ARTEMISININ AND ITS DERIVATIVES REACTIONS: CHARACTERIZATION OF THE REACTION PRODUCTS USING LC/TOF MS

Kogila Vijayan
Rowan University

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ARTEMISININ AND ITS DERIVATIVES REACTIONS:
CHARACTERIZATION OF THE REACTION PRODUCTS USING LC/TOF MS

by

Kogila Vijayan

A Thesis

Submitted to the
Department of Chemistry and Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
Master of Science in Pharmaceutical Sciences
at
Rowan University
January 15, 2023

Thesis Chair: Amos Mugweru, Ph.D., Professor, Department of Chemistry and Biochemistry

Committee Members:
Subash Jonnalagadda, Ph.D., Professor, Department of Chemistry and Biochemistry
Kandalam Ramanujachary Ph.D., Professor, Department of Chemistry and Biochemistry
Dedication

I dedicate this thesis to my beloved parents, Susila Muthusamy and Vijayan Muthappan, my beloved husband Adetola Oke, and to my daughter Ruby Oke. Thanks for the guidance, love, and unconditional support throughout my thesis studies. Thanks for being there for me.
Acknowledgement

I would like to express my deepest gratitude to my research advisor Dr. Amos Mugweru for his guidance and for the invaluable research experience I gained in his lab. Thanks for the time you dedicated to teaching me analytical chemistry and other methods of analysis. I wish to thank the members of my thesis committee Dr. Subash Jonnalagadda and Dr. Kandalam Ramanujachary for their advice on this research project and on career paths to follow this program. Also, thanks for being supportive and understanding.

Thanks to Rowan University Department of Chemistry and Biochemistry for the Research Fellowship and Teaching Fellowship positions awarded to me during this program. Fellowship provided as well departmental facilities that enabled this work.

I also want to acknowledge my family for their encouragement, patience, and support throughout my educational journey.
Abstract

Kogila Vijayan
ARTEMISININ AND ITS DERIVATIVES REACTIONS: CHARACTERIZATION OF THE REACTION PRODUCTS USING LC/TOF MS
2019-2021
Amos Mugweru, Ph.D.
Master of Science in Pharmaceutical Sciences

Artemisinin (ART) is a sesquiterpene lactone and a popular malaria drug with potential anticancer properties. In this work, LC/TOF MS was used to investigate the reaction of ART with DNA bases and estradiol. ART-deoxyadenosine and ART-deoxycytidine interactions were studied in the presence of Fe (II) ions. ART-deoxyadenosine and ART-deoxycytidine reaction mixtures gave chromatographic signatures that remained unchanged at room temperature but grew after incubation at 37°C. The change in temperature from room temperature to 37°C was the main driver of adduct formation in these reactions. ART was found to react with Fe (II) ions as observed from several new chromatographic peaks. ART-deoxyadenosine as well as ART-deoxycytidine in the presence of Fe (II) ions resulted in the formation of new chromatographic signatures of adducts consisting of DNA bases and ART. It was clear that the addition of Fe (II) to DNA base-ART mixtures gave rise to new reaction products mediated by a different reaction mechanism. Artemisinin-estradiol reactions were studied in the presence of Fe (II) ions. Artemisinin-estradiol reaction mixtures gave chromatographic signatures that remained unchanged at room temperature but grew after incubation at 37°C. Artemisinin-estradiol in the presence of Fe (II) ions resulted in new chromatographic signatures of adducts of estradiol with artemisinin. It was clear that the addition of Fe (II) to estradiol-artemisinin mixtures gave rise to new reaction products mediated by a different mechanism. Studies of ART reactions with DNA and estradiol in vitro are key in elucidating the elusive mechanism of this drug.
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Chapter 1
An Introduction to LC-MS TOF Analysis of Pharmaceuticals

Chromatographic Analysis of Pharmaceuticals

The pharmaceutical industry has relied on chromatographic techniques for many years to monitor the purity, stability, safety, and efficacy of the drug products [1]. Chromatographic techniques have been widely incorporated in the investigation of the active pharmaceutical ingredients (API), to meet standard criteria established worldwide [2, 3].

Chromatography is an analytical technique used to characterize individual components in a mixture. Chromatographic analysis has been widely used for qualitative and quantitative studies in the identification, and purification of the pharmaceutical drugs and proteins [4]. Chromatographic analysis of pharmaceuticals dates to the 1920s [5]. From the simplest forms of chromatography (Thin layer and Paper chromatography) to the advanced forms (Gas chromatography and liquid chromatography) have been applied in the separation of drug products and biomolecules [6, 7]. At present, chromatographic techniques has been incorporated in the analysis of the active pharmaceutical ingredients, drugs, impurities, final products, and others [3, 8].

Principle of chromatographic technique comprises of mobile phase, stationary phase, and an analyte [4]. Mobile phase is a liquid or carrier gas that passes through the column; stationary phase is a solid or a liquid coated onto a solid support [9]. Analyte is a substance undergoing chromatographic analysis and to be separated.
During the analysis, the sample to be analyzed are injected and carried through the system by the mobile phase. When the mobile phase passes through the stationary phase in the column, the analyte are separated according to their affinity to the phases [10].

**Chromatographic Techniques Often Used for Drug Analysis**

There are various types of chromatography including paper chromatography, thin layer chromatography, column chromatography, liquid chromatography, high pressure liquid chromatography, gas chromatography, ion-exchange chromatography, gel-permeation chromatography, affinity chromatography, hydrophobic interaction chromatography and others [4]. Chromatography often categorized into two types; liquid or gas chromatography based on the mobile phase used [11].

Liquid chromatography (LC) is a separation technique used to separate components of a mixture into their individual entity. The separation occurs based on the affinity of the substance to the mobile phase (liquid) or stationary phase [12]. Common liquid chromatographic system composed of an eluent reservoir, pump, sampler, chromatographic column, detector, data recording and processing unit.

Gas chromatography (GC) used in the characterization of volatile and semi volatile substances in a sample. GC separates components mainly based on their differences in boiling point, vapor pressure and polarity [13]. A GC system composed of a carrier gas, injector, column, detector and data recording and processing unit [14]. The separation occurs based on the affinity of the substance towards the mobile phase (gas) or stationary phase (solid or liquid) [15, 16].
Advances in Chromatography for Drug Analysis

Complexity in the drug discovery process and high regulation of pharmaceutical drug products demand for advancement in the chromatographic methods [1, 17]. Recent advances in the chromatographic methods include High performance liquid chromatography (HPLC), capillary electrophoresis, fluorescence chromatography and others [18]. Addition of mass spectrometry function to chromatography enhances the sensitivity, efficiency, and resolution of the method. Coupling of mass spectrometry to chromatography has been considered as an ideal method for analysis due to its high sensitivity and specificity [19, 20].

Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical technique used in analytical chemistry to detect and identify the amount, chemical properties and structural properties of a given analyte [21, 22]. Mass spectrometer functions by ionizing the analyte into a charged state and analyse the resulting ions and the fragments based on their mass-to-charge ratio (m/z) [19]. Mass spectrometry instrumentation composed of three primary parts; the ion source, mass analyser and detector which are responsible for three processes; ion creation, ions separation and ions detection respectively [23].

The role of ion source in the mass spectrometer is to ionize the sample [24]. Ion source generates gas phase ions where the analyte are transferred into gas phase and undergo subsequent ionization [21]. There are different types of ion sources available such as Atmospheric Ionization (API), Electrospray Ionisation (ESI), Matrix Assisted Laser Desorption Ionization (MALDI), and others. ESI and MALDI are two popular techniques frequently employed in the analysis of proteins [25].
MALDI is a soft ionization process used in the analysis of large molecules. MALDI functions by mixing the sample with a suitable organic matrix material and subsequent laser irradiation which ionizes the sample molecules and convert them into gaseous phase. The resulting ions are moved to the analyzer for separation and detection based on their mass-to-charge ratio (m/z) [26]. MALDI generates one or two charge per molecule often makes the ions fall out of the mass range. This is the reason that MALDI often used with TOF analyser. ESI on the other hand, is able to produce multiple charged ions considered to be more relevant [27]. Electrospray ionization is useful to study wide range of biomolecules from small molecules to large molecules as well noncovalent biomolecular complexes [28]. ESI can be used for protonation of a molecule and have been used to analyze biological molecules such as proteins, peptides, oligonucleotides [28, 29]. ESI functions by generating a fine spray of charged droplets attracted toward the MS inlet where the charged ions are detected based on their mass-to-charge ratio (m/z) [19].

Mass analyzer receives the ionized masses and categorize them based on their mass-to-charge to ratio and outputs them to a detector. There are various types of mass analyzer available such as Quadrupole analyzers, Time-of-flight analyzers (TOF), Ion Trap analyzers, hybrid analyzers, Orbitrap [19]. Quadrupole mass analyzer made of a set of four conducting parallel rods. The rods are electrically paired with each other via radio frequency (RF) and direct current (DC) power supplies [30]. This produces an oscillating electric field for the ions to pass through and filtered based on their m/z value. The Quadrupole analyzer function as a mass filter where it allows only single m/z value ions can reach the detector during the scanning [31]. Time-of-flight analyzer is widely employed in the mass spectrometry due to its versatility and sensitivity. It is
often paired with MALDI in the analysis of biomolecules [32]. TOF mass analyser accelerate ions through a high voltage where the ions travel through the TOF tube and characterized based on their velocity [33].

Mass detectors in the mass spectrometry detect the separated charged ions as they pass through the detector based on the charge induced or current produced. Mass detector is the final element of the mass spectrometer and considered as the eye of the instrument. There are different types of ions detectors available such as Electron Multiplier, Faraday Cups, Photographic Plates, Scintillation Counter, High Mass Detection detectors, advanced detectors and others [34]. Electron Multiplier (EM) and Faraday Cups (FC) detectors have been used in MS for many years. FC detectors are considered as simple detectors and have been used extensively in the early days to measure charged particles in vacuum. FC cup is a metal conducting cup used to measure the charged particle beams in the form of current to determine the number of ions. The mechanism involves signal amplification; when the ions hit the FC, the current produced from the electron emission is amplified and recorded [34, 35]. Electron Multiplier is the most commonly used detector in the modern MS due to its desirable output. It has low noise, cost less, and has good signal amplification [36]. EM works on the principle of ion beam amplification [31]. When ions hit the dynode, secondary electrons will be released, cascade of the signal will be amplified and sent to be output instrument [37].

**Liquid Chromatography Time-of-Flight Mass Spectrometry (LC/TOF MS)**

Liquid chromatography is a technique used in pharmaceutical industry for sample analysis. LC analysis system is frequently coupled to a mass spectrometry (MS) for an additional mass analysis feature of the compounds of interest [19]. Liquid
chromatography-mass spectrometry (LC-MS) is a powerful analytical method that combines the separation feature of the liquid chromatography to the mass detection of mass spectrometry [38]. LC-MS instrumentation comprises of a LC unit, an interface between the LC and MS, an ion source, a mass analyzer, and a detector [39]. LC separates the sample components, introduces them to the mass spectrometer. Ion source ionizes the sample attracted toward the MS inlet. ESI functions by generating a fine spray of charged droplets channeled toward the MS inlet. Nebulizer gas helps in the formation of droplets and the dry gas evaporates the solvent from the analyte [36]. Mass spectrometer forms and detects charged ions based on their mass to charge ratio (m/z) [19]. LC/TOF MS is an advanced LC-MS system equipped with Time-of-flight (TOF) for an enhanced sensitivity and mass accuracy. LC/MS data provides details on the molecular mass, chemical and structural characterization of the substance of interest [40, 41]. A schematic representation of mechanism of Electrospray Ionization (ESI) in the MS shown in Figure 1.

Figure 1. Schematic representation of Mechanism of Electrospray Ionization (ESI).
**Research Objective**

Artemisinin, prescribed and orally taken by millions of people for anti-malarial treatment, presents no observed side effects. On the other hand, conventional cancer drugs present formidable side effects. Artemisinin and its derivatives are promising potential drugs for cancer. Clinical studies have shown anticancer activity of ART towards cancer cells. The proposed mechanism of antitumor action of ARTs mainly involved in apoptotic cell death which has been confirmed by most literatures. Recognized endoperoxide bridge pharmacophore could be reduced by heme or free ferrous iron to generate carbon free radical and reactive oxygen species. Drug and DNA interaction can enhance understanding of its mechanism by unravelling structural changes of the drug.

The main aim of the current work is to investigate the MOA of Artemisinin and its derivatives reaction with DNA, proteins, and other biomolecules. Our work has been focused to investigate the reaction of the Artemisinin and its derivatives with the DNA bases in the presence of Fe (II). An active derivative of artemisinin (artesunate) was also subjected into characterization and stability study in different solvent systems. On the other hand, Artemisinin was reacted with Estradiol in presence of Fe (II) and the reaction products were analyzed. We also establish the role of the temperature in the stability of the drugs and their reactions toward the DNA bases and estrogen. LC/TOF MS was used to identify the reaction products.
Chapter 2

Artemisinin DNA Base Interaction Studies in Presence of Fe (II): LC/TOF MS

Separation of Reaction Products

Introduction

Artemisinin (ART) is a sesquiterpene lactone consisting an internal peroxide bridge also known as a 1,2,4-trioxane ring system [42]. ART is effective against malaria, a disease caused by the *Plasmodium falciparum* parasite [43]. Due to its low solubility and short half-life, other semi-synthetic derivatives have been developed [44]. Dihydro-artemisinin, one of the semi-synthetic forms of ART, was derived from the primary active metabolite of ART [45]. Other first generation semi-synthetic forms of ART are arteether and artemether (lipid soluble), and artesunate (water soluble) [46].

Several studies and case reports show ART exhibiting anticancer properties against cancer cells [47, 48] as well as providing enhanced synergistic activity with other anti-cancer agents [49]. The mechanism of action of ART is not well understood. However, the endo-peroxide bridge of ART is thought to play a key role in the overall mechanism of action [50]. Interaction of the endo-peroxide of ART with heme in proteins is speculated to form free radicals causing cell injury and apoptosis in cells [51]. The formation of carbon centered free radicals when ART interacts with heme proteins is well known [52]. However, some research indicates that ART and its derivatives go through Fenton-like reactions to kill cancer cells [53, 54]. ART has been reported to exhibit antileishmanial activity in particular against visceral leishmaniasis [55]. New reports indicate that this drug can be used as an anti-mycobacterial [56]. Despite ART’s potential uses and decades of research, knowledge gaps concerning its mechanism and
its specific targets remain glaring. For example, in a new pharmacophoric study on the role of the peroxide bond in dioxanes against Leishmania parasites no role was found for the peroxide bridge [57]. In fact, tetrahydropyran, an epoxide, was found to induce a higher increase in intracellular reactive oxygen species (ROS) levels than its endoperoxide counterpart. This implied that generation of free radicals by cleaving the O–O bond is not the main mechanism of action in these peroxides. Presence of free iron also did not affect the free radical generation [57]. This contrasts with multiple other studies done previously that indicated presence of Fe (II) ions, mainly from the decomposition of heme, are paramount in artemisinin’s Leishmania efficacy [58].

The anticancer effects of ART and its derivatives are due to the latter’s ability to induce apoptosis through cellular processes including DNA damage. The iron-mediated cleavage of the endoperoxide bridge in ART could trigger the formation of the carbon centered radicals that can induce ferroptosis [59]. In general, Fe (II) in the presence of ART can trigger generation of free radicals through a Fenton-type reaction. Studies on Drug and DNA interactions are important in chemotherapeutics [60]. Research into such interaction can enhance understanding of the mechanism involved by unravelling structural changes undergone by the drug. Products of the reaction can also help tailor new therapies. A rapid separation method to help unravel the reaction products as well as help qualitatively identify the reaction products is key to the search of the reaction mechanisms.

The aim of the current work is to develop new chromatographic methods and use them to investigate the reaction of ART with DNA bases, and to establish the role of Fe (II). LC/TOF MS was used to establish the reaction products. Reaction of ART with any
DNA base is key in unravelling part of the mechanism involved in the drug’s anticancer activity. For the first time, the LC/TOF MS technique was used to clearly show that ART’s reaction with DNA bases followed a different mechanism from that which occurs in the presence of Fe (II). The role of the temperature in the activity of the drug towards the DNA bases was also established.

**Materials and Methods**

**Chemicals and Reagents**

ART was purchased from Tokyo Chemical Company (TCI). DNA base (2-deoxyadenosine) was purchased from sigma. DNA base (2-deoxycytidine), and Iron (II) Chloride were purchased from Alfa Aesar. Methanol, acetonitrile, ammonium acetate and water, all LC-MS grade, were purchased from VWR. Solutions of ART and its derivatives containing 20 mM Ammonium acetate: Methanol, 15:85 was prepared prior to each experiment. All other chemicals were LC-MS grade.

**Chromatographic Procedure**

Standards of ART and DNA bases were analyzed using TOF/LC-MS. Separation of reaction products ART-deoxyadenosine and ART–deoxycytidine was carried out using Agilent Technologies, ZORBAX Extended C-18 column (2.1x 50 mm) with 1.8 µm diameter particles. An autosampler was used to inject the sample, and the temperature was set to 25°C. The mobile phase consisted of Methanol and water 70:30, respectively. The LC-MS analysis was carried out using Agilent 6230 series LC-MS TOF unit. The chromatographic run was performed under isocratic conditions at the flow rate of 0.300 mL/min. Every end of the run followed by a post run step that included flushing the column with mobile phase for 5 min. The column temperature was set at 25°C.
**Mass Spectrometer Procedure**

Mass spectrometry detection for the LC/MS was with a TOF 6230 series set to Dual AJS ESI positive ion mode. The settings of the mass spectrometer were as follows: scan mode (standard), range (200-3000) m/z, threshold (200), nebulizer gas (40.0 psi), and dry gas (8.0 L/min), dry temperature (325°C). The compound stability and trap drive level were set to 100%. The MS/MS fragmentation amp was set to 175 V and skimmer cone at 65 V.

**Sample Preparation**

**Artemisinin-DNA Reactions.** Solid mixtures of ART and 2-deoxyadenosine were placed in a 5 mL volumetric flask. In another 5 mL volumetric flask, ART and 2-deoxycytidine were added. A buffer solution containing 15% ammonium acetate (20.0 mM) and 85% methanol was added to the two volumetric flasks containing the solid mixtures. The final concentration of ART was $1.4 \times 10^{-2}$ M while that of 2-deoxyadenosine was $1.6 \times 10^{-2}$ M. The final concentration of 2-deoxycytidine was $1.8 \times 10^{-2}$ M. Two more solutions were made following the same procedure, but with $2 \times 10^{-3}$ M Fe$^{2+}$. Equal mixtures of ART and 2-deoxyadenosine were placed in a 5 mL volumetric flask, and 3 mL of Iron II chloride solution was added to the solid mixture of ART and 2-deoxyadenosine. A buffer solution was then used to make up the 5 mL volume mark. The concentrations of ART and 2-deoxyadenosine were $1.4 \times 10^{-2}$ M and $1.6 \times 10^{-2}$ M, respectively. The final solution contained $1.4 \times 10^{-2}$ M ART, $1.8 \times 10^{-2}$ M 2-Deoxycytidine and $2 \times 10^{-3}$ M Fe$^{2+}$. For analysis, 1 mL of the sample mixture was drawn, filtered, and transferred into individual vials. Comparisons were made between solution mixtures left at room temperature versus those incubated at 37°C.
Some sample mixtures were incubated at 37°C for different lengths of times before analysis.

**Optimization of the Reaction Conditions**

The concentration of standards and the chromatographic procedures were adjusted and optimized for the optimal reaction and elution of the reaction products. Initially, the ART, 2-deoxyadenosine and 2-deoxycytidine standards were analysed individually. Various ratios of solvents were investigated in order to optimize solubility of both the ART and DNA bases as well as to effectively separate samples. The ratio of the solvents for the sample mixture preparation, 20 mM Ammonium acetate: methanol, was adjusted to 15:85 to facilitate the solubility of the DNA base with ART. Methanol and water were used as the mobile phases for the LC-MS method; the water contained 0.1% formic acid. The ratio was adjusted to 70:30 for proper elution of the standard and reaction products. A column with the dimensions 2.1 × 50 mm, with 1.8 µm was used for faster and more precise separation of the reaction products. In this experiment, the incubation temperature was set to 37°C and the column temperature was maintained at 25°C. After optimization of the reaction conditions, each analysis were carried out three times.

**Results**

**ART-Deoxyadenosine**

Figure 2 shows the chemical structure of ART and its derivatives. Reaction of ART with deoxyadenosine was monitored by obtaining chromatograms of mixtures at room temperature and at 37°C. Figure 3 shows chromatograms of sample mixtures, first analyzed immediately after mixing and then analyzed again each day for four days. On the first day, only two chromatogram peaks corresponding to the two reactants were
obtained, at or around retention times 0.4 and 1.3 min. On the third day of incubation at 37°C new chromatographic peaks at retention times, 0.7, 0.8, 0.9, 1.1 and 1.2 min were visible. Although the intensities of these peaks were very small, they signify formation of new molecules between ART and deoxyadenosine. These new chromatographic reaction product peaks grew with time of incubation, while the parent reactant peaks reduced. The initial reactant signature peaks decreased by about 17 ± 2% to 30 ± 4%, while the new product peaks grew by about 41 ± 4% to 100 ± 5% during the time of incubation. Further analysis of these reaction products was carried out using mass spectra. Mass spectra corresponding to Figure 3 are shown in Figure 4 and indicate deoxyadenosine molecular ion \([\text{M+H}]^+\) at \(m/z\) 252. Other ions include \([\text{M+Na}]^+, [\text{M+ K}]^+, \) and \([2\text{M+Na}]^+\) corresponding to \(m/z\) of 274, 290 and 525 respectively. On the other hand ART, with a molecular weight of 282, showed molecular ions \([\text{M+H}]^+, \) at \(m/z\) 283, in addition to other ions such as \([\text{M+Na}]^+, [\text{M+K}]^+, \) \([2\text{M+Na}]^-\) corresponding to \(m/z\) of 305, 321, and 587 respectively.
Figure 2. Chemical structure of artemisinin and its derivatives.

![Chemical structures](image)

Artemisinin  Dihydroartemisinin

Artesunate  Artemether

Figure 3. TIC chromatograms of the ART-deoxyadenosine reaction mixture obtained at room temperature (day zero) and at 37 °C incubation.

![TIC chromatograms](image)
Figure 4. Mass spectrum of artemisinin-deoxyadenosine reaction products at 0.7 minutes (A), 0.8 mins (B), 0.9mins (C), 1.1mins (D) and 1.2 mins (E).

Removal of water from m/z 283 could result in m/z 265. Removal of CO from 265 could give m/z 237. Removal of C2H4 from 237 could yield m/z 209, which was also the mechanism of transition from m/z 219 to m/z 191. Dehydration of m/z 237 could also form m/z 219. These individual fragmented masses were observed in the mass spectra elsewhere [61]. Alkylation reaction most likely occurred in the ART-deoxyadenosine mixture. In principle, alkylation of 2′-deoxyadenosine can occur at nitrogen atoms N1, N6, N7, and N9. Alkylation at multiple location in deoxyadenosine may be one of the key reasons we encountered different chromatographic peaks, however at the same time these peaks showed some similar masses in the mass spectra. The new product chromatographic peaks appearing between 0.7 and 1.2 min are shown on Table 1.
Table 1

**Retention Time and Fragmentation Patterns of Deoxyadenosine Reacted with Artemisinin**

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>251, 337, 353, 399</td>
</tr>
<tr>
<td>0.8</td>
<td>237, 251, 307, 399</td>
</tr>
<tr>
<td>0.9</td>
<td>251, 307, 337, 353</td>
</tr>
<tr>
<td>1.1</td>
<td>251, 283, 305, 337, 353 and 587</td>
</tr>
<tr>
<td>1.2</td>
<td>209, 237, 251, 265, 337 and 353</td>
</tr>
</tbody>
</table>

Although these are pure molecules as observed from single chromatographic peaks. The mass spectra showed a combination of deoxyadenosine and ART, meaning that they are adducts. Possible mechanism of deoxyadenosine (dA) alkylation on the N1, N6, N7, and N9 positions by ART under temperature control was shown on Figure 5. This alkylation mechanism on deoxyadenosine was previously reported as a possibility in [62].
Introduction of Iron (II) in the reaction mixture drastically changed both the number of chromatographic peaks as well as the retention times (Figure 6). To clearly evaluate the role of Fe$^{2+}$, new reaction mixtures were made involving ART with Fe$^{2+}$, $1.4 \times 10^{-2}$ M and $2 \times 10^{-3}$ M, respectively. Figure 6a shows the chromatograms of ART and Fe$^{2+}$. ART by itself showed a peak at 2.0 min retention time. After introduction of Fe$^{2+}$, new chromatographic peaks at 0.91, 0.97, 1.2, 1.4, 1.8, 2.5, 3.1 and 3.6 min retention times were observed. From the mass spectra, the new product chromatographic peaks appearing between 0.91 and 3.6 min retention times are shown in Table 2.
Figure 6. (a) TIC chromatograms for ART vs. ART-Fe$^{2+}$ reaction obtained at room temperature and at 37°C. (b) TIC chromatograms of ART-deoxyadenosine-Fe$^{2+}$ reaction obtained at room temperature and at 37°C.

Table 2

Retention Times and Fragmentation Patterns of Artemisinin in the Presence of Fe$^{2+}$

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.91</td>
<td>265, 283, 305, 314, and 587</td>
</tr>
<tr>
<td>0.97</td>
<td>203, 209, 237, 265, 277, 283, 305, 314, 321 and 358</td>
</tr>
<tr>
<td>1.2</td>
<td>223, 300, 305, 314, 328, 356 and 587</td>
</tr>
<tr>
<td>1.4</td>
<td>203, 209, 251, 297, 332, 337, 346, 360, and 388</td>
</tr>
<tr>
<td>1.8</td>
<td>209, 251, 265, 315, 332, 337, 346, 353 and 360</td>
</tr>
<tr>
<td>2.5</td>
<td>203, 209, 224, 237, 251, 265, 279, 297, 337, 346, 358, 360 and 399</td>
</tr>
<tr>
<td>3.1</td>
<td>209, 251, 346, 360 and 374</td>
</tr>
<tr>
<td>3.6</td>
<td>203, 209, 224, 251, 265, 297, 346, 351, 360 and 374</td>
</tr>
</tbody>
</table>
From these mass spectra, it appeared that ART’s endo-peroxide bond opened up and reacted with itself to form the many ART derived products observed. Based on the short time this reaction took to happen, it appears to be occurring in a free radical fashion.

To establish whether this was the case, DNA bases were included in the mixture in sections that follow below (Figure 6b). Interaction of ART with deoxyadenosine in the presence of iron was monitored by observing chromatograms obtained from samples of ART, deoxyadenosine and Fe$^{2+}$ mixtures and after incubation at room temperature and at 37°C. The sample mixtures were first analyzed immediately after mixing and then again, each day for three days. On the first day, several small chromatogram peaks were obtained at about retention times 0.6, 0.8, 1.2 and 2.0 min (Figure 6b). The chromatographic peaks at retention times 0.6 and 2.0 min were for deoxyadenosine and ART, respectively. Chromatographic peaks at retention times 0.8 and 1.2 were product reaction peaks formed from deoxyadenosine and ART at room temperature. On incubating the mixture at 37°C, three new reaction peaks appeared at about 0.9, 1.4 and 1.8 min, with mass spectra quite different from the previous results without Fe$^{2+}$ ions. These new chromatographic reaction product peaks grew with time of incubation at 37°C. Further analysis of these reaction products was carried out using mass spectra; spectra of the reaction products corresponding to Figure 6b are shown in Figure 7. Presence of deoxyadenosine was indicated by presence of deoxyadenosine molecular ion [M+H]$^+$, similar to the previous result without Fe$^{2+}$. Other ions include [M+Na]$^+$, [M+K]$^+$, and [2M+Na]$^+$, corresponding to m/z of 274, 290 and 525, respectively. On the other hand, ART, with a molecular weight of 282 showed molecular ions [M+H]$^+$, at m/z 283, in addition to other ions such as [M+Na]$^+$, [M+K]$^+$, and [2M+Na]$^+$, corresponding to m/z of 305, 321 and 587, respectively. The retention times and mass
fragmentation patterns of products appearing between retention times 0.8 and 1.8 min are given in Table 3.
Figure 7. Mass spectrum of artemisinin-deoxyadenosine-Fe<sup>2+</sup> at 0.8mins (A), 0.9mins (B), 1.2mins (C), 1.4 (D), and 1.8 (E).

Table 3

Retrospect Times and Fragmentation Patterns of Deoxyadenosine Reacted with Artemisinin in the Presence of Fe2+

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>252, 283, 305, 337, 367, 587, and 619</td>
</tr>
<tr>
<td>0.9</td>
<td>203, 209, 237, 252, 277, 283, 293, 305, 411, 531, and 547</td>
</tr>
<tr>
<td>1.2</td>
<td>223, 255, 279, 305, 314, 321, 356, 443 and 587</td>
</tr>
<tr>
<td>1.4</td>
<td>203, 217, 251, 252, 269, 291, 293, 297, 305, 319, 328, 337, 346, and 370</td>
</tr>
<tr>
<td>1.8</td>
<td>203, 209, 251, 277, 337, 346, 353 and 360</td>
</tr>
</tbody>
</table>

22
These masses consist of those from ART as well as those from adenosine. The fact that these masses are from different molecules than those observed when no iron was included points to a different mechanism of formation for these molecules. Based on the products formed, it was hypothesized that there’s difference in the mechanism of reaction between ART and deoxyadenosine reaction when iron is present. In general, iron II in the presence of hydrogen peroxide is known to form Fenton’s reagent [63]. We speculated that ART in the presence of iron II could also form similar type of reaction. When the endoperoxide in ART interacts with iron, it forms free radicals [51]. Activated ART can react with the DNA base, forming adducts, and reacts with itself, forming dimers. As observed from the chromatogram and mass spectra, the products mediated by free radical reactions are different from the products formed without the free radical route.

**ART-Deoxycytidine**

Chromatograms of the reaction of ART and deoxycytidine obtained at room temperature and at 37°C are given in Figure 8 (a). At day 0 (at room temperature), three chromatogram peaks were obtained at about 0.5, 0.6 and 2.0 min. The peaks at 0.5 and 2.0 min belonged to deoxycytidine and ART, respectively, while the other at 0.6 min was the reaction product of ART and deoxycytidine at room temperature. On subjecting the mixture solution to 37°C, five new reaction peaks appeared at about 1.2, 1.4, 1.8, 3.1 and 3.6 min. The reaction product peaks grew with time of incubation at 37°C while the parent peaks reduced. The initial reactant signature peaks decreased by about 6 to 60%, while the new product peaks grew by about 100% during the time of incubation. The identity of the reaction products was confirmed by observing the mass spectra of these molecules. Figure 9 shows the mass spectra of chromatographic peaks shown in
Figure 8 (a). Deoxycytidine molecular ion [M+H]^+ was observed at m/z 228. Other ions including [M+Na]^+, [2M+ H]^+, and [2M+Na]^+, corresponding to m/z of 250, 455 and 477, respectively. On the other hand ART, with a molecular weight of 282, showed molecular ions [M+H]^+ at m/z 283, in addition to other ions such as [M+Na]^+, [M+K]^+, [2M+Na]^+, corresponding to m/z of 305, 321 and 587, respectively. The retention times and mass fragmentation patterns of products appearing at retention times between 1.2 and 3.6 min are given in Table 4.

![Figure 8](image)

**Figure 8.** (a) TIC chromatograms of the ART and deoxycytidine reaction obtained at room temperature and at 37°C. (b) TIC chromatograms of the ART-deoxycytidine-Fe^{2+} reaction obtained at room temperature and at 37°C.
Figure 9. Mass spectrum of artemisinin-deoxycytidine reaction products at 1.2 minutes (A), 1.4 mins (B), 1.8 mins (C), 3.1 mins (D), and 3.6 mins (E).

Table 4

*Retention Times and Main Fragmentation Patterns of Deoxycytidine Reacted with Artemisinin*

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>203, 209, 284, 291, 305, 323, 332 and 337</td>
</tr>
<tr>
<td>1.4</td>
<td>203, 224, 245, 251, 284, 291, 337, 346, 360, and 388</td>
</tr>
<tr>
<td>1.8</td>
<td>209, 251, 277, 337 and 346</td>
</tr>
<tr>
<td>3.1</td>
<td>209, 251, 277, 284, 307, 346, 351, 360 and 374</td>
</tr>
<tr>
<td>3.6</td>
<td>209, 251, 277, 297, 307, 351, 360 and 374</td>
</tr>
</tbody>
</table>

Alkylation most likely occurs in the reaction between the drug and the DNA base which leads to the formation of ART-deoxycytidine adducts. In principle, alkylation of 2'-deoxycytidine can occur at nitrogen atoms, N3 and N4, and at the oxygen atom.
Alkylation at multiple location in deoxycytidine could be one of the key reasons of encountering different molecules representing the same masses in the mass spectra. These adducts undergo mass fragmentation and are represented by the reaction peaks indicating presence of both deoxycytidine and ART mass fragmented ions. It is possible that the mechanism of deoxycytidine (dC) alkylation at N3, N4, and O2 positions by ART occurred under temperature control. Figure 10 shows deoxycytidine alkylation on the N3, N4, and O2 positions by Artemisinin (ART). This kind of alkylation mechanism on deoxycytidine was previously reported in [64, 65]. Studies show that alkylation at the N3 position is favored in reaction in water and can be reversible and less stable compared to N4, whereas under thermodynamic control N4 alkylation will be more likely to take place. Alkylation at the O2 position was proposed even though it is less likely [65].

Figure 10. Possible mechanism of deoxycytidine (dC) alkylation on the N3, N4, and 2O positions by Artemisinin (ART) under temperature control.
Free interaction of ART with deoxycytidine in the presence of iron was monitored by obtaining chromatograms of mixtures of these samples after incubation at room temperature and at 37°C. The chromatograms obtained are given in Figure 8 (b). The sample mixtures were first analyzed immediately after mixing, then analyzed again each day for four days. On the first day, several chromatogram peaks were obtained at about retention times 0.5, 0.8, 1.2 and 2.0 min. The chromatographic peaks at retention times 0.5 and 2.0 min were for deoxycytidine and ART, respectively. The chromatographic peak at retention time at 1.2 min was from ART with Fe\(^{2+}\). On incubating the mixture at 37°C, seven new reaction peaks appeared at about 0.9, 1.3, 1.4, 1.8, 2.1, 3.1 and 3.6 min. These new chromatographic reaction product peaks grew with time of incubation at 37°C. Further analysis of these reaction products was carried out using mass spectra.

The mass spectra of the chromatographic peaks shown in Figure 8 (b) are given in Figure 11. Deoxycytidine molecular ion [M+H]\(^+\) was observed at m/z 228. Other ions included [M+Na]\(^+\), [2M+H]\(^+\), [2M+Na]\(^+\) and [2M+K]\(^+\), corresponding to m/z of 250, 455, 477 and 493, respectively. On the other hand ART, with a molecular weight of 282, showed molecular ions [M+H]\(^+\), at m/z 283, in addition to other ions such as [M+Na]\(^+\), [M+K]\(^+\), [2M+Na]\(^+\), corresponding to m/z of 305, 321 and 587, respectively. The m/z for the reaction products observed at 0.8, 0.9, 1.2, 1.3, 1.4, 1.8, 2.1, 3.1 and 3.6 min are given here. The new product chromatographic peaks and fragmentation patterns appearing between 0.8 and 3.6 min are shown in Table 5.
Figure 11. Mass spectrum of artemisinin-deoxycytidine- Fe^{2+} at 0.8mins (A), 0.9mins (B), 1.2mins (C), 1.3 mins (D), 1.4 (E), 1.8 (F), 2.1 (G), 3.1 (H) and 3.6 (I).

Table 5

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>283, 305, 314, 337, 587, and 619</td>
</tr>
<tr>
<td>0.9</td>
<td>209, 237, 277, 283, 293, 305, and 531</td>
</tr>
<tr>
<td>1.2</td>
<td>223, 305, 314, 321, 356, 587 and 605</td>
</tr>
<tr>
<td>1.3</td>
<td>251, 291, 300 and 307</td>
</tr>
<tr>
<td>1.4</td>
<td>251, 291, 297, 307, 319, 337, 346, 353, 360, 374 and 388</td>
</tr>
<tr>
<td>1.8</td>
<td>251, 277, 297, 337, 346, and 353</td>
</tr>
<tr>
<td>2.1</td>
<td>209, 237, 265, 283, 305, 337, 346, 353 and 356</td>
</tr>
<tr>
<td>3.1</td>
<td>251, 346, 351, 360, 367 and 374</td>
</tr>
<tr>
<td>3.6</td>
<td>251, 277, 297, 307, 351, 360, 367 and 374.</td>
</tr>
</tbody>
</table>
Discussion

Based on the products formed, it was clear there was a difference in the mechanism of the reaction between ART and the DNA bases when iron was present. In general, iron II in the presence of hydrogen peroxide is known to form Fenton’s reagent [63]. We speculated that ART in the presence of iron II could also form similar type of reaction. When the endoperoxide in ART interacts with iron, it forms free radicals [51]. Activated ART reacts with the DNA base, forming adducts, and reacts with other molecules including itself, to form dimers. As observed from the chromatogram and mass spectra, the products mediated by free radical reactions are different from the products formed without free radical route. The two reactions most likely proceed side by side when ART is administered for the treatment of various diseases. A study of the molecular transformation of ART moiety in the presence of a host of biological molecules would open new understanding of molecular pathways involving ART and its derivatives, and hence help to tailor new therapies. ART is one the few exceptions that work non-stereo specifically, enabling easy laboratory synthesis of active forms of the drug.

Conclusions

Understanding the mechanism of reaction involving ART and DNA can help unravel potential reactions when these drugs are administered. In this work, we have explored the interactions of ART with DNA bases, ART with Fe$^{2+}$, and ART with both DNA and Fe$^{2+}$. Reaction of ART with DNA bases was found to give rise to new adducts, as observed from new chromatographic peaks showing both the DNA base and ART signatures. These reactions were only observed when the mixture was incubated at 37°C. Incubation of ART with Fe$^{2+}$ caused the formation of several new molecules,
indicating breakdown of the peroxide bond. Formation of new reaction products was not time dependent, as observed from chromatograms typical of free radical reactions. Study of ART and its derivatives with biological molecules is key to understanding the drug’s activity in vivo.
Chapter 3

LC-MS TOF Characterization and Stability Study of Artesunate in Different Solvent Systems

Introduction

ART derivatives possess a potent antimalarial activity. They are very active against all Plasmodium species and the asexual blood stage of malaria parasites [66, 67]. ART is a sesquiterpene lactone derived from the Chinese medicinal plant qinghao, also known as Artemisia annua or sweet wormwood [68]. It is a 1,2,4-trioxane ring system with an internal peroxide bridge [42]. Synthetic and semi-synthetic derivatives of ART include ARTS, which mainly improves the pharmacokinetic limitations of ART. These limitations in ART include poor solubility, low bioavailability and a short half-life [44]. DHA was one of the first semi-synthetic functional derivatives of ART [69]. Other first-generation semi-synthetic derivatives included arteeter and ARTM, which are lipid-soluble, while ARTS is water-soluble [70].

The natural 1,2,4-trioxane pharmacophore in all artemisinins is responsible for the antimalarial activity [71, 72]. The dosage is prescribed as a combination therapy to manage infection and to reduce resistance against the ARTs [73, 74]. Although the mechanisms of action of these drugs are not well understood, the endo-peroxide bridge is suggested to play a key role in the overall mechanism of action [50, 75].

Recent reports suggest that artemisinins possess anticancer properties [76, 77]. Cancer drugs are usually toxic to both cancerous cells as well as normal cells. Artemisinins present no significant cytotoxicity to normal cells according to thousands of studies so far from thousands of malaria patients [78]. They were found to be effective against
several cancers including throat cancer [79], ovarian cancer [80], breast cancer [81], colon cancer [82] and prostate cancer [83], among other cancer types [84]. In general, ART-derived monomeric molecules in the micro-molar range showed cytotoxicity to cancer cells, with the IC50 values of ART-dimers in cancer cells being much lower than doxorubicin and paclitaxel [85, 86]. Preliminary clinical research shows that after two months of treatment with ARTS, the tumor in laryngeal squamous cell carcinoma patients was significantly reduced. Just like ART-derived dimers, hybrid anticancer drugs that are chemically bound together can have more attractive properties than single-molecule anticancer drugs and can cause enhanced cytocidal pharmacological effects as well as provide synergy to inhibit cancer cell growth [87].

The ARTS moiety represents a potentially active pharmacophore showing great promise for the conjugation of other cancer drugs to form new novel drugs. The activity of the ARTS molecules is centered around the peroxide bridge; therefore, other sites within the molecule, in particular position twelve on ARTS (Figure 12), are attractive for conjugation with other drugs. Despite being water-soluble, ARTS has a low stability in neutral or acidic pH. Sodium ARTS is known to convert into biologically active metabolite DHA in vivo [88]. Other research reports that after absorption, ARTS rapidly converts to DHA and that DHA is the form that is responsible for antimalarial activity [89, 90]. In other research, the onset of hydrolysis of ARTS into DHA was observed 25 min after an intravenous administration [89]. A linear decomposition of ARTS was observed, with only about 31% and 18% of the drug remaining after 18 h of reaction, while the parent compound ARTS and the rest were converted into DHA and other products [91]. The rate of decomposition can be affected by physical parameters such as the temperature and solvent composition.
The temperature can initiate or accelerate the breakdown of pharmaceutical compounds [92]. The temperature affects the rate of drug oxidation or hydrolysis, while the rate of reaction increases proportionally with every 10 °C increase in temperature [93]. For ARTS, a thermal analysis at 100 °C for 39 h produced beta-ARTS, ARTS dimers, 9,10-anhydrodihydroartemisinin (glycal), DHA, beta-formate ester and other smaller reaction products from DHA intermediate and further thermal decomposition [94]. The study of the decomposition of ARTS in different solvent systems can help identify solvents that can delay decomposition during chromatographic analysis. The aim of the current work is to investigate the rate of decomposition of ARTS at room temperature and at a physiological temperature of 37 °C in different solvent systems using LC-/MS/TOF. ARTS decomposition was studied in methanol and water (90:10 v/v), and methanol and ammonium acetate (85:15 v/v). These are the common solvents used in chromatographic analysis [95-97]. The decomposition products as well as the rates of decomposition were compared in three solvent systems. Understanding the nature and rate of decomposition of ARTS is essential to understanding its other potential reactions as well as to tuning methods for its analysis. This work was initiated because a constant frustration during the chromatographic analysis of ARTS revealed very interesting
information regarding the mobile phases used in relation to the number and size of chromatographic signals generated.

Materials and Methods

Chemicals and Reagents

ARTS was purchased from Tokyo Chemical Company (TCI). LC-MS-grade methanol, ammonium acetate, acetonitrile and water were purchased from VWR. Other chemicals used for instrument calibration include ESI-L tuning mix and mass reference solutions (ammonium trifluoroacetate, purine, HP-0921), all LC-MS-grade from Agilent Technologies, Santa Clara, CA, USA.

Chromatographic Procedure

The LC-MS analysis was carried out using an Agilent 6230 series LC-MS/TOF unit. The separation of reaction products was carried out using Agilent Technologies, ZORBAX Eclipse Plus C-18 RRHD column (2.1 × 50 mm) with 1.8 µm diameter stationary phase particles. The mobile phase consisted of methanol and water containing 0.1% formic acid (v/v) 70:30. The chromatographic runs were performed under isocratic conditions at a flow rate of 0.250 mL/min. Every end of the run was followed by a post-run step that included flushing the column with the mobile phase repeatedly. The column temperature was set to 25 °C.

Mass Spectrometer Procedure

The mass spectrometry for the LC/MS was the TOF 6230 series set to Dual AJS ESI in positive ion mode. The settings of the mass spectrometer were as follows: scan mode (standard), range (200–3000) m/z, threshold (200), nebulizer gas (40.0 psi) and dry gas
(8.0 L/min), and dry temperature (325 °C). The compound stability and trap drive level were set to 100%. The MS/MS fragmentation amp was set to 175 V and the skimmer cone to 65 V. The instrument mode was set to high resolution for tuning and calibration.

**Sample Preparation**

Several ARTS samples in 5 mL volumetric flasks were dissolved in different solvent compositions including (i) methanol 100%, (ii) methanol with water, 90:10, and (iii) 20 mM ammonium acetate buffer with methanol, 15:85 v/v. The concentration of ARTS in the solutions was maintained at $1.04 \times 10^{-2}$ M. For the analysis, 1 mL of the sample mixtures was drawn, filtered, and transferred into individual vials using syringe filters. A comparison was made for solution mixtures left at room temperature versus those incubated at 37 °C. Sample mixtures were incubated at 37 °C at different lengths of time before analysis.

Chromatographic procedures were adjusted to optimize the separation of the elution products. Various mobile phase compositions, flow rates and injection volumes were investigated to effectively separate the samples. An isocratic solvent containing a mixture of methanol and water was used as the mobile phase for the LC-MS method. After several trials, 70:30 v/v methanol:water with 0.1% formic acid showed an optimized elution of the reaction products. In this experiment, the incubation temperature was set to 37 °C, and the column temperature was maintained at 25 °C, which was about room temperature. After the optimization of the reaction conditions, each sample was run three times.
Results

**ARTS in Methanol**

The reaction of ARTS with methanol was monitored by obtaining chromatograms of the ARTS sample dissolved in methanol and then incubated at room temperature and at 37 °C. Figure 13a shows the LC-MS obtained. The sample was first analyzed immediately after mixing (at room temperature, ~25 °C) and then analyzed again each day for twenty-one days at 37 °C. On the first day, only one chromatogram peak for ARTS was obtained at 2.1 min retention time. Upon incubating the mixture at 37 °C, other reaction product peaks appeared at about 0.9, 1.2, 1.6, 1.9, 2.8, 3.1, 3.4 and 6.0 min. These new chromatographic reaction product peaks grew with the time of incubation at 37 °C. There was no further increase of chromatographic peaks after 21 days of continuous incubation at 37 °C. The mass spectra of these reaction products are shown on Figure 14. Figure 13b shows a linear plot of the intensity as a function of time. The linear growth of the reaction peaks for DHA and artemether from day 1 to day 21 is shown.
Figure 13. (A) TIC chromatograms of ARTS in methanol for different time durations and at 37 °C. (B) Plot of intensity as a function of time of DHA and ARTM reaction peaks.

The reaction peaks observed at 1.9 and 6.0 mins corresponded to DHA and ARTM, respectively. The fragmentation pattern from the mass spectra confirmed the presence of DHA and ARTM. The reaction peak of ARTM at 6.0 min was growing rapidly during the incubation period. Based on the peak intensity of ARTS, the initial concentration of the reactant was estimated to be quite high, largely beyond the linear response region. When compared to ARTS, the percentage increases after 21 days of DHA and ARTM were 16.16% and 68.69%, respectively. The increase of ARTM and DHA followed a linear growth with $y = 3.4x −6.63$ and $y = 0.74x −1.44$, respectively.

The mass spectra of the reaction products corresponding to Figure 13a are shown in Figure14. The ARTS molecular ion is 384. Other ions include [M+Na]+, [M+ K]+ and [2M+Na]+, corresponding to m/z of 407, 423 and 791, respectively. The new product
chromatographic peaks appearing between 0.9 and 6.0 min are shown in Table 6. Table S1 shows the identification of mass fragments of ARTS reaction products in methanol.
Figure 14. Mass spectrum of artesunate at 0.9mins (A), 1.2mins (B), 1.6mins (C), 1.9mins (D), 2.8mins (E), 3.1mins (F), 3.4mins (G) and 6.0mins (H).
Table 6

*Retention Time and Fragmentation Patterns of ARTS Reaction Products in Methanol*

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>407</td>
</tr>
<tr>
<td>1.2</td>
<td>407, 791</td>
</tr>
<tr>
<td>1.6</td>
<td>221, 307</td>
</tr>
<tr>
<td>1.9</td>
<td>261, 307, and 591</td>
</tr>
<tr>
<td>2.8</td>
<td>261, 267, 285, 307, 321, 329, 341, 385, and 591</td>
</tr>
<tr>
<td>3.1</td>
<td>221, 267, 275, 321, 329, 421, 458, and 619</td>
</tr>
<tr>
<td>3.4</td>
<td>221, 249, 267, 285, 289, 311, 329, 341, and 385</td>
</tr>
<tr>
<td>6.0</td>
<td>221, 249, 267, 275, 281, 321, and 619</td>
</tr>
</tbody>
</table>

*ARTS in Methanol and Water*

The stability of ARTS in methanol:water, 90:10 (V/V), was monitored by collecting chromatograms of the sample at room temperature and at 37 °C. The sample was immediately analyzed after dissolving in the solvent and then again, each day for twenty-one days. Figure 15A–C show the obtained chromatograms. On the first day, only one chromatogram peak for ARTS was obtained at about 2.1 min. Other reaction product peaks appeared at about 0.5, 1.0, 1.6, 1.9, 2.8, 3.2, 3.5 and 6.0 mins upon incubation at 37 °C. These new chromatographic product peaks grew with the time of incubation at 37 °C. There were no changes in the chromatographic peaks after 21 days of continuous monitoring. Further analysis of these reaction products followed, using mass spectra. Figure 15D,E show the growth graph of the reaction peaks. Figure 16 shows the mass spectra of the ARTS reaction peaks.
Figure 15. (A) TIC chromatograms of ARTS reaction in methanol:water (90:10) monitored as a function of time (days). (B) Zoomed-in TIC chromatograms at 1.8 to 2.4 minutes. (C) Zoomed-in TIC chromatograms at 2.4 to 4.0 minutes. (D) Peak intensities as a function of DHA and ARTS. (E) Peak intensities as a function of time.
The ARTS peak at 2.1 min continuously decreased, while the DHA peak at 1.9 min and the ARTM peak at 6.0 min continuously grew. The parent peak of ARTS decreased to about 80% in 21 days. As compared to ARTS, the percentage increases of the DHA, ARTM and DHA dimers were 97%, 100% and 15%, respectively. The DHA increase was almost by the same margin as the reduction of ARTS. Although the initial concentration of ARTS was high, as shown on the plateau region of the detector, it was clear that it led to the formation of other product chromatographic peaks. In this solvent system, DHA and ARTM plateaued after 15 and 17 days, respectively (Figure 14D,E). The decline of the chromatographic peak of ARTS was much slower after 20 days.

The mass spectra of the reaction products corresponding to Figure 15A,B are given in Figure 16. The major fragment ions include [M+Na]+, [M+ K]+ and [2M+Na]+, corresponding to m/z of 407, 423 and 791, respectively. The new product chromatographic peaks appearing between 0.5 and 6.0 min are shown in Table 7. Table S2 shows the identification of mass fragments of ARTS reaction products in methanol:water.
Figure 16. Mass spectrum of reaction products at 0.5mins (A), 1.0mins (B), 1.6mins (C), 1.9mins (D), 2.8mins (E), 3.2mins (F), 3.5mins (G) and 6.0mins (H).
Table 7

Retention Time and Fragmentation Patterns of Artesunate Reaction Products in Methanol: Water

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>203, 217, 252, 261, 275, and 305</td>
</tr>
<tr>
<td>1.0</td>
<td>203, 217, 221, 261, 293, 305, and 321</td>
</tr>
<tr>
<td>1.6</td>
<td>203, 217, 221, and 307</td>
</tr>
<tr>
<td>1.9</td>
<td>221, 249, 261, 267, 307, 323, and 591</td>
</tr>
<tr>
<td>2.8</td>
<td>203, 217, 221, 249, 261, 267, 307, 323, and 591</td>
</tr>
<tr>
<td>3.2</td>
<td>203, 217, 221, 267, 321, 421, and 619</td>
</tr>
<tr>
<td>3.5</td>
<td>203, 217, 221, 249, 261, 289, 305, 311, and 351</td>
</tr>
<tr>
<td>6.0</td>
<td>221, 249, 267, 275, 321, 337, and 619</td>
</tr>
</tbody>
</table>

ARTS in Ammonium Acetate and Methanol

The stability of ARTS in 20 mM ammonium acetate: methanol (15:85) v/v at room temperature and at 37 °C was compared using chromatograms collected at different times. The obtained chromatograms are in Figure 17a. The Figure 17a insert shows the chromatograms between 1.8 and 2.4 min. There was a notable increase of the product peak and decrease of the reactant peak. On the first day, one chromatogram peak for ARTS occurred at 2.1 min retention time. Upon continuous incubation at 37 °C, other reaction product peaks appeared. The main peaks showed up at the following retention times: 0.5, 0.7, 0.8, 0.9, 1.2, 1.3, 1.9, 2.8 and 6.0 mins. The completion of the reaction was observed at day 21 of the continuous incubation at 37 °C, which was indicated by the reduction of the ARTS peak and the color change of the sample from clear to orange. Further analysis of these reaction products followed, using mass spectra. Figure
17B shows peaks’ growth as a function time in days for all the significant chromatographic peaks. Figure 18 shows the mass spectra of the ARTS reaction peaks.

*Figure 17.* (A) Daily TIC chromatograms of ARTS reaction in 20 mM ammonium acetate: methanol obtained at room temperature and at 37 °C. (B) Peak intensity as a function of time (days) for chromatographic peaks representing DHA, ARTS, ARTM and DHA dimers.
In this solvent system, we find some similar peaks but with different growth rates. ARTS showed up at its usual retention time at 2.1 min, and the peak intensity decreased, while the DHA peak at 1.9 min and ARTM peak at 6.0 min increased. The main compound formed in this reaction was DHA. A decrease of 97% of ARTS and an increase of about the same amount of DHA show the conversion of ARTS to DHA in this solvent system. When compared to ARTS, the percentage increase of ARTM was about 17.17%, while the percentage increase of the peak of the DHA dimer was 32.32%. Based on the mass spectra obtained, the peak at 2.8 min was assigned to the DHA dimer.

The introduction of ammonia in the solvent system resulted in the formation of a colored compound. The mechanism behind this was speculated to involve nitrogen from ammonia. This ARTS caused a breakdown of the peroxide bond resulting in a more enhanced conjugated molecule and, hence, the colored compound. This reaction was observed to be a function of time, judging from the growing chromatographic product peaks.

The mass spectra of the reaction products corresponding to Figure 17a are given in Figure 18. The ARTS molecular ion is 384. The other ions include [M+Na]+, [M+ K]+ and [2M+Na]+, corresponding to m/z of 407, 423 and 791, respectively. The new product chromatographic peaks appearing between 0.5 and 6.0 min are shown in Table 8. Table S3 shows the identification of mass fragments of ARTS reaction products in 20 mM ammonium acetate: methanol.
Figure 18. Mass spectrum of reaction products at 0.5mins (A), 0.7mins (B), 0.8mins (C), 0.9mins (D), 1.2mins (E), 1.3mins (F), 1.9mins (G), 2.8mins (H), and 6.0mins (I).

Table 8

Retention Time and Fragmentation Patterns of ARTS Reaction Products in 20 Mm Ammonium Acetate: Methanol

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>218, 230, 232, 245, 248, 260, 276, and 308</td>
</tr>
<tr>
<td>0.7</td>
<td>232, 236, and 265</td>
</tr>
<tr>
<td>0.8</td>
<td>206, 262, 307, and 329</td>
</tr>
<tr>
<td>1.3</td>
<td>214, 246, and 307</td>
</tr>
<tr>
<td>1.9</td>
<td>221, 261, 307, 591, and 573</td>
</tr>
<tr>
<td>2.8</td>
<td>261, 307, and 591</td>
</tr>
<tr>
<td>6.0</td>
<td>221, 275, 321, and 619</td>
</tr>
</tbody>
</table>
Discussion

Mobile phase buffers and solvents are not supposed to participate in any reaction during drug analysis. However, in this work, solvents played a key role in the decomposition of ARTS. The ARTS molecule degraded and formed several ARTs, including DHA, ARTM and DHA dimers, at different rates in different solvent composition systems. In methanol, ARTS mainly formed ARTM. The methyl group in methanol favors the formation of ARTM from the degradation product of ARTS. The initial reactant peak decreased by about 3.13%. When compared to ARTS, the percentage increases of DHA and ARTM were 16.16% and 68.69%, respectively. Meanwhile, ARTS in methanol and water mainly gave rise to DHA and ARTM. The growths of DHA and ARTM were quite significant in the methanol and water composition, where the initial reactant peak decreased by about 80%. When compared to ARTS, the percentage increases of DHA, ARTM and DHA dimers were 97%, 100% and 15%, respectively. DHA increased by almost the same margin as the reduction of ARTS. On the other hand, ARTS in methanol and ammonium acetate formed DHA, DHA dimer and other reaction peaks. The main compound formed in this reaction was DHA. The initial reactant peak decreased by about 97%. When compared to ARTS, the percentage increases of DHA, ARTM, DHA dimers were 98.98%, 17.17% and 32.32%, respectively. It is clear that the presence of water and ammonium acetate promoted the formation of the DHA dimer. Other studies confirmed that the IC50 values of artemisinin-derived dimers in cancer cells were significantly lower than those of monomeric ones [85, 86].

The introduction of ammonium acetate in the solvent system resulted in the formation of a colored compound at 37 °C (Figure 20). The ARTS has a peroxide bond, whereas the other molecule, ammonium acetate, acts as a nitrogen source. The mechanism
behind this reaction is speculated to be that the nitrogen from ammonia attacks ARTS and breaks the peroxide bond, which enhances the conjugation reaction that results in the formation of the colored compound. Figure 19 shows the mechanism of conjugation between ARTS and nitrogen in 20 mM ammonium acetate:methanol at 37 °C. The drug molecule ARTS’ color change occurred as a result of conjugation between the drug and ammonia in the solvent at 37 °C. Figure 20a–c show, respectively, a comparison between the ARTS reaction in methanol, methanol:water and 20 mM ammonium acetate:methanol. Figure S4 shows proton NMR of ARTS in methanol, and 20mM Ammonium acetate at room temperature and 37°C.

![Figure 19. Mechanism of conjugation between ARTS and nitrogen in 20 mM ammonium acetate: methanol at 37°C.](image-url)
Conclusions

In this work, we have explored the decomposition of ARTS in different solvent systems, namely in methanol, methanol with water, and methanol, water, and ammonium acetate. In all three solvent systems, ARTS decomposed at different rates into DHA, ARTM and DHA dimers, among major products. There were other products formed and not identified, as their peak intensities were low. The presence of ammonium acetate in the solvent system resulted in the formation of a bright orange solution at five days, a confirmation of conjugation from the breakdown of the endoperoxide bond. These degradation reactions were only observed when the mixture was incubated at 37 °C. ARTS at 37 °C in methanol with water and ammonium acetate produced DHA at the highest rate compared to the other solvent systems, while the methanol with water system produced ARTM at the highest rate. The study of the ART derivatives at this temperature is key to understanding the drug activity in vivo as well as to altering the methods that are used to analyze the derivatives.
Chapter 4

**Artemisinin Estradiol Interaction Studies in Presence of Fe (II): LC/TOF MS**

**Separation of Reaction Products**

**Introduction**

Artemisinin (ART) is a sesquiterpene lactone consisting an internal peroxide bridge also called as 1,2,4-trioxane ring system [42]. Artemisinin is known for its effectiveness against malaria, a disease caused by the *Plasmodium falciparum* parasite [43]. Due to its low solubility and short half-life, other semi-synthetic derivatives have been developed [44]. Dihydroartemisinin, one of the semi-synthetic form of artemisinin, is derived from the primary active metabolite of artemisinin [45]. Other first generation of semi-synthetic forms of artemisinin are arteeter and artemether (lipid soluble), and artesunate (water soluble) [46].

Breast cancer is the most common cancer in women and one of the leading causes of fatality among women in North America [98]. Breast cancer is caused by the abnormal growth of breast cells where the cells undergo rapid and uncontrolled cellular division. Estrogen is a class of female sex hormones which is associated with an increased risk of certain types of cancers such as breast cancer and, endometrial cancer [99]. The role of estrogen as carcinogens has been confirmed by several epidemiological studies. Estrogen promotes cancer development through several pathways such as receptor mediated hormonal activity causing cellular proliferation, cytochrome p450 mediated metabolic activation causing mutation, and aneuploidy [100]. Estrogen responsive breast cancer also known as estrogen receptor positive mediated by naturally occurring estrogen and can be treated by blocking the estrogen receptor [101, 102]. ART observed
to selectively decrease functional levels of estrogen receptor and also have some degree of antiproliferative effects in estrogen-induced breast cancer lines [98].

Several studies and case reports show ART exhibiting anticancer properties against cancer cells [47, 48] as well as providing enhanced synergistic activity with other anti-cancer agents [49]. The ART’s mechanism of action is not well understood. However, the endo-peroxide bridge in ART is thought to play a key role in the overall mechanism of action [50]. Interaction of the endo-peroxide of ART with heme in proteins is speculated to form free radicals causing cell injury and apoptosis in cells [51]. The formation of carbon centered free radicals when ART interacts with heme proteins is well known [52]. However, some research intimate that ART and its derivatives go through Fenton-like reaction to kill cancer cells [53, 54]. ART has been reported to exhibit antileishmanial activity in particular against visceral leishmaniasis [55]. New reports indicate that this drug can be used as antimycobacterial [56]. Despite these potential uses of ART and decades of research, gaps about its mechanism and its specific targets are even more glaring. For example, in a new pharmacophoric study on the role of the peroxide bond in dioxanes against Leishmania parasites showed no role of the peroxide bridge [57]. In fact, the tetrahydropyran, an epoxide, was found to induce a higher increase in intracellular ROS levels than the endoperoxide counterpart. This implied that generation of free radicals by cleaving the O−O bond was not the main mechanism of action in these peroxides. Presence of free iron also did not affect the free radical generation [57]. This contrasts with other multiple studies done before that indicated presence of iron mainly from be decomposition of heme is paramount in artemisinin Leishmania efficacy [58].
The anticancer effects of ART and its derivatives are due to the latter’s ability to induce apoptosis through cellular processes including DNA damage. The iron-mediated cleavage of the endoperoxide bridge in ART could trigger the formation of the carbon centered radicals that can induce ferroptosis [59]. In general, Fe (II) in presence of ART can trigger generation of free radicals through a Fenton type reaction.

Studies on drug and hormone interactions are important in chemotherapeutics. Drug and hormone interaction can enhance understanding of its mechanism by unravelling structural changes of the drug. Products of the reaction can also help tailor new therapies. A rapid separation method to help unravel the reaction products as well as help qualitatively identify the reaction products is key in search of the reaction mechanisms. The aim of the current work is to develop new chromatographic methods to investigate reaction of the ART with estradiol and establish the role of Fe (II). LC/TOF MS was used to establish the reaction products. Reaction of ART with any hormone is key in unravelling part of the mechanism involved in the drug anticancer activity. For the first time, LC/TOF MS technique was used to clearly show that ART reaction with estradiol followed a different mechanism from that in the presence of Fe (II). The role of the temperature in the activity of the drug toward estradiol was also established.

Materials and Methods

Chemicals and Reagents

ART was purchased from Tokyo Chemical Company (TCI). Estradiol was purchased from AK Scientific, Inc. Iron (II) Chloride was purchased from Alfa Aesar. Methanol, acetonitrile, ammonium acetate and water, all LC-MS grade were purchased from
VWR. Solutions of ART and its derivatives containing 20 mM Ammonium acetate: Methanol, 15:85 was prepared prior to each experiment. All other chemicals were LC-MS grade.

Chromatographic Procedure
Standards of ART and estradiol were analyzed using TOF/LC-MS. Separation of the reaction products of ART-estradiol were carried out using Agilent Technologies, ZORBAX Eclipse Plus C-18 RRHD column (2.1x 50 mm) with 1.8 µm diameter particles. An autosampler was used to inject the sample, and the temperature was set to 25°C. The mobile phase consisted of Methanol and water 70:30, respectively. The LC-MS analysis was carried out using Agilent 6230 series LC-MS TOF unit. The chromatographic run was performed under isocratic conditions at a flow rate of 0.250 mL/min. Every end of the run was followed by a post run step that included flushing the column with mobile phase for 5 min. The column temperature was set at 25°C.

Mass Spectrometer Procedure
Mass spectrometry detection for the LC/MS was with a TOF 6230 series set to Dual AJS ESI positive ion mode. The settings of the mass spectrometer were as follows: scan mode (standard), range (200-3000) m/z, threshold (200), nebulizer gas (40.0 psi), and dry gas (8.0 L/min), dry temp (325°C). The compound stability and trap drive level were set to 100%. The MS/MS fragmentation amp was set to 175 V and skimmer cone at 65 V.
Sample Preparation

**Artemisinin-Estradiol Reaction.** Solid mixtures of ART and estradiol were placed in a 5 mL volumetric flask. A buffer solution containing 15% ammonium acetate (20.0 mM) and 85% methanol was added to the volumetric flask containing the solid mixture. The final concentration of ART was $1.4 \times 10^{-2}$M, while that of estradiol was $4.4 \times 10^{-2}$M.

One more solution was made following the same procedure, but with $2 \times 10^{-3}$ M Fe$^{2+}$. Mixtures of ART and estradiol were placed in a 5mL volumetric flask, and 3 mL of Iron II chloride solution was added to the solid mixture of ART and estradiol. A buffer solution was then used to make up the 5 mL volume mark. The concentrations of ART and estradiol were $1.4 \times 10^{-2}$M and $4.4 \times 10^{-2}$M, respectively. The final solution contained $1.4 \times 10^{-2}$M ART, $4.4 \times 10^{-2}$M estradiol, and $2 \times 10^{-3}$M Fe$^{2+}$.

For analysis, 1 mL of the sample mixture was drawn, filtered, and transferred into individual vials. Comparisons were made between solution mixtures left at room temperature versus those incubated at 37°C. Some sample mixtures were incubated at 37°C at different length of times before analysis.

**Optimization of the Reaction Conditions**

The concentration of standards and chromatographic procedures were adjusted and optimized for the optimal reaction and elution of the reaction products. Initially, the ART, and estradiol standards were analysed individually. Various ratios of solvents were investigated to optimize solubility of both the ART and estradiol as well as to effectively separate samples. The ratio of the solvents for the sample mixture
preparation, 20 mM Ammonium acetate: methanol, was adjusted to 15:85 to facilitate the solubility of the estradiol with ART.

Methanol and water were used as the mobile phases for the LC-MS method; the water contained 0.1% formic acid. The ratio was adjusted to 70:30 for proper elution of the standard and reaction products. A column with the dimensions 2.1x 50 mm, with 1.8 µm was used for faster and more precise separation of the reaction products. In this experiment, the incubation temperature was set to 37°C and the column temperature was maintained at 25°C. After optimization of the reaction conditions, each analysis was carried out three times.

Results

ART-Estradiol

Figure 21 shows the chemical structure of ART. Reaction of ART with estradiol was monitored by obtaining chromatograms of mixtures at room temperature and at 37°C. Figure 22 shows chromatograms of sample mixtures, first analyzed immediately after mixing and then analyzed again each day for four days. On the first day at room temperature, only two chromatogram peaks corresponding to the two reactants were obtained, at or around retention times 1.7 and 2.0 minutes for estradiol and ART respectively. On the second day of incubation at 37°C, a new chromatographic peak at retention time 1.8 minutes was visible. Although the intensity of this peak was very small, it signifies formation of new molecule between ART and estradiol. The new chromatographic reaction product peak grew with time of incubation, while the parent reactant peaks reduced. The initial reactant signature peaks decreased by about 44% to 58%, while the new product peak grew by about 100% during the time of incubation. Further analysis of these reaction products was followed using mass spectra.
Mass spectra corresponding to Figure 22 are shown in Figure 23 and indicate estradiol molecular ion $[M+H]^+$ at $m/z$ 273. Removal of hydroxyl group from the molecular ion resulted in $m/z$ 255. On the other hand, ART, with a molecular weight of 282 showed molecular ions of $[M+H]^+$ at $m/z$ 283, in addition to other ions such as $[M+\text{Na}]^+$, $[M+\text{K}]^+$, $[2M+\text{Na}]^+$, corresponding to $m/z$ of 305, 321, and 587 respectively.

![Chemical structure of artemisinin](image)

*Figure 21. Chemical structure of artemisinin.*

![TIC chromatograms of artemisinin-estradiol reaction mixture](image)

*Figure 22. TIC chromatograms of artemisinin-estradiol reaction mixture obtained at room temperature (day zero) and at $37\, ^\circ\text{C}$ incubation. (a) overlaid. (b) shifted to the right.*
Removal of water from \( m/z \) 283 could result in \( m/z \) 265. Reduction of CO from 265 could give \( m/z \) 237. Reduction of \( \text{C}_2\text{H}_4 \) from 237 could yield \( m/z \) 209, which was also the mechanism of transition from \( m/z \) 219 to \( m/z \) 191. Dehydration of \( m/z \) 237 could also form \( m/z \) 219. These individual fragmented masses were observed in the mass spectra elsewhere [61].

From the mass spectra, the new product chromatographic peak showing up at 1.8 minutes produced \( m/z \) 209, 219, 237, 251, 265, 279, 297, 315, 332, 337, 346, and 360 on mass spectrometry.

**ART-Estradiol- \( \text{Fe}^{2+} \)**

Introduction of Iron (II) in the reaction mixture drastically changed both the number of chromatographic peaks as well as the retention times (Figure 24). Interaction of ART with estradiol in the presence of iron was monitored by observing chromatograms obtained from samples of ART, estradiol and \( \text{Fe}^{2+} \) mixtures and after incubation at room
temperature and at 37°C. The sample mixtures were first analyzed immediately after mixing and then again, each day for four days. On the first day, several chromatogram peaks were obtained at about retention times 0.9, 1.3, 1.7 and 2.0 minutes (Figure 24). The chromatographic peaks at retention times 1.7 and 2.0 minutes were for estradiol and ART respectively. Chromatographic peaks at retention times 0.9 and 1.3 were product reaction peak formed from estradiol and ART at room temperature. On incubating the mixture at 37°C, five new reaction peaks appeared at about 1.0, 1.4, 1.8, 3.2 and 3.6 minutes with mass spectra quite different from previous results without Fe$^{2+}$ ions. The initial reactant signature peaks decreased by about 25% to 97.2%, while the new product peak grew by about 75 to 100% during the time of incubation. Further analysis of these reaction products was followed using mass spectra.

Figure 24. TIC chromatograms of artemisinin-estradiol-Fe$^{2+}$ reaction obtained at room temperature and at 37°C. (A) overlaid. (B) shifted to the right.
Spectra of the reaction products corresponding to Figure 24 are shown in Figure 25. Presence of estradiol was indicated by presence of estradiol molecular ion $m/z$ 272. On the other hand, ART, with a molecular weight of 282 showed molecular ions $[M+H]^+$, of $m/z$ 283, in addition to other ions such as $[M+Na]^+$, $[M+K]^+$, $[2M+Na]^+$ corresponding to $m/z$ of 305, 321, and 587 respectively. The retention times and mass fragmentation patterns of products appearing between retention times 0.9 to 3.6 minutes are given in Table 9.
Figure 25. Mass spectrum of artemisinin-estradiol-Fe$^{2+}$ reaction peaks at 0.9mins (A), 1.0mins (B), 1.3mins (C), 1.4mins (D), 1.8mins (E), 3.2mins (F), and 3.6mins (G).
Table 9

Retention Times and Fragmentation Patterns of Estradiol Reacted with Artemisinin in the Presence of Fe2+

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Main Fragments (m/z)</th>
</tr>
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<tbody>
<tr>
<td>0.9</td>
<td>219, 247, 265, 283, 303, 314, 325, and 587</td>
</tr>
<tr>
<td>1.0</td>
<td>209, 219, 237, 255, 277, 283, 286, 293, and 531</td>
</tr>
<tr>
<td>1.4</td>
<td>209, 224, 239, 251, 265, 270, 293, 297, 314, 328, and 376</td>
</tr>
<tr>
<td>1.8</td>
<td>209, 223, 237, 255, 277, 286, 332, and 346</td>
</tr>
<tr>
<td>3.2</td>
<td>209, 224, 237, 251, 297, 346, 360, and 374</td>
</tr>
<tr>
<td>3.6</td>
<td>209, 219, 224, 237, 251, 265, 279, 297, 346, 351, 360, and 374</td>
</tr>
</tbody>
</table>

These masses consist of those from ART as well as those from estradiol. The fact that these masses are from different molecules from those observed when no iron was included, points to a different mechanism of formation of these molecules.

Discussion

Based on the products formed, it was clear there was a difference in the mechanism of reaction between ART and estradiol when iron was present. In general, iron II in the presence of hydrogen peroxide is known to form Fenton’s reagent [63]. We speculated that ART in the presence of iron II could also form similar type of reaction. When the endo-peroxide bond in ART interacts with iron, it forms free radicals [51]. Activated ART reacts with estradiol, forming adducts, and reacts with other molecules, including itself, to form dimers. As observed from the chromatograms and mass spectra, the
products mediated by free radical reactions are different from the products formed without free radical route. The two reactions most likely proceed side by side when ART is administered for the treatment of various diseases. A study of the molecular transformation of ART moiety in the presence of a host of biological molecules would open new understanding of molecular pathways involving ART and its derivatives, and hence help to tailor new therapies. ART is one of the few exceptions that work non-stereo specifically, enabling easy laboratory synthesis of active forms of the drug.

**Conclusion**

Understanding the mechanism of reaction involving ART and hormone can help unravel potential reactions when these drugs are administered. In this work, we have explored interactions of ART with estradiol, and ART with both estradiol and Fe$^{2+}$.

Reaction of ART with estradiol was found to give rise to new adducts as observed from new chromatographic peaks showing both the estradiol and ART signatures. These reactions were only observed when the mixture was incubated at 37°C. Incubation of ART with estradiol in the presence of iron caused formation of several new molecules indicating breakdown of the peroxide bond. Formation of new reaction products was not time dependent as observed from chromatograms typical of free radical reactions. Study of the ART and its derivatives with biological molecules is key in understanding the drug activity in-vivo.
Conclusions and Key Findings

Understanding the mechanism of reaction involving ART, biomolecules, and hormones, can help unravel potential reactions when these drugs are administered. In this work, we have explored the interactions of ART with DNA bases and estradiol.

Interactions of ART with DNA bases, ART with Fe$^{2+}$, and ART with both DNA and Fe$^{2+}$ have been studied under temperature control. Reaction of ART with DNA bases was found to give rise to new adducts, as observed from new chromatographic peaks showing both the DNA base and ART signatures. These reactions were only observed when the mixture was incubated at 37°C. Incubation of ART with Fe$^{2+}$ caused the formation of several new molecules, indicating breakdown of the peroxide bond. Formation of new reaction products was not time dependent, as observed from chromatograms typical of free radical reactions.

We have explored interactions of ART with estradiol, and ART with both estradiol and Fe$^{2+}$. Reaction of ART with estradiol was found to give rise to new adducts as observed from new chromatographic peaks showing both the estradiol and ART signatures. These reactions were only observed when the mixture was incubated at 37°C. Incubation of ART with estradiol in the presence of iron caused formation of several new molecules indicating breakdown of the peroxide bond. Formation of new reaction products was not time dependent as observed from chromatograms typical of free radical reactions.

Apart from that, understanding the reorganization reaction of Artemisinin and its derivative in body temperature in-vitro is also essential to elucidate chemical and
physical changes of the drug when administered in the human system. In this work, we have explored re-arrangement of artemunate at room temperature and 37°C. Reaction of artemunate in different solvents at 37°C gave rise to new molecules. These reactions were only observed when the mixture was incubated at 37°C. Artesunate at 37°C was primarily hydrolyzed to its active metabolite dihydroartemisinin and gave rise to other artemisinin derivatives such as artemether and other new molecules. Study of the artemisinin derivatives at body temperature is key in understanding the drug activity in-vivo.
References


44. Rudrapal, M., & Chetia, D., Endoperoxide antimalarials: development, structural diversity and pharmacodynamic aspects with reference to 1,2,4-trioxane-based structural scaffold. Drug design, development and therapy, 2016. 10: p. 3575–3590.


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**Table A1.** Identification of Mass Fragments of ARTS Reaction Products in Methanol
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| ![Artesunate 344](image1) | ![Artesunate 691](image2) |

**Table A2.** Identification of Mass Fragments of ARTS Reaction Products in Methanol:Water
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Artesunate 308

Artesunate 265

Artesunate 307

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**Table A3.** Identification of Mass Fragments of ARTS Reaction Products in 20 mM Ammonium Acetate:Methanol
Figure A1. Shows proton NMR of ARTS in methanol, and 20mM Ammonium acetate at room temperature and 37°C. (a) ARTS in MEOH:20mM Ammonium acetate at 37°C, (b) ARTS in MEOH at 37°C, (c) ARTS in MEOH:20mM Ammonium acetate at room Temperature), (d) ARTS in MEOH at room Temperature.
Appendix B

Chapter 4

Figure B1. Mass spectrum of artemisinin-estradiol at 1.7min and 2.0mins