Establishment of a simple colorimetric method for determination of Artesunate based on Diazo Coupling Reaction

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DEDICATION

To my mother, brothers, sisters

Father's Soul

With Special love to my husband

Acknowledgments

First, I thank Allah for bringing my dreams into reality.

I am extremely grateful to my supervisor

***Dr.** Mubasher Hashim Awad, for his continuous follow-up*

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Finally, my thanks also are extended to everybody helping me

in my work.

Abstract

Artesunate (ARTS) is a readily available and malarial in combination therapy, the standard method used to determine the authenticity of ARTS tablets involves high performance liquid chromatography (HPLC). In many countries, resources to purchase and maintain such equipment are expensive and not always available.

Primary aromatic amine was treated with sodium nitrite and hydrochloric acid for diazotization reaction followed by coupling with Artesunate in pH 4, 6, 8 medium to form a yellow colored azo dye compound which exhibits maximum absorption (λ_{max}) at 420 nm. These experiment was repeated twice for Artesunate tablets. The colorimetric method can be used to obtain a rapid visual assessment of tablet authenticity. The method can also be used to quantify the drug content of tablets, when used in conjunction with a spectrophotometer. The optimum reaction conditions and other analytical parameters were evaluated.

Three samples of Artesunate (raw material, Amipharma tablet, Shanghai tablet) has been analyzed by different chemical methods. The Artesunate samples were authenticated using FTIR spectrum, the results was good and showed similarity for two Artesunate tablets (Amipharama Artesunate and Shanghai Artesunate). HPLC, and TLC analysis of Artesunate Raw Material has been measured with good retention time value

مستخــلص

يعتبر عقار الارتيسيونيت Artesunate من اشهر الادوية المتداولة لمحاربة ومعالجة مرض الملاريا. حيث تعتبر تقنية كروموتغرافيا السائل عالية الضغط HPLC هي الطريقة القياسية للتعرف علي عقار الارتيسيونيت Artesunate بالأقراص الا ان هذه التقنية مكلفة وغير متوفرة في العديد من الدول.

عومل أمين أروماتي أولي مع نتريت الصوديوم وحامض الهيدروكلوريك لأزوتته (diazotization) تبع ذلك اجراء تفاعل ازدواج مع عقار الارتيسيونيت Artesunate في وسط تفاعل منظم عند اس هيدروجيني 8,6,4 لتكوين صبغة الأزو ذات اللون الاصفر التي تظهر امتصاص أقصي (λ_{max}) عند 420 nm. تم تكرار التجربة اعلاه مرتين باستخدام أقراص عقار الارتيسيونيت Artesunate. يمكن استخدام طريقة التحليل اللوني(colorimetric method) للتأكد من وجود مادة الارتيسيونيت Artesunate بالأقراص, كما يمكن تقدير كمية العقار بقرص الارتيسيونيت وذلك باستخدام تقنية الطيف الضوئي spectrophotometric. وقد تم تعيين الظروف المثلي التي تؤثر على التفاعل اللوني والعوامل التحليلية الأخرى.

تم تحليل ثلاثة عينات من عقار الارتيسيونيت Artesunate هي خام Artesunate, اقراض الارتيسيونيت انتاج كل من شركة أميفارما , و شركة شنقهاي بعدة طرق كيميائية.تم تحليل عينات عقار الارتيسيونيت Artesunate باستخدام FTIR, حيث أوضح طيف الاشعة تحت الحمراء لأقراص عقار الارتيسيونيت Artesunate المتحصل عليه من مصنعي شنغهاي و أميفارما علي نتائج متشابهة. تحليل خام عقار الارتيسيونيت Artesunate بكروموتوغرافيا السائل الضغط العاليHPLC و الطبقة الرقيقة TLC أعطت نتائج مطابقة لقيمة معامل الاعاقة القياسية.

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Abbreviations

ACT	Artemisinin based combination therapy
AM	Artemether
API	Active Pharmaceutical Ingredients
ARTS	Artesunate
CQ	Chloroquine
DHA	dihyroartemisinin
DNP	2, 4-dinitrophenylhydrazine
ELS	Evaporative light scattering
FBS	Fast Blue RR salt
FPP	Finished pharmaceutical product
FRTR	Fast Red TR reagent
FTIR	Ferial transmitted infrared spectroscopy
HPLC	High performance liquid chromatography
HPLC-EC	HPLC with electrochemical detection
HPLC-MS	HPLC with mass spectrometry detection
PDA	Photodiode array
SP	Sulphadoxine/Pyrimethamine
TOF	Time-of-flight
TLC	Thin layer chromatography
UV	Ultraviolet spectroscopy
WHO	World Health Organization
CT	Combination therapy

Chapter One

Introduction

1 Malaria

Malaria continues to be one of the most severe infectious diseases globally. Malaria is widespread in the tropical and subtropical regions. It kills more people each year than any other infectious diseases except AIDS and tuberculosis. Although it is difficult to obtain an exact figure of malaria cases, the World Health Organization (WHO) estimates that malaria is responsible for over 300 million clinical cases and over one million deaths annually. About40% of the global population is estimated to be at risk. Malaria is not just a disease commonly associated with poverty, but it is also a cause of poverty and a major hindrance to economic development [1, 2].

Antimalarial chemotherapy has been the primary option in the fight against malaria and over the years many drugs have been developed and used in the treatment of the disease. However, the burden of this disease is still very heavy, partly due to the development of multi-drug resistant *Plasmodium falciparum* strains [1]. The rate of increase in the resistance of the malaria parasite *Plasmodium falciparum* to antimalarial drugs in many parts of the world is becoming more disturbing [3], because of the resistance problems associated with Chloroquine which was considered first-line therapy globally for many years. In the year2001, WHO convened an Informal Consultation on the use of Antimalarial Drugs. This meeting reviewed and updated guidelines and recommendations on the use of antimalarial drugs for chemoprophylaxis and treatment. The purpose of the meeting was to outline the limited options for the treatment of malaria in endemic areas (especially Africa) where resources of treatment is limited. The potential value of malaria therapy using combination drugs was identified as a strategic and

viable option in improving efficacy, delaying development and selection of resistant parasites. Later on the roll back malaria program organized by WHO also considered convening a technical consultation to recommend minimum criteria for appropriate combinations selection, particularly for Africa [1-4].

The malaria community presently considers mono-therapy as an inappropriate approach for malaria treatment. African countries have recently begun to scale up their antimalarial efforts, and are deploying strategies to combat the new face of malaria.

One of these strategies is the use of Artemisinin - based combination therapies which have proven to be very effective against malaria in Africa [3, 5]. Before the use of any antimalarial or drug combination can be implemented as treatment and / or prophylaxis, it is necessary to ascertain the purity, content and other quality standards of the drug as well as its availability and pharmacokinetic properties [6]. This information can only be obtained by analytical methods. The analytical methods must be selective enough to give the true signal of a particular drug, and be able to separate each drug present from the other in any environment (in formulations or biological fluids) for quantification. Selective methods for the determination of drugs is one of the policies of the WHO expect committee reports on quality control of drugs and also a requirement under the Good Manufacturing Practices for Active Pharmaceutical Ingredients (API). [6-8].

Classical and other simple instrumental methods have been used in the analysis of pharmaceutically active ingredients, either as raw materials or as finished pharmaceutical product (FPP), and also in biological fluids. For field adaptive studies of combined antimalarial drugs, where each component needs proper elucidation, it is necessary to develop a very simple, rapid and accurate technique for analysis, which can give signals of the drug without interferences

from other drugs present. Liquid Chromatography (LC) is a revolutionary separation technique which has found its major use in the chemical and life sciences. It is used in many different fields for quantification of a variety of compounds in different matrices. In most liquid chromatographic analysis where the compound of interest is found in a complex matrix which contains compounds that may interfere with the analysis, sample preparation or cleanup prior to analysis is considered in order to separate the compound from the matrix. This has been found to be time consuming, waste of solvents and leads to reduction in quantity of the compound. In accordance with requirements for quality control of drugs, there has been the need for selective methods that can be used to determine compounds of interest from the matrices without sample clean up. In view of this, high performance liquid chromatography (HPLC) system of separation, identification and quantification has been the best option for most pharmaceutical analysis [9, 10].

1.1 Objectives of work

The Objectives of this study were

1) To authenticate the raw materials using HPLC, FTIR, and thin layer chromatography (TLC).
2) To develop a selective, simple, rapid and accurate colorimetric test to determine ARTS authenticity in tablets. ARTS does not possess reactive groups like Antimalarials such as Chloroquine and Sulfadoxine, and this study, we establishes the best conditions of the reaction between raw material ARTS with diazonium alt.

Chapter Two

Literature Review

2 Malaria The Disease and Drugs

2.1 Overview

Malaria is caused by single-celled protozoan parasites of the genus *Plasmodium*. Four species infect humans by entering the bloodstream. The most serious forms of the disease are caused by *Plasmodium falciparum* and *Plasmodium vivax*, and the other related species are *Plasmodium ovale* and *Plasmodium malariae*. This group of human-pathogenic *Plasmodium* species are usually referred to as *malaria parasites*.

Malaria parasites are transmitted by female *anopheles* mosquitoes. The parasites multiply within red blood cells, causing symptoms similar to regular influenza that include headache, fever, anemia, chills, flu-like illness, and in severe cases, coma (cerebral malaria) and death.

Malaria in humans develops via two phases: an **exoerythrocytic (hepatic) and an erythrocytic phase**. When an infected mosquito pierces a person's skin to take a blood meal, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver. Within 30minutes of being introduced into the human host, they infect hepatocytes, multiplying as equally and asymptomatically for a period of 6–15 days. During this so-called dormant time in the liver the sporozoites are often referred to as hypnozoites. In the liver they differentiate to yield thousands of merozoites which, following rupture of their host cells, escape into the blood and infect red blood cells, thus beginning the erythrocytic stage of its life cycle. The parasite escapes from the liver undetected by wrapping itself in the cell membrane of the infected host liver cell. Within the red blood cells the parasites multiply further, again asexually, periodically breaking out of their hosts to invade fresh red

blood cells. Several of such amplification cycles occur. Thus, classical descriptions of waves off ever arise from simultaneous waves of merozoites escaping and infecting red blood cells.

Some *Plasmodium vivax* and *Plasmodium. ovale*sporozoites do not immediately develop into exoerythrocytic-phase merozoites, but instead produce hypnozoites that remain dormantfor periods ranging from several months to as long as three years. After a period of dormancy, they reactivate and produce merozoites. Hypnozoites are responsible for long incubation and late relapses in these two species of malaria.

The parasite is relatively protected from attack by the body's immune system because for most of its human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. However, circulating infected erythrocytes are destroyed in the spleen. To avoid this fate, the *Plasmodium falciparum* parasite displays adhesive proteins on the surface of the infected erythrocytes, causing the blood cells to adhere to the linings of small blood vessels, thereby sequestering the parasite from passage through the general circulation and the spleen. This leads to decrease in blood flow and anemia. This "stickiness" is the main factor giving rise to hemorrhagic complications of malaria. High endothelial venules (the smallest branches of the circulatory system) can be blocked by the attachment of masses of these infected red blood cells. The blockage of these vessels cause symptoms such as in placental and cerebral malaria. In cerebral malaria the sequestrated red blood cells can breach the blood brain barrier possibly leading to coma. Active malaria infection with *P. Falciparum* is a medical emergency requiring hospitalization. Infection with *P. vivax, P. ovale* or *P. malariae* can often be treated as our patients. Treatment of

malaria involves supportive measures as well as the use of specific antimalarial drugs. When properly treated, someone with malaria can be completely cured. Preventative or prophylactic drugs can be taken continuously to reduce the risk of infection[11-13].

2.2 Antimalarial Drugs, Drug Resistance and Drug Combinations

Malaria has been treated, over the years, with numerous drugs. Cheap and effective drugs such as Chloroquine (CQ) and Sulphadoxine/Pyrimethamine (SP) was the antimalarial drug of choice for many years in most parts of the world. However, resistance of *Plasmodium falciparum* and *Plasmodium vivax* to CQ and SP has spread recently in the endemic areas, making the drug ineffective against the most dangerous *Plasmodium* strain in many affected regions of the world. This has been identified as a key factor in the increase of malaria mortality [2, 4]. There are several other drugs which are used for treatment and, partially for prevention (prophylaxis). Many drugs can be used for both purposes. Their usage depends mainly on the frequency of resistant parasites in the area where the drug is used.

Early diagnosis and prompt treatment is one of the principal technical components of the global strategy to control malaria. The effectiveness of this intervention is highly dependent on antimalarial drugs, which should not only be safe and effective, but also available, affordable and acceptable to the population at risk. The rational use of an effective antimalarial drug not only reduces the risk of severe disease and death and shortens the duration of the illness, but also contributes to slowing down the development of the parasite's resistance to antimalarial drugs. The emergence and rapid spread of *Plasmodium falciparum* resistance to commonly used antimalarial drugs poses a serious challenge to the effectiveness of early diagnosis and prompt treatment as a priority strategy within current malaria control efforts [1, 3].

Due to the high resistance of *P. falciparum,* there has been the urgent need for drug combination therapy. This has been proposed to delay the emergence and spread of drug resistance. Resistance arises from mutations. The chance that a mutant will emerge, resistant to two different drugs, is the product of the individual chances for each drug. For example, if the chance that a parasite is resistant to drug A is one in 106 and to drug B one in 1012, only one in 1018 will be resistant simultaneously to both A and B provided that resistance is not linked. In practice this means that the emergence and spread of resistant strains should be delayed and the useful therapeutic life of the combination should be much longer than its single components. There is an urgent need for new, efficient and affordable antimalarial drug combinations that can be used in epidemic areas around the world. The concept of combination therapy is based on the synergistic potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination. Combination therapy (CT) with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. In the context of this definition, multiple-drug therapies that include a non-antimalarial drug to enhance the antimalarial effect of a blood schizontocidal drug are not considered combination therapy. Similarly, certain antimalarial drugs that fit the criteria of synergistic fixed-dose combinations are operationally considered as single products in that neither of the individual components would be given alone for antimalarial therapy. An example is sulfadoxine-pyrimethamine. The most prominently used antimalarial in combination with others is Artemisinin-based combination therapies which have shown to improve treatment efficacy and also contain drug resistance in South-East Asia. The use of these combined

antimalarial drugs depends on the resistance level in a particular region and how patients react to it.

Currently, proposed combination therapy and existing single therapy antimalarial drugs include:

• Artesunate-Chloroquine (Therapy only)

• Artesunate-Amodiaquine (Therapy only)

• Artesunate-Mefloquine (Therapy only)

• Artemether-lumefantrine (Therapy only)

• Artesunate-Sulfadoxine/Pyrimethamine (Therapy only)

• Atovaquone-proguanil, (Therapy and prophylaxis)

• Amodiaquine (Therapy Only)

• Quinine (Therapy only)

• Chloroquine (Therapy and prophylaxis; usefulness now reduced due to resistance)

• Cotrifazid (Therapy and prophylaxis)

• Doxycycline (Therapy and prophylaxis)

• Mefloquine, (Therapy and prophylaxis)

• Primaquine (Therapy in *P. vivax* and *P. ovale* only; not for prophylaxis)

• Proguanil (Prophylaxis only)

• Sulfadoxine-pyrimethamine (Therapy; prophylaxis)

• Hydroxychloroquine, (Therapy and prophylaxis)

Even though all the above listed drugs exist for the cure of malaria, the choice depends on which of them resistance has not been grown to and national safety facts on a particular combination [1-4, 11-13]

2.3 Artesunate

Artemisinin is obtained from the extracts of the plant *Artemisia annua*, with several derivatives; dihydroartemisinin, its methyl ether (artemether), its ethyl ether (arteether) and its hemisuccinate ester (Artesunate) are known as more effective than its parent material Artemisinin [(14)]. These are rapidly gaining grounds as antimalarial that are used for the treatment of severe and uncomplicated multidrug-resistance falciparum malaria. Since 2001 the World Health Organization has recommended using Artemisinin-based combination therapy (ACT) as first-line treatment for uncomplicated malaria in areas experiencing resistance to older medications.

The advantages of Artemisinin based combination therapy (ACT) relate to the unique properties and mode of action of the Artemisinin component, which include the following:

• rapid substantial reduction of the parasite biomass

• rapid resolution of clinical symptoms

• effective action against multidrug-resistant *P. falciparum*

• reduction of gametocyte carriage, which may reduce transmission of resistant alleles (in areas with low or moderate malaria transmission)

• no parasite resistance documented as yet with the use of Artemisinin and its derivatives

• few reported adverse clinical effects; however pre-clinical toxicology data on Artemisinin derivatives are limited.

Artemisinin, Artesunate, Artemether and dihydroartemisinin have all been used in combination with other antimalarial drugs for the treatment of malaria. Of all these drugs Artesunate has the most documented clinical information.

WHO treatment guidelines for malaria recommend different Artesunate-based combination drugs such as:

• Artesunate-Amodiaquine

• Artesunate-Chloroquine

• Artesunate-Mefloquine

• Artesunate-Sulfadoxine/Pyrimethamine

While numerous countries, including most African countries, have adopted the change in their official malaria treatment policies, cost remains a major barrier to ACT implementation.

Because ACTs cost up to ten times as much as older medications, they remain unaffordable [2, 3, 13].

Artesunate (figure 2.1) is an antimalarial agent. It is a water-soluble hemisuccinate derivative of Artemisinin. Artesunate and its active metabolite dihydroartemisinin are potent blood schizonticides, active against the ring stage of the parasite. Artesunate is ideal for the treatment of severe malaria, including cerebral malaria. It is also active against Chloroquineand Mefloquine resistant strains of *P. falciparum*. Artesunate exist as a fine, white crystalline powder, very slightly soluble in water, freely soluble in methanol. Its chemical designation is (3*R*,5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano [4,3-*j*]-1,2-benzodioxepin-10-ol, hydrogen succinate, and its molecular formula is $C_{19}H_{28}O_8$.

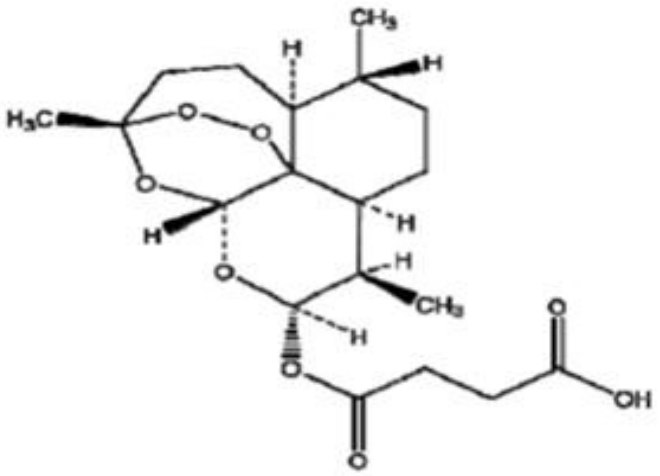


Figure2.1 Chemical Structure of Artesunate

Artesunate acts as a weak acid. An aqueous suspension of AS containing 10 mg/g has pH of 3.5–4.5, and a p*K*a value of 4.6 for the carboxyl group. AS is formulated for oral,11parenteral, (intravenous and intramuscular) and rectal administration. Combination therapy is only in oral dosage forms [15, 16].

The specific mechanism of action of Artesunate and other members of the Artemisinin in family has not been extensively studied. Artesunate in particular is incompatible with basic quinolines by virtue of proton transfer, and has intrinsic chemical instability. At pH 1.2, Artesunate conversion to dihyroartemisinin (DHA) is rapid, with $t_{1/2}$ 26 min, and at pH 7.4,t1/2 is about 10 hours. Because of rapid hydrolysis to dihyroartemisinin (artenimol), Artesunate is considered by many as a pro-drug of the latter.

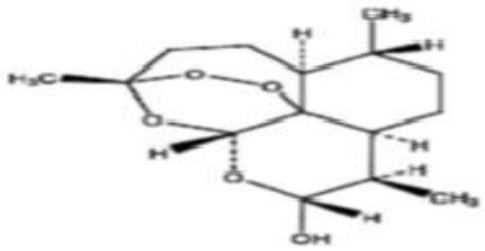


Figure 2.2 Chemical Structure of Dihydroartemisinin

With a pKa of 4.6, over 99% of Artesunate will be ionized at pH 7.4, and thus uptake by passive diffusion from the intestinal tract will be minimal. The functional group responsible for antimalarial activity of Artesunate is the **endoperoxide** bond present. When the para site that causes malaria infects a red blood cell, it consumes haemoglobin and liberates free heme, an iron-porphyrin complex. The iron reduces the peroxide bond in Artesunate generating high-valent iron-oxo species, resulting in a cascade of reactions that produce reactive oxygen radicals which damage the parasite leading to its death [13-15].

The development of selective analytical methods for determination of concentrations of Artesunate and other members of the family poses challenging problems since the drugs lacks physicochemical properties that can be used to asses them. Some of these physicochemical properties are:

• Thermal labiality

• Lack of UV or fluorescent chromophores for absorption

• Lack of functional groups for derivatization.

The remarkable activities of these drugs require assays with sensitivities in the low nanogram-per-millilitre range. Methods used to measure the concentrations of these drugs include titrimetry, HPLC with UV detection (HPLC-UV), HPLC

with electrochemical detection (HPLC-EC) and HPLC with mass spectrometry detection (HPLC-MS). The HPLC-EC has been the most widely used for the measurement of Artemisinin based drugs and their major metabolite dihydroartemisinin (DHA) in biological fluids [16, 17].

The simultaneous determination of Artesunate and other antimalarial in a combination has never been possible due to the fact that there is no UV-Chromophoric system in Artesunate. There has not been any HPLC documentation for the simultaneous determination of Artesunate and Amodiaquine using HPLC-UV technique [16, 17].

2.4 Diazonium salts

First prepared in 1858 by German born peter Griess.[18] When a primary aromatic an amine is treated with nitrous acid in well cooled solution. The product is an unstable compound known as diazonium salt. Diazo-compound may be obtained with primary aliphatic amines provided that the amino-group is attached to a carbon atom which is adjacent to a negative group. Diazonium compounds or diazonium salts are group of organic compound shairing a common functional group with the characteristic structure $R\text{-}N_2^+X^-$. Where R can be organic residue such as alkyl or aryl, and X is an inorganic or organic anion such a halogen.

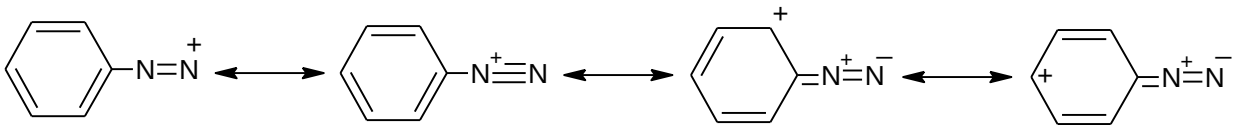
Salt in chemistry is defined as the product formed from the neutralization reaction of acid and base, salt are ionic compounds compose of cation and anion, so that the product is electrically neutral.[19] strong base diazonium hydroxide which has not yet been isolated, but is known in aqueous solution. Most diazonium salts of the inorganic acids are colourless solids extremely soluble in water. They form complex salt with many metallic salt, of which one of the most important is zinc chloride, $(Ar.N2)2\ ^{2+}ZnCl^{2-}$. These complex salts are stable in solution.[20] Diazonium salts are high sensitive and breakdown under near UV or violet light.

[21] The diazonium salts are very important synthetic reagent, being the starting point in the preparation of various aromatic compounds dyes and drugs.

2.4.1 Structural of Diazonium salts

The structural formula of the benzene diazonium $[C_6H_5\text{-}N \equiv N]^+$ ion is

The increased stability of benzenoid diazoinum salt compared with their aliphatic analogues is due to the extra resonating forms in which the ring accommodates the positive charge:



An electron-releasing substituent in the ring would be expected to stabilize the diazonium salt and this is found to be true. [22]

2.4.2 Nomenclature of Diazonium salts

The name of diazonium salt is obtained by adding diazonium chloride, diazonium sulphate, etc… to name of the parent hydrocarbon e.g $C_6H_5N_2.Cl$ benzene diazonium chloride, p-$CH_3.C_6H_5N_2HSO_4$ p-toluenediazonium sulphate.[22]

2.4.3 Preparation of Diazonium salts

There are various methods of preparing a solution of diazonium salts but the usual procedure is to dissolve “or suspend” the amine in excess dilute inorganic acid “usually hydrochloric” cooled in ice to add slowly with stirring a cooled aqueous solution of sodium nitrite. The addition of which is completed when the reduction mixture produce a blue colour with potassium iodide. Starch paper, thereby showing the presence of free nitrous acid.[23] The quantity of hydrochloric

acid must be sufficient to liberate nitrous acid from the sodium nitrite which is added, to ensure that throughout the reaction the aniline is present as aniline hydrochloride, and to keep the solution acid. If free aniline is present it reacts with the benzenediazonium chloride to give a yellow precipitate of diazo-aminobenzene.

$$C_6H_5N_2Cl + HNHC_6H_6 \rightarrow C_6H_5N_2.NHC_6H_5 + HCl$$

The quantity of sodium nitrite added must be slightly in excess of that required just to react with the aniline hydrochloride. Excess leads to the formation of coloured by products, and a deficiency leaves unchanged aniline in the solution. Throughout the preparation the temperature must be maintained between 5°C to 10°C, if it rises above 10°C the benzenediazonium chloride reacts with water to yield phenol. If it falls below 5°C the reaction between nitrous acid aniline is arrested. The reaction is exothermic and the containing vessel must, therefore must be cooled in ice-water. [24]

The stability of diazonium salt depend on type of amine, nitrogen is always evolved.

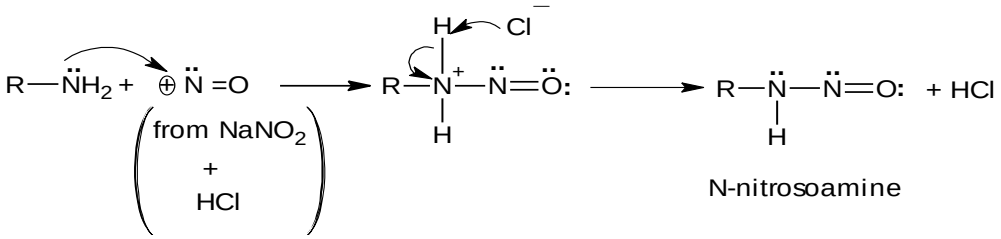
$$RNH_2 + HNO_3 \longrightarrow ROH + N_2 + H_2O$$

Since, diazonium salt decomposed event at ice bath temperature, the solution is used immediately after preparation. [25]

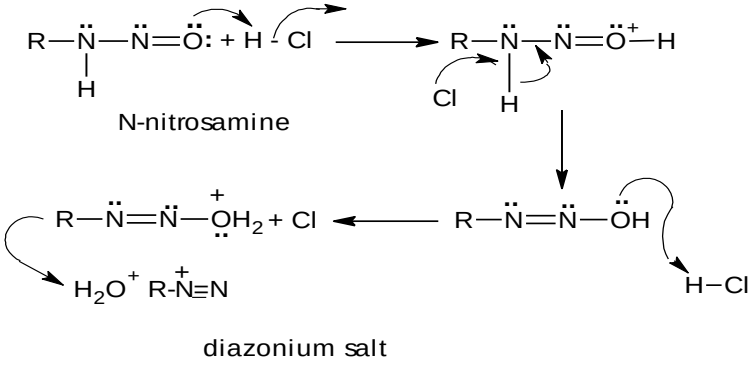
2.4.4 Mechanism of Diazonium salts

The mechanism consists of many steps, it begins with nucleophilic attach of amine on nitrosonium ion it can be conceptually divided, formation of N-nitroso amine followed by loss of H_2O.

(1) Formation of N-nitroso amine by nucleophilic attack of amine group on ^{+}NO followed by loss of a proton.



(2) Loss of H_2O to form diazonium salt, in this part transfer here a series of leads to loss H_2O and formation of the diazonium ion.



The formation of diazonium compound by the interaction of sodium nitrite, an inorganic acid and a primary aromatic amine in ice cooled solution is known as "diazotisation". The stability is an unstable compound, it is obtained in cold solution.[24]

2.4.5 Reaction of diazonium salt

These may be divided into two groups:

1) Those which involve the liberation of nitrogen gas and displacement of diazo-group, $-N_2X$, by another univalent group.
2) Those in which the two nitrogen atoms are retained.

2.4.5.1 Replacement reactions

1] Replacement by hydroxyl

When a diazonium sulphate solution is boiled or stream distilled, the diazo-group is replaced by hydroxyl group.

$$C_6H_5N_2HSO_4 + H_2O \longrightarrow C_6H_5OH + N_2 + H_2SO_4$$

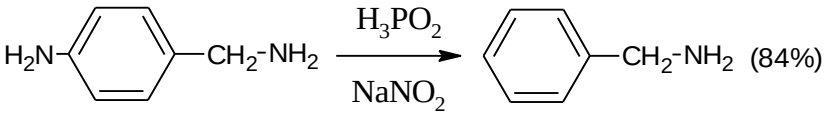
The resultant solution containing phenol which may be isolated from the tarry by product by stream distillation. [21] The hydrolysis of diazonium salt has been improved by running the aqueous solution down a chilled tube in a vessel containing boiling 20 percent. Sulphuric acid through which steam is passing. This reaction occurs by the nucleophilic unimolecular mechanism.[21]

2] Replacement by hydrogen

Replacement of diazo-group by hydrogen offers a means of preparing substituted aromatic compounds in which the substituents are in positions which they would not take up by direct substitution.

The most reliable method of replacing the diazo-group by hydrogen is by means of hypophosphorous acid. Furthermore that the aromatic primary amino group may be selectively replaced by hydrogen in aliphatic aromatic diamines by

dissolving the diamine in hypophosphorous acid adding sodium nitrite at 0-5°C. e.g



Another common method of replacing the diazo-group by hydrogen is to dissolve the amine in mixture of ethanol and concentrated sulphuric acid, add sodium nitrite and then warm. According to Griess (1984), benzeidiazonium sulphate reacts as follows:

$$C_6H_5N_2HSO_4 + C_2H_5OH \longrightarrow C_6H_6 + CH_3.CHO + N_2 + H_2SO_4$$

Remsen et al (1887) however showed that this was incorrect, the main product was shown to be phenetole together with a small amount of benzene, he obtain the following result in absolute ethanol.

$$C_6H_5N_2Cl + C_2H_5OH \longrightarrow \underset{(61\%)}{C_6H_5.OC_2H_5} + \underset{(5\%)}{C_6H_6}$$

When methanol was used instead of ethanol no benzene was obtained at all. The product was anisole $C_6H_5.O.CH_3$ (70%). Formation of ether depends on the structure of the diazonium salt and the natural of alcohol used. The mechanism of decomposition of diazonium salt with alcohols is uncertain. According to Hey and waters the reaction takes place by free-radical mechanism:

$$C_6H_5{-}N{=}N{-}Cl \longrightarrow C_6H_5^{\bullet} + N_2 + Cl^{\bullet}$$

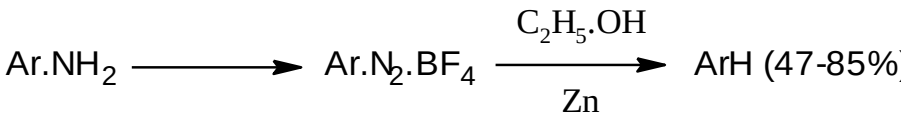
$$C_6H_5^{\bullet} + CH_3.CH_2^{\bullet}OH \longrightarrow C_6H_6 + CH_3.\dot{C}H.OH$$

$$CH_3.\dot{C}H.OH + Cl^{\bullet} \longrightarrow CH_3.CHO + HCl$$

However, that this free radical decomposition is not generally produced in aqueous solution but that their formation is possible in non-ionizing solvent. [20]

If a substituted benzene is present during this decomposition the aryl radical will attack it at the para position irrespective of the kind of substituent.

The observation conforms that the mechanism involves attack by radical rather than the ion because radicals are not subject to electronic orientation effect. This reaction may be generalized as follows:



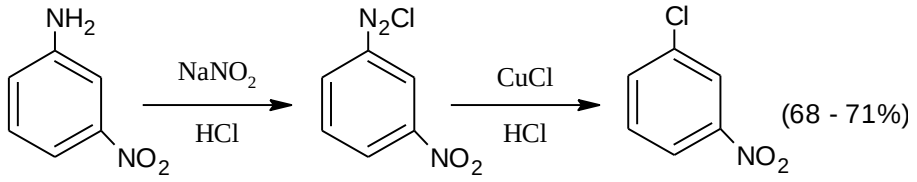
Where x may be electron attracting (NO_2, Br) or releasing (CH_3). [22]

3] Replacing by halogen

(i) Sandmeryer reaction

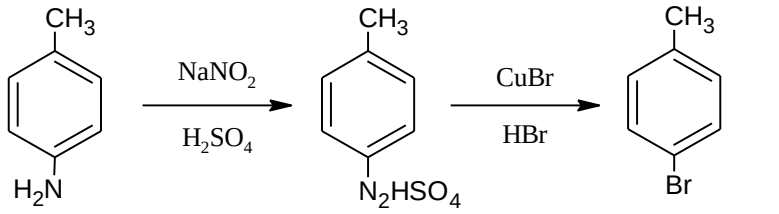
When a diazonium salt solution is run into a solution of cuprous halide dissolved in the corresponding halogen acid the diazo-group is replaced by a halogen atom the important point to note the Sandmeyer reaction is that it is the halogen joined to the copper that enters the nucleus.

m.chloronitrobenzene



(68 - 71%)

p.chloronitrobenzene



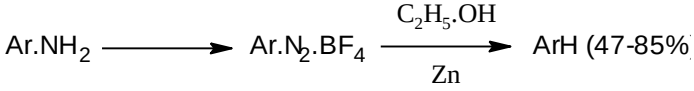
(70 - 73%)

The mechanism of the reaction is uncertain. Investigated the reaction kinetics of the Sandmeyer reaction and suggested that the mechanism is (a) slow co-ordination of the terminal N atom of $Ar.N_2^+$ to the copper in the $CuCl_2^-$ ion to form complex $Ar.N_2.CuCl_2$ (b) decomposition of this to ArCl. [20]

The reaction gives good yield and is not affected by the presence of some other substituents. [22]

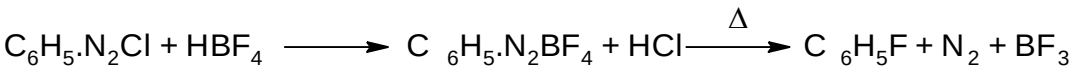
(II) Gatterman reaction

This reaction is carried out by dissolving the amine in hydrochloric acid, cooling, adding cooled aqueous sodium nitrite and then warming the diazonium salt solution in the presence of copper powder. [20] Made by reducing an aqueous solution of cupric sulpate with zinc dust. [21]



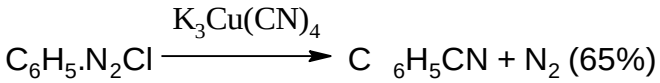
Iodo-compounds: in order to replace a diazonium group by an atoms “strong nucleophile” it is sufficient to warm the salt with aqueous potassium iodide solution.

Fluoro-compound may be prepared when borofluoric acid is added to a diazonium salt solution. The insoluble diazonium borofluride is precipitated. This is collected by filtration, dried and heated gently.



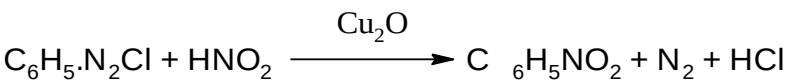
4] Replacement by a cyano-group

This is special case of Sandmeyer and Gattermann reaction and carried out by treating a diazonium salt solution with cuprous cyanide dissolved in aqueous potassium cyanide.

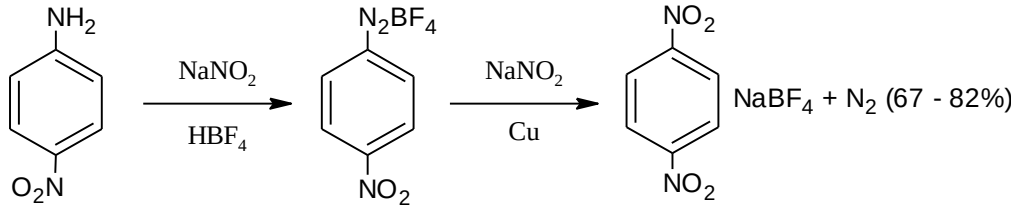


5] Replacement by a nitro-group

This was originally carried out by treating a diazonium salt solution with an equivalent amount of nitrous acid in presence of cuprous oxide.



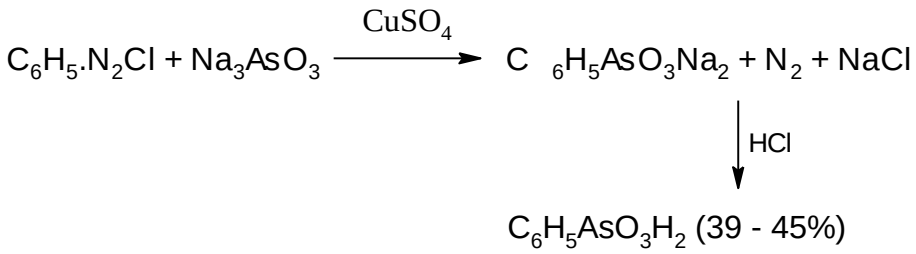
A better method is to decompose the diazonium borofluoride with aqueous sodium nitrite containing copper powder.



In certain cases the addition of the diazonium salt solution to sodium nitrite solution containing sodium hydrogen carbonate give good yield of nitro-compound, e.g o-nitro-aniline give o-dinitrobenzene (97 % yield).

6] Replacement by an arsenic acid group AsO_3H_2

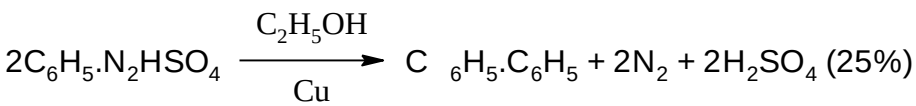
This is carried out by decomposition of a diazonium salt with sodium arsenite in the presence of a copper salt e.g phenyl arsenic acid.



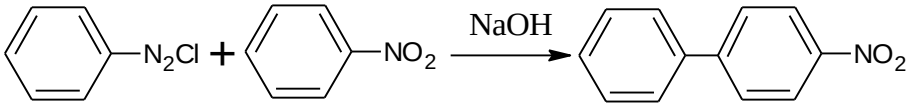
They yield may be increased by buffering $C_6H_5AsO_3H_2$ (39.45%) the solution with sodium carbonate.

7] Replacement by an aryl group

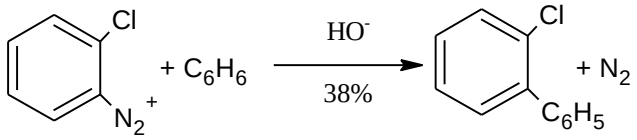
This may be carried out by treating a diazonium sulphate with ethanol and copper powder e.g diphenyl from benzene diazonium sulphate.



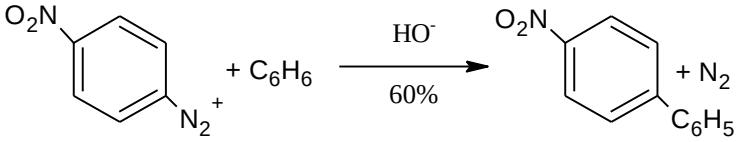
This method of preparation is known as the Gomberg reaction and experiment has shown tht whatever is the natural of substituent in the second component, o- or p-substitution always occurs, e.g benzene diazonium chloride forms p-nitrodiphenyl when treated with nitrobenznene. [20]



Diazonium salt containing a deactivating substituent give better result. [22]

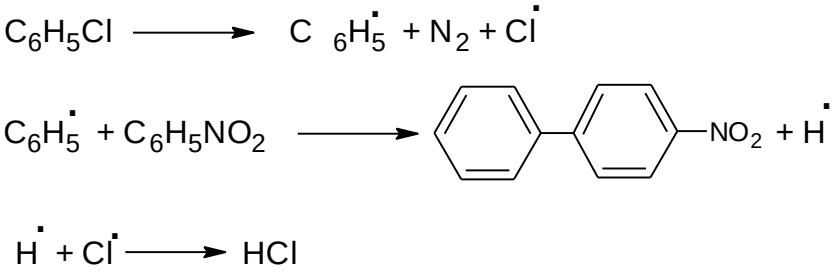


o-chlorodiphenyl



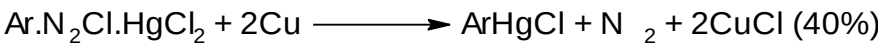
p-chlorodiphenyl

This anomalous orient effect of the nitro-group led to suggest that reaction take place by a free radical mechanism:



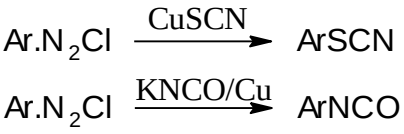
8] Replacement by a chloromercui-group

This is an example of indirect mercuration and may be carried out by heating the double compound of a diazonium choloride and mercuric chloride in acetone or ethanol solution with copper powder:



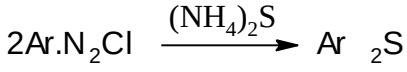
$$Ar.N_2Cl.HgCl_2 + 2Cu \longrightarrow ArHgCl + N_2 + 2CuCl\ (40\%)$$

The diazo-group may be replace by many other groups. [23]

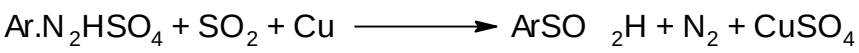


$$Ar.N_2Cl \xrightarrow{CuSCN} ArSCN$$

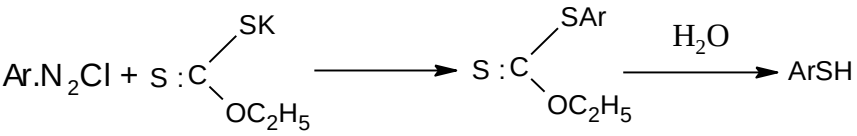
$$Ar.N_2Cl \xrightarrow{KNCO/Cu} ArNCO$$



$$2Ar.N_2Cl \xrightarrow{(NH_4)_2S} Ar_2S$$



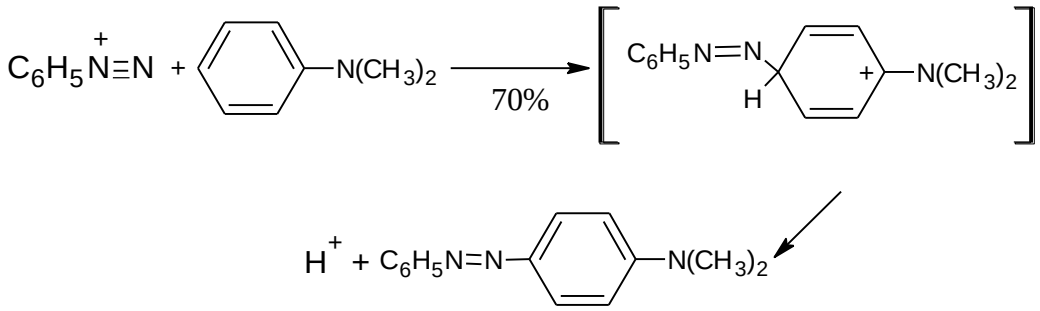
$$Ar.N_2HSO_4 + SO_2 + Cu \longrightarrow ArSO_2H + N_2 + CuSO_4$$



$$Ar.N_2Cl + S:C(SK)(OC_2H_5) \longrightarrow S:C(SAr)(OC_2H_5) \xrightarrow{H_2O} ArSH$$

2.4.5.2 Reaction of the diazonium salt in which the nitrogen atoms are retained (coupling reaction)

The most important reaction of this type is that in which the diazonium cation causes electrophilic substituent in another benzenoid ring. The diazonium cation is much weaker electrophilie than the nitronium ion and therefore attacks activated benzene ring only. For instance those of phenol or aniline to form an azo compound. [24]



p-dimethylaminoazobenzene

When the perbromide is treated with aqueous ammonia, the azide, ArN_3 is produced. Phenyl azide may also be prepared by the action of hydrozonic acid on nitrosobenzene.

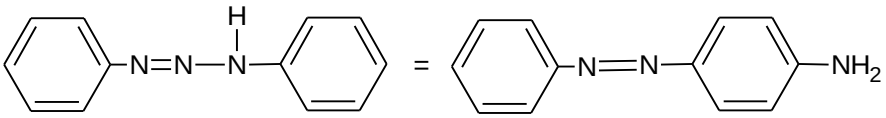
$$C_6H_5.NO + 2HN_3 \longrightarrow C_6H_5.N_3 + H_2O + 2N_2 \ (92\%)$$

When reduced with stannous chloride and hydrochloric acid, or with sodium sulphite, form phenyl hydrazines.

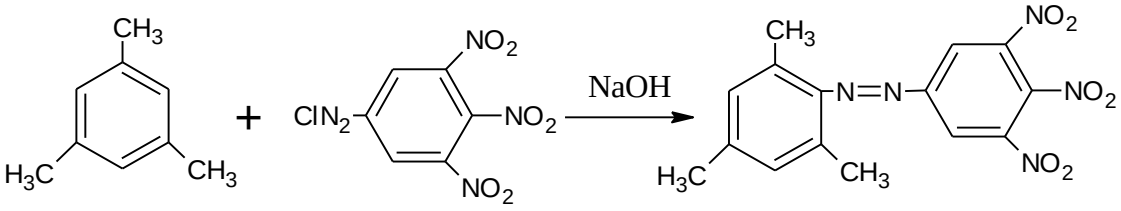
$$ArN_2Cl + 4[H] \xrightarrow{SnCl_2/HCl} ArNH.NH_2.HCl$$

Diazonium salt readily undergo coupling reaction. This is the reaction between a diazonium salt and another substance containing a labile hydrogen. The result being the formation of an azo compound with primary and secondry amines coupling may take place at the nitrogen atoms to form diazoamino-compound. [25]

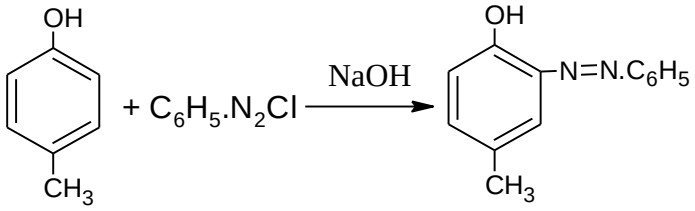
Diazo aminobenzene is a yellow crystalline solid, which is insoluble in water and slightly soluble in cold alcohol. If diazonium benzene is allowed to stand in contact with hydrochloric acid or with aniline hydrochloride. A change occurs which is apparently interamolecular and aminoazobenzene is formed. [26]



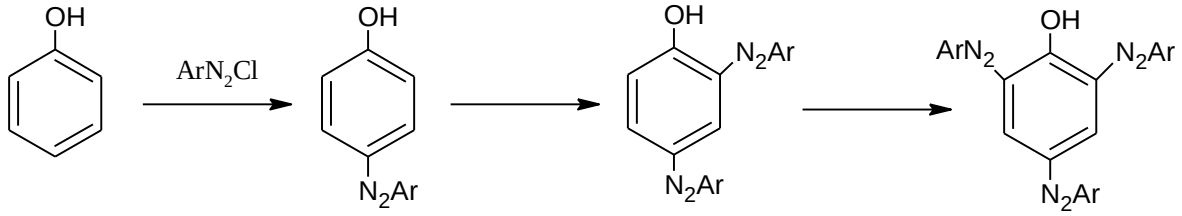
Simple diazonium salts do not couple with hydrocarbon but the nitro derivatives couple readily e.g diazotized pi cramide(s-trinitro aniline) couples with mesitylene.



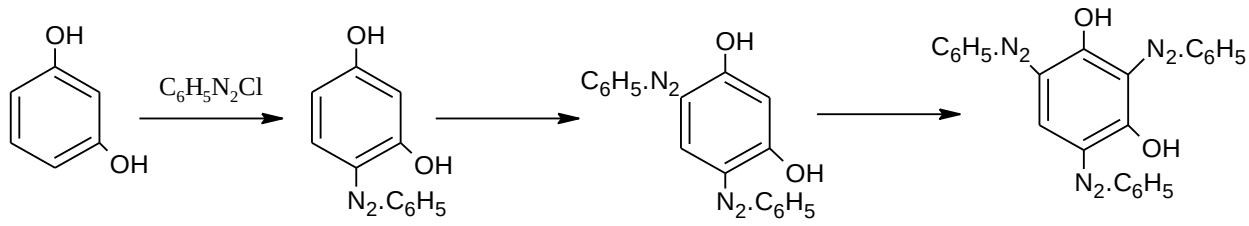
With phenol, coupling is best carried out in faintly alkaline solution. With amines in faintly acid solution, the azo-group enters mainly the p-posititon to the hydroxyl or amino-group, but if this positron is occupied, coupling takes place in the o-position. It is never occur the m-postiton e.g p-cresol give the o-azo-compound.



When an excess of diazonium salt is used, the bisazo-(o-and p-) and the triazo-compound may be formed.

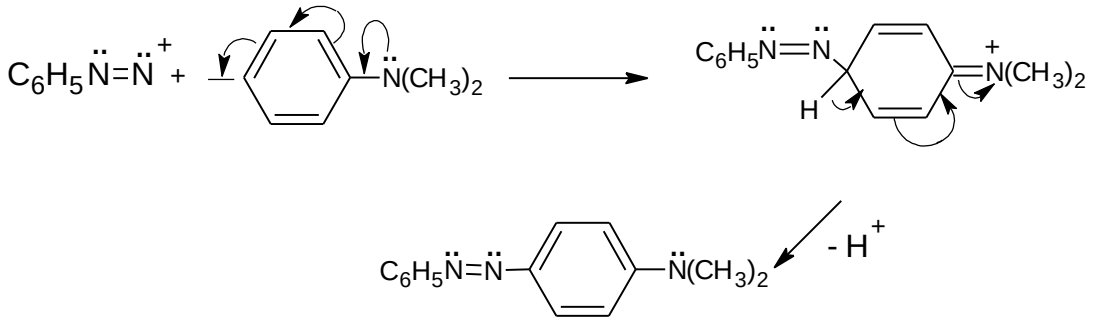


Experiment has shown that the introduction of a second azo-group is facilitated by the presence of an alkyl group in the p-position to the hydroxyl group or by two hydroxyl group in the m-position e.g resorcinol readily forms the trisazo-derivative.(27)

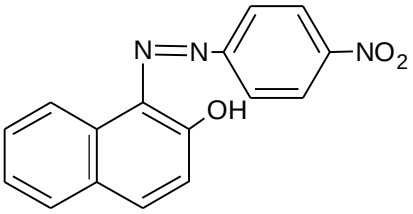


The introduction of second (and third) azo group takes place more slowly than the first and so it is possible to introduce two, or there different azo-groups.

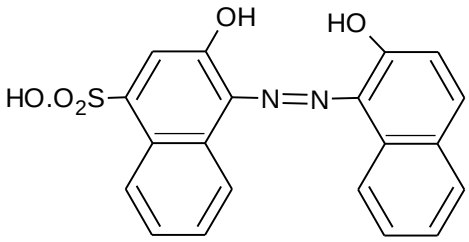
The mechanism of coupling is still a subject of discussion. Resent work indicates that coupling takes place by direct attack at the carbon atom. The active component in the coupling reaction being the diazonium cation and the free amine or the phenoxide ion. (28)



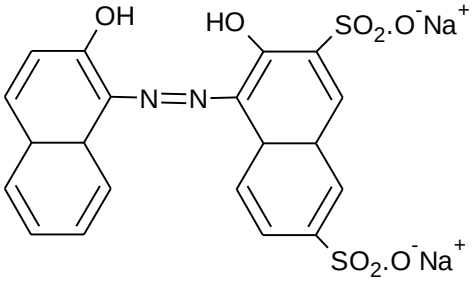
Azo compounds are highly coloured and their formation by the coupling reaction is an important one in the dyestuffs industry. The following are examples of azo dyes. (29)



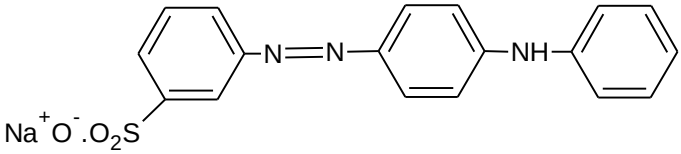
para red



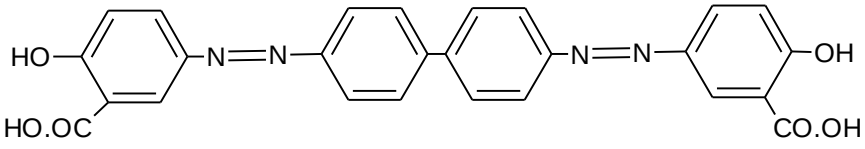
erichromic blue-black



fast red B



metanil yellow



chrysamine G

2.5 Qualitative and Quantitative analytical methods for the detection of counterfeit Acts

A broad panel of techniques has been reported for the analysis of Artemisinin derivatives, ranging from simple and cheap in-field ones (colorimetric and thin layer chromatography) to more advanced laboratory methods (mass spectrometry, nuclear magnetic resonance, and vibrational spectroscopies) through chromatographic methods, which remain the most widely used. Nowadays, the first step in detecting counterfeit drugs is to compare the physical appearance and text on packets, leaflet inserts, and blister packs (when present) of suspected samples with those of known genuine products. However, with increased counterfeiter sophistication, this careful visual inspection is not sufficient to distinguish between fake and authentic drugs. It must therefore be followed by chemical analysis, most often using high-performance liquid chromatography (HPLC), considered as the gold standard analytical method in drug analysis, but also with simple in-field assays [e.g., colorimetric test and thin-layer chromatography (TLC)] or more advanced laboratory techniques [e.g., mass spectrometry (MS), vibrational spectroscopies (Raman or IR), and nuclear magnetic resonance (NMR) spectroscopy]. Taken together, these analytical methods allow one to quantitatively determine the chemical composition of the drug [active pharmaceutical ingredients (APIs) as well as impurities and excipients] and hence to identify poor-quality medicines, which include not only counterfeit drugs but also substandard and degraded drugs.

2.5.1 Colorimetric method

Several colorimetric tests have been developed to determine the authenticity of Artemisinin derivatives. As Artemisinin derivatives do not have particular chemical groups that easily react with certain reagents to yield colored products,

they can be transformed by acid or base treatment to enolates/carboxylates or α,β-unsaturated decalones, which are more reactive compounds [30]. Thus, a diazonium salt, the commercial Fast Red TR (FRTR) salt, reacts with the alkali decomposition product of Artesunate, leading to the appearance of a distinctive yellow color at pH 4. The specificity of the test is pH-dependent. Indeed, at pH 6–8 not only ARTS but also other commonly used antimalarial drugs develop yellow (Artemisinin, Sulfadoxine) or orange (Primaquine) colors [31]. On the other hand, the FRTR salt added to the acid decomposition products of AM, DHA, or ARTS produces a yellow colour. A faint orange color appears in the same experimental conditions in the presence of paracetamol, whereas other common antimalarial (Artemisinin, Chloroquine, Sulfadoxine, etc.) appear colorless [32]. The method is thus specific to AM, ARTS, and DHA, but cannot distinguish selectively each of these drugs. The accurate quantitative analysis of the content of ARTS tablets after alkali decomposition and that of AM and ARTS tablets after acidic degradation can be accomplished, provided the yellow product is extracted into ethyl acetate and its absorbance measurement is performed at 420 nm with a spectrophotometer [32, 31]. The method is sensitive enough to require only 1% (0.5 mg) of ARTS tablets with the alkali decomposition process and 5% (2.5–5 mg) of ARTS, AM, and DHA tablets with the acidic decomposition process [32, 31].

2.5.2 Chromatographic methods

2.5.2.1 Thin-Layer Chromatography

TLC is a simple, sensitive or moderately sensitive, rapid, and inexpensive technique that is employed for the analysis of Artemisinin derivatives. Some publications deal with the specific quantitative determination of Artemisinin derivatives in different matrices, such as Artemisinin in *Artemisia annua* plant extracts [33], reconstituted mixtures of Artemisinin derivatives such as (Artemisinin,

AM, ARTS, and DHA) [34], or pharmaceutical formulations of ARTS [35] or AM [36] using normal- or reverse-phase TLC or high performance TLC. These assays can only be performed in specialized laboratories. Nevertheless, semi quantitative TLC determination using colored reaction tests is a potent technique to check the quality of AS and AM formulations in-field using the transportable Global Pharma Health Fund Minilab[38, 37]

Very recently, [39] developed two novel colour reaction assays utilizing TLC silica gel sheets and 2, 4-dinitrophenylhydrazine (DNP) or 4-benzoylamino-2, 5-dimethoxybenzenediazonium chloride hemi (zinc chloride) salt [Fast Blue RR salt (FBS)] as reagents, giving, respectively, a pink or a blue product in the presence of ARTS, AM or DHA (but not Artemisinin itself) clinically used in monoformulations as well as with ACTs. The identity of Artemisinin derivatives can be determined unambiguously from their characteristic retention factor after elution on the TLC sheet. When the colour reaction obtained with both DNP and FBS reagents is combined with the separation of the pharmaceutical components by migration on TLC plates, the other antimalarial drugs tested as well as a range of commonly used excipients and other drugs readily accessible and frequently used in malaria-endemic countries such as anti (retro) virals, antibiotics, and analgesics are not detected. The DNP and FBS tests need 2–5 mg of material and can detect as little as 10% of nominal ART content in ACTs. As for any colorimetric method, assays should be conducted using a positive control (the declared ART drug) and a negative control (the solvent used for sample extraction). The method enables the detection of counterfeit as well as substandard medicines since a semi-quantitative measure of amounts of ARTS in the formulations analyzed is given by the depth of the color compared with that of the authentic suitable ARTS at various concentrations.

The method is specific, simple to use, rapid, robust, reproducible, inexpensive, and requires no trained staff. Developed in a kit format, it could quickly become a powerful, widely used in-field tool to ensure quality of Artemisinin derivatives in ACTs. Compared with the FRTR test, the in-field method used nowadays [32, 31] these two novel reaction assays combined with TLC are not more sensitive but are much more specific. For example, AM, ARTS, and DHA cannot be discriminated in the FRTR test since they produce the same yellow colour after acidic decomposition [32]. Moreover, all the compounds colored yellow (as ACTs when co-formulated with other antimalarial such as Amodiaquine, lumefantrine, and Primaquine, etc) to orange before the reagent addition are not suitable for the FRTR test as they give false-positive results. When DNP and FBS reagents are used, only erythromycin among the 80 non-Artemisinin based drugs tested reacts in a similar way as Artemisinin derivatives, yielding a blue coloration with the FBS reagent, whereas 24 compounds treated with the FRTR reagent [32, 31] yielded yellow to orange as well as red, brown, or pink colors, which can hinder the detection of Artemisinin derivatives. It should be pointed out that the method of [39] is nevertheless specific as the second reagent (DNP) does not produce any colour with erythromycin.

2.5.2.2 High-Performance Liquid Chromatography

HPLC is the most popular instrumental technique used for the analysis of pharmaceuticals. It is regarded as the reference or gold standard method for the quantitative determination of pharmaceutical formulation contents, especially Artemisinin derivatives, and is used for validating alternative analytical methods. One of the advantages of HPLC is that many detectors can be coupled with it, such as electrochemical, evaporative light scattering (ELS), UV, photodiode array

(PDA), and MS detectors, which provides more possibilities for detecting different types of constituents.

Nowadays, HPLC-PDA detection (or HPLC-UV detection), HPLC-ELS, and HPLC-MS are the preferred HPLC techniques for the quantitative analysis of Artemisinin derivatives. Indeed, the maximum UV absorbance of Artemisinin derivatives occurs at low wavelength (192 nm for Artemisinin) and, even though it is weak, the absorption intensity is sufficiently high to allow the quantification of Artemisinin, AS, and DHA at concentrations of 10, 400, and 340 μg/mL, respectively, using a PDA detector set at 192 nm [40]. Moreover, the quasi-universal, versatile, low-cost, and very sensitive ELS detector allows the quantification of ARTs with an improved sensitivity compared with UV detection at 220 nm [39] but close to that at 192 nm [40]. Hence, HPLC-PDA detection and HPLC-ELS have proved to be sensitive (even though sensitivity is not generally a crucial issue for the analysis of pharmaceutical formulation ingredients), accurate, precise, and reproducible methods for the quantification of ARTs in a time of less than 10 min [41, 40]. HPLC coupled with the high sensitivity and selectivity of MS is the most powerful technique for the definitive structural identification of chemical entities present, even at low concentrations, in pharmaceutical formulations through detection of their major ions. Quadruple, ion trap, and time-of-flight (TOF) mass spectrometers using electrospray ionization (ESI) or atmospheric pressure chemical ionization with single ion monitoring or multiple reaction monitoring in tandem MS are currently employed for the determination of Artemisinin derivatives.

Chapter Three

Materials and Methods

3 Solvents and Chemicals

The following drug materials were procured; pure Artesunate powder (Shanghai International Trading Co. Ltd., Sudan), and authentic samples of Artesunate tablets are collected from Shanghai International Trading Co. Ltd.,- Sudan and Amipharma company- Sudan. All reagents were prepared with Distilled water and were of analytical reagent grade.

3.1 Instrumentation

The equipment's used are: Electronic balance (Metler Toledo, P31- Min 0.01 g) and UV-visible spectrophotometer (Model UNICO 2100). The HPLC (LC-2010HT) SHIMADZU consisted of a Hewlett Packard 1050 system, fitted with auto sampler, quaternary pump and variable wavelength detector. FTIR was tested by 8400S SHIMADZU and Samf: BUCK SCINTIFIC 500 infrared spectrometer of Equinox Companies.

3.2.1 Preparation of diazonium salt

18.8 m mol (1.75g) of pure aniline was dissolved in mixture of 2.5 ml of concentrated HCl and 10 ml water, the mixture was cooled below 5°C and diazotized by adding a solution of sodium nitrite 9.41 m mol (0.65g / 1.5ml water) with constant stirring, after 5 minutes[42], 2.625g of ARTS in 5 ml buffer solutions at pH 4, 6, 8 was added slowly and with stirring to the diazonium salts, A yellow precipitate was formed and filtered through Buchner funnel, washed with 25ml cold water and dried on a sheet of filter paper. The pH was adjusted to 4, 6, and 8

by the addition of 0.2 M boric acid, 0.2 M acetic acid, 0.2 M phosphoric acid solution. Water was added to maintain a consistent volume of 5 ml for each sample.

3.2.2 Quantitative determination of ARTS in genuine and tablets

One tablet of ARTS was transferred to 13×100 mm glass tubes. A standard curve was prepared with samples containing 0 (blank), 2.5, 50, 100 mg of analytical grade Artesunate. 5 ml buffer solutions at pH 4, 6, 8 was added, the tubes gently swirled and the samples were allowed to sit at room temperature for 20 min. Then 1.5 ml of diazonium alts was added and the tubes were gently swirled. Final pH of the solution was 6. After 5 min, a distinctive yellow color appeared in the genuine ARTS tablet samples. For quantitative analysis, the yellow reaction product was extracted from the water insoluble tablet excipients by adding 15 ml of ethyl acetate and transferred the mixture to 250l glass beaker, then the mixture was stirred see fig.4.1 to 4.3. After phase separation, the upper organic phase was transferred to 13×100 mm borosilicate glass tubes and the absorbance measured at 420 nm and 242nm. Absorbance measurements were 0 to the blank. Artesunate content for each tablet was determined from the standard curve.

3.2.3 UV Assay method for determination of ARTS in bulk and tablet

Two tablets were crushed into powder in a porcelain mortar with pestle. ARTS powder equivalent to 0.1g was weighed into 100 ml volumetric flask. A quantity of 1M sodium hydroxide was added to ARTS powder to 40 ml mark and allowed to stand for 20 minutes to hydrolyses the drug. The sample was filtrated with a sintered glass filter into 100 ml volumetric flask, 40 ml of 1.1M acetic acid was added to filtered solution. The resultant solution was allowed to stand for 5 minutes to form a yellowish solution. The amount of ARTS in the final solutions formed were assayed spectrophotometerically at 420 nm and 242 nm.

3.2.4 FTIR Assay method for determination of ARTS in bulk and tablet

0.008g of Artesunate was mixed with 0.15g KBr, then the IR spectra was obtained.

3.2.5 HPLC method for determination of ARTS in bulk and tablet

Twenty tablets were weighed and finely powdered. An accurately weighed powdered sample equivalent to 100 mg of Artesunate was transferred to 20 ml volumetric flask; 15 ml of mobile phase was added and the flask was ultrasonicated for 5 min. The volume was then made up to the mark with mobile phase and solutions was filtered through Whatman filter paper No.41. one ml of the filtrate was transferred to 10 ml volumetric flask,1 ml of standard stock solution was added and volume was made up to the mark using mobile phase to get final concentration of 500 µg/ml of Artesunate (500µg/ml of Artesunate as internal standard). After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the tablet sample solution was injected. Chromatogram was obtained and peak areas were recorded. The peak area ratio of Artesunate to the internal standard was calculated and the amount of Artesunate present in sample was estimated [(43)].

3.2.6 Thin layers Chromatography

The ARTS drop was spotted on pre-coated silica gel plates. The mobile phase used for separating of ARTS consisted of methanol, toluene, ethyl acetate and glacial acetic acid as mobile phase in ratio of 1:4.5:4:0.1 v/v [(44)]. spot was visualized by derivatization with an acidified 4-methoxybenzaldehyde in methanol-water.

Chapter Four

Results and Discussion

4 Introduction

The Artesunate compound do not have the particular chemical groups that easily react with certain reagents to yield colored products, however, they can be transformed acid or base treatment to more reactive compounds, i.e. enolate /carboxylates or α, β - unsaturated decalones . These compounds react readily with diazonium salts demonstrated that the alkali-decomposition product of Artesunate react with the diazonium salt, to produce a yellow product correlating well with Artesunate concentration

4.1 HPLC analysis of Artesunate raw material

The densitometry HPLC technique is quite simple, accurate, precise, reproducible, sensitive, and specific. HPLC method has been developed for quantification of Artesunate in combined tablet formulation. The validation procedure confirms that this is an appropriate method for their quantification in the plant material and formulation. It is also used in routine quality control of the raw materials as well as formulations containing any or all of these compounds.

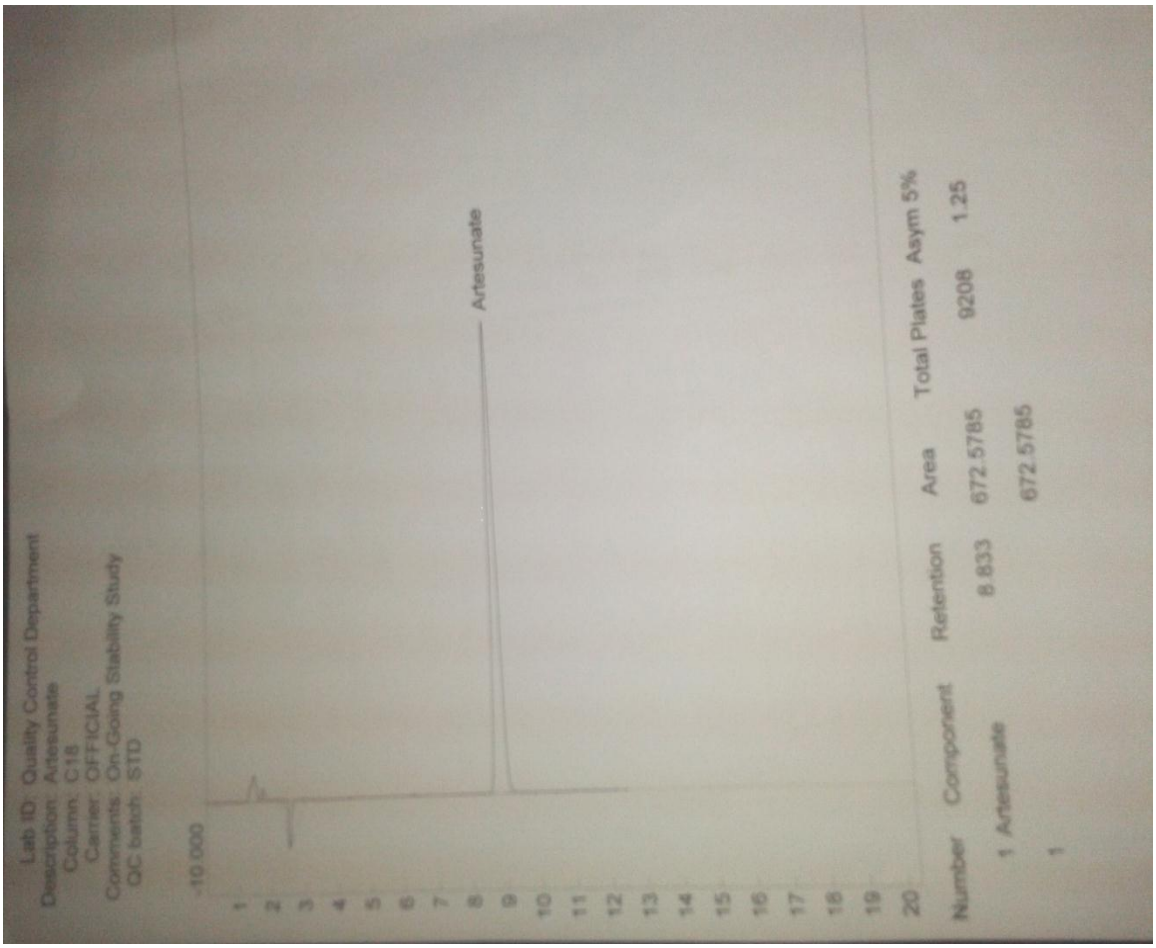


Figure 4.1 HPLC of Artesunate Raw material

Aim of the analysis was to develop a new selective high-performance liquid chromatography (HPLC) method for the quantification of amodiaquine and Artesunate in bulk and pharmaceutical dosage form. The HPLC analysis was performed on the LCGC Qualisil C8 (5 µm, 250 mm X 4.6 mm i.d.) column in isocratic mode, at 300°C temperature using a mobile phase consisting of Acetonitrile: phosphate buffer (70:30, *v/v*) at a flow rate of 0.8 ml/min. The detection was carried out at 221nm for Artesunate. Figure4.1 shows that the retention time of Artesunate raw material was 8.833 which was slightly higher than Artesunate raw material studied by (P.S.Jain, 2013) with value equal 5.6.[43]

4.2 TLC analysis of ARTS raw material

ARTS raw material was authenticated by using TLC. Figure4.2 shows that the Rf of ARTS in methanol, toluene, ethyl acetate and glacial acetic acid as solvent system equal 0.51, which was agree with standard value 0.57 [44].

Figure 4.2 TLC of ARTS Raw material

4.3 Colorimetric analysis of ARTS

Colorimetric analysis is a method of determining the concentration of a chemical element or chemical compound in a solution with the aid of a color reagent. It is applicable to both organic compounds and inorganic compounds and may be used with or without an enzymatic stage. The method is widely used in medical laboratories and for industrial purposes. The colorimetric analysis showed that was really composed or not, from figures 4.3, 4.4 and 4.5 the yellow colour indicated that the Artesunate was composed.

Figure 4.3 Amipharama tablet with diazonium solution

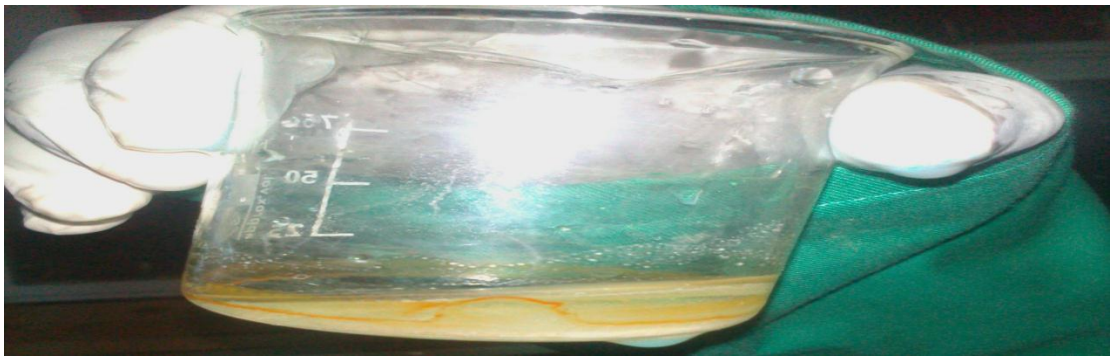


Figure 4.4 Shanghai tablet with diazonium solution

Figure 4.5 Artesunate raw material with diazonium solution

A qualitative or semi-quantitative assessment of the drug was determined visually, i.e. without the need for a spectrophotometer, by comparing the colour intensity of the sample to solutions of known drug content. The specificity and simplicity of this colorimetric method (ARTS-Benzene diazonium salt test) will certainly be useful in detecting counterfeit Artesunate.

4.4 Ultraviolet analysis of ARTS

Simple UV method has become necessary for the assay of this drug because, UV unlike HPLC is simple, rapid and readily available in malaria endemic areas of the world. This will also help to checkmate influx of fake and adulterated products into the drug market and reduce the burden of malaria [45]. In order to assay Artesunate by UV method, it is necessary to involve it in a reaction process that would break the endo peroxide ring and introduce a least one double bond in the molecule.

Table 4.1 UV of ARTS in different types and different wavelengths at pH 4

Artesunate type	Absorbance	Wavelength (nm)
Raw material	0.126	420
Raw material	0.146	242
Raw material + diazonium	0.457	420
Raw material + diazonium	0.294	242
Amipharama + diazonium	0.418	420
Shanghai + diazonium	0.417	420

Table 4.1 shows the UV-visible scan of the Artesunate raw material and in tablet samples before and after the coupling reaction with diazonium at pH 4, six samples were investigated under UV spectrophotometer, four samples were mixed

with diazonium salt, and two samples without diazonium salt in different wavelength at 242 nm and 420 nm. Table 4.1 shows showed that the absorbance values were increased when Artesunate mixed with diazonium (raw material at 242 nm = 0.294, raw material at 420 nm = 0.557, Amipharama tablet at 420 nm = 0.418, Shanghai tablet at 420 nm = 0.417) because colour increased the intensity which made absorbance increased in UV spectrophotometer. Artesunate pure has white colour, the absorbance values were (at 242 nm = 0.146, at 240 nm = 0.126).

The absorbance values of Artesunate samples with diazonium salt at wavelength 420 nm showed that the raw material has the higher absorbance value (0.457) more than Amipharama table (0.418) and Shanghai tablet (0.417) respectively which return to that the Artesunate tablets did not pure for their additive comparable to raw material.

The results obtained from the present study show that UV absorption of Artesunate could be employed for the assay of the drug especially in poorly equipped laboratories like those found in most developing countries. The proposed method is sensitive and reproducible.

4.5 Optimization of reaction variables Univariate method

A systematic study of the effect of pH and coupling reaction time on the development of yellow color products were determined.

4.5.1 effect of pH on coupling reaction

the effect of pH over the rang 4, 6, and 8 on the intensity of yellow product was estimated by measurement the absorbance of the colored reaction product at 420 nm, this yellow precipitant was extracted using ethyl acetate. Fig.4.6 shows that the absorbance value of the yellow extracted product was increased with increase pH of the medium reaction.

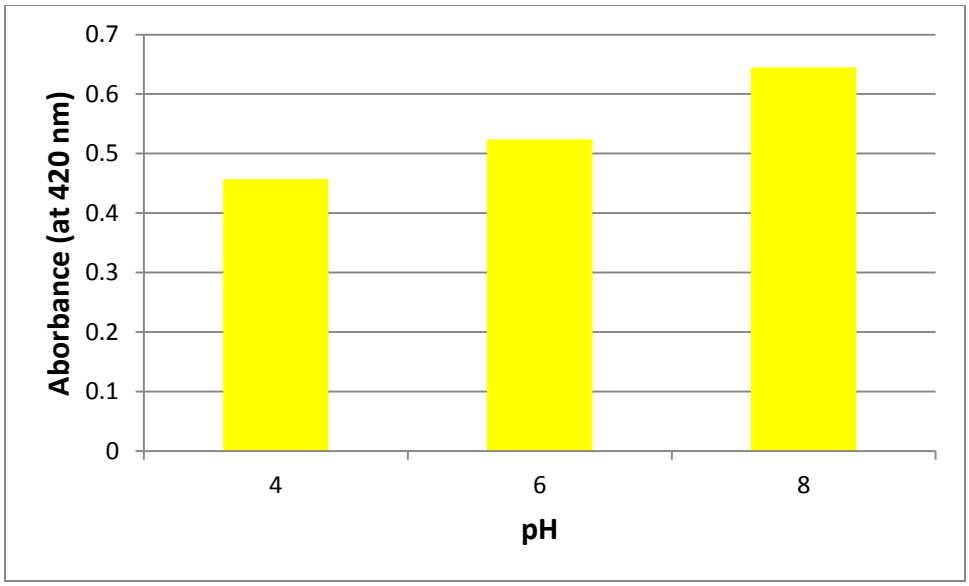


Figure 4.6 The Absorbance ARTS – diazonium salt versus pH

Under the same assay conditions, other antimalarial compounds were compared in order to evaluate assay specificity . At pH equal 6, Artemisinin develops a yellow color while Primaquine develops an intense orange color. When pH is increased to 8, sulfadoxine gives a strong yellow color. Although less intense at lower pH, Artesunate is the only drug from the compounds tested that develops a yellow color at pH 4.

4.5.2 Effect of time on coupling reaction

The optimum time of coupling reaction between diazonium salt and Artesunate in buffer solution at pH 4 was determined by recording the absorption of the reaction mixture at 420 nm in different time intervals. Table 4.2 and Figure3.7 show that 20 minutes was required for maximum color intensity development and the yellow color was stabled after 30 minutes.

Table 4.2 Effect of coupling reaction time.

Time/ min.	1	5	10	15	20	25	30	35	60
Absorption	0.130	0.234	0.356	0.402	0.457	0.455	0.454	0.454	0.454

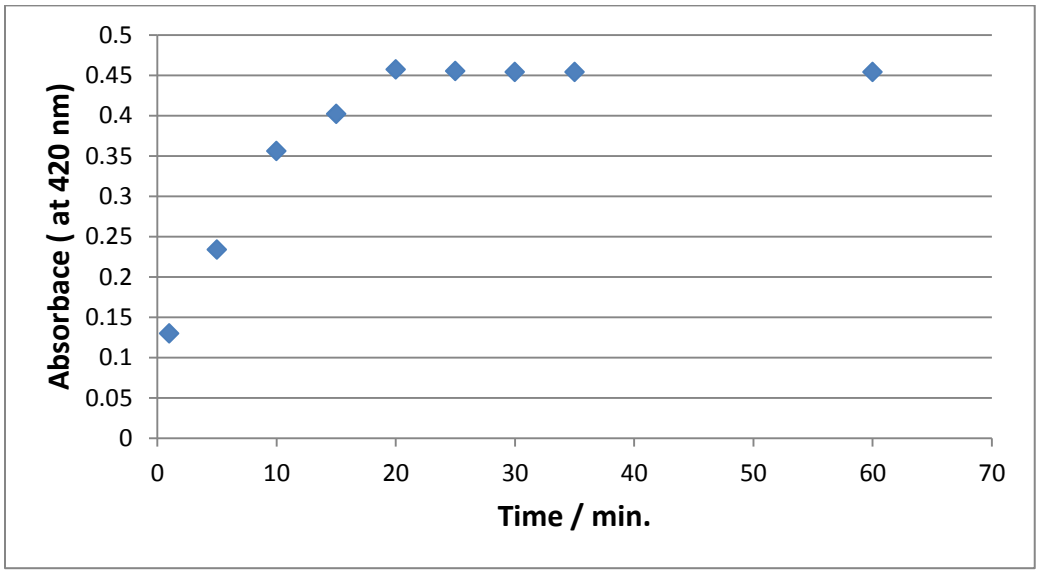


Figure 4.7 Absorbance versus Time / min of coupling reaction at pH 4

4.6 IR analysis of ARTS

The FTIR spectra of Artesunate presented here was analyzed by two different laborites (Amipharama and Samf) and the frequencies of the modes are fully consistent with earlier studies of Artesunate, and with the vibrational data reported for some 1, 2, 4 trioxanes by Jefford and co-workers Sohrabi [(46)].

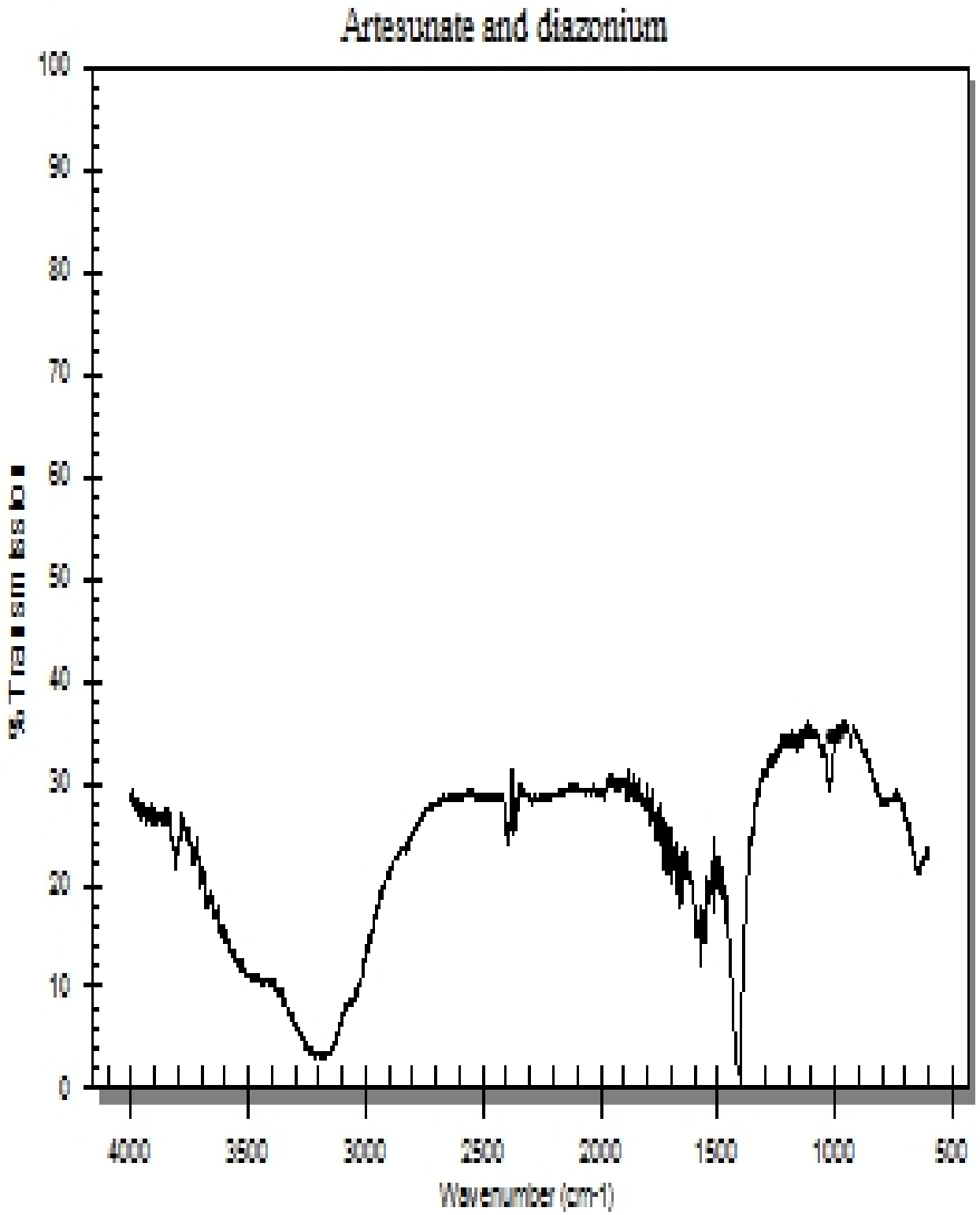


Figure 4.8 Artesunate raw material with diazonium

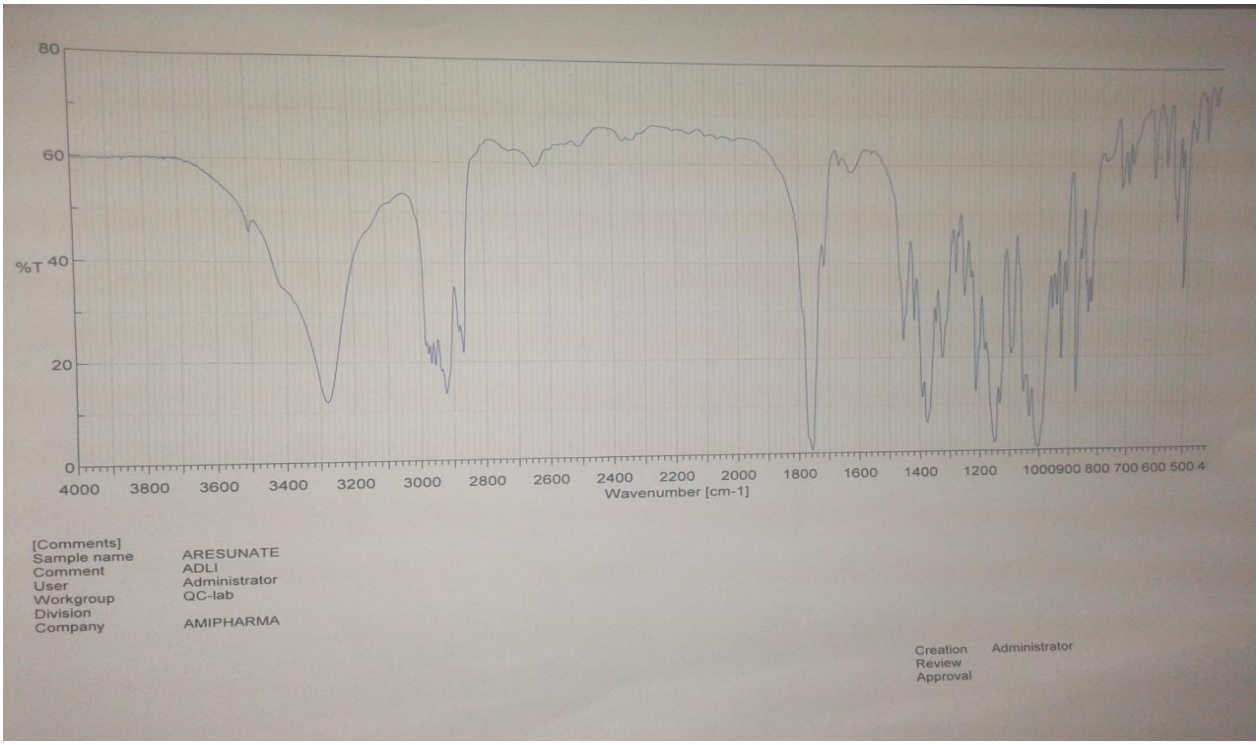


Figure 4.9 Artesunate raw material (Amipharama analysis)

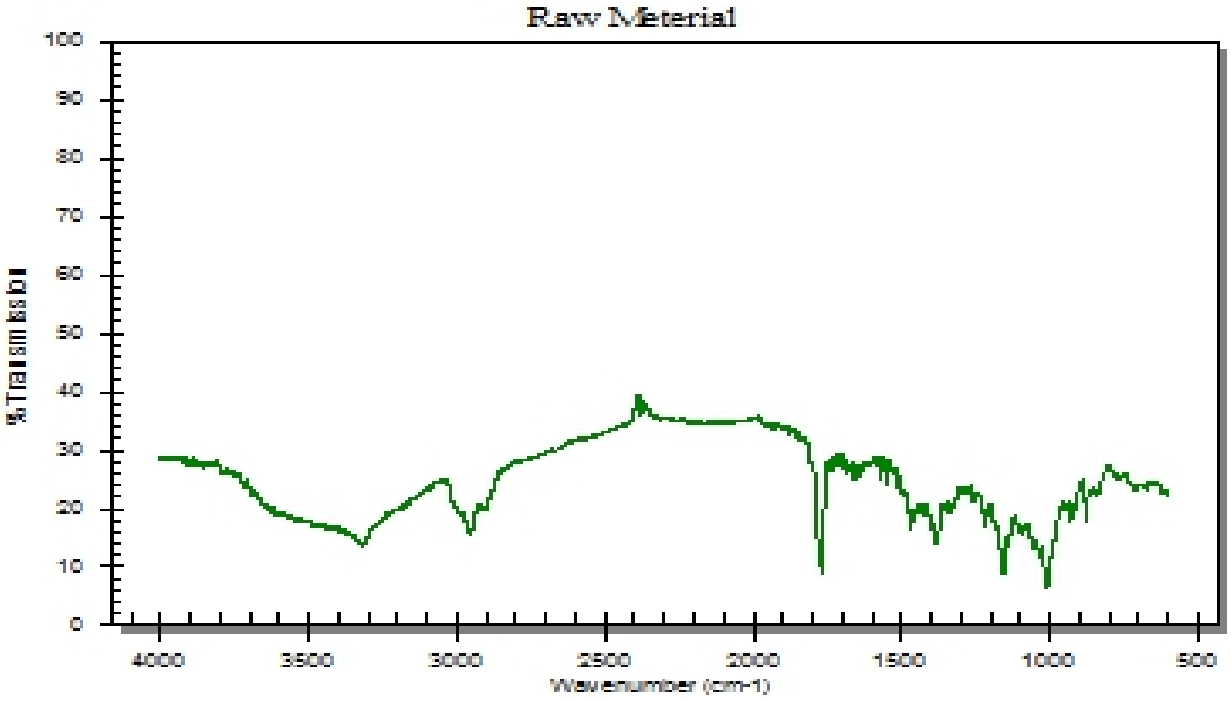


Figure 4.10 Artesunate raw material (Shanghai trading)

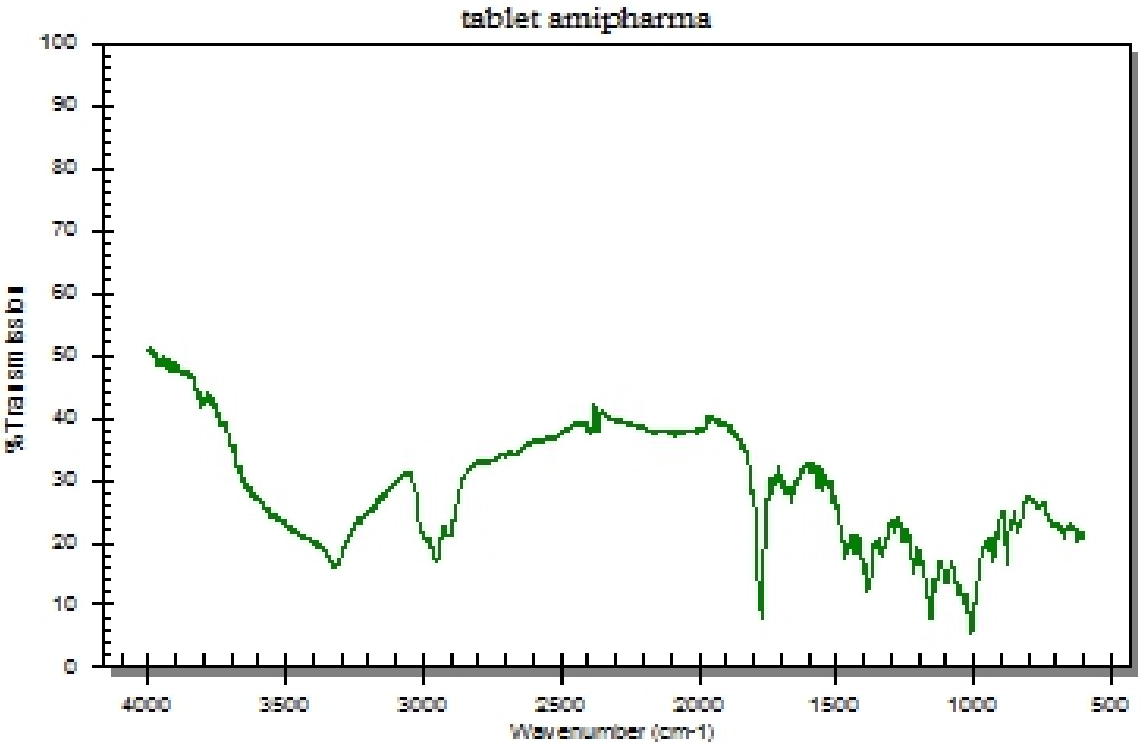


Figure 4.11 Amipharama Artesunate tablet

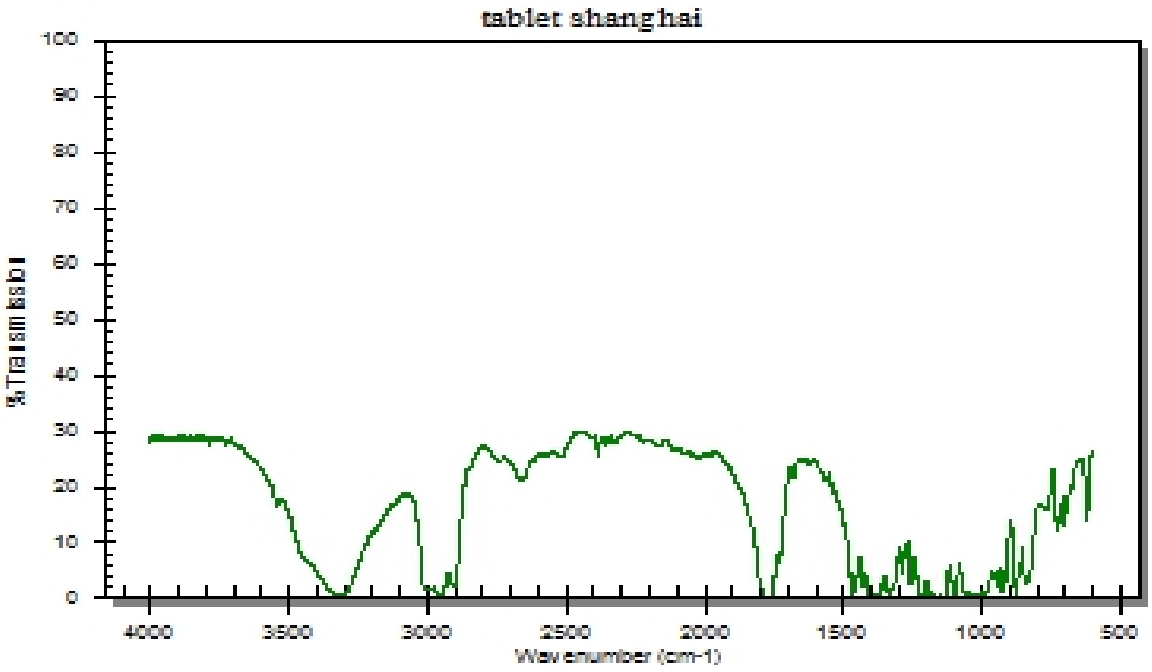


Figure 4.12 Shanghai Artesunate tablet

Figure 4.9 and 4.10 show that the FTIR spectra of pure Artesunate raw material obtained from Amipharama company and Shanghai trading respectively, the FTIR spectrum showed characteristic bands at 3279 cm–1 (O-H stretching vibrations), 2947 cm^{-1} (Fermi resonance of the symmetric CH_3 stretch with overtones of the methyl bending modes), 1093 cm^{-1} (C-O stretching), 890-820 cm^{-1} (O-O-C stretching in boat/twist form), 825 cm^{-1} (O-O stretching in boat/twist form) and 1420-1310 cm^{-1} (C=O). It indicates the properties of the O-O-C component, respectively representing the 1, 2, 4- trioxane ring [46]. It is of interest to note that; the spectra obtained from pure Artesunate presented in Figure 4.9 and 4.10 are rather similar, but some differences in resolution are observed, it return to the different types of FTIR (Amipharama: 8400S SHIMADZU, Samf: BUCK SCINTIFIC 500).

Figure 4.11 and 4.12 were showed Artesunate tablet (Amipharama and Shingahi) respectively. Their basic chemistry consists of C=O, C–O, C–H, CH_2, CH_3, C–O–O, C–O–C in both compounds and C–O–C=O, CH_2–CH_2 unique for Artesunate. Indication for the presence of these groups is supported by their finger print; the strong absorption band due to (C=O) vibration is around 1420-1300 cm^{-1}; and carbonyl C=O stretching occur at 1790 cm^{-1}; the C–H in-plane deformational vibration appear between 1225-1050 cm^{-1}; aromatic compounds contain delocalized π electrons from the resonance-stabilized double bonds, showing weak combination and overtone bands in the 2200 to 1620 cm^{-1}. The (C–O) vibration appears as a broad and strong bands at 1380-1370 cm^{-1}; followed by less intense band at around 1190 cm^{-1} and weak bands at around 700 cm^{-1} could be assigned to the CH_2 rocking vibrations [47 -49].

Figure 4.8 Pure Artesunate raw material mixed with diazonium salt, the spectra obtained showed similarity to pure Artesunate presented in Figure 4.9 and

4.10, except peak at 1555 cm^{-1} (C=C benzene ring) was appeared in Figure 4.9, The N=N stretching vibration of symmetrical trans azo compound is forbidden in the IR spectroscopy. Unsymmetrical azo- benzene (ARTS – N=N- Ar) absorbed at 1420 cm^{-1} was appeared in Figure 4.8, his band are weak because of the nonpolar nature of the bond.

Chapter Five

Conclusion and Recommendations

5 Conclusion

Diazotization reaction of primary amine group followed by coupling with Artesunate in pH 4 was found to be a simple, sensitive, accurate and economic spectrophotometric method for quantitative determination of Artesunate drug in pure form and synthetic samples. The color reaction takes place under a wide range of pH at room temperature. The classical univariate method such as effect of pH and azo coupling reaction time on development the yellow product have been used for optimizing the different variable affecting the completion of the reaction.

From the spectral analysis it can be concluded that the information obtained from FT-IR spectra of pure Artesunate in single or in combination with other drugs could be used to ascertain the presence of these drugs in formulations because no two functional groups have the same vibrational frequency and fingerprint.

5.1 Recommendations

Quantitative measurements are required for the verification of the purity of Artesunate in a combined formulation or in biological fluids.

This is necessary as the country is currently conducting researches to back up information on such an Artemisinin combined therapy as first line drug for the treatment of malaria.

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