ANALYSIS OF PYRONARIDINE AND PYRONARIDINE TETRAPHOSPHATE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

by

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LIST OF SYMBOLS

δ	Chemical shift
ΔG	Gibbs free activation energy
k_r	Exchange frequency/rotation frequency
T_c	Coalescence Temperature

LIST OF ABBREVIATIONS

$CDCl_3$	Deuterated chloroform
CD_2Cl_2	Deuterated dichloromethane
DMSO	Dimethyl sulphoxide
D_2O	Deuterated water
MeOD	Deuterated methanol
IR	Infrared
g	gram
Hz	Hertz
NMR	Nuclear Magnetic Resonance
MS	Mass Spectrometry
ppm	parts per million
s	singlet
PNDT	Pyronaridine tetraphosphate
PND	Pyronaridine
d	doublet
COSY	Correlation Spectroscopy
TOCSY	Total Correlation Spectroscopy

HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Coherence
NOESY	Nuclear Overhauser Effect Spectroscopy
DEPT	Distortionless enhancement by polarization transfer

ANALISIS PIRONARIDINA DAN PIRONARIDINA TETRAFOSFAT DENGAN MENGGUNAKAN KROMATOGRAFI CECAIR PRESTASI TINGGI DAN SPEKTROSKOPI RESONANS MAGNETIK NUKLEUS RESOLUSI TINGGI

ABSTRAK

Pironaridina (PND), 2-metoksi-7-kloro-10-[3',5'-bis-(pyrrolidinil-1-metil)-4'-hidroksianinilo]benzo[b]-1,5-naftiridina (formula molekul: $C_{29}H_{32}ClN_5O_2$) yang disintesiskan oleh Zheng et al pada awal 1970an telah digunakan sebagai ubat antimalaria selama 20 tahun. Ia berwarna merah bata, dengan rasa pahit dan tidak berbau dengan takat lebur pada suhu 174-176 °C. Bagi tujuan formulasi ubat, PND diubah kepada terbitan fosfatnya iaitu pironaridina tetrafosfat (PNDT) yang terlarut dalam air (formula molekul: $C_{29}H_{32}ClN_5O_2.4H_3PO_4$) dan mempunyai takat lebur pada 227-230 °C. Kajian yang dilakukan ke atas PNDT sebelum ini hanya menunjukkan satu puncak pada kromatogram apabila dianalisis dengan menggunakan kromatografi cecair prestasi tinggi (KCPT). Tetapi, dengan menggunakan kaedah terubahsuai Jayaraman yang dikembangkan oleh Karupiah (2003), tiga puncak diperhatikan pada kromatogram. Tiga puncak ini dilabel sebagai komponen B pada 10.0 min, X pada 21.2 min dan Y pada 24.3 min. Sebatian B diekstrak dan pencirian struktur sebatian tersebut dilakukan. Akan tetapi, untuk memastikan identiti komponen B, informasi struktur PND dan PNDT perlu ditentukan. Sehingga sekarang, data NMR yang wujud hanya daripada spektrum yang dirakam pada 2.11 Tesla (T) (90 MHz untuk ^1H).

Spektra ^1H dan ^{13}C pada 1-dimensi dan 2-dimensi dirakam pada 14.2 T (600 MHz bagi ^1H dan 151 MHz bagi ^{13}C). Spektra IR dan MS juga diperoleh untuk mengenalpasti struktur sebatian B. Keputusan yang diperoleh mencadangkan bahawa sebatian B adalah sebenarnya PND tetapi wujud pada konformasi yang berlainan. Dalam kajian ini, struktur PND dan PNDT telah ditentukan dengan menggunakan kaedah NMR yang berlainan seperti ^1H-^1H COSY, DEPT, HSQC, HMBC, TOCSY dan

NOESY dalam pelarut $CDCl_3$ dan DMSO. Spektra yang diperhatikan bagi PND adalah seperti yang dijangkakan mengikut struktur kimianya. Pada bahagian alifatik, empat puncak diperhatikan dan pada bahagian aromatik, tujuh puncak diperhatikan. Bagi PNDT, spektra NMR diperoleh dengan menggunakan kaedah yang sama tetapi dalam pelarut DMSO dan D_2O. Akan tetapi, enam puncak diperhatikan pada bahagian alifatik dan bukannya empat. Dua puncak berlebihan tersebut didapati terletak pada proton gelang pirolidina (H2"&H5" dan H3"&H4") dengan menggunakan kaedah HSQC. Kaedah NOESY juga digunakan intuk mengenal pasti interaksi tanpa ikatan antara proton. ΔG yang diperlukan untuk proton tersebut bergabung telah dihitung; bagi H2"&H5", ΔG ialah 15.03 kcal/mol dan bagi H3"&H4" ialah 15.33 kcal/mol. Nilai tersebut dihitung dengan kaedah eksperimen suhu terubah. Kehadiran dua puncak yang lebih dalam spektra PNDT adalah disebabkan oleh ikatan hidrogen antara PNDT pada posisi N-gelang pirolidina dan pelarut D_2O. Untuk membuktikan kesimpulan ini, kaedah pelarut tercampur digunakan dimana pelarut DMSO dan D_2O disediakan pada nisbah yang berbeza dan PNDT dilarutkan di dalamnya. Apabila nisbah DMSO yang ditambah dalam larutan mencukupi, maka dua puncak akan bergabung menjadi satu. Keadaan yang sama diperhatikan apabila dua pelarut lain iaitu aceton dan metanol digunakan. Eksperimen 'deuterium exchange' juga telah dilakukan dalam kajian ini bagi menentukan posisi proton bagi kumpulan hidroksi, OH dan amina, NH pada spektrum NMR bagi PND.

Kajian ini menunjukkan bahawa komponen B mempunyai struktur yang sama seperti PND tetapi pada konformasi yang berlainan. Maklumat struktur yang lengkap bagi PND dan PNDT telah diperoleh daripada kaijian ini dan dapat digunakan sebagai rujukan bagi kajian di masa hadapan.

ANALYSIS OF PYRONARIDINE AND PYRONARIDINE TETRAPHOSPHATE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

ABSTRACT

Pyronaridine (PND), 2-methoxy-7-chloro-10-[3',5'-bis-(pyrrolidinyl-1-methyl)-4'-hydroxyanilino]benzo[b]-1,5-napthyridines (molecular formula: $C_{29}H_{32}ClN_5O_2$), synthesized by Zheng et al. in the early 1970s, has been used in China for over 20 years as an antimalarial drug. It is an odourless, brick red powder with a bitter taste and decomposes at 174-176 °C. For drug formulation purposes, PND is converted to its water-soluble tetraphosphate derivative, PNDT (molecular formula: $C_{29}H_{32}ClN_5O_2.4H_3PO_4$) that has a melting point of 227-230 °C. All previous studies conducted on PND and PNDT using HPLC showed only one peak present in the chromatogram. However, the modified version of Jayaraman's HPLC method developed by Karupiah (2003) showed the presence of three peaks. These were labelled as component B at 10.0 min, X at 21.2 min and Y at 24.3 min. Component B was then extracted and characterized. However, in order to ascertain its identity, detailed structural information of both PND and PNDT must first be established. To date, the only NMR data available in literature on PND was deduced from a proton spectrum recorded at 2.11 Tesla (T) (90 MHz in ^1H).

1-Dimensional and 2-Dimensional ^1H and ^{13}C NMR spectra of both PND and PNDT were recorded at 14.2 T (600 MHz in ^1H and 151 MHz in ^{13}C) and 9.5 T (400 MHz) in this research. In addition, IR and MS were also performed to identify component B. The results suggest that component B maybe a different conformation of pyronaridine. In this research, the structure of PND and PNDT were obtained under different NMR techniques such ^1H-^1H COSY, DEPT, HSQC, HMBC, TOCSY and

NOESY in $CDCl_3$ and DMSO. The spectra correspond to the diastereotopic protons on the pyrrolidine ring with the aid of HSQC technique. Nonbonded interactions between the methylene protons at 1" and certain aromatic protons were established using NOESY technique. In addition, the free molar activation energy, ΔG required for the singlets corresponding to the diastereotopic protons to coalesce was calculated to be at 15.33 kcal/mol for H3" & H4" and 15.03 kcal/mol for H2" & H5" using variable temperature experiments. The presence of the two extra resonances is believed to be caused by the formation of hydrogen bond between D_2O and the N centre at the pyrrolidine ring. To confirm this deduction, mixed solvent experiments were employed. Different solvents namely DMSO, acetone and methanol were added individually to the PNDT sample in D_2O, and the two singlets corresponding to the diastereotopic protons were observed to coalesce to form only one peak. The same was observed in the acetone and methanol experiment respectively. This is believed to occur due to the disruption of the hydrogen bond between D_2O and the N centre. Deuterium exchange study was conducted on PND in DMSO to confirm the acidic OH and NH position in the NMR spectrum.

From this study it is concluded that component B is observed to have the same structure as PND and just conformationally different. Comprehensive structural data for PND and PNDT was established from this research and can be used as a reference to many other researchers who would be interested in studying this drug in the future.

CHAPTER ONE

INTRODUCTION

1.1 Malaria

Malaria, a protozoal disease caused by the parasite Plasmodia and spread by the Anopheles mosquito is a very old disease thought to originate in Africa. (www.rph.wa.gov.au/labs/haem/malaria/history.html). There are 500 million cases and nearly three million deaths annually, where most cases occur in sub-Saharan Africa (Beer *et al.*, 2006). The spread of malaria is usually associated with factors such as global changes including deforestation, mass migration due to war, mining and such. The main factor that contributes to the spread of this disease is the emergence of drug resistance in *P.falciparum* (Rosenthal, 1998; Taylor-Robinson, 2000). However, the actual cause of drug resistance is not known to be due to drug pressure and increase in infection reservoir (Lines *et al.*, 1991) or due to intrinsic parasite characteristics (Giha *et al.*, 2006). In the United States and Western Europe, most malaria infections are imported by travellers and due to the increase in globalisation of commerce, the number of travellers to areas of high risk is increasing each year (Wirth, 1999).

1.2 Status of Malaria in Malaysia

Malaria remains the most common vector-borne parasitic disease in Malaysia despite a decrease in the annual number of cases (Singh & Singh, 2001). The topography, the climate, and the migrations of the people due to increased economic activity are similar in peninsular Malaysia, Sabah, and Sarawak. However, the situation is different from area to area in terms of species of vectors, distribution of parasitic species, and resistance of *Plasmodium falciparum* to chloroquine. The problems faced by the suppression or control programs in the three regions are different (Rahman, 1982). In 1993, 39 890 cases were reported, 24.3 % in West (Peninsular) Malaysia and the remainder in East Malaysian states, particularly Sabah (73.0 %) where 65 % of the

infections were caused by *Plasmodium falciparum* (Hakim et al., 1996). Malaria cases in the early 1990's were around 50,000, with a steady decline to 6050, 5096 and 1770 for years 2001, 2002 and 2003, respectively (Vythilingam *et al.*, 2005)

Plasmodium falciparum is the most predominant species in Malaysia with *P.vivax* and *P.malariae* being the next widespread species (Singh & Singh, 2001). The distinction between the three geographically distinct regions: Peninsular Malaysia, Sabah and Sarawak, in the number of malarial cases and the distribution of Plasmodium species are depicted in Table 1.1 (Singh & Singh, 2001). In the study conducted by Vythilingam et al. (2005), where the impact of development and malaria control activities on the vectors in Sabah were studied, cases of malaria decreased at significant levels in three out of six of the test villages ($P < 0.05$) and no positive mosquitoes were obtained. Children were more prone to malaria as they have not developed immunity. It can be concluded that both integrated malaria control programmes coupled with the removal of habitat have acted to reduce malaria cases along with the primary vector *An. balabacensis* in Sabah.

The increase of malarial cases is linked to an increase in the incidence of drug resistant parasites. Chloroquine and sulphadoxine/pyrimethamine show widespread resistance in the endemic areas of West Malaysia where a slight increase in the overall resistance is observed for chloroquine (51 % to 64 %) and the increase for severe resistance was from 4 % to 55 % (Hakim *et al.*, 1996). Mefloquine is also used in combating multidrug resistant falciparum malaria in Malaysia and has been proven effective (Lim *et al.*, 1994), however in Thailand the development of resistance towards this antimalarial is observed (Hakim *et al.*, 1996).

Table 1.1: Distribution of the different human malaria species in Malaysia for 1995 and 1999 (Singh & Singh, 2001).

Species	Peninsular Malaysia[b]		Sabah[c]		Sarawak[d]		Malaysia	
	1995	**1999**	**1995**	**1999**	**1995**	**1999**	**1995**	**1999**
Plasmodium falciparum	3740	1712	30 999	3052	411	786	35 150	5550
Plasmodium vivax	3751	1712	15 858	1207	959	2031	20 568	4950
Plasmodium malariae	16	23	615	75	207	269	838	367
Plasmodium ovale	0	0	0	0	0	0	0	0
Mixed species	245	46	2393	124	14	69	2652	239
Total	**7752**	**3493**	**49 865**	**4458**	**1591**	**3155**	**59 208**	**11 106**

[a]Annual number of microscopy-confirmed cases of malaria for Peninsular Malaysia, Sabah, Sarawak and the total number for Malaysia.
bTaken from Vector-Borne Diseases Section, 1999 Annual Report, Division of Disease Control, Dept of Public Health, Ministry of Health
Malaysia.
[c]Taken from Sabah State Vector-Borne Diseases Control Programme 1999 Annual Report.
[d]Taken from Sarawak State Vector-Borne Diseases Control Programme 1999 Annual Report.

1.3 Malaria Pathology

1.3.1 Species

Malaria is caused by the protozoan parasite, *Plasmodium*, a member of the phylum Apicomplexan. The four species for the malarial parasite that infect man are:

i.) *P.falciparum*

ii.) *P.malariae*

iii.) *P.ovale*

iv.) *P. vivax*

P.vivax and *P.ovale* malaria invade immature young erythrocytes, whereas *P.malariae* infect mature cells and *P.falciparum* infects cells of all stages (Murray & Perkins, 1996). *P.falciparum* can cause death as a result of cerebral malaria, pulmonary or renal failure. *P.vivax* is also a common cause of human malaria, especially in South East Asia and in Central and South America. Patients who contract this form of malaria may become very ill with severe clinical symptoms. The other two forms of human malaria account for about 5-10 % of the cases of malaria. Both *P.vivax* and *P.ovale* are associated with relapsing malaria as both parasites are able to deposit a dormant form in the host's liver. This form is called the hypnozoites that may awake at anytime and produce merozoites that invade the blood (www.malariasite.com/malaria.html).

1.3.2 Life Cycle

Malaria infection is initiated by the bite of an infected Anopheline mosquito (Croft, 2000). Malaria parasites are transmitted to mosquito vectors inside the vertebrate host where the asexual-stage parasites change to gametocytes (Bannister & Mitchel, 2003). The sexual differentiation occurs during the asexual erythrocytic cycle and this then precedes to gametocyte formation (Smith, 2002). However, certain pre-erythrocytic merozoites that are derived from hepatic schizonts (Frevert, 2004) are

known to form gametocytes (Bannister & Mitchel, 2003). *P.falciparum* possesses one of the fastest DNA replication rates of all eukaryotic cells (Li & Cox, 2003). In *P.vivax* and *P.ovale* malaria the young blood cells are infected, in *P.malariae* the mature red cells are infected and in *P.falciparum* malaria cells of all stages are infected. In *P.falciparum*, the parasites multiply very rapidly and occupy 30 % or more of the red blood cells causing a very significant level of haemolysis (www.rph.wa.gov.au/labs/haem/malaria/history.html). Merozoites from erythrocytic schizonts develop into either all asexual-stage or all sexual-stage parasites. From this, each schizont produces either male or female gametocytes (Smith *et al.*, 2002). In *P.falciparum*, gametocytogenesis occurs over approximately eight days and passes through 5 morphologically different sub stages (Day *et al.*, 1998; Talman, 2004). The other plasmodium species undergo gametocyte development that is proportional to the duration of the sexual cycle, which is between one and three days (Day *et al.*, 1998).

It is during this sporozoites portion of the life cycle that genetic exchange can occur between different parasites. The obligate sexual exchange, which occurs at each transmission cycle, is thought to play a major role in the rapid spread of traits such as drug resistance throughout parasite populations (Yepuri, 2004). The asexual cycle takes about 48 hours for one cycle for all the species (72 hrs for *P.malariae*) where each merozoite divides into 8-32 (average 10) fresh merozoites (www.malariasite.com/malaria/Evolution.htm). Sexually committed merozoites escape host erythrocytes when the schizont ruptures and invade new erythrocytes where they grow into trophozoites which then transform into gametocytes (Bannister & Mitchel, 2003). All stages of development subsequent to the liver are observed in the peripheral blood for *P.vivax*, *P.ovale* and probably *P.malariae*. However, for *P.falciparum*, only ring forms and gametocytes are usually present in the peripheral blood where as the developing forms appear in the blood vessels of larger organs such as the brain, which restrict the blood flow (www.rph.wa.gov.au/labs/haem/malaria/history.html).

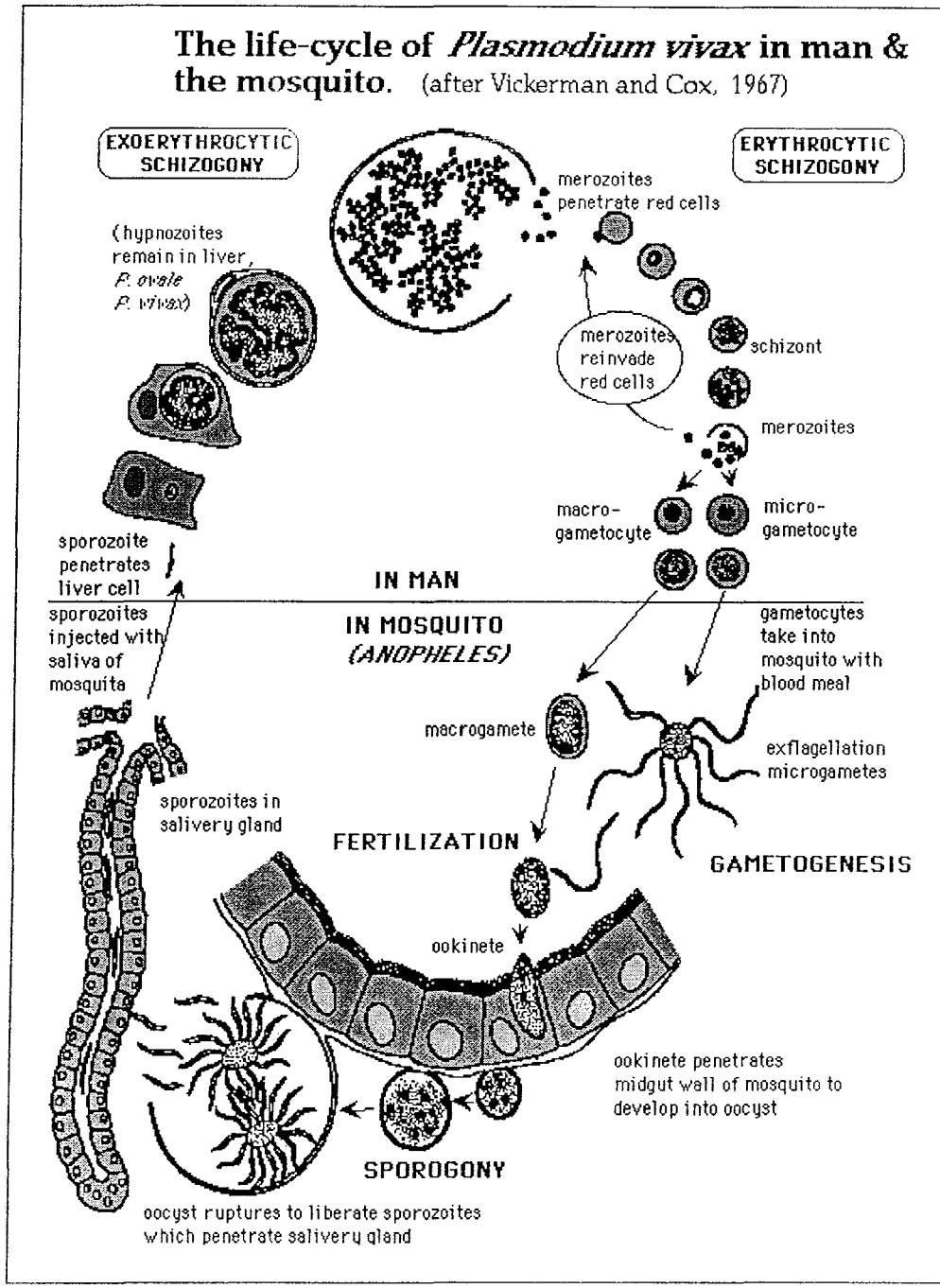


Figure 1.1: Schema of the Life Cycle of Malaria (http://www-micro.msb.le.ac.uk/224/Malaria.html)

1.4 Biological classification of antimalarial drugs

Antimalarial drugs have their own effects and modes of interaction according to the various stages of the parasites life cycle. Therefore, these drugs can be categorised according to these modes of actions on the specific stages of the malaria parasite life cycle namely:

i.) Tissue schizontocides

ii.) Hypnozoites

iii.) Schizontocides

iv.) Gametocides and

v.) Sporontocides

(Bruce-Chwatt, 1986; Kumar *et al.*, 2003)

Drugs that are classified as *Tissue schizontocides* for causal prophylaxis act on the primary tissue forms of the plasmodia. These tissue forms of the plasmodia are responsible for the initiation of the erythrocytic stage. Therefore, blocking of this stage allows prevention of further development of the infection. Pyrimethamine and primaquine are commonly used as tissue schizontocides. However since it is impossible to predict the infection before clinical symptoms begin, this mode of therapy is more theoretical than practical.

Drugs classified as *Tissue schizontocides* for preventing relapse act on the hypnozoites of *P.vivax* and *P.ovale* in the liver that cause relapse of symptoms on reactivation. Primaquine is the prototype drug and pyrimethamine also has such activity. *Blood schizontocides* drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in malarial chemotherapy and they include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulphadoxine and pyronaridine. Pyronaridine is the drug of interest in this thesis and will be discussed in detail in section 1.6.

Gametocytocide drugs destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P.malariae*, but not against *P. falciparum* whereas primaquine has gametocytocidal activity against all plasmodia, including *P. falciparum*. Drugs classified as *Sporontocides* prevent the development of oöcysts in the mosquito and thus ablate the transmission (http://www.malariasite.com/malaria/anti_malarial_drugs.htm). The antimalarial drugs that are most commonly used will be discussed in detail in the section below.

1.5 Antimalarial Drugs

This section discusses the various antimalarial drugs that are important and currently used in the prophylaxis and treatment of malaria infection.

1.5.1 Quinine

Quinine is one of the longest used drugs in the treatment of malaria. Its derivative is chloroquine and other analogues include amodiaquine, mefloquine and halofantrine. These drugs are blood schizontocides and their mechanism of action is through accumulation in the acidic food vacuoles of trophozoite-infected cells, thereby preventing haemoglobin production in the organelle. Quinoline inhibits the heme polymerase enzyme resulting in specific toxicity during the parasite development (Slater & Cerami, 1992).

Quinine is commonly used in areas where *P.falciparum* shows resistance to chloroquine and sulphadoxine/pyrimethamine combination (Wernsdorfer & Pyne, 1991). Quinine's monotheraphy has been associated with a failure rate of 40-70 % in South East Asia and Africa (White, 1992). Due to the increase in the degree of resistance towards this drug, it is usually given in combination with tetracycline for a

period of seven days. The cure rate observed for this drug is between 90 to 98 % (Looareesuwan *et al.*, 1996).

Quinine has antipyretic, antimalarial and analgesic properties and it is also a stereoisomer of quinidine and it has a bitter taste. The theorised mechanism of action for quinine is that it is toxic to the malaria parasite. It specifically interferes with the parasites ability to degrade and digest haemoglobin, thus starving the parasite. It would also cause the build-up of toxic levels of partially degraded haemoglobin in the parasite. Use of quinine in therapeutic doses may cause cinchonism (i.e. ringing in the ears, nausea, vomiting) when used in excessive doses or in rare cases it may even cause death by rapid pulmonary oedema (www.malariasite.com/malaria/quinine.htm).

1.5.2 Chloroquine

Chloroquine is a 9-aminoquinoline that has been known since 1934 and was synthesised as an antimalarial agent. However, due to its immunomodulatory properties, this drug has also been used in the treatment of autoimmune diseases such as rheumatoid arthritis (Savarino *et al.*, 2003). Chloroquine is widely used to treat all types of malarial infections. Its chemical formula is $C_{18}H_{26}ClN_3$ with a molecular weight of 391.877 g/mol. Chloroquine is also known as N(sup 4)-(7-chloro-4-quinolinyl)-N (sup 1), N (sup 1)-diethyl—1,4-pentadiamine. As an alkaline drug, it reaches high concentration within the food vacuoles of the parasite and raises its pH (www.malariasite.com/malaria/chloroquine.htm). Chloroquine interferes with the parasites haem degradative pathway and thereby it prevents detoxification of harmful products from the metabolism (Slater, 1992). *P.falciparum* is resistant to chloroquine in all endemic areas except Central America (Winstanley *et al.*, 2002).

1.5.3 Mefloquine

Mefloquine is a 4-aminoquinoline methanol structurally related to quinine (Cerami *et al.*, 1992). Mefloquine is an orally administered antimalarial and it is commonly known as mefloquine hydrochloride (formulated with HCl). Its chemical name is [2,8-bis(trifluoromethyl)quinolin-4-yl]-2(piperidyl)methanol. The chemical formula is $C_{17}H_{16}F_6N_2O$ with a molecular mass of 378.312 g/mol (http://en.wikipedia.org/wiki/Mefloquine). It is administered orally and has a long half life that results in the emergence of drug resistance (White, 1992).

Mefloquine is selectively active against the intraerythrocytic mature forms (trophozoite and schizonts) of malaria and has no activity against gametocytes (Palmer *et al.*, 1993). Mefloquine produces swelling of the *P.falciparum* food vacuoles. It may act by forming toxic complexes with free heme that damage membranes and interact with other plasmodial components. It is effective against the blood forms of *falciparum* malaria, including the chloroquine resistant types. Mefloquine is absorbed rapidly and is extensively bound to plasma proteins and it has an elimination half-life of 2-3 weeks (www.malariasite.com/malaria/mefloquine.htm).

Mefloquine is used for prophylaxis and chemotherapy of chloroquine-resistant *P.falciparum* malaria 48 hours after the parasite burden has been reduced by prior administration of an artemisinin antimalarial (White, 1999). Large trials conducted on mefloquine suggest that it is generally well tolerated. However, adverse effects have been reported such as diarrhoea, dysphoria, anorexia, vivid dreams, headache, changes in sleep, hallucinations and late vomiting (Albright *et al.*, 2002).

1.5.4 Halofantrine

Related to mefloquine is the other analogue of quinine, which is halofantrine, a phenanthrene methanol analogue that is an addition to the treatment of MDR *P.falciparum* (Dvorak *et al.*, 1975). Halofantrine is an effective drug for the treatment of malaria and is often recommended against infections with chloroquine or pyrimethamine resistant *P.falciparum* strains (Cosgriff *et al.*, 1982). Its chemicals name is 1-(1,3-dichloro-6-trifluoromethylphenanthyl)-3-*N*,*N*-dibutylaminopropan-1-ol (Kolade *et al.*, 2005).

Its mechanism of action may be similar to that of chloroquine, quinine and mefloquine that is by forming toxic complexes with ferritoporphyrin IX that damage the membrane of the parasite. The peak plasma concentration is achieved in 4 to 8 hours after the oral dose with elimination half-life of 1 to 3 days for the parent drug and 3 to 7 days for the active metabolite. It is used in the treatment of chloroquine resistant and multi-drug resistant, uncomplicated *P.falciparum* malaria (http://en.wikipedia.org/wiki/halofantrine.htm). Cardiotoxicity has become a major concern during treatment with this drug where lengthening of the QTc (Q and T wave correction in cardiac cycle) and severe cardiac arrhythmia have been observed (Touze *et al.*, 2002).

1.5.5 Amodiaquine

For the past 20 years, amodiaquine has been known to show higher cure rates even though there is cross-resistance between amodiaquine and its close congener, chloroquine (Winstanley *et al.*, 2002). Amodiaquine (AQ) is synthesized from 4,7-dichloroquinoline and 4-acetamido-diethylamino-o-cresol or an alternative synthesis from 2-aminomethyl-p-aminophenol and 4,7-dichloroquinoline. Its chemical name is 4-[7-dichloro-4-quinolyl)amino]-2-[(diethylamino)methyl]phenol dihydrochloride dihydrate and the structural formula is $C_{20}H_{22}ClN_3O,2HCl,2H_2O$. Amodiaquine hydrochloride is a

yellow crystalline powder that is odourless with a bitter taste and molecular weight of 464.8 g/mol. The decomposition point for AQ is at 150-160 °C (www.inchem.org/documents/pims/pharm/amodiaqn.htm). When used over a long period of time, amodiaquine has been observed to cause agranulocytosis and hepatitis. However, there is limited evidence as to whether amodiaquine is toxic when used for treatment in a 25/mg/kg dose over three days (Winstanley *et al.*, 2002).

1.5.6 Pyrimethamine/ Sulphadoxine

Antimetabolites are divided into two types, based on mode of action, and exert their activity on the folic acid cycle. Type one includes sulpha drugs such as sulphadoxine and type two include pyrimethamine and cycloguanil (Warhurst, 1986). Sulphadoxine-pyrimethamine is sometimes considered as a single drug rather than a combination, although they have different modes of action. Pyrimethamine inhibits the dihydrofolate reductase whereas sulphadoxine inhibits dihydropteroate synthase (DHPS). The inhibition of both these enzymes prevents the synthesis of folic acid in the parasite (Kremsner & Krishna, 2004). The resistance in parasites are increased when sulphadoxine is used as monotheraphy due to the mutations that occur in the gene coding for DHPS (Plowe, 2001). It was thus combined with pyrimethamine to inhibit folic acid formation. Sulphadoxine has an elimination half-life of 120 hours in children (Kremsner & Krishna, 2004).

1.5.7 Chloroguanide

Chloroguanide or more popularly known as proguanil was developed by the British antimalarial research in 1945. It is a biguanide derivative that is converted to an active metabolite called cycloguanil pamoate. It exerts its antimalarial action by inhibiting parasitic dihydrofolate reductase enzyme. It has prophylactic and suppressive activity against *P.falciparum* and can cure acute infection. It is effective in suppressing the clinical attacks of *vivax* malaria, however it is slower compared to 4-

aminoquinolines. Peak plasma levels are achieved within 5 hours and the elimination half-life is 16 to 20 hours (www.malariasite.com/malaria/chloroguanide.htm).

1.5.8 Artemisinin Derivatives

Antimalarial activity against both *P. falciparum* and *P. vivax* has been identified from the traditional Chinese plant *Artemisia annua* L. (Chawira *et al.*, 1987). Artemisinin is the active principle of the herb *artemisia annua*. The active antimalarial constituent of this compound was isolated in 1971 and named artemisinin. Artemisinin, a sesquiterpene lactone has a molecular formula of $C_{15}H_{22}O_5$ and molecular mass of 282.332 g/mol. Its chemical name is (3R, 5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one and has a melting point of 152-157 °C. The NMR structure of artesunate has been established (Ruscoe *et al.*, 1998; Robert *et al.*, 2002).

Structurally, artemisinin is unrelated to any other antimalarial and problems related to high rates of recurrence of the disease, its low solubility, short plasma life and poor oral bioavailability prompted the investigations into the discovery of other derivatives with better physiochemical properties (Lee & Huffard, 1990; Lin & Miller, 1995). The derivatives are artesunate, a water-soluble ester together with oil soluble artemether and arteether, all of which have *in vitro* and *in vivo* activity against erythrocytic forms (blood schizonts) of malaria (Barradell & Fitton, 1995; Lin & Miller, 1995). Artemether and artesunate have activity that decreases after one to two hours, however by giving artemisinin alongside lumefantrine or mefloquine or amodiaquine solves this drawback. Such a treatment is called ACT (artemisinin-based combination therapy) (www.malariasite.com/malaria/artemisinin.htm). The combination of artemisinin and mefloquine is more effective than artesunate monotheraphy as it decreases the rate of recurrence and improves the overall cure rate (90-100 %) (Kain, 1995).

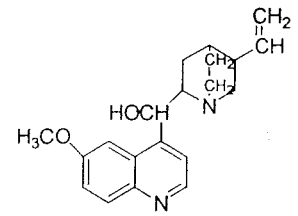
Artemisinin related compounds are concentrated in parasite-infected erythrocytes and the bridging endoperoxide group appears to be crucial for anti malarial activity (Barradell & Fitton, 1995). The antioxidant effect is critical for parasite survival, thus the anti-malarial activity of artemisinin compounds is mediated by increasing the levels of activated oxygen radicals (Barradell & Fitton, 1995; Meshnick, 1994).

1.5.9 Lumefantrine

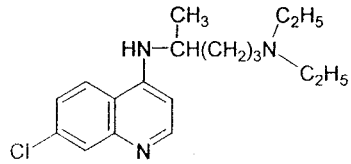
Lumefantrine was first synthesized in China and is now available commercially only in the co-formulated form with artemether (Annerberg *et al.*, 2005). It is a highly lipophilic compound and is bound to plasma proteins at more than 99.9 % (Colussi *et al.*, 1999) and has variable absorption (Ezzet *et al.*, 2000). It has proven to be well tolerated and has high efficacy in children and adults with multidrug resistant strains of *P.falciparum* (Annerberg *et al.*, 2005).

1.5.10 Primaquine

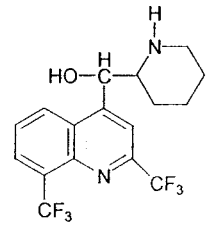
Primaquine is an 8-aminoquinoline antimalarial. Its chemical name is N-(6-methoxyquinolin-8-yl)pentane-1,4-diamine and the molecular structure is $C_{15}H_{21}N_3O$. Primaquine is active against various stages of the malaria parasite. It kills the latent and developing asexual stages in the liver and also sterilizes the gametocytes. A standard regimen of primaquine would clear the blood of asexual trophozoites of *P.vivax* (Pukrittayakamee *et al.*, 1994; Wilairatana *et al.*, 1999). However, the same is not observed for *P.falciparum* (Baird *et al.*, 2002). The mechanism of action of this antimalarial is unclear, however the various metabolites that are present in it are believed to disrupt the parasites mitochondrial membranes (Warhurst, 1984).



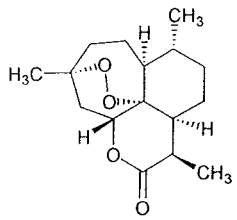
Quinine



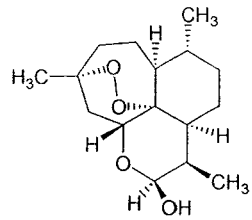
Chloroquine



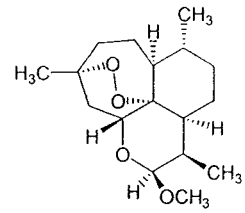
Mefloquine



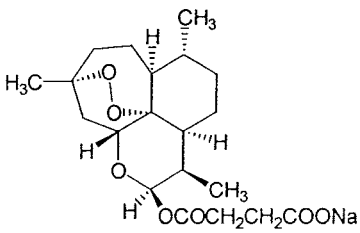
Artemisinin



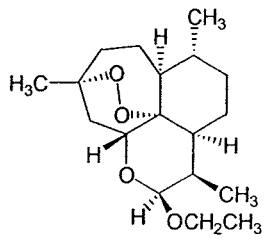
Dihydroartemisinin



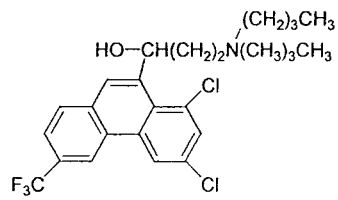
Artemether



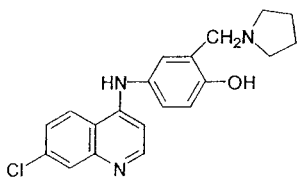
Artesunate



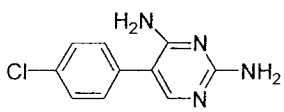
Arteether



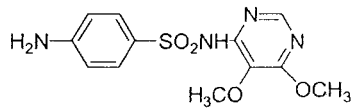
Halofantrine



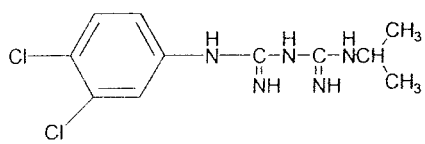
Amodiaquine



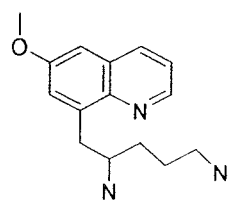
Pyrimethamine



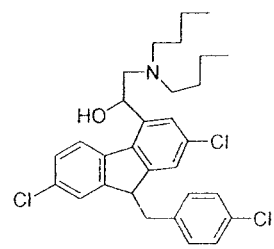
Sulphadoxine



Chloroguanide



Primaquine



Lumefantrine

Figure 1.2: Structure of main antimalarial drugs

1.6 Pyronaridine Tetraphosphate (PNDT)

1.6.1 Introduction

Pyronaridine tetraphosphate, 2-methoxy-7-chloro-10-[3',5'-bis-(pyrrolidinyl-1-methyl)-4'-hydroxyanilino]benzo[b]-1,5-napthyridines, an anti-malarial drug synthesized by Zheng et al. in the early 1970s, has been used in China for over 20 years (Zheng *et al.*, 1979; Feng *et al.*,1987). It has been reported to be effective against not only chloroquine-resistant but also multi drug resistant *P.falciparum* without major side effects (Looareesuwan *et al.*, 1996; Ringwald, 1996).

A review of novel and therapeutically used compounds revealed that many anti-malarial agents contain a 4-aminophenol group, which may influence their toxicological profile. These compounds, analogues of amodiaquine, can be classed as Mannich anti-malarial agents due to the incorporation of an amine group in their side chains. Pyronaridine tetraphosphate (PNDT), also known as a bis-Mannich anti-malarial substituted at the C-5' with a pyrrolidine group and an acridine rather than a quinoline nucleus has already been used effectively in the treatment of resistant strains of *P.falciparum* both *in vitro* (Ruscoe *et al.*, 1998) and in the clinic (Peters & Robinson, 1992).

Mannich base anti-malarial drugs are broadly classified into two groups: quinoline-type, e.g., amodiaquine and amopyroquine, and acridine-type, e.g., pyronaridine and pyracrine. Despite common chemical features, they differ substantially from chloroquine and amongst themselves in their activities, rate and stability of resistance (Shao, 1990). The presence of the hydroxyanilino moiety would cause PNDT to share toxicity problems with its congener, amodiaquine. This group has been implicated in agranulocytosis associated with the prophylactic use of the drug, but not reported for treatment (Winstanley, 1996).

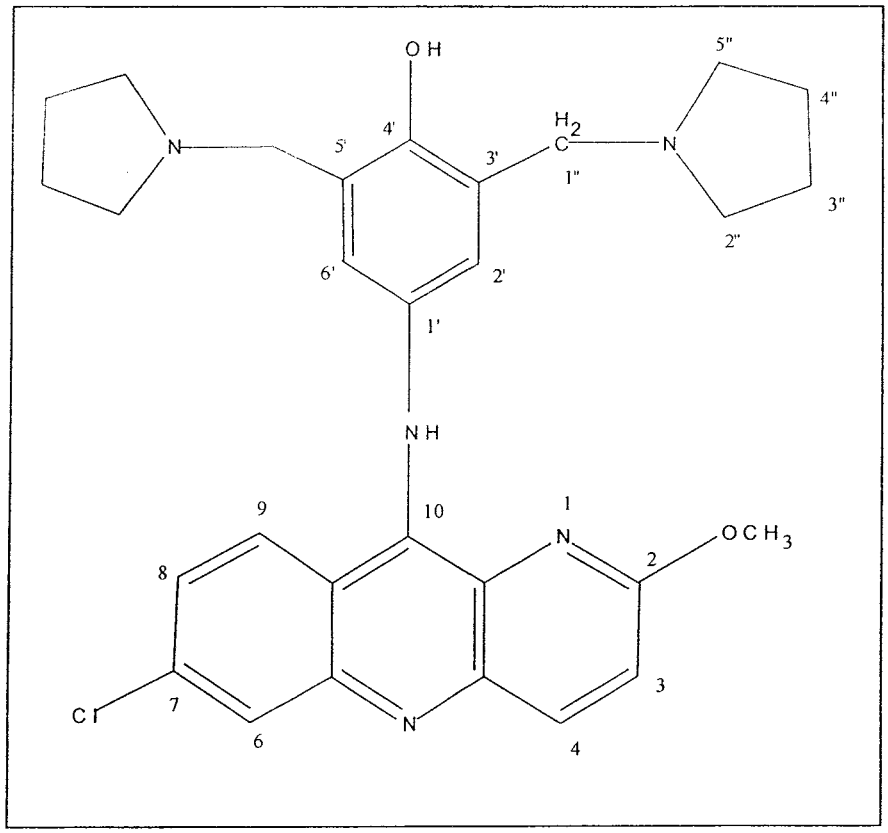


Figure 1.3: Pyronaridine (2-methoxy-7-chloro-10-[3',5'-bis-(pyrrolidinyl-1-methyl)-4'-hydroxyanilino]benzo[b]-1,5-napthyridines)

1.6.2 Chemistry of Pyronaridine (PND)

Pyronaridine (PND) is the product of a nine-step synthesis from 2-amino pyridine. The last synthetic step is a Mannich reaction to add two pyrrolidinyl-1-methyl substituents. PND is then treated with phosphoric acid to give PNDT, of molecular weight 910 (content in PND base = 57 %). An abbreviated synthesis from commercially available 2-methoxy-5-aminopyridine is also possible (Chen *et al.*, 1992).

PNDT occurs as a hygroscopic yellow powder, odourless, with a bitter taste. It is soluble in water, very sparingly soluble in ethanol, and insoluble in chloroform, ether, and other organic solvents. PNDT has been used experimentally and in clinical treatment of malaria. All dosages of PNDT have been calculated as the free base (PND). The drug has been produced as tablets and injectable liquid and it can be administered by the oral, intramuscular routes and intravenous drips (Olliaro, 2000).

Ruscoe *et al.* (1998), have studied the effects of chemical modification on the disposition of Mannich antimalarial agents in their pharmacology and toxicology. It was evident from the study that increases in the lipophilicity and basicity by the C-5' position of amodiaquine had a profound effect on the ability to accumulate and reside within tissues. The increase in the tissue residency time of C-5' substituted compounds increases the duration of their pharmacological activity through maintenance of therapeutically active concentration in plasma.

1.6.3 Synthesis of PND

The schematic representation for the synthesis of PND is shown in Figure 1.4. Pyronaridine is synthesised from 2-aminopyridine. 2-aminopyridine (1) is reacted by mononitration at position 5 to obtain 2-amino-5-nitropyridine (2). 2-hydroxy-5-nitropyridine (3) was synthesized by hydrolysis of (2). Chlorination of (3) would yield 2-chloro-5-nitropyridine (4). Compound (4) could also be obtained by diazotisation of (2)

followed by treatment with C_2Cl_2. Compound (4) can also be produced from pyridine (5) by oxidation to form N-methylpyridine-2-one (6), which was subsequently nitrated and pursued by chlorination to derive (7).

Compound (4) was then reacted with sodium methoxide to obtain 2-methoxy compound (8), which was reduced to give 5-amino compound (9). Condensation with 2,4-dichlorobenzoic acid was performed to produce 2-methoxy-5-(2'-carboxy-5'-chlorophenyl)aminopyridine (10). 2-methoxy-7,10-dichlorobenzo(b)1,5-napthyridine (11) was formed after cyclisation of (10), (11) was followed by condensation with p-aminophenol to derive 2-methoxy-7-chloro-10(4'-hydroxyphenyl)aminobenzo-(b)1,5-naphthyridine(12). The latter was reacted with Mannich reagent to produce pyronaridine (13), melting point at 174-176 °C (decomposition). This was then reacted with phosphoric acid to yield pyronaridine tetraphosphate, melting point at 233-236 °C (decomposition) (Chen *et al.*, 1992).

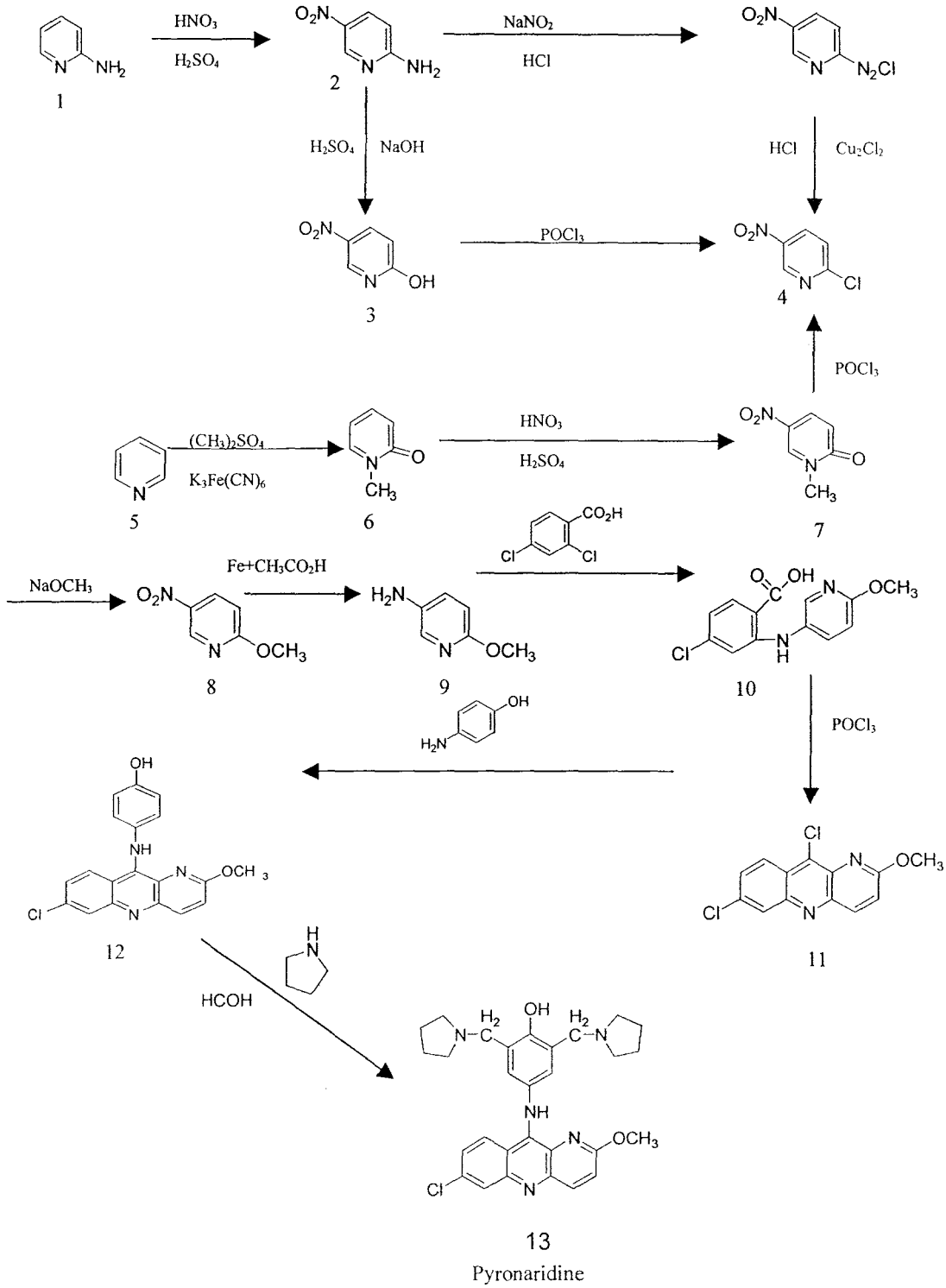


Figure 1.4: Schematic diagram of synthesis of PND and its simplified structural formula (Chen et al., 1992).

1.6.4 Analysis of PND

Wages *et al.* (1990), described a high performance liquid chromatographic method with oxidative electrochemical detection using an analogue of amodiaquine as an internal standard to quantify pyronaridine in rhesus monkey blood and urine samples. This method would be applied in support of clinical and pharmacokinetic studies to evaluate the prospects of using this drug outside of China. The chromatograms from the study showed only one peak corresponding to pyronaridine tetraphosphate in both the blood and urine samples.

Saleh and Loh, (1993), developed a reversed-phase liquid chromatographic method for the determination of the antimalarial drug pyronaridine in plasma. A UV detector was used in this study. Pyronaridine, which is basic, was shown to be very dependent on the hydrogen ion concentration for extraction or separation by reversed-phase LC. The method developed was suitable for the analysis of human, rat and monkey plasma and whole blood samples. The chromatograms produced only one peak corresponding to pyronaridine.

Jayaraman *et al.* (1997), described a method for the determination of pyronaridine in human plasma using high performance liquid chromatography with fluorescence detection. It involved the isolation of pyronaridine from plasma samples by liquid-liquid extraction followed by HPLC separation and determination. A typical chromatogram of pyronaridine standard and quinine internal standard showed one peak corresponding to each compound. The same was observed in spiked plasma samples. The assay procedure was adequately sensitive to measure 10 ng/ml pyronaridine in plasma samples with acceptable precision (<15 %) and found to be suitable for use in clinical pharmacological studies.

Chen and Fleckenstein (2001), conducted an improved assay method using HPLC for the determination of PNDT in plasma and whole blood. The internal standard used in this study was amodiaquine owing to its similar structure to PNDT, where good recovery from plasma (88.7 %) and from blood (85.4 %) and larger retention time compared to PNDT was observed. Amodiaquine was considered as less likely to interfere with identification of more water-soluble metabolites of PNDT in future pharmacokinetic studies. One peak corresponding to pyronaridine tetraphosphate was detected in the standard, in the plasma spiked with PNDT and blood sample spiked with PNDT. The method proved to be sensitive, selective and specific.

Babalola *et al.* (2003), developed a method for the determination of pyronaridine in human plasma and oral dosage form. Reversed phase HPLC with UV detection at 278 nm was described involving liquid-liquid extraction of the drug followed by basification of the deproteinized plasma with alkaline phosphate buffer. Preliminary pharmacokinetic data demonstrate the principal applicability of the method for clinical trials of the drug, especially in malaria endemic regions such as Africa. The assay could also be applied for the analysis of pyronaridine formulations without extraction process.

A new approach using solid-phase extraction technique was developed for the determination of pyronaridine and the analysis was performed using reverse phase chromatographic method with fluorescence detection. Three chromatographic peaks were resolved after injection of PNDT solution and the same was observed for the capsule formulation. These unknown peaks were labelled as 1,2 and 3. Compound 3 was successfully extracted using column chromatography and characterized using mass spectrometry and NMR to be PND. This method was found to be suitable for use in clinical pharmacokinetic studies (Ramanathan *et al.*, 2005).

Table 1.2: Summary of methods of analysis for Pyronaridine tetraphosphate

Researcher (Year)	Equipment	Extraction Solvent	Column and Mobile Phase	Limit of Detection
Feng & Wang (1986)	Spectrofluorometry, λex = 394 nm and λem = 458 nm	n-Heptane:Isoamylalcohol (98:2, v/v)	-	10 ng/ml
Wages et al., (1990)	HPLC –electrochemical detector	Ethyl acetate	Polymer PRP-1, 10 μm MeOH: H_2O (34:66,v/v), PH 2.8 with 1% TEA	20 ng/ml
Saleh & Loh, (1993)	HPLC – UV at λ = 278 nm	Diethyl ether	Partisil –10, ODS C_{18}, 10 μm, Acetonitrile: Phosphate buffer 0.08M (15:85, v/v), pH 2.0 with 1% TEA	70 ng/ml
Jayaraman et al., (1996)	HPLC – Fluorescence, λex = 267 nm and λem = 443 nm	Diethyl ether: hexane (70:30, v/v)	Nucleosil, C_{18}, 10 μm, Acetonitrile: Phosphate buffer 0.05 M (60:40,v/v), pH 6.0	10 ng/ml
Chen & Fleckenstein (2001)	HPLC – UV at λ = 275 nm	Diethyl ether	Zorbax Bonus, diisopropyl-C_{14}, Acetonitrile: Phosphate buffer 0.08M (13:87, v/v), pH 2.8	3 ng
Babalola et al., (2003)	HPLC – UV at λ = 278 nm	Diethyl ether	Microbore C_{18}, 0.1%TFA:Acetonitrile (75:25%, v/v), pH 2.2	50 ng/ml
Ramanathan et al., (2005)	HPLC - Fluorescence, λex = 267 nm and λem = 443 nm	C_{18} solid phase sorbent	Partisil 10 – ODS, MeOH:0.05M Ammonium actetate buffer (50:50, v/v), pH 4.0	10 ng/ml

1.6.5 *In vitro* and *In vivo* Activities

The nitrogen atom at position 1 and pyrrolidinyl Mannich base side chains on the structure plays an important and indispensable role for antimalarial activity of PNDT, Figure 1.1. The pyrrolidinyl Mannich bases impart increased activity to the corresponding compounds (Chen, 1993).

PNDT is a blood schizontocide, i.e., it acts on asexual blood stage parasites. Gametocytocidal activity has been reported *in vitro*, but only limited efficacy is reported *in vivo* (Shao, 1990). Its mechanism of action remains unclear. Initial data suggested that the target of PND, and other 9-anilinoacridines, was the parasite topoisomerase II (Chen, 1981). However, further experiments with acridine analogues, known inhibitors of topoisomerase II, contradicted this initial hypothesis (Chen *et al.*, 1993). Its mode of action has been studied using rats infected with *P.berghei* strain where pyronaridine was administered at a dose of 20 mg/kg (Wu *et al.*, 1998). The early changes detected were the modifications to the morphology of trophozoites. During these changes, pellicular complexes form in which multilamellate whorls appear, followed by enlargement of pigment aggregation in the food vacuoles. The mitochondria become swollen, the endoplasmic reticulum disappears and ribosomes become blurred. Thus for *in vitro*, the food vacuole of the malaria parasite may be the primary target of PNDT (Fu & Xiao, 1991).

It is possible that PNDT acts via a mechanism that is common to other 4-aminoquinolines by interfering with heme metabolism, probably by blocking hemazoin formation. Electron microscopy findings confirm that the parasites digestive system is targeted (Looareesuwan *et al.*,1996). The *in vitro* interactions of artemisinin with amodiaquine, pyronaridine and chloroquine for the treatment of *P.falciparum* were studied by Gupta *et al.* (2002). Pyronaridine generally showed at least additive activity but predominantly moderate synergism with artemisinin. The results observed in this