One important example is the offset between the time that the reaction is begun (i.e. when the substrate is added to the enzyme) and when MS data collection begins. The offset time is generally between 20-30 s, which is not necessarily a significant issue at low concentration reactions (e.g.
the 5 μM one in Figure 5.17) but makes a huge difference at higher concentrations when the reaction rate is increased. This explains the appearance of the 100 μM FBP reaction chromatogram in Figure 5.19; the non-equilibrium reaction could be occurring during the first 30 s and therefore missed because of the initial set-up time. Therefore, the procedure must be optimized in the future before any useful data can hope to be attained from higher concentration substrate reactions. Moreover, issues with precision must be resolved in order to make this continuous viable. Several factors can contribute to an overall lack of precision, including signal variability and incorrect or incomplete mixing. As shown in the chromatograms above, ion signal constantly varies scan-to-scan in a random manner and only about 10-20 scans are averaged per time point. The low scan number indicates the importance of each of the individual scans in the averaging process, and even small variations in a few scans can make a significant difference in quantitative data. Future work will take advantage of the high scan speed of the QqTOF to allow for more scans to be taken per time, hopefully dissipating the amount of random variation in intensity. In addition, as shown above in Figure 5.4, the current assay procedure combines solutions from two syringes via a mixing tee before entering the ESI source. The mixing apparatus used here is Y-shaped and mixing dead volume of 1.7 μL. The degree of mixing in the tee has a profound effect on sample consistency and the relative signal intensity between the analyte and internal standard, and a lack of appropriate mixing is another possible cause of the lack of precision for this method. Significant future effort will be directed toward optimizing the mixing process, including modifying syringe pump flow rate and trying different varieties of mixing apparatuses, to obtain the best possible results.
Conclusions

The work presented here conclusively demonstrated that a discontinuous HR-MS method using QqTOF-MS without chromatography or sample clean-up can detect and quantitate rabbit muscle aldolase activity with and without inhibitors. Results showed that kinetic parameters for enzyme activity can be elucidated using the Michaelis-Menten model. However, the $K_M$ values from this study are slightly higher than those in the literature, likely due to the low ionic strength of the reaction buffer. In addition, both AMP and ATP were confirmed to inhibit aldolase at 1 μM, albeit not in a competitive manner as reported in the literature. On the other hand, the continuous assay protocol was not shown to be viable because of issues with timing and precision.

The goal of this research is to provide improvements in sensitivity, specificity, and throughput of current aldolase assays to facilitate inhibitor screening. The exact mass measurements from QqTOF-MS significantly increase specificity and sensitivity, especially with regard to the spectrophotometric assay that is most common for this application. Additionally, the method presented here eliminates the use of auxiliary enzymes and fluorescent probes, simplifying the assay protocol compared to coupled-enzyme assays. Also, while the upfront cost for the MS is very high, the operational cost of the assay is fairly low and conditions can be optimized to save materials.

The largest disadvantage at this point in using the discontinuous MS assay is the amount of benchtop labor needed to run and quench reactions. By working to make the continuous assay a consistently-viable option, the directness, sensitivity, and specificity of the discontinuous assay can be combined with the speed and ease of the continuous assay protocol to produce a truly high-throughput alternative to colorimetric and fluorometric assays.
CHAPTER 6
CONCLUSIONS AND FUTURE WORK

The aim of this work was to develop effective chromatography and mass spectrometry methods for use in quantitation of enzyme activity and inhibition. To this end, Chapter 3 described a short, isocratic HILIC HPLC method for nucleotides in the MEP pathway. The FructoShell-N column demonstrated high separation efficiency and the run time could be varied by changing the flow rate. Additionally, preliminary data showed that the method is MS-applicable and could be transferred to aldolase with no changes. In the future, this method can be utilized for UV-active MEP pathway analytes not requiring MS, such as ADP and ATP in the IspE reaction. However, the HILIC method alone has limited applicability because other reactions like aldolase do not have UV-active substrates and products. But unlike some other methods for phosphates, this method is easily MS-applicable and future work will focus on optimizing LC-MS conditions for activity, inhibition, and biomarker assays by analyzing real samples containing MEP pathway substrates, enzymes, and buffers.

In Chapters 4 and 5, MS – specifically HR-MS – methods were used to fill the gap left by insensitive and non-specific UV detection. The HR-MS method developed in Chapter 4 is fast, specific, sensitive, uses very little materials, and has high precision and accuracy for experiments completed with FBP and its isotopically-labelled internal standard. This method could also be applied to other phosphate-containing compounds such as those in the MEP pathway with little-
to-no changes to instrument parameters. It should also be noted that the HR-MS method developed in this chapter, as well as the LC methods from Chapter 3, directly detect phosphate-containing compounds in their native state without the need for time-consuming derivatization processes. We believe that makes for an easier, faster, and cheaper assay compared to those needing analyte derivatization or tagging.

In Chapter 5, the HR-MS method was applied for kinetic studies of rabbit muscle aldolase successfully un-inhibited and in the presence of two known inhibitors, although there is still work to be done for optimizing parameters. Under the current protocol, the reaction is run on the benchtop, aliquots are removed and quenched, and the concentration of FBP is determined by HR-MS. This method represents a significant improvement in specificity compared to other methods, but improvements in benchtop labor need to be made in order for the assay to be high-throughput.

To this end, a continuous assay was developed and applied with mixed results. Specifically, precision and robustness are substantial problems. As shown in Chapter 5, there are large run-to-run differences in experimental $V_i$ values and even the shape of reaction curve. One possibility for this is insufficient mixing of the solutions from the two syringe pumps in the tee. In the future, the precision issues with this assay will be addressed by optimizing sample mixing prior to entering the ESI-MS. For example, as opposed to a simple mixing tee, turbulent mixing can be deterred and mixing efficiency can be optimized by using a channeled microfluidic device. To discourage parallel flow within the channels, agitation of the system may also be required.

Perhaps the most prolific issue with studying biological systems using ESI-MS is signal suppression caused by non-volatile buffer salts. In the ESI-MS experiments described in this dissertation, salt concentrations were intentionally kept low to ensure acceptable and reproducible signal. However, it was hypothesized in Chapter 5 that the low buffer strength contributed to lower
binding affinity between the aldolase and fructose bisphosphate, thus skewing the accuracy of kinetic parameters. Therefore, in order to obtain kinetic results that can be compared to literature values and to truly gauge the effectiveness of the method, ionic strength must be comparable to experiments described in the literature. However, recent experiments have revealed that significant ion suppression leading to imprecise quantitation is seen with even a small increase in NaCl concentration from 2 mM to 5 mM (data not shown). To reach the necessary concentrations of 50-100 mM NaCl, HR-MS will need to be combined with a separation method for pre-ESI desalting. Optimally, this would be the HILIC UPLC method developed as part of Chapter 3, which was shown to separate nucleotides and retain FBP and to be ESI-MS-compatible. The current runtime for HILIC is about 10 min per sample and offers an on-line and automated separation, desalting, and quantitation method by LC-MS. Another option is off-line desalting by solid-phase extraction (SPE), completed using single-use cartridges on the benchtop in under 5 min per sample. This technique will not separate analytes from each other in a mixture like HPLC, but act as a sample preparation technique and separate matrix components from analytes. Both of these avenues will be explored as a means of studying enzyme kinetics under realistic biological conditions. Ideally, the assay would abandon the discontinuous method of sampling and instead be able to study reactions by microfluidic mixing followed by an on-line quenching, desalting, and detection/quantitation protocol.

After optimization, the methods developed here – LC, LC-MS, and HR-MS – will be applied to in vitro and in vivo analyses of MEP pathway enzymes and aldolase. The ultimate goal is for these assays to provide the sufficient throughput needed for kinetic and biomarker studies. The specificity and sensitivity of HR-MS allows for reaction products to be conclusively used as biomarkers for the inhibition of a particular enzyme. In the presence of inhibitors in vivo, this
assay can be applied to quantitate the concentration of inhibitor present as well as confirm whether the enzyme of interest is actually being targeted by the inhibitor. This information is vital to determining whether an inhibitor is “drug-like” or not and predicting promiscuous binding effects that could lead to off-target effects.
REFERENCES


60. Rochat, B., From targeted quantification to untargeted metabolomics: Why LC-high-resolution-MS will become a key instrument in clinical labs. TrAC Trends in Analytical Chemistry 2016, 84, 151-164.


APPENDIX A

RAW DATA AND SUPPLEMENTAL INFORMATION FOR CHAPTER 4
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<td>75</td>
<td>40.175</td>
<td>0.9280</td>
<td>2.31</td>
</tr>
<tr>
<td>100</td>
<td>55.252</td>
<td>1.9266</td>
<td>3.49</td>
</tr>
<tr>
<td>500</td>
<td>233.35</td>
<td>18.278</td>
<td>7.83</td>
</tr>
</tbody>
</table>

Table A.2: Raw data from 10 μM discontinuous reaction

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>[FBP] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.460</td>
</tr>
<tr>
<td>1</td>
<td>5.900</td>
</tr>
<tr>
<td>2</td>
<td>3.863</td>
</tr>
<tr>
<td>5</td>
<td>2.593</td>
</tr>
<tr>
<td>10</td>
<td>1.615</td>
</tr>
<tr>
<td>15</td>
<td>1.051</td>
</tr>
<tr>
<td>20</td>
<td>0.985</td>
</tr>
<tr>
<td>30</td>
<td>0.889</td>
</tr>
<tr>
<td>45</td>
<td>0.807</td>
</tr>
<tr>
<td>60</td>
<td>0.865</td>
</tr>
</tbody>
</table>

Quantitation based on RF 0.163
APPENDIX B

RAW DATA AND SUPPLEMENTAL INFORMATION FOR CHAPTER 5
No Inhibitor

\[
y = -0.0153x + 0.2252 \\
R^2 = 0.7536
\]
\[ y = -0.6306x + 1.2895 \]

\[ R^2 = 0.9845 \]
\[ y = -1.3681x + 4.2556 \]
\[ R^2 = 0.9918 \]
$y = -2.7985x + 9.2065$

$R^2 = 0.9759$
15 μM Initial Rate

\[ y = -3.4557x + 8.8029 \]

\[ R^2 = 0.9836 \]

---

25 μM
$y = -7.7429x + 19.199$

$R^2 = 0.8331$
50 μM Initial Rate

\[ y = -15.395x + 44.908 \]

\[ R^2 = 0.9645 \]

1μM AMP

Control

\[ y = 0.0046x + 0.1699 \]

\[ R^2 = 0.1045 \]
2 μM

\[ y = -0.2333x + 1.9707 \]
\[ R^2 = 0.4972 \]
\[ y = -0.4807x + 5.0409 \]

\[ R^2 = 0.9991 \]
$y = -0.8264x + 8.7489$

$R^2 = 0.9999$
15 μM

![Graph showing [FBP] (μM) vs. Time (min) for 15 μM.]

$y = -1.4509x + 13.863$

$R^2 = 0.8867$

15 μM Initial Rate

![Graph showing [FBP] (μM) vs. Time (min) for 15 μM Initial Rate.]

$y = -1.4509x + 13.863$

$R^2 = 0.8867$
25 μM

\[ \text{[FBP] (μM)} \]

Time (min)

25 μM Initial Rate

\[ y = -1.5522x + 14.305 \]

\[ R^2 = 0.9776 \]
159

50 μM Initial Rate

\[ y = 4.3268x + 37.674 \]

\[ R^2 = 0.2955 \]

1 μM ATP
\[ y = -0.0236x + 0.1442 \]

\[ R^2 = 0.6408 \]

**Control**

![Control graph with equation and R-squared value](image)

**2 μM**

![Graph showing concentration over time for 2 μM](image)
2 μM Initial Rate

\[ y = -0.0619x + 1.469 \]

\[ R^2 = 0.3623 \]

5 μM

Time (min)
\[ y = -0.1991x + 2.9966 \]
\[ R^2 = 0.9374 \]

5 μM Initial Rate

\[ \text{Time (min)} \]

\[ \text{[FBP] (μM)} \]

10 μM

\[ \text{Time (min)} \]
10 μM Initial Rate

\[ y = -0.1802x + 5.8233 \]

\[ R^2 = 0.9831 \]

15 μM Initial Rate

\[ y = 0.4755x + 12.318 \]

\[ R^2 = 0.8219 \]
25 µM

\[ y = -0.4924x + 21.108 \]

\[ R^2 = 0.4559 \]
$y = 2.0853x + 44.867$

$R^2 = 0.8573$

50 μM Initial Rate