Drug Analysis

Bioanalytical Method Development and Validation

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Abstract

This thesis describes bioanalytical methods for drug determination in biological matrixes, with drugs in focus used against diseases largely affecting low-income countries.

Solid-phase extraction is used for sample cleanup, and processed samples are analyzed by liquid chromatography. Developed bioanalytical methods are validated according to international guidelines.

Efomithine (DFMO) is a chiral drug, used for treating human African trypanosomiasis. A bioanalytical method for determination of DFMO enantiomers in plasma is presented. The enantiomers are detected by evaporative light-scattering detection. The method has been applied to determination of D-DFMO and L-DFMO in rats, after intravenous and oral administration of racemic DFMO. It is concluded that DFMO exhibits enantioselective absorption, with the more potent enantiomer L-DFMO being less favored.

Sulfadoxine (SD) and sulfamethoxazole (SM) are sulfa-drugs used for malaria and pneumonia respectively. Two methods are described for simultaneous determination of SD and SM in capillary blood sampled on filter paper. The former method allows direct injection of extracts from dried blood spots (DBS), while for the latter method solid-phase extraction is added. Pre-analytical factors contributing to measurement uncertainty is also discussed, and it is concluded that it is of high importance that homogeneity in type of sampling paper and sampling volume is assured.

Piperaquine (PQ) is an antimalarial, increasingly used in artemisinin combination therapy. A method for determination of piperaquine in DBS is presented. By using a monolithic LC column, a very short LC analysis of two minutes per sample is achieved.

A method for simultaneous determination of three antiretroviral drugs i.e. lamivudine (3TC), zidovudine (AZT) and nevirapine (NVP), in DBS samples is described. The method is applied to drug determination in two subjects after receiving standard antiretroviral treatment. Conclusion is that the method is suitable for determination of 3TC and NVP, and to some extent for AZT.

Keywords: African trypanosomiasis, calibration model, chiral chromatography, dried blood spots, efomithine, evaporative light-scattering detection, HIV/AIDS, lamivudine, liquid chromatography, malaria, nevirapine, piperaquine, pneumonia, solid-phase extraction, sulfadoxine, sulfamethoxazole, validation, zidovudine

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Author Contribution

*Paper I:* I planed and performed all experiments during method development, performed the method validation and wrote the paper.

*Paper II:* This paper was planed and written by Rasmus Jansson. He also performed all the sampling and did the pharmacokinetic calculations. Together with Rasmus, I was responsible for chiral analysis of samples.

*Paper III:* I planed experiments together with Jenny Lindkvist. Jenny developed the method, and I validated it. We both participated in the analysis and interpretation of data. She wrote the paper and I revised it.

*Paper IV:* Experiments were planed in collaboration with Jenny Lindkvist whom developed the method as well as completed the experimental part of method validation. I performed evaluation during method validation. We both participated in analysis and interpretation of data. I wrote the paper and Jenny revised it.

*Paper V:* Experiments were planed together with Niklas Lindegårdh. I performed all experiments during method development as well as during validation. I wrote the paper and Niklas revised it.

*Paper VI:* I planed experiments together with Susanne Römsing. I developed the method, and Susanne validated it. We both participated in the analysis and interpretation of validation results. Celestino Obua carried out sampling of patients and I analyzed these samples. I wrote the paper with Susanne and Celestino revising it.

Professor Yngve Bergqvist introduced me to the respective project, supervised me during experimental work and method validation, and all resulting papers were revised by Yngve.
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Abbreviations

%RE  Percentage relative error
3TC   Lamivudine
AIDS  Acquired immune deficiency syndrome
ART   Antiretroviral therapy
AZT   Zidovudine
CSF   Cerebrospinal fluid
CSP   Chiral stationary phase
DBS   Dried blood spot
DFMO  Eflornithine
DHA   Dihydroartemisinin
ELSD  Evaporative light-scattering detection
FDA   Food and Drug Administration
HAT   Human African trypanosomiasis
Hib   *Haemophilus influenzae* type B
HIV   Human immunodeficiency virus
LC    Liquid chromatography
LLE   Liquid-liquid extraction
LLOQ  Lower Limit of Quantification
NNRTI Non-nucleoside reverse transcriptase inhibitor
NRTI  Nucleoside reverse transcriptase inhibitor
NVP   Nevirapine
ODC   Ornithine decarboxylase
PABA  p-aminobenzoic acid
PCP   *Pneumocystis jiroveci* pneumonia
PI    Protease inhibitor
PQ    Piperaquine
QC    Quality control
SD    Sulfadoxine
SM    Sulfamethoxazole
SPE   Solid-phase extraction
TDM   Therapeutic drug monitoring
UV    Ultraviolet
WHO   World Health Organization
1 Introduction

This thesis describes analytical methods developed for drug determination in biological matrixes. Drugs in focus are used against diseases greatly affecting people in low-income countries.

Drug determinations can be useful in various areas. When evaluating pharmacokinetic properties of a drug i.e. drug concentration over time, drug determinations need to be made. By these determinations, for instance analyte absorption and elimination and biological availability can be known. Evaluation of adherence is another aim for drug analysis. Poor adherence results in sub-optimal drug levels, increasing risk of resistance developing of parasite, virus or bacteria to the administrated drug. If the drug is present, without patient recovery, this can be an indication of resistance. Drug determinations can also be used for studies of dose versus response i.e. which dose is needed for recovery. Additionally, this approach can be important when drugs have narrow therapeutic windows. Due to inter-individual variability, being large for some drugs, dose adjustment may be needed. Many drugs are enantiomers i.e. molecular mirror images. Although being the same drug, enantiomers can behave differently with respect to efficacy, toxicity, bioavailability among others. Determination of individual enantiomers might for instance help understanding reasons for side effects of drugs. Drug determination starts with sampling and for clinical studies performed in low-income countries, this might be performed in rural settings and/or with limited equipment available. The use of capillary blood on sampling paper i.e. dried blood spots (DBS) is often the desired matrix. When determining pharmacokinetic properties of a drug, plasma is commonly selected as matrix.

Quantitative analysis determines the concentration of a specific analyte in a matrix where other compounds are present. Several analytical methods for determination are based on separating substances from one another by utilizing differences in chemical properties. As biological fluids contain several substances, apart from the analyte of interest, some purification is in most cases needed prior to analysis with liquid chromatography (LC). Solid-phase extraction (SPE) is a selective sample preparation technique suitable when the analyte is present in a complex matrix. By SPE, interfering compounds can be removed. Thereafter, LC is used for separation of the drug from remaining interfering substances. In order to correctly determine concentrations of unknown samples, a calibration model needs to be
selected. Often, ordinary linear regression has been used, although not always optimal. Various approaches to calibration model selection have been published\textsuperscript{3-6}, and some have been applied to the methods described in this thesis. After model selection, a developed bioanalytical method is evaluated thoroughly during method validation, based on international guidelines\textsuperscript{7}. Several issues are addressed such as accuracy and precision of determinations, drug stability at various conditions, and selectivity of the method when possible interferences are present etc. The objective of prevalidation and method validation is to assure that the developed method is applicable to routine analysis. The method needs to be able to make precise and accurate determinations of drug concentrations in unknown patient samples.

The overall aim of this thesis is developing analytical methods for drugs, with the resulting methods being as adapted as possible for studies performed in rural settings in low-income countries. Paper I describes a method for determination of eflornithine (DFMO) enantiomers, by SPE and LC with evaporative light-scattering detection (ELSD). This method is applied in Paper II, on determination of DFMO enantiomers. D-DFMO and L-DFMO are determined in rat plasma after intravenous and oral administration of the racemic drug. In Paper III, a method for determining two sulfa drugs simultaneously, in DBS, is described. Sulfadoxine (SD) is an antimalarial, while sulfamethoxazole (SM) is used for pneumonia. Pre-analytical factors possibly affecting error in drug determination of SD and SM are discussed in Paper IV. In this paper, SPE is added to the previous method. Paper V describes a method for determining the antimalarial piperaquine (PQ) in DBS. Simultaneous determination of three antiretrovirals i.e. lamivudine (3TC), zidovudine (AZT) and nevirapine (NVP) is described in Paper VI, where the DBS sampling technique is used. This method is applied to drug determination in two subjects on standard antiretroviral therapy (ART).
2 Diseases in low-income countries

Neglected tropical diseases, such as Human African trypanosomiasis (HAT), malaria and Acquired immune deficiency syndrome (AIDS) frequently occur as co-infections, influencing morbidity and mortality\(^8\). Also, pneumonia is a common opportunistic infection among children infected with Human immunodeficiency virus (HIV)\(^9\). Co-infection can affect response to treatment with antiparasitic drugs\(^{10}\), while infection with HIV results in greater vulnerability to malaria for children and pregnant women\(^{11}\). In addition, co-infection with HIV and parasitic infections may impact diagnosis of malaria\(^{10}\). Figure 1 shows estimated incidence of HAT, pneumonia, malaria and HIV/AIDS worldwide.

*Figure 1. Estimated incidence of African trypanosomiasis\(^{12}\), pneumonia\(^9\), malaria\(^{13}\) and HIV/AIDS\(^{14}\) worldwide (adapted). Black: African trypanosomiasis (A), pneumonia (P), malaria (M) and HIV/AIDS (H/A). Dark grey: A + P + H/A. Light grey: P + M + H/A. White: P (or missing data) + H/A.*
2.1 African trypanosomiasis

Human African trypanosomiasis (HAT), or sleeping sickness, is caused by protozoan parasites known as *Trypanosoma brucei*, transmitted by tsetse flies of *Glossina* species\(^\text{15}\). HAT occurs in two forms (chronic and acute) as well as in two different stages (early and late)\(^\text{16}\). *T. b. gambiense* causes the chronic form of HAT and infection with *T.b. rhodesiense* results in the acute form\(^\text{17}\). Gambiense HAT occurs in central and West Africa, while rhodesiense HAT arises in Southern and Eastern Africa. The World Health Organization (WHO) estimates that 300 000 to 500 000 people are affected by HAT, but no exact figures are available\(^\text{16}\). It is believed that between 50 000 and 70 000 people die from the disease\(^\text{18}\). HAT was almost eradicated some 50 years ago\(^\text{15}\), but due to lack of human and financial resources and years of conflict in the affected countries the disease was difficult to monitor and control\(^\text{19}\), with epidemics developing without intervention\(^\text{16}\). In contrary, with extensive control elimination of HAT might be possible, at least in secluded areas\(^\text{20}\). Drugs that are used to treat HAT are old, insufficient and highly toxic, with parasitic resistance developing towards them\(^\text{19}\). No vaccine is to be expected in the near future\(^\text{19}\).

2.2 Pneumonia

Pneumonia is a severe form of acute lower respiratory infection, specifically affecting the lungs, resulting in cough and fast or difficult breathing. Severe pneumonia is often the result of a bacterial infection by *Streptococcus pneumoniae* or *Haemophilus influenzae* type B (Hib). *Pneumocystis jiroveci* pneumonia (PCP), caused by a fungal organism, is a common opportunistic infection among HIV-infected children. It is estimated that more than 2 million children die from pneumonia every year. Approximately 1 million of these are children in Sub-Saharan Africa, as compared to one thousand being from industrialized countries. There are vaccines against both Hib and PCP pneumonia, available to various extent for children in low income countries\(^\text{9}\).
2.3 Malaria

Malaria is an infectious disease caused by protozoan parasites of genus Plasmodium, transmitted by the female anopheline mosquito of various species. P. falciparum, P. vivax, P. malariae and P. ovale are the species that account for almost all human infections. Falciparum malaria is common in Africa and is the most severe disease with the highest mortality rate. In vivax and ovale malaria, resting parasites can be reactivated and cause clinical relapse many months after transmission. There are over a quarter of a billion cases of malaria. At least one million people die every year, either as a direct consequence of malaria infection or due to combination with other conditions. Young children and pregnant women are especially vulnerable to malaria. More than 80% of the deaths due to malaria occur in Sub-Saharan Africa. The number of people infected by malaria are increasing, with parasite drug resistance developing being the major cause. Several vaccine candidates are under development and some have entered clinical trials, but a vaccine good enough to be an effective alternative to treatment and mosquito control remains years away. In the absence of an effective vaccine, malaria is primarily controlled by drugs.

2.4 HIV/AIDS

Acquired immune deficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV), belonging to the family Retroviridae. Viruses are transferred through sexual contact, perinatally or through breast feeding. In 2006, approximately 39.5 million people were infected with HIV, with 2.3 million being children under 15 years of age. Coverage of antiretroviral therapy (ART) is estimated to 28% of people in need in this area. Adults receive treatment at greater extent than children. The majority of children (90%) have been infected through mother-to-child transmission. Although such transmission is so very common, most pregnant women are not receiving preventive treatment. Preventive treatment includes early diagnosis, ART, nutritional support and co-trimoxazole prophylaxis. In 2005, it was estimated that only 11% of pregnant women living with HIV, received antiretroviral prophylaxis to hinder transfer to their children.
3 Pharmaceuticals in resource limited settings

3.1 Antitrypanosomal drugs

Current treatment of HAT is based on five drugs that can be divided into two groups. The first group consists of those effective for early stage disease. Drugs crossing the blood-brain barrier, and thereby being suitable for late stage disease, are found in the second group. Suramin and pentamidin are used for early stage HAT, both being developed more than a century ago. The most commonly used drug for late stage HAT, against both *gambiense* and *rhodesiense* disease is melarsoprol. Melarsoprol is becoming less and less effective. Also, the drug itself can be a cause of death. Nifurtimox has been used to treat second stage HAT, when patients do not respond to other treatments, but is currently not registered for this disease. Combination of eflornithine (DFMO) and nifurtimox is being evaluated. DFMO is the only new drug registered for treatment of HAT over the past 50 years. There is one drug in phase III clinical trials i.e. DB289, although only useful for treating first stage disease. Some drugs are being evaluated in animals.

3.1.1 Eflornithine

![Chemical structure and pKₐ-values of Eflornithine (DFMO).](image)

*Figure 2. Chemical structure and pKₐ-values of Eflornithine (DFMO).*
DL-α-difluoromethylornithine, often named DFMO or eflornithine (Figure 2), was originally developed in the early 1980s cancer research. More recently, DFMO is being used as the active component in DFMO cream, for reducing growth of unwanted facial hair. However, back in the 1980s, the drug was found to be active on trypanosomes in vivo. Later on, DFMO was nick-named “the resurrection drug” for its amazing ability of pulling people out of coma. Although DFMO is only effective for late stage T.b. gambiense infection it is becoming increasingly evident that DFMO is both safer and more effective than melarsoprol.

A drawback of DFMO is that the drug must be administered by slow drip infusions repeated every six hours during 14 days, which is not ideal for use in rural Africa. Development of oral DFMO would greatly simplify administration and enable treatment at remote sites, but oral DFMO studies have not yet been successful. As bioavailability is rather low for oral DFMO, dosage required for achieving sufficient plasma and cerebrospinal fluid (CSF) concentrations of DFMO is high. This leads to gastro-intestinal side effects, further compromising absorption. If resulting concentrations become insufficiently low relapses occur, and risk of resistance developing against DFMO increases.

Trypanosomes hide from the immune system by continuously changing their antigenic coat of variant surface glycoproteins. Decarboxylation of ornithine is a necessary stage in the polyamine biosynthesis necessary for cell differentiation and division. Inactivation of ornithine decarboxylase (ODC) by DFMO results in trypanosomes incapable of modifying their surface antigens or dividing, making the host’s immune response more effective. The two enantiomers of DFMO differ in their ability to inhibit ODC, with the L form being more potent than the D enantiomer.

DFMO has negligible plasma protein binding. Mean half-life is 3.3 h in human volunteers when receiving low oral doses (5–10 mg/kg body weight). Drug levels in CSF must be above 50 μM of D/L-DFMO to attain consistent clearance of parasites. In plasma, racemic DFMO concentration is roughly 8 times CSF concentration.

DFMO has three pKₐ values, pKₐ 0.08 for the carboxylic group, 6.4 and 10.4 for the amine groups. When comparing distribution coefficients at different pH (the logD of [drug] lipid/[drug] water) it is apparent that DFMO is highly water soluble throughout the entire pH range, with logD values ranging between -3.66 and -2.21.

To investigate the behaviour of the DFMO enantiomers, when administering oral racemic DFMO, a chiral method of determination for the individual enantiomers in biological matrix is needed. As DFMO lacks chromophores and fluorophores, it cannot be analyzed directly using ultraviolet (UV) or fluorescence detection. There are chromatographic methods published for the determination of racemic DFMO, in biological matrices such as human plasma, CSF and urine. These methods are all based...
on pre- or postcolumn derivatization with either dansyl chloride\textsuperscript{48}, O-Phtaldialdehyde\textsuperscript{36, 49, 50} or naphthalene-2,3-dicarboxaldehyde/cyanide\textsuperscript{47, 51} with fluorescence or UV detection. There are few chiral methods for determination of the DFMO enantiomers described, and D-DFMO and L-DFMO are not determined in human plasma or CSF\textsuperscript{43, 52}.

**Paper I** describes a bioanalytical method for the determination of DFMO enantiomers in human plasma. In **Paper II**, the method is used for determination of D-DFMO and L-DFMO in rats that were administered racemic DFMO orally and intravenously. A partial validation\textsuperscript{7} was performed prior to analysis due to change of species (human to rat) as well as the use of smaller sampling volume (300 μL compared to 1000 μL). It is concluded that DFMO exhibits enantioselective absorption with the more potent enantiomer L-DFMO being less favoured, as can be seen in Table 1. This could help explain why oral treatment with racemic DFMO is associated with poor response. Oral therapy with DFMO might be more successful if bioavailability of L-DFMO can be improved.

**Table 1.** Estimated bioavailability (F) after oral administration of racemic DFMO, results obtained from Paper II.

<table>
<thead>
<tr>
<th>Racemic dose DFMO (mg/kg bodyweight)</th>
<th>L-DFMO Mean F (%)</th>
<th>D-DFMO Mean F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>750–2000</td>
<td>41</td>
<td>62</td>
</tr>
<tr>
<td>3000</td>
<td>47</td>
<td>83</td>
</tr>
</tbody>
</table>
3.2 Antipneumonia drugs

The sulfonamides were first discovered in the 1930s as antibacterial agents. Folic acid is required for synthesis of DNA and RNA in both bacteria and mammals. Mammals can obtain folic acid through diet. For bacteria, folic acid requires synthesis, with p-aminobenzoic acid (PABA) being an essential part of resulting folic acid. As sulfonamides are structural analogues of PABA, their mode of action is by competing with PABA for an enzyme needed in the production of folic acid. This results in disrupted DNA and RNA synthesis and inhibition of bacterial growth\textsuperscript{53}.

3.2.1 Sulfamethoxazole

![Chemical structure and pK\textsubscript{a}-values\textsuperscript{46} of Sulfamethoxazole (SM).](image)

Sulfamethoxazole (SM) (Figure 3) is an antibacterial drug part of a fixed dose combination with trimethoprim, often used to treat children with pneumonia in low income countries\textsuperscript{9}. Also, sulfamethoxazole-trimethoprim i.e. co-trimoxazole is increasingly used as prophylaxis against PCP pneumonia for people living with HIV\textsuperscript{54}. Mode of action for SM is by disrupting folic acid synthesis, thereby eliminating growth of bacteria\textsuperscript{53}.

Plasma protein binding of SM is 63.4\%\textsuperscript{55}. SM concentrations have been determined in plasma of healthy volunteers, after a single dose of two tablets of co-trimoxazole containing 400 mg SM per tablet. Maximum concentration in plasma was approximately 156 \mu mol/L and minimum concentration 20 \mu mol/L 30 hours after dosage. Half-life was 9.6 hours\textsuperscript{56}. SM is metabolized through hydroxylation, acetylation and glucuronidation, with only the acetylated metabolite N\textsubscript{4}-acetylsulfamethoxazole being detectable in plasma\textsuperscript{57}.

SM acts both as acid and base, with the primary amine having pK\textsubscript{a} 1.39 acting as base, and the secondary amine being acidic with pK\textsubscript{a} 5.81\textsuperscript{46}. SM water solubility is increased with pH (Figure 4) and it is apparent from the logD profile\textsuperscript{46} that SM is less water soluble than sulfadoxine.
Figure 4. LogD profile for Sulfamethoxazole (SM) and Sulfadoxine (SD)\(^{46}\).

There are methods described for determination of SM in human plasma, by LC and UV detection\(^{56, 57}\), but not in DBS samples. In Paper III, a method is described for determination of sulfadoxine and SM simultaneously. This method has been applied to a clinical study evaluating caretakers’ reports on antimalarial and antibiotic use in children with severe pneumonia in Uganda\(^{58}\).
3.3 Antimalarial drugs

At the beginning of the seventeenth century “Peruvian” bark was discovered for treatment of fevers. In 1735, the tree producing this bark was given its scientific name *Cinchona* by Linnaeus. Quinine, the active compound, was not isolated until 1820. Thereafter, antimalarial drug discovery has been driven by war, resulting in development of synthetic antimalarials such as chloroquine, amodiaquine, pyrimethamine and mefloquine. Sulfadoxine was developed as an antibacterial agent in the 1930s, but it was not until some 30 years later its value as an antimalarial drug was discovered\(^{69}\). Increasing resistance of *P. falciparum* to conventional treatments such as chloroquine, sulfadoxine-pyrimethamine and amodiaquine is probably the main cause of increase in malaria mortality observed in eastern and southern Africa\(^{13}\). In China, infusions prepared from wormwood, *Artemisia annua*, were used for treating fevers over a thousand years ago. Nowadays, artemisinins have become a very important antimalarial drug group\(^{60}\). Development of drug resistance is slowed by using different antimalarial drugs in combination. Therefore recommended policy is to introduce new antimalarials as components of fixed-dose combinations. Between 2001 and 2004, forty malaria-endemic countries used artemisinin combination therapy as first- or second-line malaria treatment, and others are in process of changing their policies\(^{61}\). Also, the use of this type of drug therapy in combination with long-lasting insecticidal nets has been shown to reduce the morbidity and mortality due to malaria\(^{62}\).

3.3.1 Sulfadoxine

![Chemical structure and pK\(_a\)-values of Sulfadoxine (SD).](image)

*Figure 5. Chemical structure and pK\(_a\)-values\(^{63}\) of Sulfadoxine (SD).*
The antimalarial activity of the sulfonamide sulfadoxine (SD) (Figure 5) was discovered during the 1960s\textsuperscript{59}. Somewhat later it was noticed that the activity of SD was increased in combination with pyrimethamin, and monotherapy of SD was discontinued\textsuperscript{64}. SD mode of action is, as for other sulfonamides, by competing with PABA and disrupting folic acid synthesis, thereby inhibiting parasite growth\textsuperscript{53}.

For SD, plasma protein binding is 90\%, with SD mainly binding to albumin\textsuperscript{64}. In one study SD concentrations in DBS were 124 (93–180) μmol/L on day 7, and half-life was 6.7 days\textsuperscript{65}. SD is metabolized to a small extent, approximately 10\%, by acetylation or glucuronidation\textsuperscript{64}.

SD, as well as SM, acts both as acid and base, with the primary amine having pK\textsubscript{a} 1.52 acting as base and the secondary amine with pK\textsubscript{a} 6.01 being acidic\textsuperscript{46}. SD is only slightly soluble in water\textsuperscript{64}, but solubility is improved with increased pH (Figure 4).

Methods to measure SD in DBS\textsuperscript{66, 67}, whole blood, red blood cells and human plasma\textsuperscript{68} have been described using either liquid extraction from DBS\textsuperscript{66}, LLE\textsuperscript{68} or SPE\textsuperscript{67}, followed by LC and UV detection. Papers III and IV describe two methods for determination of SD in combination with SM. First, a direct injection method was developed, where extract from DBS is injected into the LC, thereby greatly reducing time of sample preparation and limiting the need of equipment. The resulting method is suitable for analysis of DBS samples in rural settings when monitoring adherence. However, when analyzing samples by this method, corrosive perchloric acid is injected, requiring extensive rinsing of the injector port. In Paper IV, SPE was added in order to remove the perchloric acid prior to analysis. The method described in Paper III has been used for simultaneous determination of SD and SM when evaluating caretakers’ reports on antimalarial and antibiotic use in children. Conclusion drawn is that reported drug intake has limited validity\textsuperscript{58}.

3.3.2 Piperaquine

![Chemical structure and pK\textsubscript{a}-values\textsuperscript{69} of Piperaquine (PQ).](image)

*Figure 6. Chemical structure and pK\textsubscript{a}-values\textsuperscript{69} of Piperaquine (PQ).*
Piperaquine (PQ) (Figure 6) was produced in 1966\textsuperscript{70}, replacing chloroquine in 1978 as the antimalarial drug recommended in China\textsuperscript{71}. A fixed dose co-formulation of dihydroartemisinin (DHA) and PQ was developed through an international public-private partnership\textsuperscript{72}. DHA-PQ has been evaluated in clinical trials where patients have been treated for uncomplicated \textit{falciparum} malaria, and the drug combination has proven to be safe, tolerable and effective\textsuperscript{73-75}. Additionally, DHA-PQ is cheaper than for instance artesunate-mefloquine\textsuperscript{76}. One Phase III clinical trial in Africa has been completed in 2006, and another is still ongoing in South-East Asia\textsuperscript{77}, as is required for international registration\textsuperscript{71}.

Mode of action of PQ is believed to be the same as for chloroquine, by inhibition of the heme digestion pathway in the parasite food vacuole\textsuperscript{78}. Concerns have been raised regarding whether or not cross-resistance between chloroquine and PQ might be an issue in Africa where chloroquine resistance is problematic\textsuperscript{75}. However, so far DHA-PQ is effective against multi-drug resistant strains of \textit{P. falciparum}\textsuperscript{71, 76, 79, 80}.

PQ has a plasma protein binding of \textgreater{}97\%\textsuperscript{81}. Mean elimination half-life was in one study determined to 23 days in adults\textsuperscript{82}, but this might be an underestimation as another study suggests a t\textsubscript{1/2} of 36 days\textsuperscript{83}. When determining PQ half-lives it is of great importance that such a study is performed with extended sampling, beyond 29 days\textsuperscript{84}. In one clinical study, day 7 PQ concentrations in DBS varied between 88 and 168 nmol/L\textsuperscript{76}.

There are five metabolites of PQ identified and characterized. One is a carboxylic metabolite of PQ, while another is formed by N-oxidation. Two metabolites are hydroxylated forms of PQ. The fifth metabolite is either formed by double N-oxidation or hydroxylation. PQ metabolites need to be further evaluated when determining safety of PQ\textsuperscript{85}. The first two metabolites mentioned above can be detected in plasma\textsuperscript{85, 86} as well as in urine\textsuperscript{85, 87}. In \textbf{Paper V}, two patient samples were analyzed for determination of PQ in DBS, with no interfering endogenous compounds found, although potential presence of metabolites was not investigated. It is not likely that PQ metabolites co-elutes with PQ, when determining the analyte in DBS. When analyzing PQ in plasma (similar LC settings) metabolites elute either prior to or after PQ\textsuperscript{86}.

Another concern brought up recently, is the presence of impurities in old batches of DHA-PQ i.e. Artekin\textsuperscript{®} as well as in the PQ drug substance\textsuperscript{88, 89}. The amount of impurity ranged between 1.5 and 5\%. Modification in the production process has led to significant reduction of impurity being present in batches of PQ\textsuperscript{88}. It is probable that this impurity was present in the PQ drug substance used during method development and validation presented in \textbf{Paper V}, possibly affecting accuracy of the method. A partial validation with new PQ substance is advisable before performing further clinical studies using this assay.
PQ is a basic analyte with four pKₐ values. Titrations of PQ resulted in pKₐ values 6.88 and 6.24 for the two aromatic amidinium ions, while being 5.72 and 5.39 for the respective nitrogen connected to the carbon chain. PQ is poorly soluble in methanol, but solubility is greatly increased when the methanol is acidified. The substance is practically insoluble in pure water, although it is possible to dissolve PQ in 0.05 M phosphoric acid. Solubility being pH dependent is evident when comparing logD values at different pH. At pH 7.4, logD is 5.99, at 5.2 it is 2.41 while it at pH 4.8 is only 0.97, showing that solubility of PQ in water increases with a decrease in pH.

There are methods published for determination of PQ in various matrices. Methods for determination of PQ in plasma using LLE and SPE for sample preparation have been described. A high throughput assay for PQ determination in plasma using 96-wellplates has been published. Other matrices reported are whole blood and urine. Recently, a method for determining PQ in plasma by LC and tandem mass spectrometry has been published, using 96-wellplate SPE during sample preparation.

Determination of PQ in DBS is described in Paper V. This method uses SPE combined with LC and UV detection. PQ is extracted on a mixed mode cation-exchange SPE column. PQ and the internal standard (IS) are then separated on a monolith column allowing high flow-rates, resulting in a 2 minutes analysis time per sample. The method described in Paper V has been used in a clinical study comparing DHA-PQ with artesunate-mefloquine, as well as evaluating adherence to drug therapy. Conclusions are that DHA-PQ is a good alternative to artesunate-mefloquine, with good adherence without supervision.
3.4 Antiretroviral drugs

ART for HIV infection consists mainly of combinations of nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI). PIs are used in second-line regimens. WHO recommend first-line ART to be constructed of two NRTIs and one NNRTI\textsuperscript{93}.

Therapeutic drug monitoring (TDM) can be useful when optimizing response to ART. In TDM, drug dosages are individualized so that certain drug concentrations are achieved, within the therapeutic range between suboptimal and toxic levels. If inadequate drug concentration is determined, this may reflect an insufficient dose for the patient or indicating non-adherence. Measuring antiretroviral drugs can also provide some means to hinder drug resistance from developing. The effect of ART is evaluated by monitoring viral load and measuring CD4+ cell count. These parameters aren’t sufficient for optimal prevention, as resistance may already have been developed by the time an increase in viral load is observed\textsuperscript{94}. If ART failure occurs, assessment of adherence by TDM can be highly important before resistance testing\textsuperscript{95}. Also, drug interactions might occur between antiretrovirals and other drugs present, potentially requiring monitoring, alteration of drug dosage or timing of administration\textsuperscript{96}.

3.4.1 Lamivudine, Zidovudine and Nevirapine

![Chemical structures of Lamivudine, Zidovudine and Nevirapine](image)

*Figure 7. Chemical structure and pK\textsubscript{a}-values\textsuperscript{46} of A) Lamivudine (3TC), B) Zidovudine (AZT) and C) Nevirapine (NVP).*
Lamivudine (3TC) and zidovudine (AZT) are preferred NRTIs in first-line regimens in resource-limited settings. Nevirapine (NVP) is widely available and is less costly than the alternative NNRTI efavirenz. The 3TC, AZT and NVP triplet is available as fixed-dose combination\textsuperscript{93}. Chemical structures of 3TC, AZT and NVP are shown in Figure 7.

TDM has mainly been focused on NNRTIs and PIs, as current understanding is that drug concentration measures are of little value for NRTIs. The NRTIs require intracellular activation to the active triphosphate anabolite, with anabolite concentrations correlating poorly with plasma concentrations of the parent drug\textsuperscript{97}. No quantitative relationship has been found between plasma concentrations of NRTIs, intracellular triphosphate concentrations and antiviral effect. However, this should not be taken as evidence that concentration determinations of NRTIs have no clinical value\textsuperscript{98}. On contrary, in the future it may become necessary to measure NRTIs when performing clinical trials in low-income countries\textsuperscript{99}.

The 3TC and AZT antiretroviral activity is due to conversion into lamivudine triphosphate and zidovudine triphosphate. These anabolites have the ability to inhibit reverse transcription of the HIV virus, thereby hindering viral replication. 3TC is excreted primarily as unchanged drug (68–71\%), having mean half-life of 2.5 h and protein binding of less than five percent. AZT is primarily eliminated through glucuronidation with 60–70\% being eliminated as glucuronide. 10–20\% is excreted as unchanged drug. Half-life for AZT is approximately 1 hour and plasma protein binding is about 20\%\textsuperscript{100}. Target steady state plasma concentrations of 0.71 μmol/L for 3TC and 1.9 μmol/L for AZT have been used when evaluating concentration-controlled therapy i.e. TDM\textsuperscript{101}. These concentrations for 3TC and AZT have also been used as targets when investigating lamivudine triphosphate and zidovudine triphosphate concentration-response relationships\textsuperscript{98}. Antiretroviral activity for NVP is due to the drug acting as a non-competitive enzyme inhibitor binding to reverse transcriptase\textsuperscript{102}, thereby disrupting viral replication. NVP is transformed to hydroxylated metabolites, and is largely eliminated as glucuronidated metabolites (80\%), with only a small fraction excreted as parent compound (<3\%)\textsuperscript{103}. NVP half-life is approximately 30 h and protein binding is 60\%\textsuperscript{100}. Therapeutic target concentration in plasma is 13 μmol/L for NVP\textsuperscript{104}. Correlation between plasma levels and DBS levels of NVP has been found, with DBS concentrations somewhat lower\textsuperscript{105}.

3TC acts as a basic compound with pH\textsubscript{a} 3.31, while AZT is acidic with pH\textsubscript{a} 9.36\textsuperscript{46}. NVP acts as base with pH\textsubscript{a} 2.42\textsuperscript{46}. As can be seen by the logD profile\textsuperscript{46}, Figure 8, these analytes differ greatly with respect to solubility, with 3TC and AZT being hydrophilic and NVP hydrophobic.
There are several bioanalytical methods for determination of antiretrovirals in biological fluids, including NRTIs, mostly for measurements in human plasma. LC tandem mass spectrometric methods in combination with SPE for sample clean-up have been described for determination of several antiretroviral drugs simultaneously\textsuperscript{106, 107}. Gradient LC methods with UV detection determining up to 16 antiretrovirals at once have also been described, utilizing SPE during sample preparation\textsuperscript{108, 109}. Isocratic LC has been applied to determination of AZT and NVP\textsuperscript{110}, while ion-pair LC has been practiced when determining 3TC, AZT and NVP\textsuperscript{111}. Two methods have been described for determination of antiretroviral drugs in DBS. Radioimmunoassay has been used to measure AZT only\textsuperscript{112}, and a method with LC and mass spectrometry has been described for simultaneously determining several PIs and NNRTIs but none of the NRTI drugs\textsuperscript{105}.

**Paper VI** describes a method for simultaneously determining 3TC, AZT and NVP in DBS, by SPE and gradient LC-UV. Determination of drug levels in clinical samples from two subjects on standard twice daily ART (3TC 150 mg, AZT 300 mg and NVP 200 mg) is presented. With this method it appears possible to determine 3TC and NVP in DBS patient samples, from the time dose is administered and prior to the next dose. For AZT, due to the short half-life of the drug, only the time span of 0-2 hours was possible to follow, at least for the two subjects studied in this paper.
4 Bioanalysis

4.1 The analytical chain

The analytical chain describes the process from sampling to result i.e. the drug concentration determined in the sample.

In bioanalytical drug analysis, common matrices are venous blood and plasma. Venous blood, with anticoagulant, is after centrifugation separated into plasma and blood cells. Centrifuged blood consists of 55% plasma and 45% blood cells. The plasma matrix is composed of some 90% water with remaining 10% being mostly plasma proteins\(^{113}\). Sampling of biological matrix is the first step in the analytical chain, and these samples must regularly be stored in a cooler or freezer before assay. When the samples have arrived at the laboratory, they need to be prepared for analysis. The aim is to improve the assay by removing interferences in the matrix, and often also concentrating the analyte. The need of sample preparation is determined by the complexity of the samples and by the application of the analytical method. For analytical methods developed for clinical studies, separation of analytes from matrix components and detection at low concentrations are often issues to account for.

Common methods for sample preparation are protein precipitation, LLE and SPE. As proteins present in the biological fluid can be problematic if a sample is injected directly into the chromatographic system, removal of proteins is regularly necessary. This can be done by denaturation of proteins by addition of acids, organic solvents or ionic salts\(^{114,115}\). LLE can be time demanding and difficult to automate. SPE has a higher chemical selectivity as compared to LLE since there are great numbers of stationary phases available, based on different separation mechanisms. One limitation of SPE is the possibility of clogging the column or blocking its pores with matrix components. By utilizing protein precipitation prior to SPE, this problem is minimized\(^{116}\).

SPE is based on four steps i.e. conditioning of the SPE column, sample load, washing out interferences and eluting the analyte. Often an evaporation step is added so that the analyte is concentrated or at least less diluted. Another reason for evaporation is that the elution fluid might be
incompatible with the chromatographic system and the analyte need to be transferred to a more suitable injection fluid.

By using LC, analyte and IS can be separated from endogenous compounds still present in the injected sample, as well as from each other. Sample containing analyte, IS and remaining interferences is transferred via a mobile phase through a chromatographic column. The resulting peak corresponds to the analyte concentration\textsuperscript{2}. Chromatograms from calibration standards with known concentration are evaluated, and the relationship between response and concentration is established. From this, the concentration in an unknown sample can be determined. In order to verify that the developed method will result in accurate and precise determinations, that the method is selective and the analyte is stable, the bioanalytical method is validated according to published guidelines before applying it to routine use\textsuperscript{7}.

### 4.2 Sampling in rural areas

When performing biological sampling, there must be facilities for storage i.e. cooler and freezer. However, for clinical studies concerning drugs against tropical diseases, samples can be collected at sites remotely located from the laboratory, lacking such facilities, and storage as well as transportation can be an issue. Capillary blood applied on sampling paper can be dried at room temperature and sent by ordinary mail. In a patient perspective, DBS sampling has important advantages. This sampling method is especially advantageous for children, as only 100 μL blood is needed, and no venipuncture is necessary. For the laboratory personnel, the risk of HIV infection when handling the samples is reduced\textsuperscript{117}. As for the analytical chemist developing bioanalytical methods with the DBS matrix, such small blood volume and the more complex sample (compared to venous blood or plasma), can be rather troublesome, as achieving high assay sensitivity is more difficult. Apart from extracting the analyte from the biological matrix, the analyte also needs to be extracted from the sampling paper.

Common brands of sampling papers are 903® specimen collection paper, 3MMChr and 31ETChr (Whatman International Ltd, Maidstone, UK). Another paper is 2992. The 903® paper is commonly used for neonatal screening, while both 3MMChr and 31ETChr are blotting papers. These papers consist of pure cellulose produced from cotton linters, with some variation with respect to absorption properties and thickness. While 31ETChr, being the thickest, has superior properties over 3MMChr regarding absorption resulting in more symmetric blood spots, the latter is much less costly. At least from our experience, 903® is more difficult to purchase as usage needs to be clarified when ordering. This might be due to the fact that 903® is registered as an \textit{in vitro} medical device.
In Papers IV, V and VI, comparisons were performed between sampling papers. In Paper IV, SD and SM extractions were compared for 31ETChr, 903® and 2992. Conclusions drawn are that mean extraction recoveries are significantly higher for 903® and 2992, while extraction of the analytes from blood dried on 31ETChr is more reproducible. Also, the latter paper has the best absorption properties. For PQ, described in Paper V, no significant difference is found between papers, and 31ETChr is chosen for this method. As both 903® and 2992 seemed more difficult to purchase, in Paper VI, it appeared wise to exclude these labels. For extraction of 3TC, AZT and NVP, two sampling papers are compared i.e. 31ETChr and 3MMChr. No significant difference in extraction recoveries are found, with precisions being similar between papers, although 31ETChr is somewhat more precise, as can be seen in Figure 9. As 3MMChr is much less costly, this paper is chosen for the method. Comparison is performed by calculating the statistic \( t \), at 95% confidence level, determining whether two means differ. Assumed is that variances are approximately equal. Sampling papers are compared at three concentration levels, and for all three analytes no significant difference is found between means of extraction recoveries. For 3TC there is a difference between standard deviations at the lowest concentration. However, both middle and high concentration variances are similar. Therefore, conclusion made is that there is no difference in extraction recoveries between 31ETChr and 3MMChr.

![Figure 9](image_url)

Figure 9. Comparison between extraction recoveries on Whatman 31ETChr and 3MMChr papers for antiretrovirals (Lamivudine (3TC), Zidovudine (AZT) and Nevirapine (NVP)), n=3. Evaluation was performed during method development in Paper VI.
During sample preparation, the DBSs are cut into small pieces, as described in Papers III and V. As this is laborious, some attempts are made in Papers IV and VI to improve this. In Paper IV, blood spots are punched out. Since it wasn’t possible to find a suitable and commercially available punch that could easily be obtained, in Paper VI blood spots are cut out in full with scissors. The latter approach also avoids actually cutting in the blood samples, thereby minimizing risk of transferring the analyte between samples. There is no significant difference between extraction recoveries as can be seen in Figure 10. Comparison is made by calculating the statistic $t$.

**Figure 10.** Comparison between extraction recoveries for cut blood spots and whole blood spots for antiretrovirals (Lamivudine (3TC), Zidovudine (AZT) and Nevirapine (NVP)), n=3. Evaluation was performed during method development in Paper VI.

When extracting analytes from DBSs, several fluids need to be evaluated such as salt solutions, acids and bases. In Paper V, PQ is extracted with a mixture of perchloric acid, acetonitrile and phosphate buffer, while SD and SM are extracted with perchloric acid only. For ARVs, in Paper VI, zinc sulphate with methanol is the only combination providing rather clean extracts while managing to extract the analytes in sufficient amounts.
4.3 Solid-phase extraction

SPE is a selective method for sample preparation where the analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Due to many different choices of sorbents, SPE is a very powerful technique\(^1\). SPE consists of four steps; conditioning the column, loading the sample, washing the column and finally eluting the analyte. First, the SPE column is conditioned by passing a solvent through the sorbent to wet the packing material, activate functional groups and remove impurities as well as air present in the column. In reversed-phase SPE, common solvents are methanol and acetonitrile. The organic solvent is followed by a buffer for compatibility with aqueous samples. The sample containing the analyte is then loaded on the column. In this step, the analyte is retained along with some matrix components, while other passes through. A wash step removes interferences while still retaining the analyte. Finally, the analyte is eluted from the sorbent by applying a solvent capable of disrupting analyte-sorbent interactions. Ideally, there will be no interferences present in the elute. Often evaporation and reconstitution is performed, in order to transfer the analyte into a solvent more compatible with the chromatographic settings, and also to concentrate the analyte.

Typically, sorbents used in SPE consists of 40 μm diameter silica gel with approximately 60 Å pore diameters. To this silica gel, functional groups are chemically bonded, for different modes of action. The most commonly used format is a syringe barrel that contains a 20 μm frit at the bottom of the syringe with the sorbent material and another frit on top, referred to as packed columns. Extraction disks are also placed in syringe barrels. These disks consist of 8–12 μm particles of packing material imbedded into an inert matrix. Disks are conditioned and used in a similar way as packed columns. The major advantage of disks compared to packed columns is that higher flow rates can be applied\(^113\). Packed columns and extraction disks are also available in 96-well plate format. Benefit of well plates is the possibility to process large numbers of samples, either manually via a special vacuum manifold, or via automated instruments. This format makes manual processing easier and quicker compared to working with individual cartridges. However, variation of vacuum between wells can be a problem\(^118\).

The sorbent functional groups used for SPE are similar to those used in LC i.e. normal phase, reversed phase and ion-exchange. Normal phase sorbents are polar and used for samples where the analyte of interest is present in an organic solvent. Reversed phase is used for aqueous samples, where the analyte is hydrophobic and retained on a nonpolar sorbent. Ion exchange sorbents isolate analytes based on the ionic state of the analyte\(^113\). Mixed sorbents are also available, combining these modes. Polymeric sorbents are made of divinylbenzene instead of silica, and are often comparable with reversed phase sorbents\(^118\).
Analytes can be classified into four categories; basic, acid, neutral and amphoteric compounds. Amphoteric analytes have both basic and acidic functional groups and can therefore function as cations, anions or zwitterions, depending on pH\textsuperscript{113}. Three issues are of high importance when developing a SPE method. First, one must choose the proper sorbent functional group to be able to retain and elute the analyte. Secondly, for all but neutral analytes, an efficient use of pH is needed to shift the analyte between ionic and uncharged form. Adjustment of pH is used to vary retention and improve selectivity. In some cases, functional groups in the column are affected by pH, and made ionic or uncharged. Thirdly, solubility of the analyte in all solvents used for the extraction method need consideration\textsuperscript{1}.

4.3.1 Cation-exchange sorbents

In strong cation-exchange SPE, sulphonic acid functional groups are present with protons as counter ions. When other cations enter, there is an exchange of ions. Apart from the analyte, there are additional cations present in the sample, all competing for sites on the column. The pK\textsubscript{a} of the analyte is a critical factor when using ion-exchange, since a cationic drug is ionic below its pK\textsubscript{a} and uncharged above. Strong cation-exchange SPE columns are charged in the full pH range. Other factors that affect retention in ion-exchange include the charge of the analyte that is being extracted as well as charges of competing ions. A singly charged ion has less affinity than a doubly charged one. Also, the time it takes for the analyte to diffuse and come into contact with functional groups on the SPE column depends on the size of the ions, as large ions diffuse more slowly. Additionally, the kind of matrix from which the drug is being removed need to be considered\textsuperscript{113}. With ion-exchange being a more selective mode of SPE, it could be advantageous over reversed-phase SPE for complicated matrixes\textsuperscript{118}.

In Paper I, a packed bed strong cation-exchange column is used for DFMO. In Paper V, an extraction disk mixed mode strong cation-exchange column, combined with reversed phase C8, is utilized for PQ. DFMO is a zwitterion, while PQ is a weak basic drug. They are treated as cations in both SPE methods developed. Loaded samples are acidic to facilitate retention of the analyte, keeping pH low during wash. Finally, the analyte is eluted at basic pH where the analyte is transferred into uncharged form i.e. converting positive groups into neutral. After SPE, an evaporation step eliminates the incompatible solvent and allows reconstitution in a solvent compatible with the LC settings. For DFMO the SPE step has the additional benefit of concentrating the analyte five times, thereby increasing assay sensitivity.
4.3.2 Polymeric sorbents

Polymeric sorbents contain organic polymers, styrene-divinylbenzene, instead of silica. These polymers might be hydroxylated or N-vinylpyrrolidone groups may be added, resulting in differences between phases. These sorbents are usually used similarly to silica SPE columns in reversed-phase mode, although being more capable of retaining analytes with greater polarity\(^1,113\). Also, polymeric sorbents generally don’t need to be conditioned prior to sample application, although this step is often included in order to remove residues from the manufacturing process\(^113\). Another advantage is that no secondary interaction from silanols is present, resulting in more predictable retention of the analyte. Polymeric sorbents functioning as ion-exchange sorbents are also available\(^1\).

In Paper IV, IST ENV+ was used for retention of the sulfamethoxazole (SD) and sulfisoxazole (SM) simultaneously. ENV+ is a hydroxylated polymeric stationary phase. The retention capacity of ENV+ was apparent when developing the SPE method, as it was necessary to reduce the bed mass from 100 mg to 25 mg in order to elute the analytes sufficiently. In Paper VI, Oasis HLB, containing N-vinylpyrrolidone groups, was used during SPE for the antiretrovirals 3TC, AZT and NVP. As this column had previously been used during sample clean-up for these drugs in human plasma\(^111\), and extraction of NVP from DBS had been described with a mixture of methanol and 0.2 M zinc sulphate in equal quantities\(^105\), effort was made to combine the extraction from DBS with the SPE method. It was noticed that with methanol:0.2 M zinc sulphate 50:50 (v/v), extraction recoveries of 3TC and AZT were very poor. Also, problems occurred with columns clogging during sample load. The amount of methanol present and the concentration of zinc sulphate were reduced. However, without methanol, the extraction fluid from DBS was more coloured and the sample contained more debris. Therefore, the final extraction fluid was methanol:4 mM zinc sulphate (1:99 v/v).

4.4 Liquid Chromatography

In LC, the stationary phase is composed of spherical particles with diameters ranging between 2 and 5 μm. Due to the small size of these particles high pressure is needed for the mobile phase to flow through the column, and therefore this technique is occasionally called high pressure LC.

Chromatography aims to separate analyte and IS from each other and from compounds still present in the injected sample. The LC system consists of several components interconnected by short capillaries with small internal diameters. A liquid phase is transported through the system by a pump, and the sample to be analyzed is injected into this flow by an injector. The mobile phase transports analyte and IS through a chromatographic column,
where separation from interferences occurs due to differences in affinity to the stationary phase in the column. Generally, a guard column is placed prior to the chromatographic column in order to protect the latter. The column elute reaches the detector. This results in a signal that is proportional to analyte concentration, and this signal is sent to an integrator where voltage is plotted as a function of time resulting in a chromatogram.

Chromatograms are evaluated and the peak height or area is plotted as a function of concentration in known calibration standards, with relationship between response and concentration being established. From this relationship, the concentration in an unknown sample can be determined. Often, an IS is added during sample preparation. The IS aims to balance variations that occur during the sample preparation and in the chromatographic system. When an IS is used, analyte response is divided with IS response, and the ratio is used for preparation of the calibration curve and determinations of unknown samples.

4.4.1 Reversed-phase LC

Reversed-phase chromatography is used to separate relatively hydrophobic analytes. The main interaction is between the analyte and the mobile phase. Retention is adjusted by varying the strength of the mobile phase and thereby affecting analyte interaction with the stationary phase. Separation occurs due to differences in partitioning between mobile- and stationary phase for the analytes. Highly hydrophobic, non-polar, analytes are more retained by the stationary phase and will therefore take longer time to migrate through the column. More hydrophilic, polar, analytes will be less retained. The most frequently used non-polar group bonded to the stationary support in the chromatographic column is octadecylsilane, C18. As in SPE, the support is often spherical silica, but with much smaller diameter (2–5 μm as compared to 40 μm in packed SPE-columns) and also of more homogenous quality. In reversed-phase LC, the mobile phase is polar and composed of a water phase and a water miscible organic phase. The water part is commonly a buffer, as pH control is of high importance, and the organic phase is often methanol or acetonitrile.

The monolith LC column consists of a single rod of silica-based material. This column is prepared by a sol-gel technology that creates a continuous network throughout the column. Macropores with an average diameter of 2 μm as well as mesopores of 13 nm are present. The large pores result in the possibility of using high flow-rates at limited back-pressure, whereas the small pores give the surface area needed for efficient separation. Also, monolith columns are showing great efficiency over a wider range of flow-rates, compared to 5 μm particle columns. In Paper V, the monolith column is used for analysis of the antimalarial PQ. By using this column, the time of analysis is only 2 minutes per sample, due
to the high flow-rates that is used i.e. 3.5 mL per minute. When using ordinary particle based LC columns, 1 mL per minute is common.

Gradient reverse-phase chromatography is used when a number of analytes differ widely in polarity. The initial condition is selected for retention of the most polar analyte in the sample. Thereafter organic solvent content is increased until the least polar analyte is eluted. This approach results in less time consuming LC, as retention is decreased as content of strong solvent is increased. Often, linear change with time is used\textsuperscript{118}. In Paper VI, a version of gradient chromatography is used, utilizing two isocratic compositions of mobile phase, where 3TC is eluted with the first composition and AZT and NVP with the second.

4.4.2 Chiral LC

Enantiomers are molecular mirror images of the same analyte and cannot be separated using conventional analytical methods. To discriminate between enantiomers, it is necessary to introduce a chiral environment. The chromatographic enantioselective separation can be achieved by the use of a chiral stationary phase (CSP) (direct method), a chiral mobile phase additive (direct method) or by formation of a diastereomeric derivative through a reaction with another chemical substance possessing a stereogenic centre (indirect method)\textsuperscript{116}.

Chirobiotic TAG, see Figure 11, is a macrocyclic type of CSP, based on native teicoplanin aglycone. Macro cyclic antibiotics (teicoplanin, vancomycin etc.) have the advantage, as compared to protein-based stationary phases, that they can be used with a wider range of mobile phases. The aglycone of the teicoplanin in Chirobiotic TAG is a semi-rigid basket including several aromatic rings. It contains a single carboxylic group (pK\textsubscript{a} ≈ 2.5) and a single primary amine (pK\textsubscript{a} ≈ 9.2). There are eight chiral centers (\&\&\&\&\&\&\&\&\&\&\&\&) surrounding four cavities (A–D). The structure of teicoplanin is characterized by a large variety of possible interactions sites with the deprotonated carboxylic acid group being essential in the interaction process between enantiomers and this CSP. Chirobiotic TAG has been used for chiral separation of amino acids\textsuperscript{123, 124}. 
Chirobiotic TAG is used in Papers I and II for separation of DFMO enantiomers. In Paper I the developed and validated method is presented, while Paper II describes a clinical application.

4.4.3 Absorbance detection

Absorbance detection is based on the principle that the analyte of interest absorbs light. When referring to UV-visible detectors, this light is in the ultraviolet or visible region of the electromagnetic spectrum. The LC column elute is directed through a flow cell, where light of a particular wavelength passes. The intensity of light emerging is compared to the light absorbed by the mobile phase. In order to detect the analyte, it is essential that the mobile phase possesses as little absorption as possible at the wavelength used for detection. A monochromatic absorbance detector is composed of a mercury or deuterium lamp that sends monochromatic light through the flow cell. With variable-length UV detectors wavelength can, as the name implies, be varied. Advantages of UV detectors are that they are inexpensive, reliable and easy to operate. Most compounds have some absorbance in the UV-visible region, although it may not be strong enough to result in analytical methods with enough sensitivity. At lower range of the UV region (190–220 nm) there may be many other interfering compounds also absorbing light. Additionally, commonly used solvents absorb light at these wavelengths. If needed, other detectors can be used, or derivatisation of the analyte can be performed prior to detection\(^2\).\(^{118}\) In Papers III–VI, the analytes are detected with an UV-detector.
4.4.4 Evaporative light-scattering detection

In ELSD, the column elute is nebulized into a fine aerosol. The aerosol is transferred down a drift tube by a carrying gas where the solvent is evaporated, leaving non-volatile particles. These particles enter the optical cell, where they pass through a beam of light. The intensity of scattered light is a function of particle diameter\textsuperscript{125}. The ELSD detects all analytes less volatile than the mobile phase, and therefore it is important to use mobile phases that are easily and quickly evaporated.

In ELSD, three types of light-scattering is present, see Figure 12. Rayleigh scattering occurs when particles are very much smaller than the wavelength of incident light. For particles greater than this, Mie scattering is present, efficient over a narrow range of particle diameters. As particles increase beyond this range, the principle of scattering is refraction-reflection. Therefore, smaller particles generated at low concentrations scatter light less efficiently leading to attenuated detector response\textsuperscript{126}. In Rayleigh scattering the scattered light is proportional to the squared concentration, \( C^2 \), while for Mie the scattered light is proportional to \( C^{4/3} \). Refraction-reflection results in scattered light being proportional to \( C^{2/3} \). Obviously, the ELSD signal is not linearly related to concentration, and when constructing the calibration curve, double logarithmic transformation is often used prior to applying linear regression\textsuperscript{125-127}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure12.pdf}
\caption{Principles of light-scattering in ELSD.}
\end{figure}

As DFMO lacks chromophores and fluorophores, derivatization is necessary when using conventional modes of detection i.e. UV or fluorescence\textsuperscript{47}. However, derivatization can be problematic, with for instance derivate instability, reagent interference, and increased void-volume for post-column derivatization assays\textsuperscript{127}. In Papers I and II, DFMO enantiomers are detected with ELSD, without derivatization prior to detection.
5 Method prevalidation

5.1 Calibration model selection

If the appropriate calibration model is known in advance, the concentration of the analyte in a sample can be determined at once. When developing new bioanalytical methods, it is not so, and in this situation various regression models need to be explored. Only when a suitable model is found, that correctly describes the relationship between the signal and the concentration, conclusions can be drawn regarding the models ability of determining unknown samples accurate and precise. The simplest regression model is ordinary linear regression, based on the assumption that variance is constant. However, this is frequently not true for analytical data. It is therefore important to determine whether measurement variance is constant (homoscedasticity) or not (heteroscedasticity).

5.1.1 Calibration models

Ordinary linear regression assumes that all errors are in the responses, \( y \). The line that minimizes deviations in the \( y \)-direction between the experimental points and the calculated line is sought after. This line of regression is on the algebraic form \( y = a + bx \), where \( a \) is the intercept and \( b \) the slope. It indicates how the responses, \( y \), varies when the concentration, \( x \), is set to chosen values.

Weighted linear regression is used when heteroscedasticity is confirmed or suspected. When using weights, the regression line is calculated so that values where the absolute errors are smallest are granted additional weight. By doing this, closeness of the calculated line is more important with respect to lower concentrations with smaller absolute error, than for those points representing higher concentrations with the largest errors. This is achieved by giving each point a weighting inversely proportional to the corresponding variance. However, as it would be impractical to calculate these weights in laboratory routine, empirical weights such as \( 1/x^{1/2} \), \( 1/x \), \( 1/x^2 \), \( 1/y^{1/2} \), \( 1/y \) and \( 1/y^2 \) can be used. The weighted straight line equation can easily be calculated with a commercially available statistical software.
Double logarithmic transformation means that the responses, $y$, as well as the concentrations, $x$, are recalculated by taking the logarithm of values. When the errors in $y$-direction strongly depend on $x$, this type of transformation can reduce the variability of $y$ with $x$, so that the transformed data can be fitted with ordinary linear regression equations.

5.1.2 %RE plots and Accuracy profiles

The Food and Drug Administration (FDA) guideline states that “the simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified.”

There are approaches described for calibration model selection. One method is to choose model by plotting the percentage relative error (%RE) e.g. the bias of each calibration standard versus added concentration, for each calibration model. By using fixed limits of $\pm 20$ and $\pm 15\%$ as stated by the FDA, models can be rejected or accepted. Also, the %RE sums are calculated and compared. The model with acceptable plot and the smallest $\Sigma %RE$ is selected. Another method is construction of accuracy profiles, as suggested by the French Society of Pharmaceutical Science and Technology, SFSTP. In these profiles, confidence limits at each concentration level is calculated, increasing the probability of making accurate future predictions.

When constructing accuracy profiles, concentrations are determined in replicate at several occasions. The mean values and intra- and inter-assay standard deviations are calculated, and from these, confidence intervals are determined. These confidence limits take into account both the accuracy of the mean value and the total-assay precision, with 90% confidence limits. If calculated two-sided 90% confidence interval is included within the fixed limits, the analytical method is accepted at the 95% level. Confidence intervals have been calculated by Equations 1-3.

\[
CI_j = \hat{\mu}_i \pm t(0.1, \nu df) S_i(IP) \tag{1}
\]
\[
\hat{\mu}_i = \text{mean value at concentration level } i
\]
\[
t(0.1, \nu df) = \text{value of students distribution at } \nu \text{ degrees of freedom}
\]
\[
S_i(IP) = \text{intermediate precision}
\]
\[
\nu df = p(n-1) \tag{2}
\]
\[
p = \text{number of runs}
\]
\[
n = \text{number of replicates}
\]
\[
S(IP) = \sqrt{S^2(\text{intra-assay}) + S^2(\text{inter-assay})} \tag{3}
\]
As the intermediate variance is the sum of two components, no exact degrees of freedom can exist\(^4,6\). Approximations can be made\(^132\), where both intra-assay and inter-assay variance is considered. However, it is recommended in the SFSTP guide that the intra-assay precision degrees of freedom are used\(^4,6\), due to practical reasons and for the sake of simplicity\(^4\). More recently, a new SFSTP proposal has been published, in three parts, recommending calculation of \(\beta\)-expectation tolerance intervals when constructing accuracy profiles\(^133-135\). It is taken into account that the intermediate variance consists of two parts, being independently distributed, and an approximation of the degrees of freedom are made\(^136,137\). Details on the calculations of the \(\beta\)-expectation tolerance intervals can be found in the third part of the proposal\(^135\). Construction of %RE plots as well as accuracy profiles are performed during pre-validation. It has been suggested that model selection should be performed during method validation with selection based not only on optimum with respect to the calibration standards, but of the models ability to determine quality control (QC) samples accurate and precise as well\(^3\). Figure 13 shows an accuracy profile, with confidence limits (90%) calculated as described in Equations 1–3. Additionally, the error of individual samples is displayed, indicating resulting %RE plot.

![Figure 13. Accuracy profile with 90% confidence limits, with percentage error of individual samples inserted. Calibration model is double logarithmic transformation and linear regression. Data shown is from Paper IV.](image-url)
In Paper V, the first paper chronologically, no model selection is performed. Ordinary linear regression is applied on the data, with the zero sample included in the calibration curve, in order to correct for an impurity present in all calibration standards and QC samples. As mentioned earlier, the selection should be based on pre-validation data, thereafter applying the optimal model on validation data. In spite of this, it appeared interesting to re-evaluate the PQ validation data for model selection. The model selection was based on %RE plots and Σ%RE, due to lacking replicate intra-assay calibration data. Weighted linear regression \((1/x^{1/2}, 1/x, 1/x^2, 1/y^{1/2}, 1/y \text{ and } 1/y^2)\) as well as double logarithmic transformation with linear regression were evaluated. The re-evaluation resulted in the calibration model using double logarithmic transformation being optimal. Although the original calibration model resulted in the smallest Σ%RE, double logarithmic transformation with linear regression is the only model resulting in acceptable accuracy at the highest QC level\(^7\). For comparison of precision and accuracy of the calibration model used in Paper V and the model indicated from re-evaluation, see Table 2.

Table 2. Precision and accuracy of QC samples evaluated with ordinary linear regression (OLR) as used in Paper V in comparison to double logarithmic transformation with linear regression (Log-Log).

<table>
<thead>
<tr>
<th>PQ added (nM)</th>
<th>150</th>
<th>2500</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-assay CV (%)</td>
<td>12.1</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>Inter-assay CV (%)</td>
<td>1.4</td>
<td>5.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>3.0</td>
<td>5.6</td>
<td>15.9</td>
</tr>
<tr>
<td>Log-Log</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-assay CV (%)</td>
<td>6.7</td>
<td>8.1</td>
<td>7.9</td>
</tr>
<tr>
<td>Inter-assay CV (%)</td>
<td>4.8</td>
<td>6.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>3.7</td>
<td>3.9</td>
<td>14.2</td>
</tr>
</tbody>
</table>

In Papers III, IV and VI weighted linear regression \((1/x^{1/2}, 1/x, 1/x^2, 1/y^{1/2}, 1/y \text{ and } 1/y^2)\) and double logarithmic transformation with linear regression are evaluated. In Paper I, quadratic regression (non-weighted and weighted) is also evaluated due to the ELSD being non-linear. In this paper, %RE plots and Σ%RE are used for the selection of calibration model. Optimal accuracy of QC samples is added as selection criteria, as the model with the smallest Σ%RE did not perform within acceptable limits at the lowest QC level. In Papers III, IV and VI, the selection of calibration model is based on accuracy profiles alone. Generally, double logarithmic transformation followed by linear regression is the calibration model describing data accurately.
6 Method validation

Method validation is performed to assure that the method developed provides precise and accurate results when determining unknown samples. It also offers information with respect to stabilities, useful in situations such as when determining how samples should be stored, how to handle them in the process of analyzing them, as well as other issues. Whenever possible, the same biological matrix as the matrix of the intended samples should be used for validation purposes.

Performing a full validation is important when developing a bioanalytical method. Also, this extent of validation is needed if an assay is changed, for instance if metabolites are added for quantitative purposes to an existing bioanalytical method. Partial validation is required if modifications of an already validated method is made. This type of validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Reasons for performing a partial validation can be that a fully validated method is transferred to another laboratory, or when changing species within a matrix. Cross-validation is performed when a developed bioanalytical method is compared to an existing validated method that serves as a reference method. A cross-validation could also be conducted to establish reliability between laboratories. In this case, the same bioanalytical method is used at both sites.

6.1 Full method validation parameters

In Papers I–VI, the FDA guideline for bioanalytical method validation has been applied, in full for Papers I and III–VI, and partly for Paper II. Parameters evaluated during method validation are a) selectivity, b) accuracy, precision and recovery, c) calibration curve and d) stability.

Selectivity is the ability of the bioanalytical method to quantify and differentiate analytes in presence of components that can be expected in the samples. Blank matrix from at least six donors is evaluated, as well as pharmaceuticals possibly present in the patient samples.
Accuracy describes the closeness of the mean value to the actual value i.e. found concentration versus added. It is determined by replicate analysis of samples containing known concentrations of the analyte. At least five determinations per concentration should be used, with found mean concentration being within ±15% of the added. Precision describes the closeness of individual measurements obtained from multiple sampling from the same homogenous sample. A minimum of five determinations per concentrations is made, with coefficient of variation being no more than 15%. Precision is subdivided into intra-assay precision, measuring precision during a single analytical run, and inter-assay precision i.e. precision over time. Extraction recovery describes the extraction efficiency of the analytical process, as percentage of amount of analyte carried through sample preparation.

A calibration curve expresses the relationship between the instrument response and known concentrations of the analyte i.e. $y$ as a function of $x$. Calibration standards can deviate by no more than ±15%, except at the lower limit of quantification (LLOQ) where deviation can be ±20%. The LLOQ is the lowest concentration standard in the calibration curve, where the analyte signal should be at least 5 times the response of a blank sample, and precision of replicate measurements $\leq$20%. From the calibration curve, concentrations of the analyte in QC samples and unknown samples are determined.

Drug stability in a biological matrix depends on the storage conditions as well as the chemical properties of the analyte and the matrix. The stability during sample collection and handling, long-term and short-term storage, freezing and thawing, and during the analytical process, is preferably examined. It is desirable that conditions used in the stability experiments reflect situations relevant to the actual handling of the samples.
7 Concluding remarks and future outlooks

As previously mentioned, the aim of this thesis was to develop methods for determination of a selection of drugs used against HAT, pneumonia, malaria and AIDS. Methods developed have been on demand by other research groups and/or WHO, with an intended future use, although sometimes altered over time.

When starting the DFMO project, the resulting method was aimed for determination of D-DFMO and L-DFMO in human subjects. It can be concluded that a chiral separation of DFMO has been achieved, and that the method can be used for determination of D-DFMO and L-DFMO. When the method was applied to evaluating D-DFMO and L-DFMO pharmacokinetics in rats, it was concluded that bioavailability of L-DFMO, the more potent enantiomer, was less than that of D-DFMO. This could help explain why studies with oral administration of racemic DFMO have not been all that successful, as concentrations may not be high enough in CSF when using this administration route. If ways to improve bioavailability of L-DFMO is found, oral therapy with racemic DFMO might be more successful. As the assay was originally developed for 1000 μL plasma, while being only 300 μL when sampling rats, dosages were selected with respect to reduced assay sensitivity. Also, additional error was added as volume correction was applied. Therefore, a more sensitive method for determination of D-DFMO and L-DFMO in rat plasma is desired, and is currently being developed in our research group in collaboration with Göteborg University.

The aim of the SD/SM project was to develop a method for simultaneous determination of SD and SM, with limited sample preparation prior to LC, which was achieved, although an additional method was developed as complement, with SPE added. Main focus of the latter project was evaluating and discussing the importance of uniformity of type of sampling paper, sampling volume and biological matrix, benefit of punching discs from DBS, and impact on absorption properties of different brands of sampling papers. It is concluded that pre-analytical factors need to be controlled, as they would otherwise result in increased measurement uncertainty. The SD/SM direct injection method has been successfully applied to determining SD and SM in DBS, in a clinical study evaluating caretakers’ reports on antimalarial and antibiotic use in children with severe pneumonia in Uganda. Caretakers’ were interviewed regarding treatments given prior to arriving at the hospital. It is concluded that the validity of
reported drug intake is low\textsuperscript{58}. As both antimalarial and antibiotic drug resistance is driven by frequent use of non-therapeutic dosages, adherence is important to assess.

PQ determination in DBS was chronologically the first project. The advantage when starting this project was that prior knowledge was available in our group, with a suitable LC method previously being developed, as well as a SPE method, although needing some modifications. The challenge of the method development was the DBS matrix. This PQ assay has been used in a clinical study for determination of day 7 PQ concentrations in DBS. Conclusions are that DHA-PQ is a good alternative to artesunate-mefloquine, with good adherence without supervision\textsuperscript{76}. Due to previous lack of knowledge concerning the impurity in the PQ standard as well as not using the optimal calibration model when publishing the assay, it appears wise to perform a pre-validation for model selection followed by partial validation, if setting up the PQ method in the future.

The method for simultaneous determination of three antiretroviral drugs i.e. 3TC and AZT belonging to the NRTIs, and NVP being part of the NNRTI drug class, was evaluated by determining 3TC, AZT and NVP in patient samples. Sampling was performed at Makerere University, Uganda, while analysis was performed at Dalarna University. It is concluded that the method can be useful for determination of 3TC and NVP, and to some extent for AZT. When analyzing the patient samples from the Ugandan subjects, it was noticed that NVP concentrations were higher than expected, as compared to plasma concentrations for a Western population\textsuperscript{138}. It would be of interest to further investigate this difference, although requiring partial validation of the method as the calibration range needs to be extended. Of particular concern is resistance developing to first-line ARVs, such as 3TC, AZT and NVP, as second-line drugs are much more expensive. The key to avoid resistance being developed is to achieve very high and sustained adherence to drug treatment. The ability to measure ARV drug levels is highly desirable, with methods suitable for limited laboratory facilities and restricted availability of skilled staff for venepuncture sampling. The method being developed can be useful for determining 3TC and NVP when evaluating adherence. Further needed are robust methods for determination of all other first-line drugs used, and possibly for second-line drugs as well, in DBS matrix, in order to access adherence to ART in low-income countries.
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9 Summary in Swedish

9.1 Sjukdomar i låginkomstländer

Malaria, infektion med humant immunbristvirus (HIV), lunginflammation och Afrikansk sömnsjuka förekommer i varierande grad världen över, se Figur 14, men gemensamt är att de har stor inverkan på sjuklighet och dödlighet i synnerhet i låginkomstländer. Samtidig infektion med HIV och parasitinfektioner kan försvåra diagnos av malaria, Afrikansk sömnsjuka och andra sjukdomar, samt påverka svar på behandling med antiparasitläkemedel.

Afrikansk sömnsjuka orsakas av parasiter (Trypanosoma) och sprids via tsetseflugor\textsuperscript{15}. Världshälsoorganisationen (WHO) uppskattar att mellan 300 000 och 500 000 människor påverkas av sjukdomen\textsuperscript{16} och att minst 50 000 av dem dör\textsuperscript{18}. Läkemedel som används för behandling av Afrikansk sömnsjuka är gamla och mycket giftiga\textsuperscript{19}. Dessutom utvecklar parasiterna resistens mot dem\textsuperscript{19}. Efllornitin utvecklades inom cancerforskningen under 1980-talet\textsuperscript{32} men används idag som aktivt ämne i en kräm för borttagning av oönskad hårväxt\textsuperscript{15}. Under 80-talet insågs även att efllornitin var verksam mot parasiterna som orsakar Afrikansk sömnsjuka\textsuperscript{38}. Efllornitin är det enda nya läkemedel som registrerats för behandling av Afrikansk sömnsjuka under de senaste 50 åren\textsuperscript{19} och är säkrare och mer effektivt jämfört med alternativen\textsuperscript{35}.

Lunginflammation är en allvarlig form av luftvägsinfektion som ger upphov till hosta och andningssvårigheter. Sjukdomen orsakas ofta av bakterier, men kan också bero svampinfektion, vilket är vanligt vid samtidig HIV-smitta\textsuperscript{9}. Det uppskattas att mer än 2 miljoner barn dör av lunginflammation varje år, hälften av dem från Sub-Sahara Afrika. I höginkomstländer orsakar lunginflammation omkring 1000 dödsfall årligen\textsuperscript{9}. De antibakteriella egenskaperna hos sulfonamider upptäcktes på 1930-talet\textsuperscript{53}. Sulfametoxazol är ett läkemedel som tillhör denna grupp och används i kombination med ett annat läkemedel vid behandling av lunginflammation i låginkomstländer\textsuperscript{9}. Läkemedelskombinationen används även i förebyggande syfte för den typ av lunginflammation som är vanlig vid HIV-infection\textsuperscript{93}.

Malaria är en infektionssjukdom som orsakas av parasiter (Plasmodium) och sprids via myggor. Falciparum malaria är vanligast i Afrika och är den allvarligaste formen av sjukdomen med högst dödlighet\textsuperscript{21}. Över 250 miljarder fall av malaria inträffar årligen och minst en miljon människor dör, direkt eller indirekt på grund av sjukdomen. Små barn och gravida kvinnor är speciellt utsatta\textsuperscript{16}. Till störst del (90 %) sker sjukdomss fallen i Afrika. Ett stort problem är resistensutveckling hos parasiterna mot malarialäkemedel\textsuperscript{13}. Sulfadoxin är en sulfonamid som används i kombination med ett annat läkemedel vid behandling av malaria\textsuperscript{64}. Piperakin är ett annat malarialäkemedel som används i allt större utsträckning, i kombination med dihydroarteminisin\textsuperscript{72}.

AIDS (förvärvat immunbristsyndrom) orsakas av humant immunbristvirus, HIV. Virus överförs via sexuell kontakt, perinatalt eller via amning\textsuperscript{28}. 2006 var 39,5 miljoner människor infekterade med HIV, och av dessa levde 60 % i Sub-Sahara Afrika\textsuperscript{29}. Det uppskattas att mindre än var tredje person har tillgång till antiretroviral behandling i denna del av världen\textsuperscript{30}. Antiretroviral behandlig utgörs av två olika grupper av läkemedel, nukleosidanaloger samt icke-nukleosider. Triplettan lamivudin, zidovudin och nevirapin är rekommenderad förstahandsbehandling i många låginkomstländer\textsuperscript{93}.
Läkemedelsanalys

Analys av läkemedel i biologiska prover är användbart i olika situationer. Vid farmakokinetiska studier undersöks läkemedelskoncentration över tid, hur läkemedlets absorption och elimination sker, dess biologiska tillgänglighet m.m. Ett annat område är vid utvärdering av följsamhet, där tillräcklig läkemedelskoncentration, trots uteblivet tillfrisknande, kan vara en indikation på resistensutveckling. Suboptimala koncentrationer kan i sin tur vara ett tecken på låg följsamhet och kan bidra till ökad resistensutveckling. Samband mellan ett läkemedels dos och respons används för att avgöra lämplig dos för tillfrisknande, vilket kan vara individberoende. Ibland kan bättre respons erhållas om dosen ställs in med hjälp av mätning av läkemedels koncentration. Om ett läkemedel har ett smalt terapeutiskt fönster, där spannet mellan otillräcklig dos och toxisk dos är litet, kan koncentrationsbestämning vara särskilt viktigt. Kiral analys används för studier av enantiomerer. Många läkemedel förekommer i två sådana former, vilka är molekylära spegelbilder. Dessa kan skilja åt t.ex. genom olikheter i absorption, toxicitet, effekt m.m.


9.3 Avhandlingsarbeten


**Arbete III** och **IV** beskriver två metoder för simultan kvantifiering av två sulfaläkemedel i kapillärblod intorkat på provtagningspapper. Sulfadoxin är ett malarialäkemedel, medan sulfametoxazol används vid behandling av lunginflammation. Syftet med det första arbetet var att utveckla en så enkel metod som möjligt för bestämning av de två läkemedlen, med minimal provupparbetning innan vätskechromatografi. I det andra arbetet kompletterades provupparbetning och analys med fastfasextraktion. Fokus i arbete två är vikten av att i möjlig mån kontrollera preanalytiska faktorer för god riktighet för provsvår. Exempelvis påverkar oenhetlighet i typ av papper inom en studie riktigheten på koncentrationsbestämmningar. Detta eftersom utbyte av analyter varierar beroende på provtagningspapper. Ett annat exempel är variationer i blodvolym, vilket ger variationer vid bestämning av koncentration. Stansning av blodfläckar utvärderades och resulterade i likvärdiga resultat som klippta fläckar, men med svårighet att finna en kommersiellt tillåtlig stans. Metoden i det första arbetet har använts i en studie i Uganda, där vårdnadshavares rapporterade intag av malarialäkemedel och antibiotika hos barn har utvärderats. Slutsatsen är att rapporterat läkemedelsintag har begränsad tillförlitlighet.58.

References


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)