THE DEVELOPMENT AND VALIDATION OF THE HPLC METHOD FOR DETERMINATION OF ARTESUNATE AND AMODIAQUINE IN NOVEL ANTIMALARIAL FORMULATIONS

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ABSTRACT

ASAQ combination drug used in treating malaria is bitter in taste, resulting in poor adherence and difficulty in swallowing by children. To mitigate this issue, taste masking is achieved by coating the drug with polymers to improve the taste of the dosage form. However, this produces a novel drug product which must undergo analytical evaluation to ensure that it is safe and drug content matches the label claim. The aim of this study is to develop and validate a novel analytical methodology which is suitable for this purpose.

Thus, a reversed phase high performance liquid chromatography for the quantitative determination of AS and its impurities was developed. The method was validated according to ICH guidelines for linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantification (LOQ). The method was found accurate and precise with an average retention time of 8.2 min. Good linearity was observed in the concentration range of 0.25 – 0.75 mg/ml with regression coefficient of r2 =0.999. The assay of the proposed method was found to be 101.5%. The recoveries of artesunate were found to be within 99% - 102%. The % RSD for precision was found to be <2%. Specificity was done to check potential interferences with excipients and degradation products and none of the retention times were interfering with artesunate peak.

A gradient method was developed for quantification of amodiaquine and its impurities. The method was validated according to ICH guidelines for linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantification (LOQ). The method was found accurate and precise with an average retention time of 13.75min. Good linearity was observed in the range of 0.075 – 0.225 mg/ml with regression coefficient of r2 =0.999. The assay of the proposed method was found to be 106.5%. The recoveries of artesunate were found to be within 98% - 102%. The % RSD from repeatability, intra and inter day precisions was found to be <2.0%. Specificity was done to check potential interferences with excipients and degradation products.

1. INTRODUCTION

1.1. MALARIA

Malaria is a life-threatening disease found around the world and is especially dominant in Africa. Malaria is spread to human beings by the female *Anopheles* mosquitos which are infected with these kinds of parasites. The parasites *Plasmodium falciparum*, *P. vivax, P. ovale*, and *P. malariae* are the primary cause of malaria in humans. Of these, *P. Falciparum* is the most harmful, leading to severe infections and potentially death.

As per data recorded by the WHO, in 2020 there were an estimated 241 million cases of malaria, with around 627,000 recorded fatalities. These data indicate that Africa has a large share of the global malaria burden, with 95% of recorded infections being found in this region. Of the deaths within this region, 80% occurred in children below five years of age, making them the most at risk for contracting the disease [1].

Malaria is an acute, fever-based illness. For a less immune person, the symptoms of disease are normally observed between 10-15 days after the bite of a parasite infected mosquito. The primary symptoms observed are generally fever, headache, and chills which may be acute and not easy to identify as malaria. Young children with severe malaria may possess more than one symptom, including, anaemia, respiratory troubles, metabolic acidosis, cerebral malaria and multiorgan failure [2].

Malaria is an avertable and curable disease. Malaria is controlled by early detection and treatment, which also lowers additional transmission and prevents fatalities. There are several antimalarial drugs available, which include quinine sulphate, atovaquone/proguanil, doxycycline, primaquine phosphate, chloroquine mefloquine, and artemisinin combinations. The best available first line treatment suggested by World health organisation (WHO), particularly for *Plasmodium falciparum* malaria, is artemisinin-based combination therapy (ACT) [1]. Artemether-Lumefantrine, Artesunate-Mefloquine (AS-MQ), Artesunate-Sulfadoxine/Pyrimethamine (AS-SP), Dihydroartemisinin-Piperaquine (DHA-PPQ) are some of the different ACT combination medicines used for the treatment. Artemisinin derivatives act quickly to

reduce the number of malaria parasites in the blood, leading to rapid clinical improvement. Partner drugs with different mechanisms of action work synergistically with artemisinin, enhancing overall treatment efficacy and reducing the likelihood of treatment failure. The combination of two or more drugs with different mechanisms of action helps to prevent the development of resistance. ACT regimens are usually short (typically 3 days), improving patient compliance compared to longer treatment courses. Better compliance leads to more effective treatment and reduces the risk of resistance development.

This combination therapy is proven to be more effective in treatment of acute, uncomplicated stage, because it is highly effective whilst having fewer adverse effects. The drugs used in this fixed combination is artesunate (AS) and amodiaquine (AQ), which can be administered to patients orally [3].

1.2. DRUG PROFILE OF ARTESUNATE AND AMODIAQUINE

Artesunate is a prodrug that is converted to its active form dihydroartemisinin (DHA). This process involves hydrolysis of the 4-carbon ester group via plasma esterase enzyme. It is hypothesized that the cleavage of endoperoxide bridge in the pharmacophore of DHA generates reactive oxygen species which increase stress and cause malarial protein damage via alkylation. The chemical structure of artesunate is given in Figure 1[4].

A disadvantage of artesunate is its short half-life. Rapid conversion to dihydroartemisinin takes place in vivo, with half-life of about 26 mins. Hence it is given in combination with other antimalarial drugs such as amodiaquine.

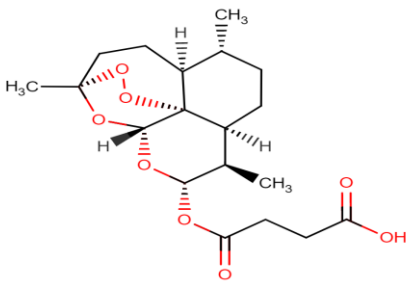

Figure 1: Structure of Artesunate $C_{19}H_{28}O$

The chemical structure of amodiaquine is given in Figure 2[5].

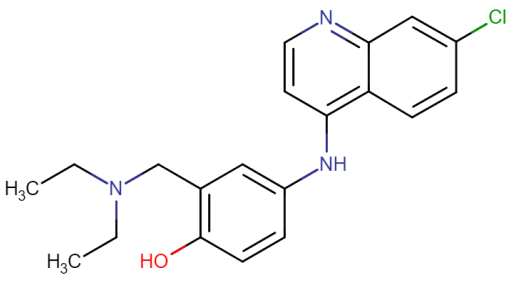

Figure 2: Structure of Amodiaquine $C_{20}H_{22}ClN_{30}$

Table 1: Properties of artesunate and amodiaquine

	Artesunate	**Amodiaquine dihydrochloride dihydrate**
Description	White crystalline powder	Yellow crystalline powder
Molecular weight	384.4 g/mol[4]	355.86 g/mol[5]
Solubility	soluble in organic solvents like ethanol, sparsely soluble in aqueous buffers.	soluble in water, very faintly soluble in alcohol, benzene, and ether.
Log P	2.61	3.8 [6]
pKa	4.6	7.1 and 8.1[6]

Amodiaquine is believed to limit the heme polymerase activity of parasite, just as other quinoline derivatives. This results in an increase of available heme, which is lethal to the parasites. This analyte connects with the free heme inhibiting the parasite, in turn changing to less lethal form and disrupts its membrane function [7].

The primary concern about AS-AQ combination is its bitter taste which results in poor adherence in children[8]. One such approach to overcome this, is by using a novel formulation approach to mask the bitter taste. By coating the active pharmaceutical

ingredient with MicroCoat™ technology developed at Fluid pharma Ltd, it is hypothesised that the bitter flavour of the drug when consumed orally will be reduced. APIs will be spray layered onto separate micropellets and taste masking is done with polymer coating (when necessary) separately using a modified fluidized bed coater. Following components were used in the formulation.

Table 2: Components used in formulation.

Components	**Mweight in g/mol**	**Chemical name**	**Layer in formulation**	**Function**
Cellets ®100	370.35	Microcrystalline cellulose	inner core	Have uniform particle size of 100 - 200 µm and large surface area which helps in uniform drug loading
PVP K30	40000	Polyvinylpyrrolidone	seal coating	It used as a binder, binding drug with Cellets and as a seal coating
Methocel™ E5	1261.4	Hydroxypropyl methyl cellulose	taste mask coating	Can help in masking the taste of bitter APIs especially for water soluble drugs and a pore former
Ethocel™ standard 20 premium	454.5	Ethyl cellulose	taste mask coating	It is water insoluble polymer and helps in controlling the drug release

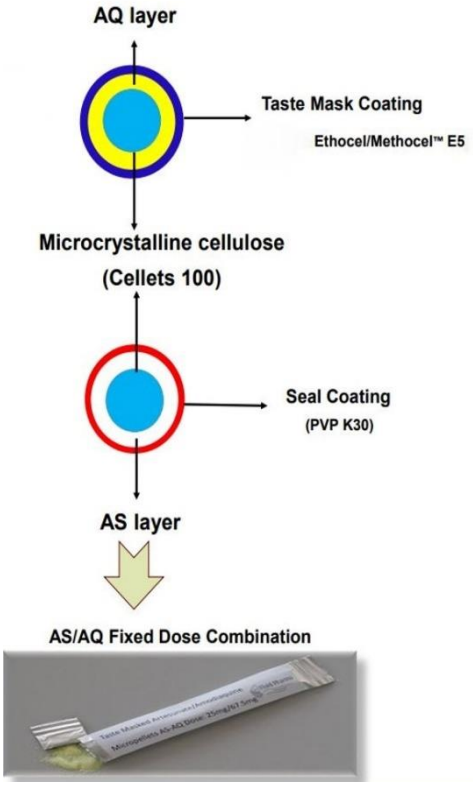

Figure 3: Schematic representation of different layers used in the formulation.

The main objective of any drug formulation is to be safe (e.g., to not cause any side effects to the consumer) and effective. To meet these criteria several safety characteristics must be assessed. The most important are strength, purity, and identity. The formulation to be developed by Fluid Pharma will differ from existing Winthrop AS-AQ tablet combination necessitating the analytical method development and validation to support novel development and safety testing.

1.3. PHARMACEUTICAL ANALYSIS

All analytical method development and validation is key part of pharmaceutical analysis it plays a significant part in the innovation, development, and mass production of drugs. These test procedures are used by quality control (QC) departments in establishing the uniqueness, purity, effectiveness, and performance of product essential for drug safety and therapeutic efficacy[9].

One method of choice for pharmaceutical analysis is high pressure liquid chromatography (HPLC) connected to an Ultraviolet (UV) detector, due to the advantages it offers over standalone UV in terms of specificity and sensitivity. The use of chromatography assists with the separation and identification of target analytes within complex mixtures. HPLC methods can therefore be used to identify, quantify analyte, and separate mixture of components present in a sample [10]. The HPLC method can be used to assess a range of analytes in combination dose forms due to the advantages of speed, selectivity, precision, ease of automation and accuracy[11]. Separation by HPLC is performed by flowing a mobile phase, with analyte dissolved within, over a stationary phase. Based on the chemical composition of the analyte, the molecules interact while moving through the stationary phase. The elution time of sample depends on the interaction of analyte with stationary phase. Thus, different components of a sample are eluted at various time points [11]. The detector unit (e.g., UV detector) detects the analytes when leaving the column. The signals are processed and verified by a computer software and subsequently displayed in a chromatogram.

In general, HPLC system contains the four major components.

1. Pump: The HPLC pump is called as solvent delivery system. The main function of pump is to deliver constant flow of liquid mobile phase all through the HPLC.
2. Injector: It is employed to inject the sample into the flow of mobile phase without affecting the system's flow rate or pressure.

3. Column: The column is the primary component responsible for separation of the analytes. When analytes present in the mobile phase move through the column, partition of analytes occur depending on their interaction with stationary phase. The most popular packing material for HPLC columns is silica. The long carbon chain of C18 provides a large surface area for interaction with analytes, leading to high selectivity and resolution in separations. This helps to achieve sharp and well-defined peaks in chromatograms. C18 is highly hydrophobic due to its long alkyl chain (18 carbon atoms). This allows a strong hydrophobic interaction with non-polar compounds, making it suitable for reversed phase HPLC, where non-polar analytes are retained and separated effectively. C18 bonded phases are chemically stable and can withstand a wide range of pH values (typically pH 2 to 8), making them durable and suitable for various analytical conditions. Other kinds of substances for the support of the stationary phase include polymers and alumina[12]. columns have different length ranging from 30mm to 250mm and particle size vary from 3-5 μ.
4. Detectors: In HPLC a UV/Vis's spectrophotometer detection unit comprises of a flow cell that is positioned beneath the column. As samples moves from the column, they travel through the flow cell and take up UV light, changing the energy level in a quantitative way. A high-pressure xenon lamp, deuterium, or a mercury lamp could be the source of the radiation.[13]. Multi-wavelength detectors can determine the absorbance at different wavelengths altogether at the same time. In DAD (diode array multi-wavelength detector) the uninterrupted radiation is projected over the analyte cell. The photodiode array

picks up each wavelength that has been independently settled. These detectors gather data on absorbance across the entire UV-visible spectrum.

Isocratic and gradient elution:

Most commonly, two elution methods are used in HPLC termed isocratic and gradient elution. An isocratic method makes use of a mobile phase ratio which remains constant throughout analysis. In contrast, gradient elution method makes use of changing mobile phase composition. Whilst isocratic methods offer simplicity in terms of method development and performance, they can often lack the ability to fully separate complex mixtures of multiple analytes[9].

Modes of Chromatography:

Whilst several modes of chromatography can be used with HPLC, the most popular method for separating target analytes in the chemical, pharmaceutical, and biological sciences is reversed phase chromatography. In this method, silica gel is hydrophobically packed with octyl or octa decyl functional groups attached, with a polar mobile phase and a non-polar stationary phase. To maintain chemical equilibrium, the aqueous mobile phase may use secondary solutes for retention and selectivity adjustment through complexation, ion pairing, ion suppression, and ion control. In this mode, the nonpolar molecules are retained for a longer period due to their strong affinity for the nonpolar stationary phase, while polar compounds are eluted first[14].

1.4. ANALYTICAL METHOD VALIDATION

It is crucial to make sure that any developed analytical method is suitable for analysis of a developed drug product, its performance characteristics must be assessed to ensure that they fulfil the requirements for the planned analytical application. Method validation is the procedure of assessing whether the developed analytical procedure is appropriate for its intended use, in providing assessment of the strength, and quality, for the quantification of the drug substances and drug products. The International Conference on Harmonization (ICH) aims to achieve harmonization for development and approval of safe, high quality and effective medicines. The ICH has developed a guideline for the validation of analytical methods, stability studies[15], impurities analysis with complete methodology[16]. As the formulation developed by Fluid Pharma will be used within global markets, the ICH guidelines will be appropriate for analytical method development and validation.

Below is a summary of the validation parameters established by the ICH and other regulatory agencies. 1) Specificity 2) Accuracy 3) Limit of detection 4) Limit of quantitation 5) Precision 6) linearity and range 7) Robustness 8) Solution stability 9) System suitability. The aim of the validation should be planned according to pre-specified approval conditions. The objective and scope of the method must always be stated as the first stage of any method validation because the type of analysis and the supplemental information of a sample have an impact on the validation. The proper validation parameters must be specified for a well-organized validation procedure. The International Conference on Harmonization has issued the specific quality requirements for method validation. [17]

1.5. LITERATURE REVIEW OF PUBLISHED AS AND AQ ANALYTICAL METHODS

Several analytical methods have been reported in the literature for the analysis of artesunate and amodiaquine which are summarised in Table 2. The published methods primarily make use of columns containing C18 material of lengths ranging from 3 to 25 cm and have diameters between 3 to 10 µm.

Particles that pack the columns have a typical diameter between 3 to 5 µ. Smaller particle size will improve the efficiency of separation, but the drawback is an increase in system back pressure [12]. These are most often coupled to mobile phases composed of phosphate buffer mixed with methanol and acetonitrile [18]. Phosphate buffer has wide application as a mobile phase in reversed phase HPLC, in part, due to its ability to be used at wavelengths below 220 nm. The use of pH modification can be used to change the ionisation state of analytes, influencing their retention by a C18 stationary phase[19].

The use of a UV detector was applied for all studies in Table 2, with wavelengths of 210 nm [20] 216 nm [21], and 220 nm [22], used for artesunate, and 223 nm[23], 211 nm[3], 339 nm[24] for amodiaquine. These are wavelengths which are observed to be the lambda max for each of these compounds, as evidenced by full UV-vis scans published in the literature [25]UV provides good sensitivity for light absorbing compounds and has seen widespread application to pharmaceutical analysis.

Within the published methodology, LODs could be observed in the range of 2.07-87.9 µg/mL for artesunate and 1.23-1.64 ug/mL for amodiaquine. The higher LODs for artesunate highlights the poorer UV absorbance of artesunate in comparison to amodiaquine. This is reported in several publications[23] .

Artesunate instability is a common theme, with stability observed to be poorer in solvents like ethanol, polyethylene glycol 400, propylene glycol [26], and aqueous solvents[27] whilst good stability was observed in solvent acetonitrile and methanol.

This instability has been overcome during sample preparation in certain publications by use of short extraction times and selection of appropriate solvents.

Artesunate is highly soluble in organic solvents and intermittently soluble in aqueous buffers. Whilst many publications detail analytical methods for the assay of APIs, the assessment of impurities is not widely observed within the literature.

The analysis of both APIs and impurities adds a level of complexity to HPLC analysis, due to the need to separate a greater number of compounds and assess impurities at trace concentrations[28]. Of the publications which have made note of impurities of AS and AQ, one has demonstrated quantification of the major impurity of artesunate, DHA [29]. Other studies have shown separation of impurities from APIs, but not quantified[30], whereas the majority have not discussed impurities. The intended application of these studies is, in part, responsible, with assay and dose content analysis (DCA) of tablets being the primary motivation for several publications.

Table 3: Reported HPLC methods for artesunate and amodiaquine

S. No	REFERENCE	COLUMN	MOBILE PHASE	INJECTION VOLUME	FLOW RATE	DETECTOR	RETENTION TIME	OBSERVATIONS	CHALLENGES
1	International Pharmacopeia for Artesunate[31]	(10 cm × 4.6 mm, 5µm) stainless steel column is used. Maintain the column at 30 °C	44 volumes of acetonitrile and 56 volumes of buffer pH 3.0 is used.	20 µL	1.0 mL /min.	Detection is carried at a wavelength of 216 nm.	Retention time about 9 mins.	Identity tests, Impurity evaluation, Assay method	No mention of Stability and Excipient study
2	Novel Stability Indicating RP-HPLC Coupled with PDA Detection for the Simultaneous Quantification of Artesunate and Amodiaquine in Bulk and its Tablet-[23]	symmetry Shield RP18 (250mm × 4.6mm, 5µm) column. Column temperature is at 30°C.	Acetic acid was used to adjust the pH of potassium dihydrogen phosphate buffer 60v (A): methanol 40v (B) mixture to 3.8.	10 µL	The elution was performed at a flow rate of 1 mL/min	PDA Detector of wavelength was selected at 223 nm.	The retention time was found 1.519 and 3.643 mins for artesunate and amodiaquine.	Method was validated according to ICH guidelines, forced degradation studies were performed and stability was done.	They have not mentioned about any impurity studies of AS and AQ
3	Simultaneous Determination of Artesunate and Amodiaquine in Fixed-Dose Combination by a RP-HPLC Method with Double UV Detection-[30]	C18 column (100 * 4.6 mm, 3 µm particle size).	The mobile phase A consist of anhydrous potassium dihydrogen phosphate buffer 10mM and sodium 1-octanesulfonate 100mM. pH was adjusted to 3.0 with phosphoric acid. Mobile phase B consists of acetonitrile.	10 µL	0.8 mL/ min is used as flow rate.	Active ingredients AS and AQ were detected at 210 and 300 nm, by UV-visible detector respectively	AQ and AS retention time is 4.3 mins and 10.7 mins.	Forced degradation studies, assay method, validation and impurities identification were done for AS and AQ.	The impurity samples are different. Trying with different diluent solutions.
4	RP-HPLC Method for Simultaneous Estimation of AS and AQ HCl in their Combined Dosage Form- [32]	Octadecyl silane column 250 mm length and 4.6mm diameter and a particle size of 5 µm is used.	Phosphate Buffer and methanol composition in the ratio of 30 and 70. (pH of Phosphate Buffer was maintained to 3 using 0.5% of Ortho-phosphoric acid).	20 µL	1.0 mL/min	Wavelength For Artesunate is 225 nm and Amodiaquine is 339 nm by uv-visible detector.	Retention time for Artesunate is 3.99 mins and Amodiaquine is 2.39 mins.	validation and assay method were performed.	Forced degradation studies and impurities identification was not done which are important parameters for developing safe and effective dosage form.
5	A validated HPLC method for determination of AS and AQ in bulk and tablet formulation[33]	column was Inertsil C18, 250 mm length and 4.6mm diameter and a particle size of 5 µm is used.	Ortho phosphoric acid, is used to adjust pH to 5.8 of mobile phase constituting phosphate buffer, acetonitrile, and methanol in the proportion of 50:30:20.	20 µL	1.0 mL/min is used as flow rate.	Detection was carried out at 208 nm by uv-visible detector.	AS and AQ HCl were eluted at 5.03 mins and 2.77 mins respectively.	validation and forced degradation studies were performed corresponding to ICH guidelines and outcomes were within the acceptance criteria.	No information about impurities studies of both artesunate and amodiaquine.
6	The initial pharmaceutical development of an AS/AQ oral formulation for the treatment of malaria[3]	AS was determined using Hypersil C4 column, 250 mm length and 4.6mm diameter and a particle size of 5 µm. AQ was determined by using PERKIN ELMER lambda 20 Spectrophotometer at 342 nm wavelength.	Mobile phase consists of Sodium acetate trihydrate and pH scale is adjusted to 5.2 using 0.05 M/NaOH and acetonitrile mixture (63:37).	100 µL	1.5 mL/min-	Detector is set to 211 nm by uv-visible detector.	Elution time for AS was 8 mins and DHA is 11 and 16 mins (the α and β DHA, respectively) and artemisinin is 10 mins.	stability studies were performed by controlling the humidity. Assay method and validation were specified.	This paper has specified about any impurity studies of artesunate and amodiaquine.

7	Analytical Method Development and Validation of AS and AQ HCl in Combined Tablet Dosage Form by HPLC [24]	Column used is 150 mm x 3.9 mm and particle size of 5 µm, made up of stainless steel.	mobile phase containing a mixture of phosphate buffer and methanol in the ration of 30 and 70 respectively, pH scale of 3.0 is used.	20 µL	Flow rate is 1.0 mL/min	Diode Array Detector (DAD) is set at wavelengths of 225 nm, 339 nm for AS and AQ-HCl, respectively.	AS, AQ HCl had retention durations of 1.3 mins, however they were extracted at different wavelengths.	This method was validated according to ICH guidelines. Forced degradation studies were conducted.	They have not mentioned about any impurity studies and stability studies of AS and AQ.
8	A report on forced degradation studies of AS and AQ tablets[20]	Column length of 10 cm, diameter of 4.6-mm, particle size of 3 µm is used	Mobile phase contains phosphate buffer with a pH scale of 3 and acetonitrile in the proportion of 5:4.	20 µL	Flow rate is set at 0.8 mL/min	UV detector is used for AS and AQ at wavelength of 210 nm and 300 nm respectively.	Retention time of artesunate was 8.5 mins and amodiaquine is 2.99 mins.	A validation of stability indicating method development was done.	There is decrease in assay value by acid and base degradation. Artesunate was completely degraded when sample is heated till 60 mins into unknown degradant.
9	A simple and sensitive RP-HPLC method for simultaneous estimation of Artesunate and Amodiaquine in combined tablet dosage form[22]	Hypersill C18 (250, 4.6 mm i.d.) is used as column.	Mobile phase is a mixture of Acetonitrile and 25 mM potassium dihydrogen phosphate buffer (70:30, v/v)	20 µL	Flow rate of 1 mL/min	Detection was carried out at 220 nm by uv-visible detector.	Retention times 5.202 min,2.983 min for artesunate and amodiaquine respectively.	Method was validated according to ICH guidelines.	Impurity analysis and forced degradation studies were not performed.
10	The forced degradation and solid-state stability indicating study and Validation of method for the determination of Assay of Artesunate by HPLC[34]	Symmetry shield RP -18, 4.6 x 150mm, 3.5µm is used as column.	The mobile phase was composed of a 30:35:35 v/v/v mixture of water, acetonitrile, and methanol.	30 µL	1.0 mL/min as a Flow rate	detection wavelength was set at 210 nm by uv detector.	retention time is 7.8 mins.	assay is stability indicating and impurities for artesunate were also spiked	This method cannot be used for amodiaquine for simultaneous estimation.
11	RP-HPLC Method for the Determination and Quantification of Artesunate [21]	C18 Promosil column (ODS, 150 × 4.6 mm, 5 µm) is used.	Mobile phase comprised of ethanol: water (65:35) having pH 4.5 was run isocratic.	20 µL	flow rate of 1 mL/min	detection wavelength was set at 216 nm.	retention time is 4.8mins.	developed method was validated.	Impurity identification is not done and forced degradation studies were not performed.

1.6. AIMS AND OBJECTIVES

The aim of this research programme is to develop HPLC methods for the analysis of artesunate and amodiaquine hydrochloride in novel antimalarial formulations that mask the taste of the drugs for the treatment of malaria in children. The objectives of the study include:

1. To develop an HPLC methods to support assay of a novel drug product (formulation) containing both artesunate and amodiaquine, to validate this method in accordance with ICH guidelines.
2. To develop an HPLC method to support the dissolution testing of novel formulations containing artesunate, and to validate this method in accordance with ICH guidelines.
3. To assess the stability of artesunate and amodiaquine in different solvents to be performed to check the stability of both drugs and this can confirm which solvent can be used for extraction of both the API from the formulation.
4. To perform forced degradation studies of both the API to know whether any degradation products formed are interfering with drug peaks.
5. To apply these methods to support development of the novel formulation.

2. MATERIALS AND METHODS

2.1. MATERIALS:

Artesunate (AS- lot no: 230001) is obtained from Huevepharma (Huevepharma, Italy) and Amodiaquine Hydrochloride (AQ- lot no: 210016) is obtained from IPCA (IPCA, India). HPLC grade solvents methanol (lot no: 1914578), acetonitrile (lot no: A9964), 96% ethanol (lot no: 1991216), potassium dihydrogen phosphate, sodium octane sulfonate, sodium acetate, PTFE, PVDF and polypropylene filters was procured from Fisher Scientific. Cellulose acetate and glass filters were sourced from Chromatography Direct, Runcorn UK). AS and AQ reference standards are obtained from Hueve pharma. Impurity reference standards of artemisinin, anhydrodihydroartemisinin, dihydroartemisinin, acetaminophen, 4,7- Di chloroquinoline (4,7- DCQ), 7- choro-4-hydroxy phenylamino quinoline and 4-acetamido-2-diethylaminomethylphenol were obtained from LGC, UK. Phosphoric acid, perchloric acid and hydrochloric acid are purchased from Honeywell. Uncoated AS micro pellets formulations one with (Artesunate, Cellets, Syloid and 1.2% PVP K30 and one with (Artesunate, Cellets, Syloid and 0.8% klucel), coated AQ micro pellets formulation with (Amodiaquine, Cellets, Syloid, Methocel, Ethocel and magnesium stearate) are obtained from Fluid Pharma.

2.2. METHOD DEVELOPMENT FOR ASTESUNATE & ITS IMPURITIES

2.2.1. PREPARATION OF SAMPLES

Buffer: Accurately weighing 1.36 g of potassium dihydrogen phosphate, it was then transferred to a volumetric flask, dissolved in 900 mL of water, and then brought to pH 3.0 by adding phosphoric acid while stirring at 200 RPM with a bibby stirrer. Finally, the volume was brought to 1000 mL by adding deionized water. Then, a 0.45 µ nylon filter membrane was used to filter this solution.

Mobile phase: It was prepared from this buffer by combining sixty-six volumes of acetonitrile and thirty-four volumes of buffer. This was used as mobile phase after filtration and degassing.

Artesunate stock solution: It is prepared by weighing 5 mg of analyte and dissolve it in 5 mL of methanol then sonicate it for 5 mins, before making to volume with methanol and vortex it for 10 sec to get a final concentration of 0.5 mg/mL.

Artesunate impurities stock solution: DHA, artemisinin, anhydrodihydro-artemisinin were prepared by weighing 1 mg of sample and dissolve it in 5 mL of methanol then sonicate it for 5 mins, before making up the volume to 10 mL with methanol and vortex it for 10 sec to get a final concentration of 0.1 mg/mL.

Amodiaquine hydrochloride stock solution: It was prepared by weighing 13.5 mg of analyte and dissolve it in methanol and sonicate for 5 mins and make up the volume up to 10 mL with methanol to get a final concentration of 1.35 mg/mL.

Amodiaquine impurities stock solution: weigh 1.35 mg of acetaminophen, 4,7- Di-chloroquinoline, 7-chloro-4-(4-hydroxy phenyl amino) quinoline and 4-acetamido-2-diethylaminomethylphenol and dissolve it in 5 mL of methanol and sonicate it for 5 mins then make up the volume up to 10 mL with methanol to get a concentration of 0.00675 mg/mL.

Forced degradation studies:

Acid degradation: AS stock solution was prepared at a concentration of 2.5 mg/mL from the stock take 5 mL of solution and add 4 mL of 0.01M HCL and heat it in water bath at 50 ^{0}C for 30 mins. Samples were then made up to volume by addition of sufficient methanol to reach 25 mL before being filtered and injected on the HPLC system.

Base degradation: AS stock solution was prepared at a concentration of 2.5 mg/mL from the stock take 5 mL of solution and add 4 mL of 0.01M NaOH and heat it in water bath at 50 ^{0}C for 30 mins. Samples were then made up to volume by addition of sufficient methanol to reach 25 mL before being filtered and injected on the HPLC system.

Oxidative degradation: Studies were done similarly 2.5 mg/mL stock solution was prepared then take 4 mL of the sample from stock solution and add 4 mL of 10 % H_2O_2 which was left for 24 h before being made to volume, filtered, and injected onto the HPLC system.

Photolytic degradation: A stock solution of 0.5 mg/mL was treated with light for 5 h by use of UV light 24 h. These samples were then injected onto the HPLC system.

Heat Degradation: A stock solution of 0.5 mg/mL sample was heated in water bath at 50 ^{0}C for 24 h. These samples were then injected onto the HPLC system.

Placebo preparation:

Placebo mixture was prepared by weighing all the excipients (Cellets, Polyvinylpyrrolidone K30, Syloid) used in the formulation without adding API and add it to 50 mL volumetric flask. Then add ¾ of methanol and stir it by placing a magnetic stirrer bar in the sample for 60 mins. After 60 mins make up the volume to 50 mL with methanol and filter the sample.

Preparation of stability samples:

A solution of Artesunate reference standard at the target concentration of 0.5 mg/mL, along with an extracted sample of formulation with and without the presence of AQ formulation, was held for 24 h and stability was assessed over that period. The concentration for the standard and sample solution at initial time point was determined. Assess the concentration of same sample and standard solution at different time points for 24 h against 24 h and report the change the API concentration over that time.

2.2.2. SIMULTANEOUS METHOD DEVELOPMENT FOR AS AND AQ:

This methodology was adapted from [30]. In this method HPLC system with SPD-20A UV Visible detector was used. A Thermo Hypersil BDS C18 column 100* 4.6 mm, 3 µm particle size (Thermo Fischer, Hemel Hempstead) was used for separation. A gradient separation was performed at a flow rate of 0.8 mL/min. Mobile phase A was 10 Mm

phosphate buffer adjusted to pH 3.0 using orthophosphoric acid and 100 Mm octane sulfonate and mobile phase B was acetonitrile. Initial conditions were 60% A held for 5 mins followed by decrease in %A to 40% after 15 mins, which was then held for 2 mins before returning to 60% over 0.1 min and holding for 5 mins. Both 210 nm and 300 nm were used as wavelength with a 10 µL injection volume.

2.2.3. EVALUATION OF METHOD FROM INTERNATIONAL PHARMA-COPEIA MONOGRAPH FOR AS ANALYSIS:

In this method separation was performed on Thermo Hypersil BDS C18 column 100*4.6mm, 3µm particle size at 30 ^{0}C. Isocratic method was used with mobile phase composition containing phosphate buffer adjusted to pH 3.0 using phosphoric acid and acetonitrile at a ratio of 44:56 respectively. A flow rate of 1 mL/min was used, a wavelength of 216 nm with a run time of 20 mins and injection volume of 20 µL is used.

Various conditions were altered based on the artesunate IP monograph to separate all the analytes. This includes changing the mobile phase composition, columns, flow rate and injection volume.

Final method development was done on Varian column (150* 4.6 mm, 3 µm) with mobile phase consisting of 66% acetonitrile and 34% phosphate buffer adjusted to pH (3.0) using phosphoric acid at a flow rate of 0.4 mL/min. The total run time was 20 mins and wavelength used was 216 nm.

2.2.4. VALIDATION OF DEVELOPED METHOD FOR ANALYSING ARTESUNATE & ITS IMPURITIES USING HPLC

The HPLC methods were validated for specificity, linearity, precision, and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005).

Specificity of an analytical method is its ability to measure accurately an analyte in the presence of interference, such as excipients, and known (or likely) degradation products that may be expected to be present in the sample matrix.

Following sample solutions were injected on to HPLC:

- Methanol(diluent)
- AS standard at 100% test concentration (0.5 mg/mL)
- Placebo mixture of final formulation without API.
- Forced degradation samples.

Linearity was determined by the correlation coefficient (R2) for calibration curves constructed following the isocratic elution method analysis (concentration range 0.25 mg/mL– 0.75 mg/mL) of artesunate standards. The linearity for the related substances (DHA, artemisinin, Anhydrodihyroartemisinin) was assessed over the range of 0.5 % to 20 % of the nominal test concentration of AS, which equates to 0.0025 mg/mL – 0.1 mg/mL. AS reference solutions were also assessed across this range, to demonstrate the acceptable linearity of AS at these low concentrations.
The standard error for the predicted y value for all x values in the regression was calculated and used to calculate the limit of detection (LOD) and limit of quantification (LOQ) of artesunate, respectively.

Determination of the precision of the analytical methods was achieved by repeatability, intra-day, and inter-day analysis. Repeatability was assessed by injecting 6 replicates of standard sample at 100 % of the nominal test concentration of 0.5 mg/mL. Intra-day precision was measured by three different standard solutions of Artesunate (0.25, 0.5, and 0.75 mg/mL) were prepared from working standard solution and injected into the system with stated chromatographic conditions and analysed, three times in a day. Inter-day precision was assessed through preparation of Standard solutions of Artesunate 0.25, 0.5 and 0.75 mg/mL from working standard solution and injected into the system with same chromatographic conditions and analysed for three consecutive days.

Accuracy was assessed by preparation of known concentrations of analytes prepared from individually prepared stocks made with artesunate reference material. The closeness of the observed concentration to the theoretical concentration, based upon weighing and dilution, was used to calculate the accuracy of the method.

The range of the method for assay is confirmed as 0.25 mg/mL to 0.75 mg/mL, over which acceptable accuracy, linearity, and precision were obtained. The range of the method suitable for related substances is set as 0.0025 mg/mL – 0.1 mg/mL for AS, artemisinin, DHA (alpha), 0.0005 – 0.1 mg/mL for anhydro artemisinin, and 0.0125 – 0.1 mg/mL for DHA (beta). Over this range, acceptable linearity has been observed for AS and its associated impurities.

2.3. METHOD DEVELOPMENT FOR ARTESUNATE DISSOLUTION SAMPLES

2.3.1. PREPARATION OF SAMPLES

Buffer: weigh 1.36 g of potassium dihydrogen phosphate, it was then transferred to a volumetric flask, dissolved in 900 mL of water, and then brought to pH 3.0 by adding phosphoric acid while stirring at 200 RPM with a bibby stirrer. Finally, the volume was brought to 1000 mL by adding deionized water. Then, a 0.45 µ nylon filter membrane was used to filter this solution.

Mobile phase: It was prepared from this buffer by combining 44 volumes of acetonitrile and 56 volumes of buffer. This was used as mobile phase after filtration and degassing.

Artesunate stock solution: 10 mg of artesunate was accurately weighed and transferred into a 10 mL volumetric flask. 5 mL of acetonitrile was added, and the sample sonicated for 5 mins using an (decon ultrasonic bath). This was then made to volume using acetonitrile.

1 mL of the solution was added into a 10 mL flask and diluted up to the mark with acetate buffer to give a target concentration of 100 µg/mL of artesunate.

Preparation of accuracy samples: They were prepared in triplicate at 50 %, 100 % and 150 % of nominal test concentration at 0.028 mg/mL. These samples were injected 3 times each on to HPLC.

2.3.2. STABILITY TESTING

Many analytes may get degraded before doing chromatographic analysis like preparation of samples, during storage of prepared vials and during extraction time hence under these conditions during method development samples should be evaluated for stability.

The stability of artesunate was evaluated in different solvents acetonitrile, acetate buffer, 96% ethanol and methanol for 6 h to know the percent degradation of artesunate in various solvents.

Around 10 mg of artesunate was measured and added to a 10 mL volumetric flask, before addition of either acetonitrile, methanol, 96 % ethanol or acetate buffer This solution was sonicated for 5 mins to dissolve the powder. After dissolving, this solution was made to volume (10 mL) with the same solvent to give stock solutions of drug in 100 % of each of the solvent mixtures. The samples were further sonicated, before being filtered by using micro syringes and 25 mm nylon syringe filters into HPLC vials. Samples were then analysed by HPLC using a composition of acetonitrile with a buffer pH scale of 3.0 in proportion of 44:56 v/v as mobile phase for 30 mins with a flow rate of 1.0 mL/min. 12 times sample was injected repeatedly for 6 h. Column used here is ACE C18, 150 × 4.6 mm,3 µ with an Injection volume of 20 µL of sample into system.

2.3.3. SOLUBILITY DETERMINATION

Solubility of artesunate was determined in deionised water, acetate buffer pH - 5.5 and simulated saliva. Known excess amount of 20 mg of artesunate was placed in 100 mL amber bottles separately then 20 mL of the solvent under investigation was added. A magnetic stirring bar was placed in each bottle. All tests were done in duplicate, and bottles are placed on a magnetic stirring plate for (1 h, 3 h and 6 h). 5 mL was withdrawn from each bottle, filtered using 0.2µm PTFE syringe filter with first 3 mL discarded and 2 mL taken for analysis using HPLC.

2.3.4. EVALUATION OF THE METHODOLOGY FROM IP MONOGRAPH OF ARTESUNATE TABLETS

In this section a Shimazu HPLC system with an SPD-20A UV visible detector was used. Methodology from IP monograph of Artesunate tablets was adapted. ACE C18 column (150 × 4.6mm)5µm particle size column was used for separation. Isocratic separation was performed at a flow rate of 1 mL/min by using potassium phosphate buffer of pH-3.0 and acetonitrile (44:56 v/v) as mobile phase and 216nm as detection wavelength and 100 µL injection volume.

2.3.5. PREPARATION OF CALIBRATION STANDARDS

A 1 mg/mL stock solution of artesunate was prepared by weighing 10 mg of the drug into a 10 mL volumetric flask and making up to volume with the diluent, acetonitrile. A series of standards were prepared by appropriate dilution of the stock solution with acetate buffer pH – 5.5 the concentration of the standards ranged from 0.01 mg/mL to 0.1 mg/mL. Drug quantification was achieved using the analytical methods detailed in Section 2.3.6 and calibration curves plotted for the detected range.

2.3.6. VALIDATION OF DEVELOPED ARTESUNATE ANALYTICAL METHOD FOR DISSOLUTION TEST:

The HPLC methods were validated for linearity, precision, and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). Linearity was determined by the correlation coefficient (R2) for calibration curves constructed following the isocratic elution method analysis (concentration range 0.01 mg/mL – 0.1 mg/mL). The standard error for the predicted y value for all x values in the regression (STEYX) was calculated and used to calculate the limit of detection (LOD) and limit of quantification (LOQ) of artesunate, respectively.

Determination of the precision of the analytical methods was achieved by instrument precision. It was assessed by injecting 6 replicates of standard sample at 100 % of the nominal test concentration of 0.028 mg/mL.

Accuracy standards were prepared by taking known concentrations of analytes from reference standards. The peaks areas generated were used to calculate concentrations of accuracy samples.

2.4. METHOD DEVELOPMENT FOR AMODIAQUINE & ITS IMPURITIES

2.4.1. PREPARATION OF SAMPLES

Buffer preparation: weigh about 13.6 g of monobasic potassium phosphate and dissolve in 2 L of water. Add 20 mL of 0.01M perchloric acid and mix, adjust the pH with phosphoric acid to 2.5±0.5. Pass the solution through a 0.45 µm membrane filter.

Mobile phase: Mixture of buffer and acetonitrile 56:44 v/v was used as mobile phase.

Stock solutions of AQ: It was prepared by weighing 1.5 mg of analyte, then add 5 mL of methanol: water (80:20) and sonicate it for 5 mins, before making the volume with methanol to get a final concentration of 0.15 mg/mL.

Stock solution of AQ impurities: 5 mg of paracetamol, 4,7-dichloroquinoline, 7-chloro-4-(4-hydroxyphenylamino) quinoline and 4-aetamido-2-diethylamino-methylphenol were weighed and dissolved in 5 mL of methanol: water (80:20) and sonicated for 5 mins then make up the volume up to the mark to get a final concentration of 0.5 mg/mL stock solution. Take 17 µL from stock solution and dilute up to 10 mL with methanol: water (80:20) to get a concentration of 0.00075 mg/mL.

Stock solution of AS: 5 mg of artesunate standard was weighed and dissolved in methanol: water (80:20) and sonicate it for 5 mins and make up the volume with methanol: water to get a final concentration of 0.5 mg/mL.

Stock solution of AS impurities: weigh 1 mg of Dihydroartemisinin (DHA), artemisinin, anhydroartemisininin and dissolve in methanol: water (80:20) and sonicate it for 5 mins and make up the volume with methanol: water to get a final concentration of 0.1 mg/mL.

Forced degradation sample stock solution: 50 mg of AQ was weighed and dissolved in water and sonicate for 5 mins and make up the volume up to mark to get a final stock concentration of 5 mg/mL.

Acid degradation sample preparation: Take 750 µL of sample from above stock and add 4 mL of 0.01M HCL was added and subsequently left in fume hood for 48 h and make up the volume up to 25 mL with water after 48 h and analyze the samples.

Base degradation sample preparation: Stock solution was prepared in methanol: water. Take 750 µL of sample from stock and 4 mL of 0.01M NaOH was added and subsequently left in fume hood for 30 mins and make up the volume up to 25 mL with water after 30 mins and analyze the samples.

Oxidative degradation sample preparation: Take 750 µL of sample from stock and 16.7 mL of 30% v/v Hydrogen peroxide(H_2O_2) was added and subsequently left in fume hood for 24 h and make up the volume up to 25 mL with water after 24 h and analyze the samples.

Heat degradation sample preparation: Take 750 µL of sample from stock and 20 mL of water was added and left in water bath at 60 ^{0}C for 24 h and make up the volume up to 25 mL with water after 24 h and analyze the samples.

Light degradation sample preparation: Take 750 µL of sample from stock and 25 mL of water was added and left under UV light for 72 h and analyze the samples.

2.4.2. EVALUATION OF THE METHODOLOGY FROM USP MONOGRAPH FOR AQ ANALYSIS

Separation was performed on pursuit Varian C18 column (150 × 4.6 mm, 3 µm) held at 25 ^{0}C and detection was carried out at 224 nm with an isocratic mobile phase composition containing phosphate buffer of pH – 2.5 and methanol at a ratio of 78: 22 v/v respectively. A flow rate of 1 mL/min was used with a total run time of 45 mins and injection volume of 10 µL.

2.4.3. MODIFICATION OF THE USP MONOGRAPH METHOD

Various method conditions were altered to develop a new separation. This includes changing the mobile phase from methanol to acetonitrile and changing the mobile phase composition from isocratic to gradient with a flow rate of 1 mL/min. changing

the oven temperature. Final developed conditions are given as follows: separation was performed on a Varian column (150 × 4.6 mm, 3 µm) held at 40 ^{0}C with a gradient mobile phase containing buffer: acetonitrile. Initial starting conditions were 95%buffer and 5% acetonitrile for 9 mins. Followed by decrease in buffer % to 80% from 9-20 mins, followed by decrease in buffer% to 60% from 20 – 40mins and from 40-45mins the mobile phase was returned to starting conditions 95% buffer and 5% acetonitrile.

2.4.4. VALIDATION OF DEVELOPED AMODIAQUINE HYDROCHLORIDE & ITS IMPURITIES METHOD:

The HPLC methods were validated for specificity, linearity, precision, accuracy, limit of detection, limit of quantification and system suitability parameters in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005).

Specificity of an analytical method is its ability to measure accurately an analyte in the presence of interference, such as excipients, and known (or likely) degradation products that may be expected to be present in the sample matrix.

Following sample solutions were injected on to HPLC:

- Methanol: Water (80:20) (diluent)
- AQ standard at 100 % test concentration (0.15 mg/mL)
- Placebo mixture of final formulation without API.
- Forced degradation samples.

Linearity was determined by the correlation coefficient (R2) for calibration curves constructed following the concentration range (0.075 mg/mL – 0.225 mg/mL) of amodiaquine hydrochloride standards. The linearity for the related substances (mannich base, 4,7-Di chloroquinoline, paracetamol, 7-chloro-4(hydroxy phenyl amino quinoline) was assessed over the range of 0.25 % to 0.75 % of the nominal test concentration of AQ, which equates to to 0.000375 mg/mL– 0.001125 mg/mL.

The standard error for the predicted y value for all x values in the regression was calculated and used to calculate the limit of detection (LOD) and limit of quantification (LOQ) of amodiaquine hydrochloride, respectively.

Determination of the precision of the analytical methods was achieved by repeatability, intra-day, and inter-day analysis. Repeatability was assessed by injecting 6 replicates of standard sample at 100 % of the nominal test concentration of 0.15 mg/mL. Intra-day precision was measured by three different standard solutions of amodiaquine hydrochloride (0.075, 0.15, and 0.225 mg/mL) were prepared from working standard solution and injected into the system with stated chromatographic conditions and analysed, three times in a day. Inter-day precision was assessed through preparation of Standard solutions of amodiaquine hydrochloride 0.075, 0.15 and 0.225 mg/mL from working standard solution and injected into the system with same chromatographic conditions and analysed for three consecutive days.

Accuracy was assessed by preparation of known concentrations of analytes prepared from individually prepared stocks made with amodiaquine hydrochloride reference material. The closeness of the observed concentration to the theoretical concentration, based upon weighing and dilution, was used to calculate the accuracy of the method.

The range of the method for assay is confirmed as 0.075 mg/mL to 0.2255 mg/mL, over which acceptable accuracy, linearity, and precision were obtained. The range of the method suitable for related substances is set as 0.000375 mg /mL - 0.001125 mg/mL for paracetamol, 4,7-Di Chloroquinoline, mannich base, 7-chloro-(4-hydroxy phenyl amino) quinoline. Over this range, acceptable linearity has been observed for AQ and its associated impurities. System suitability parameters were assessed for resolution, tailing factor and capacity factor.

Resolution is a measure of how well two peaks in a chromatogram are separated.

$Rs=2(t_R2-t_R1)/ W_1+W_2$

Where:

- t_R1 and t_R2 the retention times of the two peaks.
- W_1 and W_2 are the widths of the peaks at their bases.

The tailing factor measures the symmetry of a peak.

Where: $T = W_{0.05}/2.\ f$

- $W_{0.05}$ is the width of the peak at 5% of the peak height.
- f is the distance from the peak maximum to the leading edge of the peak at 5% of the peak height.

The capacity factor, also known as the retention factor, indicates how well a compound is retained by the stationary phase compared to the mobile phase.

$k' = t_R - t_0 / t_0$

Where:

t_R_ is the retention time of the analyte.

t_0_is the dead time, or the time it takes for an unretained compound to pass through the column.

3. RESULTS

3.1. RESULT FOR ARTESUNATE & ITS IMPURITIES METHOD

3.1.1. SIMULTANEOUS DETECTION METHOD FOR ARTESUNATE AND AMODIAQUINE HYDROCHLORIDE

Initial method developed focus on the methodology provided in Section 2.2.2, based upon a publication by [30]. This method aimed to separate all analytes of interest. Artesunate at 0.5 mg/mL and its impurities DHA, artemisinin, anhydro-dihydroartemisinin at 0.1 mg/mL, amodiaquine at (1.35 mg/mL) and its impurities acetaminophen, mannich base, 4,7-DCQ, 7-chloro-4(4-hydroxy phenyl amino) quinoline was injected onto the system and separation was assessed. Chloroquine phosphate was included in the separation for system suitability test because chloroquine and amodiaquine are both 4-aminoquinoline compounds. Their structural similarity confirms that the chromatographic behaviour of chloroquine can work as a reliable indicator of how amodiaquine will behave under similar conditions. Chloroquine phosphate is commonly available and relatively inexpensive compared to some other potential reference standards. This makes it a functional choice for regular analysis in quality control laboratories. All the peaks were identified by injecting the individual standards of each analyte separately.

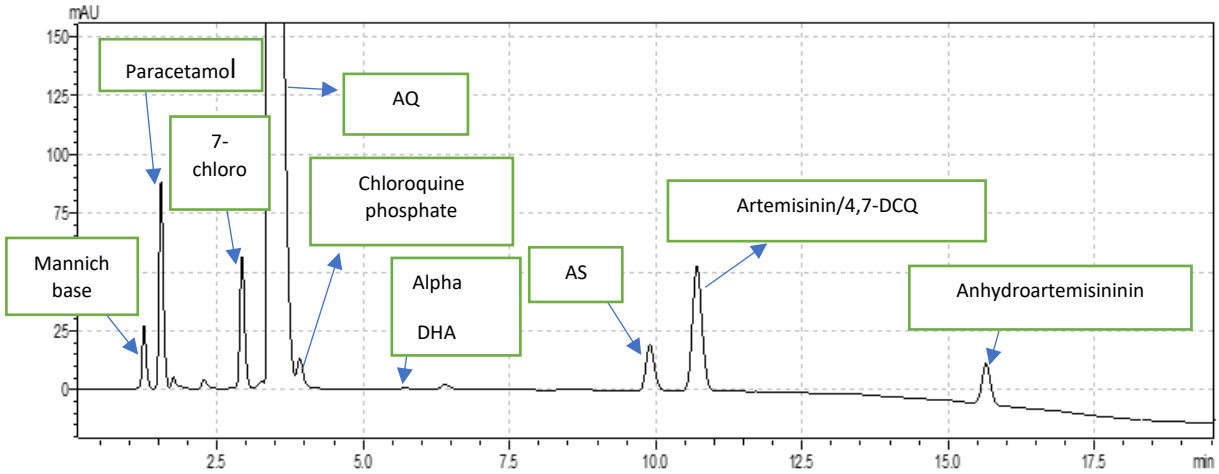

Figure 4: Separation of AS/AQ and its impurities using a mixture of analytes dissolved in buffer: acetonitrile.

Table 4: Retention times of AS, AQ and its impurities based on simultaneous method.

S.no	Analyte	Peak Area	Retention time in (mins)
1	Acetaminophen (paracetamol)	443719	1.54
2	7-chloro 4- hydroxy phenyl amino quinoline	325178	2.92
3	Amodiaquine	40086017	3.47
4	Chloroquine phosphate	50250	3.91
5	Alpha DHA	21402	5.71
6	Beta DHA	8585	8.65
7	Artesunate	196644	9.89
8	Artemisinin	609044	10.70
9	4,7-DCQ	609044	10.70
10	Anhydroartemisininin	175356	15.64

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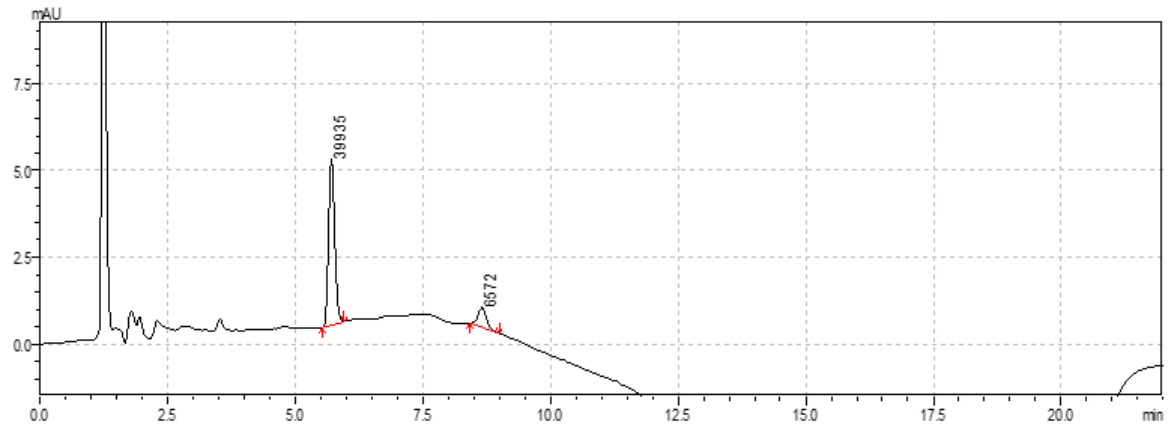


Figure 5: Increased concentration of DHA to 0.5 mg/mL to clearly see both alpha and beta DHA peaks.

Whilst the separation of most analytes was achieved, co-elution was observed between artemisinin and 4,7-di chloroquinoline. The octane sulfonate acts as an ion-pairing agent. In reversed-phase HPLC, hydrophobic compounds are retained longer than hydrophilic ones. Artesunate and amodiaquine, being antimalarial drugs, may have ionizable groups. AQ is in an ionic form, so not sufficiently retained. By adding a hydrophobic ion-pairing agent like octane sulfonate, ionic analytes form neutral or less polar ion-pairs that have greater affinity for the hydrophobic stationary phase. This

enhances the retention of AQ in a reversed phase system simultaneously to AS allowing for better separation, but the octane sulfonate used is high cost (60£ per 10 g). Hence the decision was made to develop two separate analytical for artesunate and its impurities and amodiaquine and its impurities because there are many analytes to separate.

3.1.2. INTERNATIONAL PHARMACOPEIA METHOD DEVELOPMENT FOR ARTESUNATE

The conditions given in Section 2.2.3 were used to assess the separation of a standard mixture containing AS and its three impurities as shown in Figure 6. Peaks were identified by injection of individual standards of analytes. Amodiaquine hydrochloride is also present in the final formulation therefore the impact of AQ and its impurities on the AS separation was evaluated by injection of mixture of AQ and its impurities.

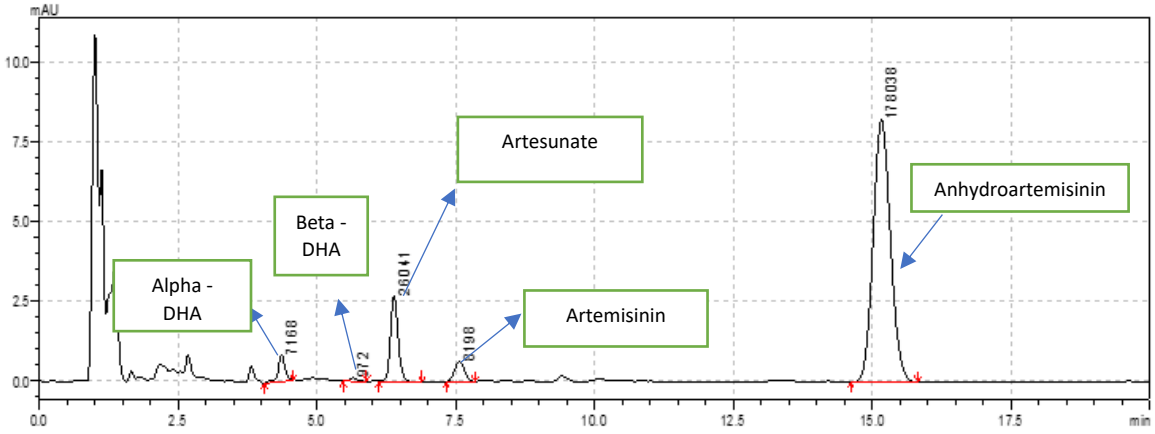


Figure 6: Artesunate and its 3 impurities dissolved in methanol at a concentration of 0.5 & 0.1 mg/mL to check their separation.

Table 5: Retention times of artesunate and its impurities

S.no	Analyte	Peak Area	Retention time in (mins)
1	Alpha DHA	7168	4.36
2	Beta DHA	972	5.65
3	Artesunate	26041	6.38
4	Artemisinin	8198	7.56
5	4,7-DCQ	8198	7.56
6	Anhydroartemisininin	178038	15.16

Whilst AQ and its impurities will not be assayed using this method, it must be demonstrated that they should not interfere with the AS and its impurities peaks. AQ and its impurities (acetaminophen, Mannich base, 7-chloro-4-(4-hydroxy phenyl amino quinoline) were not observed to interfere with AS or impurity elution. However, the elution of 4,7-DCQ took place at the same retention time as that of artemisinin, an AS impurity. This is shown in figure - 7.

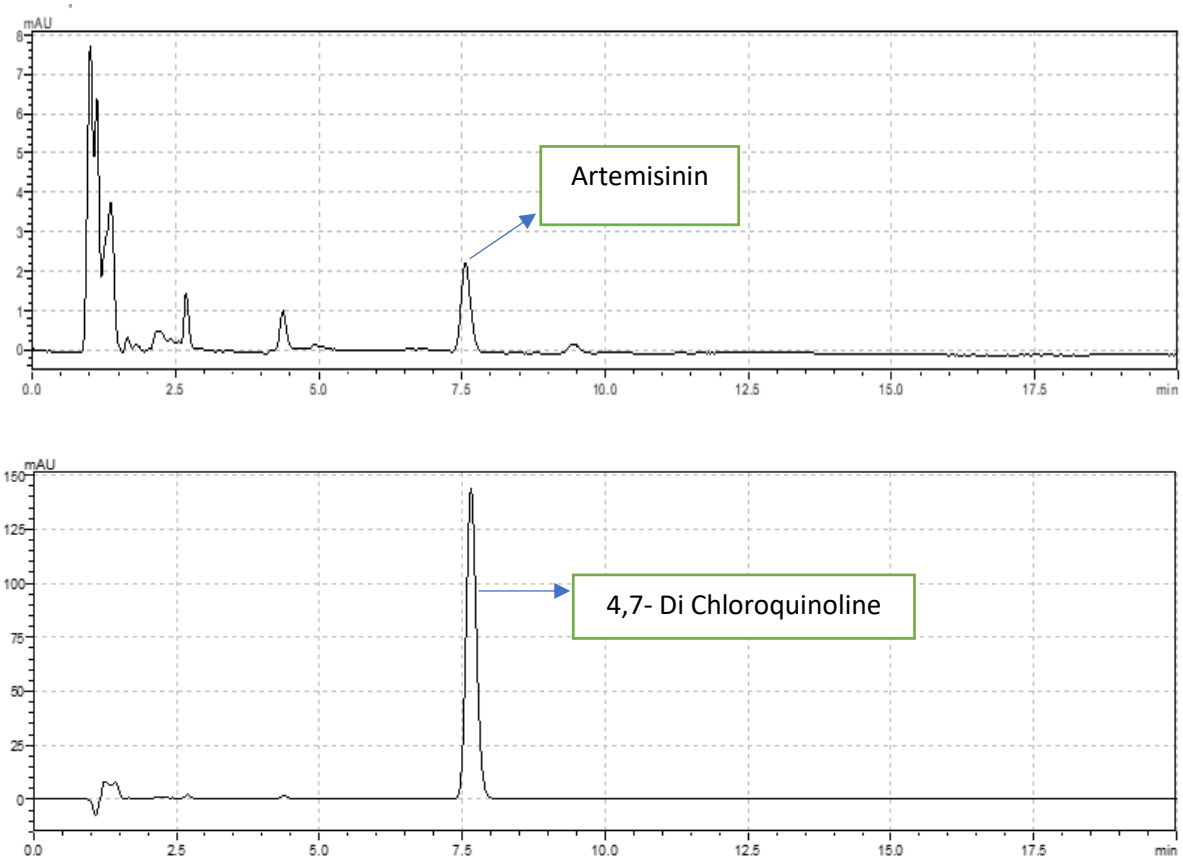


Figure 7: Co-elution of artemisinin retention time: 7.56 mins and 4,7- Di chloroquinoline retention time: 7.56 mins.

Due to this co-elution several approaches were used to separate all components by changing the mobile phase composition 50:50 with 0.8 mL/min flow rate , 56:44 buffer: acetonitrile with 0.8 mL/min flow rate, 52 % acetonitrile and 48 % buffer with 0.6 mL/min flow rate but, both analytes were coeluting when tried with these mobile phase ratios and columns (brand- Varian, thermo Hypersil with ODS or BDS material), at lengths of 10 cm, 15 cm and 30 cm at particle sizes of 3 and 5 µm. When 66 % acetonitrile and 34 % buffer with a flow rate of 0.4 mL/min is tried on 15 cm Varian column with 3 µm particle size acceptable separation is seen for all analytes. The

diluent was also changed from acetonitrile to methanol because the impurity standard dihydroartemisinin peak shape is not good, when we have used acetonitrile as a diluent and different injection volumes like 50, 75 µL were also tried but peak shape was not good, because in reversed phase HPLC, the stationary phase is typically non-polar (e.g., C18). When acetonitrile used, its elution strength might not adequately balance the interactions between DHA and the stationary phase this can lead to strong retention and peak tailing. Acetonitrile has limited hydrogen bonding capabilities compared to solvents like methanol. Since DHA contains functional groups capable of hydrogen bonding, methanol can form stronger hydrogen bonds with polar analytes like DHA, improving solubility and reducing peak tailing by facilitating more consistent elution from the stationary phase.

However acceptable separation was achieved by using the conditions mentioned in section 2.2.3 by using 15 cm Varian column with 4.6 mm diameter and 3 µm particle size by using 66 % acetonitrile and 34 % buffer at a lower flow rate of 0.4 mL/min and injection volume is 20 µL. AS separation was now acceptable with resolution observed between all the impurities.

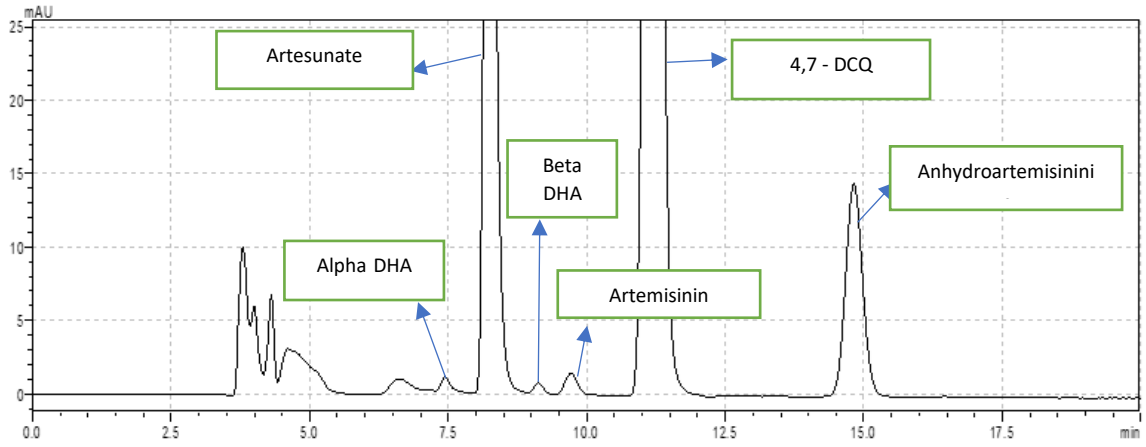


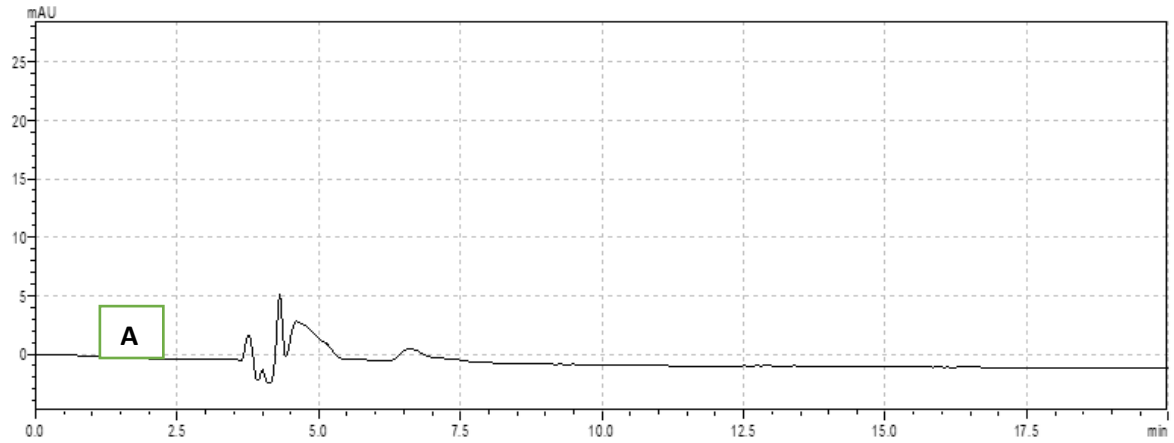
Figure 8: Chromatogram showing separation of AS from its impurities (DHA, artemisinin, anhydrodihydro artemisinin) and an AQ impurity 4,7- DCQ, at impurity concentration of 0.01 mg/mL for AS impurities, 0.0675 mg/mL for 4,7-DCQ and AS concentration at 0.5 mg/mL.

Table 6: Retention times of AS and its impurities.

S.no	Analyte	Peak Area	Retention time in (mins)
1	Alpha DHA	11712	7.44
2	Artesunate	1065382	8.2
3	Beta DHA	8286	9.12
4	Artemisinin	25370	9.71
5	4,7- DCQ	34130	11.18
6	Anhydroartemisininin	3229830	14.82

3.1.3. EFFECT OF FILTERS ON BACKGROUND PEAKS

Background peaks in the chromatogram were observed. Following investigation, by injection of a methanol blank with and without filtration with a PTFE filter, these extraneous peaks were identified as coming from the filtration process. It was hypothesised that use of an alternative filter material would remove these background peaks. Therefore, several filters containing different materials (nylon, polypropylene, glass fibre, cellulose acetate, PVDF and PTFE) were assessed by filtration of methanol blanks and subsequent injection onto the system. Filter materials containing PVDF and PTFE were found to give the cleanest chromatograms, as evidenced in Figure 9(B) & (C).



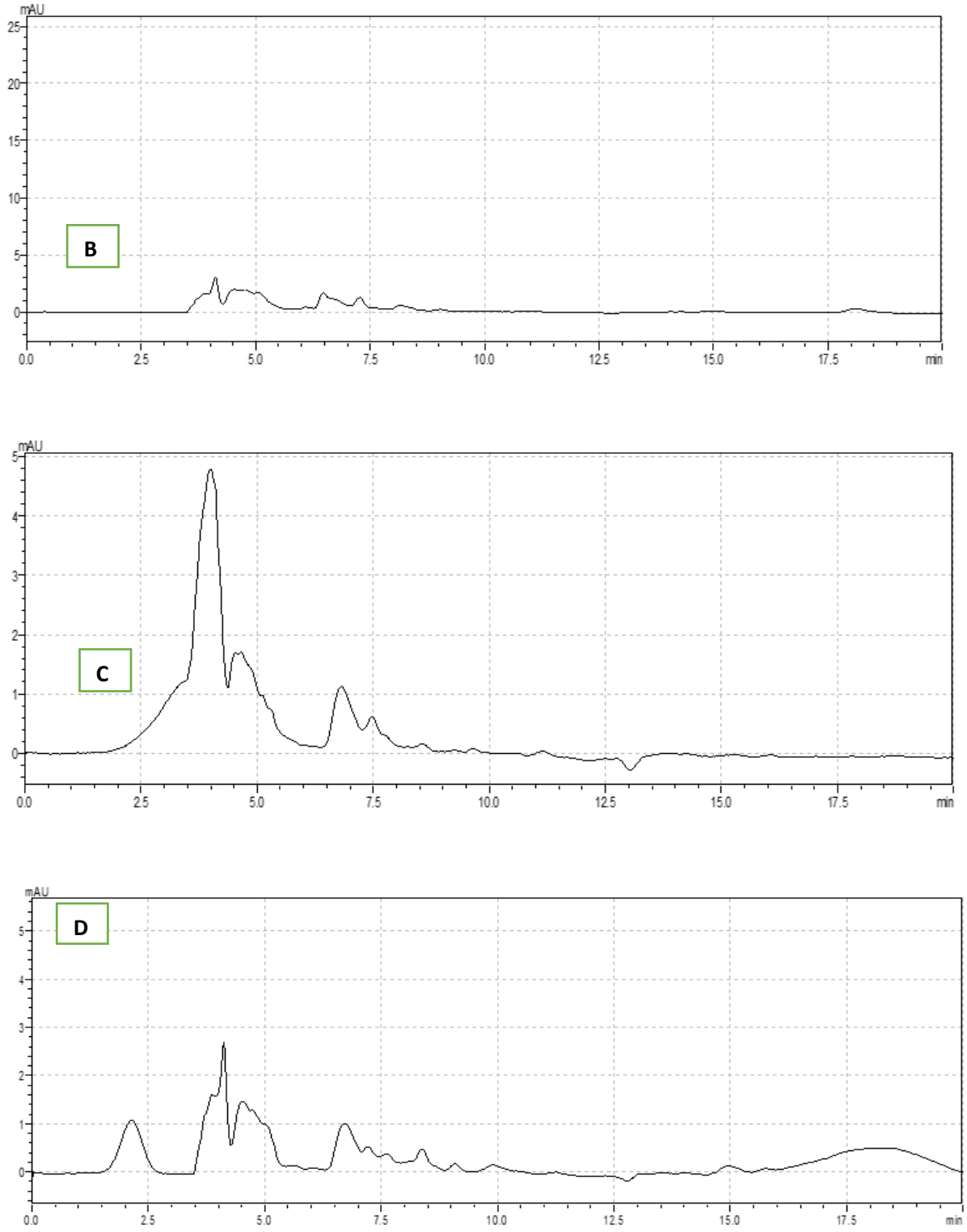


Figure 9: (A)-Chromatogram of methanol without filtration, (B)-methanol with filtration by PTFE filters (second image), (C)-methanol with filtration by PVDF filters (third image), (D)-methanol with filtration by polypropylene filters (fourth image).

The data generated suggested that both figure 9 (B) - PTFE and figure 9(C) - PVDF filter

material were suitable for preparation of samples to the presence of the fewest extraneous peaks, coupled to this interference being found at very low levels such that it was unlikely to interfere with detection of the analytes of interest. PTFE was selected and used for sample preparation moving forward.

3.1.4. EXTRACTION METHOD OF ARTESUNATE FROM FORMULATION

Extraction of AS from two formulations as mentioned in section 2.1 uncoated AS micro pellets formulations are one with Artesunate, Cellets, Syloid and 1.2% PVP K30 and another with Artesunate, Cellets, Syloid and 0.8% klucel composition. One dose of each formulation containing 25 mg of AS was weighed and transferred to a 50 mL volumetric flask. This flask was filled with methanol such that it was ¾ full and a magnetic stirrer added. Stirring took place for either 30 or 60 mins to evaluate the effect of agitation before the flask was made to volume with methanol. The solution within the flask was then vortexed. A guard column is a short column placed between the injector and the analytical column because the final ASAQ formulation contains polymers. It helps to adsorb and trap strongly retained substances or chemical impurities that could irreversibly bind to the analytical column thus prevents clogging and maintaining the integrity of analytical column. Sample is filtered before being injected and onto the HPLC system at a final concentration of 0.5 mg/mL for each PVP K30 and Klucel separately. Recovery of the sample was calculated based upon the theoretical API content of the formulation. The results of this are given in Table-7 below.

Table 7: Percentage Recovery of API by stirring at two-time points.

Formulation	Extraction Time	% Recovery	%RSD
1.2% PVP Formulation	30 min	98.62	4.52
	60 min	101.52	0.29
0.8% Klucel Formulation	30 min	103.81	7.89
	60 min	101.66	1.88

For both formulations, recovery was near 100 %. However, the % RSD of extraction was lower at 60 mins than at 30 mins. Therefore, a 60 mins extraction timepoint was used to reduce the variability of the method.

3.1.5. RESULTS FOR VALIDATION OF ARTESUNATE AND ITS IMPURITIES USING HPLC

The developed method was validated in accordance with the ICH guidelines[17]. The finalized chromatographic conditions developed are given in section 2.2.3. The analytical methods were evaluated for specificity, linearity, precision, and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). The linearity, precision, accuracy, and system suitability parameters for artesunate are summarized in Table 8. The analytical methods implemented, as detailed in Section 2.2.4, were deemed to pass all specification criteria as defined by the ICH guidelines with suitable LOD and LOQ levels for the quantification of small amounts of artesunate in samples.

Specificity:

Results for Specificity:

- Methanol (diluent): No extra peaks observed within the blank that will interfere with analysis of AS or related substances Figure 10(A).
- Placebo mixture: No interference of any peaks presents in the placebo mixtures (AQ micropellets, AS micropellets and AS dry powder blend) for AS or related substances peak analysis Figure 10(C & D)
- Forced degradation samples: separation was observed for all known and unknown degradants of artesunate Figure 11.
- The suitability of method for assessment of AS and related substances was determined in method specificity.

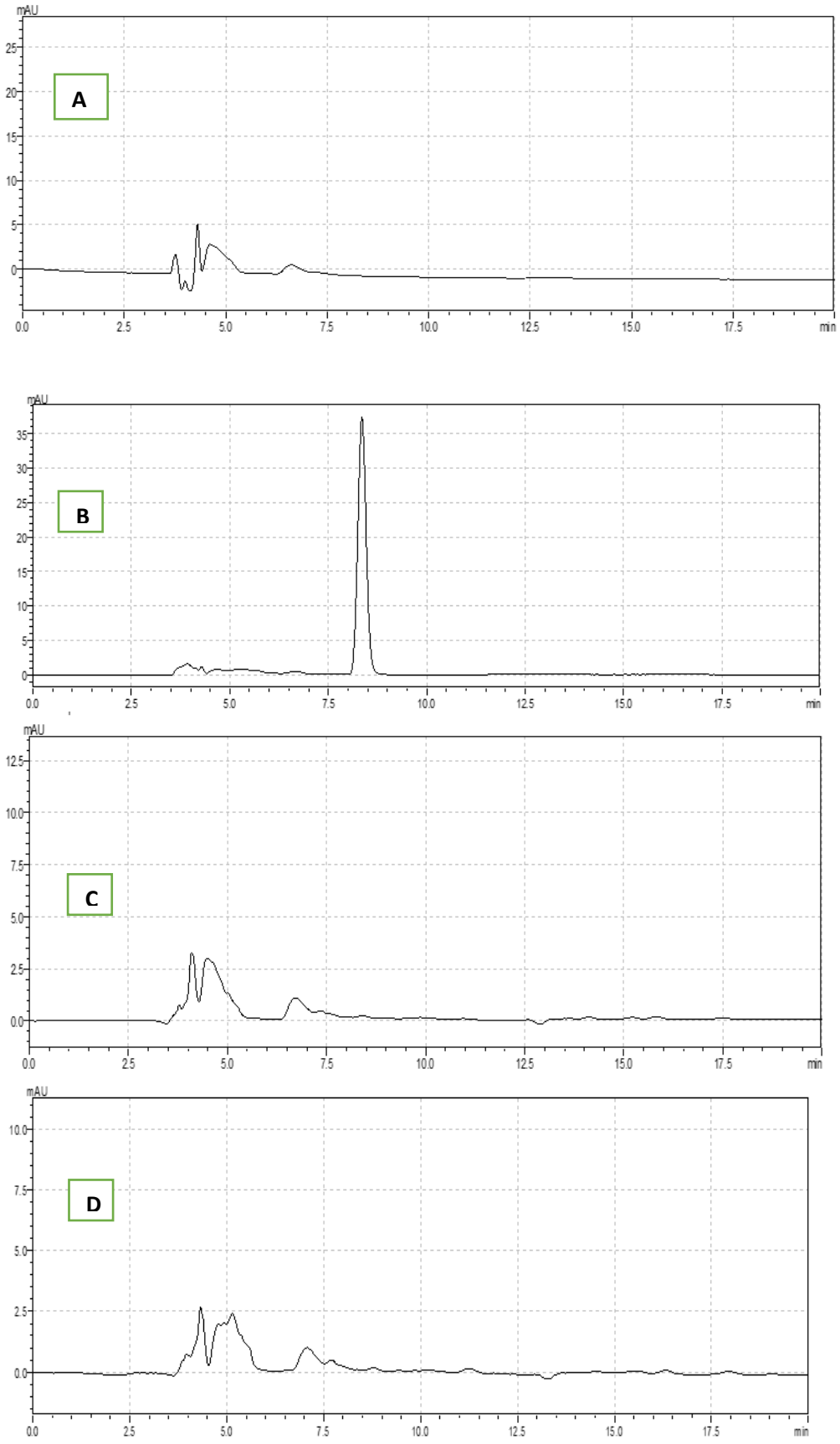
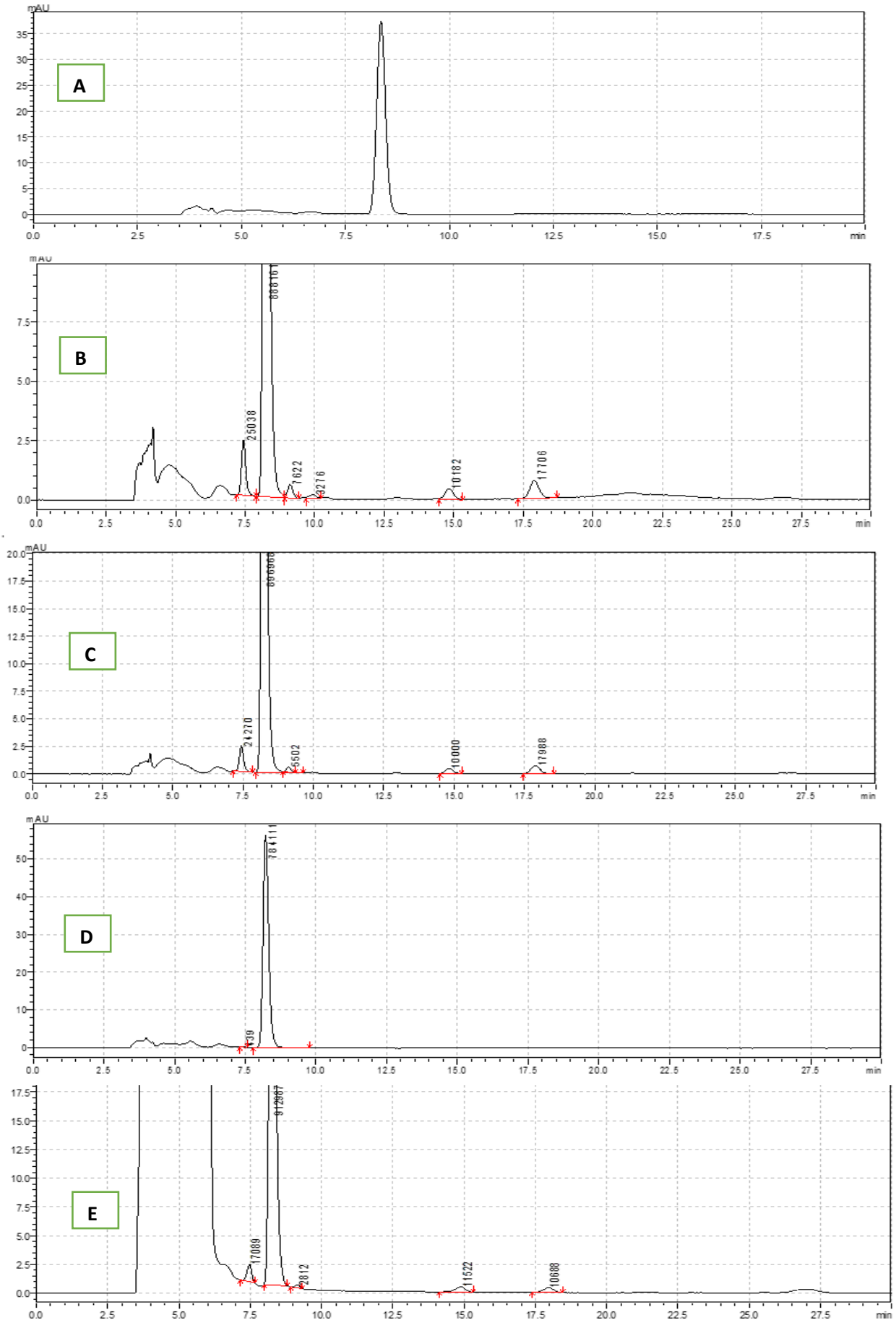


Figure 10: (A) Blank diluent (methanol), (B) AS reference standard at 0.5 mg/mL, (C) AQ placebo (3rd image) and (D) AS placebo (4th image).


A
B
C
D
E
mAU
min

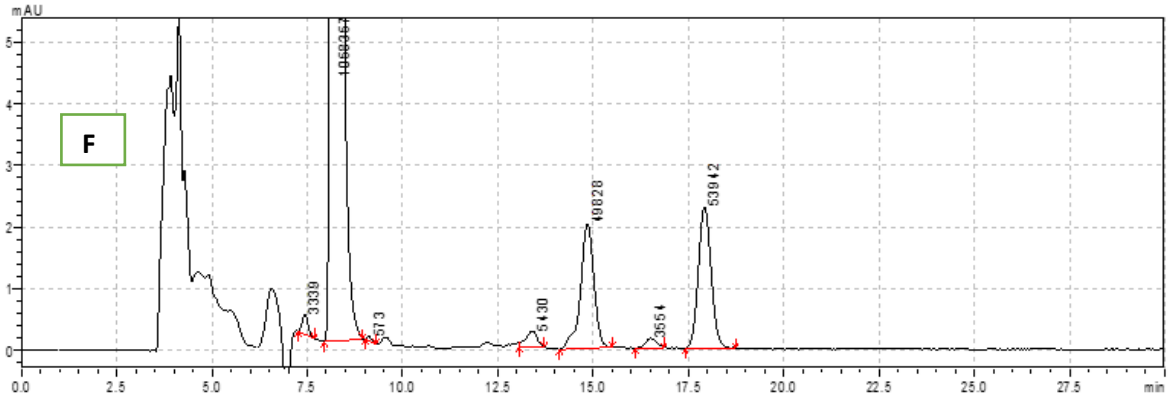


Figure 11: Artesunate control in MeOH (A), forced degradation of an AS solution at 0.5 mg/mL using 0.01 M HCl (B), 0.01M NaOH (C), degradation with light for 5 h (D), oxidation with 10% H2O2 for 24 h (E), water bath at 50°C for 24 h (F)

Table 8: Percentage degradation & peaks generated from forced degradation studies. Known peaks are identified based on impurity reference standards.

Condition	% Degradation	**Relative Retention Time (min)**								
		Unknown Peak	Unknown Peak	DHA (alpha)	AS	DHA (beta)	Artemisinin	Unknown Peak	Anhydro-artemisinin	Unknown Peak
		0.77	0.83	0.91	1	1.10	1.26	1.62	1.83	2.20
0.01M HCl	8.53%			2.91%					0.65%	2.10%
0.01M NaOH	13.59%	11.61%	5.62%	1.07%		0.19%			0.41%	
Heat	27.38%		2.25%	0.44%		0.11%	0.17%	0.58%	16.34%	23.55%
Light	20.18%		2.20%					0.25%	0.66%	
H_2O_2	2.78%			1.87%		0.31%			1.26%	1.17%

Linearity:

Calibration curves of artesunate and its related substances were constructed using the analytical methods developed to enable the quantification of artesunate in samples.

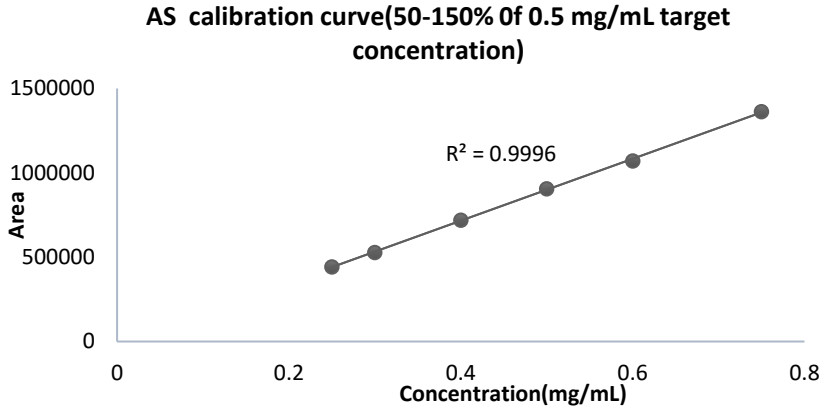


Figure 12: Linearity of Artesunate reference solutions at the concentration range of 0.25 mg/mL – 0.75 mg/mL (50% - 150% of target concentration 0.5 mg/mL).

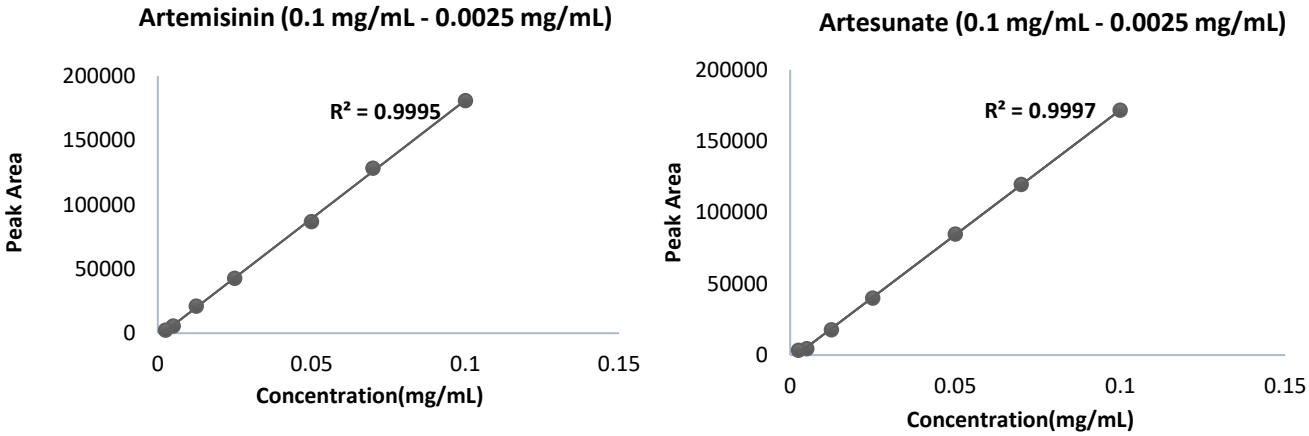


Figure 13: Linearity range of 0.0025 mg/mL – 0.1 mg/mL for artesunate and artemisinin, DHA (alpha and 0.0125 – 0.1 mg/mL for DHA (beta).

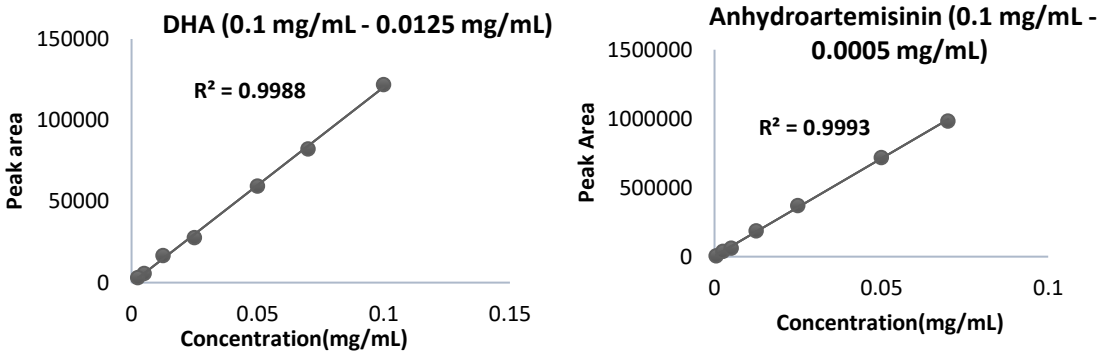


Figure 14: Linearity graph of DHA & anhydrodihydroartemisinin in the range of 0.0125 mg/mL – 0.1 mg/mL.

System suitability:

System suitability was assessed by calculating parameters of interest for the main API peak, and the impurities (DHA, artemisinin, anhydroartemisininin) of AS. These parameters were generated based on AS concentration of 0.5 mg/mL, and impurity concentration of 0.01 mg/mL. These parameters were acceptable for system suitability.

Table 9: Calculated system suitability parameters for Artesunate & its impurities.

S.no	Analyte	Retention time in (mins)	Plate count	Capacity factor	Resolution	Tailing factor
1	Artesunate	8.2	9370	1.18	1.34	1.13
2	Alpha DHA	7.44	10344	1.44	1.27	1.15
3	Beta DHA	9.2	12923	1.72	0.83	0.97
4	Artemisinin	9.7	8051	1.90	1.26	1.02
5	Anhydroartemisininin	14.8	10835	3.5	1.6	1.02

Table 10: Summary of the parameters determined for the validation of the isocratic elution HPLC methods for quantification of artesunate.

Parameter	**Artesunate Isocratic method**	**Limit as per ICH guidance**
Linearity (R2)	0.9996	> 0.999
Intra-day precision (% RSD) for 0.25 mg/mL, 0.5 mg/mL, and 0.75 mg/ mL	1.6%, 0.72%, 0.69%	RSD ≤ 2 %
Inter-day precision (% RSD) for 0.25 mg/mL, 0.5 mg/mL, and 0.75 mg/mL	0.46%, 0.90%, 0.64%	RSD ≤ 2 %
Repeatability for 0.5 mg/mL	1.10%	RSD ≤ 2 %
Accuracy (%) for 0.25 mg/mL, 0.5 mg/mL, and 0.75 mg/mL	101.59%, 99.26%, 100.51%	100 ± 2 %
Limit of detection (mg/mL)	0.0032 mg/mL	Report result
Limit of quantification (mg/mL)	0.0096 mg/mL	Report result
Tailing factor	**1.13**	**< 2**

Stability of standard and sample solutions:

Table 11: RSD of repeated injections of AS reference standard, AS formulation and AS/AQ formulation over 24 h.

Sample	Average peak area	Standard deviation	% RSD
AS Reference solution	937790.3	7741.857	0.83%
AS Formulation (1.2% PVP)	934338.3	6851.322	0.73%
ASAQ Formulation	925240.8	9312.764	1.01%

Repeated injections of standard and sample over 24 h showed an RSD of <2%, indicates little decrease in peak area and good sample stability over this period.

3.2. RESULTS OF ANALYTICAL METHOD FOR DETERMINATION OF ARTESUNATE DISSOLUTION SAMPLES

3.2.1. STABILITY

Stability testing of artesunate took place in several solvents. For artesunate no significant variation of peak area (0.001 %) was observed in acetonitrile after 6 h at room temperature. In acetate buffer this was shown to be 6.57 %, in methanol 0.01 % and in 96 % ethanol 1 % artesunate degradation was observed.

3.2.2. SOLUBILITY

Artesunate solubility was determined in three solvents: acetate buffer, deionised water, and simulated saliva. In acetate buffer pH-5.5 solubility of artesunate was 0.811± 0.004 mg/mL after 3 h. Solubility in deionised water after 3 h was 0.275 ± 0.034 mg/mL, and solubility in simulated saliva after 3 h was 1.90 ± 0.083 mg/mL.

3.2.3. DEVELOPMENT OF A METHOD FROM IP MONOGRAPH FOR ARTESUNATE

The methodology developed from IP monograph because it is standard method, and this method development is not available in United state pharmacopeia (USP) and British pharmacopeia (BP). A separate HPLC method for dissolution was developed to know the concentration of sample and the injection volume is high, and it cannot be used with other methods as it is affecting the peak shape. Acetate buffer at pH 5.5 was used for dissolution testing of artesunate and amodiaquine because it provides a mildly acidic environment hence the drugs remain stable, soluble, and in a controlled environment that closely mimics physiological conditions, providing reliable and reproducible results essential for drug development. Acetate buffer at pH 5.5 is used as a diluent because it is a dissolution medium and change in the diluent may affect the peak area hence it is used as diluent. As described in section 2.3.4 those conditions were initially applied to assess the separation of analytes. Sample containing 100 µg/mL was injected onto the system and separation was assessed. Peak identity was confirmed by injecting a blank sample (acetate buffer). The results were shown in

table-12.

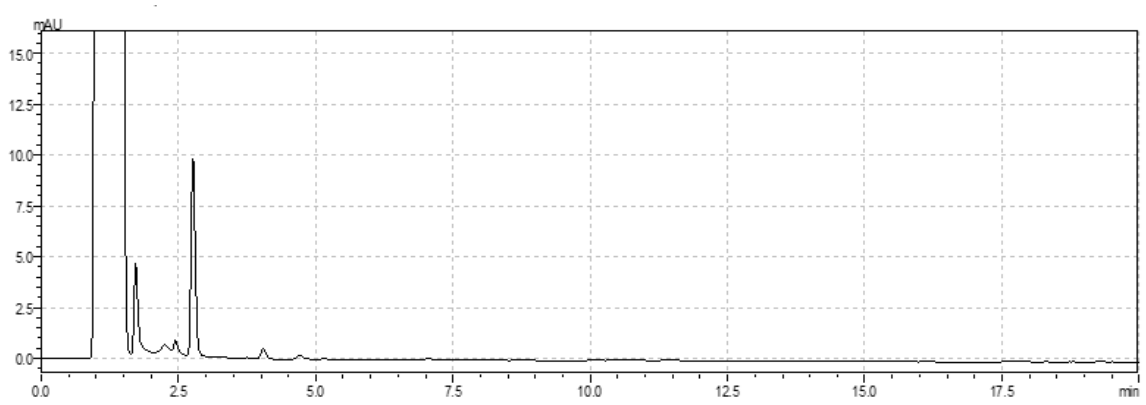


Figure 15: Chromatogram of acetate buffer blank with flow rate 1 mL/min.

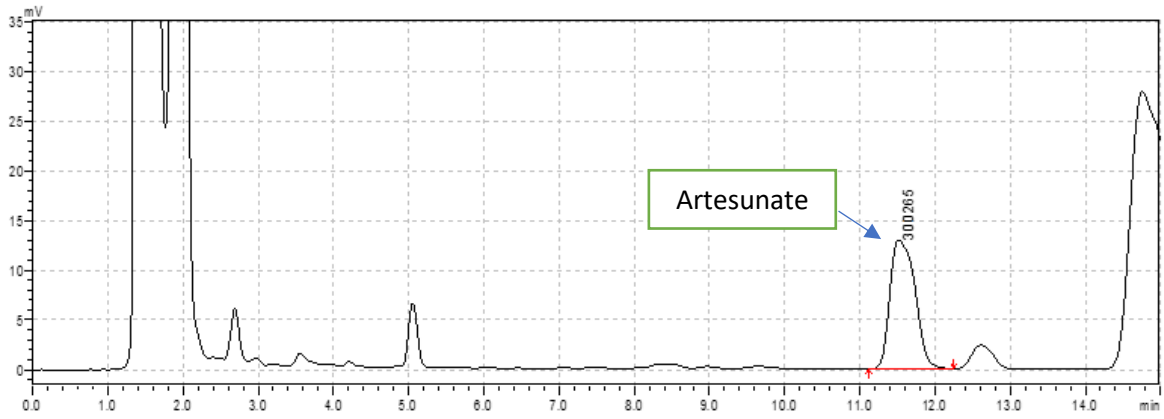


Figure16 : Chromatogram of artesunate sample with flow rate 1 mL/min.

Table 12: Results of Artesunate sample with 1 mL/min flow rate.

Sample	Peak area	Retention time in (mins)	Tailing factor
Artesunate	300625	11.5	1.373

In these conditions, the retention time for artesunate was about 11.5 mins. In the resulting chromatograph peak shape was not symmetrical, and there are two peaks coming after artesunate which were not seen in the blank.

3.2.4. TRAIL-1 FOR INTERNATIONAL PHARMACOPEIA METHOD

Hence, we have changed the column, mobile phase ratio, and flow rate to get better resolution. Then we used BDS Hypersil C18, 250 × 4.6 mm, 5 µm particle size column

by using equal volumes of buffer pH-3.0 and acetonitrile in the ratio of (50:50 v/v) as mobile phase with a flow rate of 1.5 mL/min with a run time of 15 mins at 210nm wavelength with 100 µL injection volume. The result of this is shown in Table-13.

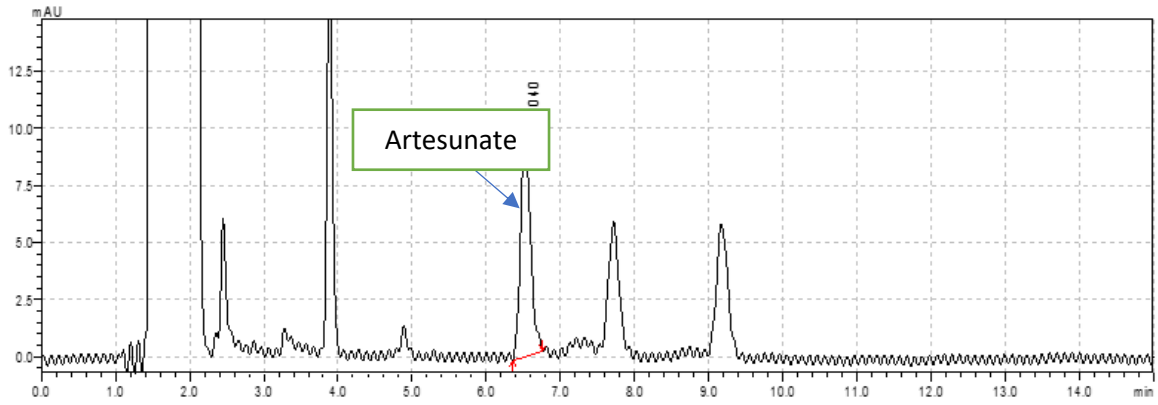


Figure 17: Chromatogram of artesunate sample with flow rate 1.5 mL/min.

Table 13: Peak table of Artesunate sample with 1.5 mL/min flow rate.

Sample	Peak area	Retention time in (mins)	Tailing factor
Artesunate	90678	6.5	1.268

The resultant chromatograph shows retention time of artesunate at 6.5 mins. It is hypothesized that peak tailing was observed due to the use of a higher injection volume. Additionally, baseline issues were observed. Hence, we have changed the flow rate from 1.5 mL/min to 1.2 mL/min to avoid baseline disturbances. To get symmetrical peaks the injection volume was also reduced.

3.2.5. TRAIL-2 FOR INTERNATIONAL PHARMACOPEIA METHOD

As there was staggered baseline and peak shape was poor. Flow rate is affecting the change in baseline. Then we have tried again with the same column BDS Hypersil C18, 250 × 4.6 mm, 5 µm particle size by using equal volumes of Acetonitrile and phosphate buffer of ph-3.0 (50:50 v/v) as mobile phase but with a decrease in flow rate to 1.2 mL/min by using 210 nm as wavelength for a run time of 15 mins with an injection volume of 75 µL. The result of this is shown in Table-14.

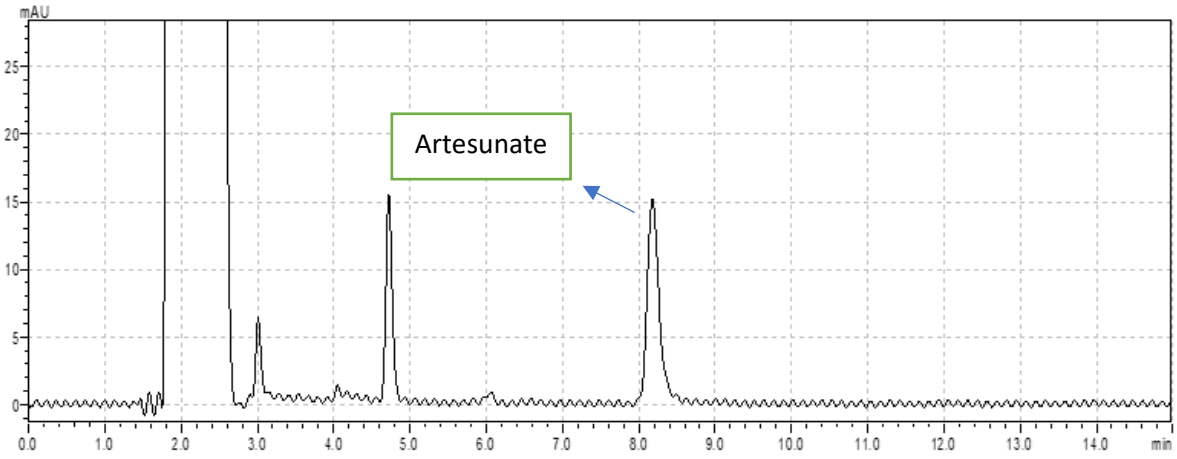


Figure 18: Chromatogram of artesunate sample with flow rate 1.2 mL/min.

Table 14: Chromatograph table of Artesunate with 1.2 mL/min flow rate.

Sample	Peak area	Retention time in (mins)	Tailing factor
Artesunate	385716	8.15	1.33

The resultant chromatogram shows the major peak at 8.15 mins, with no co eluting peaks seen. However, baseline issues were prevalent despite conditioning the column for a long time. Changes were then made to the mobile phase ratio, flow rate and column to improve the baseline. In this chromatogram the two extra peaks after artesunate were not seen, because samples were prepared in amber glassware and there is no light degradation.

3.2.6. TRAIL-3 FOR INTERNATIONAL PHARMACOPEIA METHOD

As the baseline issue is not completely resolved, a BDS Hypersil C18, 100 ×4.6 mm, 3 µ particle size column was used with a mixture of buffer pH-3.0 and acetonitrile in the ratio of (56:44 v/v). A reduction in flow rate to 1 mL/min was used, with 216 nm wavelength. Different injection volumes were used; 20, 50, 75 and 100 µL for a run time of 20 mins. It was determined that an injection volume of 75 µL was most appropriate. The result of this is shown in Table-15.

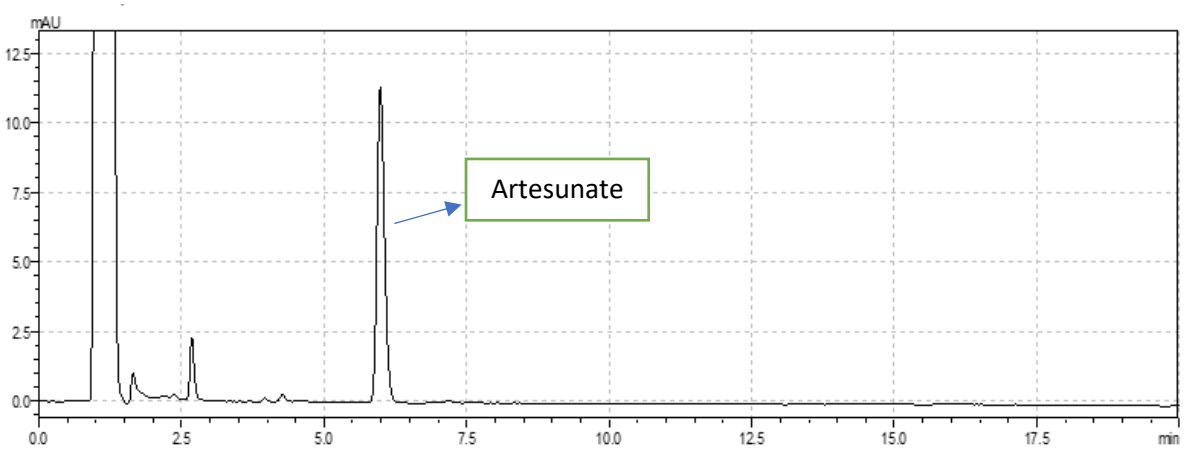


Figure 19: Chromatogram of Artesunate sample with flow rate of 1 mL/min.

Table 15: Chromatograph table of Artesunate with 1 mL/min flow rate.

Sample	Peak area	Retention time in (mins)	Tailing factor
Artesunate	384976	5.99	1.39

The resultant chromatograph shows that drug is eluted at 5.99 mins and peak shape is symmetrical. No other peaks were observed after the elution of the main peak.

3.2.7. RESULTS FOR VALIDATION OF ARTESUNATE DISSOLUTION TEST METHOD

Following the development of acceptable chromatographic conditions, the method was validated in accordance with ICH guidelines on analytical method validation. The finalized chromatographic conditions developed are given in section 3.2.6.

calibration curves of artesunate were constructed using the analytical methods developed to enable the quantification of artesunate in dissolution test samples were shown in Figure 20.

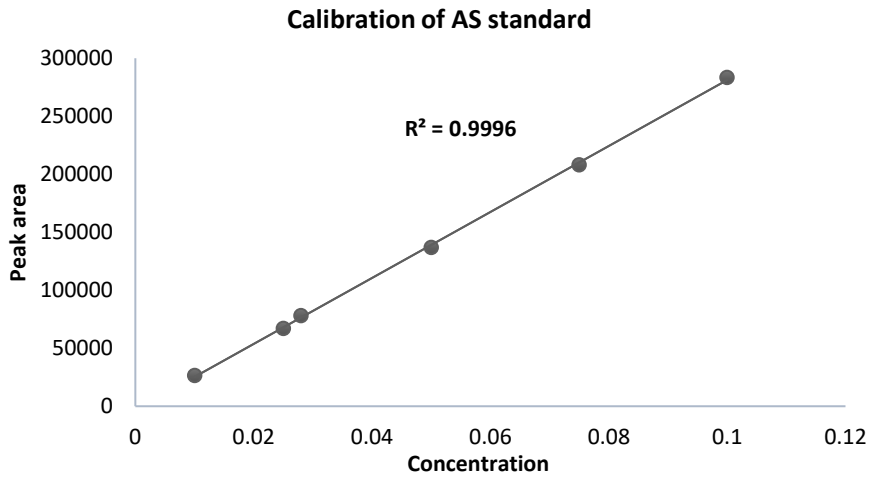


Figure 20: Calibration graphs of artesunate standards obtained following analysis in concentration range 0.01 mg/mL – 0.1 mg /mL)

The analytical methods were evaluated for linearity, precision, and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). The linearity, accuracy, and precision parameters for artesunate are summarised in Table 16. The analytical methods implemented, as detailed in Section 2.3.6, were deemed to pass all specification criteria as defined by the ICH guidelines with suitable LOD and LOQ levels for the quantification of small amounts of artesunate in samples.

Table 16: Summary of the parameters determined for the validation of the isocratic elution HPLC methods for quantification of artesunate.

Parameter	**Artesunate method**	**Limit as per ICH guidance**
Linearity (R2)	0.9996	> 0.999
Repeatability 0.5mg/mL	1.62	RSD ≤ 2 %
Accuracy (%) for 0.01 mg/mL, 0.028 mg/mL, and 0.1 mg/mL	99.73%, 100.1%, 98.2%	100 ± 2 %
Limit of detection (mg/mL)	0.006643 mg/mL	Report result
Limit of quantification (mg/mL)	0.020129 mg/mL	Report result

3.3. RESULTS FOR AMODIAQUINE & ITS IMPURITIES METHOD

3.3.1. DEVELOPMENT OF A USP MONOGRAPH METHOD

The USP monograph methodology as described in section 2.4.2 was initially applied to assess the separation of these analytes. A mixture of compounds containing all analytes of interest (AQ at 0.15 mg/mL) and associated impurities (0.00075 mg/mL) was injected on to system and separation was assessed. Peak identity was confirmed by injection of individual standards of each analyte alone.

All the analytes observed were overlayed as shown in Figure 21. This gave separation of only three analytes (Paracetamol, Mannich base and Amodiaquine).

The other two analytes (Di-chloroquinoline and 7-chloro-4(4-hydroxy phenylamino) quinoline were not eluted even after 60 mins run time indicating the high affinity of these two compounds to the stationary phase. Therefore, the use of acetonitrile was investigated due it being a more non-polar solvent with greater eluotropic strength in this instance.

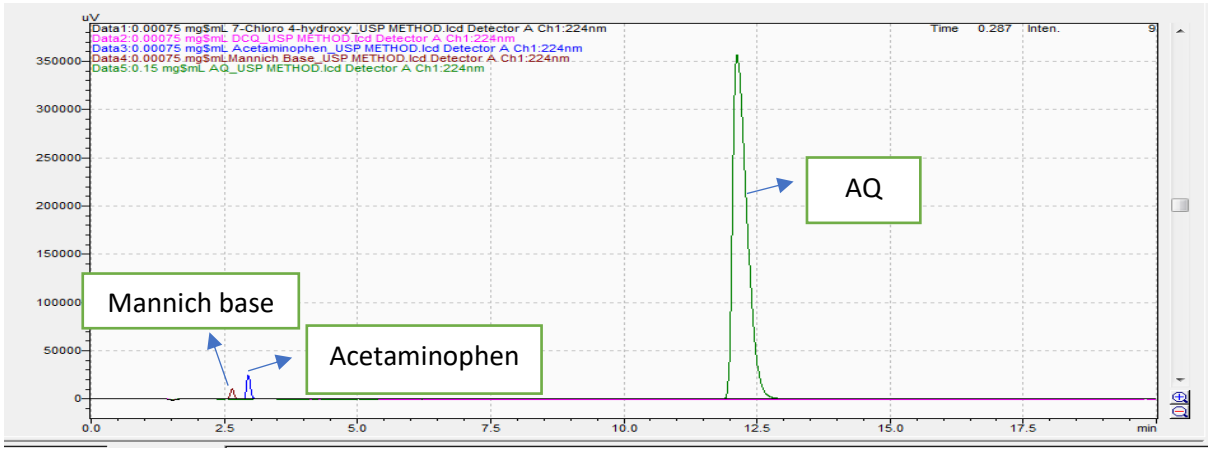


Figure 21: Overlain chromatogram of AQ and its impurities (acetaminophen, Mannich base, 4,7-DCQ, 7- chloro-4- hydroxyphenyl amino quinoline and amodiaquine)

3.3.2. MODIFICATIONS OF USP MONOGRAPH METHOD

In addition to the use of acetonitrile, a gradient method was investigated due to the varied elution profile (early and late elution) of the mixture. Flow rate was changed from 1.2 mL/min to 1 mL/min and for initial 9 mins. 95 % of buffer is used to prevent

co-elution of mannich base and acetaminophen. After 9 mins organic phase was increased to 20 % to elute other analytes but only amodiaquine and 7-chloro-4(4-hydroxy phenyl) amino quinoline were eluting within 20 mins hence organic phase was increased to 30 % but it is taking longer time to elute 4,7-Dichloroquinoline. Then further organic phase was increased to 40 % from 20 mins to elute 4,7- di-chloroquinoline and it was eluted at 34.33 mins and after 40 mins mobile phase was changed to initial conditions.

As artesunate is also present in the final product, the impact of AS and its impurities on the AQ method was evaluated by injection of mixture of AS and its impurities. AS and its impurities are not analyte of interest, but they should not interfere with AQ and its impurities peaks. AS and its three impurities (DHA, artemisinin, anhydroartemisininin) were eluted at 2.5, 3, 27 and 36 mins which was acceptable as they are not co-eluting with AQ and its impurities. Acceptable separation was achieved finally by using the conditions given in section 2.4.3.

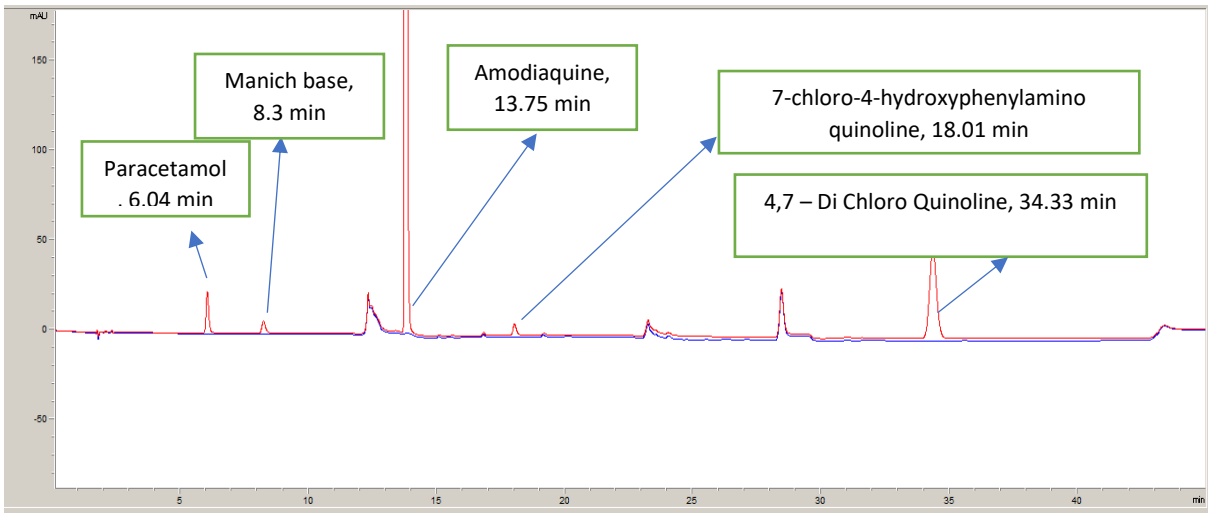


Figure 22: Chromatogram of methanol: water(blank) versus AQ & its impurities mixture.

As the separation was acceptable with resolution observed between major impurities and components. This method was further assessed and validated.

Table 17: Retention times of amodiaquine and its impurities.

s.no	Analyte	Retention time in (mins)
1	paracetamol	6.04
2	Mannich base	8.3
3	Amodiaquine	13.75
4	7-chloro-4-hydroxyphenylamino quinoline	18.01
5	4,7 - DCQ	34.33

3.3.3. DEVELOPMENT OF SAMPLE PREPARATION METHOD FOR EXTRACTION OF AQ FROM FORMULATION

A method was developed to extract AQ from formulation. A dose equivalent to 67.5 mg AQ was weighed and transferred in to 50 mL volumetric flask and filled with methanol: water (80: 20) up to ¾ of flask. A magnetic stirrer bae was added and stirring was performed for 2 h at 400 rpm. After completing the time flask was made up to the mark with methanol and sample is mixed, filtered, and injected on to HPLC system. Recovery of the API from the formulation was then calculated with the results given in table 18.

Table 18: Percentage recovery of API by stirring is 106.8.

Formulation	Extraction time	% Recovery	% RSD
AQ Formulation	2 h	106.8%	1.54%

3.3.4. RESULTS FOR VALIDATION OF AMODIAQUINE HYDROCHLORIDE AND ASSOCIATE IMPURITIES METHOD

Method Conditions:

Following the development of acceptable chromatographic conditions, the method was validated according to ICH guidelines on analytical method validation. The analytical method which was validated is stated in section 2.4.3.

The analytical methods were evaluated for specificity, linearity, precision, and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). The linearity, accuracy, and system suitability parameters for amodiaquine are summarised in Table 23. The analytical methods implemented, as detailed in Section 2.4.4, were deemed to pass all specification criteria as defined by the ICH guidelines with suitable LOD and LOQ levels for the quantification of small amounts of amodiaquine in samples.

Specificity:

Results for Specificity

- Methanol: water (80:20) (diluent): No extraneous peaks observed within the blank that will interfere with the analysis of AQ or related substances peaks (Figure-23).
- Placebo mixtures: No interference of any peaks present in the placebo mixtures (AQ micropellets, AS micropellets) for AQ or related substances peak analysis (Figure-24)
- Forced degradation chromatograms: Adequate separation was observed for all known and unknown degradants of AQ.

The suitability of the method for assessment of AQ and related substances was demonstrated in method specificity.

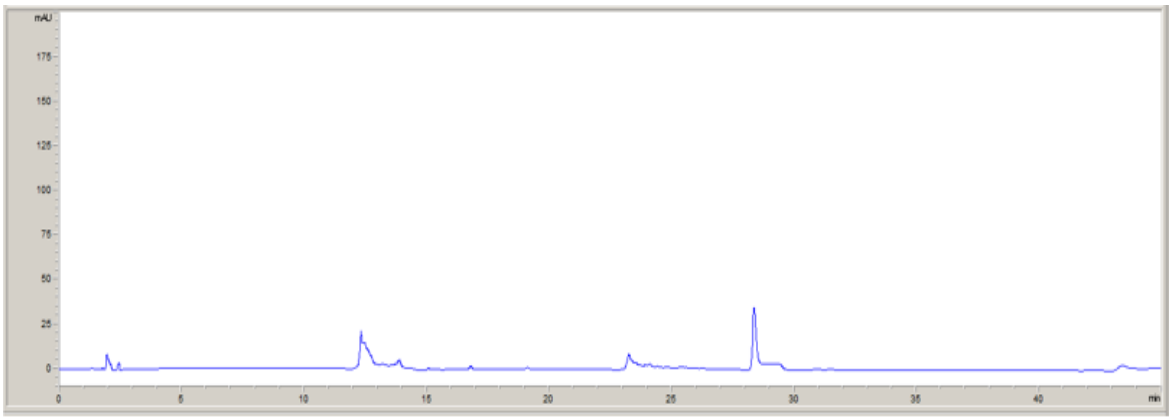


Figure 23: Chromatogram of Blank diluent (methanol: water) 80:20.

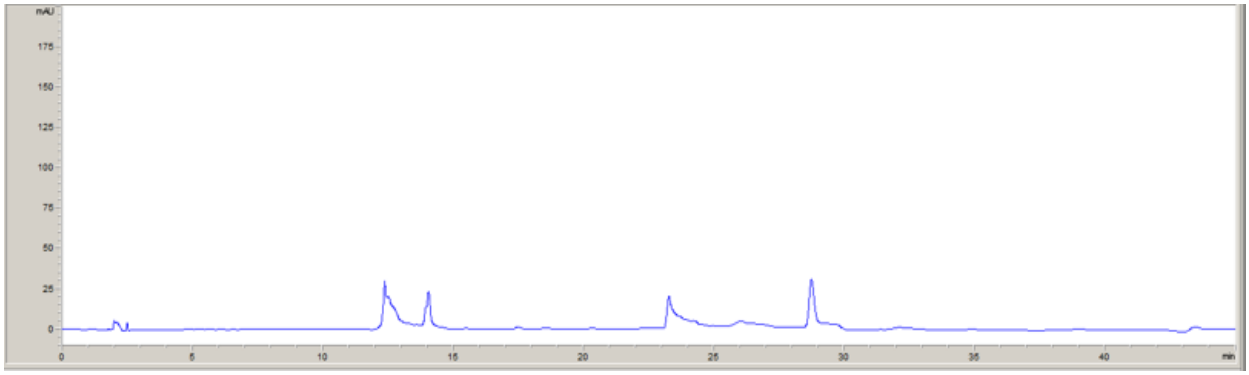


Figure 24: Chromatogram of ASAQ Placebo mixture - (methanol: water) 80:20.

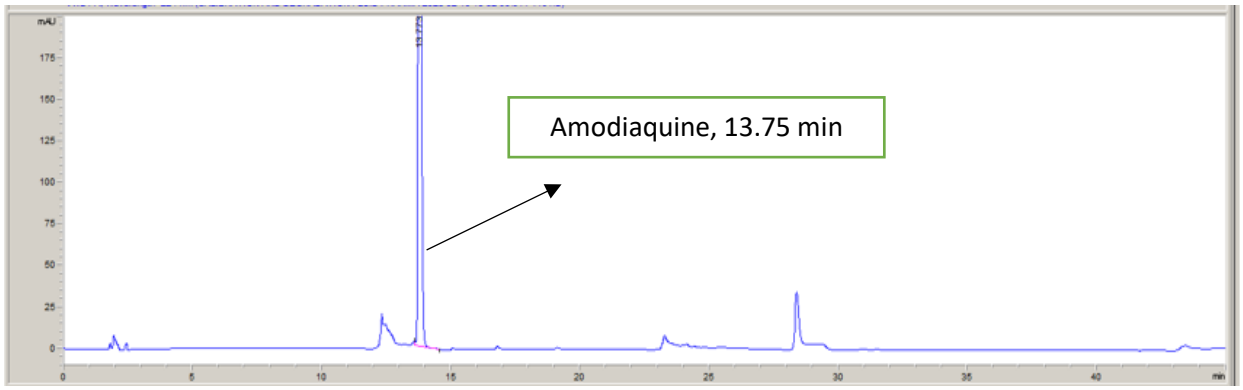


Figure 25: Chromatogram of 0.15 mg/mL AQ control - (methanol: water) 80:20.

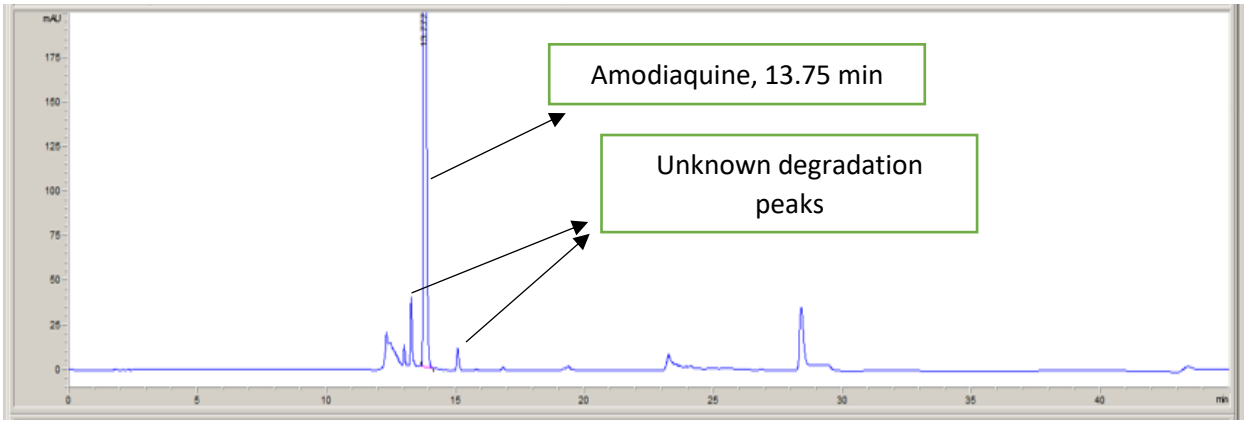


Figure 26: Heat degradation of AQ solution at 0.15 mg/mL using heat at 60^{o}C.

Table 19: Percentage Degradation of peaks observed within chromatogram generated from heat degradation sample.

Condition	% Degradation	Relative Retention Time (min)					
		Unknown Peak	Unknown Peak	Unknown peak	API	Unknown peak	Unknown peak
		0.94	0.96	1.09	1	1.14	1.40
Heat	7.34%	0.56%	2.24%	0.97%		0.10%	0.18%

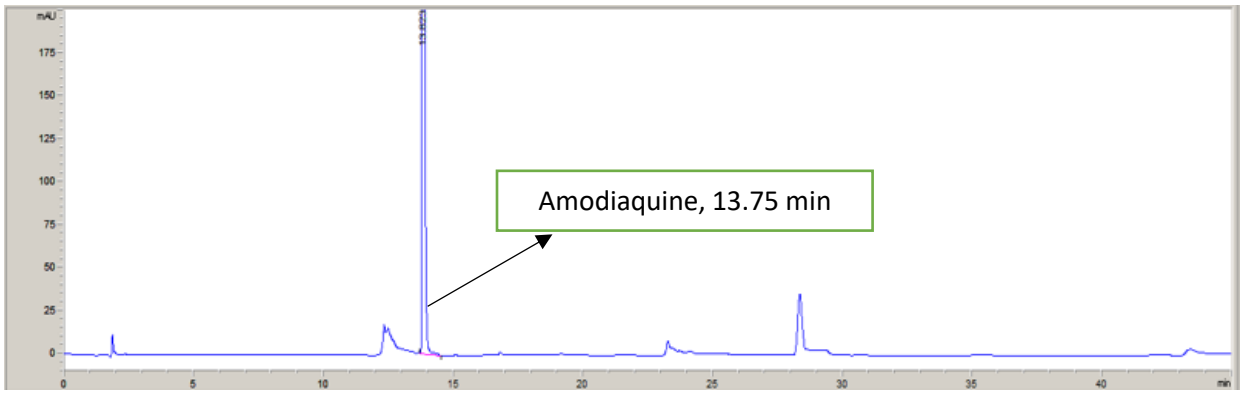


Figure 27: Acid degradation of AQ solution at 0.15 mg/mL using 2M HCl.

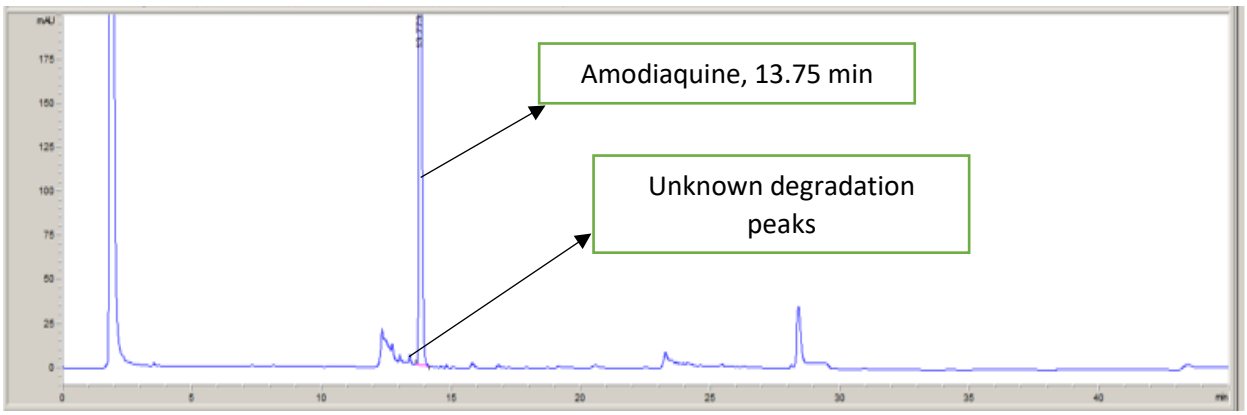


Figure 28: Oxidative degradation of AQ solution at 0.15 mg/mL using 30% H_2O_2.

Table 20: Percentage degradation of peaks observed within chromatogram generated from peroxide sample.

Condition	% Degradation	Relative Retention Time (min)					
		Unknown Peak	Unknown Peak	Unknown peak	API	Unknown peak	Unknown Peak
		0.94	0.97	1.05	1	1.07	1.14
H_2O_2	5.23%	0.21%	0.24%	0.07%		0.13%	0.33%

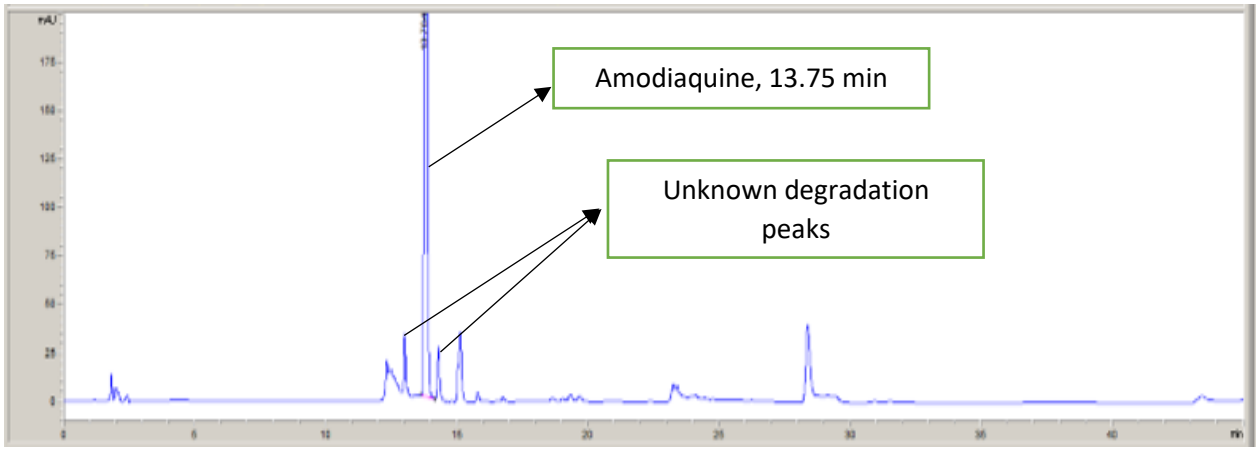


Figure 29: Base degradation of AQ solution at 0.15 mg/mL using 0.16M NaOH.

Table 21: Percentage degradation of peaks observed within chromatogram generated from base degradation sample.

Condition	% Degradation	Relative Retention Time (min)					
		Unknown Peak	Unknown Peak	Unknown peak	API	Unknown peak	Unknown Peak
		0.94	1.03	1.09	1	1.147	1.40
0.16 M NaOH	15%	2.52%	1.93%	6.52%		0.60%	0.18%

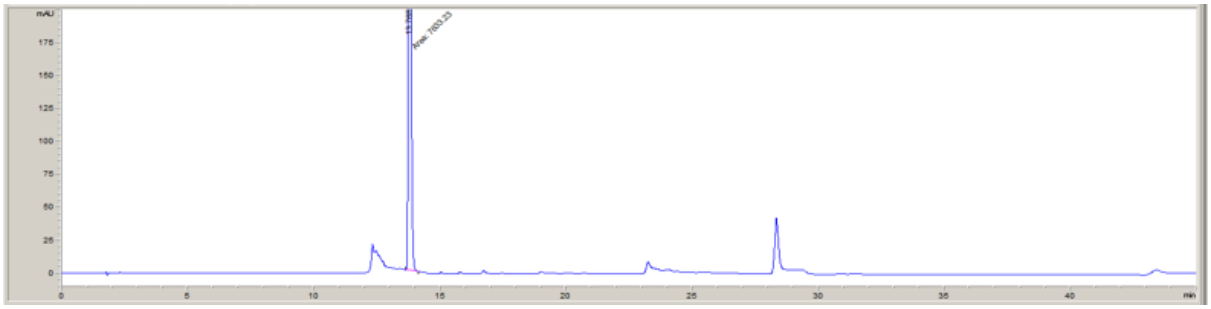


Figure 30: Light degradation of AQ solution at 0.15 mg/mL using UV lamp.

Linearity

calibration curves of amodiaquine and its related substances were constructed using the analytical methods developed to enable the quantification of amodiaquine hydrochloride in samples.

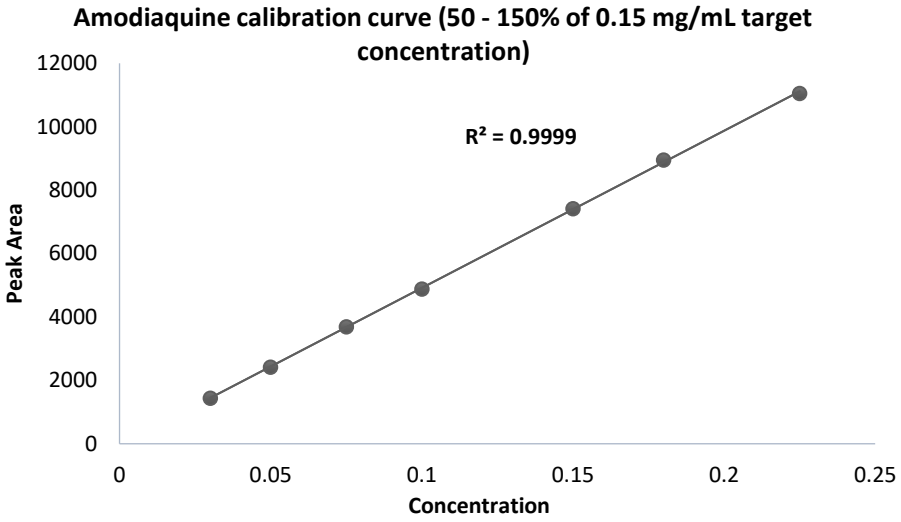


Figure 31: Linearity in response of amodiaquine reference solutions demonstrated across the concentration range 0.075 mg/mL – 0.225 mg/mL (50% - 150% of target concentration of 0.15 mg/mL.

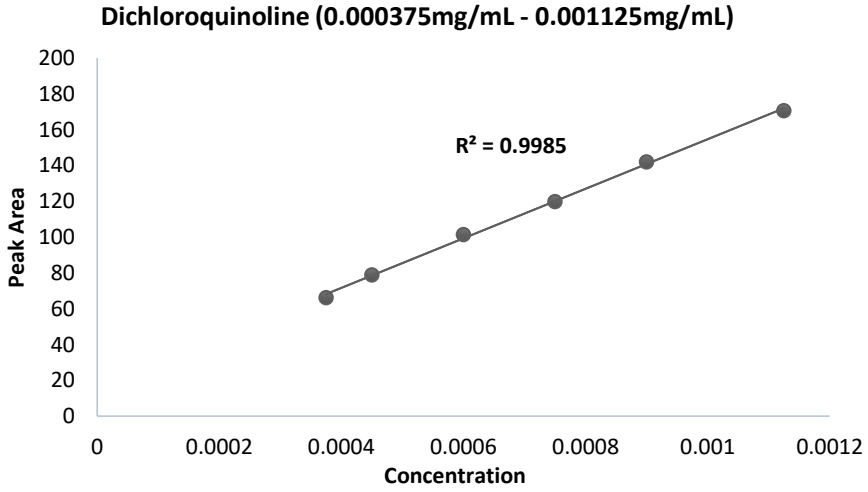


Figure 32: Linearity of Di-chloroquinoline from the range 0.000375-0.001125 mg/mL.

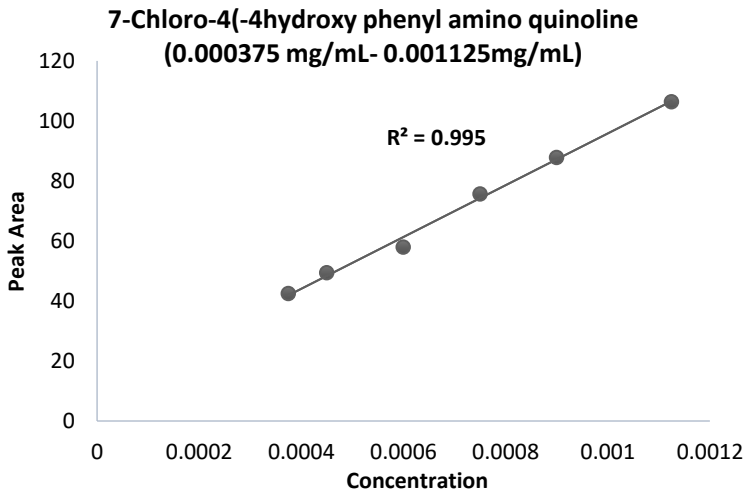


Figure 33: Linearity of 7-chloro-4-(4-hydroxyphenylamino) quinoline from the range 0.000375 mg/mL-0.001125 mg/mL.

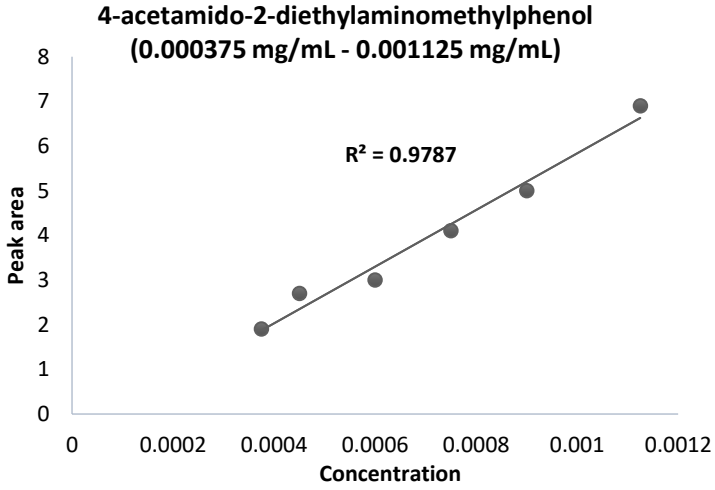


Figure 34: Linearity of 4-acetamido-2-diethylaminomethylphenol from the range 0.000375-0.001125 mg/mL.

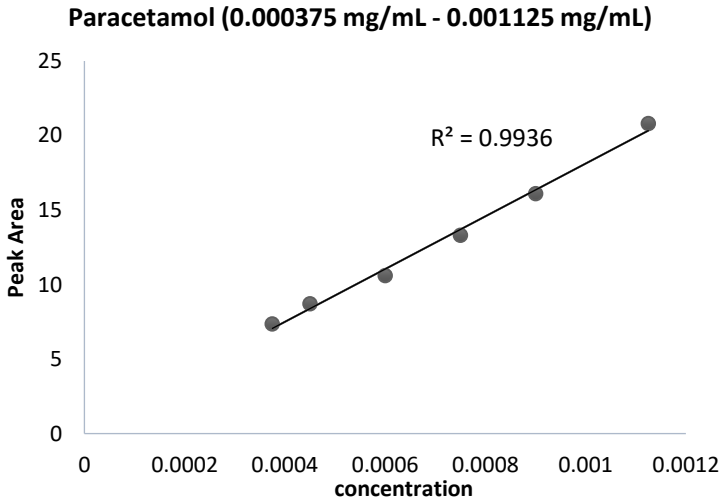


Figure 35: Linearity of paracetamol from the range 0.000375-0.001125 mg/mL.

System suitability

System suitability was assessed by calculating parameters of interest like plate count, capacity factor, resolution and tailing factor for the main API peak, and the impurities (paracetamol, 4,7-Dichloroquinoline,7-chloro-4-(4-hydroxy-phenyl-amino) quinoline, 4-acetamido-2-diethylaminomethylphenol) of AQ. These parameters were generated based on AQ concentration of 0.15 mg/mL, and impurity concentration of 0.00075 mg/mL. These parameters were acceptable for system suitability.

Table 22: Calculated system suitability parameters for Amodiaquine & its impurities.

S.no	Analyte	Retention time in (mins)	Plate count	Capacity factor	Resolution
1	Amodiaquine	13.75	9370	6.7	>1.5
2	paracetamol	6.04	10344	2.44	>1.5
3	4,7- Di chloroquinoline	34.33	12923	18.1	>1.5
4	7-chloro-4-(4-hydroxyphenylamino) quinoline	18.01	8051	9	>1.5
5	4-acetamido-2-diethylaminomethylphenol	8.3	10835	3.6	>1.5

Table 23: Summary of the parameters determined for the validation of the gradient elution HPLC methods for quantification of amodiaquine.

Parameter	**AQ method**	**Limit as per ICH guidance**
Linearity (R2)	0.999	> 0.999
Intra-day precision (% RSD) for 0.075 mg/mL, 0.15 mg/mL, and 0.225 mg/mL	1.98%, 1.18%, 1.29%	RSD ≤ 2 %
Inter-day precision (% RSD) for 0.075 mg/mL, 0.15 mg/ mL, and 0.225 mg/mL	0.46%, 0.90%, 0.64%	RSD ≤ 2 %
Repeatability 0.15 mg/mL	0.24	RSD ≤ 2 %
Accuracy (%) for 0.075 mg/mL, 0.15 mg/ mL, and 0.225 mg/mL	99.76%, 100.84%, 100.33%	100 ± 2 %
Limit of detection (mg/mL)	0.00803 mg/mL	Report result
Limit of quantification (mg/mL)	0.02678 mg/mL	Report result
Tailing factor	**1.13**	**< 2**

Stability of standard and sample solutions:

A solution of Amodiaquine reference standard at the target concentration of 0.15 mg/mL, along with an extracted sample of formulation with and without the presence of AS formulation, was held for 24 h and stability was assessed over that period. The concentration for the standard and sample solution at initial time point was determined. Assess the concentration of same sample and standard solution at different time points for 24 h against that of initial time point and report the change in API concentration over that time.

Results for Solution Stability data:

Table 24: Percentage RSD of repeated injections of AQ reference standard and AS/AQ formulation over 24 h.

Sample	Average peak area	Standard deviation	% RSD
AQ Reference solution	7836.533	88.6558	1.131314
ASAQ Formulation	8442.033	136.0736	1.611858

Repeated injections of standard and sample over 24 h showed an RSD of <2%, indicates little decrease in peak area and good sample stability over this period.

4. DISCUSSION

Within this study, we sought to develop 3 analytical methods to support testing and assessment of a novel taste-masked formulation developed by Fluid Pharma. These 3 methods were HPLC method for dissolution, method for assay and impurity profiling of an artesunate, and method for assay and impurity profiling of an amodiaquine. Initially dissolution method was developed to separate and quantify artesunate, which can be applied to the analysis of samples derived from dissolution studies of a novel ASAQ formulation. The instability of artesunate necessitates the use of HPLC, over UV alone, due to the presence of a degradant of artesunate, DHA, interfering with detection of artesunate. The stability testing performed mentioned in section 2.3.2 highlight the challenges of working with this molecule. Stability testing of artesunate was performed in methanol, ethanol, and acetonitrile for 24 h to check the percent degradation. These results agree with the literature[27] showing that artesunate is highly unstable molecule which is converted to DHA a degradation product in presence of water. However, greater stability is observed within acetonitrile and methanol when compared to aqueous solvents as observed in literature.

Assessment of analyte solubility is important for guiding analytical method development, as this influences stock solution creation and sample extraction of analyte from the formulation to be tested. The solubility of artesunate was observed be higher in organic media, and lower in aqueous media[35]

Sensitivity is a potential issue while developing HPLC methods for support of dissolution testing, as the concentration of analyte in a sample is often low. This is problematic, because samples with analyte concentrations below the limit of detection (LOD) or limit of quantification (LOQ) of the HPLC method cannot be accurately quantified. The use of a column with a smaller internal diameter and smaller particle size, helped to increase the method sensitivity. This is evidenced by an

increase in peak height, as observed in Figure 19. The use of smaller internal diameters and particle sizes to increase sensitivity has been observed in the literature previously[12]. The next stage of this work will look to validate the developed dissolution method for artesunate according to ICH (International conference on harmonisation) guidelines for accuracy, system precision, limit of detection, limit of quantitation and linearity. Precision was not over the days because artesunate is unstable in acetate buffer and samples taken from dissolution medium were analysed immediately.

Following the development of a dissolution method, development of a method for assay of the novel drug product was performed. This was used to assess the drug content of the novel formulation. Initial work attempted to adapt a method published by Le Vaillant [30] for simultaneous detection of artesunate and amodiaquine, alongside each of their impurities. However, during our method development we found that this resulted in a co-elution of two impurities (artemisinin and 4,7-dichloroquinoline). This, coupled to the high cost of the mobile phase additive octane sulfonate used for mobile phase preparation was high cost (60£ per 10g) around 21g of octane sulfonate is required for preparing 1 L of mobile phase. Attempts to decrease the amount of this additive in the mobile phase resulted in a negative effect on peak shape and loss of separation. Given the complexity of separating these analytes in one method, coupled with the need for different extraction solvents for both AS and AQ APIs and associated impurities, a decision was made to develop two methods: for AS and its impurities, and another for AQ and its impurities as it was hypothesised that this would simplify method development. International pharmacopeia method was selected as a reference for initial trails and all the artesunate and its impurities were injected individually to know their retention times. Along with artesunate samples amodiaquine is present in final formulation AQ and its impurities samples were also injected to make sure that they will not interfere with artesunate and its impurities retention time. This is evidenced by separation of all analytes, but artemisinin and Di

chloroquinoline were coeluting as observed in figure 6. Hence tried changing the different composition of mobile phase ratios and flow rates to separate all the analytes. Adequate separation was achieved when 66% of acetonitrile and 34% of buffer pH-3.0 with a flow rate of 0.4 mL/min was used. Methanol was used to extract the drug from formulation. Initially extraction was done for 30 mins, but the recoveries are less hence extraction was done for 1 h and the recoveries were within the acceptance limits. The developed method was validated according to ICH guidelines and the results were within the limits.

Initially isocratic method stated in USP monograph was taken as a reference for the method development, amodiaquine and its impurities were injected individually to know their retention times. A mixture of sample containing amodiaquine and its impurities were injected to check their separation but mannich base and paracetamol were coeluting and 7-Chloro-(4-hydroxyphenyl) aminoquinoline and 4,7-Di chloroquinoline is taking longer time for elution when methanol and buffer are used as mobile phase. Then mobile phase was changed from methanol to acetonitrile still mannich base and paracetamol were coeluting. Gradient method was used commonly when a mixture of solutes with a wide range of retention factors are to be separated. The base line peaks in the blank chromatogram are seen because of gradient elution, the solvent composition changes over time, typically becoming more non-polar (or less polar) as the run progresses. This changing composition can cause fluctuations in the baseline due to differences in refractive index, absorbance, or other properties of the solvents. These base line peaks were not due to polymers used in the formulation which was confirmed by injecting individual excipients. All the impurities were quantified by injecting standard solutions of each impurity for analysis each time.

To prove the method is stability indicating forced degradation tests were done for artesunate and amodiaquine separately using different methods.

For artesunate, acid and base hydrolysis test was done using 0.01M HCL and 0.01M NaOH and the ester bonds in artesunate was hydrolysed, which leads to the formation

of DHA (dihydro artemisinin) as a main degradation product. Oxidative degradation was done using 10% hydrogen peroxide and it caused oxidation of lactone ring in artesunate which can lead to the formation of artesunic acid and other hydroperoxides. Photolytic degradation was done by exposing sample to UV light and resulted in the formation of DHA by breaking chemical bonds. Thermal degradation done by heating the sample at 50^0C in the water bath, which lead to the breakdown of artesunate to DHA (major degradation product). The results agree with the literature [34]. Other small degradation peaks were observed but the retention times were not interfering with the artesunate peak.

Amodiaquine undergoes hydrolysis under acidic and basic conditions using 2M HCL and 0.16M NaOH which can remove the ethyl group from the side chain and forms desethylamodiaquine. Oxidative degradation was done using 30% hydrogen peroxide and can lead to the formation of various oxidized products. UV light and heat can lead to similar degradation products like hydrolysis. Analytical techniques like LC-MS, and NMR would be necessary to confirm and characterize these degradation products. The degradation products formed are not interfering with main API peak. Developed method was validated and results shown in table - 23 were within the limits according to ICH guidelines. System suitability test was done, and results were shown in table – 22 and 23. A resolution values are >1.5 which indicates that the peaks are well separated. A tailing factor of 1 indicates a perfectly symmetrical peak but the results were greater than 1 which indicates tailing this could be due to adsorption of analyte to stationary phase, with values typically below 2 being acceptable in most analyses according to ICH guidelines. The results for capacity factor were in a range of 1 and 10 that provides good separation and reasonable analysis time.

Within this current phase of work, we have developed two separate HPLC methods for Artesunate and Amodiaquine to separate it from its impurities and quantify them to support assay of a novel drug product (formulation) containing both artesunate and amodiaquine [30]

Future scope of work can be focussed on AQ, as forced degradation for AQ and its impurities method was done for different parameters like oxidation, acid and base hydrolysis, thermal, photolytic degradations. Within this phase of work some unknown peaks were observed in the chromatogram and requires detailed analysis to identify the degradation products by using LCM-MS, NMR spectroscopy. The study on the effect of polymers on AS-AQ in taste masking formulations holds significant potential to enhance patient compliance. The future scope can be comprehensive research involving polymer selection, formulation development, and rigorous evaluation to achieve effective and safe taste masking solutions for AS-AQ.

5. CONCLUSION

The ideal objective was to develop a simultaneous method for AS, AQ and its impurities. Due to the complexity as some analytes were coeluting, separate method for AS with impurities and AQ with impurities was developed. In total three analytical methods were developed for the monitoring of AS and its impurities, AQ and its impurities and HPLC method for dissolution samples for a novel taste mask coated ASAQ formulation developed by Fluid Pharma Ltd. Acceptable recovery of API from the formulation was found when using methanol as an extraction solvent for artesunate and methanol: water (80:20) as an extraction solvent for amodiaquine. Validation took place, with all tested parameters were found to be within the limits stated in the ICH guidelines. The current approach is firmly established as being unique compared to the previous methods.

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