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Development of Quantitative Bioanalytical Methods for the Pharmacological Studies of Anti-Cancer Drugs

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DEVELOPMENT OF QUANTITATIVE BIOANALYTICAL METHODS FOR THE
PHARMACOLOGICAL STUDIES OF ANTI-CANCER DRUGS

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DEVELOPMENT OF QUANTITATIVE BIOANALYTICAL METHODS FOR THE
PHARMACOLOGICAL STUDIES OF ANTI-CANCER DRUGS

YE FENG

ABSTRACT

In the anticancer drug discovery and therapy development, it is essential to understand the pharmacological properties of the drugs. Today, pharmaceutical analysis is employed throughout the whole drug discovery and development process. Pharmacokinetic-pharmacodynamic relationships are playing an increasingly important role in decisions on the rational development and use of new drugs; and they can provide a detailed knowledge of the mechanism of the drug and a better understanding of the molecular targets on which they act. Due to the significance of pharmacological analysis, sensitive analytical methods are critically needed for pharmacological studies. Therefore, our long-term goal is to provide guidelines in pharmacological studies of the anticancer drugs by quantitatively evaluating the molecular mechanisms of the drugs.

In this dissertation, the theory behind the analytical processes and modern bioanalytical technologies, together with their applications in pharmacological studies are discussed in Chapter 1. Dependent on the type of molecules analyzed, different methods were developed to achieve the accurate and reliable detection. Specifically, the development of two HPLC methods with both the UV (Chapter 2) and MS (Chapter 3)
detection for analysis of triapine, a ribonucleotide reductase inhibitor, is presented. In the UV method, the chelating nature of triapine was investigated by spectrophotometry, which provides an effective strategy to avoid the unwanted complexation reaction in the quantitation of triapine. An LC-MS/MS method was successfully applied to measurements of patients’ samples for pharmacokinetic studies of this drug. Another LC-MS/MS method was developed for quantification of fludarabine incorporated in DNA (Chapter 4). To the best of our knowledge, this is the first LC-MS/MS method developed for quantification of the amount of fludarabine incorporated. The significance of this study is that it provides an accurate method to study the fludarabine pharmacological effect. Moreover, the cytotoxicity mechanism of fludarabine was revisited by developing an LC-MS/MS method in conjunction with enzymatic digestion (Chapter 5). In this study, we found that the vast majority of fludarabine was incorporated in the internal position. The significance of this finding is that it allows physicians to develop better therapeutic strategies to use this drug for treatment of cancer. Finally, we applied the LC-MS/MS method developed in the study of Chapters 4 and 5 to elucidate the action of fludarabine on RNA metabolism (Chapter 6). In this study, we successfully determined the quantity of fludarabine incorporated into RNA. In addition, we were able to elucidate the primary incorporation position of fludarabine in RNA.
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CHAPTER I
INTRODUCTION TO PHARMACOLOGICAL STUDY OF DRUGS AND BIOANALYTICAL METHOD DEVELOPMENT

1.1. General introduction

1.1.1. Pharmacological studies of anti-cancer drugs

Cancer continues to be one of the major causes of mortality throughout the world. Common cancer treatment methods include chemotherapy, surgery and radiotherapy. Since the introduction of using nitrogen mustard for the treatment of Hodgkin's lymphoma in the late 1940s [1], the use of effective chemotherapeutic agents has been demonstrated to relieve symptoms and improve survival in patients with various types of cancer. Over recent years, rapid development of drug discovery technologies and current insights into molecular targets have increased the availability of potential new agents through integrative and innovative approaches in drug discovery and hasten the drug discovery process [2]. Since 1990, 80,000 compounds that NCI’s Developmental Therapeutics Program (DTP) have, have been screened as potential drug candidates [3]. The key of establishing effective chemotherapy strategies lines in an understanding of the
pharmacological properties of the drugs [2].

The two major areas of pharmacological study are pharmacokinetics and pharmacodynamics. Pharmacokinetics (PK) describes how the body affects a specific drug after administration. PK is divided into several areas including the extent and rate of liberation, absorption, distribution, metabolism and excretion. This is commonly referred to as the LADME scheme [4]. Liberation refers to the manner that the drug is released from specific formulations. Absorption illustrates how the drug is introduced to the body. Distribution is the dispersion or dissemination of the drug within the bodily fluids and tissues. Metabolism focuses on the transformation of the original drug into metabolites. Excretion described how the drug is removed from the body. The purpose of PK is to study the LADME processes of drugs in the body by examining the time course of the drug concentration profiles in body fluids such as plasma, urine and blood. In general, all the drug’s PK parameters including volume of distribution, half-life, and clearance can be estimated from its concentration vs. time profiles.

Pharmacodynamics (PD) is the study of the relationships between the concentrations of a drug at the receptor or target organ (effect sites) and the intensity of its pharmacological effect. It has a focus of understanding the functioning mechanisms of the drug and usually involves three major objects: receptor binding, post-receptor effects, and chemical interactions [5]. The major goal of PD study is to establish the dose-response profile, thus PD study is often carried out together with the PK study. Generally, there are three different stages to be considered when studying the overall time course of a dose-effect relationship (see Figure 1.1.) [6]. It
is crucial to build the PK/PD profiles of new drugs because they can offer 1) a better understanding of pharmacological behaviors of drugs; 2) an opportunity for recognizing the presence of active metabolites; 3) a better strategy for a therapeutic dose regimen; and 4) a better understanding of potential drug-drug interactions.

Overall, pharmaceutical analysis is employed throughout the entire drug discovery and development process. It is used to provide accurate and precise data, supporting not only drug discovery and development, but also post-market surveillance [7]. Pharmacokinetic-pharmacodynamic relationships are playing an increasingly important role in decisions on the rational development and use of new drugs, and can provide a detailed knowledge of the mechanism of the drug and a better understanding of the molecular targets on which they act. Due to the significance of pharmacological analysis, sensitive analytical methods are critically needed for pharmacological studies.
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![Diagram](image)
1.1.2. Modern analytical technologies

In the past decades, demands on the analytical support for pharmacological studies have intensified. As a result, new technologies are continually evolving to meet the demands. Since the first combination of techniques was successfully accomplished with gas chromatography-MS (GC-MS) in the 1960s [7], many hyphenated bioanalytical techniques have been successfully developed for the pharmaceutical analysis. Today, chromatographic based hyphenated techniques are widely used in pharmacological studies. Chromatography consists of a variety of techniques, which have the common feature that the components of the sample are partitioning between a stationary and a mobile phase. Among all of chromatographic technologies, gas chromatography (GC) and liquid chromatography (LC) are most commonly utilized in pharmaceutical research.

GC is a type of chromatography for separating and analyzing compounds that can be vaporized without decomposition. In GC, the mobile phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase can be either solid (adsorption chromatography) or liquid fixed onto a solid carrier (partition or absorption chromatography). The commonly used detectors in GC include Thermal Conductivity Detector (TCD), Flame Ionization Detector (FID), Electron Capture Detector (ECD), Mass Spectrometry (MS) detector and Infrared Spectrophotometric (IR) detectors. Other special detectors for GC are also available such as Photoionization Detector (PID), Flame Photometric Detector (FPD), Pulsed Flame Photometric Detector (PFPD) and Atomic Emission Detector (AED) [8].

The main drawback of GC is that it is only suitable for analysis of volatile and
thermally stable compounds. This requirement limits the applicability of the GC methods, i.e., GC cannot analyze thermally labile and high molecular weight molecules such as biomolecules and polymers. In contrast, LC can be utilized for analysis of such analyte molecules. LC is a separation technique in which the mobile phase is a liquid. It can separate an analyte from other interference compounds by introducing the analyte in a mobile phase to a stationary phase (column) and allowing its interaction to resolve the constituents. The most commonly used detection methods in LC include UV-VIS spectroscopy, fluorescence, electrochemical, or MS detectors. Other detectors based on the measurement of optical density, light scattering, polarimetry and IR spectrophotometer can also be used with LC. Combined HPLC-NMR spectroscopy is another rapidly growing technology, enabling the rapid and detailed structural characterization of the complex mixtures [9]. In general, LC is more widely used than GC in drug analysis.

When the mobile phase is a supercritical, i.e. a compound above its critical temperature and pressure, the chromatographic method is called supercritical fluid chromatography (SFC). SFC’s principles are similar to those of LC, but SFC typically utilizes carbon dioxide as the mobile phase. Because SFC can be used for the separation of chiral compounds, it is now commonly used for achiral separations and purifications in the pharmaceutical industry. However, because several high-pressure pumps are necessary for SFC, its hardware costs are higher. In addition, SFC may not be highly reproducible and robust. As a result, the pharmaceutical and quality assurance regulations limit the usage of SFC in drug analysis [10].
1.1.3. Quantitative analytical methods and pharmacological studies

Accurate measurements of drug level are essential to the pharmacological study. Drug level measurements can be used to study the mechanism of drug action, including the contribution of metabolites to the observed drug effects. Moreover, drug level measurements can provide insights of how to optimize drug dosage regimens. Furthermore, bioavailability studies also depend on drug level measurements. Drug level measurements are achieved by using analytical techniques that are suitable for the quantitative determination of drugs and their metabolites in biological samples. Several important factors should be considered when developing a quantitative method for drug analysis. The first factor is cost. The method developed should be cost-effective. The second factor is time. The total sample preparation and run time should be short to enable the high throughput operation. The third factor is simplicity. The very complex procedure should be avoided as much as possible in the method developed. The fourth factor is robustness. The method developed should be highly reliable and reproducible.

In my Ph.D study, I focused on the development of quantitative methods that utilize high performance liquid chromatography for separation in conjunction with either ultraviolet spectroscopy (HPLC-UV) or tandem mass spectrometry (HPLC-MS/MS) for detection to provide accurate and reliable data for pharmacological studies. A quantitative analytical method generally consists of three steps: biological sample preparation, HPLC separation, and detection of the analytes of interest. After method development, the analytical method shall be validated or evaluated for its performance before it can be used in pharmacological studies. In the remaining of this chapter, I will discuss each of these topics in details.
1.2. Biological sample preparation

Quantitative bioanalytical methods should be capable of detecting trace amounts of analytes in various biological specimens, *i.e.*, plasma, urine, saliva or tissue, which contain higher contents of proteins, salts, and lipids that may interfere with analysis. Therefore, extraction of the analytes of interest from a biological specimen prior to analysis is essential to the successful application of an analytical technique. Moreover, sample preparation can prevent analytical equipment from contamination by lipids, proteins, and undissolved particles. Sample cleanup is generally achieved by protein precipitation (PPT), liquid-liquid extraction (LLE), or solid phase extraction (SPE).

1.2.1. Protein Precipitation

Protein is one of the matrix molecules present in all biological samples, which often interfere with analysis. The simplest way to remove protein from a sample is to precipitate it by adding organic solvents or a combination of organic solvents and ionic salts to the sample. After mixing, the mixture is centrifuged and the supernatant containing the analytes is separated from the precipitated protein. The supernatant can be either injected directly into an analytical system or further concentrated by evaporation prior to analysis.

The underlying mechanism of protein precipitation is to reduce the hydration layer around the protein by the addition of new solvents. There are two types of forces acting upon a protein molecule in an aqueous solution, repulsive electrostatic force and attractive electrostatic force. Attractive electrostatic forces are due to induced or
permanent dipoles of protein molecules, while repulsive electrostatic forces result from the presence of the hydration layers which can prevent the likelihood of aggregation [11]. Addition of miscible organic solvents such as acetonitrile or methanol can displace water from the protein surface, decreasing the hydration layer around protein. Because hydration layers become thinner in the presence of other solvents, proteins are more likely to aggregate by attractive electrostatic forces.

Protein precipitation is the simplest and fastest sample preparation technique, which can easily be automated. Compared with other techniques, the highest recovery can be achieved by protein precipitation. The drawback of protein precipitation is that it cannot effectively remove salts and many other compounds present in the sample, some of which may still interfere with analysis [12].

1.2.2. Liquid-Liquid extraction

Liquid-liquid extraction (LLE) is an efficient technique to separate the analyte molecules from sample matrix molecules. This separation is based on the difference in the distribution of a molecule in the water-immiscible organic phase and aqueous phase. Since most of the biological samples are aqueous solution, organic solvents are often added to extract the analyte molecules from the aqueous phase. The concept “like dissolves like” works well in LLE. In general, after extraction, more hydrophilic compounds are in the aqueous phase, while hydrophobic compounds are mainly in organic solvents. After mixing two immiscible solvents, a compound dissolved in these two phases can reach to equilibrium, at which the ratio of the concentration of this compound in two solvents will no longer change.
\[ K_D = \frac{C_o}{C_{aq}} \quad (1.1) \]

Where \( K_D \) is the distribution constant, \( C_o \) is the concentration of the analyte in the organic phase, and \( C_{aq} \) is the concentration of the analyte in the aqueous phase. Unless \( K_D \) is extremely large, there may still be a substantial amount of a solute in the aqueous phase after a single extraction. Consequently, two, three, or even four extractions are carried out in sequence to extract the solute from the aqueous phase.

Several methods can be used to increase \( K_D \). First, changing the organic solvent used can increase the solubility of the analyte molecules. Extremely hydrophobic analyte molecules can be extracted more efficiently with non-polar solvents, such as hexane and carbon tetrachloride, while polar analytes can be extracted more efficiently with polar solvents, such as chloroform and ethyl acetate [13]. Second, the salting out effect can be used to decrease the analyte concentration in the aqueous phase. Moreover, if an analyte molecule can be ionized, its \( K_D \) may be increased by suppressing its ionization to make it more soluble in the organic phase.

LLE is more effective than protein precipitation in removing salts from samples. However, the drawbacks of LLE include the consumption of large amounts of solvent, the time-consuming extraction process, and the possible emulsion formation [14].

1.2.3. Solid Phase Extraction

Solid phase extraction (SPE) is a widely used chromatographic method to separate the analytes of interest from sample matrix molecules. In SPE, the analytes of interest are extracted from its biological matrix by partitioning of the analytes between a solid phase and a liquid phase, removing salts and many endogenous
compounds that may interfere with the analysis. SPE is a more efficient separation process than LLE, and can achieve a higher recovery of analyte by employing a small column or cartridge. SFE consists of five major steps: 1) selecting the SPE cartridge; 2) conditioning the solid phase matrix; 3) loading the sample; 4) washing the solid phase matrix while retaining the analytes of interests, and 5) eluting the analytes of interest from the solid phase matrix (Figure 1.2).
Figure 1.2, Five steps of SPE: (1) selection of tube, (2) conditioning of tube, (3) addition of sample, (4) washing and (5) elution
The selection of an appropriate SPE cartridge depends on the understanding of the mechanisms of the interaction between the sorbent and analyte of interest. The most common retention mechanisms in SPE are based on van der Waals forces (“non-polar interactions”), hydrogen bonding, dipole-dipole forces (“polar” interactions) and cation-anion interactions (“ionic” interactions). There are three general extraction mechanisms used in SPE and they are normal phase, reverse phase, and ion-exchange, respectively [15]. A simplified procedure for the selection of the cartridge is illustrated in Figure 1.3.

a) Reversed phase SPE involves an analyte of interest, a polar or moderately polar sample matrix (mobile phase) and a non-polar stationary phase. The analyte of interest is typically mid- to non-polar. Several SPE materials, such as the alkyl- or aryl-bonded silicas (C-18, C-8, C-4, ENVI-18, ENVI-8, and C-Ph) are used in reversed phase extraction.

b) Normal phase SPE involves a polar analyte, a mid- to non-polar matrix (e.g. acetone, chlorinated solvents and hexane) and a polar stationary phase. Polar-functionalized bonded silicas (e.g. LC-CN, LC-NH₂, and LC-Diol), and polar adsorption media (LC-Si, LC-Florisil, ENVI-Florisil, and LC-Alumina) typically are used in normal phase extraction.

c) Ion exchange SPE is used to extract the compounds that can become ions in a solution. Anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group (LC-SAX or LC-NH) bonded to the silica surface. Cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups (LC-SCX or LC-WCX) that are
bonded to the surface.

Among the three extraction methods, SPE is the most complicated one and its utility can be limited by its relatively poor reproducibility due to variations in adsorbent materials used from one batch to another. The advantages of SPE over other two methods include more effectively depleting the interfering molecules, reducing the amount of the organic solvent used, and having a relatively high recovery. Moreover, although it is more expensive, SPE can be readily automated both off- and on-line, enabling the high throughput operation.
Figure 1.3, Illustration of Selection of SPE Cartridge
1.3. High performance liquid chromatography

1.3.1. Introduction

As mentioned in the previous section, high performance liquid chromatography (HPLC) has become the most powerful analytical tool used in pharmaceutical studies. The principle of HPLC is similar to SPE, which is based on the distribution of the analytes between the liquid mobile phase and a stationary phase. HPLC separation depends on types of the stationary phase used. Four major types of liquid chromatography are normal-phase (NP), reverse-phase (RP), ion exchange, and size-exclusion chromatography. Since reverse-phase chromatography is the most widely used one in drug analysis, I will focus my discussion on this type of chromatography in this dissertation. In the remaining of my dissertation, HPLC stands for reverse-phase HPLC.

The flow diagram of an HPLC system is displayed in Figure 1.4. The principle components in the HPLC system are mobile phase, pump, sample injector, column and detector. Pumps move the mobile phase through a degassing unit and then the eluent goes into a solvent organizer, which controls its composition. Then eluents are mixed in a mixing chamber. The sample is drawn by the injector and introduced to the column along with the mobile phase. The components of the sample are separated through the column, \textit{i.e.}, the compounds that have stronger interactions with the stationary phase move slower, and tend to stay longer on the column. Finally eluents go to the detectors.
Figure 1.4, Flow diagram of HPLC setup

(M: mixing chamber; I: injector; D: detector)
1.3.2. Column Selection

A good column allows for suitable resolution of the analytes of interest, low backpressure generation, minimal solvent consumption and short analysis times. Therefore, selecting a proper column is essential for the LC method development. There are several factors to consider when selecting a column, which include column length, particle size, column diameter (internal diameter), pore size, and bonded phase [17, 18].

1) Column length is typically predicated by the resolution required from a HPLC system. The longer a column, the higher the resolution power. However as column length increases, the backpressure increases which can be unpractical for many HPLC systems. Moreover, longer column results in longer retention time. Since HPLC is a diffusion-limited technique and that above analyte retention factor (k) values of ~10, increasing retention will have little or no effect on resolution because of decreases in efficiency caused by an increase in longitudinal molecular diffusion. Therefore column is not always the longer the better. For drug analysis, 50 to 150 mm columns can provide suitable resolution.

2) Column internal diameter dictates the speed of analysis and affects the method sensitivity. The larger diameter results in larger extra column volume, which may decrease efficiency. Usually 2.1-mm i.d. columns are selected to save solvent and increase sensitivity when we are working with a limited sample.

3) The particle size of the stationary phase support affects the efficiency of a
separation and one typically needs high efficiency when trying to separate a few components in a short time. Smaller particle sizes bring higher efficiency; however, this is usually at the expense of an increase in system backpressure. The most widely used particles are 3.0 or 5.0 µm in diameter and provide very reasonable efficiencies when used with HPLC systems. Sub-2.0-µm particles, which provide higher resolution and shorter analysis times, are typically used with ultrahigh-pressure liquid chromatography (UHPLC) systems, which are capable of dealing with high system backpressures (1000 bar and greater) and can generate very high efficiencies for high resolution or very fast separations.

4) Pore size describes the average pore diameter of the pores on the surface of the silica packing material. The smaller the pore size, the larger the surface area and therefore the greater carbon loading of the column. One should choose column packing with small pore (60-100Å) when the solute molecular weight is less than about 5000Da (Note that most of the drug molecules are smaller than 5000Da). Otherwise, one should use column packing with the 300Å pore size.

5) There are lots of choices of bonded phases, which offer significant differences in selectivity. When differences in analyte hydrophobicity are large, hydrophobic stationary phases can be selected. The length of the carbon chain in hydrophobic stationary phase typically ranges from C4 to C18. As the carbon chain length increases, the hydrophobicity of the stationary phase also increases. It is best to initially select a phase in the middle of the hydrophobic
spectrum (e.g., C8), and then change to a more hydrophobic phase or more hydrophilic phase depending on initial results and solubility properties of sample. Modified alkyl phases with more polar functional groups embedded within the ligand are used when separating analytes with different (polar) functional groups. Typically, these phases can be used with 100% aqueous eluent systems and are better at retaining polar analytes. Phenyl-containing phases interact strongly with analytes containing p electron systems (aromatic, unsaturated). Amino, diol and silica phases are traditionally used for the separation of polar analytes in both reversed-phase and normal-phase modes. Lately they have also found utility in hydrophilic interaction liquid chromatography (HILIC) mode in which highly organic eluent systems are used to retain polar analytes using polar stationary phases.

1.3.3. Mobile Phase Selection

Mobile phase is one of the most important components in RP-HPLC. The type of mobile phase used may have a great impact on separation. It can also promote or suppress ionization of the analyte molecules. In general, mobile phase consists of a mixture of water or an aqueous buffer solution and various water-miscible solvents. Two main features affected by formulation of mobile phase are solvent polarity and solvent selectivity [19]. The polarity and selectivity can be optimized by changing the type of organic solvents, percentage of organic solvents, pH, buffers, and other additives.
Table 1.1, Properties of common solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Refractive Index (25°C)</th>
<th>Viscosity, cP</th>
<th>Boiling Point, °C</th>
<th>Polarity Index, P</th>
<th>Eluent Strength, $e^0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>1.333</td>
<td>0.89</td>
<td>100</td>
<td>10.2</td>
<td>Large</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>1.341</td>
<td>0.34</td>
<td>82</td>
<td>5.8</td>
<td>0.65</td>
</tr>
<tr>
<td>methanol</td>
<td>1.326</td>
<td>0.54</td>
<td>65</td>
<td>5.1</td>
<td>0.95</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>1.372</td>
<td>0.30</td>
<td>69</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>toluene</td>
<td>1.494</td>
<td>0.55</td>
<td>110</td>
<td>2.4</td>
<td>0.29</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>1.370</td>
<td>0.43</td>
<td>77</td>
<td>4.4</td>
<td>0.58</td>
</tr>
<tr>
<td>chloroform</td>
<td>1.443</td>
<td>0.53</td>
<td>61</td>
<td>4.1</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Solvents and eluents can be characterized by their elution strength (Table 1.1). Generally, if the analytes of interest are more polar, the solvent with larger eluent strength can be selected; while if compounds are more hydrophobic, the solvent with smaller eluent strength can be utilized.

Increasing the percentage of organic solvents in mobile phase increases the “elution power” of mobile phase. Scouting gradient method [20] can be used to select an optimized % of organic phase.

pH in mobile phase is also important to retention. Le Chateliers principle applies to the equilibrium when adding acidic or basic species to the mobile phase. The extent of analyte ionization can also vary with pH of the mobile phase (Figure 1.5). The ionized form is more polar-less well retained under the reversed phase conditions. Non-ionized is less polar -retained longer under the reversed phase conditions. pH which ranges from pKa -1 to pKa +1 should be avoided to prevent poor symmetry of peak. Since traditional RP HPLC is silica based, many columns are pH restricted. At pH < 2.5, the Si-C bond will be cleaved and more -OH groups will be created; at pH > 7.5, the silica gel backbone becomes soluble and flows out at the end of column. Therefore at the beginning of method development, the pH range of the column needs to be highlighted [21].
Figure 1.5, Acid base equilibrium

\[
\begin{align*}
\text{H}_2\text{A}^+ & \rightleftharpoons \text{HA} + \text{H}^+ & pK_a1 \\
\text{HA} & \rightleftharpoons \text{A}^- + \text{H}^+ & pK_a2
\end{align*}
\]
In respect to buffer choice, there are several factors to consider, including buffer capacity, concentration, solubility, and evaporability (i.e., mass spec compatibility). When the analyte is relatively polar and hard to be retained on the reverse phase column, ion-pairing agent can be added to the mobile phase. This ion-pairing agent often possesses a relative non-polar moiety that has a stronger interaction with the stationary phase. After the column is fully equilibrated with the mobile phase, the stationary phase is kinetically coated with the ion-pairing agent. As the ion-pairing agent is able to interact with the analyte ions, the analyte can also be retained on the column temporarily.

1.4. Detection of Analytes

As mentioned in the above section, there is a vast array of detectors that can be coupled to HPLC. The discussion presented here focuses on the ultraviolet spectroscopy and mass spectrometer detectors.

1.4.1. Ultraviolet Spectroscopic Detector

UV can analyze the majority of organic compounds, and almost 70% of published HPLC analyses were performed with UV detectors [22]. UV detection is the most useful and most popular detector for HPLC and widely utilized in pharmacological studies. Many substances absorb light in the UV range. A diagram of a typical UV detector is shown in Figure 1.6. Light from the deuterium lamp is collimated by two curved mirrors onto a holographic diffraction grating. The dispersed light is then focused by means of a curved mirror, onto a plane mirror and
light of a specific wavelength is selected by appropriately positioning the angle of the plane mirror. Light of the selected wavelength is then focused by means of a lens through the flow cell and, consequently, through the column eluent. The exit beam from the cell is then focused by another lens onto a photo cell which linearly responds to the intensity of the transmitted light. The method of combining HPLC with UV is sensitive, economic, and simple. In this project, an analytical HPLC-UV method is developed for quantification of Triapine.
Figure 1.6, The UV detector
1.4.2. Mass spectrometry

Mass spectrometry (MS) is a technique that identifies the molecules based on their mass to charge ($m/z$) ratio. Over the past 15 years, MS has led to remarkable advances in the fields of biomedical and biological research [23]. Currently, combining MS with chromatographic separation provides some of the most powerful techniques available for pharmaceutical analysis. Taking advantages of powerful separation and sensitive detection, LC-MS analysis is powerful for structural elucidation, accurate quantification, and metabolites prediction in complex biological matrices.

The basic working procedure of a mass spectrometer includes several steps (Figure 1.8). First, the sample is vaporized in the ion source. Then, the vaporized species in the sample are ionized in the gas phase. Afterward, these ions are separated based on their $m/z$ ratios in the mass analyzer. Finally, the detector can amplify the signal of the separated ions, and the collection of ion signals composes the mass spectrum [24].
Figure 1.7, Flow diagram of mass spectrometry
**Ion sources** used depends on the type of the molecules to be ionized. The most commonly used ionization methods coupled with liquid chromatography are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [25].

ESI is a soft ionization technique, which involves spraying of a solution of the sample through a highly charged needle (so-called capillary) at atmospheric pressure (Figure 1.8). The charged droplets are produced in which the positive or negative ions are solvated with solvent molecules. Heating gas and nebulization gas is applied to the charged droplets to cause solvent evaporation. These droplets will then be further desolvated into even smaller droplets, which creates molecules with attached protons. The protonated and desolvated molecular ions will then be transferred to the mass analyzer [26].

APCI is another important ionization source because it is capable of ionizing less polar compounds (Figure 1.9). Unlike ESI, the ionization process of APCI occurs in gas phase. Without applying high voltage, the APCI capillary enables the volatile liquid sample to be heated and spayed first, and then a corona discharge needle with a high voltage generates ions from the aerosol cloud through interaction of reagent gas and electrons [27].
Figure 1.8, Schematic diagram of ESI interface
Figure 1.9, Schematic diagram of APCI interface
The mechanisms and characteristics of these two different sources are summarized below:

- **ESI (Electrospray ionization)**
  - Ionization in solution
  - Polar compound

- **APCI (Atmospheric pressure chemical ionization)**
  - Ionization in gas phase (corona discharge region)
  - Less Polar compound
  - Less gentle ionization

**Mass analyzers:** when ions interact with the applied electric or magnetic field, the mass analyzer separates the ions according to their mass to charge ratio ($m/z$). Typical mass analyzers include quadrupole MS, ion trap MS, and time-of-flight (TOF) MS, each of which has its own characteristics and application area. I only used Q-TRAP (triple quadrupole linear ion trap mass spectrometer) and Q-TOF (quadrupole–time-of-flight tandem mass spectrometer) for my dissertation research.

Q-TRAP is two distinct types of instruments in one classis: a linear ion trap system (IT-MS) and a triple quadrupole system (QQQ) (Figure. 1.10).
Figure 1.10, A schematic of the Q-TRAP instrument. The final quadrupole Q3 can be operated as either a conventional RF/DC quadrupole mass filter or as a linear ion trap mass spectrometer.
QQQ system is a “tandem-in-space” device [28], meaning that each step of an MS-MS experiment is carried out at a spatially distinct location in the instrument. QqQ consists of two quadrupole mass filters (Q1 and Q3) in series and a non mass-resolving quadrupole (Q2) in between. For Q1 and Q3, a DC voltage is applied to two diagonal rods and a radio frequency is applied to the two opposite diagonal rods. A specific electromagnetic field is formed between the electrodes. This magnetic field only allows ions with certain m/z to go through the poles and reach the detector. Q2 is not a true quadrupole but a hexapole with RF-only. It is a collision cell that accommodates collision-induced dissociation (CID). Briefly, Q1 is used to select the parent or precursor ions. Q2 breaks parent ions into fragments and passes them to Q3. Q3 allows selected daughter ions to reach to the detector to be detected. IT-MS instrument is “tandem-in-time” fashion rather than the “tandem-in-space” manner as discussed above [28]. This means that, once the ions are introduced into the ion trap, all of the different steps of ion manipulation occur within the same volume, but at different times. IT-MS first traps the molecular ions with m/z of interest. Then, in the same trap chamber, the trapped ions are broken down with CID. Finally, IT-MS exports the fragments to the detector and generates a spectrum of the fragments. The advantage of IT-MS, relative to QQQ, lies in its high instrument duty cycles and increased scanning sensitivity.

The Q-trap instrument combines the advantages of a QQQ with those of IT-MS within the same platform. The Q3 can be operated as either a conventional quadrupole mass filter or as a linear ion trap mass spectrometer. The combination of
highly selective QQQ scans and high sensitivity IT-MS scans on the same instrument platform provides rapid identification and confirmation of complex compounds.

Q-TOF instrument has very similar configuration with QQQ, with the replacement of Q3 by TOF (Figure 1.11). TOF is a method of MS in which ions mass-to-charge ratio is determined via a time measurement. Ions are accelerated by an electric field. This acceleration causes an ion having the same kinetic energy as any other ions with the same charge. The higher charge has the higher kinetic energy. Then these ions fly freely toward the detector. The velocity of the ion depends on the mass-to-charge ratio. The ion with smaller m/z ratio flies faster. The configuration of Q-TOF is shown in Figure 1.11. The instrument consists of three quadrupoles, Q0, Q1 and Q2, followed by a reflecting TOF mass analyzer with orthogonal injection of ions. Q1 selected certain parent ions. Q0 and Q2 are not real mass filters. They are used to focus ions, and Q2 is also a collision cell. The fragments generated by CID can then be further resolved and analyzed by TOF. Due to its high mass resolution and the wide m/z analysis range, Q-TOF is often utilized in the unknown compound identification and analysis of large molecules such as peptides, proteins, and oligonucleotides [30].
Figure 1.11, Schematic diagram of Q-TOF
1.5. Method Validation

Once an analytical method has been developed, it is necessary to evaluate the performance of the method. The NTP (National Toxicology Program) guideline and the FDA (U.S. Food and Drug Administration) guideline are two most commonly accepted standards for method validation. According to FDA and NTP, there are many parameters required for validation to demonstrate the analytical method developed reliably and reproducibly. These parameters include selectivity, sensitivity, recovery, matrix factor, calibration curve, accuracy, precision, and stability. These validation parameters are described below in detail and are summarized in Table 1.2 [30].

**Selectivity** is the ability of a bioanalytical method to differentiate and quantify the analyte of interest in the presence of other components in the sample, including potential metabolites and endogenous matrix components.

**Sensitivity** (LLOQ, lower limit of quantification) is the lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy.

**Recovery** is a measurement of the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method (equation 1.2).

\[
\text{Recovery} = \frac{\text{response spiked analyte}}{\text{response extracted analyte}} \times 100\% \quad (1.2)
\]

**Matrix factor** is an evaluation of the effectiveness of both the extraction methods and LC methods and a quantitative measure of the matrix effects due to suppression or enhancement of ionization in a mass spectrometric detector (equation
Matrix factor = (response in matrix extract) / (response in neat solution) × 100% (1.3)

**Calibration Curve:** the calibrators must be prepared in the same biological matrix as the real samples. In general, the calibrators should be prepared by spiking the known amount of analyte into a biological matrix. The range of reliable response is the range of concentrations over which the response of the detector, in general, is linear.

**Accuracy** describes how close the mean test results obtained by the method are to the nominal spiked concentration of the analyte and is determined by replicate analysis of samples containing known amounts of the analyte (equation 1.5).

\[
\%RE = \left[\frac{\text{measured} - \text{nominal}}{\text{nominal}}\right] \times 100\% \quad (1.5)
\]

**Precision** describes how close individual measurements of an analyte are when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix (equation 1.6).

\[
\%CV = \left(\frac{\text{Standard deviation}}{\text{mean}}\right) \times 100\% \quad (1.6)
\]

To evaluate the accuracy and precision of a method, a set of quality control (QC) samples are needed. These QC samples usually include at least three concentration levels (low, medium, and high).
### Table 1.2, Validation Parameter Requirements [30]

<table>
<thead>
<tr>
<th>Parameter or Process</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity (matrix interference)</td>
<td>Review noninterference in at least 6 sources of matrix for non-MS assays. For MS assays determine MFs in 6 sources if the nonisotopically labeled IS is used. If isotopically labeled IS is used, demonstrate that IS-normalized MF is close to unity.</td>
</tr>
<tr>
<td>Validation batches</td>
<td>Analyze at least 3 batches for accuracy and precision. At least 1 validation batch should be made as large as the largest anticipated sample analysis batch.</td>
</tr>
<tr>
<td>QC samples</td>
<td>Concentration of QC samples should be:</td>
</tr>
<tr>
<td></td>
<td>Low QC: About 3 times the LLOQ</td>
</tr>
<tr>
<td></td>
<td>Mid QC: Middle of the range (at about the geometric mean of low and high QC concentration)</td>
</tr>
<tr>
<td></td>
<td>High QC: Near the high end of the range, ~70% to 85% of ULOQ</td>
</tr>
<tr>
<td></td>
<td>Dilution QC: Sufficient to cover highest anticipated dilution</td>
</tr>
<tr>
<td>QC acceptance criteria</td>
<td>Intra- and inter-batch precision (%CV) and accuracy (%RE) should be:</td>
</tr>
<tr>
<td></td>
<td>QC prepared at all concentrations greater than LLOQ ≤15%; QC prepared at LLOQ concentration ≤20%</td>
</tr>
<tr>
<td>Calibration standard</td>
<td>Include the following calibration standards with each batch:</td>
</tr>
<tr>
<td></td>
<td>Minimum of 6 non-zero standards</td>
</tr>
<tr>
<td></td>
<td>Matrix blank: Matrix sample without internal standard</td>
</tr>
<tr>
<td></td>
<td>Zero standard: Matrix sample with internal standard</td>
</tr>
<tr>
<td>Standard acceptance criteria</td>
<td>Acceptance criteria for calibration standards are:</td>
</tr>
<tr>
<td></td>
<td>LLOQ standard ≤20%; All other standards ≤15%</td>
</tr>
<tr>
<td></td>
<td>At least 75% of standards should meet above criteria</td>
</tr>
<tr>
<td>Matrix blank</td>
<td>Interference in matrix blank should be ≤20% of LLOQ response</td>
</tr>
<tr>
<td>Recovery</td>
<td>Extent of recovery of analyte and IS should be consistent, precise, and reproducible.</td>
</tr>
<tr>
<td></td>
<td>Determine recovery at 3 concentration levels.</td>
</tr>
<tr>
<td>Stability</td>
<td>Perform the following stability experiments:</td>
</tr>
<tr>
<td></td>
<td>Stock solution: Minimum of 6 hours at room temperature</td>
</tr>
<tr>
<td></td>
<td>Postpreparative (extracted samples/autosampler tray):</td>
</tr>
<tr>
<td></td>
<td>Longest time from preparation through sample analysis. Assess against fresh standards, except for autosampler reinjection reproducibility.</td>
</tr>
<tr>
<td></td>
<td>Benchtop: Stability at ambient temperature (or temperature used for processing of samples) to cover the duration of time taken to extract the samples (typically ~4-24 hours).</td>
</tr>
<tr>
<td></td>
<td>Freeze-thaw: QC samples at minimum of 2 concentrations, 3 cycles, completely thawed, refrozen at least 12 hours between cycles, at anticipated temperature of sample storage.</td>
</tr>
<tr>
<td></td>
<td>Long-term: Cover longest time from collection to final analysis for any sample in study.</td>
</tr>
<tr>
<td></td>
<td>Analyze 3 aliquots at low and high concentrations with fresh standard curves and compare against intended (nominal) concentrations. Long-term stability can be completed postvalidation.</td>
</tr>
</tbody>
</table>
**Stability** studies provide information about the effect of sample handling and storage on analysis. The most common stability studies include the following categories: short-term stability, long-term stability, freeze-thaw stability, post-preparative stability, and stock solution stability.

1.6. Reference


CHAPTER II
DEVELOPMENT OF A LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATIVE DETERMINATION OF TRIAPINE, A RIBONUCLEOTIDE REDUCTASE INHIBITOR, BY SPECTROPHOTOMETRIC STUDY OF TRIAPINE COMPLEXATION REACTION

2.1. Introduction

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, or 3-AP) (Figure 2.1A) is a metal-ion chelator, belonging to a class of compounds known as α-(N)-heterocyclic carboxaldehyde thiosemicarbazones (HCTs). Even though it has been reported that triapine is useful for treatments of viral or fungal infections [1] and neurodegenerative diseases [2], most of current studies have been focusing on its anti-tumor activity due to the effectiveness of this compound on suppressing tumor growth by inhibiting the activity of ribonucleotid reductase (RR) [3].

RR is an essential enzyme that catalyzes the strictly conserved reduction reaction of ribonucleotides to deoxyribonucleotides, the precursors of DNA synthesis, and
regulates the total rate of DNA synthesis so that a constant ratio of DNA to cell mass can be maintained during cell proliferation and DNA repair [4]. As an iron-ion-dependant enzyme, human RR consists of two dimers: M1 (large subunit) and M2 or p53R2 (small subunit) [5], constituting two forms of RR in human cells (i.e., M2-M1 and p53R2-M1). RR’s activity is based on the association of the two subunits. The M1 subunit contains the ribonucleotide binding sites and allosteric effector sites. The M2 (or p53R2) subunit contains a non-heme iron complexed with a tyrosyl free radical, which is essential for its catalytic activity [6]. It has been reported that tumor cells are more sensitive to the cytotoxic effect of RR inhibition than normal cells [4]. Therefore, RR is an attractive target for antineoplastic agents.

By the intrinsic nature of chelator, triapine can coordinate with ferric ions through the N–N–S tridentate ligand system and form an octahedral complex with 2:1 molar ratio of triapines to ferric ion [7]. Triapine inhibits RR’s activity by stripping ferric ion which is needed for stabilizing the iron-tyrosyl radical from the catalytic site of the small subunit of RR through the formation of triapine-Fe(III) complex [7]. Reduction of triapine-Fe(III) complex can produce reactive oxygen species (ROS) which can further quench the tyrosyl radical on the M2 or p53R2 subunit of RR and cause tumor DNA breakage [8]. Studies showed that triapine as RR inhibitor is 1000-fold more potent than hydroxyurea (HU) the only therapeutic agent currently used in chemotherapy [3]; can significantly decrease RR activity in a variety of cancer cell lines including leukemia, non-small-cell lung cancer, renal cancer and melanoma; and enhances radiation-mediated cytotoxicity in cervical and colon cancers [3, 9-13]. Recent clinical studies indicated that
triapine synergizes with other chemotherapeutic agents, particularly in cervical cancer and hematological malignancies, and acts as sensitizer radiation therapy [14-16].

Despite of significant potential of triapine for treatment of human diseases, there are few analytical studies being conducted on this compound. A recent literature search by SciFinder Scholar revealed no publication available on analytical method development and validation for this compound. Although liquid chromatographic assays were mentioned for the measurement of triapine in clinical studies [17, 18], these assays suffered from low sensitivity, poor selectivity, and irreproducibility when being repeated. The major challenge in developing a quantitative assay for measurement of triapine is stemmed from the chemical nature of triapine. Being a chelator and a weak acid, triapine has multiple equilibra in aqueous solution, which are affected by various factors including solution pH, metal ions and other competing chelators, as well as buffer composition. In this work, a liquid chromatographic assay for quantitative analysis of triapine has been developed based on the investigation and optimization of the aforementioned factors. The assay used 2-[(3-methoxy-2-pyridinyl)methylene]hydrazinecarbothioamide (Figure 2.1B) as internal standard (IS), Waters Xterra RP18 as analytical separation column, 18.0% acetonitrile and 82.0% ammonium bicarbonate buffer [i.e., 10.0 mM NH₄HCO₃ and 5.00 mM EDTA (v/v) at pH 8.5] as mobile phase, and 360 nm as the detection wavelength. The assay has a linear calibration range of 3.00-1.00 x 10³ ng mL⁻¹ with interassay precision and accuracy of 2-4% and -3-9% respectively. It provides a sensitive and reliable method for accurate measurement of triapine in aqueous solution.
Figure 2.1, The chemical structures of (A) triapine and (B) NSC 185051, the internal standard of LC-UV method.
2.2. Experimental

2.2.1 Chemicals and solutions

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone or 3-AP) was kindly provided by Vion Pharmaceuticals (New Haven, CT, USA) and used as the chemical standard for the analyte. 2-[(3-methoxy-2-pyridinyl)methylene]hydrazinecarbothioamide (CAS 51984-14-6 or NSC 185051) was obtained from the Developmental Therapeutics Program of the National Cancer Institute at the National Institutes of Health (Bethesda, MD, USA) and used as the internal standard (IS) for triapine in the LC-UV method. HPLC-grade of acetonitrile and methanol, ferric chloride, ethylenediaminetetraacetic acid disodium salt (EDTA), monoammonium phosphate, diammonium phosphate, ammonium formate, sodium hydroxide, ammonium hydroxide, carbonic acid, calcium chloride, zinc chloride, cupric sulfate, magnesium chloride, boric acid, phosphoric acid and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium bicarbonate was from EMD Chemicals (Darmstadt, Germany). Deionized water was obtained from a Barnstead Model 7148 Nanopure® ultrapure water system of the Thermo Scientific (Asheville, NC, USA).

2.2.2 Solutions for spectrophotometric investigation of triapine complexation reaction

Stock solution of triapine (5.00 mM) was prepared by dissolving accurate amount of triapine powder in an appropriate volume of methanol and kept at −20 °C before use. Stock solutions of EDTA (100 mM), ferric chloride (2.50 mM), calcium chloride (2.50 mM), zinc chloride (2.50 mM), cupric sulfate (2.50 mM), and magnesium chloride (2.50 mM) were prepared by dissolving accurate amount of each individual compound in
appropriate volume of deionized water. The stock solution of EDTA was kept at 4 °C, and the stock solutions of metal ions were prepared freshly before use.

Universal pH buffer solutions having pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 were prepared by mixing 40.0 mM H₃BO₃, 40.0 mM H₃PO₄ and 40.0 mM CH₃COOH, then the mixture was titrated to the desired pH with 1 M NaOH. Ammonium formate solutions (10.0 and 20.0 mM, pH 3.0 and 8.5) were prepared by dissolving accurate amount of ammonium formate in appropriate volume of deionized water, and the pH values were adjusted to 3.0 with the concentrated formic acid, and 8.5 with the concentrated ammonium hydroxide. Ammonium bicarbonate solutions (10.0 and 20.0 mM, pH 8.5) were prepared by dissolving accurate amounts of ammonium bicarbonate in deionized water, and the pH was adjusted to 8.5 with the concentrated ammonium hydroxide. Phosphate buffers (10.0 and 20.0 mM, pH 8.5) were prepared by dissolving accurate amounts of monoammonium phosphate monohydrate and diammonium phosphate heptahydrate in appropriate volume of deionized water.

For the study of pH effect on the UV-visible absorption spectra of triapine, eleven solutions of triapine at the concentration of 100 µM with pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 were prepared by dilution of 80.0 µL of 5.00 mM triapine stock solution with 3920 µL of the universal pH buffer at each specified pH values.

For the study of EDTA effect on the UV-visible absorption spectra of triapine-Fe(III) complex, six solutions were prepared as follows: (i) mixing 80.0 µL of 5.00 mM triapine stock solution with 3920 µL of 10.0 mM ammonium formate solution at pH 8.5 [i.e., 100 µM triapine alone]; (ii) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 2.50 mM ferric chloride stock solution with 3840 µL of 10.0 mM ammonium
formate solution at pH 8.5 \textit{[i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Fe(III)]}; (iii) mixing 80.0 
\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 100 mM EDTA stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 2.00 mM EDTA}); (iv) mixing 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution and 80.0 }\mu\text{L of 100 mM EDTA stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 50.0 }\mu\text{M Fe(III) with 2.00 mM EDTA}); (v) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 100 mM EDTA stock solution first, then 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution with 3760 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 2.00 mM EDTA and 50.0 }\mu\text{M Fe(III)}); and (vi) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution first, then 80.0 }\mu\text{L of 100 mM EDTA stock solution with 3760 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Fe(III) and 2.00 mM EDTA}). The solution vi was prepared in triplicate to study the effect of EDTA on triapine-Fe(III) complex at temperatures of 22, 37 and 90 °C.}

Other metal ions such as Mg(II), Ca(II), Zn(II) and Cu(II) were also tested, because similar with Fe(III) these metal ions commonly exist in the human body, water source and the instrument, which can be easily introduced during the experiment. For the study of the effect of other metal ions on the UV-visible absorption spectra of triapine, twelve triapine solutions at 100 }\mu\text{M were prepared as follows: (i) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM magnesium chloride stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Mg(II)}); (ii) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM zinc chloride stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Zn(II)}); (iii) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM copper chloride stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Cu(II)}); (iv) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Fe(III)}); (v) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution with 3840 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Fe(III) and 2.00 mM EDTA}); (vi) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution first, then 80.0 }\mu\text{L of 100 mM EDTA stock solution with 3760 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 2.00 mM EDTA and 50.0 }\mu\text{M Fe(III)}); and (vii) mixing 80.0 }\mu\text{L of 5.00 mM triapine stock solution and 80.0 }\mu\text{L of 2.50 mM ferric chloride stock solution first, then 80.0 }\mu\text{L of 100 mM EDTA stock solution with 3760 }\mu\text{L of 10.0 mM ammonium formate solution at pH 8.5 (\textit{i.e., 100 }\mu\text{M triapine with 50.0 }\mu\text{M Fe(III) and 2.00 mM EDTA}). The solution vii was prepared in triplicate to study the effect of EDTA on triapine-Fe(III) complex at temperatures of 22, 37 and 90 °C.}
µL of 2.50 mM calcium chloride stock solution with 3840 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 50.0 \mu M\ \text{Ca(II)}]\); (iii) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 2.50 mM zinc chloride stock solution with 3840 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 50.0 \mu M\ \text{Zn(II)}]\); (iv) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 2.50 mM cupric sulfate stock solution with 3840 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 50.0 \mu M\ \text{Cu(II)}]\); (v) mixing 80.0 µL of 5.00 mM triapine stock solution, 80.0 µL of 100 mM EDTA stock solution and 80.0 µL of 2.50 mM magnesium chloride stock solution with 3760 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 2.00 mM\ \text{EDTA} \text{ and } 50.0 \mu M\ \text{Mg(II)}]\); (vi) mixing 80.0 µL of 5.00 mM triapine stock solution, 80.0 µL of 100 mM EDTA stock solution and 80.0 µL of 2.50 mM calcium chloride stock solution with 3760 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 2.00 mM\ \text{EDTA} \text{ and } 50.0 \mu M\ \text{Ca(II)}]\); (vii) mixing 80.0 µL of 5.00 mM triapine stock solution, 80.0 µL of 100 mM EDTA stock solution and 80.0 µL of 2.50 mM zinc chloride stock solution with 3760 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 2.00 mM\ \text{EDTA} \text{ and } 50.0 \mu M\ \text{Zn(II)}]\); (viii) mixing 80.0 µL of 5.00 mM triapine stock solution, 80.0 µL of 100 mM EDTA stock solution and 80.0 µL of 2.50 mM cupric sulfate stock solution with 3760 µL of 10.0 mM ammonium formate solution at pH 8.5 \([i.e.,\ 100 \mu M\ \text{triapine} \text{ with } 2.00 mM\ \text{EDTA} \text{ and } 50.0 \mu M\ \text{Cu(II)}]\); the mixtures of triapine, EDTA and metal ions were prepared by two ways: a) mixing triapine and EDTA first before adding metal ion; b) mixing triapine and metal ion first before adding EDTA; (ix) mixing 80.0 µL of 5.00 mM triapine stock
solution with 80.0 µL of 20.0 mM ammonium bicarbonate at pH 8.5 first, then 80.0 µL of 2.50 mM cupric sulfate stock solution with 3760 µL of 10.0 mM ammonium bicarbonate at pH 8.5 [i.e., 100 µM triapine with 50.0 µM Cu(II) in 10.0 mM ammonium bicarbonate at pH 8.5]; (x) mixing 80.0 µL of 5.00 mM triapine stock solution with 80.0 µL of 20.0 mM ammonium bicarbonate at pH 8.5 first, then 80.0 µL of 2.50 mM zinc chloride stock solution with 3760 µL of 10.0 mM ammonium bicarbonate at pH 8.5 [i.e., 100 µM triapine with 50.0 µM Zn(II) in 10.0 mM ammonium bicarbonate at pH 8.5]; (xi) mixing 80.0 µL of 5.00 mM triapine stock solution with 80.0 µL of 20.0 mM phosphate buffer at pH 8.5 first, then 80.0 µL of 2.50 mM cupric sulfate with 3760 µL of 10.0 mM phosphate buffer at pH 8.5 [i.e., 100 µM triapine with 50.0 µM Cu(II) in 10.0 mM phosphate buffer at pH 8.5]; and (xii) mixing 80.0 µL of 5.00 mM triapine stock solution with 80.0 µL of 20.0 mM phosphate buffer at pH 8.5 first, then 80.0 µL of 2.50 mM zinc chloride with 3760 µL of 10.0 mM phosphate buffer at pH 8.5 [i.e., 100 µM triapine with 50.0 µM Zn(II) in 10.0 mM phosphate buffer at pH 8.5].

For the study of buffer composition on the UV-visible absorption spectra of triapine-Fe(III) complex, six triapine solutions at 100 µM were prepared as follows: (i) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 20.0 mM ammonium formate solution at pH 8.5 first, then 80.0 µL of 2.50 mM ferric chloride stock solution with 3760 µL of 10.0 mM ammonium formate solution at pH 8.5 [i.e., 100 µM triapine with 50.0 µM Fe(III) in 10.0 mM ammonium formate at pH 8.5]; (ii) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 20.0 mM ammonium formate solution at pH 3.0 first, then 80.0 µL of 2.50 mM ferric chloride stock solution with 3760 µL of 10.0 mM ammonium formate solution at pH 3.0 [i.e., 100 µM triapine with 50.0 µM Fe(III) in
10.0 mM ammonium formate at pH 3.0]; (iii) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 20.0 mM ammonium bicarbonate solution at pH 8.5 first, then 80.0 µL of 2.50 mM ferric chloride stock solution with 3760 µL of 10.0 mM ammonium bicarbonate solution at pH 8.5 \[i.e., 100 \mu M \text{ triapine with 50.0 } \mu M \text{ Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5}\]; (iv) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of 20.0 mM phosphate buffer at pH 8.5 first, then 80.0 µL of 2.50 mM ferric chloride stock solution with 3760 µL of 10.0 mM phosphate buffer at pH 8.5 \[i.e., 100 \mu M \text{ triapine with 50.0 } \mu M \text{ Fe(III) in 10.0 mM phosphate buffer at pH 8.5}\]; (v) mixing 80.0 µL of 5.00 mM triapine stock solution and 80.0 µL of deionized water first, then 80.0 µL of 2.50 mM ferric chloride stock solution with 3760 µL of deionized water \[i.e., 100 \mu M \text{ triapine with 50.0 } \mu M \text{ Fe(III) in water}\]; and (vi) mixing 80.0 µL of 5.00 mM triapine stock solution, 80.0 µL of deionized water, and 80.0 µL of 2.50 mM ferric chloride stock solution first, then diluted with 3760 µL of 10.0 mM ammonium bicarbonate at pH 8.5 \[i.e., 100 \mu M \text{ triapine with 50.0 } \mu M \text{ Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5}\]. The solution vi was prepared in triplicate to study the effect of ammonium bicarbonate on triapine-Fe(III) complex under temperatures of 22, 37 and 90 °C.

2.2.3 Solutions for liquid chromatography with ultraviolet detection of triapine

Triapine and the IS stock solutions (1.00 mg mL-1) were prepared by dissolving 0.00100 g of each chemical in 1.00 mL of methanol, separately. Each stock solution was aliquoted into 1.50 ml amber glass vials at 100 µL/vial, and kept at −20 °C before use. Working solution of triapine standard (10.0 µg mL-1) and working solution of the IS
(10.0 µg mL⁻¹) were freshly prepared by serial dilution of each stock solution with a mobile phase being tested.

Three mobile phase compositions were tested for chromatographic separation triapine and the IS. They were as follows: (i) 30% methanol and 70% ammonium bicarbonate (10.0 mM, pH 8.5) (v/v); (ii) 18% acetonitrile and 82% ammonium bicarbonate (10.0 mM, pH 8.5) (v/v); and (iii) 18% acetonitrile and 82% ammonium bicarbonate-EDTA buffer (containing 5.00 mM EDTA and 10.0 mM NH₄HCO₃, pH 8.5) (v/v). The mobile phase composition (iii) was chosen as the mobile phase for the optimized LC-UV method. We had explained the reasons in 3.2.2. Mobile-phase composition.

Triapine standard solutions (6.00, 18.0, 20.0, 60.0, 180, 200, 600, 1.80 x 10³ and 2.00 x 10³ ng mL⁻¹) and the IS standard solution (200 ng mL⁻¹) were prepared by serial dilution of the working solution of triapine (10.0 µg mL⁻¹) and the working solution of IS (10.0 µg mL⁻¹) with a mobile phase, separately. Triapine calibrators (3.00, 10.0, 30.0, 100, 300, and 1.00 x 10³ ng mL⁻¹ of triapine and 100 ng mL⁻¹ of the IS) were prepared by mixing 500 µL of a triapine standard solution at twice of a calibrator’s concentration and 500 µL of the IS solution. Triapine quality controls (QCs) (3.00, 9.00, 90.0 and 900 ng mL⁻¹ of triapine and 100 ng mL⁻¹ of the IS) were prepared by mixing 500 µL of a triapine standard solution at twice of a QC’s concentration and 500 µL of the IS solution.

2.2.4 Instrumentation

Spectrophotometric studies of triapine complexation reaction were performed on an Agilent 8453E UV-Visible Spectrophotometer (Agilent, Santa Clara, CA, USA) using
a 10 mm standard quartz cell, which was controlled by the Agilent UV-Visible ChemStation software. The pH measurements were carried out using a Fisher Scientific Accumet® Basic AB15 pH meter (Fisher Scientific, Pittsburgh, PA, USA), which was calibrated using pH calibration buffers of 4.0 and 7.0 or 7.0 and 10.0 from EMD Chemicals (Gibbstown, NJ, USA) depending on the range of pH measurements. Chemicals were weighted by Ohaus Galaxy® 160D analytical balance with the range of 30 g x 0.01 mg (Parsippany, NJ, USA).

The liquid chromatographic system used in this work consisted of a Shimadzu LC-10ADVP binary pump (Shimadzu, Columbia, MD, USA), a Shimadzu SIL-10ADVP autosampler, an inline filter (0.5 µm pore) (Upchurch Scientific, Oak Harbor, WA, USA), an analytical separation column, and a Shimadzu SPD-10AVVP UV detector. The system used Shimadzu Class-VP software (version 5.0) for program execution, data acquisition and processing.

2.2.5 LC-UV method

Chromatographic separation of triapine and the IS was performed at ambient temperature on a Waters Xterra RP18 (5 µm, 2.1 x 150 mm) column (Waters, Milford, MA, USA) with a mobile phase containing 18% acetonitrile and 82% ammonium bicarbonate-EDTA buffer (10.0 mM ammonium bicarbonate and 5.00 mM EDTA at pH 8.5) (v/v) at a flow rate of 0.200 mL/min. The analytes were detected by the UV detector at wavelength of 360 nm. Prior to initial sample analysis, the analytical column was equilibrated with the mobile phase for at least 30 min. This method had a sample
injection volume of 20.0 µL, and a total run time of 8.5 min per sample with the retention times of 4.7 and 6.4 min for triapine and the IS, respectively.

2.2.6 Stability Studies

The stability of triapine solutions was investigated with low and high QC samples (*i.e.*, 9.00 and 900 ng mL⁻¹). The studies included QC samples that were kept at 22 and 90 °C for 4, 8, and 24 h, QC samples that undergone three freeze-and-thaw cycles where the samples were frozen at −20 °C for at least 24 h and thawed at room temperature, and QC samples that were stored at -20 °C for 30 days prior to analyses, respectively. All the above experiments were run in triplicate, and the results were compared with freshly prepared QC samples.

2.3. Results and discussion

2.3.1. Understanding of factors that affect the spectrophotometric detection of triapine

Several key factors that affect the chemical equilibria of triapine in solution could affect the spectrophotometric detection of triapine, which include solution pH, metal ions, and other chelators, as well as buffer composition. In this work, the effects of these factors had been investigated.

2.3.1.1. Effect of pH on UV-visible absorption spectra of triapine

Triapine has two acidic hydrogen atoms in aqueous solution (Figure 2.2A), whose pKₐ values are 4.3 (pKₐ₁) and 10.9 (pKₐ₂), respectively [19]. Previous studies indicated that pKₐ₁ is attributed to the deprotonation of the pyridinium unit, whereas pKₐ₂ is due to
the deprotonation of the hydrazinic N–H group of the thiosemicarbazide moiety [20]. The protonated/deprotonated forms of triapine (H$_2$L$^+$, HL, and L$^-$) are determined by solution pH, which may affect the UV-visible absorption spectra of triapine.

Figure 2.3 showed the mean absorption spectra of triapine as grouped by their similarity under different solution pHs. As seen in Figure 2.3, there were three characteristic spectra which corresponded to three forms of triapine (H$_2$L$^+$, HL, and L$^-$) under three pH ranges (i.e., pH 2-4, 6-10, and 11-12). When pH was low (< pK$_{a1}$), H$_2$L$^+$ was the predominant form which yielded a mean $\lambda_{\text{max}}$ at 393 nm (Figure 2.3A); as pH increased (> pK$_{a1}$ and < pK$_{a2}$), a blue shift of spectrum with a mean $\lambda_{\text{max}}$ at 357 nm was observed (Figure 2.3B), which was associated with the dissociation of the first H$^+$ from H$_2$L$^+$ and the production of the neutral form (HL) of triapine; as pH further increased (> pK$_{a2}$), the dissociation of the second H$^+$, and the formation of L$^-$ form of triapine took place, which resulted in a red shift with a mean $\lambda_{\text{max}}$ at 367 nm (Figure 2.3C). A solution pH of 8.5 was chosen for the subsequent study and LC-UV method development. Under this pH, HL was the predominant form of triapine, which had a $\lambda_{\text{max}}$ at 360 nm (Figure 2.3D).
Figure 2.2, Chemical equilibria of triapine in aqueous solution and triapine-metal ion complexation reaction
Figure 2.3, Effect of pH on UV-visible absorption spectra of triapine. (A) pH 2-4; (B) pH 6-10; (C) pH 11-12; (D) pH 8.5. [triapine] = 100 µM in the universal pH buffer, and temperature = 22 °C,
2.3.1.2. Effect of metal ions on UV-visible absorption spectra of triapine

As discussed in the introduction section, triapine is a metal-ion chelator which inhibits RR activity by coordinating with Fe(III) ion in the enzyme M2 (or p53R2) subunit through its N-N-S tridentate ligand and forming octahedral complex with molar ratio of 2:1 triapine to ferric ion [7]. Triapine can also coordinate with other metal ions and result in both mono- and bis-ligand complexes [20, 21]. Due to its high stability, the bis-ligand complex is a more favorable complex in aqueous solution, which is illustrated in Figure 2.2B.

Figure 2.4 showed the absorption spectra of triapine and triapine-metal ion mixtures. In comparison to triapine alone (Figure 2.4A), a red shift of $\lambda_{\text{max}}$ (360 nm $\rightarrow$ 425 nm) with lower absorptive intensity was observed upon the addition of Fe(III) to triapine solution (Figure 2.4B), which was the result of triapine-Fe(III) complex formation. Our experiments revealed that the formation of triapine-Fe(III) complex was not affected by the solution pH (data not shown). Further experiments also indicated that some metal ions such as Mg(II) and Ca(II) did not affect the absorption spectra of triapine (Figure 2.4 C and D) due to no complex formation (which was confirmed by liquid chromatographic experiments – data not shown); whereas the others metal ions such as Cu(II) and Zn(II) did form complexes with triapine and caused red shifts of $\lambda_{\text{max}}$ (Figure 2.4E and F). Therefore, the preservation of triapine in its un-complex state is the key for successful quantitation of this compound.
Figure 2.4, Effect of metal ions on UV-visible absorption spectra of triapine. (A) triapine alone; (B) mixture of triapine and Fe(III); (C) mixture of triapine and Mg(II); (D) mixture of triapine and Ca(II); (E) mixture of triapine and Cu(II); and (F) mixture of triapine and Zn(II). The above solutions were prepared in 10.0 mM ammonium formate at pH 8.5. Wherever were applicable [triapine] = 100 µM, [metal ion] = 50 µM, and temperature = 22 °C.
2.3.1.3. Effect of EDTA on UV-visible absorption spectra of triapine

To preserve triapine from complexation with metal ions in solution, EDTA as a competing chelator for metal ions in solution was examined (Figure 2.5). The experimental results showed that when EDTA was added into a triapine solution, it did not change the absorption spectrum of triapine (Figure 2.5A). EDTA and metal ion \([i.e., \text{Fe(III), Mg(II), Ca(II), Cu(II), or Zn(II)}]\) complexes showed no absorptivity within the wavelength range in which triapine and its metal complexes peaked out (Figure 2.5B). If triapine was mixed with EDTA prior to metal ions \([i.e., \text{Fe(III), Cu(II), or Zn(II)}]\), no triapine-metal ion complexes were formed due to the masking effect of EDTA on metal ions (Figure 2.5C, D and E); however, if triapine was mixed with metal ions prior to EDTA, some metal ions such as Fe(III) formed complexes with triapine and caused a red shift of \(\lambda_{\text{max}}\) from 360 nm to 425 nm (Figure 2.5F), while other ions such as Cu(II) and Zn(II) did not (Figure 2.5D and E). Once triapine-Fe(III) complex was formed, heating the complex in EDTA solution at 37 °C for 2 h would not release triapine from the complex (Figure 2.5G), and thus a much higher temperature of 90 °C was needed (Figure 2.5H).

In summary, the above experiments reveal that metal ions preferably bind to EDTA if both EDTA and triapine are present; triapine forms much more stable complex with Fe(III) than with the other metal ions studied; and preservation of triapine in its uncomplex state can be achieved by adding EDTA to triapine solution prior to its reaction with metal ions.
Figure 2.5. Effect of EDTA on UV-visible absorption spectra of triapine-metal ion complex. (A) mixture of triapine and EDTA; (B) mixture of EDTA and metal ion \textit{i.e.}, Fe(III), or Mg(II), or, Ca(II), or Cu(II), or Zn(II)]; (C) mixture of triapine, EDTA and Fe(III) [sequence-specific mixing, \textit{i.e.}, mixed triapine and EDTA first, then added Fe(III)]; (D) mixture of triapine, EDTA and Cu(II) (sequence-non-specific mixing); (E) mixture of triapine, EDTA and Zn(II) (sequence-non-specific mixing); (F) mixture of
triapine, Fe(III) and EDTA [sequence-specific mixing, \textit{i.e.}, mixed triapine and Fe(III) first, then added EDTA]; (G) repeated (F), heated at 37 °C for 2 hrs; and (H) repeated (F), heated at 90 °C for 2 hrs. The above solutions were prepared in 10.0 mM ammonium formate at pH 8.5. Wherever were applicable [triapine] = 100 µM, [EDTA] = 2.00 mM, [metal ion] = 50 µM, and temperature = 22 °C unless otherwise specified.
2.3.1.4. Effect of buffer composition on UV-visible absorption spectra of triapine-metal ion complex

Buffer was used to maintain a solution pH at a relatively constant and predetermined value. However, buffer composition might affect the complexation reaction of triapine and metal ions. Hence, it was investigated in this work.

As shown in Figure 2.6, the UV-visible absorption spectra of triapine and Fe(III) mixtures in 10.0 mM ammonium formate at pH 3.0 and 8.5 (Figure 2.6A and B) showed no significant difference with the spectrum of triapine-Fe(III) complex in water (Figure 2.6C), which implied that not only ammonium formate buffer did not affect the complexation reaction of triapine and Fe(III), but also the triapine complexation reaction was not pH-dependent. However, when triapine and Fe(III) were mixed in 10.0 mM ammonium bicarbonate or 10.0 mM ammonium phosphate at pH 8.5, the UV-visible spectra of the mixtures (Figure 2.6D and E) showed no difference with the spectrum of uncomplexed triapine (Figure 2.4A), which were probably due to the precipitation of Fe(III) as Fe(OH)$_3$ and FePO$_4$ in the buffer solutions by the following reactions:

\[
Fe^{3+} (aq) + 3HCO_3^- (aq) \rightarrow Fe(HCO_3)_3 (aq) \rightarrow Fe(OH)_3 (s) + 3CO_2 (g) \quad (1)
\]

\[
Fe^{3+} (aq) + PO_4^{3-} (aq) \rightarrow FePO_4 (s) \quad (2)
\]
Figure 2.6, Effect of buffer composition on UV-visible absorption spectra of triapine-Fe(III) complex. (A) mixture of triapine and Fe(III) in 10.0 mM ammonium formate at pH 3.0; (B) mixture of triapine and Fe(III) in 10.0 mM ammonium formate at pH 8.5; and (C) mixture of triapine and Fe(III) in deionized water; (D) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0 mM ammonium bicarbonate at pH 8.5; (E) mixture of triapine and Fe(III) in 10.0
mM phosphate buffer at pH 8.5; (F) mixture of triapine and Fe(III) in deionized water, then diluted with 10.0 mM ammonium bicarbonate at pH 8.5, heated at 37 °C for 2 hrs; (G) repeated (F), heated at 90 °C for 2 hrs. Wherever were applicable [triapine] = 100 µM, [Fe(III)] = 50 µM, and temperature = 22 °C unless otherwise specified.
Further experiments showed that once triapine-Fe(III) complex was formed, neither the bicarbonate buffer nor the phosphate buffer (data not shown) could release triapine from the complex, no matter it was at 37 °C 2 h (Figure 2.6F) or 90 °C for 2 h (Figure 2.6G).

Unlike Fe(III), other metal ions such as Zn(II) and Cu(II) could form complexes with triapine in both ammonium bicarbonate and ammonium phosphate buffers (data not shown). This was probably due to the presence of ammonia an auxiliary complexing agent in the buffer solutions, which formed Zn(NH$_3$)$_2^{2+}$, Zn(NH$_3$)$_3^{2+}$, Zn(NH$_3$)$_4^{2+}$ and Zn(NH$_3$)$_5^{2+}$, and Cu(NH$_3$)$_2^{2+}$, Cu(NH$_3$)$_3^{2+}$ and Cu(NH$_3$)$_4^{2+}$ complexes in the solutions and made them available in solution for the complexation reaction with triapine.

The above experiments indicated that ammonium bicarbonate and ammonium phosphate could prevent the formation of triapine-Fe(III) complex, but they failed to prevent the formation of triapine-Zn(II) and triapine-Cu(II) complexes. Therefore, the best way to preserve triapine in its uncomplexed state is to use EDTA solution or EDTA with ammonium bicarbonate buffer.

2.3.2. LC-UV assay development and optimization

The understandings of chemical properties of triapine and the IS, and key factors that affect spectrophotometric detection of triapine were applied to the LC-UV method development and optimization.
2.3.2.1. Selection of column

Since the logP values of triapine and the IS are 0.983, and 0.937, respectively, these compounds are rather hydrophobic than hydrophilic; therefore, reverse-phase LC columns were considered for this work. Separation of triapine and the IS was initially tested on Phenomenex Gemini C18 (2.0 mm × 50 mm, 5µm particle size), Waters X-Terra RPC 18 (2.1 mm x 150 mm, 5 µm particle size), Waters YMC ODS-AQ (2.0 mm × 50 mm, 5 µm particle size), Waters X-Bridge AQ (2.0 mm × 50 mm, 5µm particle size), and Waters X-Bridge Phenyl (2.0 mm × 50 mm, 5 µm particle size) columns with a mobile-phase flow rate of 0.200 mL min⁻¹ and a UV detector set at wavelength of 360 nm. Although reasonable retention time and sufficient resolution were obtained on all columns tested, Waters X-Terra RPC 18 column was chosen for the method development because it yielded greater signal response and better peak shape to the analytes.

2.3.2.2. Mobile-phase composition

By the considerations of the retention mechanism of reverse-phase column, detectable wavelengths of deuterium lamp (190-370 nm) used in a UV detector, chemical equilibra of triapine in aqueous solution, and minimum pH for effective EDTA complexation with various metal ions [22], ammonium bicarbonate buffer at pH 8.5 was chosen for the LC-UV method development. In this work, the concentration of ammonium bicarbonate buffer, the choice of organic modifiers, as well as the percent content of the organic modifier were optimized for the separation and detection of triapine and the IS.
Figure 2.7, Effect of organic modifier in mobile phase on LC separation of triapine and the internal standard. (A) a mixture of triapine ($1.00 \times 10^3$ ng mL$^{-1}$) and the IS (200 ng mL$^{-1}$) which was prepared in a mobile phase containing 18% acetonitrile and 82% ammonium bicarbonate (10.0 mM, pH 8.5) (v/v), and separated using the same mobile phase; and (B) a mixture of triapine ($1.00 \times 10^3$ ng mL$^{-1}$) and the IS (200 ng mL$^{-1}$), which was prepared in a mobile phase containing 30% methanol and 70% ammonium bicarbonate (10.0 mM, pH 8.5) (v/v), and separated using the same mobile phase.
Four concentrations of ammonium bicarbonate (5.00, 10.0, 25.0, and 50.0 mM, pH 8.5) were examined. It was found that the concentration of ammonium bicarbonate not only affected the peak heights but also the shapes of triapine and the IS. The higher the concentration of ammonium bicarbonate was, the higher the peaks and the longer peak tailing were (data were not shown). The optimum concentration of ammonium bicarbonate was found to be 10.0 mM, which yielded good peak heights and symmetries to triapine and the IS. Furthermore, two organic modifiers, acetonitrile and methanol, were tested. It was found that acetonitrile was the better one since it resulted in greater separation efficiency and detection sensitivity for triapine and the IS. As shown in Figure 2.7, the chromatographic peaks of triapine and the IS using a mobile phase containing 18% acetonitrile and 82% ammonium bicarbonate (10.0 mM, pH 8.5) (Figure 2.7A) were not only sharper with shorter retention times, but also 25% larger than those using a mobile phase containing 30% methanol and 70% ammonium bicarbonate (10.0 mM, pH 8.5) (Figure 2.7B). Therefore, the former one was chosen as the mobile phase for the LC-UV method.
Table 2.1, Effect of EDTA concentration in the mobile phase on the chromatographic peak area of triapine

<table>
<thead>
<tr>
<th>[Triapine] (ng mL⁻¹)</th>
<th>[EDTA] Added (mM)</th>
<th>Peak Area</th>
<th>Recovery (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 x 10³</td>
<td>0.00</td>
<td>504478</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>544331</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>549881</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>550890</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>544332</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>553917</td>
<td>110</td>
</tr>
</tbody>
</table>

<sup>a</sup> Recovery (%) = {peak area at [EDTA] ≠ 0/peak area at [EDTA] = 0} x 100%
2.3.2.3. Optimization of EDTA concentration

As discussed in the section 2.3.1.3, EDTA is a stronger chelator to metal ions than triapine. If both EDTA and triapine are present in a solution, metal ions prefer to react with EDTA, leading to triapine uncomplexed. The addition of EDTA to a mobile phase could eliminate the interference of metal ions present in reagents, solvents, column, and instrument on the measurement of triapine. In this work, the concentration of EDTA in mobile phase was optimized using six EDTA concentrations (0.00, 1.00, 5.00, 10.0, 25.0 and 50.0 mM), and the results were summarized in Table 2.1. Compared to a mobile phase (18% acetonitrile and 82% 10.0 mM ammonium bicarbonate at pH 8.5) without EDTA, the mobile phase containing 1.00 mM EDTA could increase the peak area of triapine by 8% at triapine concentration of 1.00 µg mL⁻¹. The mobile phase containing higher EDTA concentrations (5.00-50.0 mM) did not further increase the peak area of triapine significantly. Therefore, 5.00 mM EDTA was added to the mobile phase for the LC-UV method.

2.3.3. LC-UV method validation

From the studies of previous sections, it was concluded that the optimized conditions for the determination of triapine by the LC-UV method included the followings: a Waters X-Terra RPC 18 column, a mobile phase containing 18% acetonitrile and 82% ammonium bicarbonate-EDTA buffer (10.0 mM ammonium bicarbonate and 5.00 mM EDTA at pH 8.5) pumped at a flow rate of 0.200 mL/min, and a UV detector set at wavelength of 360 nm. These conditions were then applied to the method validation. The representative chromatograms of triapine in solution at various
concentrations (0.00, 3.00, and 30.0 ng mL$^{-1}$) were given in Figure 2.8. As shown in the figure, a complete baseline resolution of triapine and the IS was achieved. The retention times of triapine and the IS were 4.7 min and 6.4 min, respectively; and the total chromatographic runtime was 8.5 min.
Figure 2.8, Representative liquid chromatograms of triapine and the IS. (A) Blank; (B) at LLOQ of the method, where [triapine] = 3.00 ng mL\(^{-1}\) and [IS] = 100 ng mL\(^{-1}\); and (C) [triapine] = 30.0 ng mL\(^{-1}\) and [IS] = 100 ng mL\(^{-1}\). The LC-UV method was described in Section 2.5.
2.3.3.1. Calibration curve and the lower limit of quantitation

Internal calibration was used for quantitation of triapine, which was done using one zero (i.e., mobile phase) and six non-zero triapine calibrators (3.00, 10.0, 30.0, 100, 300, and $1.00 \times 10^3$ ng mL$^{-1}$) along with the IS in each at fixed concentration (100 ng mL$^{-1}$). In this work, a linear calibration range of 3.00-1.00 $\times 10^3$ ng mL$^{-1}$ was established by plotting the peak-area ratios of triapine to the IS versus the concentrations of triapine. The calibration equation derived from three validation batches using $1/x^2$ weighted linear regression was $Y = 0.022 (\pm 0.001) X - 0.017 (\pm 0.002)$ (where $Y$ is the peak area ratio of triapine to the IS and $X$ is the concentration of triapine) with a coefficient of determination ($r^2$) of 0.999 (± 0.001). The accuracy and precision of each individual calibrator expressed as percent relative error (%RE) and coefficient of variation (%CV) were summarized in Table 2.2, which ranged from -3% to 4%, and 0.1% to 4%, respectively. The lower limit of quantitation (LLOQ) was defined by the lowest calibrator in the calibration curve, which had signal-to-noise (S/N) ratio of 13.5 by the mean peak areas.
Table 2.2, Accuracy and precision of triapine calibrators over three validation batches.

<table>
<thead>
<tr>
<th>Nominal [Triapine] (ng mL⁻¹)</th>
<th>Mean Measured [Triapine] (ng mL⁻¹)</th>
<th>Standard Deviation (ng mL⁻¹)</th>
<th>Precision (%CV)</th>
<th>Accuracy (%RE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.00</td>
<td>3.08</td>
<td>0.09</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10.0</td>
<td>9.69</td>
<td>0.4</td>
<td>4</td>
<td>-3</td>
</tr>
<tr>
<td>30.0</td>
<td>29.8</td>
<td>1</td>
<td>4</td>
<td>-0.6</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>300</td>
<td>306</td>
<td>0.3</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>1.00 x 10³</td>
<td>1.04 x 10³</td>
<td>3</td>
<td>0.3</td>
<td>4</td>
</tr>
</tbody>
</table>

Calibration equations: \( Y = 0.022 \pm 0.001 \times X - 0.017 \pm 0.002 \), \( r^2 = 0.999 \pm 0.001 \)

\%CV = \left( \frac{\text{standard deviation}}{\text{mean measured}} \right) \times 100\%

\%RE = \left( \frac{\text{mean measured} - \text{nominal}}{\text{nominal}} \right) \times 100\%
2.3.3.2. Accuracy and precision

The intra-assay accuracy and precision were determined by five replicate measurements of each QC sample at the concentrations of 3.00, 9.00, 90.0, and 900 ng mL\(^{-1}\) (i.e., LLOQ, LQC, MQC and HQC) within a validation batch. The inter-assay accuracy and precision were determined by five parallel measurements of 5 identical QC samples at the concentrations of 3.00, 9.00, 90.0, and 900 ng mL\(^{-1}\) over five validation batches. As summarized in Table 2.3, the intra-assay accuracy and precision ranged -3\% - 7\% and 1\% - 3\%; and the inter-assay accuracy and precision ranged -3\% - 9\% and 2\% - 4 \%, respectively.

2.3.3.3. Stability

The stability of triapine in solution was evaluated using both LQC (9.00 ng mL\(^{-1}\)) and HQC (900 ng mL\(^{-1}\)). The stability was determined by the percent recovery which was calculated by the mean-peak-area ratio of triapine to the IS from three parallel measurements of three identical QC samples over that of the freshly prepared QC samples. As shown in Table 2.4, the recoveries of triapine under various test conditions such as on bench top (23 °C, up to 24 h), in oven (90 °C, up to 24 h), by three freeze-and-thaw cycles (-20 °C to 23 °C), and by long-term storage (-20 °C for 30 days) were from 90\% to 105\%, which indicated that triapine solution was very stable under the test conditions.
Table 2.3, Intra- and inter-assay precision and accuracy \( \{n = 5, [IS] = 100 \text{ ng mL}^{-1}\} \)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Nominal [Triapine] (ng mL(^{-1}))</th>
<th>Mean Measured [Triapine] (ng mL(^{-1}))</th>
<th>Standard Deviation (ng mL(^{-1}))</th>
<th>Precision (%CV)</th>
<th>Accuracy (%RE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td>3.00 (LLOQ)</td>
<td>2.79</td>
<td>0.03</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>9.00 (LQC)</td>
<td>8.66</td>
<td>0.2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>90.0 (MQC)</td>
<td>93.0</td>
<td>0.9</td>
<td>1</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>900 (HQC)</td>
<td>854</td>
<td>0.3 ( \times 10^2 )</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>3.00 (LLOQ)</td>
<td>2.72</td>
<td>0.1</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>9.00 (LQC)</td>
<td>9.27</td>
<td>0.2</td>
<td>2</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>90.0 (MQC)</td>
<td>92.9</td>
<td>2</td>
<td>2</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>900 (HQC)</td>
<td>843</td>
<td>0.2 ( \times 10^2 )</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 2.4, Stability study of triapine under various conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Temperature (°C)</th>
<th>QCs</th>
<th>Recovery ± SD (%) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Bench-top</td>
<td>22</td>
<td>LQC</td>
<td>97 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HQC</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Oven</td>
<td>90</td>
<td>LQC</td>
<td>103 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HQC</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Freeze-thaw (3 cycles)</td>
<td>-20 to 22</td>
<td>LQC</td>
<td>100 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HQC</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>Long-term (30 days)</td>
<td>-20</td>
<td>LQC</td>
<td>102 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HQC</td>
<td>101 ± 2</td>
</tr>
</tbody>
</table>

The concentrations of triapine in the LQC and HQC were 9.00 and 900 ng mL⁻¹, respectively; and the concentration of the IS was 100 ng mL⁻¹.
2.4. Conclusion

An LC-UV method for the quantitative determination of triapine has been developed and validated through understanding the chemical equilibria and complexation reaction of triapine in aqueous solution. The effects of pH, EDTA, metal ions, and buffer composition on UV-visible absorption spectra of triapine were investigated. In addition, LC separation, as well as UV detection of triapine were optimized. The method developed in this work employed 2-[(3-methoxy-2-pyridinyl)methylene] hydrazinecarbothioamide as internal standard, Waters Xterra RP18 as separation column, 18% acetonitrile and 82% ammonium bicarbonate-EDTA buffer [10.0 mM (NH₄)HCO₃ and 5.00 mM EDTA (v/v) at pH 8.5] as mobile phase, and UV wavelength of 360 nm for detection. This method had a linear calibration range of 3.00-1.00 x 10³ ng mL⁻¹ for triapine, an accuracy (%RE) of ≤ ±9%, and a precision (%CV) of ≤ 4%.

2.5. References


CHAPTER III

QUANTITATIVE ANALYSIS OF TRIAPINE, AN INHIBITOR OF RIBONUCLEOTIDE REDUCTASE, IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

3.1. Introduction

As discussed in previous chapter, triapine is a potent inhibitor of ribonucleotide reductase. Compared with hydroxyurea (HU), which is the only RR inhibitor currently used in chemotherapy in vitro, triapine was shown to have significantly higher potency and a broader-spectrum antitumor activity. Studies show that triapine is 1000-fold more potent than HU [1]; can significantly decrease RR activity in a variety of cancer cell lines including leukemia, non-small-cell lung cancer, renal cancer and melanoma; and enhances radiation-mediated cytotoxicity in cervical and colon cancers [1-6]. These promising pre-clinical data have prompted the initiation of a number of clinical trials. So far, triapine has been studied in 35 clinical trials in the USA at various stages of recruitment (www.clinicaltrials.gov), including those in treatment of both hematologic and solid malignancies. In particular, there are four trials investigating the radio
sensitizing potential of triapine being published by Kunos et al. [7-10].

Despite of therapeutic importance of triapine, there is no publication available on analytical method development and validation for this important compound. Although liquid chromatographic assays were mentioned for the measurement of triapine in clinical studies [11-13], these assays suffered from low sensitivity, poor selectivity, and irreproducibility when being repeated. The difficulty in developing a quantitative assay for triapine is due to the chemical nature of triapine. As a chelator and a weak acid, triapine has multiple equilibria in aqueous solution, which are affected by various factors. In our previous study, the effects of pH, buffer composition, and EDTA, as well as other metal ions on the spectral absorptivity and chromatographic retention of triapine have been investigated [14]. Adding ammonium bicarbonate and EDTA to both mobile phase and the solvent of dissolving trapine could solve the metal ion interference problem.

Although an LC-UV based assay to quantify triapine has been developed in our lab [14], it often suffered from low sensitivity. In clinical samples such as blood and tissue, the quantity of triapine can be very low, requiring the highly sensitive assays for its quantification. Moreover, clinical samples are highly complex and even the well-developed LC condition may not be able to completely separate this analyte molecule from some of other molecules present in clinical samples. In other words, the molecules that have the retention time similar to that of triapine may interfere UV measurements of triapine.

In this work, a sensitive, accurate, and reliable HPLC-MS/MS assay was developed for the quantitative analysis of triapine. The assay used 2-[(3-fluoro-2-pyridinyl)methylene]-hydrazinecarbothioamide (NSC# 266749) (Figure 3.1B) as internal
standard (IS). Tripaine and the IS were separated on a Waters Xbridge Shield RP 18 column (3.5 µm; 2.1 × 50 mm) using 25.0% methanol and 75.0% ammonium bicarbonate buffer (10 mM, pH 8.5) (v/v) as mobile phase. This is the first validated LC-MS/MS method for the quantification of triapine in human plasma and we have successfully applied it to the measurement of triapine in patients’ samples.
Figure 3.1, The chemical structures of (A) triapine and (B) NSC 266749, the internal standard of LC-MS/MS method.
3.2. Experimental

3.2.1. Chemicals and solutions

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone or 3-AP) was kindly provided by Vion Pharmaceuticals (New Haven, CT, USA) and used as chemical standard of the analyte. 2-[3-fluoro-2-pyridinyl)methylene]-hydrazinecarbothioamide (CAS No. 31181-41-6 or NSC# 266749) was obtained from the Developmental Therapeutics Program of the National Cancer Institute at the National Institutes of Health (Bethesda, MD, USA) and used as the internal standard (IS) for triapine in the LC-MS/MS method. HPLC-grade methanol, ethylenediaminetetraacetic acid disodium salt (EDTA), and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium bicarbonate was from EMD Chemicals (Darmstadt, Germany). Deionized water was obtained from a Barnstead Model 7148 Nanopure® ultrapure water system of the Thermo Scientific (Asheville, NC, USA). 6 different lots of human plasma with sodium EDTA and 6 different lots of human serums were purchased from Innovative Research (Novi, MI, USA).

The working solvent was prepared by mixing 25.0% acetonitrile and 75.0% ammonium bicarbonate-EDTA buffer (i.e., 10 mM (NH₄)HCO₃ and 1 mM EDTA (v/v) at pH 8.5). The standard stock solutions of triapine and IS were prepared in methanol at a concentration of 1 mg/mL, respectively and kept at −20 °C before use. Working standard solution of triapine (1.00 µg/mL) and working solution of the IS (1.00 µg/mL) were freshly prepared by serial dilution of each stock solution with a working solvent. The mobile phase for liquid chromatographic separation was prepared by mixing methanol and 10.0 mM ammonium bicarbonate (pH 8.5) at a ratio of 25:75 (v/v).
3.2.2. Instrumentation

The instrumentation system used consisted of a Shimadzu SIL-20AC autosampler (Shimadzu, Columbia, MD, USA), a Shimadzu LC-20AD HPLC unit with Waters Xbridge Shield RP 18 column (3.5 µm; 2.1 × 50 mm), and an AB Sciex API 3200 turbo-ion-spray® triple quadrupole tandem mass spectrometer (AB Sciex, Foster City, CA, USA). The system was controlled by AB Sciex Analyst® (version 1.5.1) software. The API 3200 tandem mass spectrometer was operated under the positive turbo-ion-spray ionization mode.

It was tuned by a mixture of 500 ng/ml triapine and 500 ng/mL IS in 50% methanol and 50% ammonium bicarbonate (10 mM, pH 8.5) for both the compound dependent and the source-dependent parameters. MRM data were acquired with the following mass transitions and optimized instrument settings: m/z 196 > 121 for triapine and m/z 199 > 124 for the IS; curtain gas (CUR) at 40, collision assisted dissociation gas (CAD) at 8; ionization voltage (IS) at 4500 V; source temperature (TEM) at 700; sheath gas (GS1) at 50; desolvation gas (GS2) at 40; desolvation potential (DP) at 40; entrance potential (EP) at 4; collision energy (CE) at 23; collision cell exit potential (CXP) at 2; and resolution at unit.

Analytical separation of the analytes was accomplished on a Waters Xbridge Shield RP 18 column by isocratic elution with the mobile phase at a flow rate of 0.30 ml/min. Prior to initial sample analysis, the column was equilibrated with the mobile phase at the above flow rate for at least 30 min. During each run, 10 µL of reconstituted sample was injected into the system by the autosampler set at 4 °C. The two-position
switch valve on the API 3200 tandem mass spectrometer was programmed to the switch to the waste for the first 1.2 min and then switch to the mass spectrometer. Quantitation of the analytes was carried out with MRM mode of the tandem mass spectrometer. The total instrument run time for each sample analysis was 4 min.

3.2.3. Preparation of standard solutions, plasma calibrators and controls, and patients’ plasma samples

Triapine standard solutions (5.00, 10.0, 15.0, 20.0, 50.0, 100, 150, 200, 500, 800, and 1000 ng/mL) and IS standard solution (100 ng/mL) were prepared by serial dilution of triapine working solution (1.00 µg/mL) and IS working solution (1.00 µg/mL) with working solvent, respectively.

Triapine plasma calibrators (0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/mL) were prepared individually by mixing 200 µL of pooled blank plasma, 10.0 µL of triapine standard solution (at 20 times of the calibrator’s concentration), and 10.0 µL of IS standard solution at 100 ng/mL.

Triapine plasma controls (0.75, 7.5, and 40.0 ng/mL) prepared by mixing 200 µL of pooled blank plasma, 10.0 µL of triapine standard solution (at 20 times of the calibrator’s concentration), and 10.0 µL of IS standard solution at 100 ng/mL.

Patients’ samples were prepared by mixing 200 µL of blood sample, 10.0 µL of working solvent and 10 µL of IS standard solution at 100 ng/mL.

3.2.4. Sample extraction

Plasma calibrators, controls and patients’ samples prepared as described in
Section 2.2.3 were extracted using the following protocol: each sample was deproteinized with ACN at a ratio of 4 to 1 by vortex mixing for 30 s; followed by centrifugation at 15000 x g for 10 min, the supernatant was pipetted into a 1.5-mL microcentrifuge tube and dried in a TurboVap® LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA) at 30 °C under nitrogen gas. Finally, the residue was then reconstituted in 110 µL of working solvent for LC-MS/MS analysis.

3.2.5. Stability

The stability of triapine solution at two concentrations (1.00 mg/mL and 1.00 µg/mL) were carried out by leaving the test controls at bench top (23 °C) for 6 and 24 h. The stability of triapine in human plasma before and after sample preparation, and through freeze-and-thaw cycles were investigated at low and high QC concentrations (0.75 and 40.0 ng/ml). These studies included QC samples kept on bench top at 23 °C for 6 and 24 h before sample preparation and analyses, QC samples kept in autosampler at 4 °C for 6 and 24 h after sample preparation and before LC–MS/MS analyses, QC samples undergone three freeze-and-thaw cycles where the samples were frozen at −20 °C for at least 24 h and thawed at room temperature unassisted 3 times and QC samples stored at −20 °C for 30 days prior to analysis for long term stability study. All experiments were run in triplicate and the results were compared with freshly prepared triapine solutions and plasma controls.

Similarly, the stability of IS solution (100 ng/ml) at bench top (23 °C), and the stability of IS in human plasma (5.00 ng/mL) at bench top (23 °C) and in autosampler (4 °C) were investigated. All experiments were run in triplicate and the results were
compared with controls, which were freshly prepared IS in solution and in human plasma.

3.3.2. Method application

The feasibility of the method developed was tested by measuring triapine concentration in advanced cervical cancer patients. Dr. Charles A. Kunos at Case Comprehensive Cancer Center, Case Western Reserve University provided the patients samples to be analyzed. In this study, all 6 patients enrolled have been histologically confirmed primary or recurrent gynecologic malignancies not amenable to curative surgery. Triapine was supplied by Vion Pharmaceuticals to NCI-CTEP in 50-mg viscous liquid vials and was diluted in 0.9% sodium chloride to a final concentration of 0.01 to 2 mg/mL. Patients were given triapine as a 2-h *i.v* continuous infusion (25 mg/m^2^) three times weekly. Heparinized intravenous blood samples on day 1 and day 10 before and at 2, 4, 6, and 24 h after start of 2-h infusion were drawn to determine triapine concentrations. Plasma was centrifuged at 3,000 rpm (15 min) in a refrigerated centrifuge and then stored (−80°C).

3.3. Results and Discussion

3.3.1. Method development

3.3.1.1. Mass spectrometric detection

In this work, the optimization of triapine and 2-[(3-fluoro-2-pyridinyl) methylene]-hydrazinecarbothioamide (IS) responses was done using the “auto-tune” function of AB Sciex Analyst software (version 1.5.1). Since triapine and the IS were
easier to form protonated species than deprotonated species by electrospray ionization, the positive electrospray-ionization mode was used for the triapine identification and quantification. As shown in Figure 3.2A and C (the m/z scan by the first quadrupole), tirapine and the IS produced predominant molecular ions at m/z 196 for [triapine+H]^+ and m/z 199 for [IS+H]^+, respectively. These molecular ions produced were further dissociated into fragment ions by collision with nitrogen gas in the second quadrupole (Figure 3.2B and D). The predominant fragment ions of [triapine+H]^+ and [IS+H]^+ were m/z 121 and m/z 124, respectively. Therefore, the mass transition pairs m/z 196 > 121 for triapine and m/z 199 > 124 for the IS were chosen for quantification in the multiple-reaction-monitoring (MRM) mode.
Figure 3.2, The mass spectra of triapine and the internal standard. The experimental conditions were the same as those described in Section 3.2.2.
3.3.1.2. Triapine Solution Preparation

When we first started the method development, the irreproducibility issue was found. We prepared different batches of triapine at the concentration of 1.0 ng/mL in 25% MeOH and 75% water (v/v), but it was observed that the triapine ion signals varied dramatically from batch to batch. After trouble shooting, we found that the irreproducibility problem was due to the deionized water system that we used. If the samples were prepared by using bottle water (ultrapure, HPLC Grade), the results were highly reproducible. As discussed in the introduction part, triapine is a metal chelator, which can coordinate with metal ions. Although our water system met the water purification standard, trace amounts of metal ions remained in the deionized water could still interfere with the detection of triapine. Considering the difficulty of controlling the quality of water sources, and the possibility of introducing metal ions from other sources, we attempted to develop a simple, but reliable method to minimize the metal ion interference.

In our previous study [14], it was found that metal ions preferentially bonded with EDTA, leaving triapine unreacted, and that metal ions could be precipitated in the presence of high concentration of ammonium bicarbonate. It was expected that adding ammonium bicarbonate and EDTA to both mobile phase and the solvent of dissolving trapine might solve the metal ion interference problem. In this work, we first attempted to use this strategy to solve the problem, but failed due to the fact that the presence of EDTA in mobile phase could suppress the triapine ion signal by more than 20 times. We then only added EDTA to the solvent of dissolving trapine and found that the triapine ion signal was not suppressed. More importantly, the ion signal of triapine became highly
reproducible when we prepared different batches by using the same water purification system. Therefore, in the remaining of this study, only ammonium bicarbonate (10 mM, pH 8.5) was added to mobile phase A, while an ammonium bicarbonate-EDTA buffer \(i.e.,\) 10 mM \((\text{NH}_4\text{HCO}_3\) and 1 mM EDTA at pH 8.5) was added to the solvent of dissolving triapine.

3.3.1.3. Liquid chromatographic separation

In this work, several analytical columns were tested for development of this quantification method. Separation of triapine and the IS was tested on Waters X-Terra RP 18 (2.1 mm x 150 mm, 5 µm particle size), Waters X-Bridge RP 18 (2.0 mm × 50 mm, 5 µm particle size), Waters X-Bridge AQ (2.0 mm × 50 mm, 5µm particle size), and Waters X-Bridge Phenyl (2.0 mm × 50 mm, 5µm particle size) columns with a mobile-phase flow rate of 0.300 mL/min. Although reasonable retention time and sufficient resolution were observed on all columns tested, Waters X-Bridge RP 18 column was chosen for the method development because it led to a greater signal response and better peak shape.

Furthermore, two organic modifiers, acetonitrile and methanol, were tested. It was found that methanol was the better one since it resulted in a greater separation efficiency and detection sensitivity for triapine and the IS. Finally, the optimal separation of triapine and the IS was achieved on a Waters X-Bridge RP 18 (2.0 mm × 50 mm, 5 µm particle size) column at 1.6 and 1.9 min by a mobile phase containing 25.0% methanol and 75.0% ammonium bicarbonate buffer (10 mM, pH 8.5) (v/v) (Figure 3.3).
3.3.1.4. Biological sample selection and extraction

Serum and plasma are two common biological sources. In this study, both serum and plasma were tested in order to determine which one would be the better source when we analyzed triapine in clinical studies. Triapine plasma and serum samples were prepared by spiking triapine in six lots of blank human plasma and six lots of blank human serum. Three extraction methods including protein precipitation by ACN and methanol, and liquid-liquid extraction by acetyl acetate were tested to extract triapine from serum and plasma. This testing study showed that the ion signal intensity of triapine extracted from serum was significantly lower than the signal of triapine extracted from plasma. The absolute recovery of triapine by 3 extraction methods and the matrix effect in plasma and serum were summarized in Figure 3.4. The absolute recovery was calculated by comparing the mean peak area of the triapine samples prepared by spiking it to matrix before and after extraction. The sample matrix effect was evaluated with the absolute matrix factor, which is the mean peak area of triapine in the extracted matrix over the pure solution. As shown in Figure 3.4A, the recoveries of triapine in serum were lower than that in plasma. More importantly, as shown in Figure 3.4B, the matrix factors ranged from 0.75 – 0.80 and 0.12 – 0.20 in plasma and serum, respectively, indicating that triapine in serum suffered from a serious matrix effect. Therefore, after extraction, triapine extracted from plasma had a higher intensity that that extracted from serum.
Figure 3.3, Representative MRM chromatograms of human plasma: (A) double blank; (B) 0.25 ng/mL triapine in plasma with 5.0 ng/mL IS; and (C) 2.5 ng/mL triapine in plasma with 5.0 ng/mL IS.
Figure 3.4, The comparison of recovery and matrix effect of triapine in human plasma and serum by using different extraction methods. Each column represents the mean ±SD in six lots.
Furthermore, as anticoagulants, EDTA is added to whole blood to prepare plasma, and as we discussed previously, EDTA can effectively inhibit the unwanted chelation reaction involving triapine. Therefore, this study suggested that plasma was the biological source of choice when quantifying triapine for clinical trials.

In addition, three extraction methods were compared in this study. Based on this comparison study, protein precipitation by 4 times of ACN was selected for the remaining of this work because it is simple and has a highest recovery.

3.3.2. Method validation
3.3.2.1. Sensitivity and lower limit of quantification (LLOQ)

The selectivity of the quantification method developed was determined by comparing the chromatograms of six lots of blank plasma samples with those of the spiked plasma calibrator at the LLOQ. In this work, there were no endogenous interference observed at the retention times and mass transitions of triapine and the IS (Figure 3.3A).

The LLOQ of the method was defined by the lowest calibrator (0.25 ng/mL) of the calibration curve (Fig. 3B). The precision and accuracy of each lot of plasma at LLOQ were calculated based on five separate samples with one injection per sample. The data are summarized in Table 3.1. The accuracy and the precision of the method at the LLOQ were $\leq \pm 10\%$ and $\leq 7\%$.
Table 3.1. Accuracy and precision of triapine at LLOQ in six individual lots of human plasma matrices

<table>
<thead>
<tr>
<th>Plasma matrix</th>
<th>Nominal [Triapine] (ng/mL)</th>
<th>Mean measured [Triapine] (ng/mL)</th>
<th>SD(^a) (ng/mL)</th>
<th>Precision (%CV)(^b)</th>
<th>Accuracy (%RE)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot 1</td>
<td>0.250</td>
<td>0.225</td>
<td>0.004</td>
<td>2</td>
<td>-10</td>
</tr>
<tr>
<td>Lot 2</td>
<td>0.250</td>
<td>0.26</td>
<td>0.02</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Lot 3</td>
<td>0.250</td>
<td>0.26</td>
<td>0.01</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Lot 4</td>
<td>0.250</td>
<td>0.248</td>
<td>0.007</td>
<td>4</td>
<td>-1</td>
</tr>
<tr>
<td>Lot 5</td>
<td>0.250</td>
<td>0.261</td>
<td>0.007</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Lot 6</td>
<td>0.250</td>
<td>0.250</td>
<td>0.008</td>
<td>3</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

\(^a\) SD = standard deviation  
\(^b\) %CV = (SD/mean) x 100%  
\(^c\) %RE = [(measured – nominal)/nominal] x 100%
3.3.2.2. Matrix effect and recovery

The matrix effect and extraction efficiency were evaluated at three levels: 0.750, 7.50 and 40.0 ng/mL. Matrix factor was calculated by comparing the mean-peak-area ratios of triapine to the IS in the QC samples prepared by spiking the analytes after plasma deproteinization to those prepared in the mobile phase. Recovery was calculated by comparing the mean-peak-area ratios of triapine to the IS of corresponding QC samples prepared by spiking the analytes to plasma matrix before and after plasma deproteinization. As shown in Table 3.2, the absolute matrix effect at three concentrations ranged from 0.73 to 0.79, and the IS normalized matrix effect ranged from 1.03 to 1.06. Hence, the matrix suppression or enhancement of the analytical signals by plasma was no significant and could be neglected after normalization with the IS. The absolute recoveries of triapine were consistent between 89% and 93%, while the IS normalized recoveries were between 101% and 104%, indicating that deproteinization by 4 volumes of acetonitrile was sufficient to recover triapine from plasma.

3.3.2.3. Linearity

Triapine calibration curves were established using double blank (blank plasma sample with neither triapine or IS), single blank (blank plasma with IS only), and eight nonblank calibration standards at the concentrations of 0.25, 0.50, 1.0, 2.5, 5.0, 10, 25 and 50 ng/mL. The IS concentration in zero blank and the calibrations standards was 5 ng/mL. The peak area ratio of triapine to IS (y) vs triapine concentration (x) was plotted using 1/x as a weighting factor. The linear regression equation obtained in five different days was \( Y = 0.373 (\pm 0.012) x - 0.00107 (\pm 0.00034) \). The linearity was excellent over
the range of 0.25 to 50 ng/mL with the correlation coefficients above 0.999 for all
calibration curves built on different days. The accuracies and precisions of all calibrators
were summarized in Table 3.3, where accuracy ranged -3–6% and precision was 4–8%,
respectively.

3.3.2.4. Accuracy, precision and dilution integrity

Inter-assay precision and accuracy were assessed by five parallel injections fromive identical QC samples at each concentration. Intra-assay precision and accuracy were
assessed by five replica measurements of each QC samples. As shown in Table 3.4, the
intra- and inter assay accuracy and precision were within ±10%, indicating that this
method is accurate, precise and reproducible. Since some samples containing triapine
were found above the highest concentration of calibration curve, we also investigated the
accuracy and precision after 10-fold dilution of the QC at the concentration of 400
ng/mL. The results showed that the intra- and inter-accuracy of the diluted sample were
1% and 7%. The intra- and inter-precision were 4 and 7%. These results indicated that
diluting the samples that had concentrations above the upper limit of the calibration curve
would not lead to significant errors in the measurement of the triapine concentrations.
Table 3.2. Matrix factor and recovery of triapine in pooled human plasma (n = 5)

<table>
<thead>
<tr>
<th>[Triapine] (ng/mL)</th>
<th>MF\textsubscript{triapine} ± SD</th>
<th>MF\textsubscript{IS} ± SD</th>
<th>IS Normalized MF ± SD</th>
<th>Recovery\textsubscript{triapine} ± SD (%)</th>
<th>Recovery\textsubscript{IS} ± SD (%)</th>
<th>IS Normalized Recovery ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>0.74 ± 0.03</td>
<td>0.72 ± 0.02</td>
<td>1.03 ± 0.05</td>
<td>89 ± 5</td>
<td>88 ± 5</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>7.50</td>
<td>0.73 ± 0.01</td>
<td>0.69 ± 0.02</td>
<td>1.06 ± 0.03</td>
<td>91 ± 2</td>
<td>88 ± 3</td>
<td>103 ± 4</td>
</tr>
<tr>
<td>40.0</td>
<td>0.79 ± 0.02</td>
<td>0.71 ± 0.02</td>
<td>1.04 ± 0.03</td>
<td>93 ± 3</td>
<td>89 ± 2</td>
<td>104 ± 4</td>
</tr>
</tbody>
</table>

$\text{MF}_{\text{triapine}} = \frac{\text{mean peak area of triapine in extracted plasma matrix}}{\text{mean peak area of triapine in mobile phase}}$; $\text{MF}_{\text{IS}} = \frac{\text{mean peak area of IS in extracted plasma}}{\text{mean peak area of IS in mobile phase}}$; and IS Normalized MF = $\text{MF}_{\text{triapine}} / \text{MF}_{\text{IS}}$.

$\text{Recovery}_{\text{triapine}} = \frac{\text{mean peak area of triapine in plasma matrix}}{\text{mean peak area of triapine in extracted plasma matrix}} \times 100\%$; $\text{Recovery}_{\text{IS}} = \frac{\text{mean peak area of IS in plasma matrix}}{\text{mean peak area of IS in extracted plasma matrix}} \times 100\%$; and IS Normalized Recovery = $(\text{Recovery}_{\text{triapine}} / \text{Recovery}_{\text{IS}}) \times 100\%$.
Table 3.3, Accuracy and precision of triapine plasma calibrators over six validation batches.

<table>
<thead>
<tr>
<th>Nominal [Triapine] (ng/mL)</th>
<th>Mean Measured [Triapine] (ng/mL)</th>
<th>SD (ng/mL)</th>
<th>Precision (%CV)</th>
<th>Accuracy (%RE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.25</td>
<td>0.01</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>0.03</td>
<td>6</td>
<td>0.1</td>
</tr>
<tr>
<td>1.00</td>
<td>0.98</td>
<td>0.08</td>
<td>8</td>
<td>-2</td>
</tr>
<tr>
<td>2.50</td>
<td>2.5</td>
<td>0.1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5.00</td>
<td>4.9</td>
<td>0.4</td>
<td>8</td>
<td>-2</td>
</tr>
<tr>
<td>10.0</td>
<td>9.8</td>
<td>0.5</td>
<td>5</td>
<td>-2</td>
</tr>
<tr>
<td>25.0</td>
<td>24</td>
<td>2</td>
<td>8</td>
<td>-4</td>
</tr>
<tr>
<td>50.0</td>
<td>53</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 3.4, Intra- and inter-run accuracy and precision of triapine in pooled human plasma (n = 5)

<table>
<thead>
<tr>
<th>Nominal [Triapine] (ng/mL)</th>
<th>Measured [Triapine] (ng/mL)</th>
<th>SD (ng/mL)</th>
<th>Precision (%CV)</th>
<th>Accuracy (%RE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>0.74</td>
<td>0.02</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>7.50</td>
<td>8.1</td>
<td>0.1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>40.0</td>
<td>40.</td>
<td>2</td>
<td>5</td>
<td>-1</td>
</tr>
<tr>
<td>400.</td>
<td>406 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Inter-Run <sup>b</sup>

<table>
<thead>
<tr>
<th>Nominal [Triapine] (ng/mL)</th>
<th>Measured [Triapine] (ng/mL)</th>
<th>SD (ng/mL)</th>
<th>%CV</th>
<th>%RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>0.72</td>
<td>0.01</td>
<td>1</td>
<td>-4</td>
</tr>
<tr>
<td>7.50</td>
<td>7.0</td>
<td>0.3</td>
<td>4</td>
<td>-7</td>
</tr>
<tr>
<td>40.0</td>
<td>38.2</td>
<td>0.9</td>
<td>2</td>
<td>-4</td>
</tr>
<tr>
<td>400.</td>
<td>429 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each datum point was calculated by five replicate measurements of each QC sample within a validation batch.

<sup>b</sup> Each datum point was calculated by five parallel measurements of five identical QCs at each concentration over five validation batches.

<sup>c</sup> The dilution QC was measured by a 10-fold dilution.
Table 3.5, Stability studies of triapine and the IS under various conditions (n = 5)

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>Temperature (°C)</th>
<th>Nominal [triapine] (ng/mL)</th>
<th>Recovery ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>Stock solution</td>
<td>23</td>
<td>1.00 X 10^6</td>
<td>101 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00 X 10^3</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>0.75</td>
<td>88 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.0</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Autosampler</td>
<td>4</td>
<td>0.75</td>
<td>96 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.0</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>3 Freeze-thaw</td>
<td>-20 to 23</td>
<td>0.75</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>cycles</td>
<td></td>
<td>40.0</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>Long-term (30</td>
<td></td>
<td>0.75</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>days)</td>
<td></td>
<td>40.0</td>
<td>91 ± 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>Temperature (°C)</th>
<th>Nominal [IS] (ng/mL)</th>
<th>Recovery ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>Stock solution</td>
<td>23</td>
<td>100</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>5.00</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>Autosampler</td>
<td>4</td>
<td>5.00</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>3 Freeze-thaw</td>
<td>-20 to 23</td>
<td>5.00</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>cycles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-term (30</td>
<td></td>
<td>5.00</td>
<td>88 ± 7</td>
</tr>
<tr>
<td>days)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.2.5. Stability

The results of the stability study are summarized in Table 3.5. At room temperature, tiapine and the IS stock solutions were found to be stable at least 24 h. The bench-top studies shown that triapine and IS in plasma before deproteinization were stable at room temperature up to 6 h. The post-extraction stability study of tiapine and the IS indicated that they were stable in the reconstitution solvent for at least 24 h in the autosampler at 4°C.

After three freeze–thaw cycles, the recovery of triapine was 96% at LQC and 98% at HQC levels and the recovery of the IS was 92%. Moreover, there was no significant loss of triapine and the IS observed in the long-term storage stability study.

3.3.3. Application

The feasibility of using this method in clinical studies was tested by the measurement of triapine in advanced cervical cancer patients’ samples, which were provided by Dr. Charles A. Kunos at Case Comprehensive Cancer Center. In this work, the samples collected together with ten calibrators (i.e., one single-blank, one double-blank and eight nonzero) and a set of QCs at low-, mid- and high-concentrations (i.e., 0.750, 7.50 and 40.0 ng/mL) were extracted after adding the IS solution by following the procedure described in Section 3.2.6, and analyzed by the validated method. For the samples having the concentration higher than the upper limit of calibration curve (i.e., 50 ng/mL), they were analyzed again after 1:10 dilution using the pooled blank human plasma together with the dilution QC at the concentration of 400 ng/mL. Figure 3.5 shows the triapine concentration–time profile in patient blood after start of 2-hour
triapine infusion.

Compared with the results provided by Dr. Kunos’ group, which were obtained using an LC-UV method [9], our LC-MS method generated a similar triapine concentration–time profile. Moreover, Kunos’ group found that the concentration of tirapine in the samples collected at 24 h after start of infusion on day 1 and day 10, and before start of infusion on day 10 were below the LLOQ of the LC-UV method. In other words, the LC-UV could not detect triapine in the samples. In contrast, our study showed that the average concentrations of triapine in the samples were actually above the LLOQ of our LC-MS method. Clearly, this study demonstrated that the LC-MS developed by us is much more sensitive than the currently used LC-UV method.

3.4. Conclusion

This chapter describes the development and validation of a LC–MS/MS method for the quantitation of triapine in plasma. The method used a simple deproteinization method for sample preparation, and a RP column for separation. EDTA was added in the samples to inhibit the unwanted complexation reaction with metal ions. It has a linear calibration range of 0.250–50.0 ng/mL and stability needed for routine analysis. The method has been successfully applied to the measurement of triapine in the blood samples of advanced cervical cancer patients. The method developed provides a more sensitive and robust tool for quantification of triapine for future clinical studies.
Figure 3.5, Mean triapine concentration – time profile in 6 patients on day 1 and day 10 after start of 2-h infusion at the dose of 50 mg/m².
3.5. References


4.1. Introduction

Fludarabine (9-β-D-arabino-furanosyl-2-fluoradenine monophosphate or F-ara-AMP) is an antimetabolic agent, which is currently used in the treatment of hematological malignancies such as CLL, AML, and non-Hodgkins lymphomas [1-3]. After administering to blood in the form of monophosphate, fludarabine is rapidly dephosphorylated and transported to cells, where fludarabine is successively phosphorylated to the mono, di, and triphosphate (F-ara-AMP, F-ara-ADP, and F-ara-ATP), respectively. F-ara-ATP is the main intracellular metabolite known to have the pharmacological activity and can ultimately lead to cellular apoptosis in both actively dividing and resting cells [4-6]. Once F-ara-ATP was incorporated into the DNA strand, the DNA polymerases are inhibited or forced to pause at that specific site in the DNA strand [7].
It has been found that the patient’s response to the fludarabine treatment varied greatly from one patient to another patient. Since incorporation of fludarabine into DNA is essential to cellular apoptosis, quantitation of its incorporation into cellular DNA can lead to a better understanding of its pharmacological effect, which may in turn provide useful information for adjustment of treatment to improve clinical outcome. In fact, there were lines of evidence that suggested that the incorporation of fludarabine into DNA was linearly correlated with inhibition of DNA synthesis [8].

There have been two methods reported for analysis of incorporation of fludarabine into DNA in biological matrices [4, 9]. In the method developed by Plunkett et al., $^3$H-labeled fludarabine monophosphate was used and the incorporated fludarabine was measured by the radioactivity in the corresponding HPLC fraction [4]; whereas in the method developed by Kemena et al., the incorporated fludarabine was first derivatized with chloroacetaldehyde to form a fluorescent tag which was then detected by HPLC with fluorescent detection [9]. However, both methods involved laborious sample preparation and neither of them were validated for quantification. Moreover, the use of radio-active isotope labeled or derivatized fludarabine is not practicable in a clinical setting. In this paper, a novel LC-MS/MS based method to quantify incorporation of fludarabine into DNA has been developed. Figure 4.1 displays the schematic representation of this method. Briefly, drug treated cellular DNA was first extracted from a biological sample such as cultured cells. The extracted DNA is then enzymatically hydrolyzed into mono nucleosides and F-ara-A as shown in Figure 4.1. Finally, after purification and concentration of hydrolyzed products, they are separated by LC, followed by quantifying them with a MRM method. The validated method has been
applied to the study of the incorporation in DNA of HL60 and CLL-MEC1 cell lines with various fludarabine dosages. The results demonstrated that this method could readily quantify the incorporation of fludarabine into DNA and determines variation in the incorporation among different cell lines. Moreover this method has already successfully been applied to measurements of the incorporation of fludarabine into cellular DNA extracted from the blood of patients who enrolled in Case 2Y10 clinical trial.
Figure 4.1. Schematic representation of the procedure of the LC-MS/MS method and DNA hydrolysis by the enzymatic cocktail used in this study.
4.2. Experimental

4.2.1. Materials and solutions

- Formic acid, acetonitrile, 2-[Bisamino]-2,1,3-propanediol (BisTris), deoxyribonuclease I (DNase I), nuclease P1 (NP1), bovine alkaline phosphatase (ALP), 2’-Deoxyadenosine (dA), 2’-Deoxyadenosine 5’-monophosphate (dAMP), 2’-Deoxyguanosine (dG), Thymidine (T), 2’-Deoxycytidine (dC), 2-Chloro-2’deoxyadenosine (2CdA) and Tris EDTA (TE) buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA).
- Sodium chloride (NaCl), zinc chlorides (ZnCl2), phosphate buffered saline (PBS), water saturated phenol, optima gradient methanol and chloroform were purchased from Fisher Scientific (Fair Lawn, NJ, USA).
- The 10% sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA, USA).
- RPMI-1640 medium with L-glutamine was purchased from Mediatech (Manassas, VA, USA).
- RNase A and protease K were ordered from Invitrogen (Carlsbad, CA, USA).
- Snake venom phosphodiesterase I (PDE I) was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA).
- Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, USA).
- Fludarabine (F-ara-AMP) was from Ochem (Des Plaines, IL, USA).
- RiboShredder™ RNase Blend was from Epicentre Biotechnologies (Madison, WI, USA).
- Deionized water was prepared by a Barnstead Model 7148 Nanopure® ultrapure water system of the Thermo Scientific (Asheville, NC, USA).

4.2.2. Cell culture and treatment

- Human promyelocytic leukemia cells (HL60) were obtained from American Type Culture Collection (Rockville, MD, USA).
- Chronic lymphocytic leukemia (CLL) MEC1
cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator.

F-ara-AMP solutions were freshly prepared for each experiment by dissolving F-ara-AMP powder in PBS (1x, pH 7.4). For each $5 \times 10^6$ cells, the dosages of F-ara-AMP added into the culture media were 0.00, 1.00, or 10.0 µM, respectively. Cells were treated for 2 or 24 h at 37 °C in a humidified 5% CO₂ incubator.

After treatment, the cells were removed from the medium by centrifugation 1500 x g at 4 °C for 5 min, and washed with 5 mL PBS. After centrifugation (1500 x g at 4 °C for 5 min), the cell pellets collected were stored in -20 °C till DNA extraction.

4.2.3. Cellular DNA extraction

2.0 mL of TE buffer (containing 10 mM Tris and 1 mM EDTA at pH 8.0) were added to the cell pellets. After a short vortex, 240 µL of 10% SDS solution was added and mixed with the cell suspension gently. The lysates were treated with 20.0 µL of RNase A (20 mg/mL dissolved in deionized water) at 37 °C for 1 h, followed by treatment with 25.0 µL of protease K (20 mg/mL dissolved in deionized water). After 1 h incubation at 37 °C, each sample was transferred to a Phase Lock Gel tube (5 Prime, Gaithersburg, MD) and centrifuged (4000 x g for 15min) twice with 2.0 mL of phenol, followed by two times extraction with 2.0 mL of chloroform. After extraction, the aqueous phase (ca. 2.0 mL) was transferred to a clean 15 mL centrifuge tube and mixed with 10 mL of pre-chilled ethanol (-20 °C). The DNA was precipitated at -20 °C for overnight.
After recovering the DNA pellet by removing ethanol through centrifugation (15000 x g for 15 min), each sample was then washed with 1.0 mL of pre-cooled 70% ethanol (-20 °C). Thereafter, the samples were air-dried at room temperature and reconstituted with TE buffer to a final concentration of 1.0 mg/mL (based on UV absorption at 260 nm).

4.2.4. Removal of RNA

To each 100 µL of DNA sample (1.0 mg/mL), 2.00 µL of RiboShredder™ RNase Blend (1 U/µL) was added. After 30 min incubation at 37 °C, the sample was mixed with 1.0 mL of pre-chilled ethanol (-20 °C). The DNA was precipitated at -20 °C for overnight. After centrifugation (15000 x g for 15 min), each sample was washed with 1.0 mL of pre-cooled 70% ethanol (-20 °C) twice. Then each DNA sample was air-dried and reconstituted with 200 µL of 5 mM BisTris buffer (pH 7.0).

4.2.5. Preparation of calibrators and quality control

The stock standard solutions of F-ara-AMP (4.0 mM), 2CdA (IS) (2.0 mM), and dAMP (4.0 mM) were prepared in deionized water respectively. The working solutions of F-ara-AMP (2.00 µM) and dAMP (2.00 mM) were prepared by diluting their stock standard solutions with deionized waer individually. The standard working solutions of F-ara-AMP (2.00, 6.00, 8.00, 40.0, 60.0, 80.0, 400, 800, 1800 and 2000 nM) were prepared by serial dilution of F-ara-AMP working solution (2.00 µM). The standard working solutions of dAMP (20.0, 30.0, 40.0, 80.0, 120, 200, 400, 600, and 800 µM) were prepared by serial dilution of dAMP working solution (2.00 mM).
The F-ara-A calibrators (1.00, 4.00, 20.0, 40.0, 200, 400 and 1000 nM) and the quality controls containing F-ara-A (3.00, 30.0 and 900 nM) were prepared by mixing 25 µL of each F-ara-AMP standard working solution with 25 µL of 10 mM BisTris buffer (pH 7.0), respectively.

The dAMP calibrators (10.0, 20.0, 40.0, 100, 200 and 400 µM) and the dAMP quality controls (15.0, 60.0 and 300 µM) were prepared by mixing 25 µL of each dAMP standard working solution with 25 µL of 10 mM BisTris buffer (pH 7.0), respectively.

4.2.6. Patient sample preparation

In this study, 10-mL of peripheral blood samples were collected in heparinized Vacutainer® tubes (BD, Franklin Lakes, NJ) from a patient who enrolled in Case 2Y10 clinical trial.

At the time of blood drawing, a patient had been administered with 25 mg/m² fludarabine plus 90 mg/m² methoxyamine. Blood was drawn at 24, 48, 72, 96 and 168 h after fludarabine dose was given to this patient; and the blood samples were fractioned by Ficoll-Paque method. Briefly, 10-mL heparinized blood was layered on the top of 12 mL Ficoll-Paque Plus reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in a 50 mL sterile polypropylene centrifuge tube (RNase/DNase free); then the tube was centrifuged at 4 °C and 300 × g for 30 min. Cells at the interface were collected and transferred into a clean 15 mL centrifuge tube, and washed twice with 10 mL PBS (1×, pH 7.4). In each wash step, the cells were gently vortexed with the PBS for 1 min, and then centrifuged down at 4 °C and 300 × g for 10 min. After wash, the cell pellets were frozen at -20 °C till DNA extraction. DNA was extracted by using the Gentra Puregene
Blood Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol (DNA Purification from Whole Blood or Bone Marrow Using the Gentra Puregene Blood Kit, Gentra® Puregene® Handbook, Third Edition, June 2011, Page 19 - 21). Finally, the extracted DNA was dissolved with a final concentration of 2 mg/mL in TE buffer.

The extracted DNA was further treated by RiboShredder™ RNase Blend (1U/µL) to remove the RNA contaminations (See Section of 4.2.4). Finally, the purified DNA was reconstituted with 5 mM BisTris Buffer (pH 7.0) with a final concentration of 2.0 mg/mL.

4.2.7. DNA enzyme digestion

Before hydrolysis, the DNA samples, calibrators, and quality controls were incubated in boiling water for 30 min and chilled in ice for 15 min. Then an appropriate volume of enzyme solutions was added to each sample. “Enzyme cocktail” were prepared as following: DNase I was dissolved with 0.9% NaCl at the final concentration of 10 mg/mL (ca. 20000 unit/mL); NP1 was dissolved in 1 mM ZnCl$_2$ at the final concentration of 1 mg/mL (ca. 200 unit/mL); PDE I was dissolved with deionized water to the final concentration of 100 unit/mL. The “enzyme cocktail” contains 10.0 µL of DNase I, 40.0 µL of PDE I, 15.0 µL of NP1, and 0.50 µL of ALP. For each 50.0 µL of DNA (0.5 mg/mL), calibrators, and quality controls, 4.00 µL of the enzyme cocktail was added and mixed well. The hydrolysis reaction was carried out for 17 h at 37 °C.
4.2.8. Digested products extraction

4.00 µL of IS working solution (20.0 µM) or the mobile phase were added to each 50.0 µL of the digested sample, and vortex-mixed for 30 s; the sample was then deproteinized with 450 µL of HPLC-grade acetonitrile by vortex mixing for 30 s, followed by centrifugation at 15000 x g for 10 min. 450 µL of supernatant was pipetted into a 1.5-mL microcentrifuge tube and evaporated to dryness at 30 °C for 60 min in a TurboVap® LV Evaporator (Zymark, Hopkinton, MA, USA) under a pressurized stream of nitrogen gas. Finally, each sample was reconstituted in 90.0 µL of deionized water. For the fludarabine measurement, 5.0 µL of the resulting solution was then injected into LC-MS/MS for the fludarabine measurement. For the dA measurement, a separate 5.0 µL of the 400 x dilution solution was used.

4.2.9. LC-MS/MS

The liquid chromatography tandem mass spectrometry system was comprised of an AB Sciex QTRAP 5500 mass spectrometer (Foster City, CA) and a Shimadzu Prominence UFLC system (Kyoto, Japan) composed of a solvent reservoir, a degasser (DGU-20A3), a binary pump (LC-20AD), a flow controller (CBM-20A), and an autosampler (SIL-20ACHT). Data was acquired and analyzed by Analyst software (version 1.5.1).

The separation of F-ara-A, deoxyribonucleotides, and IS was performed on a Waters Atlantis® T3 (3 µM, 2.1 mm x 50 mm) column (Waters, Milford, MA, USA) by gradient elution at ambient at the flow rate of 0.20 mL/min. The eluents used consisted of mobile phase A (MPA) containing 0.1% formic acid in ultra-pure water and mobile phase
B (MPB) containing methanol. The program was initiated with 12.5% MPB from 0.0 to 1.0 min, 12.5-50% MPB from 1.0 to 4.0 min, 50-12.5% MPB from 4.0 to 4.1 min, and 12.5% MPB to 5min. The injection volume of each sample was 5 µL. The equilibrate time between each injection was 5 min.

ESI-MS/MS was operated on the positive-electrospray-ionization (ESI+) mode. The source-dependent parameters were set as follows: CUR, 30; IS, 5500; TEM, 300; G1, 40; G2, 40. The compound-dependent parameters were as follows: DP, 50; EP, 50. Detection of F-ara-A, IS, dA, dG, dC and dT was achieved on MRM with the mass transition of 286 > 154, 286 > 170, 252 > 136, 268 > 152, 228 > 112 and 243 > 127 m/z, respectively. The MRM conditions were set as follows: CAD, medium; CE, 30.0; CXP, 13.0; Dewell Time, 100 ms.

4.3. Results and Discussion
4.3.1. Method development
4.3.1.1. Enzyme digestion optimization

By referring to the work of Yamazoe et al. and Lin et al., a quaternary enzyme system consisting of DNase I, NP1, PDE I, and ALP was chosen [10, 11]. Among the four enzymes, DNase I splits phosphodiester bonds preferentially adjacent to pyrimidine nucleotides and typically yield tetranucleotides [12]. NP1 and PDE I are 5’ to 3’ and 3’ to 5’ exonuclease, respectively. The completely hydrolyzed products of these two enzymes are dNMPs [13, 14]. When the first three enzymes work together to hydrolyze the DNA, single strand or double strand, into dNMPs.
Because the sensitivity of detecting nucleosides by ESI-MS is much higher than that of detecting nucleotides, the enzymatic cocktail system also contains ALP that can effectively convert all nucleotides to nucleosides [14]. To achieve the highest fludarabine releasing efficiency with the least cost of labor and materials, several digestion methods were experimented [15].

Comparison between a sequential digestion and the enzyme-cocktail digestion with the same amount of enzymes for the same total digestion time indicated no significant difference in the digestion efficiency. Therefore the enzyme-cocktail digestion was chosen in this project. Furthermore, the best digestion time was experimented. By digesting DNA with the enzyme cocktail for 5, 10, 15, 20, and 25 h, a kinetic curve was obtained (data was not shown). From the curve, the optimized digestion time was between 15 and 20 h. As a result, a total digestion time of 17 h was adopted in this work due to its best fit to an 8 h working schedule.

4.3.1.2. Mass spectrometric characterization

As seen from Figure 4.2, the expected hydrolysis products from a DNA sample are F-ara-A along with four deoxyribonucleosides (dG, dA, dC and dT) if the sample is treated by fludarabine. Thus an LC-MS/MS method has been developed to monitor all of hydrolysis products.

In this work, full-scan (MS1) and product ion scan (MS2) spectra of F-ara-A, internal standard (IS) 2-chloro-2′deoxyadenosine and four deoxyribonucleosides (dG, dA, dC and dT) were acquired. The optimization of the six species responses was done using the “auto-tune” function of AB Sciex Analyst software (version 1.5.1). Since all of the
analytes could easily form the protonated species than deprotonated species by electrospray ionization, the positive electrospray-ionization mode was used for the identification and quantification. Figure 4.2 displays the typical fragmentation patterns of the six species and their respective main fragmentation ions.

The spectra showed very similar patterns. Therefore, the mass transition pairs of m/z 286/154, 286/170, 268/152, 252/136, 228/112 and 243/127 were chosen for the detection of F-ara-A, IS, dG, dA, dC and dT, respectively, to monitor the LC separation in MRM.

4.3.1.3. LC separation of enzyme digestion

Figure 4.3A displays LC-MS/MS chromatograms of the 6 molecules with a loading amount of 1 nmol each. The LC elution conditions were optimized by using 0.1% formic acid in water and methanol on a Waters Atlantis® T3 (3 µM, 2.1 mm x 50 mm) column. The final gradient shown in Materials and Methods was selected because it provided the shortest separation time and the highest separation efficiency. Figure 4.3A demonstrates complete separation of all 6 molecules within 5 min. Furthermore, the hydrolysis products from fludarabine treated and untreated cellular DNA sample were analyzed by LC-MS/MS. IS was added to the sample after hydrolysis for the MRM analysis. Figure 4.3B displays the result of detecting the products resulted from hydrolysis of an untreated DNA sample. As expected, dG, dA, dT, dC along with IS were detected, but F-ara-A was not detected. Figure 4.3C displays LC-MS/MS chromatograms of hydrolysis products of a DNA sample that was treated by 10 µM fludarabine for 24 h. As seen from Figure 4.3C, F-ara-A along with dG, dA, dT, dC, and IS, was detected from
this treated sample, demonstrating that this new method can detect the incorporation of fludarabine into DNA.
Figure 4.2, Fragmentation spectra of F-ara-A (A); IS (B); dG (C); dA (D); dC (E); and dT (F).
Figure 4.3, Representative MRM chromatograms of standard mixture and enzymatic digested products: (A) the standard mixture of dC, dA, dG, dT, F-ara-A along with IS; (B) enzymatic digested products from cellular DNA without drug treatment along with IS; (C) enzymatic digested products from cellular DNA with 10 µM of drug treatment for 24 h and IS.
4.3.1.4. Using dA as internal standard in sample preparation

Most reported methods have expressed the amount of drugs incorporation into DNA as pmol of the drug per mg of DNA, wherein the amount of DNA was determined by UV spectrometer [4, 9]. However, the OD method has limitations that may contribute to inaccuracy of DNA concentration estimates. For example, DNA quantity can be misestimated due to contaminations from RNA, protein, lipids, and so forth. Moreover, DNA is very sticky and the bonding of DNA to pipette tips can dramatically decrease the accuracy and precision of the experiment. Furthermore, insufficient DNA hydrolysis can also impair the accurate determination of the incorporated drug. To circumvent these disadvantages, dA was utilized as the internal standard in sample preparation to cancel out the experimental errors. We quantified both F-ara-A and dA from the same DNA sample after hydrolysis reaction and expressed the amount of the drug incorporation as [F-ara-A]/[dA]. In addition, the DNA quantity also can be calculated based on the amount of dA by using Eq.1.

\[
[\text{DNA}] = [\text{dA}] \times 617 \text{ (g/mol)} / 0.61 \quad \text{(Eq. 1)}
\]

Wherein [DNA] is the concentration of DNA in mg/L; [dA] is the measured concentration of dA in mM; 617 (g/mol) is molar molecular weight of A/T pair; and 0.61 is the percentage of A/T pair in human DNA [16].

4.3.2. LC-MS/MS method validation

4.3.2.1. Calibration curves

Calibration standards for F-ara-A were prepared by serial dilution of F-ara-AMP working solution. After incubation with the tetra-enzyme cocktail, IS was added to each
sample. The linear calibration ranges (1.00 to 1000 nM) were established by plotting the peak area ratios of F-ara-A to the IS versus the concentrations of F-ara-A using $1/x^2$ weighted linear regression (Figure 4.4A). Importantly, linearity and slope of calibration curve did not change when assessed in calibration standard mixtures of F-ara-AMP and drug-free DNA (Figure 4.4B). In other words, the similar slope and intercept of the calibration curves for measuring F-ara-A were obtained in absence or presence of DNA hydrolysis products and matrix. Although calibrators should ideally be prepared by using matrix identical to that in the tested samples, our study demonstrated that the presence of DNA hydrolysis products and matrix did not affect the signal intensity of F-ara-A. Therefore, to simplify the experimental procedure, we prepared calibrators in solvent instead of spiking them into DNA hydrolysis matrix. Consequently, the calibration equation for F-ara-A derived from three validation batches using $1/x^2$ weighted linear regression was $Y = 0.000479 \pm 0.000003X + 4.82e^{-5} \pm 1.37e^{-5}$ with a coefficient of determination ($r^2$) of 0.9999. In this work, the lower limit of quantification (LLOQ) was defined by the lowest calibrators of the calibration curves, which was at 1nM. The accuracy and precision of each individual calibrator as summarized in Table 4.1, were all $\leq \pm 13\%$. These values were well within the FDA guidelines (i.e., $\leq \pm 15\%$ at all concentrations except at LLOQ where $\leq \pm 20\%$).
Figure 4.4, Fludarabine calibration curves using (A) fludarabine (1.00-1000 nM) spiked in buffer; (B) fludarabine (1.00-1000 nM) spiked in drug free DNA sample, (C) dA (10.0-400 µM) spiked in buffer; and (D) $[5',5''-2H^2]2'$-dA (10.0-400 µM) spiked in drug free DNA sample.
Table 4.1, Calibration equations of F-ara-A (1.00 to 1.00 x 10^3 nM).

\[ Y = 4.79 \times 10^{-4} (\pm 0.03 \times 10^{-4}) X + 4.82 \times 10^{-5} (\pm 1.37 \times 10^{-5}) \ (r^2 = 0.9999 \pm 0.0000). \ (n=3) \]

Each datum point was based on three separate measurements in different days.

%RE = \frac{\text{measured [F-ara-A]} - \text{nominal [F-ara-A]}}{\text{nominal [F-ara-A]}} \times 100%.

%CV = \left( \frac{\text{standard deviation}}{\text{mean value}} \right) \times 100%.

<table>
<thead>
<tr>
<th>Nominal [F-ara-A], nM</th>
<th>Measured [F-ara-A], nM</th>
<th>SD</th>
<th>Accuracy (%RE)^a</th>
<th>Precision (%CV)^b</th>
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Table 4.2, Intra- and Inter-run accuracy and precision of F-ara-A.

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<th>Inter-run (n=5)(^b)</th>
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<td></td>
<td>Accuracy (%RE)</td>
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\(^a\) Each datum point calculated by five replica measurements of each QCs sample

\(^b\) Each datum point calculated by five parallel measurements of five identical QCs samples
Table 4.3, Calibration equations of dA (10.0 to 400 µM).
Calibration equations: $Y = 1.21 (±0.05)X + 7.58 (±0.72) (r^2 = 0.999 ±0.002)$. (n=3)

<table>
<thead>
<tr>
<th>Nominal [dA], µM</th>
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<th>SD</th>
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<th>Precision (%CV)</th>
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</table>

Each datum point was based on three separate measurements in different days.
Table 4.4, Intra- and Inter-run accuracy and precision of dA.

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<td>Precision</td>
<td>Accuracy</td>
<td>Precision</td>
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<tr>
<td></td>
<td>(%RE)</td>
<td>(%CV)</td>
<td>(%RE)</td>
<td>(%CV)</td>
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</table>

*a Each datum point calculated by five replica measurements of each QCs sample

*b Each datum point calculated by five parallel measurements of five identical QCs samples
Similarly, dA calibration curves were constructed by using six non-zero dA calibrators along with IS. As described in Section of Experimental, the calibrators were prepared in BrisTris buffer. The linear calibration ranges (10.0 to 400 µM) were established by plotting the peak area ratios of dA to the IS versus the concentrations of dA using 1/x weighted linear regression (Figure 4.4C). The calibration equation was Y = 1.21 (±0.05)X + 7.58 (±0.72) (r² = 0.999 ±0.002). To assess the interference of DNA hydrolysis products and matrix, we also built the calibration curve by mixing isotope standards, [5',5''-2H²]2'-deoxyadenosine ([5',5''-2H²]2'-dA) and drug free DNA with the same calibration range (10.0 to 400 µM). The presence of DNA hydrolysis products and matrix did not affect the signal intensity of dA as evidenced by the fact that the slope and intercept of the calibration curves for dA, which was obtained in solution (Figure 4.4C), were similar to those for [5',5''-2H²]2'-dA that was obtained in the presence of DNA hydrolysis matrix. The accuracy and precision of each individual calibrator were given in Table 4.3, which were well within the FDA guidelines.

4.3.2.2. Accuracy and precision

The inter-run precision and accuracy for F-ara-A were assessed by five parallel injections from five identical QC samples at low- (3.00 nM), mid- (30.0 nM) and high-concentration (900 nM) levels (LQC, MQC and HQC). The intra-assay precision and accuracy were assessed by five replica measurements of each QC samples. As shown in Table 4.2, the intra-run accuracy expressed as percent relative error (%RE) ranged from -1 to 8%, and the inter-run accuracy values ranged from -5 to 2%. The precision of the assay expressed as percent standard deviation or coefficient of variation (%CV) varied
from 1 to 10%.

Similarly, the inter- and intra-run precision and accuracy for dA were summarized in Table 4. QC samples of dA were at the concentrations of 15.0, 60.0 and 300 µM. As shown in Table 4, the intra- and inter-run accuracy and precision were within ±11%, indicating that this method is accurate, precise and reproducible.

4.3.3. RNA interference & removal

A potential problem in the measurement of fludarabine incorporated into DNA is interference from RNA contamination. It is known that F-ara-A can also incorporate into RNA with a higher rate [17]. We conducted a study to determine the effect of RNA on measurements of F-ara-A. RNase A is widely utilized to remove RNA in DNA extraction procedure, however RNase A cleaves only on the 3’ side of pyrimidine residues, yielding a range of smaller RNAs that may not be fully hydrolyzed, depending on the sequence of RNA [18]. By contrast, RNase Blend can more effectively hydrolyze RNA [18]. In other words, RNA would be more fully hydrolyzed when both RNase A and RNase Blend were used to treat the DNA sample, while some RNAs may not be completely hydrolyzed if the sample was treated only by RNase A. In this study, treated DNA sample was divided into two parts. The first part was treated only by RNase A, while the second part was treated by both RNase A and RNase Blend. Thereafter, all four Ns (products from RNA hydrolysis), and F-ara-A were detected by LC-MS/MS. As shown in Figure 4.5A, loading of DNA hydrolysis products from the sample treated by RNase A resulted in all four Ns peaks observed, indicating RNA contamination. Moreover, an unknown interference peak was eluted at 5.1 min in F-ara-A MRM channel. By contrast, when the sample was
treated by both RNase A and RNase Blend, the quantity of Ns was reduced by over 20 folds, indicating that treatment by both RNase A and RNase Blend led to more complete hydrolysis of RNA (Figure 4.5B). Importantly, the F-ara-A interference peak at 5.1 min disappeared, indicating that this peak was related to RNA interference. Our study suggests that after treatment of DNA by both RNase A and RNase Blend, the contribution of RNA to the measured quantity of F-ara-A is insignificant. In other words, the F-ara-A molecules detected by this method originated mainly from DNA, not from RNA. Therefore, in the remaining of this study, all samples were treated with both RNase A and RNase Blent.
4.5, DNA hydrolysis products from the sample (A) treated by RNase A and (B) both RNase A and RNase Blend
4.3.4. Enzyme digestion efficiency

Whether the enzymatic hydrolysis procedure used can fully hydrolyze DNA into individual nucleosides was tested. As shown in the “Enzyme digestion optimization” section, when the digestion time was above 15 h, the intensity of digestion products (i.e., individual nucleosides) was no longer significantly increased with the incubation time, indicating that the digestion efficiency has reached the maximum under our condition.

Then, a comparison of the calculated and UV measured DNA quantity was performed to further validate the digestion efficiency. The rationale for the study is that the quantity of DNA can be both measured by UV and calculated from the amount of a nucleoside released from hydrolysis. We used the calibration curve derived above to determine the amount of dA produced from hydrolysis of 250 mg/L (measured by UV) of calf DNA. The quantity of dA measured was 0.260 mM. By using Eq. 2, the amount of DNA to produce 0.260 mM of dA was 267 mg/L if DNA was completely hydrolyzed into individual nucleosides.

\[
[\text{DNA}] = [\text{dA}] \times 617 \ (\text{g/mol})/ \ 0.581 \quad \text{(Eq. 2)}
\]

Wherein \([\text{DNA}]\) is the concentration of DNA in mg/L; \([\text{dA}]\) is the measured concentration of dA in mM; 617 (g/mol) is molar molecular weight of A/T pair; and 0.581 is the percentage of A/T pair in calf DNA [19]. It was seen that the amount of DNA calculated (267 mg/L) from the quantity of dA was similar to the amount of DNA measured by UV (250 mg/L), indicating that the enzymatic hydrolysis procedure used can essentially completely hydrolyze DNA into individual nucleosides. Importantly, this result also shows that our new method that combines enzymatic hydrolysis with LC-
MS/MS can accurately determine the quantity of nucleosides released from DNA hydrolysis.

4.3.5. Quantification of F-ara-A in fludarabine treated HL-60 & CLL-MEC1

Table 4.5 lists the result of measuring the F-ara-A incorporation into cellular DNA of the cells cultured under the different drug treatment conditions. In the drug treatment time study, HL-60 cells were incubated with 10 µM fludarabine for 2 and 24 h, respectively. An increase in incorporation with the treatment time was observed. The measured incorporation rate was only 15.9 pmol F-ara-A/mg DNA after 2 h treatment, but it was 468 pmol/mg DNA after 24 h treatment. In the dosages study, it was seen that the amount of F-ara-A incorporated into DNA increased with the drug concentration. For instance, when HL-60 cells were treated by 1 or 10 µM fludarabine for 24 h, the amount of F-ara-A increased from 306 pmol F-ara-A/mg DNA (1 µM treatment) to 468 pmol F-ara-A/mg DNA (10 µM treatment). We also observed the difference in incorporation among cell lines. For example, after the same treatment, the amount of F-ara-A incorporated into CLL-MEC1 was 32% less than that into HL-60. This result shows that this new method can be used to quantitatively measure the incorporation of fludarabine into DNA of cultured cells, leading to assessment of the efficacy of the drug.

4.4.6. Quantification of F-ara-A in patient’s Lymphocytes

In this study, a patient was recruited and administered with 25 mg/m² fludarabine plus 90 mg/m² methoxyamine. Blood was drawn at 24, 48, 72, 96 and 168 h after fludarabine dose was given. The results were summarized in Table 4.6. As shown in
Table 4.6, after treated by fludarabine for 24 h, the amount of F-ara-A incorporated into DNA was 2.5 pmol F-ara-A/mg DNA. After 48 h and 72 h, the amount of F-ara-A incorporated into DNA was increased to 10.6 and 52.7 pmol F-ara-A/mg DNA. The highest amount of F-ara-A incorporated into DNA was 76.0 pmol F-ara-A/mg DNA when the patient had been administered with fludarabine after 168 hr. This result shows that this new method can be used to quantitatively measure the incorporation of fludarabine into cellular DNA of blood collected from the patient who is treated with fludarabine.
Table 4.5, Drug incorporated rates for different cell lines, drug treatment concentrations and treated times.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>[Fludarabine] treated, µM</th>
<th>Treated Time, Hr</th>
<th>[F-ara-A] nM</th>
<th>[dA] µM</th>
<th>Drug Incorporated Rate (F-ara-A/dA)</th>
<th>F-ara-A /DNA, pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL-MEC1</td>
<td>0</td>
<td>24</td>
<td>&lt;0</td>
<td>152</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CLL-MEC1</td>
<td>10</td>
<td>24</td>
<td>43.9</td>
<td>134</td>
<td>3.28/10,000</td>
<td>319</td>
</tr>
<tr>
<td>HL60</td>
<td>0</td>
<td>24</td>
<td>&lt;0</td>
<td>264</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HL60</td>
<td>10</td>
<td>24</td>
<td>96.8</td>
<td>201</td>
<td>4.82/10,000</td>
<td>468</td>
</tr>
<tr>
<td>HL60</td>
<td>1</td>
<td>24</td>
<td>75.6</td>
<td>240</td>
<td>3.15/10,000</td>
<td>306</td>
</tr>
<tr>
<td>HL60</td>
<td>10</td>
<td>2</td>
<td>6.24</td>
<td>382</td>
<td>0.16/10,000</td>
<td>15.9</td>
</tr>
</tbody>
</table>
Table 4.6, Drug incorporated rates in patient’s lymphocytes after fludarabine treatment.

<table>
<thead>
<tr>
<th>Time After Treatment Hr</th>
<th>[F-ara-A] nM</th>
<th>[dA] µM</th>
<th>Drug Incorporated Rate (F-ara-A/dA)</th>
<th>F-ara-A /DNA, pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.09</td>
<td>380</td>
<td>0.287/10,000</td>
<td>2.8</td>
</tr>
<tr>
<td>48</td>
<td>2.73</td>
<td>255</td>
<td>1.07/10,000</td>
<td>10.6</td>
</tr>
<tr>
<td>72</td>
<td>22.8</td>
<td>428</td>
<td>5.33/10,000</td>
<td>52.7</td>
</tr>
<tr>
<td>96</td>
<td>7.95</td>
<td>293</td>
<td>2.71/10,000</td>
<td>26.8</td>
</tr>
<tr>
<td>168</td>
<td>33.9</td>
<td>441</td>
<td>7.69/10,000</td>
<td>76.0</td>
</tr>
</tbody>
</table>
4.4. Conclusion

We developed a simple, sensitive, and reliable method for quantifying the incorporation of fludarabine into cellular DNA, in which the incorporated F-ara-A is first released from DNA by enzymatic hydrolysis and then quantitated by LC-MS/MS analysis. This new method can accurately quantify nucleosides and F-ara-A released from DNA hydrolysis. Moreover, quantitation by this method is highly reproducible. On the application front, this work demonstrated that this new method could quantitatively measure the incorporation of fludarabine into DNA of cultured cells, which in turn can be used to assess the efficacy of the drug. To the best of our knowledge, this is the first non-radioactive assay that can quantitatively measure the incorporation of fludarabine into cellular DNA. More importantly, we have successfully demonstrated that this new method can be used to measure the amount of fludarabine incorporated into cellular DNA of blood collected from the patient who was treated with fludarabine.

Fludarabine can also incorporate into RNA [17]. Although this method is proposed for measurements of the incorporation of fludarabine into DNA, it can certainly be used to study the incorporation of fludarabine into RNA as well.

Moreover, Fludarabine is one of many nucleoside analogues that are an important class of anti-cancer drugs [20]. Although this method is proposed to quantify the incorporation of fludarabine into cellular nucleic acids, it can be modified to quantify the incorporation of other nucleoside analogues into cellular nucleic acids.
4.5. References


[8] Huang, P.; Plunkett, W. Phosphorolytic Cleavage of 2-fluoroadenine from 9-beta-D-arabinofuranosyl-2-fluoroadenine by Escherichia Coli. A Pathway for 2-fluoro-


[16] The Human Genome Discovery.


5.1. Introduction

As mentioned in the previous chapter, fludarabine is widely used for the treatment of chronic lymphocytic leukemia (CLL) [1]. As a single agent, fludarabine has produced superior response rates and progression-free survival than standard therapy with chlorambucil and alkylator-based regimen. Although fludarabine is effective in the treatment of CLL, the response to fludarabine is still limited [2, 3].

The principal action of fludarabine is in the inhibition of DNA synthesis [4] and incorporating fludarabine (in place of adenosine) into DNA is a major mechanism to inhibit DNA synthesis. The exact position of fludarabine incorporated into DNA dictates the mechanism of action. Based on their study that showed the majority of fludarabine (95%) was incorporated to the terminal position of DNA [5], Plunkett and co-workers suggested that fludarabine was a DNA extension terminator. In addition, when fludarabine was incorporated at the 3’-terminus, DNA ligase I would be unable to join it
to an adjacent piece of DNA [6]. Together, incorporation of fludarabine into the terminal position could effectively terminate both DNA extension and ligation. Subsequently, the actions can result in inactivation of DNA synthesis [7-9].

Recent studies by our group suggested that fludarabine also can be incorporated into the internal position of the DNA chain and activates the base excision repair (BER) pathway [10]. In that work, we demonstrate that (1) the BER pathway is involved in processing incorporated fludarabine in DNA through the enzymatic activity of UDG; (2) MX binding of AP-sites generated directly and indirectly by fludarabine induces un-repairable DNA damage that can block the BER pathway; and (3) MX potentiates the therapeutic efficacy of fludarabine, allowing for the possibility of a novel therapeutic strategy to combine inhibitors of BER with fludarabine for clinical treatment. Targeting BER as a target-based therapeutic strategy can be extended to the combination of MX with a number of other drugs that incorporate into DNA either by acting as nucleotide analogs or through the manipulation of the nucleotide pools.

Clearly, an accurate determination of the exact incorporation position of fludarabine in DNA is essential to elucidating the mechanism of action of fludarabine, from which one can improve the fludarabine therapeutic efficacy. The traditional method utilized radioactive isotope-labeled fludarabine to study its incorporation into DNA [5]. In that method, the incorporated DNA was first hydrolyzed, followed by separation and detection with HPLC-liquid scintillation counting. The drawback of that method is its inability to directly determine the identity of the products detected and to distinguish two products with the same retention time. Moreover, that method is not highly accurate when used for quantification. In this part of our dissertation research, we developed a
novel LC-MS/MS method in conjunction with enzymatic reactions to quantitatively determine the amount of the fludarabine incorporated in either the terminal or internal position in DNA. More importantly, our result suggests that the majority of fludarabine is incorporated into the internal position of DNA, which is contradictory to the previously accepted view that fludarabine was mainly incorporated in the 3’-terminal position of DNA [5]. Reporting this new method and our new result constitute the focus of this chapter.

5.2. Experimental

5.2.1 Chemicals and solutions

Formic acid, acetonitrile, ammonium acetate, acetic acid, 2’-deoxyadenosine (dA), 2’-deoxyadenosine 5’-monophosphate (dAMP), 2’-deoxyguanosine (dG), thymidine (T), 2’-deoxycytidine (dC), 2-chloro-2'deoxyadenosine (2CdA), thymidine 5’-monophosphate (TMP) and TE buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS), N,N-dimethyl hexylamine, water saturated phenol, optima gradient methanol and chloroform were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium glycinate, calcium chloride (CaCl$_2$), monopotassium phosphate (KH$_2$PO$_4$), dipotassium phosphate (K$_2$HPO$_4$), potassium fluoride (KF) and hydrochloric acid (HCl) were ordered from VWR (West Chester, PA, USA). The 10% sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). RPMI-1640 medium with L-glutamine was purchased from Mediatech (Manassas, VA, USA). Rnase A and Protease K were ordered from Invitrogen (Carlsbad, CA, USA). Micrococcal nuclease (MN), phosphodiesterase II (PDE II), bovine alkaline phosphatase
(ALP), and snake venom phosphodiesterase I (PDE I) were obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, USA). Fludarabine (F-ara-AMP) was from Ochem (Des Plaines, IL, USA). RiboShredder™ RNase Blend was from Epicentre Biotechnologies (Madison, WI, USA). Deionized water was prepared by a Barnstead Model 7148 Nanopure® ultrapure water system of the Thermo Scientific (Asheville, NC, USA).

5.2.2 Cell culture and treatment

Human promyelocytic leukemia cells (HL60) were obtained from American Type Culture Collection (Rockville, MD, USA). Chronic lymphocytic leukemia (CLL) MEC1 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator.

F-ara-AMP solutions were freshly prepared for each experiment by dissolving F-ara-AMP powder in PBS (1x, pH 7.4). For each 5 × 10⁶ cells, the dosages of F-ara-AMP added into the culture media were 0.00 or 10.0 µM, respectively. Cells were treated for 24 h at 37 °C in a humidified 5% CO₂ incubator. After treatment, the cells were removed from the medium by centrifugation 1500 x g at 4 °C for 5 min, and washed with 5 mL PBS. After centrifugation (1500 x g at 4 °C for 5 min), the cell pellets collected were stored in -20 °C till DNA extraction.
5.2.3 Cellular DNA extraction

2.0 mL of TE buffer (containing 10 mM Tris and 1 mM EDTA at pH 8.0) were added to the cell pellets. After a short vortex, 240 µL of 10% SDS solution was added and mixed with the cell suspension gently. The lysates were treated with 20.0 µL of RNase A (20 mg/mL dissolved in deionized water) at 37 °C for 1 h, followed by treatment with 25.0 µL of protease K (20 mg/mL dissolved in deionized water). After 1 h incubation at 37 °C, each sample was transferred to a Phase Lock Gel tube (5 Prime, Gaithersburg, MD) and centrifuged (4000 x g for 15 min) twice with 2.0 mL of phenol, followed by two times extraction with 2.0 mL of chloroform. After extraction, the aqueous phase (ca. 2.0 mL) was transferred to a clean 15 mL centrifuge tube and mixed with 10 mL of pre-chilled ethanol (-20 °C). The DNA was precipitated at -20 °C for overnight.

After recovering the DNA pellet by removing ethanol through centrifugation (15000 x g for 15 min), each sample was then washed with 1.0 mL of pre-cooled 70% ethanol (-20 °C). Thereafter, the samples were air-dried at room temperature and reconstituted with TE buffer to a final concentration of 1.0 mg/mL (based on UV absorption at 260 nm). To each 100 µL of DNA sample (1.0 mg/mL), 2.00 µL of RiboShredder™ RNase Blend (1 U/µL) was added. After 30 min incubation at 37 °C, the sample was mixed with 1.0 mL of pre-chilled ethanol (-20 °C). The DNA was precipitated at -20 °C for overnight. After centrifugation (15000 x g for 15 min), each sample was washed with 1.0 mL of pre-cooled 70% ethanol (-20 °C) twice. Then each DNA sample was air-dried and reconstituted with 100 µL of deionized water.
5.2.4 Enzymatic hydrolysis of DNA

Enzymatic hydrolysis was performed with a 3-step reaction by modifying the procedure described by Manor et al. [11]. Three enzyme solutions were prepared as following: MN was dissolved in 1 stage buffer solution, which contains 50 mM sodium glycinate, 2 mM CaCl₂, 4 mM TMP, and 0.8 mM TdR at the final concentration of 50 unit/mL; PDE II was dissolved in 2 stage buffer solution, which contains 100 mM potassium phosphate (pH 6.0), 20 mM TMP, 200 mM ammonium acetate (pH 6.0), 80 mM acetic acid and 50 mM KF at the final concentration of 4 unit/mL; ALP was diluted in deionized water at the final concentration of 500 unit/mL. For each 50 µL of DNA sample (1.0 mg/mL), 50 µL of MN solution was firstly added and mixed well. Incubation was carried out at 37 ºC for 30 min. The reaction mixture was then cooled in ice, and followed by adding 10 µL of PDE II solution. After incubation at 37 ºC for 3 h, sample was identically divided into two vials. One vial was added with 4 µL of ALP and the other was added 4 µL of water. The last step hydrolysis was carried out for 2 h at 37 ºC.

5.2.5 Preparation of calibrators and quality controls

The stock standard solutions of F-ara-AMP (4.0 mM), and 2CdA (IS) (2.0 mM) were prepared in deionized water respectively. The working solution of F-ara-AMP (2.00 µM) and IS (1.00 µM) were prepared by diluting their stock standard solutions with deionized water individually. The standard working solutions of F-ara-AMP (4.00, 6.00, 8.00, 40.0, 60.0, 80.0, 400, 800, 1800 and 2000 nM) were prepared by serial dilution of F-ara-AMP working solution (2.00 µM).

The F-ara-A calibrators (2.00, 4.00, 20.0, 40.0, 200, 400 and 1000 nM) and the
quality controls containing F-ara-A (3.00, 30.0 and 900 nM) were prepared by mixing 25 µL of each F-ara-AMP standard working solution with 25 µL of MN solution, 5 µL of PDE II solution and 4 µL of ALP. Incubation was carried out at 37 ℃ for 5.5 h.

5.2.6 Digested product extraction

4.00 µL of IS working solution (1.00 µM) was added to each above digested sample, and vortex-mixed for 30 s; the sample was then deproteinized with 450 µL of HPLC-grade acetonitrile by vortex mixing for 30 s, followed by centrifugation at 15000 x g for 10 min. 450 µL of supernatant was pipetted into a 1.5-mL microcentrifuge tube and evaporated to dryness at 30 ℃ for 60 min in a TurboVap® LV Evaporator (Zymark, Hopkinton, MA, USA) under a pressurized stream of nitrogen gas. Finally, each sample was reconstituted in 90.0 µL of deionized water. 5.0 µL of the resulting solution was then injected into LC-MS/MS for the measurement.

5.2.7 LC-MS/MS

The liquid chromatography tandem mass spectrometry system was comprised of an AB Sciex QTRAP 5500 mass spectrometer (Foster City, CA) and a Shimadzu Prominence UFLC system (Kyoto, Japan) composed of a solvent reservoir, a degasser (DGU-20A3), a binary pump (LC-20AD), a flow controller (CBM-20A), and an autosampler (SIL-20ACHT). Data was acquired and analyzed by Analyst software (version 1.5.1). The separation of F-ara-A, and IS was performed on a Waters Atlantis® T3 (3 µM, 2.1 mm x 50 mm) column (Waters, Milford, MA, USA) by gradient elution at ambient at the flow rate of 0.20 mL/min. The eluents used consisted of mobile phase A
(MPA) containing 0.1% formic acid in ultra-pure water and mobile phase B (MPB) containing methanol. The program was initiated with 12.5% MPB from 0.0 to 1.0 min, 12.5-50% MPB from 1.0 to 4.0 min, 50-12.5% MPB from 4.0 to 4.1 min, and 12.5% MPB to 5 min. The injection volume of each sample was 5 µL. The equilibrate time between each injection was 5 min. ESI-MS/MS was operated on the positive-electrospray-ionization (ESI+) mode. The mass spectrometry parameters were set as follows: CUR, 30; IS, 5500; TEM, 300; G1, 40; G2, 40; DP, 50; EP, 5.0; CAD, medium; CE, 30.0; CXP, 13.0; and Dewell Time, 100 ms. Detection of F-ara-A and IS was achieved on MRM with the mass transition of 286 > 154 and 286 > 170 m/z, respectively.

The measurement of F-ara-AMP was performed on a Waters YMC-AQ® (5 µM, 2.1 mm x 50 mm) column (Waters, Milford, MA, USA) by isocratic elution with 5 mM N,N-dimethyl hexylamine in water (pH 7.0)/acetonitrile (90/10,v/v) at a flow rate of 0.20 mL/min. The injection volume of each sample was 5 µL. ESI-MS/MS was operated on the negative-electrospray-ionization (ESI-) mode. The mass spectrometry parameters were set as follows: CUR, 20; IS, -4500; TEM, 700; G1, 20; G2, 20; DP, -100; EP, -5.0; CAD, medium; CE, -70; CXP, -14; and Dewell Time, 100 ms. Detection of F-ara-AMP was achieved on MRM with the mass transition of 364 > 79 m/z.

5.3. Results and Discussion

5.3.1 Experimental design and enzymatic digestion

In this work, a LC-MS/MS method in conjunction with enzymatic hydrolysis of DNA was developed to quantitatively determine the amount of fludarabine incorporated
at the terminal and internal positions in DNA. The enzymes used were MN, PDEII and ALP.

MN is an endo-exonuclease that can hydrolyze both DNA and RNA. The typical products by MN digestion are mono- or di-nucleotides with a free 5’-end hydroxyl group and a free 3’-end phosphate group [12]. PDE II is a 5’ to 3’ exonuclease. The action of PDE II on DNA requires prior removal of the 5’-end phosphate group and the generation of a free hydroxyl group. Then it successively hydrolyzes DNA or RNA and releases single nucleotides with 3’-phosphate [13]. A combination of these two enzymes leads to the complete hydrolysis of DNA into single nucleotides with 3’-phosphate (from internal positions) and single nucleosides without phosphate (from terminal positions). In other words, the product generated from the internal nucleosides would be different from that produced from the terminal nucleosides. As shown in Figure 5.1, the terminally incorporated fludarabine is released from the DNA backbone in the form of F-ara-A, while the internally incorporated fludarabine is released in the form of 3’-F-ara-AMP.
Figure 5.1, Schematic representation of DNA hydrolysis by 3-step enzymatic digestion including MN, PDE II and ALP.
In this study, the F-ara-A molecules released from the terminal position were directly quantified by the LC-MS/MS method developed in our previous study (CHAPTER IV). However, for quantification of the 3’-F-ara-AMP molecules released from the internal position, because there were no 3’-F-ara-AMP molecules commercially available as standard, we used ALP to further digest the hydrolyzed products produced by MN/PDE II digestion. ALP is a hydrolase that can effectively remove phosphate from phosphate-containing molecules. As a result, after digestion by ALP, all the incorporated fludarabine molecules are converted to F-ara-A regardless of the incorporation positions and we were able to use LC-MS/MS to quantify the total amount of fludarabine incorporated after this 3-enzymes hydrolysis reaction. Experimentally, we first divided an extracted DNA sample into two aliquots. The first aliquot was subject to enzymatic hydrolysis by only PDE II and MN. After this hydrolysis, we quantified F-ara-A by LC-MS/MS, which yielded the amount of fludarabine incorporated in the terminal position. In contrast, the second aliquot was subject to hydrolysis by MN, PDE II and ALP. After hydrolysis, we quantified F-ara-A again by using the same LC-MS/MS method, which corresponded to the total amount of fludarabine incorporated.

5.3.2 Identification and confirmation of the enzymatic hydrolysis products

In this study, cells were first treated by 10 uM fludarabine for 24 h. Cellular DNA was extracted and divided to two identical parts. The first part was incubated with MN and PDE II only. After this enzymatic hydrolysis reaction, the hydrolysis products were identified by MRM. The mass transition pairs of 364/79 and 286/154 were chosen for the MRM detection of F-ara-AMP and F-ara-A, respectively. As discussed above, after
hydrolysis by MN and PDE II, the incorporated fludarabine would be released from the DNA backbone in the form(s) of 3’-F-ara-AMP and F-ara-A. As shown in Spectra A1 and A2 of Figure 5.2, a peak was observed at a retention time of 6.6 min in the F-ara-AMP mass transition channel (364/79), while another peak was seen at a retention time of 3.5 min in the F-ara-A mass transition channel (286/154). To confirm that the two observed peaks corresponded to 3’-F-ara-A and F-ara-A, respectively, several control studies were performed. In one control study, hydrolysis products from cellular DNA without drug treatment were studied by LC-MS/MS. As shown in Chromatogram B1 and B2 of Figure 5.2, no peak was observed in these two mass transitions, indicating that the peaks shown in Chromatogram A1 and A2 resulted from treatment of DNA by fludarabine. In addition, we attempted to detect F-ara-AMP and F-ara-A from a treated DNA sample that was not hydrolyzed. It was seen that without hydrolysis, no peak was detected (data not shown here), suggesting that the peaks in Chromatogram A1 and A2 of Figure 5.2 originated from the drug molecules that had been incorporated to cellular DNA. Furthermore, we incubated pure drug molecules with MN and PDE II. Although 5’-F-ara-AMP and 3’-F-ara-AMP are isomers, it is expected that they would have different retention times because of difference in their phosphate position. As expected, fludarabine (5’-F-ara-AMP) was eluted at a retention time of 4.7 min, further suggesting that the peak at 6.6 min in Chromatogram A1 of Figure 5.2 was not due to the unincorporated drug molecules. Moreover, there was no peak observed in F-ara-A mass transition channel, indicating that there is no dephosphorylation occurred under the experimental condition.
Figure 5.2, Representative MRM chromatograms of enzymatic-digested products from drug treated DNA and controls. (A1 & A2): Fludarabine treated HL60 DNA digested by MN & PDE II; (A3 & A4): Fludarabine treated HL60 DNA digested by MN & PDE II + ALP; (B1 & B2): No drug treated HL60 DNA digested by MN & PDE II; (B3 & B4): No drug treated HL60 DNA digested by MN & PDE II + ALP; (C1 & C2): Fludarabine incubated by MN & PDE II; (C3 & C4): Fludarabine incubated by MN & PDE II + ALP.
The second part of the DNA sample aliquot was incubated with MN and PDE II along with ALP, followed by analysis by LC-MS/MS. The result was shown in Chromatogram A3 and A4 of Figure 5.2. After the 3-enzymes hydrolysis reaction, all of the incorporated fludarabine molecules are expected to convert to F-ara-A. As expected, the peak at 6.6 min disappeared in Chromatogram A3 of Figure 5.2, while the peak at 3.5 min increased greatly in Chromatogram A4 of Figure 5.2. In a control study, we also incubated fludarabine with MN, PDEII and ALP. As shown in Chromatogram C3 and C4 of Figure 5.2, after this 3-enzymes hydrolysis reaction, the peak of 5’-F-ara-AMP was disappeared, while the peak of F-ara-A was observed at 3.5 min in the mass transition channel of 266/154, indicating that fludarabine (5’-F-ara-AMP) was successfully converted to F-ara-A by ALP.

The advantage of LC-MS/MS lies in its high specificity. Moreover, mass spectrometry can provide the structure information, which, in turn, can be utilized for identification of unknown molecules. Therefore, to further identify hydrolysis products, collision-induced dissociation (CID) was utilized to elucidate the fragmentation pattern of the molecules corresponding to the eluted peaks. Figure 5.3A displays a fragmentation spectrum of the drug standard (5’-F-ara-AMP), while Figure 5.3B displays the fragmentation spectrum of the molecules corresponding to the peak at 6.6 min. Highly similar fragmentation patterns were seen in both Figure 5.3A and B, even though the retention times of their parent molecules are different. Moreover, the mass positions of daughter ions were consistent with those of the expected fragments. This fragmentation study indicated that the molecule corresponding to the peak at 6.6 min shared the same structural composition of 5’-F-ara-AMP. The difference in the retention time was due to
the difference in their phosphate position. Similarly, the fragmentation pattern of the molecule corresponding to the peak at 3.5 min was also acquired (Figure 5.3C), which is the same as that of the F-ara-A standard (Figure 5.3D). Because the hydrolysis product molecule corresponding to the peak of 3.5 min has the same retention time, be in the same mass transition channel, and shares the same fragmentation pattern as the F-ara-A standard, we believe that this hydrolysis product molecule is F-ara-A.

In summary, our study clearly suggested that the hydrolysis products produced by MN/PED II digestion were 3’-F-ara-AMP originated from the internally incorporated fludarabine (Chromatogram A1 in Figure 5.2) and F-ara-A originated from the terminally incorporated fludarabine (Chromatogram A2 in Figure 5.2), respectively. Moreover, our study confirmed that the 3-enzymes hydrolysis reaction successfully converted all incorporated fludarabine into F-ara-A (Chromatogram A4 in Figure 5.2).
Figure 5.3, Identification of the enzymatic hydrolysis products by MS2 (A) Chromatogram and fragmentation spectrum of fludarabine (5’-F-ara-AMP) incubated with MN & PDE II; (B) Chromatogram and fragmentation spectrum of the digested product (retention time 6.6 min) from a drug treated cell sample incubated with MN & PDE II; (C) Chromatogram and fragmentation spectrum of fludarabine (5’-F-ara-AMP) incubated with MN, PDE II and ALP; (D) Chromatogram and fragmentation spectrum of the digested product from a drug treated cell sample incubated with MN, PDE II and ALP.
5.3.3 Quantification of enzymatic hydrolysis products

After identifying hydrolysis products, we utilized the LC-MS/MS method developed in our previous study (CHAPTER IV) to quantify the amount of F-ara-A incorporated. The LC separation condition and mass spectrometry parameters were described in Section 5.2.6. The calibration curve of F-ara-A was developed with seven non-zero F-ara-A calibrators (1.00, 4.00, 20.0, 40.0, 200, 400 and 1000 nM) along with IS in each at a fixed concentration (80 nM) (Table 5.1). The calibration equation derived from three validation batches using 1/x^2 weighted linear regression was Y = 0.0107 X + 0.0137 (where Y is the peak area ratio of fludarabine to IS and X is the concentration of fludarabine) with a coefficient of determination (r^2) of 0.999. The method was validated by three replicate measurements of each QC sample at the concentrations of 3.00, 30.0 and 900 nM. The accuracy and precision of each individual calibrator and QC sample expressed as the percent relative error (%RE) and coefficient of variation (%CV) were summarized in Table 5.1, which ranged from -3% to 10%, and 1% to 6%, respectively. With this new enzymatic hydrolysis method, we also observed an excellent reproducibility in quantification. This result indicates that the LC-MS/MS method used can accurately quantify F-ara-A.

As discussed in section 5.3.1, the amount of fludarabine incorporated at the terminal position and the total amount of incorporated fludarabine can be quantified, respectively, by using two different hydrolysis approaches, from which the amount of fludarabine incorporated at the internal position could be deduced. In this study, two different cell lines CLL-MEC 1 and HL-60 were analyzed. 3 batches of cells for each cell line were analyzed repeatedly under the same conditions to obtain more accurate results.
Specifically, each batch of cells was treated with 10 μM of fludarabine for 24 h. After DNA extraction, each DNA sample was divided to two identical parts. To quantify the amount of fludarabine at the terminal position, the extracted DNA was incubated with MN and PDE II only. To quantify the total amount of incorporated fludarabine, the extracted DNA was incubated with MN and PDE II along with ALP. The results were summarized in Table 5.2. This study showed that 96.9% of fludarabine was actually incorporated in the internal position in CLL Mec-1 cellular DNA, while only 3.1% of fludarabine was incorporated at the terminal position. Similar results were obtained with HL 60 cells. 97.8% of fludarabine was incorporated at the internal position, while only 2.2% at the terminal position.
Table 5.1, Calibration equation of F-ara-A (1.00 to 1.00 x10³ nM). Calibration equations: 
\[ y = 0.0107 x + 0.0137 \ (r = 0.999). \]

<table>
<thead>
<tr>
<th>Nominal nM</th>
<th>[F-ara-A], nM</th>
<th>Measured [F-ara-A], nM</th>
<th>Accuracy (%RE)</th>
<th>Precision (%CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.98</td>
<td>-2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>4.16</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>20.1</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>40.0</td>
<td>39.3</td>
<td>-2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>199</td>
<td>-1</td>
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<td></td>
</tr>
<tr>
<td>400</td>
<td>410</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1.00 x 10³</td>
<td>991</td>
<td>-1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LQC (3.00)</td>
<td>3.31</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MQC (30.0)</td>
<td>31.2</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HQC (900)</td>
<td>873</td>
<td>-3</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2, Quantification of fludarabine incorporated at different postions of DNA in different cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>[Terminal incorporated drug], nM</th>
<th>[Total incorporated drug], nM</th>
<th>F-ara-A Ratio (Terminal/Total) x 100%</th>
<th>F-ara-A Ratio (Internal/Total) x 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL-Mec1</td>
<td>2.26</td>
<td>61.1</td>
<td>3.1 ± 0.5 %</td>
<td>96.9 ± 0.5 %</td>
</tr>
<tr>
<td></td>
<td>2.81</td>
<td>112</td>
<td>3.27 ± 0.1 %</td>
<td>97.8 ± 0.1 %</td>
</tr>
<tr>
<td></td>
<td>3.31</td>
<td>157</td>
<td>3.38 ± 0.05 %</td>
<td>97.8 ± 0.1 %</td>
</tr>
<tr>
<td>HL60</td>
<td>3.04</td>
<td>132</td>
<td>2.2 ± 0.1 %</td>
<td>97.8 ± 0.1 %</td>
</tr>
<tr>
<td></td>
<td>3.38</td>
<td>154</td>
<td>3.38 ± 0.05 %</td>
<td>97.8 ± 0.1 %</td>
</tr>
</tbody>
</table>
We also carried out a control study to determine the accuracy of the method developed in this study. In this control study, we utilized the same hydrolysis strategy to quantify the nucleotides (i.e. 2′-deoxyadenosine, 2′-deoxyguanosine, thymidine, and 2′-deoxycytidine) released from both the terminal and internal positions of cellular DNA. It was found that, as expected, the vast majority of these nucleosides were at the internal position. For example, about 1.4 % of the 2′-deoxyadenosine released from DNA of HL 60 cells were found to be at the terminal position. This control study clearly shown that this method is highly reliable to quantify the amount of nucleosides incorporated at the internal and terminal sites, suggesting that the amount of fludarabine incorporated at different sites of DNA measured by this LC-MS/MS method was accurate.

5.3.4 Detecting the Fludarabine Incorporation in DNA Extracted from Blood

The ultimate application of the method developed in this work is the detection of the fludarabine incorporation sites in cellular DNA extracted from blood, because this will allow for the study of the correlation of incorporation with the patient’s response to the drug at the molecular level. After developing and validating the method, we applied it to study of the fludarabine incorporation in cellular DNA extracted from the blood of a patient who had been subjected from the fludarabine treatment, to demonstrate the feasibility of using this method for clinical application.

Briefly, the blood was drawn at 24 hr after this patient was administrated with fludarabine. Then our collaborators at University Hospitals extracted raw DNA from the blood. After receiving the raw DNA, we further purified the DNA and divided an extracted DNA sample into two aliquots. The first aliquot was subject to enzymatic
hydrolysis by only PDE II and MN. After this hydrolysis, we quantified F-ara-A by LC-MS/MS, which yielded the amount of fludarabine incorporated in the terminal position. In contrast, the second aliquot was subject to hydrolysis by MN, PDE II and ALP. After hydrolysis, we quantified F-ara-A again by using the same LC-MS/MS method, which corresponded to the total amount of fludarabine incorporated. However, in this study, we didn’t observe any fludarabine from the terminal position, which indicated that the majority of fludarabine were incorporated into the internal position of patient’s DNA.

5.4. Conclusion

Fludarabine is an important drug used to treat several cancers, especially chronic lymphocytic leukemia (CLL). The accurate determination of the incorporation position of fludarabine in cellular DNA is critically important to a better understanding of the mechanism of action of this drug, which can in turn allow doctors to develop better therapeutic strategies. The main discovery of this work is that we found that the vast majority of fludarabine was actually incorporated in the internal position of DNA. This result is contradictory to the study of Plunkett et al. [5], in which they found that the overwhelming majority of fludarabine was incorporated in the terminal position.

As described in the introduction section, Plunkett et al., utilized the similar MN/PED II enzymatic system to first hydrolyze DNA extracted from the cell lines that had been treated with the radio-active isotope labeled fludarabine, followed by HPLC separation and liquid scintillation counting. LC-MS/MS is a “gold-standard” method to quantify small molecules, particularly drug molecules, because the LC-MS/MS
quantification is highly accurate and robust. More importantly, an unmatched advantage of LC-MS/MS over liquid scintillation counting is its capability of identifying the products produced from enzymatic hydrolysis. In contrast, liquid scintillation counting is based on detection of molecules containing radio-active isotopes in a given retention time window. In other words, any molecules containing radio-active isotopes will be considered as the products if they have retention in the selected detection window. Although we are not sure that this is the case in the study of Plunkett et al., some of their own experimental results did suggest that their quantification result was not accurate. In their control experiment, Plunkett et al. found that ~15% of the incorporated dTMP appeared at the terminal positions, which suggested that their method greatly over estimated the amount of dTMP in the terminal position because it is hard to imagine as many as 15% of dTMP in the terminal position of DNA.

In this work, an LC-MS/MS method in conjunction with enzymatic hydrolysis has been developed to elucidate the incorporation position of fludarabine in DNA. Fludarabine is one of many nucleoside analogues that are an important class of anti-cancer drugs [14]. Although this method is developed for fludarabine, it can be modified to determine the incorporation positions of other nucleoside analogues drugs into DNA. Fludarabine can also incorporate into RNA [15]. Our preliminary study showed that the incorporation position of fludarabine in RNA could also be determined using the same method developed in this work, suggesting that this method could be used to elucidate the incorporation sites of nucleoside analogous drugs in RNA as well.
5.5. References


Pharmacol. 1995, 36, 181-188.


6.1. Introduction

As described in Chapters 4 and 5, fludarabine, an adenosine analogue, is widely used for the treatment of hematological malignancies including chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and non-Hodgkins lymphomas [1-3]. The principal action of fludarabine is in the inhibition of nucleic acid synthesis [4]. In addition to the incorporation into DNA, fludarabine can also incorporate into RNA [5-7]. In fact, the ability to incorporate into RNA is one of the properties of fludarabine that differ from those of ara-A.
Plunkett et al. was the first to study the action of fludarabine on RNA metabolism [8]. In that study, they utilized the same method [9] involving the radioactive isotope-labeled fludarabine to study the fludarabine incorporation. With that method, they were able to quantify the amount of fludarabine incorporated into RNA. They found that fludarabine was preferentially incorporated into the (poly)A⁺ RNA fraction over the (ploy)A⁻ RNA fraction. In the same study, they also found that approximately 78% of the incorporated fludarabine were located at the terminal position of the RNA chain. Based on this evidence, they suggested that the incorporation of fludarabine into mRNA (as an RNA synthesis terminator) resulted in premature termination of the RNA transcript and impaired its functioning as a template of protein synthesis.

As pointed out above, the method that Plunkett et al. utilized for their RNA study was also based on HPLC-liquid scintillation counting in conjunction with enzymatic digestion of nucleic acids. As described in Chapter 5, the drawback of that method is its inability to directly determine the identity of the products detected and to distinguish two products with the same retention time. In other words, any molecules that contain radioactive isotopes and have the retention time in the detection window would be considered as the target products. Moreover, that method is not highly accurate when used for quantification. Therefore, in the study of Chapters 4 and 5, we developed a LC-MS/MS method in conjunction with enzymatic hydrolysis to study the incorporation of fludarabine into DNA. We found that some of our results generated by using our LC-MS/MS method were different from those resulted from the study of Plunkett et al. For example, we found that the overwhelming majority of fludarabine was incorporated into
the internal positions of the DNA chain, while Plunkett et al. suggested that it was mainly incorporated into the terminal position.

Considering the drawbacks of the conventional method, which led to the different conclusion on the incorporation of fludarabine into DNA, we decided to revisit the incorporation of fludarabine into RNA by using the same LC-MS/MS method developed in the study of Chapters 4 and 5. With this new method, we were able successfully to determine the total quantity of fludarabine incorporated into RNA. In addition, we were able to elucidate the primary incorporation position of fludarabine in RNA in cultured cells. Unlike Plunkett et al.’s result, our study suggested that the majority of the fludarabine incorporated was in the middle position. Reporting the results arising from the use of our LC-MS/MS based method to study the incorporation of fludarabine into RNA constitutes the focus of this chapter.

6.2. Experiment

6.2.1. Materials

Formic acid, acetonitrile, ammonium acetate, acetic acid, adenosine (A), guanosine (G), thymidine (T), cytidine (C), 2-chloro-2′deoxyadenosine (2CdA), thymidine 5′-monophosphate (TMP) and TE buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS), N,N-dimethyl hexylamine, water saturated phenol, optima gradient methanol and chloroform were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium glycinate, calcium chloride (CaCl2), monopotassium phosphate (KH2PO4), dipotassium phosphate (K2HPO4), potassium fluoride (KF) and hydrochloric acid (HCl) were ordered from VWR (West Chester, PA, USA). The 10%
sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). RPMI-1640 medium with L-glutamine was purchased from Mediatech (Manassas, VA, USA). Micrococcal nuclease (MN), phosphodiesterase II (PDE II), bovine alkaline phosphatase (ALP), and snake venom phosphodiesterase I (PDE I) were obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, USA). Fludarabine (F-ara-AMP) was from Ochem (Des Plaines, IL, USA). TRIzol® Reagent was obtained from Life technologies (Grand Island, NY, USA). Deionized water was prepared by a Barnstead Model 7148 Nanopure® ultrapure water system of the Thermo Scientific (Asheville, NC, USA).

6.2.2. Cell Culture

Human promyelocytic leukemia cells (HL60) were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified 5% CO₂ incubator.

F-ara-AMP solutions were freshly prepared for each experiment by dissolving F-ara-AMP powder in PBS (1x, pH 7.4). For each 5 × 10⁶ cells, the dosages of F-ara-AMP added into the culture media were 0.00 or 10.0 µM, respectively. Cells were treated for 24 h at 37 °C in a humidified 5% CO₂ incubator. After treatment, the cells were removed from the medium by centrifugation 1500 x g at 4 °C for 5 min, and washed with 5 mL PBS. After centrifugation (1500 x g at 4 °C for 5 min), the cell pellets collected were stored in -20 °C till RNA extraction.
6.2.3. RNA Extraction

1.00 mL of TRIzol® Reagent was added to the cell pellets. Lyse cells in sample by pipetting up and down several times. After incubating for 5 min at room temperature, 0.2 mL of chloroform was added to the sample. After shaking vigorously by hand for 15 seconds, the sample was incubated at room temperature for 2-3 min, followed by centrifugation at 12.000 x g for 15 min at 4 °C. The mixture separated into a lower red phenol- chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase contained RNA was removed and placed into a new tube. RNA was isolated by adding 0.5 mL of 100% isopropanol. After incubation at room temperature for 10 min, the sample was centrifuged at 12000 x g for 10 min at 4 °C. The RNA pellet was collected and washed with 1 mL of 75% ethanol. Then each RNA sample was air-dried and resuspended in RNase free water at 1.00 mg/mL.

6.2.4. Remove DNA contamination

To each 50 µL of RNA sample (1.0 mg/mL), 5.00 µL of 10X Baseline-ZERO DNase Reaction Buffer and 2.5 µl of Baseline-ZERO DNase were added. After 30 min incubation at 37 °C, the sample was mixed with 1.0 mL of pre-chilled ethanol (-20 °C). The DNA was precipitated at -20 °C for overnight. After centrifugation (15000 x g for 15 min), each sample was washed with 1.0 mL of pre-cooled 70% ethanol (-20 °C) twice. Then each DNA sample was air-dried and reconstituted with 50 µL of deionized water.
6.2.5. Enzymatic Hydrolysis of RNA

Enzymatic hydrolysis was performed with a 3 step reaction by modifying the procedure described by Manor et al [10]. Three enzyme solutions were prepared as following: MN was dissolved in 1 stage buffer solution, which contains 50 mM sodium glycinate, 2 mM CaCl₂, 4 mM TMP, and 0.8 mM TdR at the final concentration of 50 unit/mL; PDE II was dissolved in 2 stage buffer solution, which contains 100 mM potassium phosphate (pH 6.0), 20 mM TMP, 200 mM ammonium acetate (pH 6.0), 80 mM acetic acid and 50 mM KF at the final concentration of 4 unit/mL; ALP was diluted in deionized water at the final concentration of 500 unit/mL. For each 50 µL of RNA sample (1.0 mg/mL), 50 µL of MN solution was firstly added and mixed well. Incubation was carried out at 37 °C for 30 min. The reaction mixture was then cooled in ice, and followed by adding 10 µL of PDE II solution. After incubation at 37 °C for 3 h, sample was identically divided into two vials. One vial was added with 4 µL of ALP and the other was added 4 µL of water. The last step hydrolysis was carried out for 2 h at 37 °C.

6.2.6. Preparation of Calibrators and Quality Controls

The stock standard solutions of F-ara-AMP (4.0 mM), 2CdA (IS) (2.0 mM), and AMP (4.0 mM) were prepared in deionized water respectively. The working solutions of F-ara-AMP (2.00 µM) and AMP (2.00 mM) were prepared by diluting their stock standard solutions with deionized water individually. The standard working solutions of F-ara-AMP (0.80, 2.00, 4.00, 8.00, 40.0, 80.0, 120 and 200 nM) were prepared by serial dilution of F-ara-AMP working solution (2.00 µM). The standard working solutions of AMP (200, 400, 600, 800, 1200 and 2000 µM) were prepared by serial dilution of dAMP
working solution (2.00 mM).

The F-ara-A calibrators (0.40, 1.00, 2.00, 4.00, 20.0, 40.0, 60.0 and 100 nM) were prepared by mixing 25 µL of each F-ara-AMP standard working solution with 25 µL of MN solution, 5 µL of PDE II solution and 4 µL of ALP.

The AMP calibrators (100, 200, 300, 400, 600 and 1000 µM) were prepared by mixing 25 µL of each dAMP standard working solution with 25 µL of MN solution, 5 µL of PDE II solution and 4 µL of ALP.

6.2.7. Digested Product Extraction

4.00 µL of IS working solution (1.00 µM) was added to each above digested sample, and vortex-mixed for 30 s; the sample was then deproteinized with 450 µL of HPLC-grade acetonitrile by vortex mixing for 30 s, followed by centrifugation at 15000 x g for 10 min. 450 µL of supernatant was pipetted into a 1.5-mL microcentrifuge tube and evaporated to dryness at 30 °C for 60 min in a TurboVap® LV Evaporator (Zymark, Hopkinton, MA, USA) under a pressurized stream of nitrogen gas. Finally, each sample was reconstituted in 90.0 µL of deionized water. For the fludarabine measurement, 5 µL of the resulting solution was then injected into LC-MS/MS for the fludarabine measurement. For the adenosine (A) measurement, a separate 5 µL of the 400 x dilution solution was used.

6.2.8. LC-MS/MS

The liquid chromatography tandem mass spectrometry system was comprised of an AB Sciex QTRAP 5500 mass spectrometer (Foster City, CA) and a Shimadzu
Prominence UFLC system (Kyoto, Japan) composed of a solvent reservoir, a degasser (DGU-20A3), a binary pump (LC-20AD), a flow controller (CBM-20A), and an autosampler (SIL-20ACHT). Data was acquired and analyzed by Analyst software (version 1.5.1). The separation of F-ara-A, and IS was performed on a Waters Atlantis® T3 (3 µM, 2.1 mm x 50 mm) column (Waters, Milford, MA, USA) by gradient elution at ambient at the flow rate of 0.20 mL/min. The eluents used consisted of mobile phase A (MPA) containing 0.1% formic acid in ultra-pure water and mobile phase B (MPB) containing methanol. The program was initiated with 12.5% MPB from 0.0 to 1.0 min, 12.5-50% MPB from 1.0 to 4.0 min, 50-12.5% MPB from 4.0 to 4.1 min, and 12.5% MPB to 5min. The injection volume of each sample was 5 µL. The equilibrate time between each injection was 5 min. ESI-MS/MS was operated on the positive-electrospray-ionization (ESI+) mode. The mass spectrometry parameters were set as follows: CUR, 30; IS, 5500; TEM, 300; G1, 40; G2, 40; DP, 50; EP, 5.0; CAD, medium; CE, 30.0; CXP, 13.0; and Dewell Time, 100 ms. Detection of F-ara-A, IS, A, G, C, U and dT was achieved on MRM with the mass transition of 286 > 154, 286 > 170, 268 > 136, 284 > 152, 244 > 112, 245 > 113, and 243 > 127 m/z respectively.

6.3. Results

6.3.1. Experimental Design and Enzymatic Digestion

In this work, an LC/MS/MS method in conjunction with enzymatic hydrolysis of RNA was developed to quantitatively determine the total amount of fludarabine incorporated into RNA as well as the amount of fludarabine incorporated at both the terminal and internal positions in RNA. The enzymes used were MN, PDEII and ALP,
which were the same to those described in Chapter 5. The enzymatic system can hydrolyze both DNA and RNA. A combination of MN and PDEII leads to the complete hydrolysis of RNA into single nucleotides with 3’-phosphate (from internal positions) and single nucleosides without phosphate (from terminal positions). In other words, the product generated from the internal nucleosides would be different from that produced from the terminal nucleosides. A combination of MN, PDE II and ALP leads to the complete hydrolysis of RNA into single nucleosides without phosphate. In other words, all the incorporated fludarabine molecules are converted to F ara-A regardless of the incorporation positions. We used LC-MS/MS to quantify the total amount of fludarabine incorporated after this 3-enzymes hydrolysis reaction.

The similar experimental procedure described in Chapter 5 was used in this study. Briefly, we first divided an extracted RNA sample into two aliquots. The first aliquot was subject to enzymatic hydrolysis by only PDE II and MN. After this hydrolysis, we quantified F ara-A by LC-MS/MS, which was the amount of fludarabine incorporated in the terminal position of RNA. In contrast, the second aliquot was subject to hydrolysis by MN, PDE II and ALP. After hydrolysis, we quantified F ara-A again by using the same LC-MS/MS method, which yielded the total amount of fludarabine incorporated.

6.3.2. Determination of the Enzymatic Hydrolysis Products

Since the 3-enzyme digestion can hydrolyze an RNA sample into A, G, U and C along with F ara-A, we first established the separation and detection conditions for monitoring all digestion products. In this work, we used 2-Chloro-2’deoxyadenosine (2CdA) as internal standard (IS) for quantification of F ara-A, and dT as IS for
quantification of A. By using the LC-MS/MS condition described in Section 6.2.8, all 7 molecules were well separated and detected. Fig. 6.1 shown the results of hydrolysis of an RNA sample that was treated by 10 μM fludarabine for 24 h. dT was added to the enzyme buffer system. 2CdA was spiked into the sample after hydrolysis by the 3-enzyme system. As expected, F-ara-A along with G, A, U, C, dT and 2CdA, was detected from this treated sample, demonstrating that our enzyme system could digest RNA well and the expected digestion products could be well separated and detected. We also conducted a control experiment to ensure that the F-ara-A molecules detected resulted from hydrolysis of RNA, not from the residual fludarabine used to treat cells. In this quality control experiment, we attempted to detect F-ara-A from a treated RNA sample that was not hydrolyzed. It was seen that without hydrolysis, no F-ara-A was detected (data not shown here), suggesting that the F-ara-A molecules detected was originated from the F-ara-A molecules that had been incorporated to cellular RNA.

Next, we further studied hydrolysis by either the 2-enzyme or 3-enzyme systems. In this study, HL60 cells were first collected. RNA was extracted and divided to two identical parts. The first part was incubated with MN and PDE II only. As discussed above, after hydrolysis by MN and PDE II, fludarabine incorporated at the terminal site was released in the form of F-ara-A. In other words, the F-ara-A molecules detected from hydrolysis of RNA by MN and PDEII was those incorporated at the terminal position. The second part of the RNA sample aliquoted was first incubated with MN and PDE II along with ALP, followed by analysis by LC-MS/MS. After the 3-enzymes hydrolysis reaction, all of the incorporated fludarabine molecules were expected to convert to F-ara-A. Therefore, the amount of the F-ara-A molecules detected from hydrolysis of RNA by
MN, PDEII and ALP corresponded to the total amount of fludarabine incorporated into RNA.

As shown in Spectra of Fig. 6.2A1 and A2, when we studied RNA collected from the cells that were not treated by drug, as expected, no F-ara-A was observed. Next, we studied RNA collected from the cells treated with 10 µM fludarabine for 5 or 24 h. For the 5hr treatment sample, F-ara-A (Fig. 6.2B2) was detected when RNA was hydrolyzed by the three enzymes system, but not observed with the two enzymes system (Fig. 6.1B1). For the 10 hr treatment sample, F-ara-A was detected with both enzyme systems (Figs. 6.2C1 and C2). Clearly, those results demonstrated that (1) the fludarabine incorporated into RNA could be released by our enzyme systems and detected by our LC-MS/MS method; 2) the fludarabine incorporated at the different positions can be distinguished by using two different enzymatic systems.
Figure 6.1. Representative MRM chromatograms of enzymatic digested products from cellular RNA with 10 µM of drug treatment for 24 h and IS by MN & PDE II + ALP.
Figure 6.2. Representative chromatograms of enzymatic-digested products from drug treated RNA and controls. (A1): No drug treated HL60 RNA digested by MN & PDE II Fludarabine; (A2): No drug treated HL60 RNA digested by MN & PDE II + ALP; (B1) 5 hr treated HL60 RNA digested by MN & PDE II; (B2): 5 hr treated HL60 RNA digested by MN & PDE II + ALP; (C1): 24 hr treated HL60 RNA digested by MN & PDE II; (C2): 24 hr treated HL60 RNA digested by MN & PDE II + ALP.
6.3.3. Quantification of enzymatic hydrolysis products

In this study, we utilized the LC-MS/MS method developed in the study of Chapters 4 and 5 to quantify the amount of F-ara-A incorporated. The calibration curve of F-ara-A was developed with seven non-zero F-ara-A calibrators (0.40, 1.00, 2.00, 4.00, 20.0, 40.0, 60.0 and 100 nM) along with IS in each at a fixed concentration (100 nM) (Fig 6.3A). The calibration equation derived from three validation batches using 1/x^2 weighted linear regression was \( Y = 0.00771 X + 0.0137 \) (where \( Y \) is the peak area ratio of fludarabine to IS and \( X \) is the concentration of fludarabine) with a coefficient of determination \( (r^2) \) of 0.997.

As discussed above, the amount of fludarabine incorporated at the terminal position and the total amount of incorporated fludarabine can be quantified, respectively, by using two different hydrolysis approaches, from which the amount of fludarabine incorporated at the internal position could also be deduced. In this study, 3 batches of HL60 cell line were analyzed repeatedly under the same conditions to obtain more accurate results. Each batch of cells was treated with 10 uM of fludarabine for 24 h. After RNA extraction, each RNA sample was divided to two identical parts. To quantify the amount of fludarabine at the terminal position, the extracted RNA was incubated with MN and PDE II only. To quantify the total amount of incorporated fludarabine, the extracted RNA was incubated with MN and PDE II along with ALP. This study showed that 96.7% of fludarabine was incorporated in the internal position in cellular RNA extracted from HL60, while only 3.3% of fludarabine was incorporated at the terminal position.
Figure 6.3, Calibration curves (A) F-ara-A (0.4 -100 nM) and (B) Adenosine (100 -1000 uM).
In this study, we also studied the drug incorporation rate, which was expressed as the ratio of \([\text{F-ara-A}]/[\text{A}]\). Therefore, we quantified both total F-ara-A and A from the same RNA sample after hydrolysis of RNA by the 3-enzyme system. A calibration curves were constructed by using six non-zero A calibrators (100, 200, 300, 400, 600 and 1000 µM) along with dT. The calibration equation derived from three validation batches using \(1/x^2\) weighted linear regression was \(Y = 0.0042X + 0.0675\) (where \(Y\) is the peak area ratio of A to dT and \(X\) is the concentration of A) with a coefficient of determination \((r^2)\) of 0.9994.

HL60 cells were treated with 10 µM fludarabine for 5 and 24 hr, respectively. The results were summarized in Table 6.1. The measured incorporation rate expressed as the ratio of \([\text{F-ara-A}]/[\text{A}]\) ratio was 0.43/10,000 after 5 h treatment, while it was 1.45/10,000 after 24 h treatment. For comparison, we also measured the amount of F-ara-A incorporated into DNA from the same cell samples. The DNA incorporated rate expressed as the ratio of \([\text{F-ara-A}]/[\text{dA}]\) was 0.44/10,000 after 5 h treatment, and 4.70/10,000 after 24 h treatment.
Table 6.1, The rate of fludarabine incorporated into both RNA and DNA

<table>
<thead>
<tr>
<th>Sample Type (HL60)</th>
<th>[Fludarabine] treated, µM</th>
<th>Treated Time,</th>
<th>Drug Incorporated Rate (F-ara-A/A) pmol/mg</th>
<th>Drug Incorporated Rate (F-ara-A/dA) pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>10</td>
<td>5</td>
<td>0.43/10,000</td>
<td>N/A</td>
</tr>
<tr>
<td>RNA</td>
<td>10</td>
<td>24</td>
<td>1.45/10,000</td>
<td>N/A</td>
</tr>
<tr>
<td>DNA</td>
<td>10</td>
<td>5</td>
<td>N/A</td>
<td>0.44/10,000</td>
</tr>
<tr>
<td>DNA</td>
<td>10</td>
<td>24</td>
<td>N/A</td>
<td>4.70/10,000</td>
</tr>
</tbody>
</table>
6.3.4. DNA interference and removal

Since fludarabine can incorporate into both DNA and RNA, a potential problem associated with measurements of fludarabine incorporated into RNA is interference from the DNA contamination. After extracting RNA from cell lysates by TRIzol® Reagent, we hydrolyzed the RNA samples by MN, PDEII and ALP. Deoxyribonucleosides including dA, dC, and dG were observed in hydrolysis products, indicating the presence of the DNA contamination. To solve this problem, Baseline-ZERO DNase was added to our RNA sample. In this study, extracted RNA was divided into two parts. The first part was not subject to further treatment, while the second part was treated by Baseline-ZERO DNase. As shown in Table 6.2, when the RNA sample was treated with Baseline-ZERO DNase, the quantity of dNs was reduced by over 20 folds, indicating that treatment by Baseline-ZERO DNase led to effectively remove the DNA contamination. Moreover, after removing the DNA contamination, the amount of F-ara-A was reduced by about 3 folds, indicating that without the Baseline-ZERO DNase treatment, the amount of F-ara-A measured was due to incorporation to both DNA and RNA. This study also suggested that after treatment of RNA by Baseline-ZERO DNase, the contribution of DNA to the quantity of F-ara-A measured was insignificant. In other words, with the Baseline-ZERO DNase treatment, the F-ara-A molecules detected by this method originated mainly from RNA, not from DNA. Therefore, in the remaining of the study of this chapter, all samples were treated with Baseline-ZERO DNase.
Table 6.2, Comparison of Treatment with and without Baseline-ZERO DNase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>dG Rel. Int. (%)</th>
<th>dA Rel. Int. (%)</th>
<th>dC Rel. Int. (%)</th>
<th>F-ara-A Rel. Int. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without DNase</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>With DNase</td>
<td>4.8</td>
<td>5.3</td>
<td>4.3</td>
<td>20.6</td>
</tr>
</tbody>
</table>
6.4. Conclusion

In this work, an LC-MS/MS method in conjunction with enzymatic hydrolysis has been utilized to elucidate the incorporation position of fludarabine in RNA. Fludarabine is an important drug used to treat several cancers, especially chronic lymphocytic leukemia (CLL). The accurate determination of the incorporation position of fludarabine in cellular RNA is also critically important to a better understanding of the mechanism of action of this drug, which can in turn allow doctors to develop better therapeutic strategies. The main discovery of this work is that we found that the vast majority of fludarabine was actually incorporated in the internal position of RNA. This result is contradictory to the study of Plunkett et al. [8], in which they found that the majority of fludarabine was incorporated in the terminal position.

As described in Chapter 5, Plunkett et al., utilized the similar MN/PED II enzymatic system to first hydrolyze nucleic acids extracted from the cell lines that had been treated with the radio-active isotope labeled fludarabine, followed by HPLC separation and liquid scintillation counting. An unmatched advantage of LC-MS/MS over liquid scintillation counting is its capability of identifying the products produced from enzymatic hydrolysis. In contrast, liquid scintillation counting is based on detection of molecules containing radio-active isotopes in a given retention time window. In other words, any molecules containing radio-active isotopes will be considered as the products if they have retention in the selected detection window. As discussed in Chapter 5, the inability of their method to identify hydrolysis products may be a potential source of overestimating the amount of fludarabine incorporated in the terminal position by Plunkett et al. Fludarabine is one of many nucleoside analogues that are an important
class of anti-cancer drugs [14]. Although this method is developed for fludarabine, it can be modified to determine the incorporation positions of other nucleoside analogues drugs into RNA.

6.5. Reference


