Development of Analytical Methods for the Determination of Antimalarials in Biological Fluids

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Dissertation presented at Uppsala University to be publicly examined in Clas Ohlson room, Humanistgatan 2, Tenoren, Högskolan Dalarna, Borlänge, Thursday, November 12, 2009 at 13:00 for the degree of Doctor of Philosophy. The examination will be conducted in Swedish.

Abstract

The aim of this thesis was to develop analytical methods for measuring antimalarial drugs in biological fluids. Solid phase extraction (SPE) was used for the enrichment and purification of the drugs. Automatic extraction procedures using a SPE robot were developed to reduce the workload for the analyst and to minimize variations in the extraction procedure. Liquid chromatography (LC) with either UV or mass spectrometric (MS) detection was used to determine sample concentrations.

Determination of Pyronaridine in whole blood utilised a weak cation exchanger to extract Pyronaridine from blood. To improve LC separation between Pyronaridine and the internal standard, ion-pairing was utilized.

For the simultaneous quantification of the highly lipophilic Atovaquone and the strong basic drug Proguanil with metabolites, a novel mixed mode solid phase extraction column was used. It combines the properties of a carboxylic acid (CBA) column and a non-polar octyl-silica (C8) column to extract the compounds from plasma; it also required a gradient LC separation.

Stability is an important factor when developing new methods. A new approach was used to evaluate the stability of Amodiaquine in blood and plasma. This included the use of a stability marker, a stable compound which was added together with Amodiaquine when preparing the stability samples. This eliminated between-run variations and variations associated with preparation of new stock solutions.

Lumefantrine (LF) is one of the active components in a new drug combination recommended by the World Health Organization as a replacement for older drugs which have lost their effect. The first of the two methods described for this compound is the determination of LF and a possible metabolite in plasma with a calibration range suitable for pharmacokinetic studies. In the second method, a capillary sampling technique is used where the blood is dried on a sampling paper and sent to the laboratory where the extraction and determination of LF concentrations take place. This method facilitates sample collection and will enable drug efficacy studies conducted in rural settings.

To monitor a current change in treatment policy and self medication, a screening assay was developed. Its purpose is to be a complement to interviewing patients about their previous medication (in the previous few weeks) and to detect some of the more common drugs which might have been used.

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


VI Blessborn, D., Römsing, S., Bergqvist, Y., Lindegårdh, N. Assay for screening self medication of common antimalarial drugs using the dried blood spot technique. *Manuscript*.

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Author’s contribution

**Paper I:** Participated in the planning and carried out all experiments and wrote most of the paper.

**Paper II:** Participated in the planning and performed most of the experiments and wrote the experimental section of the paper.

**Paper III:** Took part of the planning and performed most of the experiments and participated in writing the paper.

**Paper IV:** This paper was written by N. Lindegårdh and A. Annerberg. They also did most of the planning and performed most of the experiments. I took part in the initial development i.e. characterisation of the physico-chemical properties of Lumefantrine and development of the LC method.

**Paper V:** Planned and performed most of the experiments together with S. Römsing and wrote most of the paper.

**Paper VI:** Participated in the planning and performed most of the experiments and wrote most of the manuscript.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>4-CPB</td>
<td>4-chlorophenylbiguanide</td>
</tr>
<tr>
<td>ATQ</td>
<td>Atovaquone</td>
</tr>
<tr>
<td>AQ</td>
<td>Amodiaquine</td>
</tr>
<tr>
<td>AQm</td>
<td>Monodesethylamodiaquine</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
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<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>CBA</td>
<td>Carboxylic acid</td>
</tr>
<tr>
<td>CG</td>
<td>Cycloguanil</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<tr>
<td>DBS</td>
<td>Dried blood spots</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
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<tr>
<td>FDA</td>
<td>US food and drug administration</td>
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<tr>
<td>ICH</td>
<td>International conference of harmonisation</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LLIN</td>
<td>Long lasting impregnated nets</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
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<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>PG</td>
<td>Proguanil</td>
</tr>
<tr>
<td>PND</td>
<td>Pyronaridine</td>
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<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
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<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>Elimination half life</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Time to reach maximum concentration</td>
</tr>
<tr>
<td>TDR</td>
<td>Special programme for research &amp; training in tropical diseases</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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1 Introduction

Malaria is present mainly in tropical areas of Africa, Asia and Latin America. It is probably the most important parasitic infection in people and more than 250 million people fall ill every year. More than 90% of malaria cases and the great majority of malaria deaths occur in tropical Africa. The most severe cases of malaria are caused by the *Plasmodium falciparum* parasite and the parasite is transferred when a female mosquito of the *Anopheles* family is feeding blood.

There are several ways to protect oneself from mosquito bites. Protective clothing, spraying household with insecticide repellents and using bednets are some protective measurements that are easily performed. Once an infection has occurred, then antimalarial drug treatment is used. Drug resistance is widespread, so it is important that the drug used is effective against the local parasite population. Therefore, it’s important for travellers to know in what area the infection was contracted to decide what drug is appropriate for treatment. For the local population it’s important to know if a newly developed parasite resistance is emerging.

One of the tools to confirm drug resistance or other problems resulting in treatment failure is to determine drug concentration levels in the blood i.e. to verify if an appropriate drug level is reached. For this, liquid chromatography is often used. Accurate quantification of drug levels is dependent of several steps, also known as the “Analytical Chain”. This includes the following steps: Sampling, sample preparation, separation, detection and evaluation of the results. The only step that the analytical chemist has very limited control over is the sampling step, which includes taking a blood sample from the patient, storage and transportation of samples to the laboratory.

Before drug concentration can be measured by liquid chromatography, the samples have to be purified from all other components present in the blood sample. This is done by extracting the drugs from the sample matrix by solid phase extraction. The sample is passed through an extraction column where the drug adsorbs to the surface and most of the sample components pass right through the column. Different solvents are used to wash and purify the sample and finally a strong solvent is used to release the drug to be collected for further analysis. Solid phase extraction is used in all developed methods in this thesis as well as reversed phase liquid chromatography for separation.
When a method has been developed, it has to be evaluated through a validation process to establish that the quantitative measurements are reliable and reproducible in the selected matrix, in this case blood or plasma. If the method passes this test, it can then be implemented for analysis of actual patient samples.

The aim of this thesis was to develop analytical methods to measure antimalarial drugs in biological fluids. Determination of Pyronaridine in whole blood (paper I) also describe the use of an alternative detection method i.e. electrochemical detection that improves selectivity over UV detection. The drug Malarone™ is a combination of two drugs, Atovaquone and Proguanil. Previously described methods had only been able to measure the drugs separately. Paper II describes a method for simultaneous quantification of the two drugs and their metabolites extracted from plasma samples.

One important part of method development is to assess stability of the drug in the matrix. In paper III, a new approach that included the use of a stable marker was evaluated to assess stability of Amodiaquine in blood and plasma. If this approach is carried through to other drugs, the difficulty may be to find a suitable stable marker that performs in a similar way as the drug of interest when extracted from the matrix (i.e. acts as a proper internal standard).

Both paper IV and V deal with the measurement of Lumefantrine, a relatively new drug that is used in combination with Artemether in the drug Riamet™, Coartem™. The method in paper IV outlines the measurement of Lumefantrine and a putative metabolite in plasma. Earlier described methods did not cover the entire concentration range reached during therapy and they did not include the metabolite to Lumefantrine.

To evaluate the efficacy of the drug and possibly detect upcoming resistance, it is important to complement blood film microscopic examination for parasite clearance with drug concentration determination. In paper V, a simplified sample collection technique (i.e. Dried Blood Spots) was used. Capillary blood is collected by finger prick and is then applied onto a sampling paper that is left to dry. This technique is especially useful in rural settings without electricity (that is needed for refrigerating blood samples) and the sampling is far less invasive than vein-puncture.

Malaria parasites are putting up a good fight against antimalarial drugs and have developed resistance against several drugs. When drug resistance has emerged, new drugs have to be implemented and the old drugs phased out. New drugs are often more expensive than the old ones and could therefore be in continued use in some areas. To monitor the changed treatment policy and self medication, a screening assay was developed in paper VI. Its purpose is to be a complement to interviewing patients about their previous medication (last few weeks) and to detect some of the more common drugs that might have been used.
2 Malaria

2.1 Mosquitoes

Malaria is an infection caused by a parasite and carried from person to person by certain mosquitoes of the *Anopheles* family (fig. 1). There are about 3000 species of mosquitoes [1] grouped into 42 genera (plural for “genus”). The genus *Anopheles* contains about 430 known species and only 40 transmits malaria (i.e. vectors) in nature. Some species prefer to feed on humans “anthropophily” and others favour animals “zoophily”. *Anopheles* mosquitoes locate their human hosts primarily through odorant receptors that respond to a component in human sweat [2].

*Figure 1. Anopheles mosquito WHO/TDR*

The *Anopheles* vectors are present almost all over the world. In large part of Europe, mainly in the coastal areas from England, France, Germany, Portugal and central Europe and to the north of the Caspian sea, the main vector is *Anopheles atroparvus*. Adults bite both humans and domestic animals, both indoors and outside. During the winter season, females enter partial hibernation, sheltering in houses and animal sheds. There has been no malaria transmission since eradication in the previously endemic areas, but potential
capability is still present. In Scandinavia and large parts of Russia is *Anopheles messeae* the main vector [3]. The world most efficient vector is probably the *Anopheles gambiae*, found in nearly all African countries south of Sahara.

### 2.2 Malaria

Malaria is an infection caused by a one-celled parasite called *Plasmodium*. There are four types of human malaria, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium falciparum* and *Plasmodium ovale*. The most common types are *Plasmodium falciparum* and *Plasmodium vivax* where *falciparum* is the most deadly type of malaria infection [4] and the most common infection in Africa. There are also other *Plasmodium* species that infect certain animals. Several mammals, birds and reptiles have their own type of malaria [5].

The malaria parasites enter the human host when an infected female *Anopheles* mosquito bite and the parasites mix in with its saliva and enter the human blood stream. Once in the blood, the parasites travel to the liver and enter liver cells to grow and multiply. The infected person has no symptoms during the incubation period that may last between 8 – 14 days depending on *Plasmodium* specie even up to several months in some cases. The parasites then leave the liver cells and enter the red blood cells. Once inside the red blood cell, they continue to grow and multiply. After they have matured, the infected red blood cells rupture, freeing the parasites to invade other red blood cells. When red blood cells rupture, toxins are released, giving typical malaria symptoms like fever, chills, headache, vomiting and flu-like symptoms. The parasites have now developed into a form that is able to infect a mosquito again when it bites an infected person.
The malaria parasites have undergone a series of changes as a part of their complex life-cycle to elude the immune system. If left untreated, the infection can rapidly become life threatening. Infected and destroyed blood cells (anaemia) may be clogging the capillaries that carry blood to the brain (cerebral malaria) or other vital organs. If a mosquito bites an infected person to obtain blood and ingests malaria parasites in the form of gametocytes, the cycle of transmission will continue. Parasites grow and mature in the mosquito’s gut for a week or two until it reaches the sexual stage and then travels to the mosquito’s salivary glands ready to infect a human host when the mosquito takes its next blood meal. How fast the parasites grow inside the mosquito depends on several factors. Most important are ambient temperature and humidity (higher temperatures accelerate the parasite growth in the mosquito) and whether the *Anopheles* mosquito survives long enough for the parasites to complete their life cycle [4, 6-8].

Malaria is present in almost all tropical and sub-tropical regions around the world, from Asia to Latin America and Middle East as well. Most countries in Europe, the USA and some others (i.e. Singapore, Taiwan and Caribbean islands) have successfully eradicated malaria thanks to economic development and public health efforts. However the *Anopheles* mosquito is still present although the *Plasmodium* parasite has been eradicated [7]. Nowhere is the malaria disease so severe and wildly spread as in Africa. There are at least 250 million cases of malaria in the world and over a million deaths, about 90% of these deaths occur in Africa, south of the Sahara, and mostly among young children [9].
2.3 Protection

The best way to prevent malaria transmission is to protect people during the hours when mosquitoes are most active i.e. from dusk to dawn. The World Health Organisation considers the best and most affordable way of protecting people is by having them sleep under insecticide treated nets (fig. 3) that will kill mosquitoes or make them unable to bite [8, 10]. Newly developed Long Lasting Impregnated Nets (LLIN) that last the life span of the net, have shown to be effective and is a good solution opposed to continuous re-inpregnation of conventional bednets [11]. LLIN also have a deterrent effect that reduces the amount of mosquitoes entering the house. Other ways to protect people is to use repellents, spraying insecticides on walls, wear protective clothing and try to eliminate places around their homes where mosquitoes can breed [12]. *Anopheles gambiae* larvae occur mainly in temporary habitats such as puddles and pools but also in other places such as rice fields [1].

![Figure 3. Insecticide treated bednet. WHO/TDR/Crump](image)

There are some difficulties with insecticides and bed nets. Families at risk of malaria is often among the poorest in the world and even if an insecticide treated net costs about US $2-5, the price is too high for people that have an income of less than one dollar a day [8]. Even if they have an insecticide treated net, many people don’t have the habit of using them or, as with older types of nets, is not in the habit of re-treating the nets with insecticides to achieve their expected impact. There is also an increasing problem with insecticide resistance in mosquitoes. Pyrethroid is the most common insecticide for indoor use and the chemical recommended for treatment of mosquito nets, but there is an increasing resistance against the insecticide despite its rapid toxicological action [13, 14].
When preventive measurements fail, antimalarial drugs are the last line of defence. In Africa, the cheapest and most widely used antimalarial drug is Chloroquine [9]. Unfortunately Chloroquine resistance is increasing and many countries have to change to more expensive drugs for treatment. Another cost effective drug is Amodiaquine which cost about 20-30 US cents per adult treatment course. Since Amodiaquine is structurally similar to Chloroquine there will be some cross-resistance between these two drugs. However, Amodiaquine is still an effective drug with higher cure rates than Chloroquine and is therefore used in many countries as a second line drug [15].

There are other more effective drugs, but unfortunately, these are often more costly. One of these drugs is Atovaquone-Proguanil (Malarone), a treatment course with Malarone ends up to at least US $40 per adult treatment course. There are other combination drugs at similar price range were the manufacturer has an agreement with WHO to manufacture the drug as “non profit”. One of those drugs is Artemether-Lumefantrine (Riamet™) by Novartis and is sold under the name “Coartem™” for about US $3 for an adult treatment course [16].

It has recently become more common to combine different antimalarial drugs in order to increase efficacy. Another benefit is that the treatment duration often can be shortened which will decrease the risk of parasite resistance arising as a result of mutation during therapy [17].

In Africa, due to distance and limited health service, it’s common with “over the counter” distribution and self medication. This often results in poor compliance with dosing schedules and this is one of the major factors of drug resistance [15]. There are also increasing problems with counterfeit drugs that contains none or too low amount of antimalarial drug and this will speed up drug resistance [18].

Malaria vaccine is unlikely to be available for endemic population before 2020 [15]. There is currently no malaria vaccine approved for human use. Since the malaria parasite has a very complicated life cycle and constantly changing its antigens, there will be difficulties developing a single efficient vaccine against these varying antigens [7, 19].

2.4 Antimalarial drugs in this thesis

2.4.1 Pyronaridine

Pyronaridine (fig. 4) was developed in China in the 1970s. It is based on the structure of mepacrine since various derivates of this nucleus have shown activity against Chloroquine resistant parasites. After synthesizing and
screening of several substitutions, a side chain similar to Amodiaquine was selected for greatest activity with fewest adverse effects [20].

Figure 4. Structure of Pyronaridine  M = 518.1 g/mol

At the time of the publication of our article on Pyronaridine (paper I), preliminary studies were mainly done on animals and information regarding concentration levels in human blood was very limited. Studies in rabbit showed a blood to plasma ratio ranging from 4.9 to 17.8 [21]. These results implied that blood was the preferred matrix. However, recently (2003) a study on a single healthy volunteer administered an oral dose of Pyronaridine tetraphosphate (6.15 mg/kg) was performed in plasma with maximal concentration \( C_{\text{max}} \) of 76.2 ng/ml that occurred 1 h \( (t_{\text{max}}) \) after administration of the drug [22].

In 2002 WHO signed an agreement with a medical company in Korea to develop a new antimalarial combination medicine Pyronaridine-Artesunate [23]. One of the advantages of the Pyronaridine-Artesunate combination is that there is no pre-existing resistance to either component. It is therefore expected, that this combination will be able to be deployed in areas with both sensitive and multi-drug resistant malaria. When combining two antimalarial medicines with different modes of action and different biochemical targets in the parasite, the effects of both medicines may be added together, resulting in improved clinical efficacy and delay in development of drug resistance. However, Pyronaridine and Artesunate or Dihydroartemisinin (the active metabolite of Artesunate) have shown evidence of weak antagonism drug interaction in vitro (combined drug effect is not entirely added) [24-26] but it is considered to be without any clinical significance [27].

The drug combination has just finished Phase I studies with evaluation of its safety, safe dosage range, and identification of side effects. It is currently undergoing Phase II trials, to evaluate safety and efficacy in larger patient groups. During the Phase I study, Pyronaridine-Artesunate was given in a fixed 3:1 ratio for the treatment of uncomplicated malaria and was administered once daily × 3 days. The dose range was 6:2 to 15:5 mg/kg Pyronaridine:Artesunate and was well tolerated up to 15:5 mg/kg. Some pharmacokinetic data given for Pyronaridine implied fast absorption with \( t_{\text{max}} \) of 1.6
to 4.8 h and C$_{\text{max}}$ 171 ng/ml at 6 mg/kg and 774 ng/ml at 15 mg/kg. The elimination half-life ranged from 6.6 to 9.7 days. It was also stated that Pyronaridine concentrations accumulated in blood with repeated daily doses [28]. A method for determining Pyronaridine in blood was used in that study [21].

2.4.2 Atovaquone-Proguanil (Malarone™)

Atovaquone (fig. 5) is a highly lipophilic molecule with very low aqueous solubility. The drug is protein bound (>99%) but causes no significant displacement of other highly protein-bound drugs [29, 30]. Atovaquone is poorly absorbed from the gastrointestinal tract but bioavailability after oral administration can be improved by taking the drug with fatty foods or fatty milk [30]. Maximum plasma concentration after a single 750 mg dose in four healthy volunteers, C$_{\text{max}}$ was about 5.3 μg/ml at the time t$_{\text{max}}$ of 2-4 h with an elimination half-life of 54-100 hours [31]. A study on three Caucasian volunteers showed a slightly longer elimination half-life of 5 to 6 days after treatment with 1000 mg Atovaquone daily for 3 days [32], whereas two studies on pregnant women with similar treatment regimens showed C$_{\text{max}}$ of about 2 to 8 μg/ml at the time t$_{\text{max}}$ of 2-9 h with an elimination half-life ranging from 60 to 130 hours [33, 34]. Atovaquone is excreted almost exclusively in the faeces as unchanged drug [35].

![Figure 5. Structure of Atovaquone, M = 366.8 g/mol](image)

Atovaquone is frequently used in combination with other agents e.g. Proguanil. Proguanil (fig. 6) is a prodrug with a weak anti-plasmodium activity and is metabolized in the body via the polymorphic cytochrome P450 enzyme CYP2C19 to the active metabolite, Cycloguanil. Approximately 3% of Caucasian and African populations and 20% of Oriental people are “poor metabolizers” [36] and have considerably reduced biotransformation of Proguanil to Cycloguanil. In vitro studies show a synergistic effect between Proguanil and Atovaquone enhancing the effectiveness of the combination [37].

Proguanil is a water soluble compound and is easily absorbed from the gastrointestinal tract following oral administration; around 75% is bound to plasma proteins. Maximum plasma concentration $C_{\text{max}}$ for Proguanil was about 0.4-0.7 μg/ml at the time $t_{\text{max}}$ of 4.5 h with an elimination half-life of 16-17 hours [33, 34] and for cycloguanil $C_{\text{max}}$ was 0.04 μg/ml at $t_{\text{max}}$ of 6.9 h with an elimination half-life of 22 hours [33].

Peak plasma levels of Proguanil occur at about 4 h while peak plasma levels of Cycloguanil occur approximately at 5 h. The elimination half-lives of both Proguanil and Cycloguanil is approximately 20 h. Elimination is about 50% in the urine, of which 60% is unchanged drug and 30% Cycloguanil, and a further amount is excreted in the faeces. Small amounts are present in breast milk [35].

Malarone™ is administered as film coated tablets containing 250 mg of Atovaquone and 100 mg of Proguanil hydrochloride for adults and tablets containing 62.5 mg of Atovaquone and 25 mg of Proguanil hydrochloride for paediatric (child) use. Patients are treated with four tablets of Malarone™ once daily for 3 consecutive days taken together with food or milk for maximum absorption.

Malarone™ is generally very well tolerated with adverse effects not differing from symptoms commonly seen with malaria itself [38]. Malarone™ was registered for prophylaxis in several European countries including Sweden in 2001.

![Chemical structures of Proguanil, Cycloguanil, and 4-Chlorophenylbiguanide](image_url)

*Figure 6. Structure of Proguanil M = 253.7 g/mol, Cycloguanil M = 251.7 g/mol and 4-Chlorophenylbiguanide M = 211.6 g/mol*
2.4.3 Amodiaquine

Amodiaquine (fig. 7) is a 4-aminoquinoline antimalarial drug with a mode of action similar to that of Chloroquine. Amodiaquine is effective against many chloroquine-resistant strains of *P. falciparum*, although there is cross-resistance. Amodiaquine is distributed as tablets containing 200 mg of Amodiaquine base as hydrochloride or 153.1 mg of base as chlorohydrate [35]. Amodiaquine is administered once daily over 3 days with total doses ranging between 25 mg to 35 mg of Amodiaquine base per kg body weight [39].

![Figure 7. Structure of Amodiaquine. M = 355.9 g/mol](image1)

![Figure 8. Structure of Monodesethyl-amodiaquine, M = 327.8 g/mol](image2)

Amodiaquine hydrochloride is easily absorbed from the gastrointestinal tract. It is rapidly and extensively metabolized in the liver to the active metabolite Monodesethylamodiaquine (AQm, fig. 8), which contributes to nearly all of the antimalarial effect. Orally administered Amodiaquine remains detectible only for a short time, with a half-life of about 8 hours after administration of a single dose of 200 mg. Maximum plasma concentration $C_{\text{max}}$ was 16 ng/ml at the time $t_{\text{max}}$ of 0.6 h. The metabolite AQm reached a $C_{\text{max}}$ of 51 ng/ml at $t_{\text{max}}$ of 5.5 h [40]. Elimination half-life after for AQm after an oral dose of 10 mg/ml Amodiaquine was between 9 and 18 days [41, 42] and the concentration in whole blood was 4-6 times higher than in plasma [42]. A study in Gabon of 114 children under the age of 10, given a dose of 30 mg/kg during 3 days, was a plasma concentration level higher than the breakpoint of 135 ng/ml on day 3 associated with treatment success [43].

Amodiaquine is now undergoing trials for combinations with other drugs to increase efficacy. A study published in 2006 [44] combined Amodiaquine with Sulphadoxine-Pyrimethamine. The study was performed on 351 adults in Rwanda and it was concluded that the combination was not well tolerated with a substantial portion of the patients experiencing adverse effects such as fatigue, nausea, dizziness and vomiting. It was concluded that this would decrease compliance and could compromise a first line treatment implementation at national level. Another combination, Amodiaquine and Artesunate
seems to be highly efficacious for treatment of uncomplicated malaria and well tolerated [45, 46].

Stability of Amodiaquine and the metabolite AQm has earlier been investigated by Minzi et al. [47] and the drugs were reported not to be stable at any temperature between -20 °C to +37 °C and applies to both spiked blood samples and samples from volunteer. Slowest decrease was seen at +4 °C, at this temperature the sample was stable for one week. Repeated freezing and thawing did not markedly affect the stability. The authors recommend keeping whole blood refrigerated at +4 °C for up to 1 week alternatively that plasma is separated and frozen as soon as possible and stored at -70 °C. If facilities are not available, storage at -20 °C is recommended but not for more than 3 months. Standards and stock solutions were stored at -70 °C and were found to be stable for more than one year. Mount et al. 1986 [48] tested the stability in blood withdrawn from volunteer given a single dose by refrigerated the samples at +5 °C and analyzed after 1, 2, 4, 8 and 16 weeks. No detectable decrease in concentrations was seen.

Our findings (paper III) supports Minzi et al. that Amodiaquine and the metabolite AQm have limited stability, and that +4 °C is more stable than -20 °C in whole blood. These results are possibly explained by a protective accumulation of the drug within blood cells. Both AQ and AQm are lysosomatropic and accumulates by a pH gradient into the acidic lysosomes of the cells where they become protonated and trapped [49]. Thus at moderate temperatures above zero the stability is possibly enhanced in blood because AQ and AQm are protected within the white cells. At moderate low temperatures below zero haemolysis caused by freezing instead enhances the degradation.

2.4.4 Lumefantrine

Lumefantrine (fig. 9, formerly known as Benflumetol) was originally synthesized by the Academy of Military Medical Science in Beijing, People’s Republic of China in the 1970s and was registered in China for antimalarial use in 1987 [50, 51]. Lumefantrine have poor solubility in water and oils but is soluble in unsaturated fatty acids [51]. It belongs to the aryl-aminoalcohol group of antimalarial drugs, which also include Mefloquine, Halofantrine and Quinine [35, 50].
A major advantage with Lumefantrine compared to other antimalarial drugs is that it has never been used in monotherapy, but only in co-formulation with Artemether. The combination drug was registered in 1992 and is now distributed under the name of Coartem™ or Riamet™ manufactured by Novartis [52]. Artemether with an elimination half life of 1 h provides the initial rapid reduction of parasites and clears most of the infection whereas Lumefantrine is slow acting with an elimination half life of 3 to 6 days eliminating the remaining parasites in the blood [53]. Lumeфантрин is a racemic mixture and normal synthesis yields the racemate of both enantiomers. A study was performed in Tanzania, east Africa, to assess if there were any differences in activity between the enantiomers compared to the racemic mixture. Fresh isolates collected from persons with *P. falciparum* mono infections showed that there was negligible difference in activity between the three compounds (+)-Lumefantrine, (-)-Lumefantrine and the racemic Lumeфантрин and they are all very active antimalarial compounds with almost identical potency [54].

Artemether-Lumefantrine is formulated in a 1:6 fixed dose ratio and each tablet contains 20 mg Artemether and 120 mg Lumefantrine. Recommended dose is 1.5 mg/kg Artemether and 9 mg/kg Lumefantrine. Initially a 4 dose regimen was evaluated where each dose was given at 0, 8, 24 and 48 h [50] but it turned out having a cure rate of less than 81%, far below the WHO recommended >90% cure rate [35]. The dose schedule was revised and a 6 dose regimen was evaluated, where each dose is given at 0, 8, 24, 36, 48 and 60 h. The two following efficacy studies performed by Vugt et al. showed a day 28 cure rate of >95% [55, 56] and patients showed good tolerance to the drug with adverse effects no different that of malaria it self [55, 57].

Lumefantrine is a highly lipophilic compound and were found to be highly bound to proteins in serum (>99%) and primarily to high density lipoproteins [58]. During fasting conditions the absorption of Lumefantrine is low and variable but the absorption is drastically increased when administered together with high fat food. So how much fat is necessary? That was investigated by Ashley et al. The aim of the study was to describe the dose response relationship between co-administration of fat and relative Lumefan-
trine bioavailability, in order to determine the minimum amount of fat necessary to optimise absorption. The study was performed on healthy volunteers and Lumefantrine was co-administered with 0-500 ml of soya milk equivalent to 0-16 g of fat. There was a dose response relationship between volume soya milk administered and the bioavailability of Lumefantrine. Administration with soya milk increased the Lumefantrine plasma concentration curve (AUC) more than five fold [59]. To obtain 90% of maximum effect one would need about 36 ml soya milk (corresponding to 1.2 g of fat). Today the general guideline is to administer Artemether-Lumefantrine together with milk or fatty food to enhance absorption of Artemether and especially Lumefantrine [35].

Good effectiveness of the drug in controlled trials does not always reflect field use, where efficacy could be compromised by poor adherence, incorrect timing of doses or insufficient intake of fatty food. This has been studied by Piola et al. where 313 patients were supervised (all doses observed with fatty food intake) and compared with an unsupervised patient group (n=644) with first dose supervised followed by outpatient treatment with nutrition advice. The cure rate were about 98% in both treatments and they concluded that Artemether-Lumefantrine has a high cure rate irrespective of whether given under supervision with food or under conditions of routine clinic practice [60]. Another article [61] achieved similar results but they also measured the day 7 concentration and found lower concentration levels in the unsupervised arm compared with the supervised but this did not affect the day 28 cure rate outcome.

There have been limited data on efficacy and safety for treatment of infants and children with Artemether-Lumefantrine, but these last two years several studies have [62-66] shown good efficacy and safety. There is also a new dispersible tablet out for trial [67] for easier administration compared to crushed commercial tablets.

Another population group with limited data is pregnant women. Most studies are made during the second and third trimester and available data is showing good tolerance to the drug with no adverse effects but with poor cure rate compared with non-pregnant patients [68-71]. This is probably due to lower drug concentrations due to altered pharmacokinetic properties in later pregnancies [69]. A longer treatment (e.g. five days) is suggested for pregnant women but further studies are needed to determine optimum dose regimen.

Available pharmacokinetic data for Lumefantrine after a standard 6 dose regimen gives a C_{max} of 7 to 28 μg/ml at t_{max} of about 3 days (i.e. about 5 – 10 h after last dose). Elimination half-life t_{1/2} is about 3 days in healthy volunteers and slightly longer in malaria patients [39, 52, 72, 73]. In a recent paper with whole blood spotted on filter paper from malaria patients, they measured day 3 concentrations in the range of 980 – 9250 nmol/l (515 – 4880 ng/ml) and at day 7, 575 – 1085 nmol/l (305 – 575 ng/ml) and it’s in
the same range as our results after our small trial in Tanzania. Concentration levels below 280 ng/ml on day 7 is often associated with treatment failure [74].

2.4.5 Drugs in the Screening Method

2.4.5.1 Chloroquine
Chloroquine (fig. 10) was developed by the allies during World War II and has been one of the most important antimalarial drugs ever since. It is a low cost drug and is relatively well tolerated. It is often given as a prophylaxis and adverse effects at prophylactic doses are rare [75]. Chloroquine is one of the drugs considered safe to use in pregnancy in the first trimester [35]. Due to a long use as monotherapy, parasite resistance started to emerge in 1957 in Thailand and a few years later in South America [75, 76]. In the beginning of the 1990s parasite resistance had spread to almost all countries in the world [77] and as a result the use of the drug drastically diminished during the years 2000 to 2006.

Chloroquine is easily and rapidly absorbed from the gastrointestinal tract and has an initial half life of 3 to 6 days with a terminal elimination half life of 1-2 months [78, 79]. At an oral dose of 10 mg/kg, C_{max} ~ 200 to 300 ng/ml in serum for Chloroquine and about 100 ng/ml for its metabolite Desethylchloroquine (fig. 11) [80, 81]. After long period of prophylactic treatment (6 weeks) with a daily dose of 100 mg/day (1 tablet Savarine™) a steady state concentration of 100 to 150 ng/ml in dried blood spots was observed in 10 healthy subjects [82].

\[
\begin{align*}
\text{Figure 10. Structure of Chloroquine} & \quad M = 319.9 \text{ g/mol} \\
\text{Figure 11. Structure of Desethylchloroquine} & \quad M = 291.8 \text{ g/mol}
\end{align*}
\]

2.4.5.2 Quinine
Quinine (fig. 12) is an old antimalarial drug that has been used for several hundred years in South America to cure fever. It’s obtained by extracting the compound from cinchona tree bark [35, 79]. Quinine is readily and rapidly absorbed when taken orally and is distributed throughout body fluids and is
highly protein bounded (80-90%) [35, 39]. The drug is extensively metabo-
lized in the liver and polar metabolites are excreted by renal elimination. 
Peak plasma concentration after oral intake occurs after 1 – 3 hours and 
elimination half-life is about 11 hours in healthy volunteers but increases to 
16 or 18 hours in uncomplicated or severe malaria cases [35, 39, 79]. A com-
mon treatment for non-immune patients is 8 mg of base/kg body weight 
three times a day (every 8 hours) for 7 days. After a dose of 8 mg/kg quinine 
base, a maximum concentration C\text{max} is in the range of 3 to 7 μg/ml in 
plasma and dried blood spots [35, 83-86]. 

Quinine is a potentially toxic drug and side effects commonly seen in 
therapeutic concentrations are mild form of tinnitus, impaired high tone 
hearing, headache, nausea, dizziness and sometimes disturbed vision [35, 39, 
79, 83]. Quinine is also one of the drugs used in pregnancy in the first tri-
merster [35].

\[ \text{Figure 12. Structure of Quinine, M = 324.4 g/mol} \]

2.4.5.3 Sulfadoxine/Pyrimethamine
Sulfadoxine (fig. 13) is easily absorbed from the gastrointestinal tract and is 
widely distributed in body tissues and fluids with about 90 – 95 % bound to 
plasma proteins. Peak blood concentrations after oral intake occurs after 
about 4 hours with a peak concentration C\text{max} of about 200 μg/ml in whole 
blood [87]. Sulfadoxine is slowly eliminated from the body with a t_{1/2} of 7 – 
9 days and is mainly excreted with the urine, primarily unchanged. Sulfadox-
ine is given as a fixed combination with Pyrimethamine (fig. 14) in tablets 
containing 500 mg Sulfadoxine and 25 mg Pyrimethamine. Dosage for pro-
phyaxis is 1 tablet once a week. Dosage for treatment is 3 tablets as a single 
dose. However, this combination is no longer recommended for prophylaxis 
due to the risk of severe liver and skin reactions during long periods of 
treatment [39].

Pyrimethamine is almost completely absorbed from the gastrointestinal 
tract. It is metabolized in the liver and slowly excreted via the kidneys. Peak 
blood concentrations after oral intake occurs within about 2 – 6 hours with a 
peak concentration C\text{max} of about 500 ng/ml in whole blood. The elimination 
half-life is about 4 days [35, 87, 88]. Blood concentration after 3 days was 
about 235 ng/ml.
The drug combination Sulfadoxine/Pyrimethamine is generally well tolerated when used at the recommended doses for malaria therapy. The more severe side effects are related to sulfadoxine and as a sulfa component it can cause hypersensitivity reactions like skin rash or more serious reactions affecting different organ systems [35].

Sulfadoxine/Pyrimethamine is one of the drug combinations considered safe to use in pregnancy in the first trimester [35]. This drug combination is unfortunately on the same route as chloroquine and its efficacy has become increasingly compromised in several countries [89-92].

![Figure 13. Structure of Sulfadoxine](Image)

![Figure 14. Structure of Pyrimethamine](Image)

M = 310.3 g/mol                   M = 248.7 g/mol

2.4.5.4 Mefloquine

During the 1960s, United States Army synthesized and tested several drugs for protection of their soldiers against multi drug resistant *falciparum* malaria and hence, Mefloquine was born. Mefloquine (fig. 15) is reasonably well absorbed from the gastrointestinal tract and administration together with food intake will enhance absorption [75]. Mefloquine is about 98% bound to plasma proteins and is widely distributed throughout the body. Mefloquine is metabolized in the liver and is mainly excreted in the faeces.

Mefloquine is administered as an oral dose with 150 mg base/tablet. For treatment, the recommended dose is 25 mg/kg body weight split into two doses, 15 mg/kg on the first day and 10 mg/kg on the second day. As a prophylaxis, the recommended dose is 5 mg/kg base once a week (equals 1 tablet once weekly for an adult). Prophylaxis should start at least 2 – 3 weeks before departure [35]. Parasites resistant to Mefloquine are found in South-East Asia, but are rare elsewhere in the world.

Mefloquine has a relatively long half-life of 12 to 17 days. Maximum concentration is reached about 20 to 40 hours after administration (treatment with 25 mg/kg) with a peak concentration \( C_{\text{max}} \) of 2200 to 2700 ng/ml in whole blood [93-97]. Concentration levels at day 28 is in the range of 200 – 370 ng/ml [95].

Side effects associated with Mefloquine treatment include nausea, vomiting, abdominal pain, diarrhoea, headache, loss of balance and sleeping disorders like insomnia and abnormal dreams. Some more serious but rare side effects are neuropsychiatric disturbances like psychosis or hallucinations etc.
[35, 79]. Mefloquine is not recommended in the first trimester of pregnancy as it is associated with an increased risk of giving still birth. And due to its long half-life, pregnancy should be avoided at least 3 months after completing chemoprophylaxis [39].

\[ \text{Figure 15. Structure of Mefloquine} \]
M = 378.3 g/mol

\[ \text{Figure 16. Structure of Mefloquine metabolite} \]
M = 309.2 g/mol
3 Method development

3.1 Compound properties

In method development, it’s important to understand the physical and chemical properties of the compound. Searching the literature for previously developed methods, or other compounds with similar structure is a useful help [98]. There are also computer programs that predict solubility in organic and aqueous solvent (LogD), charge (neutral, ionic-neutralizable or ionic-permanent) with pKa-values and so forth.

An example of LogD is given in figure 17, where the lipophilic drug Atovaquone (ATQ) with LogD of 6.2 at pH 2, is compared to the more hydrophilic Proguanil (PG) and its metabolites with LogD ranging from 0.6 to about -1. In reversed phase liquid chromatography, Atovaquone would require a lot more organic modifier to achieve a reasonable retention time compared to Proguanil and its metabolites. Therefore, in paper II, a step gradient was needed for the separation of these compounds within a reasonable time.

![LogD diagram of Atovaquone, Proguanil and metabolites](image)

*Figure 17. LogD diagram of Atovaquone, Proguanil and metabolites*
3.2 Liquid chromatography

Liquid Chromatography (LC) is the most commonly used technique for separation and quantification of drugs [99]. The principal of LC-separation is based upon the interaction of analyte with the stationary phase (packing material in the column) and the mobile phase (the liquid passing through the column). In reversed phased LC, a non-polar stationary phase is used and a polar mobile phase flows through the column. Separation is based on the partitioning of the lipophilic portion of the molecule between the stationary phase and the mobile phase. The mobile phase consists of an aqueous-organic solvent mixture where the aqueous part often is a buffer to obtain a certain pH. Retardation of a compound is controlled by the amount of organic solvent in the mobile phase. More organic solvent and the compound will pass faster through the column. LogD diagram (Fig 17) can be useful to predict the compounds elution order in reversed phase LC, and pH of the mobile phase may be used to improve separation.

If it’s not possible to obtain sufficient separation and good peak symmetry when changing pH or organic solvents, ion-pairing may be an option. In paper 1, there were difficulties obtaining good separation between Pyronaridine and the internal standard (Amodiaquine). Both are ionic compounds at pH 2.5 and react in the same way upon variations of pH and acetonitrile content in the mobile phase. The addition of a counter-ion (with opposite charge to the analyte) to the mobile phase [100] may improve separation, and in this case sodium perchlorate as counter-ion improved separation between Pyronaridine and Amodiaquine (fig. 18 & 19).

![Figure 18. pH 2.5, No separation between Pyronaridine (PND) and Amodiaquine (AQ)](image1)

![Figure 19. pH 2.5, Separation achieved with addition of counter-ion](image2)
3.2.1 The LC-system

A standard instrumental system for Liquid Chromatography consists of a solvent reservoir with the mobile phase, a pump that pushes the liquid through the system, an injector for injecting samples into the system, a column for separation of compounds, a detector and a data capture system (integrator), often a computer. All components are shown in fig. 20. Additional items can be connected to the system such as pre-column (i.e. a short disposable column) to protect and prolong the life of the more expensive analytical column. A solvent saver connected to the detector will recycle the mobile phase until the detector detects a sample peak. It will then switch over the solvent flow to waste keeping the mobile phase free of contaminants.

![Common liquid chromatographic system](image)

*Figure 20. Common liquid chromatographic system*

3.2.2 Detection

One of the most commonly used detectors is the absorbance detector since it is cheap, robust and easy to handle. Detection is based on the absorption of UV-light of a compound at a specific wavelength. A typical UV detector has a narrow cell of about 1 mm in diameter and 10 mm in length with an internal volume of about 8 μl. Wavelength selection is based on literature reference values or a wavelength scan in a spectrophotometer. The absorbance maximum at one or a few wavelengths is selected and tested in the LC system for maximal sensitivity and/or minimal interference.

All papers in this thesis except the screening assay used UV-detection. In *paper I*, electrochemical detection (ECD) was also evaluated. ECD is based on the production of electrons when the sample is oxidised. The electrochemical detector requires three electrodes; working electrode (where the oxidation takes place), reference electrode and auxiliary electrode (which compensate for any changes in the background conductivity of the mobile phase). A fixed potential is applied between the working electrode and the reference electrode. The current flowing across the detector cell between the
working and the auxiliary electrode is measured. The current which is produced by the electrochemical reaction is proportional to the concentration of the analyte which is passing the cell at the moment. Electrochemical detectors generally have higher sensitivity and better selectivity than UV-detectors. In paper I, Pyronaridine have quite good UV absorbance and the advantage of the electrochemical sensitivity was not sufficient to replace the UV detector. The electrochemical detector was therefore used as a backup detector connected in serial after the UV detector to show its higher selectivity as an alternative detector.

In paper VI, the screening assay, was the first attempt to use a diode array detector. It is similar to an UV-detector but has several cells and is able to monitor several wavelengths simultaneously. This is especially suitable if there are several compounds with absorbance maximum at different wavelengths. However, there were big difficulties in extracting and cleaning up all the drugs in the dried blood spot sample due to their large differences in physico-chemical properties. It was always a couple of drugs that had some issues with interferences from the paper-blood matrix that could not be separated or cleaned enough for adequate sensitivity of the assay. In order to solve this, the detection technique was switched to mass spectrometry (MS). The advantage with MS is the ability to separate compounds according to their mass to charge ratio (m/z).

The first step is to evaporate the mobile phase and ionise the compound of interest. This requires that the mobile phase is composed of volatile components e.g. water, organic solvents and volatile buffers. A common and popular ionisation technique coupled to LC is electrospray ionisation (ESI). It is a soft ionisation technique that produces limited fragmentation. After the evaporation and ionisation of the compound, it will enter the mass analyser where the ionised compounds will be filtered according to their mass to charge ratio. The detector is the final destination for the ionised compounds and when they strike the detector surface they will create a weak ion current that is measured. With computer software, it is possible to extract a particular ion chromatogram and in this way get a second separation from interfering compounds as described in figure 21 below.

Figure 21. Left: Total ion chromatogram from an extracted whole blood spot spiked with 100 ng/ml Quinine. Figure on the right: Extracted ion chromatogram (m/z 325), when the m/z has been extracted, a Quinine peak is clearly visible at 3.85 min.
3.3 Solid phase extraction

Biological samples need cleanup before injection into a LC-system. Large proteins and other compounds present in blood/plasma could otherwise precipitate in the LC-column and lead to blockage of the column and/or deterioration of the column performance. The sample cleanup step should both clean the sample from particles that can cause blockage and minimize the content of compounds that could interfere with the LC-analysis. It is also often possible to concentrate the sample to improve detection of compounds at low concentrations. The clean-up method used in all papers in this thesis was Solid Phase Extraction (SPE). The use of a SPE-robot often improves precision and accuracy compared to manual SPE extraction using vacuum.

3.3.1 Sample pre-treatment prior SPE

The most common sample collection for the determination of drugs is venous blood, collected in tubes containing an anticoagulant (e.g. EDTA or heparin). The blood is then often centrifuged to obtain plasma.

It is often necessary to pre-treat a biological sample before SPE. Even though SPE is a cleanup step, it cannot always handle the amount of proteins and other compounds in blood/plasma without the risk of clogging the extraction column. Sample pre-treatment often involves steps like sample dilution or haemolysis of blood, protein precipitation of plasma proteins and centrifugation. If the compound of interest has a high degree of plasma protein binding, precipitation can often improve recovery since the compound will be able to bind to the stationary phase in the SPE bed to a higher extent. Often the samples need to be buffered to a certain pH to give suitable conditions for the desired interaction between the solid phase and the compounds.

3.3.2 Conditioning

*Activation:* The SPE column need to be activated with an organic solvent e.g. methanol, for two reasons: I, methanol acts as a wetting agent, opening up the hydrocarbon chains and hence increasing the surface area available for interaction with the analyte; II, remove residues and contaminants from the manufacturing process and packing material that otherwise could interfere with the analysis.

*Column equilibration:* This step is used to prepare the SPE column for the sample and also removes excess methanol. Solvent used here should resemble the biological sample with respect to pH and modifier content.
3.3.3 Sample loading

The samples are applied onto the SPE column where the compound of interest will bind to the sorbent and ideally most of the sample matrix discarded to waste. Flow rate is of importance and the interaction speed between analyte and sorbent bed is dependent on the type of sorbent used. Ion-exchange sorbent interactions take longer time compared to interactions with non-polar sorbents.

3.3.4 Washing

The column is washed with a suitable solvent to remove interfering matrix compounds that might interfere with the following chromatographic step.

3.3.5 Elution

The samples are eluted with a suitable solvent. If the collected eluent is for immediate analysis, then it’s recommended to elute with the smallest volume possible in order to avoid dilution of the sample. The elution solvent should also be compatible with the mobile phase in the LC system. If an evaporation step is used, it’s possible to elute with larger volumes and stronger solvents to improve recovery. As for the loading step, flow rate is also an important factor to consider.

![General solid phase extraction procedure](image)

*Figure 22. General solid phase extraction procedure*

All of the SPE steps above are subjected to optimisation during method development with regards to e.g. flow rate, solvents, solvent strength, pH and type of sorbent used. All steps are dependent on the matrix used and the physical and chemical properties of the compound.
Throughout this work, the main extraction procedure has been solid phase extraction, mainly because it’s easy to automate the extraction procedure with improved precision compared to manual sample extraction. In the first three papers, a weak cation exchanger with a carboxylic acid (CBA) was used to extract the basic compounds from the sampling matrix. The CBA column (fig. 23) is particularly useful when extracting basic compounds from biological matrix i.e. blood/plasma due to the column’s ability to change its sorbent charge. The CBA column is negatively charged above pH 6.8 and is neutral below pH 2.8 enabling the CBA to ion-bound to the cation (basic compound) and then releasing it when pH is changed to pH 2.8 or below.

In **paper II** the properties of the CBA column was combined with a non-polar octyl-silica (C8) column to a novel mixed mode CBA-C8 column (fig. 24). This enabled the extraction of both non-polar and basic drugs at the same time. This has become more and more useful with the new combinations of drugs currently available such as the antimalarial drug Malarone. A C8 disk column was used in **paper IV** and **V** to extract the lipophilic compound Lumefantrine. It’s the same principal extraction mechanism as the C8 packed bed sorbent used earlier but compressed as a disk it’s possible to use smaller solvent volumes during the conditioning and elution steps.

In **paper VI**, a multi-mode (M-M) extraction column provided the best performance. The M-M column contains a non-polar (C18), strong cation exchange (-SO$_3^-$) and strong anion exchange (-NR$_3^+$) functional groups that all proved to be necessary to retain all the compounds from the screening assay.

![Figure 23. SPE column with carboxylic acid (CBA) functional groups](image)

![Figure 24. SPE column with mixed-mode CBA-C8 functional groups](image)

### 3.4 The sampling paper technique

The most common sampling technique to collect blood samples is vein-puncture. Extraction of drug from the blood is fairly simple and usually involves sample dilution or haemolysis of blood, protein precipitation of plasma proteins followed by centrifugation. This sampling technique requires trained medical personnel to obtain the blood sample and a refrigeration...
tor or freezer to avoid degradation during storage or transportation of the samples.

Sample collection of antimalarial drugs often occurs in rural settings in Africa or South East Asia where electricity may not be accessible that will complicate storage and transportation. A sampling technique that is very sought-after by research physicians involved in therapeutic drug monitoring is the sampling paper technique commonly known as dried blood spot (DBS) sampling. The advantage with this technique is that it’s a capillary sampling method that is less invasive compared to vein-puncture and can be performed by almost anyone and after clear instructions and adequate training even by the patients themselves. Another advantage is that many analytes improve their stability when dried in a blood spots and there is a reduced risk of transferring infections e.g. HIV, making it safer to handle [101].

The drawback with the dried blood spot method is the laboratory part. The introduction of another matrix, the paper, which creates possibilities for additional interactions with the drug, makes it more difficult to extract the drug from the blood with high recovery. The sample volume is also quite small, about 100 μl, making it more difficult to achieve adequate sensitivity for drugs with low concentrations in blood. Some methods have used 200 μl capillary blood, although more than 100 μl is difficult to collect, especially in small children.

It is sometimes necessary to modify the paper surface or the sample before applying it onto the sampling paper to improve recovery or stability. Earlier reported methods have used glass microfiber strips [102], acidification of whole blood with phosphoric acid before applying it to the paper [103] or pre-treating the cellulose based sampling paper with dodecyl dimethyl ammonium bromide [104] to alter the surface binding properties of the paper and increasing the recovery. In paper V, we used tartaric acid to modify the surface properties on the sampling paper that would improve both recovery and stability of the samples.
4 Method validation

4.1 Validation parameters

Method development involves method evaluation and optimisation of the various stages in sample preparation, LC-separation, detection and quantification. Method validation is the process used to establish that the quantitative measurements in the selected matrix are reliable and reproducible. A validation includes the following parameters: Accuracy, Precision, Recovery, Selectivity, Linearity and range, Quantification limit and Stability. Robustness is also a factor to investigate that includes testing of small variations in the solvents (mobile phase or extraction mixtures), extraction time, temperatures etc. The same results should be obtained even if the method is transferred to another LC-system and another analyst is performing the experiment on an analytical column of the same type. There are several articles [105-108] covering this topic and guidelines from US Food and Drug Administration (FDA) [109] and International Conference of Harmonisation (ICH) [110] suggest general recommendations on how to perform a method validation.

4.1.1 Precision, Accuracy and Recovery

The precision of an analytical method describes the closeness of individual measurements of an analyte from several aliquots of a single homogenous volume of biological matrix when a procedure is applied repeatedly. It is expressed as the Relative Standard Deviation (RSD) or percentage Coefficient of Variation (% CV) of replicate measurements.

\[
\text{RSD} = \left( \frac{\text{standard deviation}}{\text{mean}} \right) \times 100.
\]

RSD for each concentration level should not deviate more than ± 15 % to be acceptable except at the Lower Limit of Quantification (LLOQ) where it should not exceed 20 %. Precision can be divided into within-run deviation (i.e. the ability to repeat the same methodology in a short period of time) and between-run deviation (i.e. the ability to repeat the same procedure with new reagents and equipment or analysts between the runs or over longer periods of time). A typical example of a validation setup in our laboratory with minimal requirements is presented in Table 1. Often the table is extended to
6 analytical runs and 4 concentration levels with 3 to 5 determinations per concentration. This approach will allow the data for individual analytes to be analysed with one-way analysis of variance (ANOVA) enabling estimations of within-run and between-run variations for each concentration level.

Table 1. An example of a validation study with three concentration levels over five days.

<table>
<thead>
<tr>
<th>Calibration samples</th>
<th>QC (Low)</th>
<th>QC (Medium)</th>
<th>QC (High)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run: 7 calibration levels and a blank sample</td>
<td>P&amp;A Rec. F/T</td>
<td>P&amp;A Rec. F/T</td>
<td>P&amp;A Rec. F/T</td>
</tr>
<tr>
<td>1 8 3 3 3 3 3 3 3</td>
<td>3 3 3 3 3 3 3 3 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 8 3 3 3 3 3 3 3</td>
<td>3 3 3 3 3 3 3 3 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 8 3 3 3 3 3 3 3</td>
<td>3 3 3 3 3 3 3 3 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 8 3 3 3 3 3 3 3</td>
<td>3 3 3 3 3 3 3 3 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 8 3 3 3 3 3 3 3</td>
<td>3 3 3 3 3 3 3 3 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total: 40 15 15 15 15 15 15 15 15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

QC = Quality Control samples (at three different concentrations)
P&A = Precision and accuracy determinations
Rec. = Recovery are direct injections with concentrations equal to 100 % recovery in extracts
F/T = Repeated freezing and thawing of samples on each run.

Accuracy is a measure of the systematic error and is defined as the agreement between the measured value and the true value.

\[
\text{\% Deviation} = \frac{(\text{measured value} - \text{true value})}{\text{true value}} \times 100.
\]

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. The same samples used for precision can be used for the accuracy determinations and calculations with the same acceptance criteria (±15 % except LLOQ ± 20 %).

Recovery is measured as the ratio between the response of a processed spiked biological matrix standard and the response of a pure standard not subjected to any sample pre-treatment.

\[
\text{Absolute recovery} = \frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of analyte as pure standard}} \times 100.
\]

Although high recovery of analytes from the matrix is desirable, it is not always possible to achieve close to 100 % recovery. But it’s rarely a problem as long as the sensitivity is adequate and the criteria for precision and accuracy are reached.
4.1.2 Selectivity

Selectivity of an analytical method is the ability to differentiate and quantify the analyte in the presence of other components in the sample. This involves analysis of blank samples of the selected matrix from at least six sources where each sample is evaluated for interference. Selectivity is also investigated in case of a metabolite or co medication of the most common antimalarial drugs.

4.1.3 Linearity and range and limit of quantification

A calibration curve is the relationship between instrument response and known concentration of the analyte. A calibration curve is generated for each analyte in the sample and is prepared in the same biological matrix as the samples. The concentration range of the curve is based on the expected range of the study and a calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ.

Lower Limit of Quantification (LLOQ) is the lowest standard on the calibration curve and should meet the following conditions (according to FDA): The analyte response at the LLOQ should be at least 5 times the response compared to blank response and should be identifiable and reproducible with a precision of less than 20 % and accuracy of 80-120 %.

The calibration curve used should be the simplest model that adequately describes the concentration-response relationship, and has the following conditions to meet (FDA): Not deviate more than ± 15 % from the nominal concentration except for LLOQ that may deviate up to ± 20 %. At least four out of six non-zero standards should meet the criteria including the LLOQ and the calibration standard at the highest concentration level. If a standard is excluded, it should not change the model used.

4.1.4 Stability

Stability is an important part of the method validation and should be evaluated at all steps in the analytical chain from sampling to analysis. It’s important that the conditions used in stability studies imitate the situations samples might encounter during actual sample handling. FDA recommends evaluating the following:

Freeze and Thaw Stability: The effects of repeated freezing and thawing should be evaluated. Stability of the analyte should be determined during at least three freeze and thaw cycles at two concentrations with samples in triplicate. The samples should be stored at the intended temperature for 24 hours and thawed unassisted at room temperature. When the samples have thawed
completely, they should be refrozen at the selected temperature for 12 to 24 hours.

**Short-term stability:** Stability in biological matrix during a short period of time should be determined using a high and low concentration at room temperature. The duration is dependent upon sample handling, e.g. from sample collection to freezing and from thawed to sample preparation, normally about 4-24 hours.

**Long-term stability:** Long-term stability in the selected matrix should be evaluated for samples stored under the same conditions as the study samples and the evaluation should exceed the expected time between the date of the first samples collected and the date of the last sample analysis. There should be enough samples to perform analysis at no less than three separate occasions using a low and high concentration with samples in triplicate.

**Stock solution stability:** FDA guideline states that the stock solution stability should be evaluated for at least 6 hours at room temperature. Additionally it is necessary to evaluate the stability in stock solution for a relevant period of time at the intended storage temperature. Good stability of the stock solution (i.e. several months) eliminates the need of preparing new stock solutions for every new analysis or clinical study.

**Post preparative stability:** Stability of the analyte in processed samples is investigated during the validation. In the papers included in this thesis, it was part of the robustness testing and included steps similar to that stated in the FDA guide. Steps often included are, stability of samples in an autosampler for 24 to 48h, stability of the SPE extracts etc. and should cover the anticipated runtime for a large batch of samples including some extra time for sorting out small equipment problems.
5 Conclusions and future aspects

This thesis describes a brief background of the malaria problem, a short introduction of the drugs used in paper I-VI, an introduction to method development and the final validation process. The starting point of method development is generally to find the expected concentration levels and elimination half-life of the drug and to investigate the physico-chemical properties of the drug. This is important information in the development of a suitable method.

For the drug PND, whole blood was chosen as the preferred matrix because of the high ratio of drug in whole blood compared to plasma and since only a slight haemolysis during sample handling could drastically alter the plasma concentration. The extraction method proved to be accurate and reproducible but blood samples showed poor stability when refrigerated. Freezing and thawing samples did also have a negative effect on stability. In the future, a sampling paper method would be suitable, where 100 μl of capillary blood is applied onto sampling paper. A dried blood spot would probably drastically increase the stability, making it easier to transport and store samples.

Sometimes two antimalarial drugs are combined in a new co-formulation to improve efficacy and this was done in the drug Malarone™. To simultaneously determine concentrations of the highly lipophilic ATQ and the hydrophilic PG with metabolites proved to be a challenge due to the differences in physico-chemical properties of the compounds. The developed method turn out to be a compromise between analyte recovery and reproducible quantification.

Stability is an important factor to investigate and should simulate the environments that the sample might encounter from the time of sampling until it gets analyzed at the laboratory. A new approach to the recommended FDA guidelines was investigated. The new approach used a stability marker within each sample, and it proved to be a useful tool, minimizing day-to-day variations during a long-term stability study.

Parasites developing drug resistance is a major problem and new drugs have to be developed and new drug combinations have to be evaluated for efficacy and safety. One of the new drug combinations recommended by WHO is Artemether-Lumefantrine. It is given in a fix dose combination in the drug Coartem™/Riamet™. A method was developed for determining LF concentrations in plasma with a calibration range suitable for pharmacokinetic studies. It has also been shown that LF concentrations of day 7 are an
important indication of treatment outcome where concentrations below 280 ng/ml are associated with treatment failure. For this reason, a LF method with capillary blood sampling on sampling-paper was developed that is suitable for drug studies in rural settings and will be a complement to microscopic studies on parasite clearance. It is a sought after sampling method that has been used in several studies.

The aim of the screening assay is to determine drug use and self medication in areas where a change in treatment policy has taken place. This change may not always occur simultaneously in both the public and the private sector. In some areas, over the counter sale is common and interviewed patients are sometimes not sure what drug they used. This assay will be a complement to interviewing patients to increase the reliability of the survey.
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Malaria är en parasitsjukdom som finns i över 100 länder i världen men det är framför allt den Afrikanska kontinenten som är värst drabbad med de flesta malariafallen. WHO uppskattar att det förekommer ca 250 miljoner fall av malaria varje år och det resulterar i ca en miljon dödsfall. En härt utsatt grupp är små barn som har hög dödlighet i malaria om den inte behandlas men en annan grupp som också drabbas är gravida kvinnor där det kan uppstå komplikationer under graviditeten på grund av malaria.

Den myggart som sprider malaria parasiter är av släktet *Anopheles* och kännetecknas av att den sitter i vinkel mot huden när den suger blod. *Anopheles* myggen finns i stort sett i hela världen, även här i Sverige, men här finns inte några parasiter som kan spridas vidare. Myggor infekterade med malaria kan ibland spridas via flygplatser, men det krävs vissa betingelser för att parasiterna ska kunna utvecklas i myggen. Bland annat ska det vara tillräckligt varmt och fuktigt, helst över 20°C och då tar det ca 1 till 2 veckor för parasiten att växa och mogna inne i myggen. I Sverige är det för kallt vilket gör att det tar längre tid för parasiterna att utvecklas och chansen är ganska stor att myggen dör innan parasiterna är färdigutvecklade. Efter att man har blivit stucken av en malaraiinfekterad mygga brukar det ta ca 8 till 14 dagar innan symptomen för sjukdomen uppträder t.ex. feber, huvudvärk, frossa.

I de malariadrabbade områdena pågår mycket förebyggande arbeten som att röja tät vegetation och torrlägga områden nära bebyggelse för att minska myggans spridning nära människan, samt att använda impregnerade myggnät över sängar som tar död eller stöter bort myggorna.

Ett stort problem de senaste åren är den snabba ökningen av resistenta parasiter som gör många av de vanligaste läkemedlen i stort sett verkningslösa. Resistensutvecklingen beror till stora delar på att läkemedlen givits som mono-terapi, dvs de innehåller bara en aktiv substans samt att patienter inte fullföljt behandlingen och hoppar över de sista doserna för att de känner sig bättre. Det gör att de parasiter som är mest motståndskraftiga mot läkemedlet överlever och förökar sig vilket kan påskynda resistensutvecklingen. Men det finns också falska läkemedel som säljs billig och som innehåller för låg halt aktiv substans vilket även det påskyndar resistensutvecklingen.

När läkemedel ska utvärderas behövs det analysmetoder för att kunna mäta läkemedelshalten i blodet. Till att börja med används analysmetoder främst för farmakokinetiska studier där man tittar på hur snabbt läkemedlet
tas upp, vilka koncentrationer som uppnås och hur snabbt läkemedlet elimineras ur kroppen. Man tittar också på i vilka nivåer som läkemedlet är verksamt, ligger det för högt kan patienten få biverkningar och för lågt är det verkningslöst och kan påskynda resistensutveckling. Senare analysmetoder är oftast anpassade för enkel provtagning i fält, t.ex. kapillärprovtagning där en droppe blod appliceras på ett provtagningspapper som sedan skickas till laboratoriet. Denna typ av metod är främst avsedd för uppföljning av läkemedelsstudier där man i första hand tittar på blodet i mikroskop för att se om det finns några parasiter kvar i blodet efter behandlingen. Finns det parasiter så tittar man på analyssvaret från laboratoriet om patienten har rätt läkemedelsnivå i kroppen. För låg halt kan tyda på att patienten inte följt upp behandlingen fullt ut eller att doseringen behöver justeras. Men är nivån tillräcklig kan det tyda på resistensutveckling hos parasiten och ett annat läkemedel bör i så fall provas.


Avhandlingen bygger på dessa delarbeten:

I Bestämning av Pyronaridine i blod. Det här var ett relativt nytt läkemedel där det i stort sett endast fanns information från djurförsök. Därför utvecklades denna mätmetod som kunde användas för framtida studier.

II Simultan bestämning av Atovaquone och Proguanil. Sedan tidigare fanns det metoder för att bestämma varje läkemedel för sig men eftersom det numera ges tillsammans som kombination utvecklades här en metod för att bestämma koncentrationen av båda ämnena samtidigt.

III Det är viktigt att veta hur hållbart ett läkemedel är, om den klarar av kylförvaring och transporter under längre tider eller om det försämras med tiden. En ny metod för att studera hållbarheter testades på läkemed-
let Amodiaquine. Metoden gick ut på att tillsätta ett stabilt ämne till pro-
vet som sedan jämfördes med Amodiaquine efter olika tidpunkter för att
följa förändringen. Den nya metoden visade sig fungera och ge mindre
mätvariationer än tidigare metoder.

IV Bestämning av Lumefantrine i plasma. Det fanns sedan tidigare metoder
för bestämning av Lumefantrine men de var inte fullt anpassade för det
mätområde som behövdes för en farmakokinetiskstudie på läkemedlet.

V Bestämning av Lumefantrine i kapillärblod på provtagningspapper.
Denna metod utvecklades för att användas i uppföljningsstudier där lä-
kemedlets effektivitet och möjlig resistensutveckling studeras. Studierna
görs ofta långt ute på landsbygden där förhållandena kan vara mycket
primitiva. Där är det svårare att genomföra venösprovtagning samt kyl-
förvara och transportera prover. I sådana situationer är det mycket enklä-
re att ta kapillärprov än venöst prov, samt att torka in blodet på papper
gör det betydligt lättare att förvara och transportera proverna. Det är
också betydligt enklare att få tillåtelse av föräldrar att ta kapillärprov på
deras barn.

VI I områden med stor resistensutveckling är det hög tid att byta läkeme-
delsrekommendation till ett annat läkemedel. Den här förändringen går
ofta fortast i den offentliga sektorn medan förändringen går betydligt
långsammare i den privata sektorn. Ibland sker också försäljning i vanli-
ga affärer utan krav på läkemedelsordination. När patienter tillfrågas om
vilka malarialäkemedel de tagit, vet de ibland inte. Därför utvecklades
denna screening metod för att kunna detektera de vanligast förekom-
mande malarialäkemedlen, för att avgöra hur utbredd användningen av
vissa läkemedel är samt för att se om den nya läkemedelsrecommenda-
tionen följs.
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