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Development and Validation of Bioanalytical Methods

Application to Melatonin and Selected Anti-Infective Drugs

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Abstract
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This thesis describes bioanalytical methods for measuring melatonin and some anti-infective drugs in biological fluids. Solid-phase extraction (SPE) or protein precipitation was used for enrichment and purification of the analytes and Liquid Chromatography (LC) was used to analyze the samples. Developed methods were validated according to international guidelines.

Melatonin is a hormone secreted by the pineal gland with a robust circadian rhythm. Bioanalytical methods for determination of melatonin in plasma and saliva have been developed which were used for monitoring melatonin levels in volunteers and patients suffering from sleep related diseases.

Eflornithine (DFMO) is a chiral drug used for the treatment of human African trypanosomiasis. A bioanalytical method for determination of the DFMO enantiomers in plasma, after precolumn derivatization with *o*-phtalaldehyde and N-acetyl-L-cystein has been developed. The method has been used to study the L- and D-DFMO pharmacokinetics, in order to investigate the possible development of an oral treatment of DFMO.

A method for simultaneous determination of three antiretroviral drugs i.e. Lamivudine (3TC), Zidovudine (AZT) and Nevirapine (NVP) in dried blood spots (DBS) was developed. The method was used for drug determination in two subjects after receiving standard antiretroviral treatment. The method seemed well suitable for the determination of 3TC and NVP and in some extent for AZT.

Lumefantrine (LF) is one of the active components in a new fixed drug combination recommended by the WHO as a replacement to older drugs that has lost their effect. A method for the determination of LF in DBS was developed. The method is suitable for monitoring of drug treatment in rural settings.

Tafenoquine is a new promising antimalarial drug under development. A method for the determination of Tafenoquine in plasma and in DBS is described. The method may be useful in future clinical studies in laboratory environment as well as in rural settings.

Keywords: african trypanosomiasis, melatonin, malaria, antiretroviral drugs, lumefantrine, tafenoquine, lamivudine, nevirapine, zidovudine, sampling paper, dried blood spots, capillary blood, antimalarial drugs, solid-phase extraction, liquid chromatography

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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.

I Römsing. S., Ulfberg, J., Bergqvist, Y. (2003) Determination of melatonin in plasma with solid-phase extraction, high-performance liquid chromatography and fluorescence detection. *Scandinavian Journal of Clinical & Laboratory Investigation*, 63(1):81–89.

II Römsing. S., Bökman, F., Bergqvist, Y. (2006) Determination of melatonin in saliva using automated solid-phase extraction, high-performance liquid chromatography and fluorescence detection. *Scandinavian Journal of Clinical & Laboratory Investigation*, 66(1):181–190.

III Jansson-Löfmark, R., Römsing, S., Albers, E., Ashton, M. (2010) Determination of eflornithine enantiomers in plasma, by precolumn derivatization with o-phtalaldehyde-N-acetyl-L-cysteine and liquid chromatography with UV detection. *Biomedical Chromatography, 24(7):768-773.*

IV Malm, M., Römsing, S., Obua, C., Bergqvist, Y. (2009) Determination of Lamivudine, Zidovudine and Nevirapine in Capillary Blood Sampled on Filter Paper by LC. *Journal of Chromatographic of Science,* 47(10):855-862.

V Blessborn, D. Römsing, S., Annerberg, A., Sundquist, D., Björkman, A., Lindegårdh, N., Bergqvist, Y. (2007) Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of Lumefantrine in capillary blood on sampling paper. *Journal of Pharmaceutical and Biomedical Analysis, 45*(2):282–287.

VI Römsing, S., Lindegårdh, N., Bergqvist, Y. Quantification of Tafenoquine in plasma and dried blood spots using liquid chromatography and fluorescence detection. *In manuscript.*

Author's contribution

Paper I: Planned and performed all experiments and wrote the paper.

Paper II: Planned and performed all experiments and wrote the paper.

Paper III: Performed some experiments e.g. pre-validation and interference study. Revised the paper.

Paper IV: Planned the experiments together with M. Malm. Validated the method. Participated in the analysis and interpretation of validation results. Revised the paper.

Paper V: Planned and performed most of the experiments together with D. Blessborn. Participated in writing the paper.

Paper VI: Planned and performed all experiments and wrote the paper.

Contents

Abbreviations

3TC	Lamivudine
ACT	Artemisinin based combination therapy
AIDS	Acquired immune deficiency syndrome
AL	Artemether-Lumefantrine
ART	Antiretroviral therapy
ARV	Antiretroviral
AZT	Zidovudine
CSF	Cerebrospinal fluid
DBS	Dried blood spot
DFMO	Eflornithine
EFV	Efavirenz
EML	Essential medicines list
FDA	US food and drug administration
FDC	Fixed dose combination
HAT	Human African trypanosomiasis
HIV	Human immunodeficiency virus
LC	Liquid chromatography
LOD	Limit of detection
LLOQ	Lower limit of quantification
LOQ	Limit of quantification
NECT	Nifurtimox-Eflornithine combination therapy
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevaripine
ODC	Ornithine decarboxylase
OSAS	Obstructive sleep apnea syndrome
PI	Protease inhibitor
RLS	Restless legs
RP	Reverse phase
SCN	Suprachiasmatic nucleus
SPE	Solid-phase extraction
TDM	Therapeutic drug monitoring
WHO	World health organization

1 Introduction

In analytical chemistry the main goal is to determine the identity and/or concentration of one or more species in a sample. The samples analyzed are often natural occurring samples in our environment or body fluids.

The aim during method development is to achieve a robust and reproducible method, with high accuracy and precision, to determine analytes in a matrix. With accurate planning of all the steps in the analytical chain i.e. sampling, sample treatment, separation, detection and evaluation of the results, the analytical errors can be minimized.

Reversed-phase (RP) is today the most commonly used technique in liquid chromatography (LC) methods, for separation and determination of chemical components in complex biological mixtures. Compounds that differ in their molecular properties, like hydrophobicity, polarity and ionic character can be separated and analysed with the large number of techniques that LC offers. Compounds to be analysed is generally contained in a complex biological matrix and can rarely be measured direct without sample pretreatment. Interfering compounds have to be eliminated. This becomes particularly important during trace analysis in the presence of abundant matrix components. Solid-phase extraction (SPE) is a commonly used technique for sample clean up. It is especially useful when high sensitivity is needed and it is easy to automate and can often achieve high sample throughput. SPE and LC are both based on differential migration processes in which compounds are adsorbed and eluted as they are traveling through a porous medium carried by a mobile-phase flow. The time it takes depends on the compounds differential affinities between the sorbent material and the mobile phase. Finally, a selection of an accurate detection method has do be done based on the properties of the compounds to be analysed, of the required concentration level and the nature of the matrix.

When a method has been developed, it is evaluated during method validation, based on international guidelines. This is a process to establish that the method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte in the selected matrix. Also to ensure that the method can be implemented for analysis of actual patient samples. Typical parameters to validate are; accuracy, precision, selectivity, sensitivity, linearity, recovery and stability.

The aim of this thesis was to develop analytical methods for measuring a variety of analytes in various biological matrices.

Paper I and **paper II** describe methods for determination of the endogenous hormone melatonin in plasma and saliva, with fluorescence detection.

Paper III describes a method for the determination of the antitrypanosomal drug eflornithine (DFMO) enantiomers after precolumn derivatization with *o*-phtalaldehyde and N-acetyl-L-cystein on two serially connected monolith C-18 columns and UV-detection.

In **paper IV** a method for simultaneously determining three antiretrovirals i.e. lamivudine, zidivudine and nevirapine is described. Capillary blood from a fingertip is applied onto sampling paper, left to dry and packed in plastic bags. The dried blood spot (DBS) method is very useful when conducting therapeutic drug monitoring (TDM) in rural areas where facilities may not be available such as refrigerator of freezer. DBS is also used in **Paper V** for the determination of antimalarial drug Lumefantrine. Lumefantrine is used in combination with Artemether in the drug Riamet™/Coartem™. In **Paper VI** a method for determination of a promising new drug against malaria, Tafenoquine, in DBS and plasma is described.

2 Analytes

2.1 Melatonin

Melatonin, N-acetyl-5-methoxytryptamine (fig. 1), is an indoleamine secreted by the pineal gland with a robust circadian rhythm. Its secretion is stimulated by dark and inhibited by light and the endogenous circadian rhythm is driven by the suprachiasmatic nucleus (SCN). Melatonin entrains the circadian timing system and works as a chronobiotic "a substance that adjusts the timing of internal biological rhythms" [1]. The circadian rhythm is the "internal body clock" that regulates the (roughly) 24-hours cycle of biological processes in animals and plants. The term circadian comes from the Latin circa, meaning "around" and dies, "day", meaning literally "around a day" [1-2].

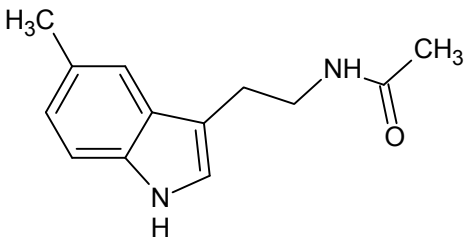

Figure 1. Melatonin, M = 232.3 g/mol

Melatonin is both lipid and aqueous soluble which results in that melatonin seems to distribute to all sub cellular compartments, with highest levels of the indoleamine being measured in the nuclei of cells [3]. After release in the circulation it gains access to various fluids, tissues and cellular compartments (saliva, urine, cerebrospinal fluid, preovulatory follicle, semen, amniotic fluid and breast milk). As no pineal storage of melatonin is available, the plasma hormone profile faithfully reflects the pineal activity [4]. The ability of the pineal gland to produce and release melatonin varies greatly with age [5] and between subjects, even though it is very reproducible from one day to another in the same subject [6].

The biosynthesis of melatonin occurs in four steps, starting from the amino acid Tryptophan and the major regulating step in the synthesis is the N-acetyltransferase-mediated conversion of Serotonin to N-acetylserotonin. This step is inhibited by light and stimulated by darkness, creating the

marked rhythm which is a distinguishing feature of the pineal output of melatonin [7] (fig. 2). Melatonin is rapidly metabolized in the liver to 6-hydroxymelatonin, which is conjugated as 60-70% sulphate and 20-30% glucuronide. Circulating plasma melatonin has a relatively short metabolic half-life, 45–60 minutes depending on distribution way i.e. intravenously or orally, with about 90 % being cleared during a single hepatic passage [2, 7]. The 24-h urinary excretion of 6-hydroxymelatonin sulphate highly correlates with plasma melatonin levels [8] and can be used for non-invasive clinical determinations. About 1% melatonin remains unchanged in the urine [4]. Maximum blood plasma concentrations are normally in the low nanomolar range, levels from 50 pmol/l down to 8 pmol/l are normal daytime and from 300 pmol/l down to 50 pmol/l night time [8-9]. The saliva melatonin concentration is about one-third of that in plasma. In some body fluids, e.g. bile and cerebrospinal fluid (CSF), measured melatonin concentrations are in orders of magnitude greater than in circulation [3].

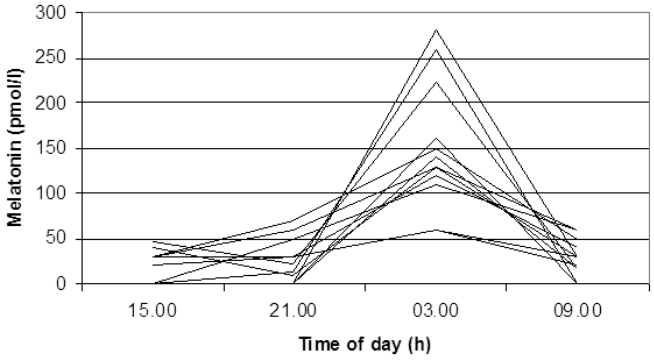

Figure 2. Diurnal saliva melatonin rhythms in 12 volunteers

The role of melatonin in the human body has been investigated in various fields. Cancer, neurological disorders, psychiatric diseases and cardiovascular diseases are all areas where melatonin in some way seems to influence [4]. The hormone also appears to have potential usefulness in a number of therapeutic areas such as those related to the resynchronization of biological rhythms e.g. shift work, jet lag, and delayed sleep phase syndrome [2] and to sleep related disorders like Obstructive Sleep Apnea Syndrome (OSAS), Restless Legs (RLS), to seasonal affective disorders and also to depressions [9-12].

Melatonin functions as an antioxidant and scavenges a large number of radicals and modulates the activity of enzymes which alter the oxidant/antioxidant balance. Melatonin has been compared with classic vitamin antioxidants, like vitamin C and E in term of relative antioxidative

capacity and results indicate that it is equivalently effective or even more effective in subduing free radical-based molecular damage [3].

In recent years there been a hypothesis that low levels of endogenous melatonin are associated with an increased risk for developing breast cancer. Women with night shift work are a risk group and the cause would be the exposure to light at night with subsequent melatonin suppression [13-14]. Some reports indicate that melatonin may have a protective effect [15-16] while other studies found no evidence that the level of melatonin is associated with the risk for breast cancer [17]. Additional studies are still needed to fully assess the potential of melatonin as a cancer scavenger.

Several chromatography methods have been reported for the determination of melatonin concentration in human biological fluids. However, when monitoring melatonin in biological fluids for clinical studies, the use of immunological methods like Enzyme-Linked Immunosorbent Assay (ELISA) and Radioimmunoassay (RIA) are the most widespread. Although the methods are highly sensitive with LLOD of 0.5 – few pmol/l, they inherently suffer from a potential risk of cross-reactivity with structurally similar compounds [18-19] .

The most used methods for determination of melatonin in plasma or serum at the time for **paper I** was fluorescence and electrochemical detection coupled to liquid chromatography. These methods had high sensitivity with detection limits from around 2–65 pmol/l [20-23] using 1–2 ml plasma or serum. For sample clean-up liquid/liquid extraction has traditionally been used. However the technique is difficult to automate for routine analysis and result in large volume of organic disposal. In **paper I** plasma was used as a matrix. This method allowed a large volume plasma, 3 ml, to be applied on the SPE column, OASIS HLB, compared with contemporary published methods where 1–2 ml sample was used. With the developed method it was possible to detect daytime levels of the hormone and to use the method for evaluation of the melatonin level variation in samples from patients suffering from sleep related disorders.

There has been a growing interest in less invasive methods for the determination of melatonin, why publications where saliva as matrix has been reported. Both LC-MS [24] and LC-MS/MS [25] methods have been described. These methods used 10 µl–1 ml sample and could detect concentration levels down to 4.5 pmol/l. A HPLC method for saliva determination has also been reported [26] in which melatonin was oxidized before analysis with fluorescence detection. The method used only 20 µl saliva but still required a relatively complicated sample preparation and clean-up process before analysis. In **paper II** saliva was used as matrix with fluorescence detection. A large volume of saliva was necessary compared to other contemporary published works which is a weakness of the developed method. Some patients are not able to excrete 2 ml saliva at the same time. Elderly people have a reduced salivary excretion and it can be difficult to

sample the required volume at night. The method was used for monitoring melatonin levels in saliva samples from patients and volunteers.

Since monitoring the circadian rhythm of melatonin is the only way to study melatonin, multiple collections must be done. This means that one blood/saliva sample is collected at four different times throughout the day, resulting in four separate sample tubes each representing a different collection time. This will result in four different hormone levels – each representing a particular time of day. If the results are plotted against time, a profile of the melatonin variation over 24-hours can be visualized.

2.2 Antitrypanosomal drugs

Human African Trypanosomiasis (HAT) or sleeping sickness, is a fatal disease caused by protozoa parasite, *Trypanosoma Brucei* (*T.b.*), transmitted to humans by the bite of an infected tsetse fly (Glossina Genus) in rural part of sub-Saharan Africa. There are two subspecies of the parasite, *T. b. Gambiense* found in west and central Africa and *T. b. Rhodesiense* found in eastern and southern Africa [27-28] .

HAT occurs in two clinical phases, one early hemolymphatic and one late encephaliticstage stage, and also in two forms; one chronic caused by *T.b. Gambiense* which represents more than 90% of reported cases and one more acute *T.b. Rhodesiense*. Approximately sixty million peopled are at risk of being infected and it is considered a neglected disease [29]. In 1998 WHO estimated that between 300 000 and 500 000 people were infected by HAT but only 40 000 cases were reported [30]. In 2009 there were for the first time in 50 years, less than 10 000 cases reported [31]. However, the real number is probably higher since only a minority of cases are still being reported [32]. The reduced transmission of the disease is a result of the work of National sleeping sickness programmes in endemic countries by the establishment of strengthened control and improved surveillance and case reporting. The ultimate objective of WHO´s work is to eliminate HAT.

There are few drugs available for the treatment of HAT, Pentamidine and Suramin for first stage treatment and Melarsoprol and Eflornithine for treatment of the second stage, where Eflornithine is effective only for *T.b. Gambiense*. The drugs used in the first stage are less toxic, easier to administer and more effective than drugs used in the second stage which are complicated to administer and toxic, sometimes fatal [33]. In 2009 WHO included a new combination therapy for the second-stage of HAT in the Model List of Essential Medicines (EML) [30, 34]. The new combination therapy (consists of Nifurtimox, a drug used for the treatment of Chagas disease (American trypanosomiasis) and Eflornithine. Nifurtimox-Eflornithine combination therapy (NECT) has high efficacy and good safety profile and is easier to administer.

2.2.1 Eflornithine

Eflornithine (fig. 3) (DL-α-difluoromethylornithine, DFMO) was developed in the 1970s as a potential anticancer drug [35]. In the early 1980s DFMO was found to be effective in treating mice infected with Trypansoma *brucei*, a parasite normally infecting cattle [36]. The first trials in humans were preformed in 1985 [37]. In 1990 the drug was registered for the treatment of Sleeping Sickness under the trade name Ornidyl. It is the only new drug registered for the treatment of HAT over the past 50 years [38].

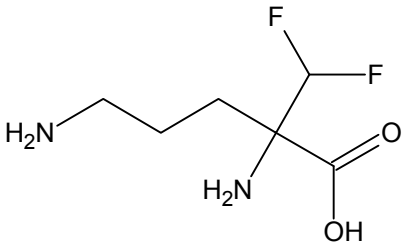

Figure 3. Structure of Eflornithine, M = 182.2 g/mol

The most commonly used dosage regimen for the treatment of late stage HAT consist of 100 mg/kg body weight at intervals of 6 h for 14 days given as short infusions [38]. For children a higher dose levels is required, 150 mg/kg body weight, to achieve sufficient plasma concentrations [39]. When eflornithine is given in combination with nifurtimox fewer infusions is required, 14 compared to 56, i.e. infusion every 12 h for 7 days v*s.* every 6 h for 14 days [40] which makes the administration less difficult and cumbersome for both the patient and care providers [34]. An oral drug formulation for treatment of sleeping sickness would be a major improvement since the complicated mode of administration leads to logistical problems leaving numerous patients untreated.

Antigenic variation of the African trypanosome surface coat has long been considered the main defense mechanism against the host immune system [41]. DFMO exerts its trypanostatic effect by inhibiting ornithine decarboxylase (ODC), an enzyme needed for the synthesis of polyamines involved in cell multiplication and differentiation [36, 42]. The inhibition of ODC activity makes the trypanosomes incapable of modifying their surface antigens or dividing, making them more exposed to the hosts immune attack [43]. Therefore, a sufficiently intact immune system is required to eliminate the parasite. There are indications that DFMO not can cure HAT patients that are HIV-positive [38].

DFMO does not bind significantly to plasma proteins [38] it has a mean half-life of 1.5–5 hours and it penetrates the blood-brain barrier [42]. The CSF DFMO levels must exceed 50 µmol/l to attain reliable clearance of parasites and CSF/plasma ratios between 0.13 and 0.51 have been reported.

DFMO has a renal clearance of approximately 80% [38]. DFMO is administered as a racemate and the enatiomers of DFMO have different potency with the L-form having 20-fold higher ability to inhibit the target enzyme ODC [44]. Under physiological conditions, DFMO is a zwitterion, resulting in it being poorly absorbable and rapidly excreted in the urine, which explains its moderate activity and brief duration of action [43].

There have been a few of methods published for the determination of racemic DFMO in biological matrices [45-50] but only two for stereospecific determination. In the first method described [51] a chiral stationary phase, Chirasil-Val, was used with L-proline/copper as chiral additive for a semi-preparative resolution of several ornithine analogs with GC after derivatization into the monofluoroacyl derivatives of their corresponding lactams. The method was however not developed for determination of L- and D-DFMO in plasma. The second method available [52] is based on solid-phase extraction and liquid chromatography where separation of the L- and D-enantiomers are achieved on a chiral stationary phase, Chirobiotic TAG, with evaporative light-scattering detection.

Paper III describes a bioanalytical method for the separation of L- and D-DFMO enantiomers after pre-column derivatization with *o*-phtalaldehyde and *N*-acetyl-L-cysteine on two serially connected monolith C18 columns. The method was about 15-fold more sensitive and required 10 times less sample volume, compared to methods available for the determination of the enantiomers of DFMO in plasma. The developed assay enables collection and analysis of small samples volumes and can be used for pharmacokinetic studies in both small animals and humans.

2.3 Antiretroviral drugs

The human immunodeficiency virus (HIV) is a retro virus that attacks the human immune system, causing a gradually reduction of the host's cellular immunity (especially of $CD4^+$ T lymphocytes) culminating in Acquired Immune Deficiency Syndrome (AIDS). The virus is transmitted through sexual contact, transfusion of contaminated blood, by breastfeeding or during pregnancy [53]. WHO and UNAIDS estimated in 2008 that 33.4 million people were living with HIV, two third in sub-Saharan Africa. The same year almost 3 million people became infected and 2 million died as a result of their AIDS infection [54]. In 2008 the antiretroviral therapy range was estimated 42% in low- and middle income countries representing a 36% increase in one year [55].

The standard antiretroviral therapy (ART) consists of the use of at least three antiretroviral (ARV) drugs. First-line therapy recommended by the WHO should consist of a non-nucleoside reverse transcriptase inhibitor (NNRTI), either Nevirapine (NVP) or Efavirenz (EFV) plus two nucleoside

reverse transcriptase inhibitors (NRTIs) one of which should be Lamivudine (3TC) or Emtricitabine and the other Zidovudine (AZT) or Tenofovir Disoproxil Fumarate. For first-line failure one second-line treatment is proposed. For pregnant women preferred first-line ART combination consists of AZT, 3TC and NVP or EFV (EFV not to be initiated during first trimester) [56]. The triple-nucleoside combination therapy can be administered as a fixed-dose combination (FDC) which gives enhanced adherence to treatment, makes it easier do distribute the drugs and decreases the high pill burden and daily doses for the patients [57]. Drug interactions and toxicity are common in patients with AIDS and include altered absorption, altered protein binding, induction or inhibition of liver enzymes responsible for the metabolism of drugs, and impaired renal function [58].

2.3.1 Lamivudine

3TC (fig. 4) is an enantiomer of a cytosine nucleoside analogue and a competitive inhibitor of HIV-1 virus (NRTI). The antiviral activity is due to intracellular phosphorylation to its active 5´triphosphate anbolite which inhibits reverse transcriptase of the HIV virus acting as a chain terminator [59]. 3TC is a cell activation-independent agent which means that the drug is phosphorylated in resting cells with 3TC triphosphate concentration related in a linear manner to the apparent oral clearance of the drug from plasma [60]. A combination of cell activation-dependent and independent agents may have additive-to synergistic activity, which have been shown for 3TC and AZT [61].

3TC is rapidly absorbed, has a good bioavailability (<80%) and is mainly excreted as unchanged drug (68–71%) [59, 62]. 3TC has a mean half-life of 2.5 hours and has a protein bounding of less then 5% [58]. Target steady state plasma concentration for 3TC is 0.71 μmol/l [63].

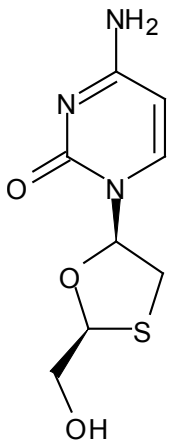

Figure 4. Structure of Lamivudine, M = 229.3 g/mol

2.3.2 Zidovudine

AZT (fig. 5) also acts as a NRTI. AZT is primarily eliminated through glucuronidation (60–70%) while 10–20% is eliminated unchanged [57]. The drug has a half-life of approximately one hour in plasma and the plasma protein binding is about 20% [58].

AZT is a cell activation-dependent nucleoside which is preferentially phosphorylated in activated cells and yield higher ratios of intracellular triphosphate to endogenous deoxynucleoside triphosphate than in resting cells. There is a small relationship between circulating concentrations of AZT in plasma and intra cellular concentration of its active 5´triphosphate anabolite. This does not mean that there are none since higher dose levels of AZT result in higher concentrations of its anabolite. The amount of AZT triphosphate formed is related in a non linear manner to the concentration of the parent drug. The relationship between concentration of the parent drug and the anabolite is complex and multifactorial [64]. AZT Target steady state plasma concentrations for AZT is 1.9 µmol/l [63].

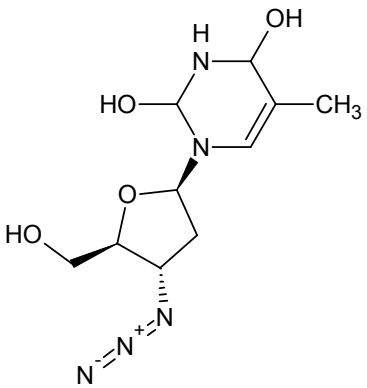

Figure 5. Structure of Zidovudine, M = 267.2 g/mol

2.3.3 Nevirapine

NVP (fig. 6) is a NNRTI and exerts its antiviral activity by blocking the RNA-and DNA-dependent DNA polymerase and it does not require intracellular activation like NRTIs [57]. NVP is well absorbed orally (>90%) and distributes well to nearly all tissues. More than 80% is excreted in urine as glucuronides and only a small fraction (>3%) is excreted unchanged in the urine. Approximately 60% is bound to plasma proteins and it has a half-life of approximately 30 hours [62]. Therapeutic target plasma concentration is 13 µmol/l [65-66].

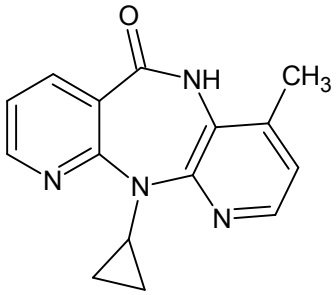

Figure 6. Structure of Nevirapine, M = 266.3 g/mol

ARVs are typically administered in standard fixed doses without dose adjustment for factors that may affect drug concentrations (e.g. drug absorption, distribution, metabolism and elimination based upon the persons age, general health or concomitant medication) [67]. Therapeutic drug monitoring (TDM) can therefore be useful in optimization of ART, to improve efficacy and safety by maintaining ARV concentrations within the therapeutic range. TDM can also identify non compliance, drug interactions and help to adapt dosages to fit the current needs of a specific patient [68]. TDM is based on the principle that there is a close relationship between blood drug concentrations and efficacy of treatment. In fact, for NNRTIs like NVP, this relationship has been established. The concentrations of NRTIs, like 3TC and AZT, are on the other hand, not correlated due to effects to their intracellular metabolism [69]. However, there has been shown that there is a potential interest of concentration-controlled regimens of NRTIs [63-64].

There are several bioanalytical methods published for the determination of antiretrovirals in biological fluids, mostly for measurement in plasma. For the quantification of antiretrovirals in DBS, only two methods have earlier been described. AZT was monitored with a radioimmunoassay [70] and seven PIs and two NNRTIs (one of them NVP) was quantified by liquid chromatography/tandem mass spectrometry [71].

In **paper IV** a method for the simultaneous determination of 3CT, AZT and NVP in DBS is described. The method was used to determine drug levels in two patients on standard twice daily ART dose (3TC 150 mg, AZT 300 mg and NVP 200 mg) and the results indicate that de method would be usable for TDM of the drugs.

2.4 Antimalarial drugs

Malaria is a parasite infection caused by a protozoon of the genus Plasmodium, transmitted by the bite of a female Anopheles mosquito. There are mainly four types of human malaria, *Plasmodium malariae*, *Plasmodium*

ovale, *Plasmodium vivax* and *Plasmodium falciparum* [72]. The most common species are P. *vivax* and P. *falciparum* where P. *falciparum* gives the most deadly type of infection and are responsible for most of the mortalities [73]. In 2008, there were approximately 250 million cases of malaria reported and nearly one million deaths – mostly children living in Africa [74]. Malaria is present in over 100 countries worldwide.

The last decades there has been a rapid spread of drug resistance by the parasite against antimalarial drugs [75]. Today, resistance to antimalarial drugs poses a significant challenge to malaria control programs. Resistance has been developed to all antimalarial drug classes except for one – the artemisinins [76]. However, recently there are worrying signs from South East Asia that resistance is emerging [77-78]. There are also other factors that limit the use of current drugs for malaria such as low efficacy, safety issues, poor compliance and high cost [79]. To increase the efficacy of antimalarial drugs it is common to combine different drugs, with different mechanisms of action. Artemisinin-based combination therapy (ACT) is recommended as first-line treatment for uncomplicated P. *falciparum* malaria by the World Health Organization (WHO). There are, today, five ACTs recommended for use: artemether-lumefantrine (AL), artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine pyrimethamine and dihydroartemisinin-piperaquine. Selection of ACT should be based on efficacy in the area, or country, and of intended use [80]. WHO criterion for adequate efficacy of ACT in malaria is the achievement of an average cure rate of ≥95% in clinical trials [80].

The increased development of resistance against antimalarials also requires the development of new drugs, ideally with novel modes of action. A new drug should have low production cost, high efficacy against drug-resistant malaria and be safe. It should also ideally have short treatment course to improve patient compliance, since poor compliance is one of the major factors contributing to drug resistance [81]. The resistance against insecticides, like DDT, also constitute a threat in controlling malaria [75].

There is a number of ways to prevent malaria; elimination of the place of mosquito breeding, insecticides, chemoprophylaxis and insecticide treated nets [75]. So far it has not been possible to develop an effective malaria prophylactic vaccine since the host-parasite interactions are complex. However, several vaccines that instead target different stages of the malaria parasite life cycle are in clinical trials [79, 82]. New antimalarial drugs are sought for and one that has reached an advance stage of clinical evaluation is Tafenoquine.

2.4.1 Lumefantrine

Lumefantrine, previously known as Benflumetol (fig. 7), was discovered by Chinese scientists at the Beijing Academy of Military Medical Sciences in

the early 1980s [83]. Lumefantrine was registered in China as oral combination drug with Artemether (AL) in 1992. Clinical trials, outside China, began in 1995 and in 1999 it had regulatory approval in the treatment of adults and children for uncomplicated P. *falciparum* distributed under the name of Coartem™ or Riamet™ manufactured by Novartis [84-85]. Lumefantrine has never been used as monotherapy for the treatment of malaria which is good in the perspective of resistance.

Oral formulation of AL is available as tablet and as dispersible formulation, specially developed for infants and young children, and they have similar pharmacokinetic properties and safety profile [86-88]. Artemether is quickly absorbed and peak plasma concentrations of Artemether and its main active metabolite, Dihydroartemisinin, are reached approximately two hours post-dose. This leads to an initial rapid reduction in parasite mass and most malaria-related symptoms such as fever. Lumefantrine, on the other hand, is absorbed and cleared more slowly with terminal elimination half-life of 3–4 days, eliminating remaining parasites from the blood thus preventing recrudescence [89-90]. Each tablet of AL contains a 1:6 fixed combination with 20 mg Artemether and 120 mg Lumefantrine and the standard regimen consists of six doses, administrated twice-daily for three days. 77 of the 83 countries with endemic P. *falciparum*, have adopted the WHO recommendation of ACT as regimen for acute, uncomplicated P. *falciparum* malaria in adults and in children with a body weight ≥5 kg [88]. A majority have approved the six-dose AL regimen as treatment regimen. The combination drug is well tolerated and effective with 28-day parasitological cure rate of >95% [90].

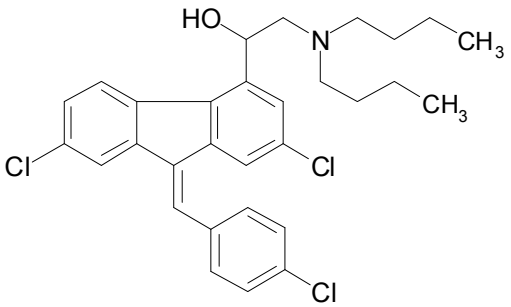

Figure 7. Structure of Lumefantrine, M = 528.9 g/mol

Lumefantrine is an aryl-amino alcohol that prevents detoxification of heme, and belongs to the same family as Mefloquine, Halofantrine and Quinine [91]. It is a highly lipophilic compound with poor solubility in water but soluble in unsaturated fatty acids. It is highly bounded to serum proteins, primarily high density lipoproteins (≥99%), with the free fraction of 8% being bound to erythrocytes [85, 92]. The bioavailability of Lumefantrine vary with food, high-fat meal increase the bioavailability 16-fold compared

fold compared with fasted state in healthy volunteers [85]. In a trial in Uganda [93], patients randomized received AL either under supervision with a meal or unsupervised at home, advised to take the drug with a meal or breast milk. The results showed that both groups had an identical 28-day cure rate (98%). A pharmacokinetic substudy revealed that Lumefantrine plasma concentration was higher in the supervised group, however the identical 28-day cure rate showed that food intake at home was enough to reach sufficient Lumefantrine exposure for optimal efficacy [93-94].

The pharmacokinetics of Lumefantrine has been well characterized. Pharmacokinetic data for Lumefantrine after a standard six-dose regimen gives a C_{max} of 13 to 53 µmol/l at t_{max} of 3–4 days i.e. about 5–10 hours after last dose. Elimination half-life $t_{1/2}$ is about 6 days in healthy volunteers and 3–4 days in malaria patients [85, 95]. With plasma Lumefantrine concentration on day 7 over 745 µmol/l a cure rate of 94% is achieved. With concentrations levels below 529 µmol/l, 49 % had recurrent infections [85].

Lumefantrine is predominantly metabolized by cytochrome P450 3A4 (CYP3A4) isoenzyme [85]. Anti-retroviral drugs are metabolized through the same pathway why pharmacokinetic studies evaluating drug-drug interactions between anti-retroviral drugs and Lumefantrine are important in the future.

There are a few methods published for the determination of Lumefantrine in plasma [96-101] and one for quantification of Lumefantrine in whole blood on filter paper [102]. In the DBS method [102] the blood is sampled and thereafter exactly 100 µl blood is mixed with the same volume of phosphoric acid before applying 50 µl onto the filter paper.

Paper V describes a method for the determination of Lumefantrine in capillary blood applied onto sampling paper. Lumefantrine exhibits a time dependent adsorption to filter paper, with recovery changing from initially 60% to 15–20% 4–7 days later. The sampling paper was therefore treated with L-(+) tartaric acid to prevent the recovery change over time. The tartaric acid is considered safe to use. With the treatment of 0.75 mol/l tartaric acid a recovery of 60–65% was achieved and accurate day 7 level determinations of Lumefantrine in blood samples from nine malaria patients in Tanzania was possible.

2.4.2 Tafenoquine

Tafenoquine, previously known as WR-238605 (fig. 8), is an 8-amonoquinoline and an analog to Primaquine. Tafenoquine is currently being co-developed by GlaxoSmithKline (GSK) Research & Development Limited and the Walter Reed Army Institute of Research, as a replacement for Primaquine as well as for the prevention of malaria [103].

Tafenoquine was developed during the 1980s by researchers at Walter Reed Army Institute in the search for new 8-amodioquinolines that could be

used for prophylaxis against P. *falciparum* since increased parasite resistance against available blood-stage schizonticidal drugs had been developed [104]. Tafenoquine was developed for oral administration and the absorption is increased by approximately 40% with a standard high-fat meal, it is the amount of Tafenoquine absorbed that is affected by food rather than the rate of absorption [105]. Tafenoquine acts on all stages of the malaria parasite and protects against all species of malaria parasites [103, 106-107].

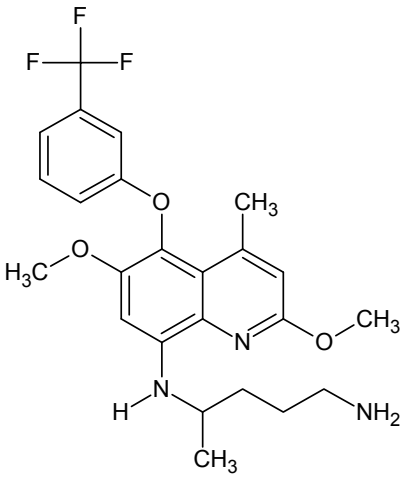

Figure 8. Structure of Tafenoquine, M = 463.5 g/mol

Tafenoquine is slowly absorbed, maximum plasma levels is achieved 9 to 13 hours after dose and it has a mean half-life of approximately 14 days [105, 108]. The bioavailability in humans is unknown but studies in dogs have found a bioavailability of 60–100%, which would result in a volume of distribution of 1530–2550 l in humans, suggesting a high degree of tissue binding. Tafenoquine is eliminated by biliary excretion with enterohepatic re-circulation, but it is not eliminated in the urine [109]. A mean plasma steady-state concentration of 0.69 μmol/l was achieved following weekly dosing of 200 mg Tafenoquine for 6 months in healthy Caucasian subjects [110]. Whole blood concentrations of Tafenoquine are 1.8-fold higher than plasma concentrations and there is an accumulation of the drug in red blood cells with a red blood cell concentration 2.8 times higher than that found in plasma [109].

Gender differences in Tafenoquine plasma concentrations have been found. The mean plasma concentration 12 h after the last dose of Tafenoquine is approximately 1.3 fold higher in females than in males [111]. This may be attributed to weight differences between the genders since women with higher concentrations levels also experienced gastrointestinal disturbances. However further studies are required to determine if there is a need to reduce the dose for females to minimize gastrointestinal disturbance

[111]. Tafenoquine produces hemolysis in glucose-6-phosphate dehydrogenase (G6PD)-deficient recipients like primaquine [112-113].

There are a few bioanalytical methods published for the determination of Tafenoquine in biological fluids, most of them in human plasma [109-110, 114-117]. One of the methods, a liquid chromatography method with fluorescence detection, allows the measurement of Tafenoquine in both venous and capillary blood [117]. There are no published methods described for the determination of Tafenoquine in DBS, of knowledge.

Paper VI describes a bioanalytical method for the determination of Tafenoquine in plasma and DBS. The paper was pre-treated with 0.6 mol/l tartaric acid before application of the sample to enhance the extraction recovery of Tafenoquine, from approximately 10% to 40%.

3 Method development

Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required and necessary equipment. The analytical chain describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results.

3.1 Sample collection

The first step in the analytical chain is to decide which matrix to utilize. Most often venous blood, withdrawn from the arm, or capillary blood, withdrawn from the fingertip, or urine is used. More rarely, saliva and cerebrospinal fluid are utilized. If the venous blood is withdrawn into tubes with an anticoagulant, plasma is obtained after centrifugation. Plasma was used as a matrix in **paper I** for the determination of melatonin and **paper III** for the determination of Eflornithine. Plasma is an accepted sampling method and it allows sample volumes in the range of ml.

In **paper II** saliva was used since a less invasive method was required. The levels of melatonin in saliva reflect the level in blood daytime and 30% of it night time [1-2]. Saliva may be sampled with saliva-collecting tubes, by spitting in a tube or by chewing on parafilm or gum to stimulate saliva production and then spit in a tube. In **paper II** saliva samples were collected using Salivette sampling device (fig 9). The procedure is straightforward and involves chewing on a cotton roll that absorbs the saliva. The cotton roll is placed in the sampling device which is stored in refrigerator until brought to the laboratory for centrifugation and later analysis. The use of saliva for biochemical analysis has a number of advantages compared to blood. Collection is non-invasive, stress free, very convenient and cost effective. In addition, the patient can collect the samples either at home or at work, without the assistance or supervision of medical personnel since instructions are simple and contain few precautions. These advantages makes it possible to study/ reach larger group of patients than if a more invasive method would be used.

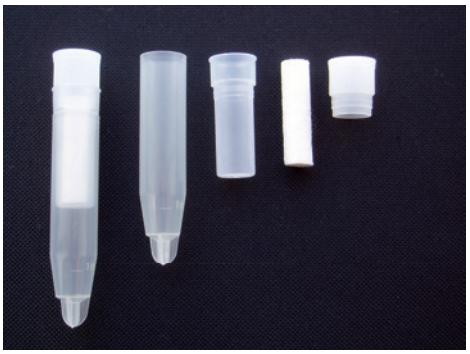

Figure 9. Salivette sampling device

When performing therapeutic drug monitoring (TDM) in rural areas, facilities may not be available (i.e. centrifuges or freezers) for storage of blood samples. This problem can be overcome by capillary blood sampling applied onto sampling paper i.e. dried blood spots, DBS. In DBS, blood is obtained by puncturing a fingertip (or heal/earlobe) with a lancet. The blood is collected with a capillary pipette of fixed size and the blood is applied on to sampling paper (fig. 10). The DBS is dried completely (at ambient temperature) before storing the samples in plastic bags, for transportation to the laboratory, since moisture may cause bacterial or fungus growth [118].

The DBS sampling technique, compared with vein-puncture, requires minimal training to perform, it's less invasive and the DBS represents also a low infectious risk from viruses such as HIV-1 and -2 and hepatitis C as they are dried on the sampling paper. The hepatitis B virus can remain infectious for at least seven days [119-121]. With DBS normally only 50 or 100 µl blood (sample) is collected, compared to 500-5000 µl for whole blood/plasma, which is an advantage when collecting blood from children.

The dried blood spot method was used in **paper IV V** and **VI**.

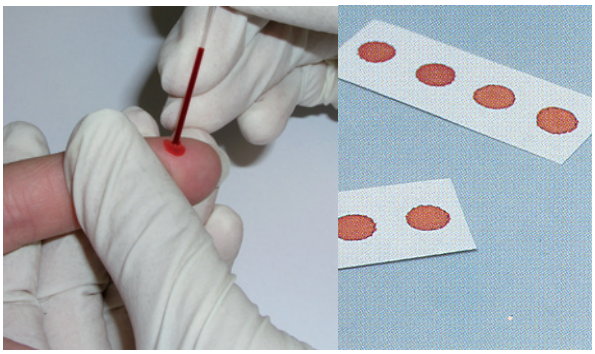

Figure 10. Capillary blood collection and dried blood spots

3.2 Sample preparation

Good sample preparation is often the key to successful analytical results. It has a direct impact on accuracy, precision and quantification limits and is often the rate determining step for many analytical methods. The purpose of sample preparation is to clean up the sample before analysis and/or to concentrate the sample. Material in biological samples that can interfere with analysis, the chromatographic column or the detector includes proteins, salts, endogenous macromolecules, small molecules and metabolic byproducts [122]. Injection of matrix substances can also cover up and hide the drug or analyte being analyzed, making quantification difficult or even impossible. A goal with the sample preparation is also to exchange the analyte from the biological matrix into a solvent suitable for injection into the chromatographic system. General procedures for sample preparation are dilution followed by injection, filtration, liquid/liquid extraction, solid-phase extraction (SPE), protein precipitation or desalting.

3.2.1 Extraction from DBS

Over the past decades, many applications with the DBS method have been reported [118]. It is a sampling method that offers a number of advantages over conventional whole blood sample collection. Low sample volumes, non-invasive sampling compared to vein-puncture, no need for cooling (e.g. freezer) and easy to transport. There are also some disadvantages with the method. The low amount of blood in the dried spot gives a reduced sensitivity in detecting drugs at low concentration. There is no control over the volume and origin of the blood. Interactions between the paper and the drug can give raise to low recovery and it is also an additional step in the analytical chain which can result in additional errors in precision and accuracy.

There are a few parameters that have to be optimized when the DBS method should be used for a drug. Sampling paper should be selected with respect to extraction recovery and reproducibility of the drug and also availability and cost. The liquid used for the extraction of drug from the blood spot should be optimized and organic modifier, ionic strength and pH should be selected. Some drugs interact strongly with the sampling paper why it sometimes is necessary to modify the surface to get high and reproducible recovery. In some earlier reported methods the papers have been modified with a solution of plasma–protein, formic acid and ammonium acetate [123] or dodecyl dimethyl ammonium bromide [124] to achieve enhanced recovery of the drugs.

In **paper IV** the sampling paper used was 3MMChr and 3TC, AZT and NVP was extracted with zinc sulphate and methanol which gave rather clean extracts with a satisfying extraction recovery. In **paper V** and **VI**, extraction

of the drugs was achieved with a solution of acetonitril and acetic acid. To improve the recovery of the drugs the sampling paper, 31ETChr, had to be modified with tartaric acid, which also enhances the stability of the analyte in **paper V**.

3.2.2 Solid-phase extraction

Over the past decade SPE techniques have largely replaced liquid/liquid extraction methods as the preferred technique to extract drugs from biological fluids prior to detection. Advantage with SPE over liquid/liquid extraction is reduction in the consumption of organic solvents which leads to lesser disposal of large quantities of organic solvents. Other benefits with SPE include high enrichment factor, reduced laboratory work, purified extracts, ease of automation, compatibility with chromatographic analysis, and easily adaptable for very selective extraction. Another advantage compared to liquid/liquid extraction is the wide range of sorbents available which makes it possible to apply SPE to a variety of substances e.g. highly polar, ionic and non-polar compounds [125].

In SPE a separation or extraction of one or more compounds from a mixture is achieved by selective distribution between a solid phase (sorbent) and a liquid phase (solvent). The extraction is performed by absorbing the analyte(s) from matrix onto a solid support (sorbent) by chemical attraction. Retention is achieved through strong, but reversible, interactions between the analyte and the sorbent. Typical interactions in SPE are hydrophobic (van der Waals forces), polar (hydrogen bonding and dipole-dipole forces) or ion exchange interactions [125]. All steps (fig. 11) in SPE are subjects to optimization during the method development.

Conditioning;
the column is activated with an organic solvent that acts as a wetting agent on the packing material and solvates the functional groups of the sorbent. Water or aqueous buffer is added to activate the column for proper adsorption mechanisms.

Sample loading;
after adjustment of pH, the sample is loaded on the column by gravity feed, pumping or aspirating by vacuum.

Washing;
interferences from the matrix are removed while retaining the analyte.

Elution;
disruption of analyte-sorbent interaction by appropriate solvent, removing as little of the remaining interferences as possible.

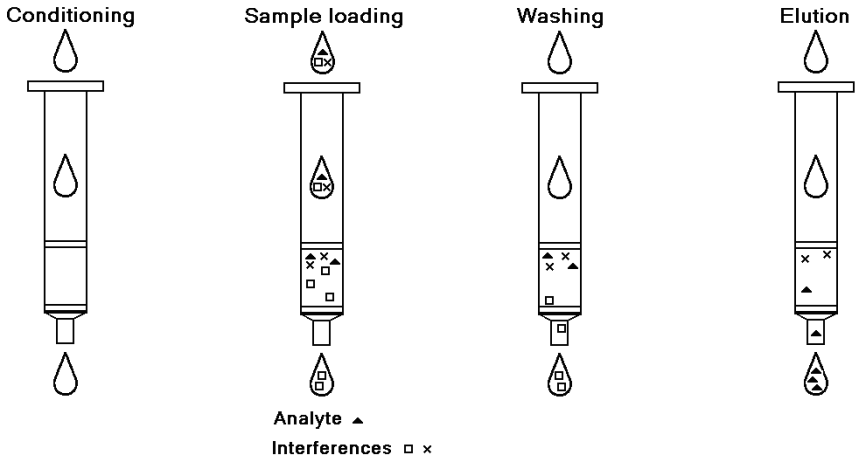

Figure 11. General solid-phase extraction procedure

When the SPE sorbent is selected it is necessary to take into account some physico-chemical considerations such as the functional groups, LogD and pKa of the analytes, the nature of the bonded phase, the strength of the interactions, the secondary interactions between the analyte and the sorbent, the interactions between the sorbent and the components of the matrix and the interactions between the analytes and the sample matrix.

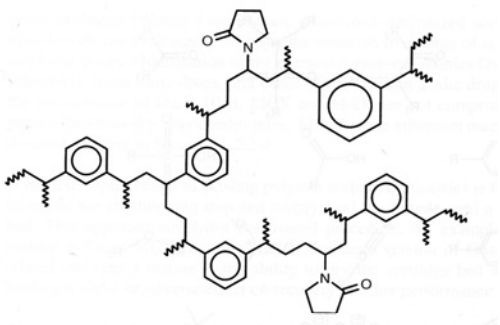

Figure 12. Oasis HLB SPE sorbent

In **paper I and IV**, a synthetic polymer Oasis® HLB (Hydrophilic Lipophilic Balance) (fig. 12), was used. The polymeric sorbents contain an organic polymer, styrene-divinylbenzene, instead of silica with an N-vinylpyrrolidone group added. These sorbents do not give rise to silanol effects, they are water-wettable and not affected by sorbent drying and has also more capacity for polar compounds than compared to silica C-18 phases [125]. Melatonin has pKa of 16.51 and -0.69 and is uncharged in the entire pH-range [126], which means that the forces to retain melatonin on the sorbent are strictly hydrophobic.

Although 3 ml plasma sample was applied on the SPE column a recovery of almost 100 % was achieved. The eluate did not contain more than about 15 mg/l albumin after the extraction on the Oasis SPE column, i.e. the sorbent did successfully remove a large amount of the proteins. The normal range of albumin concentrations in human blood is 35 to 50 g/l, and albumin normally constitutes about 60% of the plasma proteins. Oasis HLB was also tested for the extraction of melatonin from saliva. The column was not suitable, since it resulted in unclean sample i.e. other endogenous compounds were eluted and detected at the same retention time as melatonin. The Oasis HLB column was also used in **paper IV** for the antiretrovirals 3TC, AZT and NVP.

In paper **II** and **VI** an IST Multi-mode (MM) SPE was used. It is a mixed-mode column containing non-polar (C18), strong cation exchanger ($-SO_3^-$) and strong anion exchanger ($-NR_3^+$) functional groups (fig 13). In **paper II** the sorbent was used as a simple reverse phase sorbent and the unwanted cationic and anionic interferences were adsorbed on the sorbent bed. A strong and permanent retention of unwanted charged materials on the SPE sorbent was achieved. In the washing step, only water was used since methanol or acetonitrile would break the hydrophobic interactions between melatonin and the sorbent. The elution of the melatonin occurred in pure methanol, leaving charged endogenous components on the sorbent bed.

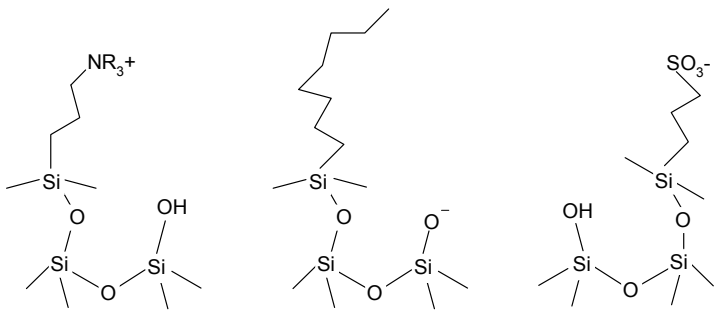

Figure 13. Multiple (hydrophobic, cation- and anion exchange) interactions are possible on ISOLUTE Multi-Mode SPE

In **paper VI** the cation part of the sorbent was used to adsorb TQ. Strong-cation exchange SPE columns contain ion-exchanges sites consisting of sulfonic acid groups. The columns are always charged in the normal pH range [125]. Tafenoquine (TQ) has pKa values of 10.20, 3.49 and 0.86 and is a relatively strong base. When TQ was applied on the SPE column the pH was below pKa of the basic group, to get retention with the ionic form of the analyte. A high organic content in combination with high pH was used to elute TQ, to get the analyte into an uncharged form.

In **paper V** a non-polar octyl-silica (C8) disk column was used. The shorter chain length (compared to C18) allows secondary interactions

between the analyte and the silica gel. For basic compounds, containing nitrogen atoms, the retention may increase as hydrogen bonds to the silica gel arise [125]. The disk makes it also possible to use smaller volumes during the conditioning and elution steps.

3.2.3 Protein precipitation

Protein precipitation is often used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modifier, a salt or by changing the pH which influence the solubility of the proteins [127]. The samples are centrifuged and the supernatant can be injected into the LC-system or be evaporated to dryness and thereafter dissolved in a suitable solvent. A concentration of the sample is then achieved. There are some benefits with the precipitation method as clean-up technique compared to SPE. It is less time consuming, smaller amounts of organic modifier or other solvents are used. But there are also disadvantages. The samples often contain protein residues and it is a non-selective sample cleanup method, there is a risk that endogenous compounds or other drugs may interfere in the LC-system. However the protein precipitation technique is often combined with SPE to produce clean extract.

In **paper III** 300µl cold methanol was added to 75µl plasma, the samples were mixed and kept at 4ºC for 30–60 minutes, mixed again and centrifuged for 10 minutes at 12000 x g. The samples were then kept at -37 ºC to freeze the precipitated proteins, transferred into new glass tubes and evaporated to dryness. The samples was dissolved in 75 µl deionised water and derivatizated before analysis. In **paper VI** 250 µl methanol was added to 100 µl plasma to precipitate the proteins. After centrifugation the samples was evaporated to dryness, dissolved in mobile phase and 50 µl was injected.

3.2.4 Derivatization

Derivatization is a technique used to modify an analyte in order to change its properties like solubility, polarity or boiling point. Generally a specific functional group of the drug participates in the derivatization reaction resulting in a derivative. There are a number of reagents on the market, targeting specific functional groups. An additional reason for derivatization is if the analyte is absence of chromophoric moiety i.e. is absent of UV- or fluorescence chromophores. The derivatization is then preformed in order to be able to detect the analyte of interest with a suitable detector.

In **paper III** eflornithine, who lacks chromophores, was pre-column derivatizated with the optically active thiol N-acetyl-N-cysteine in presence of o-pthalaldehyde. The resulting two diasteromers where detected with UV-detector.

3.3 Separation

3.3.1 Chromatography

Chromatography is an analytical method that is widely used for the separation, identification and determination of chemical components in a complex biological mixture. In chromatography one phase is stationary while the other, the mobile phase, passes through the chromatographic bed. Molecules in a sample will have different interactions with the stationary support and the mobile phase, leading to separation of similar molecules. Molecules that display stronger interactions with the support will tend to move more slowly than those molecules with weaker interactions. In this way, different types of molecules can be separated from each other as they move over the support material, fig 14. The stationary phase may be a solid, porous, surface-active material in small-particle form or a thin film of liquid coated on a solid support or column wall. The mobile phase may be a liquid, a mixture of liquids, a gas or a supercritical fluid with the characteristics of gas and liquid, depending on the chromatographic conditions [128]. As in SPE there are several types of forces that individually, or in combination, result in retention of the analyte by the stationary phase; non polar, Van der Waals forces, dipole-dipole interactions, hydrogen bonding, dielectric interactions and electrostatic attractions, i.e. ion-ion and ion-dipole [127].

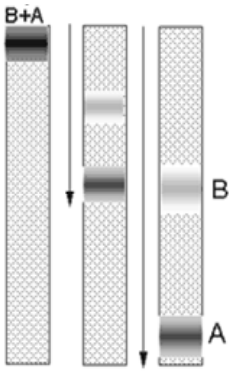

Figure 14. Illustration of chromatographic separation of a mixture of components A and B in a column.

3.3.2 Liquid Chromatography

Liquid chromatography (LC) is today the main tool for analysis of various substances in different matrices in which the mobile phase is liquid. LC is suitable to separate compounds over a wide range of polarity, without previous derivatization. The analyte is forced through a column by a liquid (mobile phase) at high pressure, which decreases the time the separated components remain on the stationary phase and thus the time they have to

diffuse within the column [129]. The interaction of the analyte with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases which results in a high degree of versatility compare to other chromatographic systems. A wide variety of chemical mixtures can easily be separated with LC.

The basic LC system consists of six units; the mobile phase, the pump, the injector, the column, the detector and a data handling system. A block diagram is shown in figure 15.

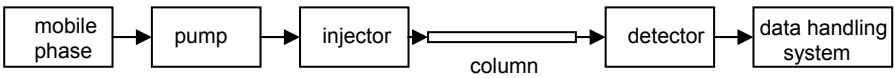

Figure 15. Basic liquid chromatography system

In reversed-phase chromatography a non polar stationary phase is used in conjunction with polar, aqueous mobile phases. Most stationary phases are silica-based bonded phases, but polymeric phases, phases based on inorganic substrates other than silica and graphitized carbon have found their place as well. There are also chiral stationary phases used for the separation of enantiomers [127]. In the selection of stationary phase for a particular application, two things have to be considered. The chemistry of the packing material in the column which should ideally be the most appropriate for the separation in mind and the physical properties of the column, especially particle size and column dimensions since it influences the resolution, the speed of the analysis, the column backpressure, the detectability and the solvent consumption per analysis [130]. The LC column is typically 5, 10, 15 or 25 cm in length and the internal diameter of the column varies from 0.5 to 5 mm. For analytical purposes flow rates between 0.2–1.5 ml/min are normal [130].

To increase the lifetime of the column, a precolumn or guard column is often used. These columns are recommended for samples that contain material/substances that can strongly adsorb on the column, such as proteins. The guard column is normally 0.4 to 1 cm long and most often of the same stationary phase as the analytical column [129].

In paper I a SymmetryShield RP-18 (5 µm, 250 x 4.6 mm) column with a flow rate of 1 ml/min was used for the separation of melatonin and IS from endogenous compounds. 100 µl was injected into the system and since 3 ml was sampled originally from the patients a concentration factor of 15 was achieved. The column gave acceptable resolution, separation and retention of melatonin and internal standard**.** In **paper II** a column with smaller dimensions was used; HyPurity C18, 3 µm (150 x 2.1 mm). The flow rate was set to 0.25 ml/min and the injection volume 38 µl which gave a concentration factor of 45. Apart from the more concentrated sample, the solvent consumption decreases with reduced inner diameter (i.d.) of the

column. In **paper V** and **VI** a Zorbax SB-CN (3.5µ, 150 x 3.0mm) column which has a moderately polar stationary phase was used. The column is specially designed to reduce or eliminate strong adsorption of basic drugs. It is well suited for aggressive mobile phases with pH<2 as well as high ionic strength and ion-pair additives. To prevent strong adsorption for the strong bases TQ and LF, mobile phases with low pH, around 2, was used and the ion-pair agent sodium perchlorate was also added.

When complex sample mixtures are separated, instead of an isocratic system, a gradient LC-system can be used. This means that the solvent composition changes over time during analysis. Initial the organic content in the mobile phase is selected for retention of the most polar analyte in the sample. Thereafter the organic content increases over time until the most hydrophobic analytes are eluted. This can be necessary when the analytes differ in properties i.e. polarity, like with 3TC, AZT and NVP in **paper IV**. Two isocratic compositions of mobile phase were used where the first one eluted 3TC and the second one AZT and NVP. In **paper III** a form of gradient was also used. The two diastereomers of eflornithine were separated on two serially connected C18 Chromolith Performance columns. These columns have highly porous rods of silica which allows high flow-rates at low back-pressure. The mobile-phase organic content was increased during the analysis to elute highly hydrophobic substances, which interact strongly with the columns, in order to shorten the LC-analysis time.

3.4 Detection

There are several different detectors suitable for detection of analytes after the chromatographic separation. Some detectors used in LC are: ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis. The ideal detector has the following characteristics; good sensitivity, good stability, reproducibility, linear response over a few orders of magnitude, short response time and ease of operation [129].

3.4.1 Fluorescence detection

Many compounds have the ability to absorb UV-light of a given wavelength, followed by the emission of light at a longer wavelength. The excited electron will return to its ground state, with the emission of electromagnetic energy producing fluorescence. As some energy is always lost in the process the fluorescent light always has a longer wavelength than the excitation light [131]. Fluorescence has been shown to be extremely useful as detection process and detectors based on fluorescent measurement have provided some

of the highest sensitivities available in LC. The sensitivity may be up to 1000 times greater than with UV-detection [129]. In addition, many substances can, by forming appropriate derivatives, get the ability to fluoresce. In **paper I** the most suitable excitation and emission wavelength were set to 285 and 345 nm respectively which also were used in **paper II**. Florescence detector was also use in **paper VI** at 262 and 470 nm respectively.

3.4.2 Absorbance detection

Absorbance detection is based on the principle that functional groups, of a chemical compound, can absorb light at one or more wavelengths in the UV or the visible light range, 190–600 nm. The absorbance detector is one of the most commonly used detectors. It is relatively inexpensive, robust and easy to operate. A majority of organic compounds have some absorbance in the UV-visible light range and a high molar absorptivity (ε) of the compound allows low concentration levels to be measured. UV-detector was used in **paper III –V.**

4 Method validation

4.1 Validation parameters

Method validation is a process used to verify/confirm that an analytic method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte. Typical parameters to validate are; accuracy, precision, selectivity, sensitivity, linearity, recovery and stability. General recommendation for analytical method validation, i.e. for pharmaceutical methods, can be found in The US Food and Drug Administration (FDA) guideline [132].

4.1.1 Accuracy and Precision

The precision of an analytical method describes the distribution of an analyte, when it is analysed repeatedly, to multiple aliquots of a single homogeneous volume of biological matrix i.e. the closeness of a series of replicates measurements to each other. The precision is usually expressed as the Relative Standard Deviation (RSD) or Coefficient of Variation (CV%) of a series of measurements divided into repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision).

RSD = (standard deviation/mean) x 100

The RSD should not deviate more than 15% for each concentration levels except for the Lower Limit of Quantification (LLOQ) where it should not exceed 20%.

The accuracy of an analytical method is the closeness of the measured value to the true value for the sample. It is determined by replicate analysis of samples of known concentration and compared with reference methods and by found versus added analyte.

%Deviation = ((measured value – true value)/true value)) x 100

The sample used for precision can be used for accuracy determinations with same acceptance criteria (± 15% except LLOQ ± 20%).

4.1.2 Selectivity

Selectivity is the extent in which a method can determine the analyte in a complex mixture without interference from other components in the mixture. The selectivity is evaluated by processing blank samples from independent sources in the same matrix to test for interferences. In **paper I-VI** the selectivity of the system was investigated by comparing chromatograms of pure matrix and chromatograms from possible interfering analytes i.e. metabolites and drugs from possible co-medication.

4.1.3 Sensitivity

The sensitivity of a method is often expressed as Lower Limit of Quantification (LLOQ), Limit of Detection (LOD) and Limit of Quantification (LOQ). The lowest standard on the calibration curve can be accepted as the LLOQ if the analyte response is 5 times the response compare to blank and can be quantified with acceptable accuracy (80-120%) and precision (under 20%). The LLOQ is incorrectly called LOQ in **paper I**. The LOD can be defined as the smallest amount that is clearly distinguishable from background or blank. The LOQ is set at higher concentration than LOD, 10 x signal-to-noise ratio (S/N) and 3 x S/N respectively, thus presenting a greater probability that a value at the LOQ is "real" and not just a random fluctuation of the blank reading. LOQ is the smallest analyte concentration for which a method can be validated with specified accuracy and precision.

4.1.4 Linearity and Range

A calibration curve is the relationship between instrument response and known concentration of the analyte. The calibration curve should be prepared in the same biological matrix as the samples and a calibration curve should be generated for each analyte. The range of the method is the concentration interval where accuracy, precision and linearity have been validated.

The used calibration curve should be the simplest model that adequately describes the concentration-response relationship. The deviation should not exceed more than 20% from the nominal concentration of the LLOQ and not more than 15% from the other standards in the curve.

4.1.5 Recovery

The recovery of an analyte from sample matrix is measured by comparing the response from extracted samples with the response from non extracted standards with the same nominal concentration as the extracted sample. An

alternative is to compare the response from extracted samples with the response from extracted blank matrices to which the analyte at the same nominal concentration has been added, or to extracted pure samples (in aqueous solution).

The recovery of melatonin in **paper I** was calculated by using a standard addition method, to take into consideration the contribution of the endogenous melatonin. The standard addition method is often used when it is not possible to prepare a blank sample matrix without the presence of the analyte; one example is the analysis of endogenous compounds in body fluids. The calibration curves is prepared by taking equal volumes of the sample, containing an unknown concentration of the analyte, and then adding known and different amounts of the analyte to all but one. After the chromatographic analysis the peak heights or areas of the analyte are plotted as a function of the added concentration, or weight, of the analyte (fig. 16).

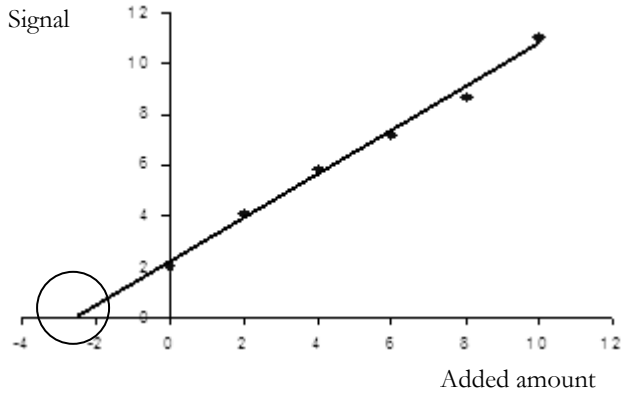

Figure 16. The standard addition method

Extrapolation of the calibration plot, to the point on the x-axis at which y = 0, gives a negative intercept which corresponds to the unknown concentration of the analyte in the sample [133]. If a calibration curve, without the analyte, is prepared and the results are plotted as a function of the added concentration, ideally, an intercept at origo is achieved. If curves, one with the unknown compound present and one without, are compared and found parallel, i.e. the slopes are the same, there is no systematic error in the developed method.

4.1.6 Stability

The stability of the analyte under various conditions should also be studied during method validation. The conditions used in stability experiments should reflect situations likely to be encountered during actual sample

handling and analysis. The following stability conditions are required by FDA and are advisable to investigate;

Stock solution stability
The stability of the stock solution should be evaluated at room temperature for at least 6 hours.

Short-Term Temperature Stability
The stability of the analyte in biological matrix at ambient temperature should be evaluated. Three aliquots of low and high concentration should be kept for at least 24 hours and then analysed.

Long-Term Temperature Stability
The stability of the analyte in the matrix should exceed the time period from sample collection until the last day of analysis.

Freeze and Thaw Stability
The stability of the analyte should be determined, after three freeze and thaw cycles. Three aliquots of low and high concentration should be frozen for 24 hours and then thawed at ambient temperature.

Post-Preparative Stability
The stability of the analyte during stages of the analysis process should be evaluated.

5 Conclusion and future perspectives

This thesis gives a short introduction of the analytes in **paper I-VI** and a short background of the underlying diseases. The methodologies used in the papers are also descried and an introduction to method development and the final validation process is given.

When the melatonin project started, a method for the determination of plasma melatonin levels in patients suffering from sleep related problems like OSAS and RLS, was needed. The method should be used for monitoring the circadian rhythm of melatonin in this patient group. The sampling procedure, with vein-puncture, was soon proved to be inconvenient since the patients, who were at the sleep lab for diagnosis of sleep patterns, woke up when the blood sampling was performed. To reach this group of patients a less invasive method was demanded why a method for the determination of melatonin in saliva was developed. The sampling could be preformed at home and the samples sent by mail to the laboratory for analysis. The method was used to screen melatonin levels in healthy volunteers and in patients. To overcome the problem with the large volume saliva that the method requires, a quantitative liquid chromatography method for analysis of melatonin with reinforced fluorescence would be of interest.

The need of a more sensitive DFMO method, requiring small sample volume, resulted in a method for the determination of the DFMO enantiomers in plasma from rat and humans with late-stage HAT. The method was used for investigation of the L- and D-DFMO pharmacokinetics, to investigate the possible development of a simplified mode of administration of DFMO i.e. oral treatment. The derivatization products formed with o-phtalaldehyde-N-acetyl-L-cysteine are however not stable over time. This problem was solved by using an autoinjector which was programmed to add the derivatization reagent, mix the samples and to stand for a duration of 4.85 minutes prior to injection. A new method with more stabile derivatization products would perhaps be of interest for further investigation of the pharmacokinetic absorption of DFMO.

When performing therapeutic drug monitoring (TDM) studies in rural settings, dried blood spot (DBS) sampling may be an attractive technique. It is a rapid and simple technique with few safety concerns and costs are significantly reduced compared to traditional vein-puncture.

The method for simultaneous determination of the three antiretroviral drugs 3TC, AZT and NVP in DBS was developed to be used in TDM when

optimizing the response to antiretroviral therapy (ART). The method was used to determine concentration levels of the three antiretrovirals in two patients, with the DBS sampling preformed at Makerere University in Uganda. The method seemed well suitable for the determination of 3TC and NVP and to some extent for AZT that has a short half-life resulting in concentration levels below the LLOQ of the method, within two hours. The TDM has also potential use in monitoring of adherence and in drug-drug interactions. Poor adherence may result in drug resistance and the drug- drug interactions may result in alteration of drug concentration in the blood with side effects as a result or reducing the effectiveness of the drug/drugs. It may also result in a change in adsorption, distribution, metabolism or elimination of the drugs or the concurrently administered medication. Interactions studies between ARVs and antimalarials would be of great interest and methods for the determination of both ARVs and antimalarial drugs at the same time would be required. Preferably field adapted methods like DBS.

The problem with parasites developing drug resistance against antimalarial drugs makes the development of new effective and safe drugs or drug combinations important. These drugs need to be evaluated with respect to pharmacokinetic properties like adsorption, biological availability, elimination and dose versus response

A new antimalarial drug combination recommended by WHO is Artemether-Lumefantrine, given as a fixed dose combination. A method for the determination of Lumefantrine (LF) in DBS was developed. The method is suitable for drug studies in rural settings and has been used in several studies. It has been shown that LF concentrations of day 7 are an important indication of treatment outcome where concentrations below 529 nmol/l are associated with treatment failure.

A method was also developed for the new promising antimalarial drug Tafenoquine in plasma and in DBS which may be useful in future clinical studies in laboratory environment as well as in rural settings.

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All my love is for you!

Give me five!

7 Summary in Swedish

Utveckling och validering av bioanalytiska metoder – applicering på melatonin och några utvalda infektionshämmande läkemedel

Analytisk kemi är den gren av kemin som innefattar metoder för att kvalitativt identifiera samt kvantitativt koncentrationsbestämma ämnen i olika matriser. Målsättningen hos den analytiska kemisten är att utveckla nya metoder som ger repeterbara, korrekta analysresultat. Vid utveckling av nya analysmetoder följer man därför en flerstegsprocess, den analytiska kedjan, där varje steg optimeras. Kedjan innefattar provtagning, provupparbetning, separation, detektion och utvärdering.

Det första steget i kedjan är provtagning där vanliga matriser är blod, plasma, urin och saliv. Vid kliniska studier av läkemedel mot till exempel malaria, afrikansk sömnsjuka eller HIV kan provtagningen ske i en miljö, ute i fält, där möjlighet till centrifugering och kylförvaring av venöst blod är i stort sett obefintlig. Man använder sig då istället av kapillärblod som appliceras på ett provtagningspapper från ett stick i fingret. Proverna kan sedan skickas till ett laboratorium med post för analys. Vid nästa steg, som är provupparbetning, isoleras analyten från den biologiska matrisen. Detta kan göras med enkel proteinfällning och/eller fastfasextraktion. Den analyt man är intresserad av separeras sedan från kvarvarande föroreningar med ett vätskekromatografiskt system. Slutligen detekteras analyten där storleken på detektorsignalen är proportionell mot analytens koncentration.

Melatonin är ett endogent hormon som huvudsakligen produceras av tallkottkörteln med en robust cirkadisk rytm. Produktionen stimuleras av mörker och inhiberas av ljus vilket leder till höga halter av hormonet i blodet under nattetid och låga dagtid [2]. Förutom att fungera som en biologisk klocka, som reglerar dygnsrytmen och växlingen mellan vakenhet och sömn, har melatoninets roll undersökts inom flera olika områden. Bland annat vid cancer- och psykiatriska sjukdomar men även vid sjukdomar som är relaterade till sömn samt resynkronisering av den cirkadiska rytmen som skiftarbete, jet-lag, förskjuten sömnfas, obstruktiv sömnapné (OSAS) samt rastlösa ben (RLS) [1, 9].

I **arbete I** och **II** har metoder för att mäta det endogena hormonet melatonin i plasma respektive saliv utvecklats. Plasmametodens syfte var att mäta koncentrationsförändringen av melatonin över dygnet hos patienter

med sömnrelaterade sjukdomar som OSAS och RLS. Dock framkom det ganska snart att metoden inte var lämplig då patienter som låg inne på sömnlaboratoriet för utredning av sömnen vaknade av provtagningen. En metod för provtagning i hemmet efterfrågades varpå en metod för att mäta melatonin i saliv utvecklades. Den tillåter patienten att själv utföra provtagningen och sedan med post sända proverna till laboratoriet för analys.

Afrikansk sömsjuka är en sjukdom orsakad av parasiter (*Trypanosoma brucei*) som sprids via bett av tsetseflugor, främst i områden söder om Sahara och norr om Zambezi [28]. 2009 rapporterades mindre än 10 000 fall av sjukdomen till Världshälsoorganisationen (WHO) men denna siffra är troligtvis missvisande då de flesta fall inte rapporteras. 60 miljoner människor lever med risken att bli infekterad [29, 31]. De läkemedel som idag används mot Afrikansk sömnsjuka är få. De är dessutom gamla, mycket giftiga och resistens har utvecklats mot vissa av dem [33]. Under de senaste 50 åren har endast ett nytt läkemedel mot Afrikansk sömnsjuka registrerats, Eflornitin som utvecklades i början av 1980-talet inom cancerforskningen [38].

Syftet med **arbete III** var att utveckla en metod för att koncentrationsbestämma läkemedlet eflornitins två enantiomerer i plasma. Metoden har använts vid studier av den kirala farmakokinetiken för eflornitin i råtta och slutligen människa för att utvärdera möjligheten att ta fram en förenklad behandling mot afrikansk sömnsjuka. Idag ges läkemedlet via dropp var 6:e timme i 14 dagar.

Humant immunbristvirus (HIV) är ett retrovirus som angriper den mänskliga kroppens immunförsvar och orsakar AIDS (förvärvat immunbristsyndrom). Viruset sprids via sexuell kontakt, blodtransfusioner eller kontakt med infekterat blod, via amning samt under graviditeten [53]. 2008 beräknades ungefär 33.4 miljoner människor världen över vara smittade varav två tredjedelar i Africa söder om Sahara. Ungefär 3 miljoner människor beräknades bli smittade av HIV och 2 miljoner dog av AIDS [54]. En normal antiretroviral behandling utgörs av en kombination av två olika grupper av läkemedel bestående av en nukleosidanalog samt två icke-nukleosider. Tripletten lamivudin, zidovudin och nevirapin är rekommenderad förstabehandling i många låginkomstländer [56] .

Arbete IV beskriver en metod för att simultant koncentrationsbestämma de tre antiretrovirala läkemedlen lamivudin, zidovudin samt nevirapin i kapillärblod applicerat på papper. Metoden har använts för att bestämma koncentrationen av läkemedlen i två patienter på antiretroviral standardbehandling där det framkom att metoden är bäst lämpad att användas för att mäta lamivudin och nevirapin men i viss utsträckning även zidovudine.

Malaria är en sjukdom orsakad av parasiter (*Plasmodium*) som sprids via bett av en infekterad malariamygga. De vanligaste arterna är *Plasmodium vivax* och *Plasmodium falciparum* där *falciparum* ger den allvarligaste formen av sjukdomen samt flest dödsfall [75]. Ungefär 250 miljoner fall av malaria inträffar varje år varav ca en miljon dör, främst barn i Afrika, men malaria finns i över 100 länder i världen [74]. Ett stort problem de senaste årtiondena har varit den snabba ökningen av resistenta parasiter som lett till att många av de vanligaste läkemedlen blivit verkningslösa. Ett sätt att motarbeta utvecklingen av resistens är att kombinera två läkemedel med olika verkningssätt samt krävs att nya läkemedel utvecklas.

I **arbete V** har en metod för att mäta lumefantrin i kapillärblod på provtagningspapper utvecklats. Metoden är främst framtagen för att användas i uppföljningsstudier där läkemedlets effektivitet samt möjlig resistensutveckling studeras.

I **arbete VI** beskrivs en metod för att mäta det nya lovande malarialäkemedlet tafenoquine i blod applicerat på papper samt i plasma. Metoden kan användas i framtida kliniska och farmakokinetiska studier av läkemedlet.

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