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Development of an UFLC/MS/MS method for the comparative analysis of oxytocin and artesunate-amodiaquine for validation of field detection systems

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Thesis

**DEVELOPMENT OF AN UFLC/MS/MS METHOD FOR THE COMPARATIVE
ANALYSIS OF OXYTOCIN AND ARTESUNATE-AMODIAQUINE FOR
VALIDATION OF FIELD DETECTION SYSTEMS**

by

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B.S., United States Military Academy, 2007

Submitted in partial fulfillment of the
requirements for the degree of
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DAVID ANDREW GODIN

ABSTRACT

Spurious, falsely-labeled, falsified or counterfeit (SFFC) pharmaceuticals are a health concern that claims hundreds of thousands of lives annually¹, a violation of intellectual property rights which cost legitimate companies billions², and a low-risk high yield revenue stream for organized crime². While ports of entry and border control points are the primary access control points for SFFC^{3,4}, advances in field portable detection and equipment offers an increasingly effective method for the assessment of pharmaceuticals at regional centers and points of distribution. This is particularly important for less developed countries (LDC) who do not maintain satellite or regional testing facilities.

As part of a proposed protocol to assess field portable detection equipment, an ultrafast liquid chromatography, tandem mass spectrometry (UFLC-MS/MS) method for the quantification of liquid formulation Oxytocin was developed. The six minute method was found to have a within run %bias of +/- 16%, a linear dynamic range of 150-1000 nanograms/milliliter (ng/ml), and an accuracy within acceptability criteria for all tested concentrations.

The effectiveness of three identified transition ions, 723.1, 86.2 and 70.1

Daltons, for the analysis of oxytocin by mass spectrometry was assessed across several figures of merit to include signal to noise ratio, %CV, calibration sensitivity, and analytical sensitivity. The 723.1 ion fragment was recommended for quantification, while the 70.1 dalton ion was recommended as a qualifier ion, although 86.2 also performed within acceptability criteria.

A method for the UFLC-MS/MS assessment of degradation products for oxytocin was proposed for specificity testing. Degradation of oxytocin by exposure to highly acidic, basic, and thermal conditions for one hour was attempted. Formation of degraded products was not observed.

Additionally, existing High Performance Liquid Chromatography (HPLC) methods for the simultaneous assessment of Artesunate and Amodiaquine HCl were modified to assess compatibility with UFLC. No method assessed produced sufficient quality signal to continue with method development.

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LIST OF ABBREVIATIONS

CAN	Acetonitrile
ACT	Antimalarial Combination Therapy
API	Active Pharmaceutical Ingredient
AOI	Analyte of Interest
Asn	Asparagine
AT	Artesunate
AQ	Amodiaquine
CV	Coefficient of Variance
Cys	Cysteine
DI	Deionized Water
DTT	Dithiothreitol
ESI	Electrospray Ionization
EU	European Union
FTIR	Fourier Transform Infrared Spectrometers
G	Grams
GC/MS	Gas Chromatograph/Mass Spectrometer
Gln	Glutamine
Gly	Glycine
GPHF	German Pharma Health Fund
HCl	Hydrochloric Acid

HPLC	High Pressure Liquid Chromatography (alternatively High Performance Liquid Chromatography)
Ile	Isoleucine
IM	Intramuscular
IV	Intravenous
Kg	Kilogram
LDC	Less Developed Country
LDR	Linear Dynamic Range
Leu	Leucine
LOD	Limit of detection
Mg	Milligram
M+H	Precursor Ion
MS	Mass Spectrometer
MW	Molecular Weight
ml	Milliliter
Ng	Nanogram
NFPA	National Fire Protection Association
NMR	Nuclear Magnetic Resonance
OT	Oxytocin
PCR	Polymerase Chain Reaction
Pro	Proline
Q1	Quadrupole 1

Q2	Quadrupole 2
Q3	Quadrupole 3
RP-	Reverse Phase
Rt	Retention Time
SFFC	Spurious/Falsely-labelled/falsified/counterfeit
TIC	Total Ion Chromatograph
Tyr	Tyrosine
UFLC	Ultra Fast Liquid Chromatography
UNICRI	United Nations Interregional Crime and Justice Research Institute
US	United States
USD	United States Dollars
USP	United States Pharmacopeia
UV-VIS	Ultraviolet Visible Light Spectrometry
WHO	World Health Organization
µg	Microgram

1. INTRODUCTION

1.1 Counterfeit and Substandard Medicines

Counterfeit and substandard medicines are a serious global health concern, estimated to contribute to hundreds of thousands of deaths per year^{1,3}. In China alone 200,000-300,000 persons die each year due to counterfeit or substandard medicines³. Referred to as “spurious/falsely-labelled/falsified/counterfeit (SFFC) medicines by the World Health Organization (WHO), these medicines may vary from harmful or toxic substances labelled as legitimate medicine, to preparations containing incorrect, inactive, or ineffective doses of active pharmaceutical ingredients (API)⁴.

The impact of counterfeit drugs is felt in wealthy countries as well as less developed countries (LDC). Industrialized countries are protected by strict manufacturing guidelines, regulations, and importation controls. In just two months in 2008, 34 million illegal pills were seized across the external borders of the European Union (EU)⁵ alone, while United States authorities are estimated to prevent approximately 75 billion dollars’ worth of counterfeit medicines from entering circulation annually². Because of the effectiveness of these systems, only an estimated 1% of medicine in legal circulation is counterfeit⁴. In these industrialized countries, the internet is the primary source of SFFCs. The WHO reports that medicine purchased from illegal internet sites were counterfeit in more than 50% of observed cases⁴.

Less developed countries do not benefit from the controls and protective regulations and robust systems enjoyed by wealthy countries, the dangers posed by SFFCs are ubiquitous and difficult to measure. The WHO estimates that 25% of medicines in LDCs are counterfeit^{3,4}, although this varies by geographic region and product. Chinese authorities report counterfeit rates between 50 and 85% for some products, while in Nigeria and Thailand, an estimated 36.5% of antibiotics and antimalarials in circulation are counterfeit or substandard³. Antimalarial and antibiotic courses of treatment rely on steady, regular dosing over long periods of time. Interrupting the dosing with substandard or counterfeit medications can cause the treatment to fail and contributes to the resistance of those infections against future treatment.

In addition to a global health concern, SFFC is a billion dollar black market with links to organized crime, and represents a significant loss of income to legitimate pharmaceutical companies. For example, counterfeit Viagra (sildenafil) is estimated to cost Pfizer Inc. two billion United States Dollars (USD) annually in sales². The United Nations Interregional Crime and Justice Research Institute (UNICRI) notes that pharmaceutical counterfeit is a low risk, high profit illegal activity that is used to fund the production and trafficking of controlled substances, illegal arms dealing, money laundering, and human trafficking². One kilogram (kg) of counterfeit Viagra has a lower production cost than 1 kg of heroin, possess a higher street value than heroin, is not as heavily policed or

penalized as the distribution of a controlled substance², and represents a low risk, high yield revenue stream for organized crime.

1.1.1 Combatting Counterfeit Pharmaceuticals

Any strategy for combatting counterfeit pharmaceuticals must functionally rely on a method of determining the authenticity of medicine in circulation. This is done primarily at ports of embarkation and debarkation, or at regional collection and distribution centers. Additionally, the United States Pharmacopeia (USP) has published drug quality monitoring procedures for assessing medicine in local circulation. These guidelines cover sampling, transportation, and storage protocols, as well as testing⁶. This methodology begins with a physical and visual inspection of the sample and its associated packaging compared to published standards. Many pharmaceutical companies have distinct manufacturer logos, markings, and water marks incorporated into their packaging to make forgery difficult. Second, a disintegration test is conducted to verify the label's stated enteric-coating and time release claims. Artesunate, for example, should dissolve in pure water at 37°C in less than 30 minutes or it is considered a major defect⁷. Next, the active pharmaceutical ingredient's presence is verified, typically through nonspecific colorimetric reagent testing. Finally an assessment by thin layer chromatography is made to verify the potency of the drug in question against a generated standard curve. Once these initial tests are

complete, the USP then recommends confirmatory and validation testing based upon current approved methods in regional satellite laboratories⁶.

This field monitoring program relies chiefly on German Pharma Health Fund's (GPHF) Minilab® to perform field analysis⁶. This Minilab is a reagent-based testing kit specifically designed for counterfeit pharmaceutical detection in developing world nations, and has an associated cost of \$6/assay⁸ in US dollars. These tests still require laboratory confirmation at satellite laboratories or regional laboratories.

1.1.2 Field Portable Analytical and Screening Technology

Improvements in miniaturization and portability of laboratory technology allow for confirmatory testing, normally associated with regional laboratories, to be employed directly at the testing sites. Field portable Fourier Transform Infrared Spectrometers (FTIR), Raman Spectroscopy, Gas Chromatography/Mass Spectrometry (GC/MS), and even Polymerase Chain Reaction (PCR) are available⁹⁻¹¹. These portable systems allow forward confirmatory testing in regions without satellite laboratories.

Developing technology may feasibly replace the GPHF Minilab® as a direct analytical tool for forward field screening. One portable screening system in development is the PharmaChk. The device is capable of providing field screening and quantification¹. It consists of four basic components: A tablet dissolving chamber, a fluorescent tag, a microfluidic chip and the signal detector¹. The fluorescent tag utilized by the system gives the platform its

modularity. A different probe is developed for each active pharmaceutical ingredient (API). Some analytes are detectable with well-studied chemical interactions, such as the luminol interactions used to detect artesunate¹. Other probes utilize highly specific aptamer binding interaction to create a detectable fluorescent response¹. Each method must be assessed in its ability to selectively bind to the target analyte of interest. This requires that each method's specificity be assessed against common counterfeit adulterants, and the analyte's own natural degradation products.

1.1.3 Assessing Field Portable Analytical and Screening Technology

As new and emerging technologies are developed, their effectiveness for screening and identifying counterfeit pharmaceuticals must be evaluated. This evaluation should address the technology's ability to detect the API in question at a therapeutic concentration, the ability to differentiate between API and common adulterants found in counterfeit pharmaceuticals, and also the method's ability to discriminate between active and inactive pharmaceutical ingredients.

For this assessment two medications were chosen from the World Health's Organization's essential medicine list¹¹⁻¹³; Oxytocin (OT) and Artesunate/Amodiaquine (AT/AQ). Oxytocin represents a high risk counterfeit drug, as it is an immediate use therapeutic drug. A counterfeit or substandard formulation could fail to induce labor, or fail to reduce post partum hemorrhage, endangering both child and mother. Thus, oxytocin is utilized in order to test the

feasibility of the proposed field-device on liquid formulations.

Artesunate/Amodiaquine, on the other hand, is a commonly encountered counterfeit drug representative of the normal challenges expected by a screening device, and measures the proposed device's ability to assess a tablet³.

1.2 Oxytocin

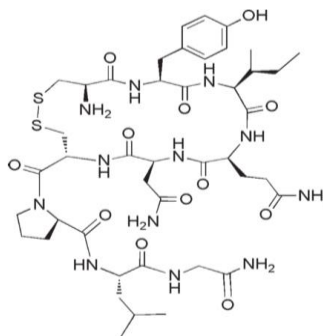


Figure 1. Oxytocin H-c[Cys-Tyr-Ile-Gln-Asn-Cys]-Pro-Leu-Gly-NH₂, showing the biologically active disulfide bond between Cys¹-Cys⁶.¹⁵

Oxytocin is a cyclic nonapeptide with a molecular weight (MW) of 1007.16 Daltons, and is the most commonly utilized peptide drug in medicine^{15,16}. Its structure is depicted in Figure 1. Oxytocin is used therapeutically to induce labor through the stimulation of uterine contractions¹⁷. It is also used at higher doses to reduce post-partum hemorrhage, a significant source of maternal deaths in developing countries¹⁸. Counterfeit or substandard oxytocin can fail to stimulate uterine contractions, elongating the birthing process and endangering both mother and child¹⁷.

Oxytocin is inactivated in the gastrointestinal tract¹⁷ and is therefore administered in solution through either intramuscular (IM) injections or through intravenous (IV) solution. Its therapeutic range is from .02 units per milliliter (units/ml) to .12 units/ml, or 40-240 nanograms per milliliter (ng/ml)¹⁹. Because oxytocin is only stable in saline for approximately 27 days¹⁶, it is typically encountered in 1 ml ampules of 10 units/ml, or 17-25 micrograms, designed for injection into saline solution. Oxytocin may also be transported as a powder, but this powder formulation was determined to be unfit for tropical climates by the WHO¹³ due to its poor stability, and was excluded from assessment for this reason.

Despite the fact that synthetic oxytocin has been utilized in medicine since the early 1950s^{15,18}, little is known about its degradation products or mechanism. Hawe et. al demonstrated in 2009 that oxytocin degradation was pH- and temperature- dependent and followed first order kinetics¹⁸. It was further demonstrated that the degradation products were resolvable from the precursor compound through reverse phase high performance liquid chromatography (RP-HPLC) and detectable by ultraviolet-visible light spectroscopy (UV-VIS)¹⁸. Hawe identified the observed degradation products as species of deamidated oxytocin. A deamidation reaction, or $R-CONH_2 \rightarrow R-COOH$, creates a mass difference of 0.985 daltons¹⁸. Hawe further identified three sites at which deamidation occurred by collecting each RP-HPLC peak fraction separately, linearizing oxytocin's ring structure through reduction with dithiothreitol (DTT), and then

conducting structural analysis by MS/MS fragmentation analysis. Have identified three sites for deamidation; the C-terminal Gly⁹, the Asn⁵ or the Gln⁴.

The mechanism by which Oxytocin decays was studied by Wisniewski et al in 2013. Wisniewski utilized sixteen synthesized OT analogues with modified structures and labeled ³⁴S and ¹³C, which were incubated in an aqueous solution and stressed to facilitate degradation. The structure of the reaction products was examined by mass spectroscopy (MS) and nuclear magnetic resonance (NMR)¹⁵. Wisniewski suggests that the degradation of oxytocin begins by cleavage of the disulfide bridge between the Cysteine¹ and Cysteine⁶ peptides through β -elimination. The resultant intermediate is a linear peptide which has a persulfide (S-thiocysteine), which Wisniewski suggests donates a sulfur atom to intact oxytocin molecules to create oxytocin trisulfide and other monomeric polysulfides¹⁵. These products were demonstrated to be resolvable by HPLC, and detectable by MS, but it is of note that the oxytocin polysulfides appear during incubation, but do not accumulate in concentration as oxytocin concentration decreases, suggesting that the persulfide and polysulfide products serve as intermediates, with the major degradation product being a dimer with a proposed structure shown in Figure 2¹⁵. The deamidated products noted by Have are not addressed by Wisniewski's degradation mechanism. It is unclear if the degradation products identified in either study are biologically active, although the 20-membered, six amino acid ring is considered a key structural feature of OT¹⁵.

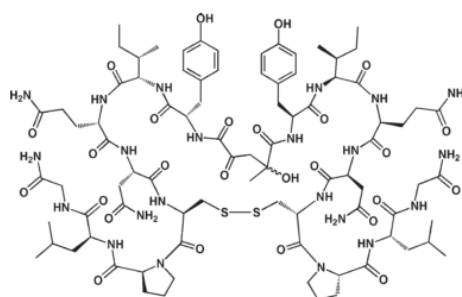


Figure 2. Proposed structure of the major dimeric product of oxytocin degradation in an aqueous environment proposed by K. Wisniewski et. al in 2013¹⁵.

1.3 Artesunate

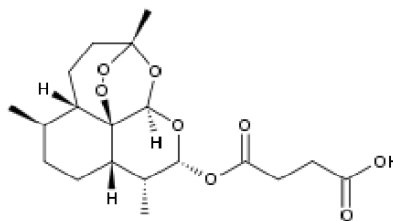


Figure 3. Chemical structure of artesunate²⁰

Artesunate (Figure 2) is an anti-inflammatory, anti-malarial compound²⁰. Artesunate (AT) is a semi-synthetic derivative of artemisinin²¹, which is isolated from the herb *Artemisia annua*. It is the most commonly used treatment to combat malaria, the infection spread by the parasite *Plasmodium falciparum*, and is most effective against the ring stage of the parasite²¹. Malaria is a serious global health concern, with over 247 million cases reported in 2008 alone²². Malaria results in nearly one million deaths annually, with a majority of those deaths reported in African children under 5 years of age²².

Artesunate has relatively well studied degradation products. Haynes et al demonstrated in 2007 that, when heated, artesunate degrades into β -artesunate, artesunate dimers, and 9,10-anhydrodihydroartemisinin²³. Haynes utilized NMR to identify products, rather than HPLC. Existing methods using HPLC-UV/VIS demonstrate that artesunate may be resolved from common impurities (with the exception of chloroquine phosphate and monodesethylamodiaquine dihydrochloride)^{22,23}. Artesunate has a short biological half-life, and is often paired with a second medication as an antimalarial combination therapy (ACT).

1.4 Amodiaquine Hydrochloride

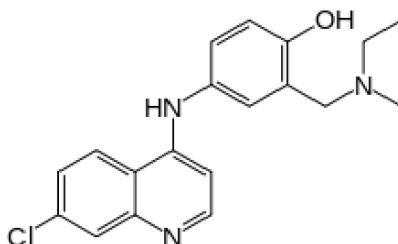


Figure 3. Chemical Structure of Amodiaquine Hydrochloride

Amodiaquine hydrochloride (AQ) is an antimalarial chemically similar to chloroquine, and is commonly used to treat resistant strains of *Plasmodium falciparum*. Artesunate-Amodiaquine combination therapy (ACT) is a widely used treatment for malaria²². RajaRao et al conducted forced degradation of artesunate and amodiaquine in 2013, but did not observe additional product peaks through HPLC/UV-VIS analysis. Further, no significant shift in the

retention times (RT) of precursor compound peaks²⁰ was observed. This indicates that products may not have formed, or that any products that formed were either not resolved through HPLC, or not detected by UV-VIS.

1.5 Ultrafast Liquid Chromatography

Several methods for the analysis of AT, AQ, and AT/AQ simultaneously are available. A number of these methods utilize HPLC coupled with UV-VIS detection^{20–23}. Early degradation studies were conducted using UV-VIS detection, which may detect and quantify a compound, but offers limited structural information^{20,22}.

Ultrafast liquid chromatography (UFLC), like high pressure liquid chromatography (HPLC), exploits differences in the affinity of an analyte of interest (AOI) with a stationary phase²⁴. The stationary phase typically contains functionalized silica. Liquid chromatography is based upon the premise that two different chemical compounds will have different affinities for the stationary phase resulting in different RTs. If RT are sufficiently different between two compounds, they may be resolved or purified by HPLC. This travel time, or RT is further managed in liquid chromatography by selectively altering the liquid mobile phase. By controlling the mobile phase's polarity, pH, or ion content in controlled gradients, it is possible to alter the RT of the analytes of interest. This, combined with high pressure and increased column efficiency over HPLC and LC systems, allows for shorter method times, smaller samples, and decreased solvent use²⁴.

1.6 Electrospray Ionization

As the analyte of interest reaches the end of the UFLC column, it is ionized by electrospray ionization (ESI). ESI is a soft ionization technique which occurs under atmospheric conditions^{25,26}. The sample in the mobile phase is ionized at the ESI source by a coronal discharge of energy, which aerosolizes the solvent into charged droplets²⁶. The net charge of the droplet causes each individual droplet to explode due to accumulated coulombic force, forming ions which enter the mass spectrometer²⁵. ESI was utilized in positive ion mode for the duration of protocol development, creating ions of molecular weight (M) plus a single hydrogen (H) or M+H²⁶. Because this process occurs under atmospheric conditions, rather than a vacuum, conditions between two similar instruments may vary. The ions created from the ESI process are then directed into a mass spectrometer.

1.7 Tandem Mass Spectrometry

The ions created during the ionization process are separated and detected based upon their mass to charge ratio (m/z). This is accomplished using a quadrupole, or four parallel poles conducting direct current or radio frequency current to create a field through which only the correct m/z may pass²⁶. The applied current separates larger ions from smaller ions, allowing for a separation of ions of different masses based upon their mass to charge ratio. Further for this

experiment, a triple quadrupole was utilized. In this arrangement, three quadrupoles are arranged in series. The first quadrupole (Q1) allows only the precursor ion (M+H) to pass, filtering all other ions out and greatly increasing precision while decreasing noise from unassociated ions. The second quadrupole (Q2) fragments this precursor ion into additional “product” or fragment ions, which are then selectively analyzed in (Q3)²⁵. This resulting precursor to product fragmentation pattern allows for the creation of a chemical fingerprint which may be used to identify a compound as well as offer some insight to its chemical structure. The triple quadrupole mass spectrometer was selected for this study due to its ability to not only detect degradation products which may not have been detected through UV-VIS analysis, but also provide insight to the structure and identity of any degradation products identified in this study.

2. MATERIALS AND METHODS

2.1 Standards

2.1.1 Oxytocin

Oxytocin was purchased from Caymen Chemical (Ann Arbor, Michigan) as a vial of 1 milligram (mg) lyophilized powder. A stock solution was prepared by adding either 1 milliliter (ml) of Deionized water or 1 ml of methanol, depending on the experiment. Oxytocin prepared with Deionized water was stored in a refrigerator at a temperature of 5°celsius (°C), while oxytocin prepared in methanol was stored in a freezer at a temperature of -25 °C. Certificates of analysis were received for all batches from Caymen Chemical, certifying a purity of 100% by UV-VIS analysis.

2.1.2 Artesunate

100 mg of powdered artesunate was purchased from Sigma Aldrich (St. Louis, MO) and stored at room temperature (22 °C) in original packaging. Standards of artesunate were prepared by dissolving 1-2 mg in methanol to reach a concentration of either 1 mg/ml or 2 mg/ml, depending on the experiment.

2.1.3 Amodiaquine

Five grams (5 g) of powdered amodiaquine was purchased from Sigma Aldrich and stored at room temperature in original packaging. Standards of amodiaquine were prepared by dissolving 1-2 mg in a sufficient volume methanol to reach a concentration of either 1 mg/ml or 2 mg/ml, depending on the experiment.

2.2 Mobile Phase Preparation

All mobile phases were prepared using American Chemical Society certified HPLC grade or higher reagents. Deionized water with a resistivity of at least 18 m Ω was used for these experiments and aliquoted daily.

For the analysis of Oxytocin, mobile phase A consisted of deionized water with .1% formic acid by volume. This was prepared by the addition of 1 ml of formic acid to 900 ml of deionized water, before filling to a volume of 1000 ml with deionized water. Mobile phase B consisted of HPLC grade acetonitrile (ACN) with .1% formic acid. This was prepared by the addition of 1 ml of formic acid to 900 ml of acetonitrile before filling to a volume of 1000 ml with ACN.

2.3 Instrumentation

The UFLC utilized in this protocol is a Shimadzu Prominence ultrafast liquid chromatography system from Shimadzu Corporation (Kyoto, Japan). Mass

spectrometry was conducted using a 4000 QTrap® series from SCIEX (Framingham, MA). A Phenomenex (Torrence, CA) Kinetex® 2.6 µm EVO C18 100 Å LC Column (150x3.0 mm) normal phase column was used for the analysis and method development.

2.4 Compound Optimization

Initial compound optimization was conducted by utilizing the automated compound optimization feature of the instrument's operating software, Analyst® (version 1.6.2). Values obtained from this automated optimization were used as the basis for performing a manual compound optimization by the protocol outlined in the operator's manual by SCIEX for manual compound optimization²⁷. Compound optimization was conducted at a concentration of 10 ng/ml of standard, with a flow rate of 10-25 ml/minute by direct infusion pump. Ions selected were based primarily upon intensity, literature^{17,18}, and reproducibility over multiple iterations of optimization. This process is described in further detail in Section 3.

2.5 Mobile Phase Selection

2.5.1 Oxytocin

The mobile phases selected for the analysis of oxytocin (Mobile Phase A: deionized water with .1% formic acid, Mobile Phase B: acetonitrile with .1%

formic acid) were selected based upon numerous existing and validated HPLC methods in the literature^{17-19,22,28}. These methods were adapted for a binary pump UFLC by separating organic phases (acetonitrile) as mobile phase B and replacing buffer solutions with Deionized water as mobile phase A, based upon manufacturer recommendations²⁹, instrument configuration, and a previous optimization conducted by M. Liu³⁰.

2.5.2 Artesunate/Amodiaquine

Several mobile phases were tested for use in the simultaneous analysis of artesunate and amodiaquine. The mobile phases tested are included as Appendix A.

2.6 Gradient Optimization

2.6.1 Oxytocin Standard Curve

The gradient selected for the generation of the oxytocin calibration curve and analysis is given in Table 1. This gradient was selected by adapting an HPLC method³⁰ to the shorter run time and higher pressures associated with UFLC, and further optimized by expanding the upper and lower limits of the gradient to reflect the region for which the peak shape remained in a Gaussian distribution.

Table 1. Oxytocin elution gradient and flow settings used for quantification

Time (min)	Gradient (%B)
0.1	20
1.5	20
3.0	50
4.5	50
5.0	20
Total Flow	.5 mL/min
Maximum Pressure	5000 psi

2.6.2 Oxytocin Degradation Analysis:

The method used for the degradation analysis of oxytocin was three times the length of the quantification method. This allowed for a longer, slower alteration of gradient that would be more likely to resolve degradation products of oxytocin, which are expected to have similar chemical structure and properties to the precursor compound. The lower end of the gradient was expanded, while the upper limit of the gradient remained consistent due to a large increase in noise observed at high concentrations of mobile phase B during testing. The gradient for the final method for the analysis of oxytocin degradation is provided as Table 2.

Table 2. Elution gradient and flow settings for the assessment of oxytocin degradation products.

Time (min)	Gradient (%B)
0.1	5
1.0	5
13	50
14	5
15	5
Total Flow	.5 mL/min
Maximum Pressure	5000 psi

2.7 Source and Acquisition Parameters

2.7.1 Oxytocin Quantification

Source and Acquisition Parameters were optimized according to manufacturer protocols²⁹ to maximize signal within normal operating parameters. The final source parameters used in the production of the calibration curve and subsequent analysis are described in Table 3. The acquisition parameters used for the calibration curve are listed in Table 4.

Table 3. Source Parameters for the 4000 QTrap® used to analyze Oxytocin

Source Parameter	Setting
Curtain Gas	20
Collision Gas	High
Ion Spray Voltage	3500 volts
Temperature	550°C
Ion Source Gas 1	50
Ion Source Gas 2	50

Table 4. Acquisition parameters used for Oxytocin Quantification and Analysis

Q1 (Daltons)	Q3 (Daltons)	Scan Time (milliseconds)	Compound ID	Declustering Potential (v)	Entrance Potential (v)	Collision Energy (v)	Cell Exit Potential (v)
1007.529	723.2	150	Oxytocin 1	161	10	43	18
1007.529	86.2	150	Oxytocin 2	161	10	123	0
1007.529	70.1	150	Oxytocin 3	161	10	129	10

2.7.2 Oxytocin Degradation Study

Source parameters for the analysis of degradation products were the same as those described in Table 3. Acquisition parameters were altered from selected ion monitoring mode to total ion chromatograph (TIC) mode with a scan range of 80 to 2800 Daltons. The lower limit was chosen to minimize noise identified during initial development of this method, while the maximum limit was selected based upon the highest value allowed by the mass spectrometer.

2.8 Oxytocin Calibration Curve

A calibration curve for oxytocin was created from a stock solution of 1 µg/ml prepared by adding 4 µl of a 1 mg/ml stock solution prepared in methanol to 3,996 ml of methanol. This stock solution was then used to create the calibrators as described in Table 5. Calibrators were assessed from lowest to highest concentration, with a blank between each concentration. Two blanks were used after the most concentrated calibrator to assess carry over. This

series was repeated five times such that each single concentration preparation was measured in five replicate injections of the same preparation to create an external calibration curve.

Table 5. Preparation of oxytocin calibrators from a 1 µg/ml standard.

Final Concentration (ng/ml)	Standard Volume (µl)	Methanol Volume (µl)
1000	500	0
900	450	50
800	400	100
700	350	150
600	300	200
500	250	250
400	200	300
300	150	350
250	125	375
200	100	400
150	75	425
100	50	450
50	25	475
20	10	490
10	5	495

2.9 Oxytocin Forced Degradation Study

To assess degradation levels and products of oxytocin, a solution of 10 µg/ml which was prepared by adding 80 µl of a 1 mg/ml oxytocin stock solution in 7,960 ml of deionized water was utilized. Four 1 ml aliquots of this 10 µg/ml solution were used. Two samples were adjusted to a pH of 2 by the dropwise addition of hydrochloric acid (HCl), and two samples were adjusted to a pH of 11 by the dropwise addition of ammonium hydroxide. Negative controls were created by adjusting 1 ml of deionized water to a pH of 2 and 11 by the dropwise addition of

HCl and ammonium hydroxide, respectively. The acidic and basic degradation process was allowed to take place for a total of sixty minutes at ambient temperature and pressure. Aliquots of 100 ml were removed from the stock vial at 0, 15, 30, 45 and 60 minutes. Each aliquot was added to 400 ml of dionized water to reduce damage to the HPLC column from extreme pH, and then analyzed immediately. This experiment was duplicated at a temperature of 90°C.

3.0 Results And Discussion

3.1 Oxytocin Quantification

3.1.1 Compound Optimization

Figure 4 is a Q1 mass spectrometer scan of an optimization solution of oxytocin (10 ng/ml) infused directly into the mass spectrometer, and represents the M+H ion of all compounds present. As demonstrated in Figure 4, a Q1 mass spectrometer scan of a solution of pure oxytocin in methanol produces a series of 1-dalton quartets at M+H, M+5, and M+22. The quartet at 1007.9, 1008.8, 1009.9 and 1010.9 was observed across multiple optimizations, with 1007.9 being the maximum signal observed in the quartet. This observation is likely indicative of oxytocin in various states of deamidation. As described by Hawe¹⁸, a deamidation reaction, or $R-CONH_2 \rightarrow R-COOH$, creates a mass difference of

0.985 daltons. Oxytocin has three sites at which deamidation may occur; the C-terminal Gly⁹, the Asn⁵ or the Gln⁴.

Because of the presence of this quartet, the automated compound optimization feature was tested in order to select the three most abundant product ions when the mass spectrometer was set to allow for a tolerance of +/- 2 daltons from the precursor ion (M+H) of 1007.9. This allowed for the inclusion of mono and bis-deaminated oxytocin (up to two sites deamidated). The presence of these ions were then confirmed through several iterations of manual compound optimization²⁷ to confirm that they were consistently present in high intensities. Product ions selected were 723.2, 86.2, and 70.1 daltons. Of these three ions, the 723.2 ion was observed in a previous protocol utilized by Hawe et al in 2009 for their research into the degradation kinetics of oxytocin, and represents the loss of the Pro-Leu-Gly-NH₂ tripeptide from the cyclic region (OT minus H-C₄H₇NCONHC₅H₁₀CONHCH₂CONH₂)^{17,18}. The structure of the remaining two product fragments selected is unknown.

Also of note in Figure 4 are significant quartets clustered around M+H+5 Daltons, and M+H+22 Daltons. While no structure is proposed for these observations, their presence may indicate a loss of precursor compound during storage, sample preparation, ionization, or some other source which could contribute to a signal loss.

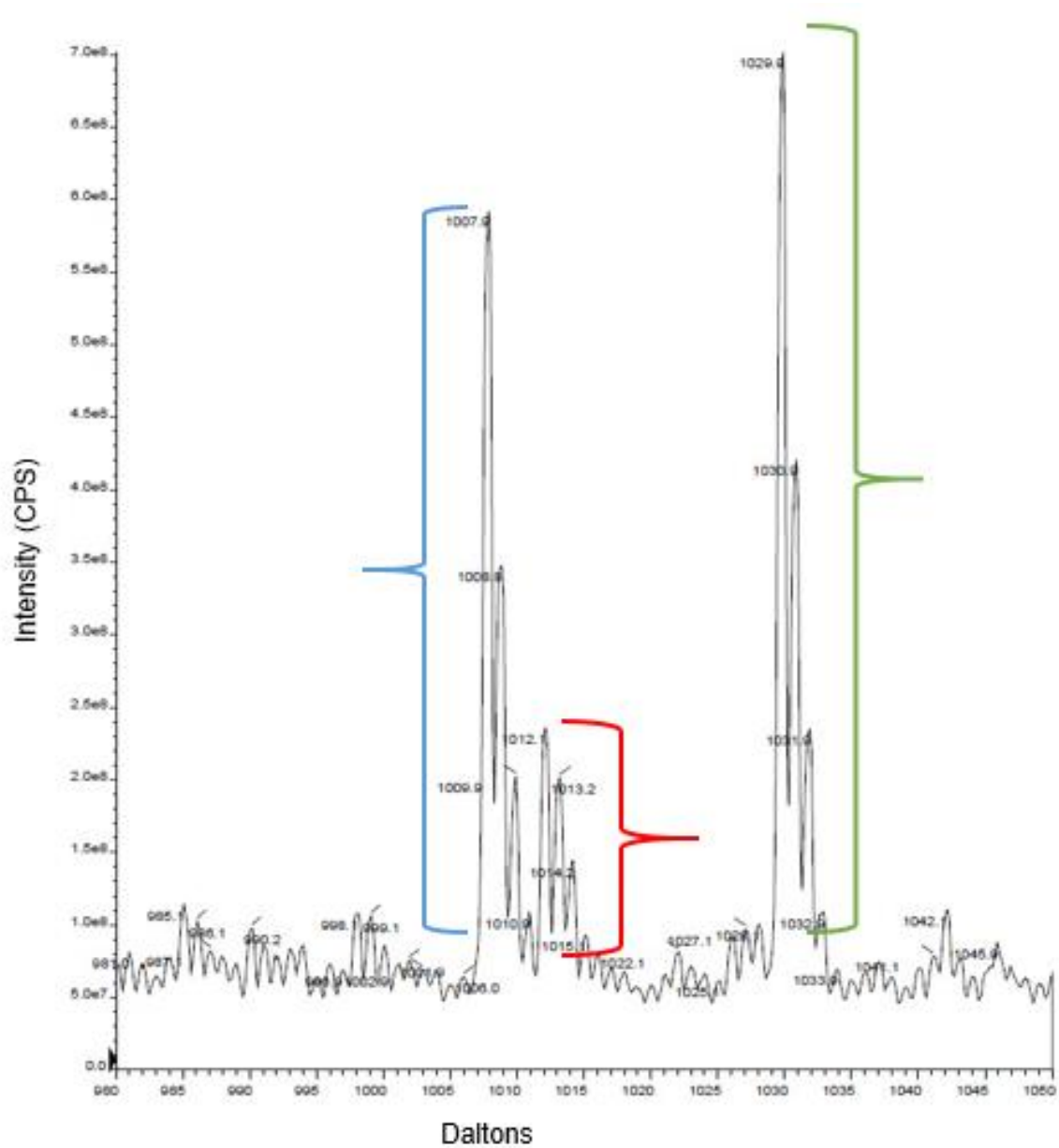
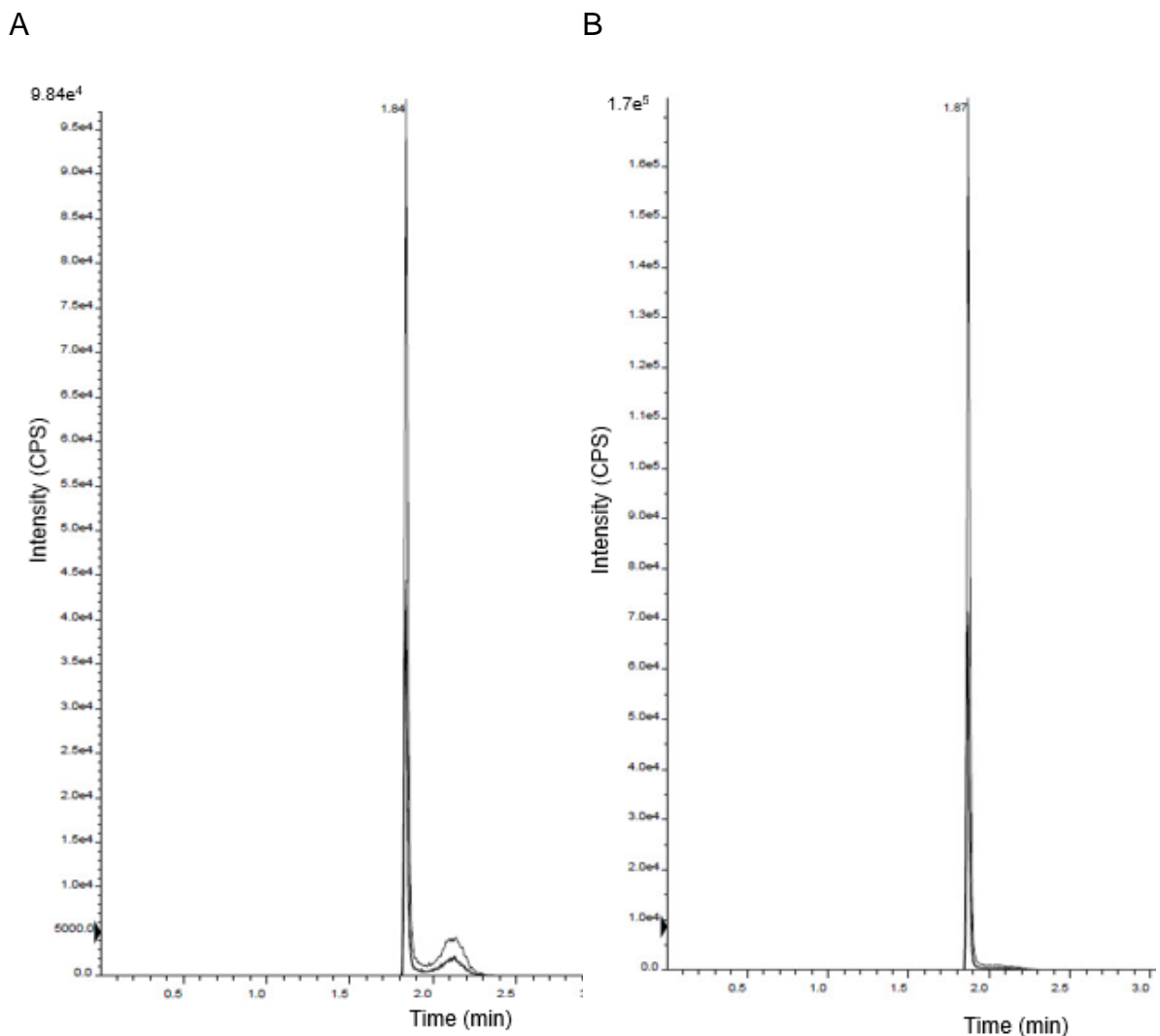


Figure 4. Q1 MS Scan of Oxytocin standard highlighting three 1-dalton quartets. In blue, a quartet beginning with Oxytocin precursor ion (m/z) of 1007.9, in red m/z+5, and in green m/z+22.

3.1.2 Oxytocin Gradient Assessment

The method for the quantification of oxytocin was initially kept as short as

possible to facilitate the rapid analysis of field samples for the active pharmaceutical ingredient. An initial assessment of the method, as described, produced a single, intense peak at a retention time of 1.84 minutes with no visible distortion in a five minute run. The upper and lower limit of this gradient was then explored by slowly altering the concentration of mobile phase B until peak shape became visibly distorted. This occurred at 40:60 (A:B) and 60:40 (A:B) respectively for the quantification method, as depicted in Figure 5.



C

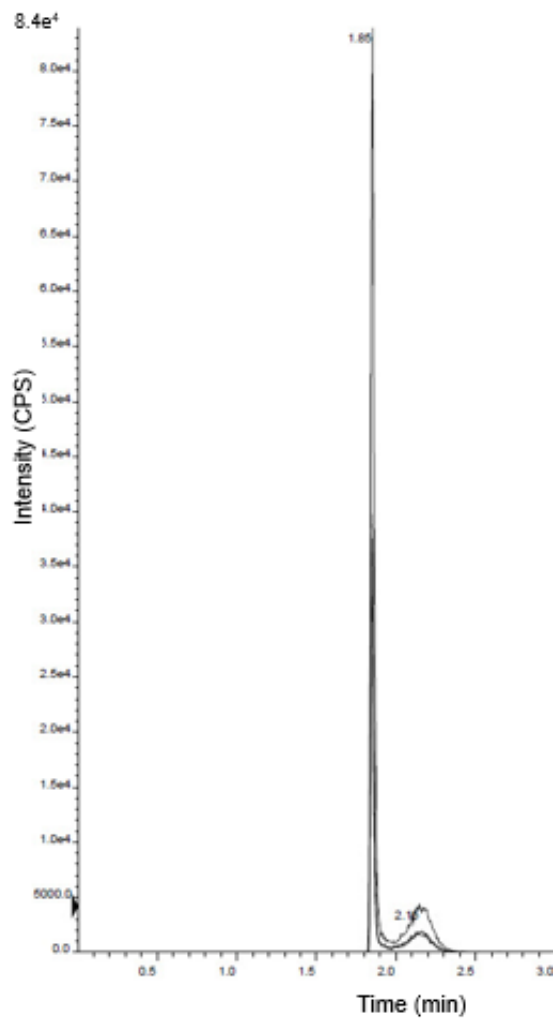


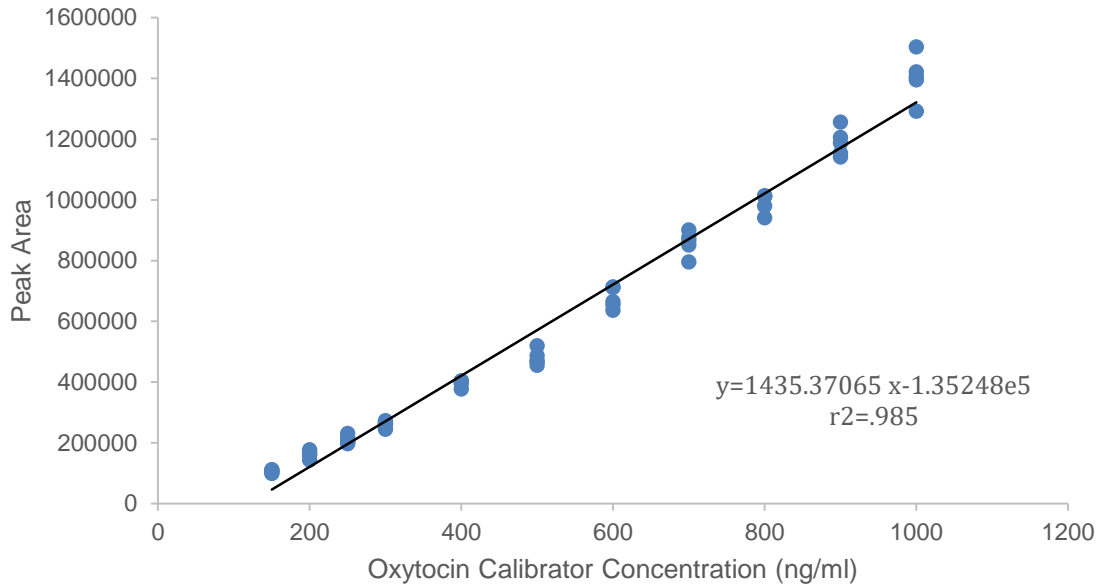
Figure 5. Oxytocin Gradient Assessment

- A.** Retention time and peak distortion begins to occur at 40:60 Acetonitrile:H₂O
- B.** Retention time and peak distortion upper limit occurs at 60:40 Acetonitrile:H₂O.
- C.** Retention time and peak shape of oxytocin at 50:50 acetonitrile:H₂O

3.1.3 Oxytocin Calibration Curve

A calibration curve was generated with five replicates of 15 non-zero calibrators. The peaks were integrated using Multi Quant® version 3.0 and the area of those peaks versus the known concentration is included in Figure 6. In order to determine the linear dynamic range (LDR) of the method, the within run precision was calculated at each concentration. This is calculated by obtaining the standard deviation of the area of peak detected at each concentration, divided by the mean peak area of that concentration to determine the within-run coefficient of variation (CV) of peak area. This value is expressed as a % (%CV), and is shown in Table 6. The highest % variation was present at lower concentrations across all three ion transitions.

A.)



B.)

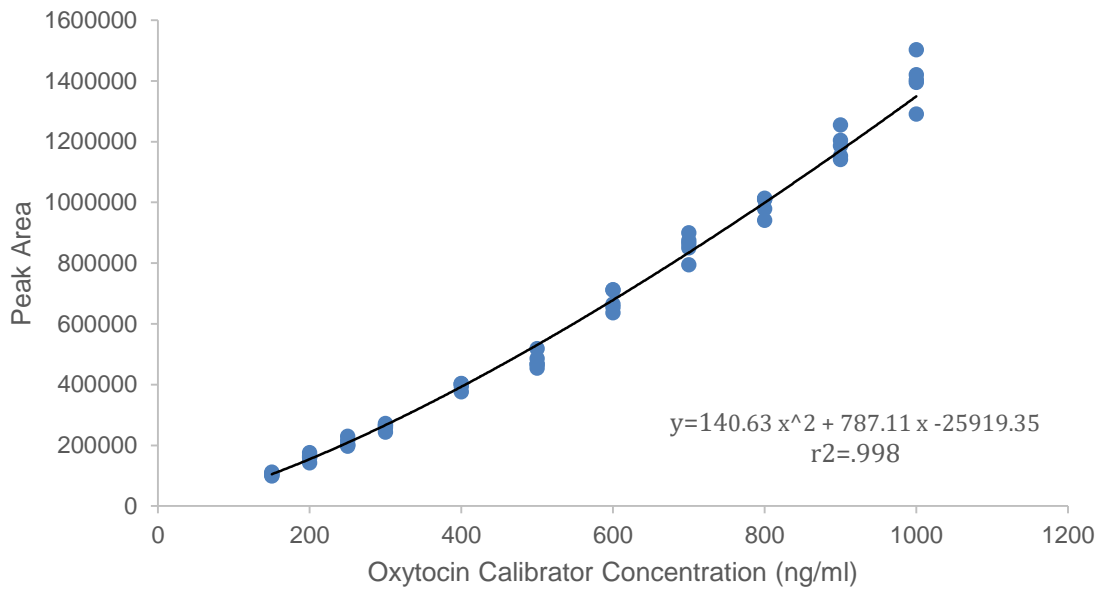


Figure 6. Signal area vs known concentration of all assessed points for oxytocin (calibrators and unknowns). Product ion 1, (1007.6->723.2) is shown. A. Linear Fit. B. Nonlinear Fit

Table 6. Peak area % CV by ion based upon five data points per ion per concentration.

Concentration (ng/ml)	Product Ion (daltons)		
	723.2	86.2	70.1
10	7.71	8.05	6.56
20	10.03	9.32	8.53
50	6.67	4.76	5.88
100	3.75	4.23	2.21
150	5.03	3.64	4.74
200	7.66	6.60	6.81
250	6.48	5.28	5.86
300	4.11	5.12	4.56
400	2.84	2.40	3.88
500	5.17	5.99	6.47
600	5.11	4.88	5.32
700	4.56	5.56	4.33
800	3.17	2.66	2.45
900	3.81	3.49	4.32
1000	5.38	4.15	4.21

3.1.4. Linear Dynamic Range

A calibration curve was generated using a weighted least squares regression fitting and the Multiquant™ 3.0 software package. The linear range was defined as one in which five or more calibrators could be modeled linearly such that the r^2 value was at a minimum .990, according to US Pharmacopeia Validation Parameters³¹. A summary of the r^2 value obtained across different concentration ranges of oxytocin is provided in Table 7. For this assessment, calibrators and quality controls were treated equally to better assess linearity. No region of the data met acceptability criteria for linearity under US Pharmacopeia standards. The range 150-1000 ng/ml, with a high r^2 value covering the largest number of tested data points was instead selected as reference. A calibration

line was generated using 6 calibration concentrations within this range. This line was used to quantify unknowns and compared to a nonlinear model which was created with the same six concentrations. The r^2 of these two fits are located in the bottom of Table 7.

Table 7. Coefficient of determination (r^2) values of calibration curves generated for tested concentrations. No region of tested data met acceptability criteria of $>.990$ when a linear relationship is assumed. Values were determined using all calibrators and QC samples within associated range. A linear and nonlinear fit were created using six calibrators across the indicated range.

Range (ng/ml)	Product Ion (daltons)			Range (ng/ml)	Product Ion (daltons)		
	<u>723.2</u>	<u>86.2</u> R ²	<u>70.1</u>		<u>723.2</u>	<u>86.2</u> R ²	<u>70.1</u>
10-1000	0.968	0.968	0.967	500-1000	.982	.983	.984
20-1000	.970	.970	.969	300-900	.983	.982	.982
50-1000	.974	.975	.974	200-800	.982	.983	.982
100-1000	.980	.980	.979	150-700	.979	.980	.979
150-1000	.982	.983	.982	100-600	.982	.985	.980
200-1000	.982	.984	.982	50-500	.980	.977	.977
250-1000	.983	.984	.983	50-400	.973	.970	.970
300-1000	.982	.983	.983	20-400	.973	.970	.970
400-1000	.980	.980	.980	10-300	.965	.961	.963
Final Linear Calibration Curve				150-1000	.985	.988	.987
Final Nonlinear Curve				150-1000	.998	.998	.998

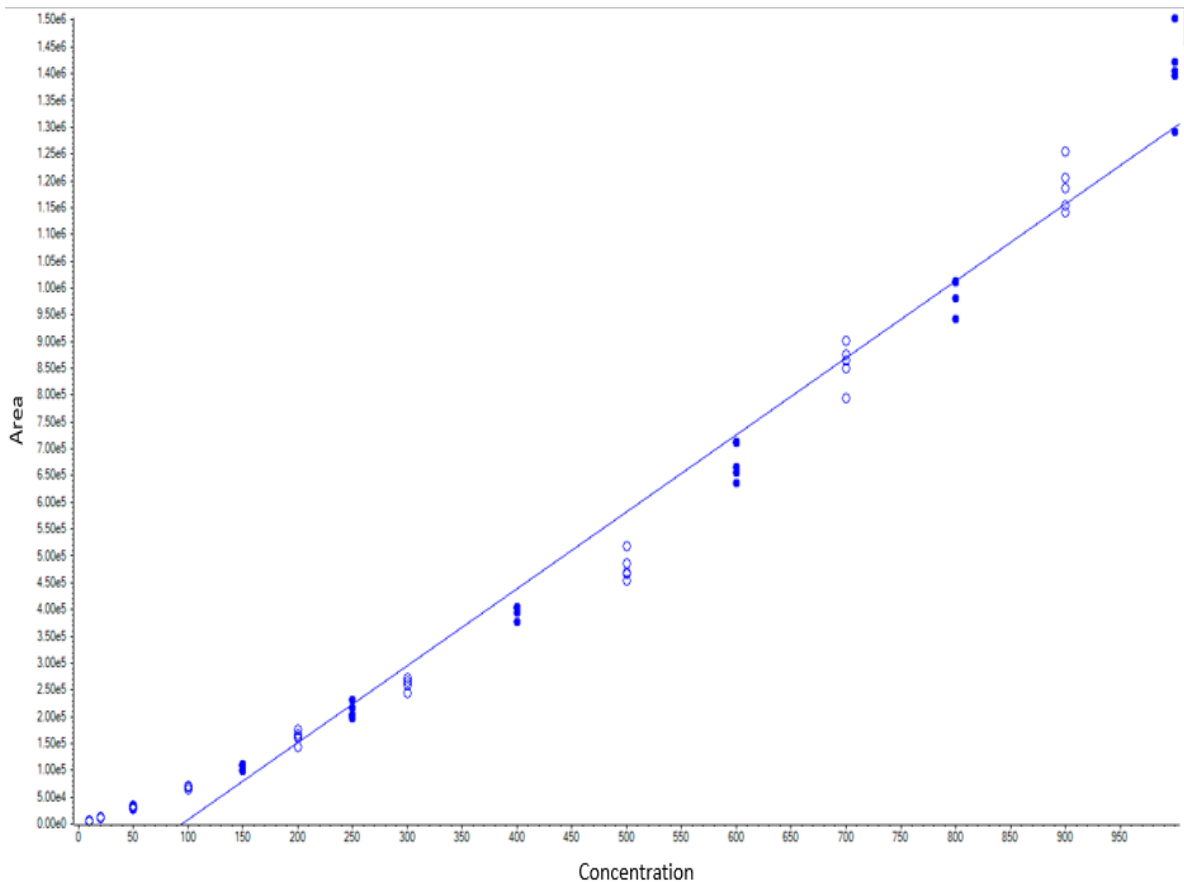


Figure 7. Final Calibration Curve. Final range of 150-1000 ng/ml. Shaded circles represent points used to generate curve. Empty circles represent test concentrations.

3.1.5 Limits of Quantification

The limit of quantitation is defined as the lowest non zero calibrator utilized by the method according to standards set forth by both the scientific working group on toxicology (SWGTOX) and the USP^{31,32}. By this definition the lower limit of quantification for this method is 150 ng/ml, while the upper limit is 1,000 ng/ml.

3.1.6 Accuracy (Bias)

Accuracy, or bias, was determined by calculating the difference between the mean of the calculated concentration and the known concentration, divided by the known concentration, and expressed as a percentage³² as shown in equation 1, and summarized in Table 8. Acceptability using SWGTOX guidelines is defined as +/- 20%³², while acceptability under USP is defined as +/- 2%³¹. All values in this method were within +/- 15% when modeled linearly, leading to acceptance under SWGTOX criteria but requiring additional optimization to meet USP criteria. This data suggests mid-concentration range is consistently underestimated, while high and low concentrations are overestimated. The accuracy is improved when a nonlinear model is employed, where the largest bias again seen in the mid-range (Table 8).

Eq 1 **Bias** (%) =

$$\left[\frac{\text{Grand Mean of Calculated Concentration} - \text{Nominal Concentration}}{\text{Nominal Concentration}} \right] \times 100$$

Table 8. % Bias by ion per concentration of both the linear and nonlinear models. All values for both models calculated with five data points/concentration

Concentration	Linear Model			Nonlinear Model		
	Q3: 723.2	Q3: 86.2	Q3: 70.1	Q3: 723.2	Q3: 86.2	Q3: 70.1
200	3.50	2.22	2.64	2.41	0.87	1.31
300	-7.86	-8.87	-9.24	-1.61	-3.59	-3.60
500	-14.36	-14.72	-14.86	-6.64	-7.82	-7.46
700	-1.21	-2.17	-2.29	1.90	0.79	0.87
900	2.47	2.99	3.48	-0.34	0.26	0.49

3.1.7 Carry Over and Minimum Distinguishable Signal

For the 75 blanks, the area of the largest signal peak observed at the appropriate retention time was 6 counts while the smallest area of the lowest non-zero calibrator of 10 ng was 4,752 counts. No significant carry over was observed in the blanks following any of the concentrations analyzed in this method. The median peak area of blank samples per product ion, and their range are provided in Table 9.

Table 9. Analysis of sample blanks based upon a sample of 75 blanks per ion.

	Product Ion		
	723.2	86.2	70.1
Median Peak Area	18.60	15.50	9.30
Maximum Peak Observed	62.00	50	31.00
Minimum Peak Observed	0	0	0

Blank values were evaluated in a histogram in Figure 9. Visual analysis of these histograms suggested that the signal may not be normally distributed. The Kolmogorov-Smirnov (KS) test is a non-parametric way to test the hypothesis that the data sets do not originate from the same distribution. In this case, the data set was tested against the normal and log-normal distribution³³. In both cases the P value was less than .01 suggesting these data are not well characterized by the normal or log-normal distribution classes. Since the data is not well classified as normal or log normal, the traditional methods to determine

the minimum distinguishable signal described by Curie and Mocack^{34,35} are not applied, instead the minimum distinguishable signal is defined as the maximum noise peak height for each ion, which is 62, 50 and 31.00 counts for product ions 723.2, 86.2 and 70.1 respectively.

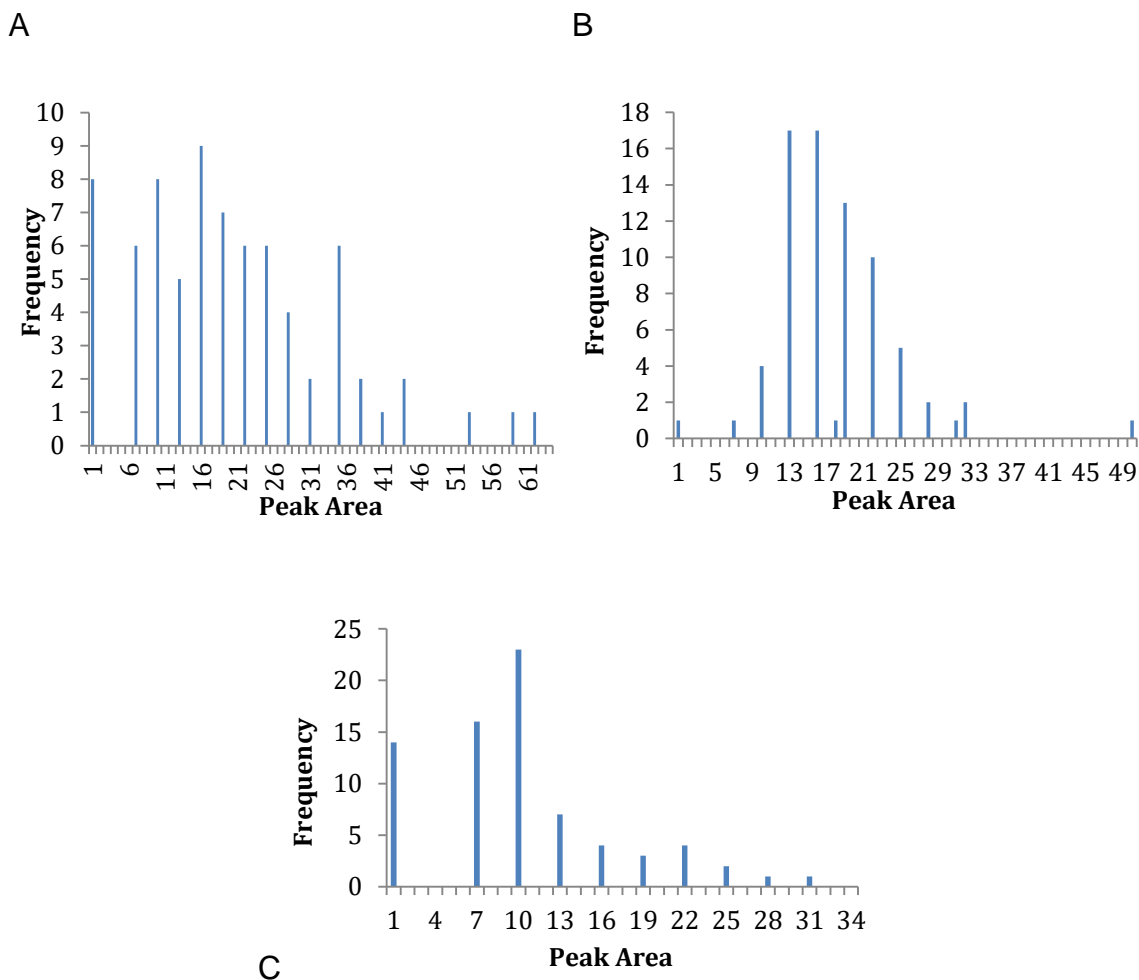


Figure 9. Histogram of Blank Signal Area based upon 75 observations per ion. For Ions A.) 723.2, B.) 86.2 and C.) 70.1 Daltons.

To further explore the minimum distinguishable signal, the region of the calibration curve from 10-150 ng, representing five calibrators nearest to the

origin, was fitted using a weighted least squares regression linear model the MultiQuant™ software as described in section 3.1.3, and an online tool³⁶. The regression lines, their r^2 values, and the y-intercept are included in Table 10. For all product ions the y-intercept is negative. Additionally, the y-intercept plus three times its standard error was -107.06, 210.3, and -58.12 for ions 723.2, 86.2 and 70.1 respectively, indicating that this calibration-based method is unsuitable for determining minimum distinguishable signal. These results again suggest that there may be a confounding factor associated with these signal measurements and it emphasizes the need for additional protocol development.

Since the noise exhibited in this method is neither normally nor log normally distributed, and has a y intercept value that is negative the minimum distinguishable signal will be defined as any value greater than the maximum blank signal observed, as noted in Table 9.

Table 10. Minimum Distinguishable Signal

Ion	723.2	86.2	70.1
Slope	689.2x	233.61x	228.44x
r =	0.993	0.994	0.994
y intercept	-1700	-567	-601
Standard Error	530.98	259.1	180.96

3.1.8. Calibration and Analytical Sensitivity

Calibration sensitivity is defined as the expected change in signal per unit

change in concentration, or slope of the calibration curve. Analytical sensitivity provides a measure of the change in concentration that can be discerned by the method³⁵. This is determined by dividing the calibration sensitivity by the standard deviation at that concentration. The calibration sensitivity over the range of the calibration curve is depicted in Table 12. The 723.2 product ion had the highest calibration sensitivity, nearly three times that of the other ions assessed. Its high standard deviation, however, resulted in comparable analytical sensitivity.

Table 12. Analytical and Calibration Sensitivity by product ion.

	Analytical Sensitivity		
	723.2	86.2	70.1
150	0.27	0.38	0.29
200	0.12	0.14	0.13
250	0.11	0.13	0.12
300	0.13	0.11	0.12
400	0.13	0.15	0.09
500	0.06	0.05	0.05
600	0.04	0.04	0.04
700	0.04	0.03	0.04
800	0.05	0.05	0.06
900	0.03	0.03	0.03
1000	0.02	0.02	0.02
Calibration Sensitivity	1435	494	486

3.2 Oxytocin Degradation Analysis

Oxytocin prepared in an acidic and basic environments were allowed to sit at room temperature and at 90 degrees Celsius for a period of one hour. Samples were compared to control samples containing no oxytocin, and evaluated for the presence of degradation products. The samples were analyzed in Q1 MS or TIC mode. Oxytocin was identified in all noncontrol samples. The sample that was allowed to sit at 90°C for 60 minutes was not evaluated due lack of remaining sample volume.

As the samples of prepared oxytocin had concentration well above the regions of linearity identified in section 3.1.4, and also well above the concentration at which the detector exhibits a linear response, the value does not correlate directly to a concentration. There was no significant reduction in peak height over time noted in any observed conditions, although due to detector saturation no conclusions may be drawn from this observation. Further, no additional degradation products were identified in basic conditions. One additional peak was observed when oxytocin was allowed to sit in acidic conditions, as seen in Figure 10, but this peak was identified in the acidic control sample as well and may be representative column-bleed due to the caustic conditions. All samples exhibited a high degree of baseline noise associated with the Q1 MS scan mode. Because of this, the samples were additionally analyzed by filtering the spectra for the m/z of known degradation products^{15,16,18}, which also identified no peaks of interest.

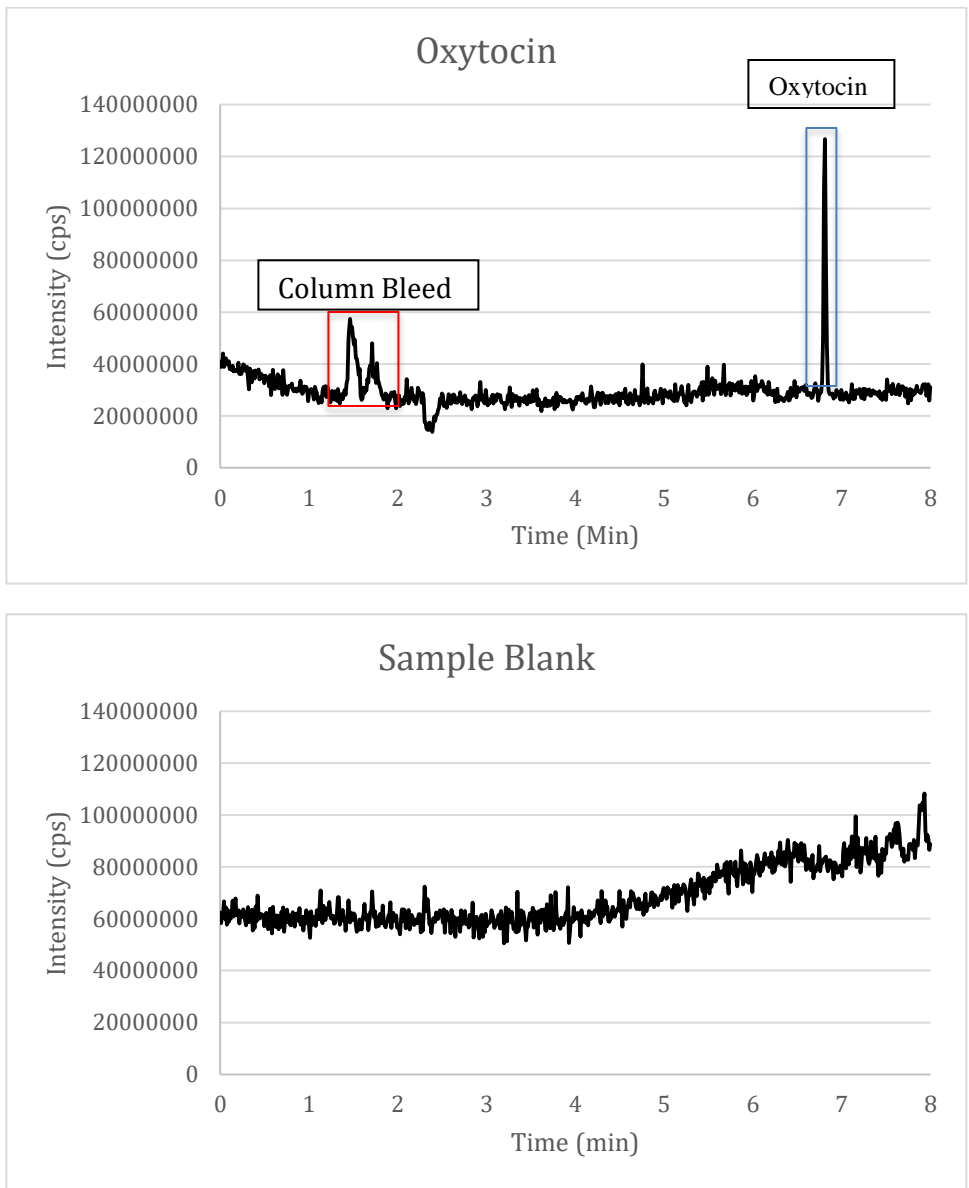


Figure 10. Oxytocin degradation after 1 hour at room temperature in acidic conditions (top) vs its blank (bottom). The only observed peaks are Oxytocin (labeled, and indicated), and two poorly shaped peaks that are believed to be column bleed due to the caustic solution.

4. CONCLUSIONS

Table 13. Summary of evaluated metrics across all product ions.

	723.2	86.2	70.1
Signal %CV (Min)	2.85	2.40	2.21
Signal %CV (Max)	10.04	9.32	8.53
Average STD (150 ng/ml)	5323	1299	1691
Calibration Sensitivity (linear)	1435	494	486
Average Analytical Sensitivity (linear)	0.07	0.07	0.07
Median Signal (150ng/ml)	5230	1760	1696
Noise (MDS)	62.00	49.61	31.00
Median Signal (150ng/mL) : Median Noise	84.36	35.49	54.71
%Bias (150-1000 ng) Max (Linear)	14.37	14.72	14.86
%Bias (150-1000 ng) Max (Non-Linear)	6.64	7.82	7.47

A summary of all metrics evaluated for comparative analysis between the three product ions selected is provided in Table 14. Of the three product ions assessed for oxytocin, the 1007.3→723.2 ion was observed to have the highest signal across all concentrations. While it does demonstrate a calibration sensitivity three times that of the other ions assessed, it had a correspondingly high standard deviation, resulting in an analytical sensitivity that was not sufficiently different across the observed product ions. The 723.2 ion's large mass is unusual amongst pharmaceuticals, and placed it well above the noise threshold in the degradation analysis. It was therefore selected as the quantitation ion for this method. While the remaining two ions were comparatively similar across all assessed metrics, the 1007.3→70.1 was selected as the qualifier ion.

This method demonstrates a low minimum distinguishable signal, and was successful in detecting oxytocin in concentrations as low as 10ng/ml. This is similar to the range achieved by Karbiwnyk et al in 2008, which had a LOD of 2.1 ng/ml and a linear range of 7.3-115 ng/ml using LC- ion trap mass spectrometry¹⁷. While the UFLC-MS/MS method may be used to screen for the presence or absence of oxytocin to identify counterfeit material containing no active ingredient, this method does not demonstrate sufficient accuracy by USP guidelines to be considered a validated method for the quantification of oxytocin³¹. Further protocol development, and identification of factors reducing linearity may be required in order to categorize this method as fit-for-purpose. It is presently more effective to model this protocol using a nonlinear model, but even the nonlinear model is not within validation parameters identified by the USP.

5.0 Future Research

5.1 Oxytocin

While reducing the method from three to two ions will increase sensitivity, additional optimization is required to bring this method into compliance with USP guidelines of +/-2% accuracy³¹. Further, the structure of the 70.1 ion is unknown at this time. Elucidating the structure of this fragment may provide insight into the specificity of these fragments to biologically active oxytocin.

Identifying the structure of the quartets observed during compound optimization in the Q1 mass spectra may also provide insight into the reduction of linear response observed in this experiment. The observation in a pure standard of what is proposed to be oxytocin in various states of deamidation, and the occurrence of the two unknown quartets at $m/z+5$ and $m/z+22$ could be relevant to the variation in biological effectiveness between equivalent doses of oxytocin.

Degradation analysis of oxytocin revealed no peaks of interest that could be used for specificity testing. While it is known that oxytocin can form trimers^{15,18}, which are above the upper detection limit of the mass spectrometer, other poly sulfonated products described in literature¹⁵, as well as dimers, are within mass detection limits. This suggests that these products did not form, or were unable to be resolved from the precursor peak of oxytocin with this method.

5.2 Artesunate/Amodiaquine

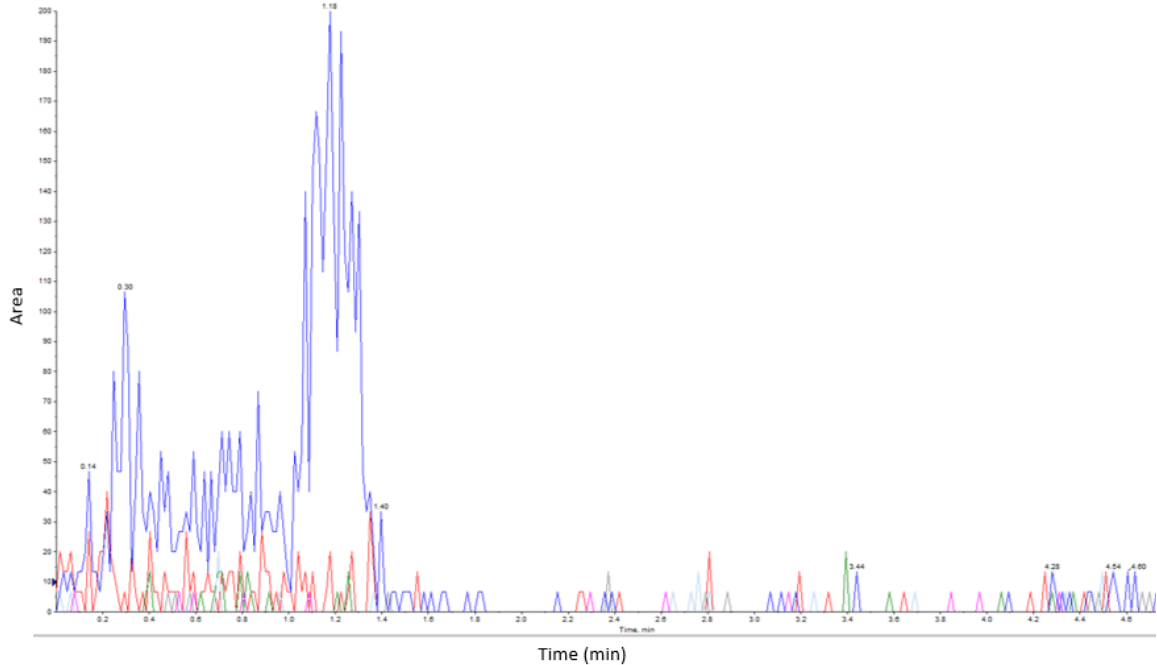
At this time no mobile phase tested was compatible with UFLC manufacturer guidelines. Each mobile phase tested has not produced sufficient signal to continue development of a UFLC/MS/MS protocol for the quantification of artesunate or amodiaquine. Additional testing of compatible mobile phases is required before method development may continue.

APPENDIX A: Mobile phases tested for artesunate/amodiaquine which yielded no significant signal during protocol development.

Mobile Phase A	Mobile Phase B	Observed Signal
2mm Formic Acid	Methanol	None
10 mm Formic Acid	Methanol	None
Deionized Water	Acetonitrile	None
Deionized Water	Methanol	None
2mm Ammonium Formate	Acetonitrile	None
10 mm Ammonium Formate	Acetonitrile	None

All mobile phases noted were tested within a 10 minute analytical period at gradients ranging from 10-90% B for any observed signal across 6-12 proposed transition ions of artesunate/amodiaquine. Samples were assessed at a concentration of 1000 ng/ml. A representative spectra indicative of a negative result is included below as Figure 11. Note poor signal quality (200 cps), a lack of shape, and a lack of correlation amongst ions. At this time no mobile phase compatible with a C18 column has been identified.

Figure 11. A Representative Negative Result for Artesunate/Amodiaquine Mobile Phase selection.



LIST OF JOURNAL ABBREVIATIONS

Anal Biochem	Analytical Biochemistry
Anal Chem	Analytical Chemistry
Anal Methods	Analytical Methods
Ann Agric Environ Med	Annals of Agricultural and Environmental Medicine
Biopolymers	Biopolymers
ChemMedchem	ChemMedChem
Chromatographia	Chromatographia
Drug Disc Ther	Drug Discoveries and Therapeutics
J Anal Toxicol	Journal of Analytical Toxicology
J Appl Pharm Sci	Journal of Applied Pharmaceutical Science
J Pharm Biomed Anal	Journal of Pharmaceutical and Biomedical Analysis
J Res Pharm Biotechnol	Journal of Biotechnology and Pharmacological Research
Pharm Res	Pharmaceutical Research
Pure Appl Chem	Pure and Applied Chemistry

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Professional Experience:

2007-2009: Analytical Team Leader, 110th CM BN(TE). United States
Army, Joint Base Lewis-McChord, WA. Lead a team of
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analysis of chemical, biological, radiological, nuclear and
high explosive materials.
2009-2010: Officer In Charge, Combined Explosive Exploitation Cell-N,
United States Army, Tikrit, Iraq. Supervisor of northern
division of CEXC, responsible for the forensic exploitation of
all improvised explosive devices (IED) for northern Iraq.
2010-2012: Assistant Operations Officer, 110th CM BN(TE). United
States Army, Joint Base Lewis-McChord, WA. Responsible
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laboratory operations.
2013-2014 Emergency Medical Technician, Basic. Olympic Ambulance.
911 response and interfacility transport. Over 1,000 patient
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Advanced Technical Education and Training

2008 Chemical Officer Basic Course
Hazmat Awareness
Hazmat Operations
2009 Hazmat Technician
Live Toxic Agent Training
Technical Escort
Permit Required Confined Space
NET "Street" Chemistry for Emergency Responders
Advanced Chemical and Biological Agent Overview (40 hr)

2012 Production and Use of Biological Agents (40 hr)
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Teaching Experience and Responsibilities (UNCLASS)

2010 Officer In Charge, Warrior Forge Chemical Training Section
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