METHODS AND TECHNIQUES FOR ASSESSING EXPOSURE TO ANTIMALARIAL DRUGS IN CLINICAL FIELD STUDIES

INFORMAL CONSULTATION ORGANIZED BY THE WORLD HEALTH ORGANIZATION WITH THE TECHNICAL SUPPORT OF THE WORLDWIDE ANTIMALARIAL RESISTANCE NETWORK

22-24 FEBRUARY 2010, BANGKOK, THAILAND

World Health Organization

WWARN

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Abbreviations

AUC	area under the concentration–time curve
DBS	dried blood spot
EDTA	ethylenediaminetetraacetic acid
HPLC	high-performance liquid chromatography
ISR	incurred sample re-analysis
LC	liquid chromatography
LLOQ	lower limit of quantification
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PK	pharmacokinetics
RBC	red blood cell
ULOQ	upper limit of quantification
UV	ultraviolet radiation
WHO	World Health Organization

Introduction

Achieving adequate concentrations of antimalarial drugs in the blood is pivotal to curing malaria. Accurate measurement of drug concentrations is essential to ensure optimal dosing of the currently available and newly introduced antimalarial drugs and for differentiating inadequate exposure to a drug from true resistance to the drug. For each drug, answers are needed to two questions: 'What exposure (concentration over time) is necessary to ensure the required therapeutic effect?' and 'Should the recommended dosage be modified for important target populations, such as infants, pregnant women and people with co-morbid conditions (especially HIV/AIDS and malnutrition), to ensure the required therapeutic effect?' Lower concentrations of several antimalarial drugs have been found in pregnant women and young children than in non-pregnant adults.

With increased laboratory capacity for accurate measurement of antimalarial drug concentrations, it is hoped that such measurements will become a routine component of most studies of therapeutic efficacy. Increased capacity is also needed to:

- characterize the pharmacokinetics (PK) (concentration–time profile) of drugs and the relevant covariates (e.g. young age, pregnancy, co-morbidity, drug interactions);
- understand the pharmacodynamics (effect) of drugs and the contributions of relevant covariates (e.g. acquired immunity, parasite resistance);
- characterize the therapeutic response, i.e. the relation between PK and pharmacodynamics;
- assess concentration-related toxicity;
- assess resistance and prevent it by identifying the optimal dosages for target populations; and
- assess adherence to precribed dosing regimen.

As the determinants of the therapeutic response are multi-factorial, studies of the PK of antimalarial drugs often have inadequate power to define the optimal dosage. The therapeutic blood or plasma concentration ranges have been defined for only a few antimalarial drugs. Furthermore, the ranges change as resistance to the drugs increases in the parasite population. Clearly, much more information is needed to improve dose regimens.

Unless drug concentrations are measured, it is impossible to distinguish clinical treatment failure resulting from an inadequate drug concentration from failure due to drug-resistant parasites. Low plasma or blood drug concentrations increase the risk for treatment failure, and high concentrations are toxic. The dose regimen should result in blood concentrations that exceed the minimum parasiticidal concentration in order to have a maximum parasite-killing effect; and, to ensure cure, the blood concentration must exceed the minimum inhibitory concentration until the last malaria parasite is killed. This requirement highlights the importance of defining therapeutic

concentrations and PK and adequately characterizing the terminal elimination phase of the drug. Recommended doses of antimalarial drugs are generally based on the central tendency (e.g. mean, median) of the drug concentration; however, distributions are particularly important, as it is patients with extreme values who are prone to treatment failure or toxicity.

The bioavailability of certain antimalarial drugs, such as lumefantrine, varies widely. Dosage has often been recommended in the absence of information on blood concentrations in important patient subgroups, such as children and pregnant women. As a result, the recommended doses have often been too low. Patients with high parasite loads and low drug concentrations are important drivers of resistance. Common errors in dosing are due to extrapolation of doses recommended for uncomplicated malaria to patients with severe malaria or from adults to children or pregnant women, deriving PK–pharmacodynamics relations from studies in immune people and failure to characterize adequately the elimination phase of a drug. For example, resistance to sulfadoxine–pyrimethamine might have been partially due to systematic under-dosing of children.

Individual studies almost invariably have inadequate power to characterize adequately the factors that influence the PK of antimalarial drugs. The main limitation of pooling data on individual patients is differences in assay and analysis methods between studies. Differences in clinical protocols (e.g. eligibility criteria, standardization of diet, presence of other drugs, comparison groups, source and quality of drug) also contribute to variance in PK estimates. A further limitation is the use of different biological matrices (plasma, serum, venous blood, venous capillary blood) in different studies. The concentration relationships between different matrices are not always straightforward. This further complicates the comparison of results from different studies, pooling of data and meta-analysis of results, sometimes making it impossible to know whether the PK in study populations actually differ or whether the apparent differences reflect differences in the matrices or in the accuracy of the methods used.

Some of these difficulties can be addressed by method standardization, the introduction of quality-control systems and, for more stable antimalarial medicines, establishing external quality assurance schemes, in which blinded samples are sent to participating laboratories and the results of their assays compared. Such a procedure will be part of a quality assured–quality control proficiency testing scheme planned within the WorldWide Antimalarial Resistance Network (www.wwarn.org). The aim of this network is to provide comprehensive, timely, quality-assured, integrated information for tracking the emergence and spread of antimalarial drug resistance worldwide. The network is collaborating on a 3-year pilot project with the WHO Global Malaria Programme, involving transfer of data and exchange of information on the development of tools to facilitate the monitoring of antimalarial drug efficacy and resistance. Global participation in the quality assured–quality control proficiency testing scheme would be facilitated by the availability of

a guidance document for sample collection and storage, core protocols for studies of PK (and pharmacodynamics) and acceptance and evaluation criteria for each bioanalytical assay.

In order to achieve these objectives, a consensus meeting was organized by WHO, with technical support from the WorldWide Antimalarial Resistance Network, and held in Bangkok in February 2010. The aim was to reach consensus on methods for assessing exposure to antimalarial drugs, including:

- criteria for assessing performance during bioanalytical method validation and criteria for acceptance during routine use of a bioanalytical method;
- requirements for accuracy and reproducibility and other essential validation parameters, as outlined in guidelines from the European Medicines Agency, the United States Food and Drug Administration and other agencies;
- appropriate sampling schemes for studies of the PK of therapeutic drugs, including monitoring at single times (usually on day 7), intense sampling for full profiling and sparse sampling for population-based PK models;
- sampling strategies, including type of matrix, type of anticoagulant, sample storage and sample transport;
- analytical methods and their applicability to analysing antimalarial drugs; and
- clinical and methodological pitfalls.

This document is the result of the consensus meeting. It can be used as a reference by investigators conducting clinical trials and by laboratories performing antimalarial drug assays as well as by national malaria control programmes, study sponsors and regulatory authorities responsible for evaluating antimalarial drugs.

Chapter 1. Analytical strategies

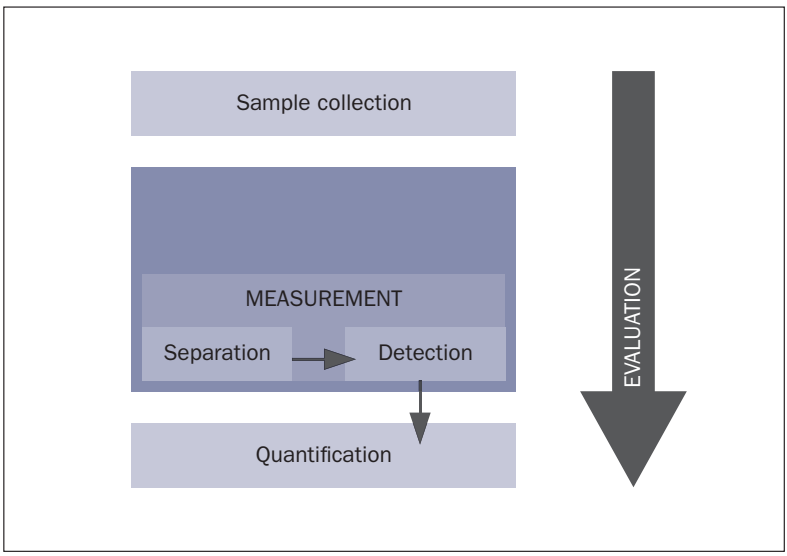
1.1 OVERVIEW

Most analytes can be measured by many different techniques. The choice of method involves several considerations, including:

- At what concentrations do I expect to find the analyte in the study population?
- What matrix will I be analysing?
- What levels of precision and accuracy are required?
- Is the analysis cost–effective? Is there sufficient funding to perform these analyses?
- What equipment is required, and are there qualified personnel to perform the analyses?
- Where will the method be used? For instance, will it have to be adapted for use in the field?
- How quickly are results required?

It is usually preferable to begin with a simple, robust method that does not require the purchase of expensive equipment or equipment that is difficult to maintain. Developing an analytical strategy is the first step in implementing a new analytical method (Figure 1.1). Collection and storage of samples are essential parts of the analysis and are covered in detail in Chapter 2.

Figure 1.1. Factors to consider in developing an analytical strategy



1.2 SAMPLE PREPARATION

The purpose of sample preparation is to eliminate as many contaminants from blood or other physiological samples as possible, in order to simplify chromatographic separation *(1-2)*. Protein precipitation is often the first (and sometimes the only) sample preparation step performed to eliminate interfering endogenous substances.

The sampling matrix is the first element to be considered before choosing a sample preparation technique. Complex matrices such as biological fluids often require a more selective method and more extensive sample preparation than only protein precipitation. This is particularly important if the target concentrations are very low. Liquid–liquid extraction and solid-phase extraction are the other two main sample clean-up techniques. Filtration may be required if the free fraction of the drug is the target. The sample preparation procedure contributes significantly to the total cost of analysis and is quite often also the bottleneck for assay throughput, so the final choice depends on many factors.

1.2.1 Protein precipitation

Protein precipitation is based on the principle that reduction of the solvation potential of a liquid phase will lower the solubility of plasma proteins, thereby precipitating them out of solution. The solubility of proteins in aqueous buffers depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein surfaces. Proteins with a high hydrophobic amino acid content on the surface are poorly soluble in an aqueous solvent. Charged and polar surface residues interact with ionic groups in the solvent and increase solubility. Knowledge of the amino acid composition of a protein helps in determining the ideal precipitation solvent for that protein. The two most common solvents used for precipitation alone or in combination with salts are methanol and acetonitrile. They have different properties and cannot be used interchangeably. Acetonitrile has been shown to be more effective in removing protein, while methanol is more effective in solubilizing hydrophilic analytes. Protein precipitation is followed by vacuum filtration or centrifugation to compress the precipitant and generate a clear supernatant.

Although protein precipitation is the most cost–effective sample preparation approach, generating the least organic waste, it has some drawbacks. This technique produces samples that contain protein residues, which can clog tubing or adsorb onto the stationary phase in the analytical column. As it is a nonselective clean-up method, a highly selective analyte detection system is needed, such as liquid chromatography–tandem mass spectrometry (LC–MS/MS); however, LC–MS/MS is highly susceptible to matrix effects (suppression or enhancement of the signal). Matrix effects are more likely to occur with nonselective sample preparation techniques such as protein precipitation than with more selective methods such as solid-phase extraction.

1.2.2 Liquid–liquid extraction

Liquid–liquid extraction, also known as 'solvent extraction' or 'partitioning', is used to separate analytes on the basis of their relative solubility in two different immiscible liquids, usually a water-based phase and an organic solvent. Extraction recovery is a function of the octanol–water partitioning coefficient, which is influenced by sample pH *(1)*.

In general, although liquid–liquid extraction often provides good recovery for a wide range of analytes, it is relatively nonselective. Thus, the final extract usually contains the hydrophobic contaminants that were present in the sample originally (e.g. phospholipids). Phospholipids have been shown to be a major cause of ion-suppression during LC–MS/MS analysis *(3)*. Liquid–liquid extraction is also quite time-consuming, difficult to automate and generates large volumes of organic solvent waste. Recently, various solid-phase extraction manufacturers have begun producing solid-supported liquid–liquid extraction columns, which makes automation easier *(1)*. Liquid–liquid extraction in a scaled-down version is still widely used, particularly in combination with LC–MS.

1.2.3 Solid-phase extraction

Solid-phase extraction is used to separate analytes from a mixture on the basis of their physical and chemical properties. The technique was introduced in the early 1970s as an alternative to liquid–liquid extraction. It is more efficient, simpler to automate and much faster because it is based on multi-step equilibrium extraction, whereas liquid–liquid extraction has only a single equilibrium step. Solid-phase extraction can be used to isolate analytes of interest from a wide variety of liquid biological matrices, such as urine, blood, plasma, serum, saliva and breast milk. The principle is the same as that for LC. The main difference is that, while the goal with LC is to ensure intermediate retention and as rapid a separation as possible, the goal with solid-phase extraction is to ensure either 100% retention (sample loading and wash) or no retention (sample elution). The liquid phase is changed at each step (load, wash and elution) to ensure extraction of the analyte of interest, while interfering components are either washed off before elution or retained on the column after the final elution step. There are two common solid-phase extraction protocols. One approach is to let the sample pass through the extraction column and to collect the liquid fraction filtering through. Conditions are then chosen to retain as many interfering compounds on the column as possible, while analytes with no or very low affinity for the stationary phase are pulled through. The commonest approach, however, is to retain the analyte of interest and pass interfering substances to waste. A wash step with an appropriate solution is often performed before the analyte of interest is eluted into a clean fraction.

Solid-phase extraction columns are available with a variety of stationary phases. The four main mechanisms for binding are reversed phase (i.e. hydrophobic interactions), normal phase, hydrophilic interaction LC and ion exchange. These methods can be subdivided further into strong and weak acid cation exchange and strong and weak base anion exchange. Most stationary phases are based on silica with a specific functional group bonded to the surface. These functional groups include hydrocarbon chains of variable lengths (for reversed-phase and solid-phase extraction), quaternary ammonium or amino groups (for anion exchange) and sulfonic acid or carboxyl groups (for cation exchange). A variety of polymer-based sorbents with better pH stability than bonded silica are available.

The stationary phase can be contained in a packed syringe-shaped cartridge, a 96-well plate or a 47- or 90-mm flat disc, each of which can be mounted on a specific type of extraction manifold. A typical cartridge solid-phase extraction manifold can accommodate up to 24 cartridges, while the typical well-plate manifold can accommodate a single plate at a time. Most manual solid-phase extraction manifolds are equipped with a vacuum port. Application of vacuum speeds up extraction, but it must be controlled throughout the procedure. Too high a vacuum during sample loading can lead to less efficient extraction and loss of recovery. The same holds true for the elution step.

Solid-phase extraction is a selective, versatile technique but can be prohibitively expensive.

1.3 MEASUREMENT

Measurement of a given analyte can be divided into a separation step and a detection step. For pharmaceutical solutions, the separation step can sometimes be excluded because the composition of the solution is controlled. The same approach cannot be applied to biological samples, which have a more complex matrix, even if a sample clean-up method such as solid-phase extraction has been used. Sometimes, the analytes are present in only trace amounts, and selective measurement is essential.

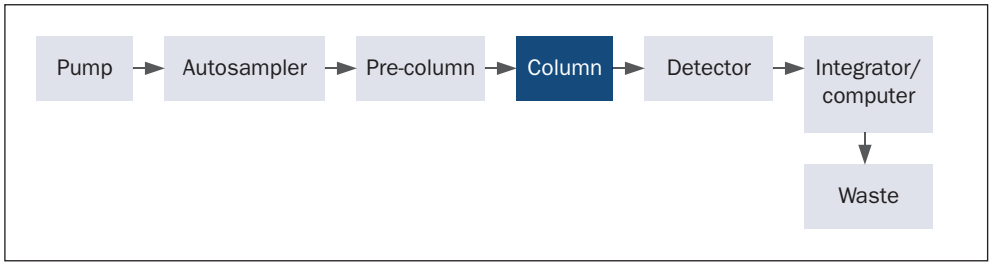
The choice of analytical technique for measuring the concentration of an antimalarial drug in biological fluids depends mainly on the concentration range of interest. A brief, focused review of LC-based analytical approaches for antimalarial drug and metabolite bioanalysis is given here. A comprehensive review of this subject is available *(4)*.

1.3.1 Separation

Analytes must be separated before their detection. Chromatography in its various forms has become the main analytical method for separating components from a mixture. The principle is the partitioning of analytes between a stationary phase and a mobile phase.

High-performance LC (HPLC) and LC are the most widely used techniques for the analysis of complex mixtures (Figure 1.2).

Figure 1.2. Column in simple liquid chromatography system



LC is highly automated, allowing comparatively high-throughput analysis with sophisticated autosamplers and data management systems for independent sample analysis and reporting *(5)*. It is the commonest method for accurate quantification of antimalarial drugs in body fluids in malarious, resource-poor settings. Bell et al. *(6)* described an LC–ultraviolet (UV) detection method for simultaneous determination of sulfadoxine, pyrimethamine, chloroquine, amodiaquine and desethylamodiaquine in plasma, developed particularly for resource-poor settings.

The first publications in which modern chromatography was used appeared in about 1940 *(7)*. LC is a separation process in which a solution of analytes is injected onto an analytical column and distributed between two phases. The analytical column is the site of separation; it contains the stationary phase, through which flows a mobile phase. The analytical sample is injected onto and passed through the column in order to separate the analytes of interest from the sample matrix. The separation is determined by the composition of the stationary and mobile phases. The two main types of column chromatography are normal-phase and reversed-phased chromatography.

Normal-phase chromatography

In normal-phase chromatography, the stationary phase is polar, typically comprising silica with active silanol groups. The mobile phase is less polar, often consisting of solvents such as chloroform, ethyl acetate and hexane. The solvent in the mobile phase can sometimes form weak associations with the active sites present in the stationary phase, and the analyte has to compete for binding sites (adsorption mechanism). A molecule will be adsorbed only when it adsorbs more strongly onto the adsorbent in the stationary phase than the solvent. Alternatively, the liquid phase forms an immobilized layer, and the analyte partitions (partitioning mechanism) between this layer and the flowing mobile phase. With bonded phases, both mechanisms are usually involved. These interactions also depend on steric factors, making normal-phase chromatography particularly suitable for separating isomers and non-polar analytes *(7)*. One disadvantage of this technique, however, is that the polar surfaces may be contaminated *(5)*. Normal-phase chromatography methods have been used to analyse samples containing pyrimethamine and chloroquine *(8-10)*.

Reversed-phase chromatography

In reversed-phase chromatography, the stationary phase is less polar than the mobile phase. Methanol and acetonitrile mixtures are commonly used as solvents. Bonded phases are made up of silica that has been modified to present carbon chains of varying length and carbon atoms to the passing mobile phase. Common phases include C2, C4, C6, C8 and C18, all producing a hydrophobic stationary phase. The phases with a short carbon chain (e.g. C2) usually also present a secondary ion-exchange binding mechanism because of the interaction between the analyte and free silanol groups on the silica surface. Unlike normal-phase chromatography, reverse-phase chromatography ensures retention of relatively polar analytes on the column, thereby separating them from the rest of the mixture. This technique is ideally suited for water-soluble analytes of medium polarity; however, it can also be used for certain non-polar and ionic analytes in combination with ion-pairing techniques *(5)*. Most assays for antimalarial drug involve reversed-phase chromatography *(4)*.

Other, less commonly used modes of LC include:

- **affinity chromatography,** based on receptor–ligand interactions, used mainly to purify the ligands via immobilized receptors in the stationary phase. This technique is suitable only for the analysis of hormones, enzymes and proteins.
- **size-exclusion chromatography:** Molecules are separated according to their size and shape after passing through a porous stationary phase. The technique is designed to allow larger molecules to pass through while retaining smaller molecules in the pores of the stationary phase. This is the principle behind on-line solid-phase extraction methods used for sample clean-up.
- **ion-exchange chromatography:** The stationary phase has ionic charges as active sites, and counter ions in the mobile phase neutralize these charges. The molecules of interest compete for the charged sites, thereby separating themselves from the rest of the mobile phase. This technique can be used to analyse acidic and basic analytes, e.g. proguanil and its metabolites.
- **chiral chromatography:** Enantiomers can be separated with this technique, in a chiral-specific stationary phase, for example in the assay developed to separate the enantiomers of mefloquine *(11)*.
- **supercritical fluid chromatography:** Pressurized supercritical fluids (e.g. carbon dioxide with a polar organic solvent) make up the mobile phase in this technique, resulting in a hybrid between gas chromatography and LC. It is particularly useful in preparative methods, as the mobile phase is easily evaporated *(5)*.

1.3.2 Detection

Several techniques exist for detecting analytes after chromatographic separation. This section focuses on analyte detection after LC separation. The commonest techniques are UV, MS, fluorescence and electrochemical detection.

Ultraviolet detection

Absorbance detectors measure the absorbance at one or more wavelengths in the UV or visible light range. These are the most commonly used form of detection, as they are cheap, robust, easy to operate and sensitive for analytes with high molar absorbtivity. UV absorption detectors are sensitive to molecules that absorb light in the range 200–350 nm; below 200 nm, there is interference from the solvent, and the practical lower limit is 210–220 nm, depending on the method. In reversed-phase chromatography, UV-transparent solvents such as acetonitrile, tetrahydrofuran, methanol and water are usually used, while tetrahydrofuran, aliphatic alcohols, *n*-paraffins and methylene dichloride are the solvents used in normal-phase chromatography.

Many molecules absorb UV or visible light. The absorbance of solutions of these molecules increases with increasing attenuation of the beam and is directly proportional to the path length *(b)* and the concentration *(c)* of the absorbing species, as summarized in Beer's law:

$$A = \varepsilon bc,$$

where A is absorbance and ε is a constant of proportionality, known as the absorption or the extinction coefficient.

Drugs containing UV-absorbing functional groups (chromophores) have valence n, p or s electrons with low excitation energy, and, when the molecule absorbs energy (i.e. light), the electrons are promoted from their ground state to an excited state. A major disadvantage of UV detection is that this approach does not allow quantification of drug molecules that do not have a chromophore group. This includes the artemisinins, which have an estimated molar absorption coefficient of 163–183 l/mol per cm, with the maximum response at 210 nm. Because of this small absorption coefficient and as absorption is concentration-dependent, the low concentrations of artemisinins present in biological fluids cannot be quantified by UV detection. The currently recommended method for quantification of artemisinins and their derivatives in biological fluids is MS.

Many LC–UV methods have been developed for analysing antimalarial drugs that are accurate, precise and reproducible when used for clinical samples. The level of sensitivity that may be obtained and the high sample volume required for analysis, however, may limit the usefulness of these methods.

The dose of antimalarial treatment must be optimized for vulnerable populations (such as children and pregnant women), which presents challenges with regard to the sampling of blood and plasma, as collecting large blood samples by venepuncture is generally not acceptable for these populations. It is becoming routine to collect much smaller sample volumes (50–100 µl) for

these studies. Many successful methods have been published in which LC–UV was used with limited sample volumes *(12-13)*; however, analytical methods that are more selective and sensitive than LC–UV are often required for small volumes.

Today, most laboratories have replaced fixed-wavelength UV detectors (usually 254 nm) with variable-wavelength detectors, which are robust and are sensitive for 0.01–100 µg of an analyte on a column. A more advanced UV detector that can be coupled to LC is the diode array detector, which allows monitoring across the full UV range simultaneously, by use of photodiodes that detect light scattered by a fixed monochromator set over a range of wavelengths to a resolution of about 1 nm. Use of LC–diode array detection permits quantification at several wavelengths simultaneously, so that each analyte can be monitored at its absorbance maximum. The system can also be used for identification, as it records entire spectra for an analyte. Diode array detection can also be used to confirm the purity of the analyte, verifying that there are no obvious co-eluting compounds, and can be used to detect unknown metabolites. When sensitivity down to low nanogram per millilitre concentrations is required, however, as for certain antimalarial drugs during the terminal elimination phase, the method of choice is LC–MS/MS.

Mass spectrometric detection

LC–MS and LC–MS/MS are increasingly popular analytical techniques and are now considered the gold standards in the pharmaceutical industry owing to their very high sensitivity and selectivity in comparison with conventional UV detection. A mass spectrometer can be used to identify and quantify various compounds by their mass-to-charge ratio. Typically, a sample to be analysed must first be vaporized before being introduced into the mass spectrometer. A MS detector has three main parts: the interface, where ions are generated (ionized), the mass analyser (separation) and the electron multiplier (detector). Ionization can be brought about by various techniques, such as electrospray ionization or atmospheric pressure chemical ionization. The ions are drawn through the mass spectrometer due to a voltage differential between the inlet (orifice) and the exit lens. The mass analysers (e.g. quadrupoles) sort the ions before they are detected. Because of its high specificity for identifying particles according to mass-to-charge ratios, this technique is useful in PK studies on slowly eliminated antimalarial drugs at low concentrations in the terminal elimination phase. Both the sensitivity and the selectivity of MS analysis can be increased substantially by the addition of consecutive mass spectrometer units (e.g. triple quadrupole).

Mass spectrometers are, however, too expensive for most institutions in resource-poor settings and require highly skilled technical operators and a high level of maintenance. A laboratory in which MS is used also depends on good infrastructure, as these instruments require a tightly controlled environment (i.e. free of dust and contamination, with humidity and temperature control). Trained LC–MS operators are a scarce resource in developing countries.

Appendix 1 lists analytical methods for the quantification of drugs in various matrices by LC–UV and LC–MS. MS requires much smaller sample volumes than the other detection methods that may be coupled to LC, is more sensitive or has a lower limit of quantification and allows the characterization of unknown metabolites or analytes.

Although MS is one of the most selective detection methods, successful detection depends on very careful sample preparation and chromatography. LC–MS is prone to matrix effects (i.e. ionization suppression or enhancement). Inappropriate conditions (nonselective clean-up and poor chromatography) can introduce large bias in study results and render them useless.

Fluorescence detection

If the structure of the analyte is suitable, fluorescence can be more analytically sensitive than UV detection. Fixed- and variable-wavelength fluorescence detectors are available. The sensitivity of a fixed-wavelength fluorescence detector (minimum detectable concentration at an excitation wavelength of 254 nm) can be 1 ng/ml or better, with a linear dynamic range of about 500. Chloroquine, hydroxychloroquine and quinine are highly fluorescent drugs and can thus be detected with high sensitivity. The main problem with this detection method, however, is that background fluorescence interference is common and few other drugs fluoresce. Another problem associated with fluorescence detection is quenching or matrix effects: the signal in a fluorescence detector may be suppressed if the analyte co-elutes with an unknown compound that quenches the signal. Both dissolved oxygen and impurities can quench fluorescence. Thus, this approach is not widely used *(10, 14)*.

Electrochemical detection

Although not commonly used, a number of LC methods combined with electrochemical detection to quantify antimalarial drugs in biological fluids have been reported *(15-19)*. Some studies attribute it with greater sensitivity than UV detection. On average, electrochemical detection requires larger volumes of sample (0.5–1.0 ml) than LC–MS/MS. It can be performed in the oxidative or reductive mode (i.e. to detect analytes that are oxidized or reduced); the reductive mode is less robust than the oxidative mode. In particular, electrochemical detection is prone to problems resulting from dissolved oxygen and metal leakage from tubing. Oxidative electrochemical detection is sometimes used for detecting primaquine. While reductive electrochemical detection was previously commonly used for detecting artemisinins, it has become less popular since the introduction of MS techniques.

1.4 METHODS FOR MEASURING ANTIMALARIAL DRUG CONCENTRATIONS

A wide variety of validated LC methods can be used to measure antimalarial drugs in biological fluids. Assays are becoming more and more sensitive, allowing better characterization of the PK of these drugs. Appendix 1 provides a comprehensive review of published assays for antimalarial drugs. The required sensitivity of an assay depends on the matrix and the target concentrations of the drug being investigated. For slowly eliminated antimalarial drugs, the use of LC–MS/MS with selective preparation techniques is required in order to characterize their long terminal elimination phase adequately.

Chloroquine, piperaquine and amodiaquine are present in plasma at very low concentrations because they are preferentially distributed to the red blood cells (RBCs). If plasma is to be analysed, a highly sensitive assay is required; by day 56, chloroquine is present in plasma at a concentration of only 1–2 ng/ml. Blood concentrations are 5–10-fold higher, so that UV detection is acceptable *(20)*. Like chloroquine, piperaquine is preferentially distributed to tissue and has a long (multiphasic) terminal elimination phase. Accurate estimation of the PK therefore requires an assay that allows quantification of very low concentrations in plasma *(21)*.

With the introduction of artemisinin-based combination therapies, LC–MS assays with adequate sensitivity have been developed for artemisinin *(22-24)*, artemether *(19, 25-29)*, artesunate *(18, 26, 30-32)* and dihydroartemisinin (the active metabolite of artemether and artesunate). Several methods of determination for artemisinin and its derivatives by electrochemical detection have been published *(17, 33-34)*. As the artemisinins do not have UV-absorbing chromophores, they cannot be analysed by straightforward LC–UV *(30)*. The limit of detection of electrochemical detection methods is 5–20 ng/ml in 0.5–1 ml of plasma. The introduction of LC–MS and LC–MS/MS simplified the analysis of artemisinin derivatives substantially, but low concentrations and problems of stability continue to hamper the move towards analysis of dried blood spots (DBSs). Atovaquone, proguanil, clindamycin and dapsone are present at relatively high concentrations in plasma, so LC–UV is an appropriate choice of analytical technique *(8, 35-48)*. Doxycycline can also be accurately determined with LC–UV *(49-53)*. The same may be said for sulfadoxine and mefloquine, which have also been quantified in DBS with LC–UV techniques *(4, 54-57)*.

The choice of matrix to be analysed should always be carefully considered. Apart from facilitating comparisons of the results of studies, it is wise to choose the matrix in which the highest concentrations can be measured, so that simpler, more cost-effective analytical techniques can be used. The choice should be balanced against measuring concentrations at the drug's target site. The presence of active metabolites will also affect the choice of analytical technique. Proguanil can be measured accurately with LC–UV, but, as the active metabolites have been reported to be present in small amounts, LC–MS/MS is a better choice *(58-59)*.

With selective sample preparation techniques, pyronaridine, pyrimethamine and sulfadoxine can easily be analysed by LC–UV *(60-69)*. These drugs have also been accurately quantified with LC–MS after protein precipitation *(26, 70-73)*, allowing simple, cheap analysis of large numbers of samples.

1.5 RECOMMENDATIONS

An appropriate, validated method should be used for any bioanalysis. The success of an analytical method relies strongly on the sample preparation procedure and the extent to which the method has been appropriately tested and validated.

A thorough clean-up step prior to analysis is highly recommended for all analytical techniques, as it will go a long way to preventing carryover, contamination and unforeseen problems in clinical studies. Protein precipitation is a cheap, rapid technique, although its applicability to analytical methods such as LC–UV is questionable. With increased levels of contamination, this type of extraction is more appropriate for use in combination with LC–MS/MS; however, careful evaluation of matrix effects, including re-analysis of incurred samples (see Chapter 4), is essential.

Liquid–liquid extraction is appropriate for use with both LC–UV and LC–MS systems, as it provides a balance between efficient sample preparation and cost. Whereas protein precipitation can be cheaper, liquid–liquid extraction produces cleaner samples and can be used to concentrate analytes. It is thus particularly appropriate for sensitive assays.

More expensive solid-phase extraction usually produces clean, contaminant-free samples. It can also be used to concentrate analytes and is a good choice for assays in which sensitivity is required.

LC–UV and LC–MS/MS are recommended as the main analytical techniques for quantifying antimalarial drugs in biological fluids. LC–UV methods should be used if throughput and sensitivity are not issues. These systems are less complex and less expensive than other detection methods, such as electrochemical detection and LC–MS/MS.

Table 1.1 provides guidance on the detection techniques recommended for each antimalarial agent and shows the approximate sensitivity of each.

Table 1.1. Typical sensitivity of detection techniques for quantifying antimalarial drug concentrations

Analyte	Metabolite	Sample volume (ml)	UV (ng/ml)	Sample volume (µl)	MS/MS entry level (ng/ml)	Sample volume (µl)	MS/MS state-of-the-art (ng/ml)	Preferred detection method
Amodiaquine	Desethylamodiaquine	1	5	250	2.5	50	1	MS
Artemether	Dihydroartemisinin	1	50	250	2.5	50	1	MS
Artemisinin	–	1	50	250	2.5	50	1	MS
Artesunate	Dihydroartemisinin	1	50	250	2.5	50	1	MS
Atovaquone	–	1	50	250	2.5	50	1	UV/MS
Chloroquine	Desethylchloroquine	1	5	250	5	50	1.5	MS
Lumefantrine	Desbutyl-lumefantrine	1	10	250	5	50	1.5	UV/MS
Mefloquine	Carboxymefloquine	1	15	250	5	50	1.5	UV/MS
Piperaquine	–	1	5	250	2.5	50	1	MS
Primaquine	Carboxyprimaquine	1	5	250	7.5	50	2.5	UV/MS
Proguanil	Cycloguanil	1	10	250	2.5	50	1	UV/MS
Pyrimethamine	–	1	10	250	2.5	50	1	UV/MS
Pyronaridine	–	1	25	250	7.5	50	2.5	UV/MS
Quinine	3-OH Quinine	1	75	250	7.5	50	2.5	UV/MS
Sulfadoxine	–	1	25	250	7.5	50	2.5	UV/MS

MS, mass spectrometry; MS/MS, tandem mass spectrometry; UV, ultraviolet radiation.

Laboratory conditions in resource-poor settings present numerous challenges and obstacles. Often, the conditions cannot be readily altered, and it is up to clever, resourceful technicians to adapt to the conditions in order to produce good-quality results. Many validated methods for measuring antimalarial drugs are available, the choice depending on the available resources. The development and validation of new techniques suitable for the available resources is to be encouraged.

It is recommended that reversed-phase LC methods be used whenever possible, as water, the main component of the mobile phase, is much cheaper and easier to acquire than HPLC-grade organic solvents for normal-phase LC methods. The use of smaller-diameter analytical columns (e.g. 2.1 mm internal diameter) decreases the consumption of expensive solvent even further. It is prudent to evaluate the availability and quality of different solvents periodically in case supply becomes a problem.

1.6 RATIONALE

Balancing cost, method efficiency and analytical throughput is challenging and highly specific to each site and study. It is therefore difficult to make an overall recommendation on sample preparation. With highly sensitive techniques such as LC–MS/MS, matrix effects (i.e. the influence of the composition of the matrix on ionization of the analyte of interest) play a major role in the success or failure of the analysis. It is essential that these effects be thoroughly investigated *(74)*. This limits the use of 'dirty' sample preparation to highly controlled, validated assays. The choice of an analytical technique should be appropriate for the environment in which it is to be used, but the simplicity of the technique should be balanced against the level of sensitivity required and adjusted by improving the sample preparation.

Recommendation of an analytical technique for a particular analyte is not always straightforward. Different variables affect how useful a method will be in a laboratory. For example, low concentrations in the terminal elimination phases of some drugs (e.g. chloroquine and piperaquine) can often be quantified correctly only with a sensitive technique such as LC–MS/MS, but this may not be necessary. The type of study should also be taken into account. A full PK profile requires a sensitive assay that covers the range of concentrations from the highest expected to those expected after at least three elimination half-lives (see Chapter 3 for concentration estimates). If the study requires single high to mid-range measurements (e.g. a single day 7 concentration measurement for efficacy or compliance studies), then a less sensitive assay would suffice.

In order to operate efficiently in a resource-poor setting, the laboratory should:

- **be supplied with simple, inexpensive equipment:** Modulated LC components are more useful than large integrated systems. Servicing complicated systems can be challenging, and it may be difficult to train in-house staff in these procedures. When a component of an LC system fails, it is much easier to replace it in-house than to wait for a service engineer

to visit the laboratory, which can result in unforeseen delays and financial consequences due to instrument downtime. Running isocratic mobile phases requires a single pump, which minimizes the cost of the instrument.

- **be adequately equipped to prevent electrical problems:** Surge protectors, uninterruptable power supply packs and adequate grounding are essential, as electrical surges can destroy equipment. Poor wiring quality and circuitry overload are often causes of operational downtime.
- **be able to perform basic routine maintenance:** A few invaluable techniques can be used to test and service instruments and to identify potential problems. These include pressure checks, the use of a pre- or guard columns, column washes and filtered mobile phases. All these checks will protect the instrument from unnecessary failures.
- **be able to simplify their analytical methods:** Use of HPLC with a single isocratic pump is the simplest option. UV detection, especially with diode array detection, offers a wider scope of application. The use of reversed-phase systems is optimal, as it eliminates the need for excessive volumes of costly, sometimes unobtainable HPLC-grade organic solvents. For instance, during the economic crisis of 2009, it became very difficult to obtain acetonitrile, a common LC solvent; production problems led to a large price increase. Furthermore, certain solvents such as ethyl ether have been strictly regulated, owing to their use in the production of illicit drugs. Methods can be altered to suit solvents that are more readily available. Depending on the analyte, methanol can sometimes be used as an alternative to acetonitrile after some assay redevelopment and validation.

If factors such as environmental conditions, electrical supply and service contracts are difficult to negotiate, it would be wise not to invest in LC–MS/MS instrumentation. Success in a small laboratory with limited resources depends strongly on the laboratory's ability to overcome challenges with experience and initiative. Generally, using simple, inexpensive equipment is the best option.

Appendix 1 lists the assay conditions for measuring various antimalarial drugs.

APPENDIX 1. ASSAY CONDITIONS FOR MEASURING ANTIMALARIAL AGENTS IN VARIOUS MATRICES

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Amodiaquine Desethylamodiaquine	SD, PYR, CQ	0.2 ml	B	PP + LLE	RP–HPLC–UV	Quinidine	100 ng/ml 100 ng/ml	15	*(6)*
Amodiaquine Desethylamodiaquine	–	0.2 ml	B	PP	IPLC–MS/MS	Hydroxychloroquine	0.15 ng/ml 1.5 ng/ml	2	*(75)*
Amodiaquine Desethylamodiaquine Bis-desethylamodiaquine	–	0.5 ml	P	LLE	NP–HPLC–UV	*N*-Acetylprimaquine	5 ng/ml[f] 5 ng/ml 5 ng/ml	25	*(76)*
Amodiaquine Desethylamodiaquine	–	0.2 ml	DBS	LLE	RP–HPLC–UV	Quinidine	LOD: 5 ng/ml LOD: 10 ng/ml	20	*(13)*
Amodiaquine Desethylamodiaquine	ARM, ARS, DHA, LF, LFm, PQ, PYR, MFQ, PND, SD, CQ, CQm	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	0.3 ng/ml 0.3 ng/ml	17	*(26)*
Amodiaquine Desethylamodiaquine	ARS, DHA	0.5 ml	P	SPE	RP–HPLC–ECD	Isobutyl analogue of desethylamodiaquine dihydrochloride	20 ng/ml 20 ng/ml	20	*(15)*
Amodiaquine Desethylamodiaquine Bis-desethylamodiaquine	CQ	0.1 ml	DBS	SPE	RP-HPLC-UV	Amodiaquine analogue	100 nmol/l 100 nmol/l 100 nmol/l	30	*(12)*
Amodiaquine Desethylamodiaquine	CQ, CQm	0.1 ml	B, P, U	LLE	RP–HPLC–UV	4-(4-Dimethylamino-1-methylbutylamino)-7-chloroquinoline	100 nmol/l 100 nmol/l	15	*(77)*
Amodiaquine Desethylamodiaquine Bis-desethylamodiaquine	–	1 ml	B	LLE	RP–HPLC–ECD	Isobutyl analogue of desethylamodiaquine dihydrochloride	LOD: 1 ng/ml LOD: 1 ng/ml LOD: 3 ng/ml	16	*(16)*
Amodiaquine Desethylamodiaquine	–	0.1 ml	DBS	LLE	RP–HPLC–UV	4-(4-Dimethylamino-1-methylbutylamino)-7-chloroquinoline	50 nmol/l 50 nmol/l	8	*(78)*
Amodiaquine Desethylamodiaquine	–	1 ml 0.5 ml U	P, B, RBCs, U	LLE	RP–HPLC–UV	6,8-Dichloro-4-(1-methyl-4-diethylamino butylamino)-quinoline (WR7547)	LOD: 3.6 ng/ml LOD: 3.3 ng/ml	12	*(79)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Amodiaquine Desethylamodiaquine	–	1.0 ml	P, B, RBCs, U	PP + LLE	RP–HPLC–UV	6-Methoxy-8-aminoquinoline	LOD: 5 ng/ml P, U 12.5 ng/ml B, RBCs LOD: 5 ng/ml P,U 25 ng/ml B, RBCs	6	*(80)*
Artemether Dihydroartemisinin	–	0.05 ml	P	SPE	RP–HPLC–MS/MS	Stable isotope-labelled artemether	1.43 ng/ml 1.43 ng/ml	7	*(25)*
Artemether	–	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	5 ng/ml	17	*(26)*
Artemether Dihydroartemisinin	–	0.5 ml	P	SPE	RP–HPLC–MS/MS	Artemisinin	2 ng/ml 2 ng/ml	7	*(27)*
Artemether α- and β-Dihydroartemisinin	–	1 ml	P	LLE	RP–HPLC–ECD	Artemisinin	LOD: 5 ng/ml LOD: 3 ng/ml[g]	20	*(34)*
Artemether Dihydroartemisinin	–	1 ml	P	SPE	GC–MS	Artemisinin	5 ng/ml 5 ng/ml	21	*(81)*
Artemether Dihydroartemisinin	–	0.5 ml	P	Acid hydrolysis LLE	RP–HPLC–UV	Progesterone	LOD: 10 ng/ml LOD: 10 ng/ml[h]	6	*(82)*
Artemether Dihydroartemisinin	–	1 ml	P	LLE	RP–HPLC–ECD	Artemisinin	LOD: 2.5 ng/ml LOD: 1.25 ng/ml	20	*(83)*
Artemether Dihydroartemisinin	–	1 ml P 2 ml U	P, U	LLE	RP–HPLC–MS	Artemisinin	10 ng/ml P, 5 ng/ml U 10 ng/ml P, 5 ng/ml U	20	*(28)*
Artemether Dihydroartemisinin	–	1 ml	P	LLE	RP–HPLC–ECD	Artemisinin	10.9 ng/ml 11.2 ng/ml	15	*(17)*
Artemether Dihydroartemisinin	–	0.1 ml	P	LLE	RP–HPLC–MS/MS	Artemisinin	5 ng/ml 5 ng/ml	5	*(29)*
Artemether Dihydroartemisinin	–	0.5 ml	P	LLE	RP–HPLC–MS	Artemisinin	5 ng/ml 5 ng/ml	18	*(19)*
Artemether Dihydroartemisinin	–	1.0 ml	P	LLE + SPE	RP–HPLC–UV	Progesterone	LOD: 25 ng/ml LOD: 25 ng/ml	30	*(84)*
Artemisinin	–	0.1 ml P 1 ml saliva	P, saliva	–	RP–HPLC–UV + PCD	–	10 ng/ml P 2 ng/ml saliva	10	*(85)*

Artemisinin	–	0.1 ml	P	PP + LLE	RP–HPLC–MS/MS	Artemether	1 ng/ml	7	*(24)*
Artemisinin	–	0.1 ml	S	PP	UPLC–MS/MS	Arteether	4 ng/ml	3	*(22)*
Artemisinin	–	0.05 ml	P	SPE	RP–HPLC–MS/MS	Artesunate	1.03 ng/ml	5	*(23)*
Artesunate Dihydroartemisinin	–	1 ml	P	SPE	RP–HPLC–UV + PCD	Artemisinin	LOD: 30 ng/ml LOD: 20 ng/ml	15	*(86)*
Artesunate Dihydroartemisinin	–	0.1 ml	P	LLE	RP–HPLC–MS/MS	Indomethacin	4.28 ng/ml 2.31 ng/ml	15	*(18)*
Artesunate Dihydroartemisinin	–	0.25 ml	P	SPE	RP–HPLC–MS/MS	Stable isotope-labelled artesunate	0.119 ng/ml 1.96 ng/ml	6	*(30)*
Artesunate	–	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	2 ng/ml	17	*(26)*
Artesunate Dihydroartemisinin	MFQ	0.5 ml	P	SPE	RP–HPLC–ECD	Artemisinin	20 ng/ml 20 ng/ml	15	*(87)*
Artesunate Dihydroartemisinin	AQ, AQm	0.5 ml	P	SPE	RP–HPLC–ECD	Artemisinin	20 ng/ml 20 ng/ml	20	*(15)*
Artesunate Dihydroartemisinin	–	0.05 ml	P	LLE	RP–HPLC–MS/MS	Indomethacin	4.3 ng/ml 2.6 ng/ml	–	*(31)*
Artesunate Dihydroartemisinin	CP, CG, DS, MDS	0.05 ml	P	PP	HPLC–UV–MS/MS	–	1 ng/ml 1 ng/ml	–	*(32)*
Artesunate Dihydroartemisinin	–	1 ml	P	LLE	RP–HPLC–ECD	Artemisinin	5 ng/ml 3 ng/ml[g]	10	*(88)*
Artesunate	–	0.5 ml	P	SPE + PCD	RP–HPLC–UV	2-Naphthoic acid	40 ng/ml	25	*(89)*
Atovaquone	–	1 ml	S	LLE	CZE–UV (DAD)	–	2 µg/ml	11	*(90)*
Atovaquone	–	0.1 ml	P	LLE	RP–HPLC–UV	–	250 ng/ml	11	*(35)*
Atovaquone	–	0.5 ml	P	LLE	RP–HPLC–UV	59C80	100 ng/ml	10	*(36)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Atovaquone	–	0.2 ml	P	PP	RP–HPLC–UV	*trans*-2-Hydroxy-3-(4-phenylcyclohexyl)-1,4-naphthalenedione	LOD: 500 ng/ml	12	*(37)*
Atovaquone	–	0.5 ml	P, B	PP + SPE	RP-HPLC–UV	Atovaquone analogue (59C80)	150 nmol/l	5	*(38)*
Atovaquone	–	0.1 ml	DBS	SPE	RP–HPLC–UV	Atovaquone analogue (59C80)	1.0 µmol/l	5	*(39)*
Atovaquone	–	0.1 ml	P	LLE	RP–HPLC–UV	Atovaquone analogue (59C80)	250 ng/ml	12	*(40)*
β-Arteether	–	1 ml	P	Acid hydrolysis LLE	RP–HPLC–UV	Progesterone	60 ng/ml	25	*(91)*
β-Arteether Dihydroartemisinin	–	1 ml	P	LLE	RP–HPLC–ECD	Artemisinin	5 ng/ml 5 ng/ml[h]	15	*(92)*
Chloroquine Desethylchloroquine	–	1.0 ml	P	LLE	NP–HPLC–fluorescence	7-Chloro-4-(1-dimethylamino-4-pentylamino)-quinoline	LOD: 1 ng/ml LOD: 5 ng/ml	15	*(10)*
Chloroquine	–	0.15 ml	B	LLE	RP–HPLC–UV	Quinidine	150 ng/ml	15	*(6)*
Chloroquine Desethylchloroquine	–	2.0 ml	P, U	LLE	GC–NPD	7-Iodo-4-(1-methyl-4-diethylaminobutylamino)-quinoline (WR7910)	60 ng/ml 60 ng/ml	10	*(93)*
Chloroquine Desethylchloroquine	–	0.5–2 ml	P, U	LLE	RP–HPLC–fluorescence	6,8-Dichloro-4-(1-methyl-4-diethylaminobutylamino)-quinoline (WR7547)	0.6 nmol/l 0.5 nmol/l	15	*(14)*
Chloroquine Desethylchloroquine Bis-desethylchloroquine	QN	1 ml	P, RBCs, U	SPE	RP–HPLC–fluorescence	Hydroxychloroquine	5.6 ng/ml 3.3 ng/ml 3.5 ng/ml	30	*(94)*
Chloroquine Desethylchloroquine	–	0.5 ml	P, RBCs	LLE	NP–HPLC– fluorescence	4-(4-Dimethylamino-1-methylbutylamino)-7-chloroquinoline	20 ng/ml 20 ng/ml	25	*(95)*

Chloroquine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PQ, PYR, MFQ, PND, SD, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	1.25 ng/ml	17	*(26)*
Chloroquine Desethylchloroquine	PG, CG	0.08 ml	DBS	LLE	RP–HPLC–UV	Bis-demethyled cycloguanil	50 ng/ml 50 ng/ml	40	*(96)*
Chloroquine Desethylchoroquine	AQ, AQm, AQm2, AQm3	0.1 ml	DBS	SPE	RP–HPLC–UV	Sontochin	100 nmol/l 100 nmol/l	30	*(12)*
Chloroquine Desethylchloroquine	AQ, AQm	0.1 ml	B, DPS	LLE	RP–HPLC–UV	4-(4-Dimethylamino-1-methylbutylamino)-7-chloroquinoline	100 nmol/l 100 nmol/l	15	*(77)*
Chloroquine Desethylchloroquine	–	0.1 ml	DBS	LLE	RP–HPLC– fluorescence	7-Chloro-4-(1'-methyl-4'-propylaminobutylamino)-quinoline	LOD: 5 ng/ml LOD: 5 ng/ml	15	*(97)*
Chloroquine Desethylchloroquine	AQ, AQm	1 ml (0.5 ml U)	P, RBCs, B, U	LLE	RP–HPLC–UV	6,8-Dichloro-4-(1-methyl-4-diethylaminobutylamino)-quinoline (WR7547)	LOD: 3.2 ng/ml LOD: 2.9 ng/ml[i]	15	*(79)*
Chloroquine Desethylchloroquine	PG, CG, 4-CPB	1 ml	P, U	SPE	RP–HPLC–UV	Chlorproguanil	1.4 ng/ml 1 ng/ml[g]	25	*(98)*
Chlorproguanil Chlorcycloguanil	DS, MDS, ARS, DHA	0.05 ml	P	PP	RP–HPLC–UV–MS/MS	–	2 ng/ml 4 ng/ml	–	*(32)*
Clindamycin	–	0.5 ml	S (dog)	PP + LLE	RP–HPLC–UV	–	80 ng/ml	15	*(41)*
Clindamycin	–	0.5 ml	P	PP	RP–HPLC–UV	Phenobarbital	200 ng/ml	25	*(42)*
Clindamycin	–	0.2 ml	P, S	PP	RP–HPLC–UV	Triazolam	170 ng/ml	13	*(43)*
Clindamycin	–	0.5 ml	P, S	SPE	RP–HPLC–UV	Propanolol	50 ng/ml	22	*(44)*
Clindamycin	–	1 ml	S	PP	RP–HPLC–MS	Lincomycin	100 ng/ml	5	*(99)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Clindamycin	–	0.2 ml	P	LLE	RP–HPLC–MS/MS	d_1-*N*-Ethylclindamycin	50 ng/ml	3	*(100)*
Clindamycin	–	0.1 ml	P	PP	RP–HPLC–MS/MS	Verapamil	50 ng/ml	2	*(101)*
Dapsone	–	0.5 ml	P	LLE	RP–HPLC–UV	Pyrimethamine	100 ng/ml	–	*(102)*
Dapsone Monoacetyldapsone	PYR	1 ml	P	LLE	RP–HPLC–UV	Quinine	LOD: 5 ng/ml LOD: 5 ng/ml	15	*(45)*
Dapsone Monoacetyldapsone	–	0.5 ml	P, U	LLE	RP–HPLC–UV	*m*-Aminophenylsulfone	LOD: 10 ng/ml LOD: 10 ng/ml[h]	9	*(46)*
Dapsone Monoacetyldapsone	PYR	2 ml	P	LLE	NP–HPLC–UV	Metoprine	LOD: 1.25 ng/ml LOD: 1.25 ng/ml[j]	8	*(8)*
Dapsone Monoacetyldapsone	PYR	0.15 ml	B, P	LLE	RP–HPLC–UV	Monopropionyldapsone	10 ng/ml 8 ng/ml	15	*(65)*
Dapsone Monoacetyldapsone	–	0.05 ml	B, DPS	LLE	RP–HPLC–UV	Acetanilide	LOD: 15 ng/ml LOD: 10 ng/ml	8	*(103)*
Dapsone	–	2 g	Tissue	PP	RP–UPLC–MS/MS	Demeclocycline	4.4 ng/g	8	*(51)*
Dapsone Monoacetyldapsone	CP, CCG, ARS, DHA	0.05 ml	P	PP	RP–HPLC–UV–MS/MS	–	100 ng/ml 100 ng/ml	–	*(32)*
Dapsone	–	0.2 ml S 0.1 ml saliva	S, saliva	PP, S DIL, saliva	RP–HPLC–UV	Diazoxide	25 ng/ml	12	*(104)*
Dapsone	–	0.2 ml S 0.1 ml saliva	S, saliva	SPE	RP-HPLC–ECD	Practolol	0.2 ng/ml	12	*(104)*
Dapsone Monoacetyldapsone	–	0.2 ml	S, saliva	LLE DIL, saliva	NP–HPLC– fluorescence	–	LOD: 5 µg/ml LOD: 10 µg/ml[g]	3	*(105)*
Dapsone Monoacetyldapsone	–	0.5 ml	S	PP	RP–HPLC–UV	Monopropionyldapsone	0.2 µg/ml[k] 0.2 µg/ml	10	*(47)*

Dihydroartemisinin	ARM, ARS, AQ, AQm, LF, LFm, PQ, PYR, MFQ, PND, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	1 ng/ml	17	*(26)*
Dihydroartemisinin	–	0.3 ml	P	LLE	RP–HPLC–MS/MS	Artemisinin	1.01 ng/ml	5	*(106)*
β-Dihydroartemisinin	–	0.1 ml	P	PP + LLE	RP–HPLC–MS/MS	Artemisinin	0.2 ng/ml	4	*(107)*
Doxycycline	–	1 ml	P	SPE	RP–HPLC–UV	Oxytetracycline	125 ng/ml	5	*(49)*
Doxycycline	–	0.5 ml	S, U	LLE	RP–HPLC–UV	Demeclocycline	LOD: 50 ng/ml	18	*(50)*
Doxycycline	DS	2 g	Tissue	PP	RP–UPLC–MS/MS	Demeclocycline	160 ng/g	8	*(51)*
Doxycycline	–	0.02 ml	S	PP	RP–HPLC–UV	Piroxicam	80 ng/ml	14	*(52)*
Doxycycline	–	0.25 ml	P (turkey)	LLE	RP–HPLC–UV	Demeclocycline	0.2 µg/ml	8	*(53)*
Halofantrine	–	0.5 ml	P	PP + LLE + deriv.	RP–HPLC–UV	Desbutyl-halofantrine	12.5 ng/ml	25	*(108)*
(+), (–) Halofantrine Desbutyl-halofantrine	–	0.5 ml	RBCs	PP	NP–HPLC– fluorescence chiral	(±)-Dichloro-[2-(dibutylamino) Methyl]-6-(trifluoromethyl)-9-phenanthrenemethanol	25 ng/ml 25 ng/ml	50	*(109)*
Halofantrine Desbutyl-halofantrine	–	1.0 ml	P	PP + LLE	RP–HPLC–UV	Chlorprothixen	20 ng/ml 20 ng/ml	17	*(110)*
Lumefantrine	–	0.25 ml	P	PP + SPE	RP–HPLC–UV	Lumefantrine analogue	25 ng/ml	15	*(111)*
Lumefantrine	–	0.1 ml	DBS	SPE	RP–HPLC–UV	TA3039	0.25 µmol/l	20	*(112)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Lumefantrine Desbutyl-lumefantrine	ARM, ARS, DHA, AQ, AQm, PQ, PYR, MFQ, PND, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	4 ng/ml 4 ng/ml	17	*(26)*
Lumefantrine Desbutyl-lumefantrine	–	0.25 ml	P	PP + SPE	RP–HPLC–UV	Lumefantrine analogue Metabolite analogue	24 ng/ml 21 ng/ml	25	*(113)*
Lumefantrine Desbutyl-lumefantrine	–	0.1 ml	DBS	PP + LLE	RP–HPLC–UV	Halofantrine	300 nmol/l 300 nmol/l	10	*(114)*
Lumefantrine	–	0.1 ml	P	LLE	RP–HPLC–MS/MS	Halofantrine	2 ng/ml	5	*(115)*
Mefloquine Carboxymefloquine	–	0.5 ml	P, B	PP + LLE	RP–HPLC–UV	Racemic α-(2-pyridyl)-2,7-bis(trifluoromethyl)-4-quinoline	30 ng/ml 30 ng/ml	15	*(116)*
Mefloquine	–	0.1 ml	B, P, DBS	PP + LLE + deriv.	GC–ECD	Mefloquine analogue	9.5 ng/ml	30	*(117)*
Mefloquine Carboxymefloquine	–	0.6 ml	P	PP + LLE	RP–HPLC–UV	WR184806	0.25 µmol/l 0.25 µmol/l	30	*(61)*
Mefloquine Carboxymefloquine	–	0.1 ml	DBS	LLE	RP–HPLC–UV	WR184806	0.5 µmol/l 0.25 µmol/l	20	*(54)*
(+)-, (–)-Mefloquine	–	0.1 ml	P, DBS	LLE	RP–HPLC–UV chiral	WR226253	0.25 µmol/l plasma	30	*(55)*
(+)-, (–)-Mefloquine	–	0.1 ml	P, DBS	LLE	RP–HPLC– fluorescence chiral	WR226253	0.1 µmol/l plasma	30	*(55)*
Mefloquine	–	1 ml	P	LLE + deriv.	GC–ECD	*erythro*-α-(2-Piperidyl)-2,7-bis(trifluoromethyl)-4-quinoline methanol	10 ng/ml[g]	10	*(118)*

(+)-, (–)-Mefloquine	–	0.5 ml	P, B	PP + LLE	RP–HPLC–UV chiral	Chloroquine	LOD: 50 ng/ml	30	*(119)*
Mefloquine Carboxymefloquine	–	0.2 ml	B, P, S	PP + SPE	RP–HPLC–UV	WR184806	80 ng/ml B, 70 ng/ml P, 60 ng/ml S 70 ng/ml B, 50 ng/ml P, 60 ng/ml S	15	*(120)*
Mefloquine	–	5.0 ml	B, P, U	LLE	NP/RP–HPLC–UV	WR184806	0.05 µg/ml B, P 0.25 µg/ml U	10	*(121)*
Mefloquine	–	1 ml	P	LLE	RP–HPLC–UV	Nitrazepam	10 ng/ml	15	*(64)*
Mefloquine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PQ, PYR, PND, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	2.5 ng/ml	17	*(26)*
Mefloquine	–	1.0 ml	B, P	LLE	RP–HPLC–UV	WR184806	10 ng/ml[k]	10	*(122)*
Mefloquine	ARS, DHA	0.5 ml	P	SPE	RP–HPLC–UV	Chlorpromazine	50 ng/ml	15	*(87)*
(+)-, (–)-Mefloquine	–	1 ml	P	LPME	Chiral–HPLC–UV	Mirtazapine	50 ng/ml	15	*(11)*
(+)-, (–)-Mefloquine Carboxymefloquine	–	1 ml	P	LPME	RP–HPLC–UV chiral	–	50 ng/ml 50 ng/ml	15	*(123)*
Mefloquine	–	0.1 ml	B	LLE	SFC–ECD	*D,L-erythro-cu*-(2-Pipridyl)-2.6-bis(trifluoromethyl)-4-quinolinemethanol dihydrochloride	LOD: 7.5 ng/ml[g]	14	*(124)*
Mefloquine	–	0.1 ml	B, DBS, U	LLE + deriv.	RP–HPLC– fluorescence	*D,L-erythro*-α-(2-Piperidyl)-2-trifluoromethyl-6,8-dichloro-4-quinoline methanol	50 ng/ml[g]	6	*(56)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
(+)-, (–)-Mefloquine	–	0.2 ml	B, P, U	LLE + deriv.	NP–HPLC–UV	Bupranolol	0.2 µg/ml B, P	30	*(125)*
(+)-, (–)-Mefloquine	–	1.0 ml	P, U	LLE	RP–HPLC–UV chiral	–	1.6 mmol/l	15	*(126)*
Piperaquine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PYR, MFQ, PND, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	2 ng/ml	17	*(26)*
Piperaquine	–	1 ml	P	LLE	RP–HPLC–UV	Chloroquine	5 ng/ml	15	*(127)*
Piperaquine	–	0.25 ml 1 ml	P	SPE	RP–HPLC–UV	3-Methyl-4-(3-hydroxy-4-diethylaminopropyl)-7-chloroquiniline	10 ng/ml 2.5 ng/mll	3	*(128)*
Piperaquine	–	0.05 ml	P	SPE	RP–HPLC–MS/MS	*D*6-Piperaquine	1.5 ng/ml	3	*(129)*
Piperaquine	–	0.1 ml	DBS	PP + MPC + SPE	RP–HPLC–UV	3-Methyl-4-(3-hydroxy-4-dimethylaminopropyl)-7-chloroquine	0.05 µmol/l	2	*(130)*
Piperaquine	–	0.05 ml	P	PP	RP–HPLC–MS/MS	Piperazine bischloroquiniline	1.0 ng/ml	2.5	*(131)*
Piperaquine	–	1.0 ml	U	SPE	RP–HPLC–UV	3-Methyl-4-(3-hydroxy-4-diethylaminopropyl)-7-chloroquinoline	3 ng/ml	2.5	*(132)*
Primaquine Carboxyprimaquine	–	0.5 ml	P	PP	RP–HPLC–ECD	WR6026	5 ng/ml 20 ng/ml	40	*(133)*
Primaquine Carboxyprimaquine	–	0.5 ml	P, RBCs	LLE	RP–HPLC–UV	6-Methoxyprimaquine	10 ng/ml 10 ng/ml	24	*(134)*
Primaquine	–	0.1 ml	P	LLE	RP–HPLC–MS/MS	3-Bromoprimaquine	7.81 ng/ml	10	*(135)*
Primaquine	–	1 ml	B	LLE + deriv.	GC–ECD	WR5991	8 ng/ml	15	*(136)*
Proguanil Cycloguanil 1-(4-Chlorophenyl)-biguanide	–	0.1 ml	DBS	SPE	RP–HPLC–UV	Chlorcycloguanil	125 nmol/l 50 nmol/l 50 nmol/l	12	*(137)*

Proguanil Cycloguanil	CQ, CQm	0.08 ml	DBS	LLE	RP–HPLC–UV	Bisdemethyled cycloguanil	100 ng/ml 100 ng/ml	40	*(96)*
Proguanil Cycloguanil 1-(4-Chlorophenyl)-biguanide	–	0.05 ml	B, P (rat)	PP	RP–HPLC–MS/MS	Chlorproguanil	1 ng/ml 1 ng/ml 5 ng/ml	7	*(58)*
Proguanil Cycloguanil 1-(4-Chlorophenyl)-biguanide	–	1 ml	S (bovine)	PP	RP–HPLC–UV	Phenylbenzoate	LOD: 60 ng/ml LOD: 60 ng/ml LOD: 60 ng/ml	10	*(138)*
Proguanil Cycloguanil	–	0.5 ml	P	SPE	RP–HPLC–MS/MS	Riluzole	1.5 ng/ml 0.5 ng/ml	3	*(59)*
Proguanil Cycloguanil 1-(4-Chlorophenyl)-biguanide	CQ, CQm	1ml	P, U	SPE	RP–HPLC–UV	Chlorproguanil	LOD: 1.0 ng/ml LOD: 0.5 ng/ml LOD: 0.5 ng/ml[f]	25	*(98)*
Pyrimethamine	SD	1.0 ml	P	SPE	RP–HPLC–UV	Sulfadimethoxine	10 ng/ml	20	*(60)*
Pyrimethamine	SD, CQ, AQ, AQm	0.15 ml	B	LLE	RP–HPLC–UV	Proguanil	50 ng/ml	10	*(6)*
Pyrimethamine	SD	1–2 ml	P	LLE	RP–HPLC–UV	Sulfamethoxazole	13 ng/ml	15	*(62)*
Pyrimethamine	SD, MFQ, MFQm	0.6 ml	P	PP + LLE	RP–HPLC–UV	WR184806	0.05 µmol/l	30	*(61)*
Pyrimethamine	SD, SDm	0.5 ml	P	LLE	RP–HPLC–UV	Quinine	5 ng/ml	12	*(139)*
Pyrimethamine	DS, MDS	1 ml	P	LLE	RP–HPLC–UV	Quinine	LOD: 5 ng/ml	15	*(45)*
Pyrimethamine	SD	0.1 ml	DBS	SPE	RP–HPLC–DAD	Sulfadimethoxine	1.0 µg/ml	12	*(63)*
Pyrimethamine	MF	1 ml	P	LLE	RP–HPLC–UV	Nitrazepam	10 ng/ml	15	*(64)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Pyrimethamine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PQ, MFQ, PND, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	0.5 ng/ml	17	*(26)*
Pyrimethamine	–	0.025 ml	P	PP	RP–HPLC–UV/MS	Sulfamethoxazole	20 ng/ml	12	*(140)*
Pyrimethamine	DS, MDS	2 ml	P	LLE	NP–HPLC–UV	Metoprine	2.5 ng/ml[j]	8	*(8)*
Pyrimethamine	DS, MDS	0.15 ml	B, P	LLE	RP–HPLC–UV	Monopropionyldapsone	10 ng/ml	15	*(65)*
Pyrimethamine	SD, SDm	1.0 ml	S, U	LLE	RP–HPLC–UV	*p*-Aminopropionphenone	1 ng/ml[j]	10	*(141)*
Pyrimethamine	–	0.1 ml	DBS	LLE	RP–HPLC–UV	Trimethoprim	40 ng/ml	11	*(66)*
Pyrimethamine	–	1 ml	P	LLE	RP–HPLC–UV	Quinine	LOD: 3 ng/ml[g]	15	*(67)*
Pyrimethamine	SD	0.25 ml	P	PP	RP–HPLC–MS/MS	Sulfamerazine	10 ng/ml	10	*(70)*
Pyrimethamine	SD	0.25 ml	P	LLE	RP–HPLC–MS/MS	Sulfaquinoxaline	1.01 ng/ml[i]	12.5	*(71)*
Pyrimethamine	–	0.5 ml	P	LLE	NP–HPLC– fluores-cence	–	LOD: 10 ng/ml[g]	8	*(9)*
Pyronaridine	–	0.5 ml	P	LLE	RP–HPLC–UV	Papaverine	50 ng/ml	30	*(68)*
Pyronaridine	–	0.2 ml	P, B	LLE	RP–HPLC–UV	Amodiaquine	25 ng/ml	20	*(69)*
Pyronaridine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PQ, PYR, MFQ, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	1 ng/ml	17	*(26)*

Pyronaridine	–	0.25 ml	P	LLE	RP–HPLC–fluorescence	Quinine	10 ng/ml	10	*(142)*
Pyronaridine	–	0.3 ml	B	LLE	RP–HPLC–MS	Amodiaquine	5.7 ng/ml	15	*(72)*
Pyronaridine	–	0.2 ml	U	LLE	RP–HPLC–MS	Amodiaquine	14.3 ng/ml	14	*(73)*
Pyronaridine	–	0.25 ml	P	SPE	RP–HPLC–fluorescence	Quinidine	10 ng/ml	30	*(143)*
Pyronaridine	–	0.5 ml	B, U	LLE + SPE (B) LLE (U)	RP-HPLC–ECD	4-(7-Chloro-4-quinolinyl)-2-[[(1-methylpropyl) amino]methyl]-phenol	20 ng/ml	10	*(144)*
Quinine	CQ	1 ml	P, RBCs, U	SPE	RP–HPLC– fluorescence	Hydroxychloroquine	35.5 ng/ml	30	*(94)*
Quinine	–	0.02 ml	P (rat)	LLE	RP–HPLC–UV	Quinidine	–	7	*(145)*
Quinine	–	1 ml	P	SPE	GC–MS	Cyproheptadine	40.6 ng/ml	13	*(146)*
Quinine	–	0.5 ml	P, S, RBCs, DBS	LLE	NP–HPLC–fluorescence	–	–	15	*(147)*
Quinine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PQ, PYR, MFQ, PND, SD, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	2.5 ng/ml	17	*(26)*
Quinine 3-OH-Quinine	–	0.1 ml	DBS	LLE	RP–HPLC–fluorescence	Quinidine	10 nmol/l 10 nmol/l	15	*(148)*
Quinine	–	0.1 ml	DBS	LLE	RP–HPLC–UV	Quinidine	1000 ng/ml	10	*(149)*
3-OH-Quinine	–	0.1 ml	P, U	LLE	RP–HPLC–fluorescence	Quinidine	4.5 nmol/l P 204.6 nmol/l U	15	*(150)*
Quinine 3-OH-Quinine	–	1.0 ml	P	LLE	RP–HPLC–UV	Pyrimethamine	70 ng/ml 70 ng/ml	20	*(151)*

Analytes	Other analytes[a]	Sample volume	Matrix[b]	Extraction method[c]	Detection method[d]	Internal standard	Lower limit of quantification[e]	Run time (min)	Reference
Sulfadoxine	PYR	1.0 ml	P	SPE	RP–HPLC–UV	Sulfadimethoxine	22 ng/ml	20	*(60)*
Sulfadoxine	PYR, CQ, AQ, AQm	0.05 ml	B	LLE	RP–HPLC–UV	Sulfisoxazole	5 µg/ml	15	*(6)*
Sulfadoxine	PYR	1–2 ml	P	LLE	RP–HPLC–UV	Sulfamethoxazole	16 µg/ml	15	*(62)*
Sulfadoxine	PYR, MF, MFm	0.6 ml	P	PP + LLE	RP–HPLC–UV	Sulfadimethoxine	75 µmol/l	30	*(61)*
Sulfadoxine *N*-Acetylsulfadoxine	–	0.5 ml	P	LLE	RP-HPLC-UV	Sulfamethoxazole	2.5 ng/ml 2.5 ng/ml	35	*(152)*
Sulfadoxine *N*-Acetylsulfadoxine	PYR	0.5 ml	P	LLE	RP–HPLC–UV	Quinine	50 ng/ml 3 ng/ml	12	*(139)*
Sulfadoxine	–	0.1 ml	DBS	LLE	RP–HPLC–UV	Sulfisoxazole	5 µg/ml[k]	12	*(153)*
Sulfadoxine	PYR	0.1 ml	DBS	SPE	RP–HPLC–DAD	Sulfadimethoxine	3400 ng/ml	12	*(63)*
Sulfadoxine	ARM, ARS, DHA, AQ, AQm, LF, LFm, PQ, PYR, MFQ, PND, CQ, QN	0.2 ml	P	PP	RP–HPLC–MS	Trimipramine-D3 Artemisinin	0.5 ng/ml	17	*(26)*
Sulfadoxine	–	0.1 ml	DBS	PP	RP–HPLC–UV	Sulfadimethoxine	5 µmol/l	10	*(57)*
Sulfadoxine *N*-Acetylsulfadoxine	PYR	1.0 ml	S, U	LLE	RP–HPLC–UV	Monobutyrylsulfadoxine	5 ng/ml 5 ng/ml[j]	10	*(141)*
Sulfadoxine	PYR	0.25 ml	P	PP	RP–HPLC–MS/MS	Sulfamerazine	2.7 µg/ml	10	*(70)*
Tetracycline	–	5 g	Tissue	Ultracentrifu-gation	RP–HPLC–MS/MS	Demeclocycline	2 ng/g	5	*(154)*
Tetracycline	–	0.5 ml P 0.25 ml U	P, U	PP, P DIL, U	RP–HPLC–UV	–	–	9	*(155)*

Tetracycline	–	0.1 ml	S	PP	RP–HPLC–fluorescence	–	LOD: 30 ng/ml	10	*(156)*
Tetracycline	–	2 g	Tissue	PP	RP–UPLC–MS/MS	Demeclocycline	130 ng/g	8	*(51)*

[a] Analyte abbreviations: AQ, amodiaquine; AQm, amodiaquine metabolite; ARM, artemether; ARS, artesunate; CCG, chlorcycloguanil; CG, cycloguanil; CP, chlorproguanil; 4-CPB, 4-chlorophenylbiguanide; CQ, chloroquine; CQm, chloroquine metabolite; DHA, dihydroartemisinin; DS, dapsone; LF, lumefantrine; LFm, lumefantrine metabolite; MDS, monoacetyldapsone; MFQ, mefloquine; MFQm, mefloquine metabolite; PG, proguanil; PND, pyronaridine; PQ, piperaquine; PYR, pyrimethamine; QN, quinine; SD, sulfadoxine; SDm, sulfadoxine metabolite.

[b] Matrix abbreviations: B, blood; DBS, dried blood spot; DPS, dried plasma spot; P, plasma; RBCs, red blood cells; S, serum; U, urine.

[c] Extraction abbreviations: deriv., derivatization; DIL, dilution; LLE, liquid–liquid extraction; LPME, liquid-phase microextraction; MPC, minimum parasiticidal concentration; PCD, post-column derivatization; PP, protein precipitation; SPE, solid-phase extraction.

[d] Detection method abbreviations: CZE, capillary zone electrophoresis; DAD, diode array detection; ECD, electrochemical detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; IPLC, ion-pair liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NP, normal phase; NPD, nitrogen-phosphorus detector; PCD, post-column derivatization; RP, reversed-phase; SFC, supercritical fluid chromatography; UPLC, ultra-performance liquid chromatography; UV, ultraviolet.

[e] LOD, limit of detection.

[f] Signal-to-noise ratio, 5:1.

[g] Signal-to-noise ratio, 3:1.

[h] Signal-to-noise ratio, 4:1.

[i] Signal-to-noise ratio, 10:1.

[j] Signal-to-noise ratio, 2:1.

[k] Lowest level calibrator.

[l] Increasing the sample volume decreases the lower limit of quantification.

Chapter 2. Pre-analytical variables in antimalarial drug assays

2.1 OVERVIEW

The aim of this chapter is to provide information on pre-analytical factors that affect the reliability of methods for measuring the concentration of antimalarial drugs and their metabolites in biological fluids. Pre-analytical variables are all the events before determination of a drug concentration, such as food intake, drug intake, sampling methods and sample handling, storage and transport to the laboratory. Even the most selective and sensitive analytical method will give biased and erroneous results if a single step in the pre-analytical procedure is done incorrectly. It is important that the same matrix be used if drug concentrations in different studies or different patients are to be compared. If the drug is bound to cellular components, the concentrations in blood, serum and plasma will differ. For instance, the concentrations of chloroquine in serum are twice as high as those in plasma because of release of the drug from blood cells during clotting *(157)*.

2.2 BIOLOGICAL MATRICES

The biological matrices used most commonly in assays for determining concentrations of drugs are venous blood and venous plasma. Venous blood can be drawn into tubes with an anticoagulant (e.g. ethylenediaminetetraacetic acid (EDTA), heparin or fluoride–oxalate) to inhibit clot formation and prevent blood cell lysis. The blood itself can be used for analysis, or plasma can be obtained by centrifugation. Plasma obtained after high-speed centrifugation of anticoagulated blood is, in principle, cell-free. If the blood is drawn into tubes without anticoagulant, serum is obtained after blood clotting and centrifugation. Serum is enriched with components from lysed cells (e.g. platelets and leukocytes) and can to some extent be regarded as an artefact (i.e. plasma enriched with cellular components and metabolic products). To obtain serum, fibrinogen is reacted with platelets to form a fibrin clot, which is discarded with the RBC fraction after centrifugation. Results obtained with plasma are more representative than those obtained with serum, as serum concentrations can vary considerably, depending on the amount of lysed blood cells. For drugs that are concentrated in blood cells (e.g. erythrocytes, platelets), the centrifugation speed and time are important. To obtain plasma, blood should be centifuged within 60 min of sampling at 1000–3000 × *g* for 7–15 min. If samples are centrifuged after more than 60 min or at low speed, drugs such as chloroquine that accumulate inside platelets are released, giving a falsely high plasma drug concentration *(157-158)*.

2.3 SAMPLING CONTAINERS

Most antimalarial agents contain a nitrogen atom in their structure (e.g. in the quinoline group), which, under physiological conditions, becomes positively charged. This functional group can cause the drug to adsorb onto glass surfaces during sampling and extraction. Piperaquine, for instance, with its four nitrogen atoms, readily adsorbs onto glass surfaces *(159)*. Therefore, it is preferable to use polypropylene tubes during storage and extraction and to avoid contact with glass as much as possible; avoiding glass also has a safety advantage. Storage of arteether in plasma in plastic containers was found to result in considerable loss of the drug over 24 h *(91)*; however, it is unclear whether this was due to nonspecific binding or degradation, as other authors have not reported problems of nonspecific binding to plastic material *(19, 23, 25-26, 34, 160)*. In order to minimize any adsorption of artemisinin derivatives, all glassware, including extraction tubes, is usually silanized with dichlorodimethylsilane in toluene (5%, v/v) *(34, 81, 161)* or hexamethyldisilazane in toluene (1%, v/v) *(17)* before use or alternatively prepared and stored in polypropylene tubes *(19)*. For assaying amodiaquine and its major metabolite desethylamodiaquine, venous blood samples can be collected into lithium–heparin siliconized glass tubes to limit adsorption *(162)*. Lumefantrine, one of the more lipophilic antimalarial drugs, has been shown to adsorb onto plastic surfaces, particularly during evaporation of solutions containing protein residues *(112-113)*; however, lumefantrine in plasma did not show any signs of nonspecific binding to plastic containers. In general, polypropylene cryovials are preferred for storage of plasma samples containing most antimalarial drugs.

2.4 FOOD INTAKE

Several antimalarial drugs, such as atovaquone *(163)*, halofantrine *(164)*, piperaquine *(165)* and mefloquine *(166)*, are highly lipophilic. Care should be taken to ensure that the assay methods are unaffected by the lipids that will be present if the drugs are taken with food. The recovery of lumefantrine was unaffected when plasma was taken pre- or post-prandially *(113)*.

2.5 ANTICOAGULANTS AND STABILIZERS

An important aspect in the choice of an anticoagulant is the quality of the resulting plasma. Anticoagulation is achieved either by the binding of calcium ions (e.g. by EDTA, citrate or fluoride) or by inhibition of thrombin (e.g. with heparin). Heparin results in plasma that contains lipid and fibrinogen clots after freezing and thawing, which makes it difficult to take aliquots of these samples after they have been thawed and also to automate sample processing. EDTA results in better-quality plasma, with fewer clots, which makes it easier to take aliquots, especially when the procedure is automated *(167)*. EDTA is a metal chelator and can potentially chelate drugs, which would reduce the apparent plasma concentration. Fluoride–oxalate generally results in clear plasma, even after repeated freeze–thaw cycles, making this anticoagulant ideal for use in automated methods. Another advantage of fluoride–oxalate is that the plasma can be heated (e.g. for virus deactivation) for at least 90 min without compromising its integrity *(168)*, whereas plasma samples treated

with EDTA or heparin become viscous, making pipetting impossible after 90 min of heating.

Cells shrink during anticoagulation with EDTA or with fluoride–oxalate salts, resulting in a slightly more 'diluted' plasma sample after centrifugation, which can affect plasma concentrations; for example, the concentrations of artesunate and dihydroartemisinin were on average 9.5% and 8.2% lower in fluoride–oxalate plasma than in heparin plasma *(160)*. This is an important aspect to consider in choosing an anticoagulant. The plasma concentration of primaquine was independent of the anticoagulant used (EDTA or heparin) *(169)*.

Some assays for the determination of artesunate in plasma samples specify use of fluoride–oxalate in order to avoid esterase-mediated (enzymatic) conversion to dihydroartemisinin. Fluoride–oxalate and EDTA *(170)* do not, however, inhibit chemical hydrolysis *(171)*. Both enzymatic and chemical hydrolysis are minimized if the temperature is lowered (e.g. by working on ice).

2.6 HAEMOLYSIS

Malaria is associated with the lysis of erythrocytes, and the resulting haemolysate is present in the plasma of patients. Antimalarial drugs themselves, particularly primaquine and quinine *(172-173)*, may also precipitate haemolytic episodes. As artemisinin and its derivatives degrade if haemoglobin is precipitated during analysis *(30, 174)*, analytical methods for artemisinin and dihydroartemisinin are particularly affected by haemolysis of blood samples *(175)*. The risk for haemolysis is increased by capillary sampling, potentially confounding the measurement of artemisinin derivatives (e.g. artesunate and dihydroartemisinin). Lindegardh et al. *(160)* reported problems in the analysis of the endoperoxide antimalarial drugs dihydroartemisinin and artesunate in haemolysed plasma samples with an LC–MS/MS method, due to the reactivity of the analytes with haemoglobin and haemolytic products in clinical samples and the presence of organic solvent during extraction. Addition of organic solvents during sample processing or even addition of a small volume of the internal standard in an organic solvent caused degradation *(30)*. Experiments with control and haemolysed plasma from uninfected and malaria patients showed that artemisinin is more vulnerable to the effects of haemoglobin than dihydroartemisinin, artesunate or artemether *(26)*. The problems associated with analysis of artemisinin derivatives in haemolysed plasma can, however, be overcome, which, in the future, should allow their analysis in blood.

2.7 DRUG CONCENTRATIONS IN DIFFERENT MATRICES

Differences in the concentrations of various common clinical chemical constituents in capillary and venous serum were studied in healthy young adults in the fasting state and found to be less than 5% *(176)*. Blumenfeld et al. *(177)* studied the concentrations of 12 chemical constituents in capillary serum, capillary plasma and venous serum obtained from healthy adults, with or without warming the skin before puncture. They found no significant difference in capillary plasma or serum taken from warmed or unwarmed

skin; however, there were important differences in the concentrations of glucose, potassium, total protein and calcium between capillary plasma and serum and venous serum. A comparison of haematological parameters in capillary and venous blood from healthy adults showed lower platelet counts and higher leukocyte counts in capillary blood *(178)*. This could lead to large differences in the concentrations in these two matrices if an antimalarial drug accumulated in any of these cells. Quantification of sulfadoxine in DBS was shown to depend strongly on the erythrocyte volume fraction, the concentrations increasing as the fraction decreased *(179)*.

Table 2.1 gives an overview of the correlations of the concentrations of various antimalarial drugs in realtion to the matrix.

Table 2.1. Matrix correlations for antimalarial drugs

Drug and metabolite	Matrix comparison	Correlation	Ratio	Comment	Reference
Amodiaquine and desethylamodiaquine	Venous blood vs venous plasma	–	3.0	–	*(180)*
Artemisinin	RBCs vs venous plasma	–	0.49	–	*(181)*
Artemisinin	Capillary plasma vs saliva	–	8.0	–	*(85)*
Atovaquone	Venous blood vs DBS	–	1.3	–	*(39)*
Chloroquine and desethylchloroquine	Venous plasma vs venous serum	CQ, r=0.89 CQm, r=0.76	CQ, 2.0 CQm, 4.0	–	*(157)*
Chloroquine and desethylchloroquine	Venous blood vs capillary blood	–	0.9	–	*(96)*
Lumefantrine	Venous plasma vs capillary plasma	Concentrations highly correlated	0.75	–	*(182)*
Mefloquine and mefloquine metabolite	Venous blood vs venous serum	–	MQ, 1.28 MQm, 2.25	–	*(183)*
Mefloquine and mefloquine metabolite	DBS (capillary and venous blood)	Good correlation between capillary and venous blood (r > 0.94)	–	–	*(54)*
Mefloquine	Venous blood vs venous plasma	Good correlation	1.0	–	*(184)*
Mefloquine	RBCs vs venous plasma	–	2.0	–	*(185)*
Pyronaridine	Venous blood vs venous plasma	–	4.9–17.8	Data only for rabbits	*(69)*
Pyronaridine	Venous blood vs capillary blood	Venous blood vs capillary blood, r=0.947	–	–	*(186)*
Quinine	Venous blood vs capillary blood	–	1.0	Mean concentration of quinine: 1.3 ± 1.0 in capillary blood; 1.3 ± 0.78 in venous blood	*(147-148)*

Drug and metabolite	Matrix comparison	Correlation	Ratio	Comment	Reference
Quinine metabolite	Venous blood vs capillary blood	–	1.3	–	*(148)*
Quinine	RBCs vs venous plasma	–	1.89	–	*(187)*
Proguanil	Venous plasma vs capillary plasma	–	1.0	–	*(137)*
Piperaquine	Venous plasma vs venous serum	–	1.6	Released from leukocytes and platelets	*(169)*
Piperaquine	Venous blood vs capillary blood	–	1.7	Ratio positively correlated with parasitaemia on day of sampling but negatively correlated with time after dose	*(188)*
Piperaquine	Venous blood vs venous plasma	–	2.2	Ratio negatively correlated with plasma concentration and parasitaemia on day of sampling but positively correlated with time after dose	*(188)*
Piperaquine	Venous plasma vs capillary blood	–	3.5	No strong correlations	*(188)*
Primaquine and primaquine metabolite	RBCs vs venous plasma	–	PRQ, 1.0 PRQm, 0.01–0.2	–	*(134)*
Primaquine	Venous blood vs venous plasma	–	0.8	–	*(189)*
Sulfadoxine	Venous blood vs venous plasma	–	1.9	–	*(179)*
Sulfadoxine	Venous blood vs venous plasma	–	0.56	–	*(62)*
Sulfadoxine	Capillary blood vs capillary plasma	$r=0.95$	0.72	–	*(190)*

CQ, chloroquine; CQm, chloroquine metabolite; DBS, dried blood spot; MQ, mefloquine; MQm, mefloquine metabolite; PRQ, primaquine; PRQm, primaquine metabolite; RBCs, red blood cells.

2.7.1 Plasma protein binding and unbound concentrations

Artemisinin and its derivatives bind modestly to human plasma proteins, the degree of binding being 43% for dihydroartemisinin, 59% for artesunate, 64% for artemisinin, 76% for artemether and 78.7% for arteether *(191-193)*. The acute-phase protein α-1-acid glycoprotein is the major binding protein for both artemisinin (33% vs 17% for albumin) and arteether *(191)*. Dihydroartemisinin binds predominantly to albumin *(193)*. Lumefantrine binds to plasma proteins at a level of 99.9%, mainly to high-density lipoproteins (77%) *(194)*. The binding of piperaquine to plasma protein is estimated to be around 97% but could be higher *(169)*. Mefloquine is extensively (98%) bound to plasma proteins, with high affinity to α-1-acid glycoprotein *(185, 195)*. Approximately 90% of quinine is bound to protein, predominantly to α-1-acid glycoprotein, which increases with increasing disease severity.

Traditionally, the total concentration (bound plus unbound) of antimalarial drugs is measured and used for PK analysis or therapeutic drug monitoring. Unbound concentrations might in fact provide more useful information than total concentrations, as only unbound drug can cross the RBC membrane. Many antimalarial drugs are, however, highly lipophilic and demonstrate a high degree of protein binding, complicating the development of methods for quantifying the free fraction. Attention has centred on the unbound concentration of quinine, which is high enough to make measurement feasible (i.e. about 10%), because there is significant inter-individual variation in unbound quinine concentrations during malaria infection *(196-202)*. One study showed that the pharmacodynamics (prolongation of the QT interval) is better linked to the free fraction than to the total quinine drug concentration *(203)*. One problem in measuring the free fraction is that binding may be pH dependent, and there can be significant ex vivo changes in the free fraction unless collection and storage are well controlled *(201)*.

Unbound fractions of artemisinin have been determined in venous plasma samples obtained from patients with malaria by ultrafiltration at 37 °C. The samples were centrifuged at $1110 \times g$ for 5 min through YMT membranes (Amicon) prewashed with purified water, in an Amicon MPS-1 micropartition system *(204)*.

2.7.2 Capillary samples vs venous samples

Capillary plasma can be used as an alternative to venous plasma in investigations of the PK of artemisinin, as capillary and venous plasma concentrations are highly correlated ($r = 0.92$). Van Vugt et al. *(182)* showed that the capillary and venous plasma concentrations of lumefantrine were highly correlated but were about 25% higher in capillary plasma. A good correlation was observed for mefloquine and its metabolite in venous blood and capillary blood applied to chromatographic paper ($r > 0.94$) *(54)*. The venous and capillary blood concentrations of proguanil and its metabolites have also been found to be similar *(137)*. Close correlations were found between the concentrations of sulfadoxine *(179)*, pyrimethamine *(186)* and

mefloquine *(54)* in venous and capillary blood samples collected on filter paper, and for chloroquine and its metabolite (only four samples) *(97)*. Several studies have shown that sampling of venous and capillary blood on filter paper gives comparable results for many antimalarial agents (see Table 2.1), although Ashley et al. *(188)* reported that the capillary blood concentrations of piperaquine were approximately 1.7-fold higher than the venous blood concentrations, and this difference increased with time. Overall, the relation between the concentrations of piperaquine in venous plasma, venous blood and capillary blood was variable and unpredictable at low concentrations; however, within the range of concentrations usually present in patients 3–21 days after treatment with currently recommended doses, the relation between capillary and venous blood concentrations was predictable. Capillary blood sampling could therefore be used in field assessments. Most other studies have investigated correlations rather than systematic differences, despite the fact that a high degree of correlation can be obtained in the absence of directly comparable results in two matrices. Proper comparisons with Bland–Altman plots have rarely been performed.

Some drugs are present at different concentrations in arterial and venous blood *(205)*. Bass et al. *(206)* showed differences in the composition of fatty acids in arterial and venous blood, indicating distribution into muscles and tissue. Thus, correlations between capillary and venous blood could be affected by the sampling technique. In 'warm' capillary vessels with good flow, the blood is more like arterial blood than venous blood. In 'cold' capillary vessels or when a tourniquet is applied, the blood is more like venous than arterial blood *(207)*.

2.7.3 Saliva vs plasma or capillary blood

Saliva is an attractive alternative biological matrix for measuring artemisinin, as samples can be obtained easily; however, its use requires greater assay sensitivity, as only unbound artemisinin is measured. Furthermore, increased variability in results can be expected. The concentrations of artemisinin in saliva are comparable to its unbound concentrations in plasma *(85)*. The concentrations in saliva are approximately eight times lower than those in plasma, but they have a comparable concentration–time profile *(181)*. Saliva concentrations are more closely correlated to unbound capillary plasma ($r = 0.85$) than to unbound venous plasma concentrations ($r = 0.77$) *(85)*. Saliva is currently not recommended as a replacement for blood/plasma.

2.7.4 Venous blood vs venous plasma

No assays for the quantification of artemisinin and derivatives in blood have been published. Some data indicate that artemisinin and dihydroartemisinin bind moderately to RBCs (RBC:plasma ratio, 0.49 and 0.28, respectively), with 11% artemisinin and 8% lumefantrine bound to RBCs *(194)*. Mefloquine has a strong affinity for RBC membranes *(208-209)*, indicating that the drug may concentrate more in RBCs than in plasma *(210)*. In vivo, the intra-erythrocytic mefloquine concentrations were twice as high as the plasma concentrations *(185)*; however, other studies suggest that the blood and plasma concentrations

of mefloquine are similar *(184, 211)*. As blood is more convenient to collect, particularly in the field, it is the preferred biological matrix for determining mefloquine. The concentration of piperaquine in serum is about 58% higher than that in plasma, as the drug is released from leukocytes and platelets *(169)*. A similar finding has been reported for chloroquine *(157)*. Primaquine does not accumulate significantly in RBCs, as evidenced by a blood:plasma ratio of around 0.8 *(189)* and a plasma:RBC ratio of around 1 *(134)*. In vitro, primaquine binds to α-1-acid glycoprotein, and the RBC concentrations are inversely proportional to the concentration of this protein *(212)*. Physiological variations in α-1-acid glycoprotein concentrations (which increases with disease severity and decreases during convalescence) therefore expects to influence the distribution of primaquine in venous blood. This should be taken into account in interpreting or comparing the plasma concentrations of primaquine. In contrast, carboxyprimaquine, the main metabolite of primaquine, concentrates in RBCs, and its RBC:plasma ratio shows wide inter-individual variation, ranging between 5 and 100 *(134)*.

The venous blood:venous plasma ratio of sulfadoxine concentrations is 0.56 *(62)*. The whole-blood concentrations of chloroquine and its metabolite are 5–10 times higher than those observed in plasma because of uptake into platelets and granulocytes *(20)*. Determination of plasma concentrations is therefore unreliable unless strictly standardized pre-analytical conditions are adhered to (e.g. time from sampling to centrifugation and centrifugation speed) *(157)*. For this reason, blood obtained by venous or capillary sampling is preferable to plasma or serum for determining chloroquine. A similar conclusion was reached for desethylamodiaquine, the active metabolite of amodiaquine *(180)*. The concentration of desethylamodiaquine has been reported to be about three times higher in venous blood than in plasma. The chemical structures of chloroquine and desethylamodiaquine are closely related, and it seems logical to determine the concentration in venous blood instead of plasma, thereby eliminating variations due to variable uptake into cells *(180)*.

2.7.5 Filter paper sampling (dried blood spots)

Several studies indicate that sampling of venous blood and capillary blood (applied onto filter paper) produces similar results for antimalarial drugs; however, for some drugs there can be large discrepancies according to the matrix. Capillary blood, by virtue of the sampling technique, contains interstitial and intracellular fluids and therefore differs from both arterial and venous blood. Substances with widely different concentrations in arterial and venous blood or in blood and intracellular and extracellular fluids are likely to be found in widely different concentrations in capillary and venepuncture samples. Collection of capillary blood specimens onto filter paper should cause minimal discomfort and is therefore an attractive alternative for sampling children. Finger-prick sampling of blood for studies of the PK and efficacy of antimalarial drugs can be done at the same time as blood smears are collected for microscopy and genotyping (i.e. to distinguish recrudescence from reinfection) *(213)*. DBS methods have been developed for

amodiaquine *(12, 78)*, atovaquone *(39)*, chloroquine *(97, 214)*, lumefantrine *(112, 114)*, mefloquine *(54)*, piperaquine *(130)*, proguanil *(137)*, pyrimethamine *(63, 186)*, quinine *(148)* and sulfadoxine *(153, 179)*. No DBS method is currently available for artemisinin or its derivatives. Most of these methods have been validated according to the United States Food and Drug Administration regulatory requirements; however, in all the validations, it was assumed that clinical samples arrive in precise, accurate volumes, which is rarely the case. Such pre-analytical error has not been assessed quantitatively. To date, there is no available method for determining artemisinin derivatives in capillary blood after sampling on paper.

Capillary blood is obtained by finger puncture with a lancet, and semi-skilled field workers can perform this procedure after only minimal training. The technique has been used routinely since the 1960s for neonatal screening *(215)*. The use of blood dried on filter paper is highly suitable for field epidemiological studies, particularly in remote locations, because facilities for refrigerated storage of samples are not needed. Storage and transport of DBS samples do not require controlled temperatures, as for plasma samples, as the DBS technique stabilizes many drugs. The biohazard for laboratory workers is minimal, and blood samples can be sent by post. This simplified sample handling could also lead to cost savings in clinical PK studies.

Many types of safety lancet are available for blood spot sampling, with a needle depth of about 2 mm for adults and a shallower depth for children. By application of gentle pressure around the finger, but without milking or massaging the area around the puncture site, free-flowing drops of blood can be aspirated into capillary or microtubes and then applied onto filter paper. The first drop of blood after skin puncture should be discarded, as it is likely to be contaminated with tissue fluids or sweat. It is important to dry the filter paper samples before storage and transport, as moisture may lead to bacterial growth and drug degradation. Blood spot specimens should be dried at ambient temperature and humidity for at least 3–4 h and stored individually with a desiccant in a plastic zip bag. Contamination of filter paper is a risk and must be avoided. The person who draws blood samples should not handle antimalarial tablets, so as to prevent contaminating the filter paper with the drugs that are to be measured *(216)*.

The choice of filter paper is important to achieve rapid drying, uniform absorption and a small blood spot diameter. Whatman ET 31 CHR and 3 MM papers have both been used successfully and produce homogeneous blood spots. Whatman ET 31 CHR paper has good blood saturation, absorption and elution properties and is easier to handle than glass fibre. Glass-fibre paper does not absorb blood well and consistent saturation cannot be obtained, leaving the edges of the spot uneven. It is essential that the analytical method be validated with the same type of paper as that used for blood sampling. The recovery of proguanil and sulfadoxine is different with different brands of paper *(137, 217)*. Dilution of blood with phosphoric acid before application to paper resulted in high recovery of lumefantrine but

nevertheless complicates the collection procedure *(114)*. Another method is to pretreat the filter paper with tartaric acid solution before applying the blood *(112)*. In the field, it is essential to follow the standard operating procedures provided by the bioanalytical laboratory.

Use of punched out blood spots rather than cutting spots into strips during sample preparation has provided satisfactory results for sulfadoxine *(217)*. This approach can be useful in the field, where exact sample volumes cannot be collected. It is important to keep in mind when punching that the blood spots should be homogeneous. This depends on the sampling paper used and should be carefully evaluated and made uniform for the study. Sample labels should contain identifiers such as the patient code, time of sampling and site of sampling.

2.8 TRANSPORT AND STORAGE OF SAMPLES TO MAINTAIN STABILITY

2.8.1 Plasma and blood samples

The length of time before transfer of the plasma sample to the laboratory freezer is another important pre-analytical variable, as certain antimalarial agents, such as artemisinin derivatives, amodiaquine and pyronaridine, are poorly stable at room temperature. Even though more and more guidelines for sample collection are being issued, the analytical laboratory should be contacted for instructions on handling samples before a new study is begun.

In field trials, after prompt blood centrifugation, plasma samples for the measurement of most antimalarial agents can be stored at 4 °C for up to 48 h before final storage at –80 °C. The short- and long-term stability of biological samples containing various antimalarial drugs is summarized in Table 2.2.

Table 2.2 Short- and long-term storage stability of biological samples for analysis of antimalarial drug concentrations

Drug and metabolite	Matrix	Anticoagulant	Stability		Reference
			Long-term	Short-term	
Amodiaquine and desethylamodiaquine	Plasma	Heparin	–80 °C: ≥ 35 days –20 °C: ≥ 35 days	4 °C: ≤ 14 days 22 °C: ≤ 3 days	*(218)*
Amodiaquine and desethylamodiaquine	Whole blood	Heparin	–80 °C: ≥ 35 days 4 °C: ≥ 35 days	22 °C: ≤ 3 days –20 °C: ≤ 14 days	*(218)*
Amodiaquine and desethylamodiaquine	DBS	Heparin	Room temperature: ≤ 4 months	4 °C: ≤ 30 days	*(13)*
Atovaquone	Plasma	Heparin	–40 °C: ≥ 6 months	Heat inactivation to decrease HIV activity had no effect	*(35)*
Atovaquone	DBS	Heparin	–	–86 °C, 4 °C, 23 °C, 60 °C: ≤ 60 days sealed in plastic bags	*(39)*
Chloroquine and desethylchloroquine	Plasma	Heparin	–86 °C, –20 °C, 4 °C: ≥ 35 days	37 °C: ≥ 6 days	*(218)*
Chloroquine and desethylchloroquine	DBS	Heparin	20 °C: ≤ 7 weeks	–	*(214)*
Chloroquine and desethylchloroquine	DBS	Heparin	Room temperature: decreased by 20% after 20 years	–	*(219)*
Chloroquine and desethylchloroquine	Plasma	Heparin	4 °C: ≥ 3 months	–	*(95)*
Chloroquine and desethylchloroquine	Plasma	–	–	–80 °C: ≤ 1 year	*(220)*
Lumefantrine	Plasma	Heparin	–86 °C, –20 °C: ≥ 4 months	–	*(113)*
Lumefantrine	DBS	Heparin	Room temperature: ≤ 5 months	–	*(114)*
Lumefantrine	DBS	Heparin	Room temperature: ≤ 2.5 years	–	*(221)*
Lumefantrine	Plasma	–	–	–80 °C: ≥ 2 years	*(220)*
Mefloquine	Venous blood	Heparin	–80 °C: ≤ 1 year	–20 °C: ≤ 3 months	*(222)*

Mefloquine	Plasma	Heparin	–80 °C: Slight degradation after 12 months	Room temperature: stable up to 1 h –80 °C: ≤ 3 months Freeze–thaw stability (–80 °C and 25 °C): ≥ 1 cycle	*(87)*
Mefloquine and mefloquine metabolite	DBS	EDTA	–	–20 °C: ≥ 50 days; 37 °C: <10% decrease in concentration after 50 days	*(54)*
Artemisinin	Serum (rat)	–	–	Room temperature: ≥ 6 h (serum) and 12 h (autosampler) Freeze–thaw: ≥ 3 cycles	*(22)*
Artemether	Plasma	–	–	–80 °C: ≥ 2 years	*(220)*
Artesunate and dihydroartemisinin	Plasma	–	–80 °C: ≤ 3 months Only slight degradation seen after 12 months	Room temperature: slight reduction after 3 h Freeze–thaw stability (–80 °C and 25 °C): ≥ 1 cycle	*(15)*
Artesunate and dihydroartemisinin	Plasma	Fluoride–oxalate	–	Room temperature: poor stability at 1 h Ice: good stability for 24 h –80 °C: ≥ 2 months	*(160)*
Artesunate and dihydroartemisinin	Plasma	Heparin	–70 °C: ≥ 6 months	–	*(88)*
Artesunate and dihydroartemisinin	Plasma	Heparin	≤ 20 °C: ≥ 6 months	–	*(86)*
Artesunate and dihydroartemisinin	Plasma	–	–	Room temperature: not stable for 24 h or 48 h –80 °C: ≥ 60 days Freeze–thaw: ≥ 1 cycle	*(223)*
Artesunate and dihydroartemisinin	Plasma	Fluoride–oxalate	–	–80 °C: ≥ 2 months Freeze–thaw: ≥ 3 cycles for ≥ 39 h 60 °C: unstable in autosampler	*(30)*
Artesunate and dihydroartemisinin	Plasma	–	–	37 °C: ≥ 2 h Distribution of artesunate to blood cells was fast and complete after 1.5 h	*(224)*

Drug and metabolite	Matrix	Anticoagulant	Stability		Reference
			Long-term	Short-term	
Artemether and dihydroartemisinin	Plasma	–	–	Plasma extracts Room temperature: ≤ 5 h 4 °C: ≤ 22 h –80 °C: ≤ 3 months	*(17)*
Artemether and dihydroartemisinin	Plasma	–	–	–80 °C: ≥ 2 years	*(220)*
Arteether	Serum (rat)	Heparin	–	Room temperature, 4 °C: ≥ 12 h Freeze–thaw: ≤ 3 cycles	*(225)*
Arteether	Venous blood, venous plasma	–	–	Storage in plastic containers resulted in considerable loss over 24 h	*(91)*
Arteether and dihydroartemisinin	Plasma	–	–	–20 °C, –80 °C: ≥ 8 months	*(92)*
Artemether and dihydroartemisinin	Plasma	Citrate–phosphate–dextrose, heparin	≤ –70 °C: ≥ 8 months	Plasma extracts Room temperature: ≥ 14 h	*(19)*
Artemether and dihydroartemisinin	Plasma	Heparin	–70 °C: ≥ 6 months	–	*(34)*
Pyronaridine	Venous blood	Heparin	–	Room temperature: poor stability (1 day) 6 °C: limited stability (1 month) Freeze–thaw: poor (8–12% loss after 1 cycle and 35% loss after 3 cycles)	*(144)*
Pyronaridine	Venous blood	Heparin	–	Room temperature: concentrations decreased by 10% within 12 h and 35% within 24 h Freeze–thaw: 10% decrease at each cycle	*(226)*
Pyronaridine	Venous blood	–	–80 °C: ≤ 7 months	Room temperature: ≤ 48 h	*(73)*
Pyronaridine	Plasma	–	–	–20 °C: plasma extracts ≤ 2 days –80 °C: ≤ 3 months Freeze–thaw: ≤ 3 cycles	*(143)*

Sulfadoxine and sulfadoxine metabolite	Plasma	Heparin	–	Room temperature, –20 °C: ≤ 45 days Freeze–thaw stability: 2 cycles of freezing (–20 °C) and thawing	*(152)*
Sulfadoxine and sulfadoxine metabolite	DBS	Heparin	–20 °C, room temperature, 37 °C: ≤ 105 days	–	*(179)*
Sulfadoxine and sulfadoxine metabolite	DBS	Heparin	4 °C, 22 °C, 37 °C: 40 days –20 °C: 4 years	Freeze–thaw stability: 3 cycles of freezing (–86 °C) and thawing	*(57)*
Pyrimethamine	Venous plasma	Heparin	–20 °C: 4 months	–	*(60)*
Quinine and quinine metabolite	Venous plasma	Heparin	–20 °C: 79 days	–20 °C, room temperature, 4 °C: 1 week (except 2′-quininone, which is stable for 4 days)	*(227)*
Quinine	DBS (capillary blood)	Heparin	Room temperature: ≥ 3 months	–	*(228)*
Quinine and quinine metabolite	DBS (capillary blood)	–	–	Room temperature: 2 months 56 °C: 1 h 30 °C: 3 months	*(148, 228)*
Proguanil	DBS (capillary blood)	–	37 °C: ≥ 30 days	50 °C: 50% decrease after 5 days	*(96, 137)*
Piperaquine	Whole blood	Heparin	–17 °C, 8 °C: ≥ 2 months	–	*(229)*
Piperaquine	Plasma	–	–20 °C: ≥ 6 months	Room temperature: ≥ 48 h	*(26, 127)*
Piperaquine	DBS (capillary blood)	–	37 °C: ≥ 30 days	–	*(130)*
Piperaquine	Plasma	–	–	–80 °C: 1 year 30% decrease after 3 years	*(220)*

DBS, dried blood spot; EDTA, ethylenediaminetetraacetic acid.

The stability of both artesunate and dihydroartemisinin is poor at ambient and higher temperatures, but stability is guaranteed if all steps in sample preparation and analysis are performed on ice *(30, 230)*. Artesunate degrades to dihydroartemisinin by both chemical and esterase-mediated hydrolysis, and both are minimized at low temperatures. Both artesunate and dihydroartemisinin in human plasma are unstable at room temperature but are stable for at least 2 months at –80 °C *(231)*. Artemether and dihydroartemisinin are stable in plasma if kept at –80 °C for at least 2 years *(220)*, and artemisinin and dihydroartemisinin are stable for at least 22 h at 4 °C and for at least 2 years at –80 °C *(17, 19, 220)*. Plasma extracts containing artemisinin and dihydroartemisinin were stable for at least 5–14 h at room temperature in an autosampler *(19)*.

Amodiaquine and its metabolite were stable in plasma and blood at –80 °C for at least 1 month, at 4 °C for 14 days and at 22 °C for 1 day *(218)*. No change in chloroquine or desethylchloroquine values was found when plasma samples were stored for up to 11 months at –20 °C *(93)*, and the stability of both at –80 °C for at least 1 year has been confirmed *(220)*. Piperaquine in whole blood samples was stable for at least 2 months at temperatures between –17 °C and 8 °C, and the concentration of piperaquine (within the precision of the assay) was not altered by heating at 60 °C for 60 min *(229)*. Piperaquine was also found to be stable in plasma at room temperature for up to 48 h *(26)* and for at least 1 year at –20 °C and –80 °C *(127, 220)*. Pyronaridine in blood appeared to have limited stability when stored in a refrigerator (at about 6 °C): after 1 month, the concentration had decreased by 10–15%; the stability was even poorer at room temperature and samples could be kept for less than 1 day. Conflicting results were reported on the stability of lumefantrine in plasma at –20 °C *(113, 232)*. It is recommended that plasma samples containing lumefantrine be stored at –20 °C for shorter periods and thereafter preferably at –80 °C, at which stability for up to 2 years has been verified *(113, 220)*.

2.8.2 Filter paper samples

Antimalarial drugs are generally very stable in DBS. In dried blood on filter paper strips, amodiaquine and desethylamodiaquine were stable for up to 30 days at 4 ˚C and for up to 24 h at room temperature *(13)*. Another study showed no significant decrease in concentration at room temperature for about 4 months *(78)*. The concentration of mefloquine in DBS was unaltered for at least 50 days at either room temperature or higher (37 ˚C): the concentration decreased by less than 10% when the paper was stored at 37 ˚C for 50 days *(54)*. Sulfadoxine was stable on filter paper for up to 15 weeks at 37 ˚C, at room temperature or at –20 ˚C *(179)*. No decrease in the concentration of proguanil and its metabolites on filter paper was found during storage for at least 30 days at 37 ˚C *(137)*. Pyrimethamine was stable for up to 3 months at room temperature and for about 14 days at 35 ˚C *(186)*. Lumefantrine on filter paper was very stable over the concentration range tested (300–3000 nmol/l) for 5 months. DBS samples containing lumefantrine have been kept at room temperature for 2.5 years with no decrease in concentration *(219)*. No significant change in lumefantrine concentration (< 10%) was observed during

storage at room temperature for 2 months *(114)*. Primaquine was stable at temperatures ranging from – 86 °C to 37 °C for at least 30 days *(130)*. There was no loss of quinine or 3-hydroxyquinine after storage of filter paper samples for 2 months at room temperature or at 37 °C *(148)*. The stability of chloroquine in blood spots on paper at room temperature had decreased by approximately 20% after 20 years at room temperature *(219)*.

In conclusion, most DBS samples can be stored for long periods with no significant decrease in drug concentration at room temperature. As the water present in biological samples plays an important role as an active reagent in hydrolysis and other degradation reactions, use of filter paper to store samples often results in better drug stability, because of dehydration of the samples.

2.8.3 Freeze-and-thaw stability

Minimal variation is generally seen in the concentrations of antimalarial drugs and their metabolites after one or two freeze–thaw cycles. Even the labile compounds artesunate and dihydroartemisinin are stable during three freeze–thaw cycles. Apparent loss of some antimalarial agents (artemisinin, amodiaquine and quinine) has been reported after two cycles, and there have been contradictory reports regarding the stability of lumefantrine and desbutyl-lumefantrine during multiple freeze–thaw cycles *(26, 113)*. For repeated analyses of samples, it is advisable to place plasma aliquots into separate vials to minimize unnecessary freezing and thawing *(231)*. Piperaquine has good freeze–thaw stability in plasma and blood samples *(229)*. Pyronaridine has poor freeze–thaw stability, with a decrease in concentration of about 10% after one cycle and 35% after three cycles *(113, 144)*. Adequate mixing of deep-frozen samples after thawing is critical; if this is not done carefully, there will be large variation between analyses, especially if aliquots are taken from the same tube, owing to the formation of concentration gradients during thawing.

2.9 RECOMMENDATIONS

2.9.1 Sample collection

All samples, especially those containing drugs that preferentially accumulate in cells, such as chloroquine, piperaquine, amodiaquine and mefloquine, should be centrifuged at high speed (1000–3000 × *g*) within 1 h of collection. The centrifugation time should be 7–15 min. No significant differences have been reported with different anticoagulants (e.g. EDTA and heparin), except with artesunate. Artesunate is targeted by esterases, which are inhibited by the presence of fluoride in the anticoagulant. Therefore, when sampling for artesunate, it is recommended that fluoride anticoagulants be used. In the interests of safety and accuracy, all samples should be drawn into plastic specimen collection vials. After centrifugation, aliquots of samples should be placed in polypropylene cryovials to ensure the integrity of the sample during storage and to prevent adsorption of the analyte onto the container. The effects of high lipid concentrations and haemolysis on drug concentrations can vary and should be investigated during validation.

2.9.2 Matrix selection

The matrix chosen should be the same as that in the studies with which the data are to be compared. The study question might indicate which matrix should be used (e.g. venous blood or venous plasma). Some drugs concentrate in cells, resulting in increased concentrations in blood samples, so that the drug can be followed for longer without an improvement in assay sensitivity.

2.9.3 Stability

As the results of studies on the stability of antimalarial drugs are contradictory and scant, it is recommended that the stability of a specific sample type be ascertained during assay validation, before field sampling. Most antimalarial drugs are stable in plasma at 4 °C for up to 48 h. Venous blood samples should not be stored for more than 1 h at room temperature; if blood is to be used for the assay, it can be stored at –20 °C for up to 3 months. Long-term storage at –80 °C is usually sufficient for at least 1 year. If the storage facilities are unreliable or the stability of the sample is unknown, it is recommended that spiked quality control samples be stored with the untested samples to allow intermittent evaluation of sample stability. Most drugs are stable for two to three freeze–thaw cycles; however, storage of at least two aliquots allows for repeated or additional analyses without submitting the samples to unnecessary freeze–thaw cycles. Artemisinin derivatives are thermolabile drugs, and working on ice when collecting the samples and when preparing samples for analysis significantly improves the quality of the results.

2.9.4 Protein binding

Most methods are for the total drug concentration, while some study questions might require a distinction between free and bound drug. Benet et al. *(233)* argued that a change in the free fraction might be important clinically only when the drug is highly (> 95%) protein bound and has a high extraction ratio or index. Unfortunately, assaying for free drug concentrations is technically difficult when a drug is highly protein bound, presenting problems such as assay insensitivity and nonspecific adsorption of the drug. The general recommendation is to measure the total drug concentration while measurement of free fraction should be considered on a case by case basis.

2.9.5 Dried blood spots

It can be difficult to select a sampling technique for field studies, especially when resources are limited. Although the use of DBS is attractive in terms of cost, storage, safety and stability, both the method and the sampling collection technique should have been validated before their use at a clinical site, as the collection technique is difficult to validate in a laboratory. Another problem is that field conditions may differ widely between sites.

Certain drugs should not be analysed in DBS, which is more suitable for monitoring the therapeutic effect of antimalarial drugs in the field. No DBS method is available for artemisinin or its derivatives, although one might become available if a method for analysing these drugs in blood is found.

The use of DBS for detailed PK investigations is difficult to regulate and control and is therefore not recommended at this stage. If the complete process (i.e. from sample collection to end result) can be standardized, characterized and validated, it might replace venous sampling in PK investigations in the future.

2.10 RATIONALE

It is critical to minimize the variation associated with sampling during PK investigations in order to ensure that the results of an analysis are accurate, reliable and comparable.

Rapid, high-speed centrifugation is essential for measuring drugs such as chloroquine, piperaquine, mefloquine and amodiaquine, which may leak from cells if the samples are left for too long before centrifugation, and this will have a significant effect on the resulting plasma drug concentration. The selection of analytical matrix is also important. Because of the high uptake of chloroquine into platelets and leukocytes, it is preferable to sample venous or capillary blood rather than serum or plasma. Analysing whole blood also minimizes errors due to drug leakage from cells before matrix separation. Whole blood is also more representative of the effective drug concentration after malaria treatment in the field. A clear difference has been shown in drug concentrations in capillary and venous blood, indicating that the results cannot be compared directly and the two matrices cannot be used interchangeably.

Use of plastic sampling and storage containers minimizes the risk for analyte adsorption and improves safety at the site.

Dried capillary blood spots represent an attractive sampling system, as the finger-prick technique is rapid, simple, safe and cheap. The method has been used in many clinical studies *(234-236)*. Nevertheless, more pre-analytical errors are associated with this technique than with conventional sampling. The volume and homogeneity of the spot markedly affect the results, and inaccuracies during sampling at clinical sites cannot be rectified. If the technique can be standardized and the errors characterized and minimized, it will be an alternative to venous sampling in PK studies in the future. It is mainly the pre-analytical variables that preclude recommendation of DBS methods for regulatory-level PK studies.

Chapter 3. Optimizing sampling schemes for pharmacokinetics studies

3.1 CLINICAL INDICATIONS FOR MEASURING ANTIMALARIAL DRUG CONCENTRATIONS IN BLOOD

The profile of antimalarial drug concentrations must be characterized over time in order to optimize dosing, and thereby optimize cure rates, and to reduce the emergence of resistance, diminish gametocyte carriage and limit toxicity. Antimalarial PK often differs substantially between patients. The PK of antimalarial drugs must therefore be quantified precisely for all target populations, especially young children, pregnant women and patients with prevalent co-morbid conditions.

The usefulness of a PK study of antimalarial agents depends on whether a representative target population is included, sufficient people are recruited to characterize variations in PK and an adequate number of samples is collected at appropriate times. The aim of this chapter is to provide guidance on sampling schemes for studies of antimalarial PK. In designing sampling schedules, prior knowledge of the PK of the drug, the statistical methods to be used to analyse the data and the practical constraints of sampling the study population should be considered. The ranges of the published PK of each antimalarial drug are summarized in Table 3.1, and the important covariates that influence these parameters are summarized in Table 3.2. Visual representations of the concentration–time profiles for each antimalarial drug currently recommended by WHO, for which there are adequate clinical data, are given in Appendix 2.

Table 3.1. Pharmacokinetics parameters (reported as ranges of mean or median) in studies of currently recommended dosages of antimalarial agents used for the treatment of patients with acute malaria

Drug (metabolite)	Reference	C_{max}	T_{max} (h)	AUC	K_a (per h)	V/F (l/kg)	CL/F (l/h per kg)	$T_{½}$
Intravenous artesunate	*(237-240)*	11 346–29 681 ng/ml	–	558–1146 ng/ml × h	–	0.08–0.24	1.63–4.26	0.03–0.19 h
(dihydroartemisinin)		(1279–3011 ng/ml)	(0.01–0.2)	(739–2559 ng/ml × h)	–	(0.47–1.01)	(0.73–2.16)	(0.3–1.1 h)
Rectal artesunate	*(239, 241-243)*	269–507 ng/ml	0.8–0.9	692 ng/ml × h	0.3	2.1	6.0	0.9 h
(dihydroartemisinin)		(682–896 ng/ml)	(1.5–2.3)	(2402–2786 ng/ml × h)	(0.7)	(2.1–4.4)	(2.2–2.64)	(0.7–1.3 h)
Oral artesunate	*(244-247)*	51–244 ng/ml	0.5	113–232 ng/ml × h	3.3	6.8	19.2	0.36 h
(dihydroartemisinin)		(251–488 ng/ml)	(2)	(740–1671 ng/ml × h)	(1.19–4.27)	(2.3–4.6)	(0.6–4.0)	(0.64–2.5 h)
Oral artemether	*(245, 248-249)*	34–186 ng/ml	1–2	65.6–211 ng/ml × h	–	49.5	25.9	1.5–3.9 h
(dihydroartemisinin)		(101–165 ng/ml)	(1–3)	(357–604 ng/ml × h)	–	(7.8)	(4.5)	(1.3–1.9 h)
Primaquine 15 mg daily for 14 days	*(250-253)*	50.7–57.7 ng/ml	2.2–2.3	480–547 ng/ml × h	–	4.8–5.1	0.2–0.5	5.6–6.4 h
(carboxyprimaquine)		(291–432 ng/ml)	(2.6–7.3)	–	–	–	–	(21.8 h)
Primaquine 45 mg, single dose	*(254)*	167 ng/ml	2.0	–	–	–	0.5	6.1 h
(carboxyprimaquine)		(890 ng/ml)	(6.1)	(12 737 ng/ml × h)	–	–	–	–
Proguanil	*(255-257)*	363–750 ng/ml	4.5–5.2	5.7–13.5 µg/ml × h ($AUC_{48h-\infty}$)	0.41–0.51	15.8–29.7	0.76–1.23	8.0–17.6 h
(cycloguanil)		(26–67 ng/ml)	(6.4–6.9)	(0.71–1.8 µg/ml × h) ($AUC_{48h-\infty}$)	–	–	–	(6.4–22.6 h)
Quinine	*(196, 258-266)*	4–15.3 µg/ml	2.0–16.1	52.6–332 µg/ml × h	0.93–3.50	0.53–1.84	0.05–0.138	7.0–19.7 h
Atovaquone	*(255-257)*	2.07–13.3 µg/ml	5.1	63–663 µg/ml × h ($AUC_{48h-\infty}$)	0.26–0.46	4.7–10.2	0.07–0.32	31.3–72.9 h
Amodiaquine	*(234, 247, 267)*	14.1–15.5 ng/ml	–	(28.9–44.3 µg/ml × h)	–	322.7	14	3.3 h
(desethylamodiaquine)		(235–1185 ng/ml)	(46.9–47.9)	–	(0.13–0.87)	(75.2–123)	(0.61–0.86)	(104–216 h)
Lumefantrine	*(245, 248-249, 268-275)*	5.72–25.7 µg/ml	4–544	210–636 µg/ml × h	0.06–0.17	3.7	0.12	32.7–79.2 h

Pyrimethamine	*(276-277)*	280 ng/ml	12–19.8	38.0–89.1 μg/ml × h	–	4.25–4.46	0.0174–0.0417	2.8–3.4 days
Sulfadoxine	*(276-278)*	63.9–130 μg/ml	5.7–13.5	–	0.3	0.242–3.13	0.00068–0.00190	4.1–8.9 days
Chloroquine (desethylchloroquine)	*(278-281)*	283–1430 ng/ml (89–220 ng/ml)	6.5–6.9 –	37–140 μg/ml × h (44–64 μg/ml × h)	0.14 –	31.8–154 –	0.23–0.80 –	4.5–9.7 days (7.3–8.5 days)
Mefloquine	*(282-288)*	1.62–2.70 μg/ml	15–45	307.2–1497 μg/ml × h	0.292	8.14–31.8	0.017–0.174	8.5–19.3 days
Piperaquine	*(280, 289-293)*	568 ng/ml	5.7	46.9–56.4 μg/ml × h	0.08–0.72	164–614	0.85–1.85	12–28 days

Only metabolites that have been reported are included in this table; some antimalarial agents have active metabolites (e.g. desbutyl-lumefantrine for lumefantrine) that have not been reported in any of the studies summarized here.

C_{max}, maximum plasma concentration; T_{max}, time after administration to maximum plasma concentration; AUC, area under the concentration–time curve; K_a, first-order absorption rate constant; V/F, apparent volume of distribution; CL/F, apparent total clearance after oral administration; $T_{½}$, terminal elimination half-life.

Table 3.2. References that report covariates that affect the pharmacokinetics of and exposure to antimalarial drugs

Drug	Covariate	Clearance			Volume of distribution			Exposure (e.g. AUC, day 7)		
		↑	↔	↓	↑	↔	↓	↑	↔	↓
Artemether	Lopinavir–ritonavir								*(294)*	*(295)*
	Ketoconazole							*(295)*		
	Mefloquine								*(295)*	
	Quinine									*(295)*
	Grapefruit juice							*(296-297)*		
	Fat							*(295)*		
	Pregnancy									*(249)*
	Time dependence									*(296, 298)*
Artesunate	Mefloquine								*(299)*	
	Time dependence								*(300-301)*	
Atovaquone	Pregnancy	*(257)*			*(257)*					*(257)*
Chloroquine	Age (children)									*(278, 302)*
Dihydroartemisinin	Lopinavir–ritonavir									*(294-295)*
	Ketoconazole							*(295)*		
	Mefloquine								*(295)*	

	Quinine									*(295)*
	Amodiaquine		*(303)*		*(303)*					*(303)*
	Grapefruit juice							*(296-297)*		
	Fat							*(295)*		
	Time dependence	*(304)*	*(300-301, 305)*					*(296, 298)*	*(300-301, 305)*	*(304)*
	Malaria			*(306)*			*(306)*	*(306-307)*		
	Pregnancy									*(249)*
Desethyl-amodiaquine	Efavirenz									*(308)*
	Body weight	*(267)*								*(267)*
Lumefantrine	Pregnancy				*(271)*					*(271)*
	Smoking									*(249)*
	Fat							*(295, 309)*		
	Age (children)									*(275)*
	Lopinavir–ritonavir							*(294-295)*		
	Mefloquine									*(295, 310)*
	Dose dependence									*(272)*

Drug	Covariate	Clearance			Volume of distribution			Exposure (e.g. AUC, day 7)		
		↑	↔	↓	↑	↔	↓	↑	↔	↓
	Body weight								*(269)*	
	Ketoconazole							*(295)*		
	Quinine								*(295)*	
Mefloquine	Ketoconazole							*(311)*		
	Fat							*(308)*	*(287)*	
	Malaria		*(312)*			*(312)*	*(306)*		*(312)*	
	Gender								*(313)*	
	Primaquine		*(314)*			*(314)*			*(314)*	
	Sulfadoxine–pyrimethamine		*(314)*			*(314-315)*			*(314-315)*	
	Dose dependence	*(284)*			*(284)*				*(287)*	*(284)*
	Pregnancy		*(316)*		*(316)*					
	Body weight			*(283)*						
	Temperature on admission						*(283)*			
Piperaquine	Diarrhoea									*(293)*
	Anaemia									*(293)*

	Age (children)	*(290)*				*(290)*			*(317)*	*(293)*
	Fat		*(318)*	*(165)*		*(318)*	*(165)*	*(165, 319)*	*(318)*	
	Body weight	*(292)*			*(292)*				*(317)*	*(292)*
	Gender								*(317)*	
	Dose dependence								*(292)*	
Sulfadoxine	Age (young children)	*(276)*	*(276)*		*(276)*				*(276)*	*(276, 278)*
	Pregnancy	*(277)*			*(277)*		*(320)*			*(277, 320-321)*
	Artesunate								*(322)*	
Pyrimethamine	Age (young children)	*(276)*	*(276)*		*(276)*				*(276)*	*(276)*
	Pregnancy	*(277)*		*(320)*	*(277)*		*(320)*	*(320)*	*(321)*	*(277)*
	Artesunate								*(322)*	
Quinine	Rifampicin	*(323)*								*(323)*
Proguanil	Pregnancy	*(257)*			*(257)*					*(257)*
Primaquine	Quinine									*(254)*

Only papers in which these covariates were investigated are included. Parameters normalized to body weight were used in assessing the covariate 'Age (children)'. ↑, significant positive correlation; ↔, nonsignificant correlation; ↓, significant negative correlation.

AUC, area under the concentration-time curve.

3.2 COVARIATES THAT AFFECT THE PHARMACOKINETICS OF ANTIMALARIAL DRUGS

PK varies considerably among individuals, and when the differences are large in a given study population the sampling schedule might have to be altered. Important covariates that are known to influence apparent clearance, volume of distribution or exposure are summarized in Table 3.2, although the completeness and accuracy of this summary cannot be assured. As most of the comparisons were made with previously published results, the statistical significance of the observed differences cannot be tested, and they are not necessarily a result of the covariate to which they are attributed but may be due to differences in study or assay methods. The testing and reporting of the effects of each covariate are not standardized, and nonsignificant covariate effects are often due to the inadequate power of PK studies. It should also be noted that drug quality (i.e. active ingredient content and dissolution properties) can have a significant impact on the pharmacokinetics.

3.2.1 Food intake

The oral bioavailability of lipophilic drugs often varies widely, but that of several of these drugs, such as atovaquone *(163)*, halofantrine *(164)* and piperaquine *(165, 324)* improves markedly when they are administered with a high-fat meal or drink. Food has been reported to increase the bioavailability of mefloquine in healthy volunteers *(166)*, but a fatty meal did not increase its bioavailability in patients with malaria and should therefore not affect the response to treatment. Lumefantrine is highly lipophilic, and its oral bioavailability increases substantially if it is administered after a meal rich in fat *(270)*. The most lipophilic drugs, such as lumefantrine, bind predominantly to lipoproteins in plasma *(325-326)*.

3.2.2 Age and body weight

Age and body weight were correlated with clearance in several studies, generally resulting in lower concentrations in younger than in older children or adults given the same dose in milligrams per kilogram (Table 3.2). Young children achieve substantially lower concentrations of lumefantrine, sulfadoxine, pyrimethamine and chloroquine than older patients *(275-276, 302)*. In some cases, the effect of age on drug disposition is complex: both age and body weight affect exposure to piperaquine. The concentrations at the beginning of the terminal elimination phase are generally lower in children than in adults *(290, 292)*. A low plasma concentration of piperaquine on day 7 has been associated with treatment failure in children *(293)*; however, one study of 11 children found that they had lower clearance of an oral dose than adults (normalized for body weight), resulting in higher total exposure to piperaquine *(292)*. Hatz et al. *(269)* reported that heavier patients achieved lower doses of lumefantrine after the standard dose regimen. Only the PK of quinine has been studied in obese patients: the results (normalized for ideal body weight) were similar to those for people of normal weight, suggesting that dosing of obese patients should be based on ideal and not observed body weight *(327)*. One of the primary objectives of increasing available PK data is to provide better guidance on dosing by age or weight.

3.2.3 Malnutrition

Quinine clearance was reported to be reduced in two studies of patients with kwashiorkor or severe global malnutrition, with a contraction in the volume of distribution in the latter case *(266, 328)*. Another study of quinine in patients with malnutrition, however, showed an unchanged volume of distribution and accelerated clearance *(329)*. Limited data suggest that the PK of chloroquine is unaffected in malnutrition *(330)*.

3.2.4 Pregnancy

The concentrations of artemether, chloroquine, lumefantrine, mefloquine, atovaquone, proguanil (and cycloguanil) and sulfadoxine are markedly lower in women in the second or third trimester of pregnancy *(249, 257, 271, 281, 316)* (Table 3.2). As in young children, suboptimal dosing could contribute to the poorer treatment responses seen among pregnant women.

3.2.5 Plasma protein binding

Plasma protein binding influences the apparent volume of distribution of drugs. Several antimalarial drugs (e.g. amodiaquine, mefloquine, quinine and primaquine) bind to α-1-acid glycoprotein, the level of which increases in acute infection *(212, 331)*. Thus, the total plasma concentrations of these drugs are higher in acute malaria than in convalescence. As a consequence, concentrations of quinine that would be associated with toxicity in self-poisoning (> 10 mg/l) are usual in the treatment of severe malaria, and there is no serious toxicity because the free fraction is relatively low. Some antimalarial agents have chiral centres and are given as a racemate (e.g. mefloquine); these drugs display stereoselective protein binding *(326)* and, as a consequence, stereospecific PK *(285, 332-333)*.

3.2.6 Pharmacokinetics interactions

Concomitant medication administration can influence exposure to a drug substantially. Many antimalarial agents are metabolized by cytochrome P450 isoenzymes, and concomitant intake of drugs metabolized by the same isoenzymes can lead to induction or inhibition of metabolism. Exposure to lumefantrine is increased by co-administration of inducers and inhibitors of cytochrome P450 isoenzymes, such as ketoconazole, nevirapine and lopinavir–ritonavir, but is decreased by concurrent use of mefloquine *(294, 310, 334-335)*. Exposure to quinine was decreased markedly by the cytochrome P450 enzyme inducers rifampicin, phenobarbital and phenytoin *(323, 336)*.

3.2.7 Dose-dependent pharmacokinetics

None of the antimalarial drugs shows capacity-limited elimination, but absorption can be saturated. Exposure to lumefantrine is lower when the recommended total dose is given as three daily doses rather than in the recommended six doses over 3 days *(272)*. Exposure can be increased even more by splitting the same recommended total dose over 5 days *(270, 337)*. The bioavailability of mefloquine is 20% higher when the recommended dose of 25 mg/kg is divided into two doses over 24 h (15 mg/kg and 10 mg/kg) *(284)*, and exposure was increased by an additional 40% when these two doses

were split into three doses of 8 mg/kg per day *(283)*. Auto-induction causes a marked, time-dependent increase in the metabolism of artemisinin and artemether *(204, 296, 298, 338-339)*, but a similar effect was not seen for artesunate or dihydroartemisinin *(300-301, 305)*. Increased metabolic capacity due to auto-induction has been well characterized for artemisinin, with values for the maximum observed concentration (C_{max}) and the area under the concentration–time curve (AUC) about six times higher after the first dose on day 0 than on day 5 *(340)*.

3.3 SAMPLING SCHEMES FOR PHARMACOKINETICS STUDIES

Studies of PK have usually involved intensive sampling strategies. These provide rich data, which can be evaluated by non-compartmental analysis or modelled in a standard two-stage approach. Such strategies are, however, costly and time-consuming and usually cannot be used to determine the sources of inter-patient variation in large numbers of patients. Now, small studies with intensive sampling are conducted during the early stages of drug development. For studies of many patients in the target population (known as population PK studies), investigators are using 'sparse sampling', in which PK data for all patients are analysed simultaneously by nonlinear mixed-effects modelling. This type of sampling has considerably increased the feasibility of conducting PK studies in large target population groups or in field studies. Increasingly, drug concentrations are determined at a single time as a simplified measure of exposure. Measurement of concentrations of longer-acting antimalarial drugs on day 7 following initiation of treatment should be considered a routine part of trials.

3.3.1 Intensive sampling

The PK of drugs is characterized initially in intensive sampling studies in a small number of healthy volunteers, and then in patients. Each participant provides sufficient blood samples so that the PK can be estimated for each one separately, with good precision. The individual estimates are then combined to obtain summary statistics that describe the distribution of the PK for this subset of the population. Intensive sampling studies provide guidance for sparse sampling schedules and are also used to evaluate the PK in subgroups, such as pregnant women and patients with compromised renal function, especially if the PK is expected to differ significantly from those of normal patients. Bioequivalence studies and comparisons of drug formulations or groups of patients usually involve intensive sampling in order to satisfy regulatory requirements. The precision of estimates of PK depends on both the number of people in the study and inter-individual variation. In many intensive sampling studies therefore, an attempt is made to minimize inter-individual variation by restrictive inclusion and exclusion criteria or by using a complex study design, such as cross-over studies or longitudinal studies with convalescent patients acting as self-matched controls. Variation among individuals is seldom characterized adequately in intensive sampling studies.

3.3.2 Sparse sampling

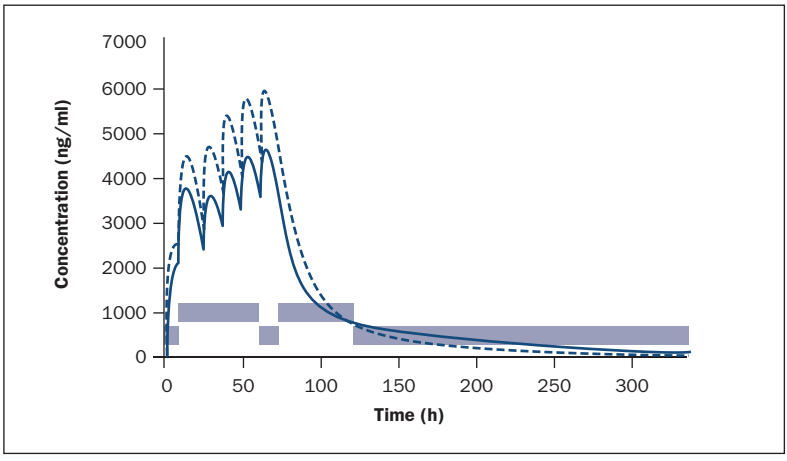
An intensive sampling strategy is often not feasible in malaria-endemic countries in which clinical trials are conducted with outpatients and only a

few follow-up visits. Intensive sampling may also not be possible for vulnerable populations, such as pregnant women, infants and young children with malaria. Under such conditions, it may still be possible to characterize drug disposition by taking fewer samples (sparse sampling) from larger populations. Sparse data on the PK of the drug in the population can be analysed by nonlinear mixed-effects modelling. The population approach is designed to cover *(341)*:

- collection of relevant information on PK in patients who are representative of the target population to be treated with the drug;
- identification of factors of demographic, pathophysiological, environmental or concomitant drug-related origin that may change the PK of a drug; and
- quantitative estimation of the magnitude of the unexplained (random) variation in the patient population, an increase in which may decrease the efficacy and safety of a drug.

Sparse blood samples can be collected in specifically designed population PK studies or as an additional component of large trials of efficacy and safety to maximize the information obtained from the study. A population PK study should be designed carefully, with adequate power, so that the PK and inter-individual variation are characterized sufficiently to be representative of the population being studied. Sparse blood samples can be collected at fixed sampling times or randomly in preselected sampling windows. The sampling window approach produces more informative data than fixed times, for a better description of the PK of the drug at a population level (Figure 3.1). Collection of samples randomly within preselected windows is the most flexible design, with the fewest possible samples while maintaining high-quality data. Optimal design methods (see section 3.4) are valuable for setting the sampling schedule in population PK studies.

Figure 3.1. Example of sampling windows selected for determining the pharmacokinetics of lumefantrine



adults in general, venous plasma *(270)*

pregnant women, capillary plasma *(271)*

The concentration–time profiles in this example are for simulated populations based on published pharmacokinetics *(270-271)*. The five sampling windows recommended are shown as blue boxes. Each sampling window should contain an equal number of samples randomly distributed over the duration of the window.

3.4 OPTIMAL DESIGN METHODS

Optimal design methods result in sampling schemes for population PK studies that are robust and efficient by taking into account the concentration–time profile of the antimalarial drug, the statistical methods to be used to analyse the data (e.g. nonlinear mixed-effects modelling), practical constraints to sampling and uncertainty in the PK values and structural PK models. Optimal design methods can also be used to evaluate particular sampling schemes, which can help researchers when sampling schemes are 'insufficient' (i.e. not all PK parameters can be estimated) or 'inefficient' (i.e. the sampling scheme provides imprecise estimates of one or more PK parameters).

Optimal design methods specify sampling times or windows at the most 'information-rich' areas of the concentration–time profile and are therefore particularly useful for studying populations for which only sparse sampling is possible. A survey of 16 malaria researchers (including clinicians, pharmacologists and health workers) showed that the number of blood samples that could be collected from children and pregnant women in the first 24 h after dosing was considered limited (one to four samples), especially for people attending outpatient clinics. Optimal design methods allow evaluation and comparison of various sampling schemes that can be used in a limited 'design space' (e.g. only three samples allowed per patient; minimum time allowed between consecutive samples, 1 h).

To determine the optimal sampling scheme, the following must be specified:

- the structural PK model (e.g. one-compartment model with first-order absorption and elimination);
- the approximate values of the PK parameters (e.g. absorption rate constant, clearance and apparent volume of distribution);
- the distribution of inter-individual variation in each PK parameter and the value of the variance of each of these distributions;
- the residual variability model and the values of the variance(s); and
- the study 'design space', determined by sampling constraints, such as the maximum number of patients who can be recruited into the study, the maximum number of samples allowed per patient, the minimum time allowed between consecutive samples from one person, constrained times for the collection of blood samples (e.g. 9:00–17:00) and cost limitations.

The next steps are to choose the statistical model that will be fitted to the data and to determine an optimal sampling design that will allow precise estimation of all the parameters (i.e. PK and inter-individual and residual variation). This is done mathematically by computing the Fisher information

matrix of the population PK model on the basis of the parameters of the structural PK model, inter-individual variation and the residual variation specified by the researcher *(342)*. The Fisher information matrix has been determined for several candidate sampling designs, and the optimum sampling design (known as the D-optimal design) chosen is that which maximizes the determinant of the Fisher information matrix. In other words, basing a design on the determinant of the Fisher information matrix will ensure the highest precision (smallest standard errors) of the parameters to be estimated.

The many open-source software packages available for evaluating sampling designs and determining optimal designs include: POPT/WinPOPT, with the MATLAB platform (http://www.winpopt.com/); PFIM, with the R platform (http://www.pfim.biostat.fr/); PopDes, with the MATLAB platform (http://www.pharmacy.manchester.ac.uk/capkr/popdes/); and PopED, with the MATLAB platform (http://poped.sourceforge.net/).

3.5 SIMPLIFIED MEASUREMENT OF EXPOSURE TO A DRUG

Exposure is usually measured on day 7 following initial treatment or another single time point depending on the drug being used. Measuring antimalarial drug concentrations should become a routine component of most therapeutic efficacy studies, although it is not always feasible to collect multiple blood samples, particularly from the most vulnerable populations. Measurement of the blood, serum or plasma concentration of slowly eliminated antimalarial drugs (i.e. terminal elimination half-life > 2 days) at a single time is simple and might be a better determinant of therapeutic response than the total AUC.

White et al. *(343)* outlined the reasons why day 7 is a particularly suitable single time for assessing exposure to an antimalarial drug:

- **Feasibility:** Prospective studies of the therapeutic efficacy of antimalarial drugs routinely include an assessment of clinical and parasitological response on days 3 and 7 *(344)*. The concentrations of most antimalarial drugs are still above the lower limit of quantification of the method on day 7.
- **Pharmacodynamics:** The drug concentration on day 7 is predictive of the outcome because it reflects the concentrations to which the small numbers of residual parasites are exposed. Therapeutic concentrations must be sustained for four 48-h asexual life cycles of *Plasmodium falciparum*, *P. vivax* and *P. ovale*. If the concentrations on day 7 of slowly eliminated drugs are at least twice the minimum parasiticidal concentration, all the infecting parasites should be eliminated.
- **Pharmacokinetics:** In current antimalarial drug regimens, the main pharmacodynamics effect initially is that of the artemisinin component. Variations in the concentrations of partner drugs resulting from differences in the rate and extent of absorption, initial distribution rates and disease-related changes in the apparent volume of distribution and elimination all have little effect initially; however, by the fourth day after the start of the 3-day regimen, only the partner drug remains. If parasites are still present at this time (as they usually are), the treatment outcome is determined by

the residual partner drug concentrations. By day 7, most patients are no longer febrile or ill, and the distribution phase is completed. Consequent exposure is thus determined only by variations in the elimination rate constant.

It is not surprising therefore that a strong correlation between day 7 concentration and AUC has been shown for most slowly eliminated antimalarial drugs *(270, 272, 276, 337)*. Relations between exposure and therapeutic response become more apparent when resistance develops or the doses are inadequate. Studies of the effect of concentrations below the therapeutic threshold on day 7 on cure rates are summarized in Table 3.3. Other studies have also found significantly higher concentrations on day 7 (or in some cases day 3) of sulfadoxine, pyrimethamine, desethylamodiaquine, lumefantrine and piperaquine in cured patients than in treatment failures (without defining a precise therapeutic threshold) *(234, 276, 345-346)*. For slowly eliminated antimalarial drugs, samples collected for assay on the day of failure and on day 28 (provided the assay is sensitive enough) provide further information to help differentiate resistance from inadequate exposure to the drug.

Table 3.3. Associations between concentrations of antimalarial drugs on day 7 and therapeutic response

Drug	Therapeutic cut-off concentration (ng/ml)	Treatment failure rate (%) below vs above therapeutic concentration cut-off	Reference
Piperaquine	> 30	36 vs 6.8 ($p<0.001$)	*(293)*
Lumefantrine	> 280/500	49 (< 280) vs 6 (> 500)	*(337)*
	> 280	49 vs 25 (p value not reported)	*(270)*
	> 600	22 vs 0 ($p<0.001$)	*(347)*
	≥ 400	5.9 vs 0 ($p=0.001$)	*(275)*
	> 280	7.7 vs 1.4 ($p=0.027$)	*(274)*
	> 175	24 vs 1.1 ($p<0.001$)	*(348)*
	> 500	25 vs 6.5 ($p<0.01$)	*(349)*
Mefloquine	> 500	28 vs 0 ($p=0.0003$)	*(350)*
Desethylamodiaquine	> 75	25 vs 0 ($p=0.096$)	*(247)*
	> 16	44 vs ~22 (p value not reported)	*(234)*

3.6 RECOMMENDATIONS FOR INTENSIVE STRATEGIES

Intensive sampling schemes for all antimalarial drugs are suggested in Table 3.4. The number of participants in a study should usually be between 10 and 20 in each group. The exact number depends on factors such as expected difference between group means or, in bioequivalence studies with cross-over designs, the coefficients of variation.

Table 3.4. Recommended minimum sampling times for intensive sampling in pharmacokinetics studies

Drug (metabolite)	Sampling time, after standard dosage regimen (time after first dose)	Samples (n)	References
Amodiaquine (desethylamodiaquine)	**Three doses (0, 24, 48 h):** h: 0, 1, 1.5, 2, 3, 6, 10, 12, 26, 50 days: 3, 5, 7, 14, 28	15	*(234, 247, 267)*
Artesunate, rectal	**Single dose:** h: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 3, 4, 6, 8	11	*(239, 241-242)*
Artemisinins, oral	**Single dose:** h: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 3, 4, 6, 8	11	*(246-247, 298, 351-352)*
Artesunate, intravenous	**Single dose:** h: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.25, 2, 4	11	*(238, 240, 353-355)*
Atovaquone and proguanil (cycloguanil)	**Three doses (0, 24, 48 h):** h: 0, 1, 1.5, 2, 3, 4, 5, 28, 54, 60, 66 days: 3, 4, 5, 7	15	*(255-257)*
Chloroquine (desethylchloroquine)	**Three doses (0, 24, 48 h):** h: 0, 0.5, 1, 1.5, 2, 4, 8, 12, 28, 60 days: 3, 4, 7, 14, 35, 63	15	*(278, 281)*
Lumefantrine	**Six doses (0, 8, 24, 36, 48, 60 h):** h: 0, 1, 2, 4, 6, 12, 16, 28, 52, 64 days: 3, 4, 7, 14	14	*(270-271)*
Mefloquine	**Single dose:** h: 0, 1, 2, 3, 4, 6, 8, 10, 16, 24 days: 5, 7, 14, 28, 42 **Split dose (0, 24 h):** h: 0, 1, 2, 3, 4, 6, 8, 32, 38, 48 days: 5, 7, 14, 28, 42 **Three doses (0, 24, 48 h):** h: 0, 1, 2, 3, 4, 6, 8, 32, 58, 96 days: 5, 7, 14, 28, 42	15 15 15	*(283-285)*
Piperaquine	**Three doses (0, 24, 48 h):** h: 0, 1, 2, 4, 6, 8, 26, 50, 56 days: 3, 4, 7, 14, 35, 63	15	*(290-292)*
Primaquine (carboxyprimaquine)	**Single dose:** h: 0, 0.5, 1, 1.5, 2, 3, 6, 8, 12, 18, 24	11	*(251-253)*
Quinine	**Single oral dose:** h: 0, 1, 2, 3, 4, 6, 10, 12, 24, 48 **Single intramuscular dose:** h: 0, 0.5, 1, 1.5, 2, 4, 6, 12, 24, 48, 72	11 11	*(259-260)*
Sulfadoxine–pyrimethamine	**Single dose:** h: 0, 0.5, 1, 1.5, 2, 4, 6, 12 days: 3, 4, 5, 7, 10, 14	14	*(276-278)*

The sampling points were selected from data presented in the references listed, to extend beyond three terminal elimination half-lives in order to facilitate model validation and precise estimation of PK parameters (assuming that the assay used is sensitive enough to measure the terminal elimination phase accurately). The sampling schedule should capture the concentration–time profile of the antimalarial drug and follow it for a minimum of three, and ideally five, elimination half-lives. It is important that the method that is used for quantification be adequately sensitive for the chosen sampling scheme.

Table 3.5 shows simulated population mean concentrations from a number of published studies. These estimates can indicate to investigators the sensitivity of the method required for a particular study. The values presented are population means and do not take into account inter-individual variation. In order to cover most of the participants in a study, it is recommended that the assay that is selected has a lower limit of quantification (LLOQ) at least 10 times lower than the population mean. For example, in a study to characterize the PK of oral artemether with the sampling scheme in Table 3.4, for a population mean concentration of oral artemether at $C_{5*t½}$ = 9.4 ng/ml and a factor for interindividual variation of 10, the LLOQ should be around 1 ng/ml to adequately capture all the data.

Table 3.5. Approximate population mean concentrations at various times after administration of antimalarial drugs

Drug (metabolite)	$C_{3*t½}$ (ng/ml)	$C_{5*t½}$ (ng/ml)	C_{Last} (ng/ml)	Reference
Artesunate, intravenous (dihydroartemisinin)	8.0–5000 (40–1100)	2.0–2900 (15–210)	0 (1.0–89)	*(238, 240, 355-356)*
Artesunate, rectal (dihydroartemisinin)	770 (290–990)	660 (240–680)	142 (160–380)	*(239, 241-242)*
Artemether, oral (dihydroartemisinin)	25 (36)	9.4 (17)	5.9 (13)	*(298)*
Artesunate, oral (dihydroartemisinin)	– (230–660)	– (110–330)	– (70–200)	*(246-247)*
Artemisinin, oral	27–31	7.1–7.8	16–43	*(352)*
Primaquine (carboxyprimaquine)	5.2–17 –	1.3–4.0 –	2.8–16 –	*(251-253)*
Proguanil (cycloguanil)	39–87 –	7.0–27 –	0–3.0 –	*(255, 257)*
Quinine	2700–7000	670–1790	1140–4590	*(259-260)*
Atovaquone	330–780	78–190	170–2200	*(256-257)*
Amodiaquine (desethylamodiaquine)	– (9.0–12)	– (1.0–3.0)	0 (2.0–12)	*(234, 247, 267)*
Lumefantrine	110–160	28–39	81–110	*(270-271)*
Pyrimethamine	6.0–31	1.0–7.0	11–76	*(276-277)*
Sulfadoxine	6.5–13	1.3–3.3	11–31	*(276-278)*
Chloroquine (desethylchloroquine)	5.0–21 –	1.0–18 –	1.0–11 –	*(278, 281)*
Mefloquine (carboxymefloquine)	51–300 –	13–68 –	25–370 –	*(283-285)*
Piperaquine	1.0–4.0	0–1.0	0–5.0	*(290-292)*

$C_{3*t½}$, estimated concentration range after three elimination half-lives from maximal concentration; $C_{5*t½}$, estimated concentration range after five elimination half-lives from maximal concentration; C_{Last}, estimated concentration at the last sampling time in the intensive sampling schedule for each antimalarial drug. The estimated concentrations were taken directly from the simulated mean concentration–time profiles in the references listed for each antimalarial drug or its metabolite.

The number of samples required and the timing of sampling were based on prior knowledge of the PK of the antimalarial agent: for example, drugs with long terminal elimination half-lives require extended sampling times, while those best described by more complicated models (two- or three-compartments) require an increased number of samples after the maximum concentration. The most influential times for measuring the main PK parameters, as assessed by Fisher information matrices, were identified and included in the sampling schedule suggested in Table 3.4. Our aim was to provide sampling schemes that should work reasonably well in most situations. Therefore, they are not necessarily 'optimal' for a specific population or to answer a specific question. An optimal sampling scheme could be constructed once the target population and study question have been defined. The number of people required will depend on the study objective. For example, for comparisons between two groups, a minimum of eight (10, 12, 16, 20) people in each group gives 80% power, with a two-sided significance level of 0.05, to detect differences between means that are equal to or more than 1.5 (1.35, 1.20, 1.05, 0.95, respectively) times the standard deviation. Studies with eight (10, 12, 16, 20) people have an 80% chance of observing (at least once) a phenomenon that occurs with an incidence of 20% (15%, 13%, 10%, 8%, respectively) in the general population. In bioequivalence studies with a cross-over design, for a coefficient of variation of 15% (20%, 25%, 30%), a sample size of 10 (16, 23, 32, respectively) patients would be required, assuming 80% power and a two-sided significance level of 0.05.

3.7 RECOMMENDATIONS FOR POPULATION PHARMACOKINETICS STRATEGIES

In order to characterize population PK properly, sampling should be done across the whole concentration–time profile of the drug. Table 3.6 lists suggested sampling windows for population PK studies for each recommended antimalarial drug. These are illustrated in Appendix 2 as sampling windows overlaid on simulated concentration–time profiles from published PK studies. Each sampling window should contain an equal number of samples randomly distributed over the duration of the window. A common sample size of 100 patients was agreed on in order to obtain data that are representative of the studied population. Each patient should give at least one sample randomly distributed within each of the three to five suggested sampling windows. This should provide high-quality data suitable for nonlinear mixed-effects modelling. It is important, however, that the assay be capable of determining low concentrations in the terminal elimination phase accurately (see Table 3.5 for guidance on recommended assay sensitivity).

Table 3.6. Recommended sampling windows for population pharmacokinetics studies of antimalarial drugs

Drug (metabolite)	Sampling windows (time after first dose)	No. of samples per patient	Reference
Amodiaquine (desethylamodiaquine)	0–8 h, 8–48 h, 48–60 h, 60–96 h, 4–28 days	5	*(234, 247, 267)*
Artesunate, rectal	0–1.5 h, 1.5–4 h, 4–10 h (three samples after first dose and two samples covering subsequent doses)	3 + (2)	*(239, 241-242)*
Artemisinins, oral	0–1.5 h, 1.5–4 h, 4–10 h (three samples after first dose and two samples covering subsequent doses)	3 + (2)	*(246-247, 298, 351-352)*
Artesunate, intravenous	0–0.25 h, 0.25–1 h, 1–4 h (three samples after first dose and two samples covering subsequent doses)	3 + (2)	*(238, 240, 353-355)*
Atovaquone and proguanil (cycloguanil)	0–8 h, 8–48 h, 48–60 h, 60–120 h, 5–14 days	5	*(255-257)*
Chloroquine (desethylchloroquine)	0–8 h, 8–48 h, 48–60 h, 60–168 h, 7–63 days	5	*(278, 281)*
Lumefantrine	0–8 h, 8–60 h, 60–72 h, 72–120 h, 5–14 days	5	*(270-271)*
Mefloquine	**Single administration:** 0–24 h, 24–168 h, 7–63 days **Split administration at 0 and 24 h:** 0–24 h, 24–48 h, 48–192 h, 8–63 days **Repeated administration at 0, 24 and 48 h:** 0–12 h, 12–48 h, 48–72 h, 72–216 h, 9–63 days	3 4 5	*(283-285)*
Piperaquine	0–6 h, 6–48 h, 48–60 h, 60–168 h, 7–63 days	5	*(290-292)*
Primaquine (carboxyprimaquine)	0–2 h, 2–6 h, 6–24 h, 24–48 h, 48–72 h **Reduced sampling for primaquine only after a single dose:** 0–2 h, 2–6 h, 6–24 h	5 3	*(251-253)*
Quinine	**Single oral dose:** 0–3 h, 3–24 h, 24–96 h **Single intramuscular dose:** 0–1 h, 1–24 h, 24–96 h	3 3	*(259-260)*
Sulfadoxine–pyrimethamine	0–4 h, 4–12 h, 12–24 h, 1–14 days, 14–42 days **Reduced sampling after a single dose:** 0–8 h, 8–36 h, 1.5–42 days	5 3	*(276-278)*

3.8 RATIONALE FOR SUGGESTED TIME WINDOWS

The suggested sampling windows for each drug were based on population concentration–time profiles simulated from published data (Appendix 2). Generally, the first sampling window was designed to cover the absorption phase of the first dose, the second to cover all subsequent doses except the last dose, the third to capture the absorption phase and the peak concentration of the last dose, the fourth to cover a potential distribution phase and the fifth to characterize the terminal elimination phase. For drugs administered as a single dose, only three windows were selected, to capture the absorption phase and peak concentration, a potential distribution phase and the terminal elimination phase of the drug.

3.9 RECOMMENDATIONS FOR SIMPLIFIED MEASUREMENT OF EXPOSURE TO ANTIMALARIAL DRUGS

This recommendation applies for monitoring the therapeutic concentrations of slowly eliminated antimalarials such as lumefantrine, mefloquine, piperaquine, chloroquine, sulfadoxine, pyrimethamine and desethylamodiaquine on day 7.

Measurement of antimalarial drug concentrations should become a routine component of most therapeutic efficacy studies. When it is not feasible to collect multiple blood samples, measurement of the blood, serum or plasma concentration of slowly eliminated antimalarial drugs on day 7 is a simple indicator of exposure and might be a better determinant of therapeutic response than the total AUC. Collection of a sample for assaying an antimalarial drug on the day of failure and on day 28 or 42 (provided the assay is sensitive enough) gives further information to help differentiate resistance from inadequate drug exposure.

3.10 RATIONALE FOR RECOMMENDED SIMPLIFIED MEASUREMENT OF DRUG EXPOSURE

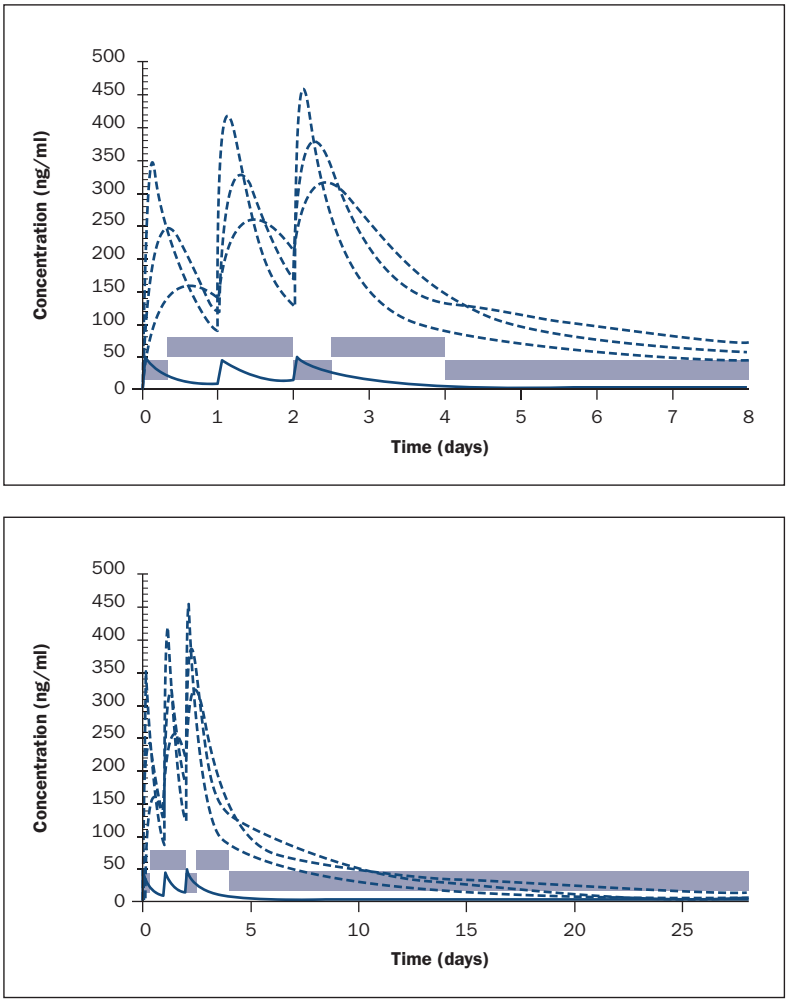
A strong correlation has been shown between the concentration on day 7 and the AUC for most slowly eliminated antimalarial drugs, including lumefantrine, mefloquine, piperaquine, sulfadoxine, pyrimethamine and desethylamodiaquine. Day 7 is a particularly suitable single time for assessing exposure, as studies of the therapeutic efficacy of antimalarial drugs routinely include assessments of clinical and parasitological response on days 3 and 7 *(344)*, when the concentrations of most of the drugs are generally still within the lower limits of quantification of assays. The drug concentration on day 7 can predict outcome because it reflects the concentrations to which the few residual parasites are exposed. By day 7, most patients are no longer febrile or ill, and the distribution phase is completed. Subsequent exposure is thus determined only by variation in the elimination rate constant. For slowly eliminated antimalarial drugs, samples collected for assay on the day of failure and on day 28 (provided the assay is sensitive enough) give further information to help differentiate resistance from inadequate drug exposure.

APPENDIX 2. SUGGESTED SAMPLING WINDOWS FOR POPULATION PHARMACOKINETICS STUDIES OF ANTIMALARIAL DRUGS

Amodiaquine and desethylamodiaquine

Suggested sampling windows for population pharmacokinetics studies: 0–8 h, 8–48 h, 48–60 h, 60–96 h, 4–28 days

Figure 3.2. Pharmacokinetics of amodiaquine and desethylamodiaquine after a standard oral treatment



Amodiaquine *(234)*

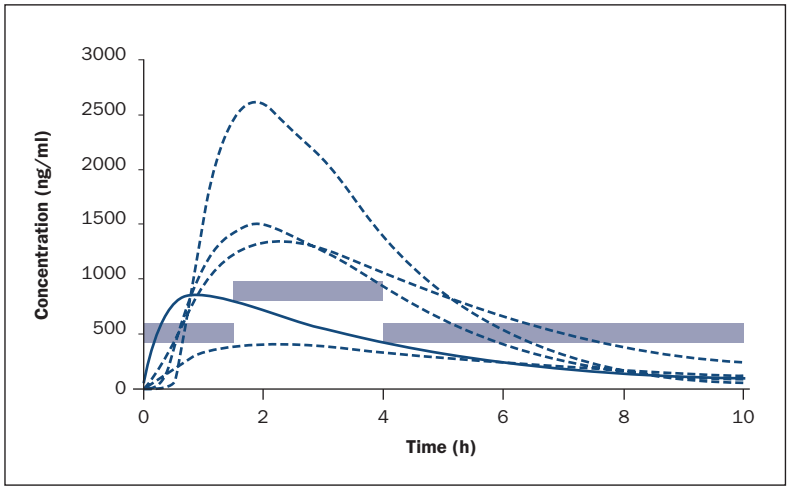
Desethylamodiaquine *(234, 247, 267)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Artesunate (rectal administration)

Suggested sampling windows for population pharmacokinetics studies:
0–1.5 h, 1.5–4 h, 4–10 h
Additional windows can be sampled to capture additional doses.

Figure 3.3. Pharmacokinetics of artesunate and dihydroartemisinin after a standard single rectal dose of artesunate



Artesunate *(241)*

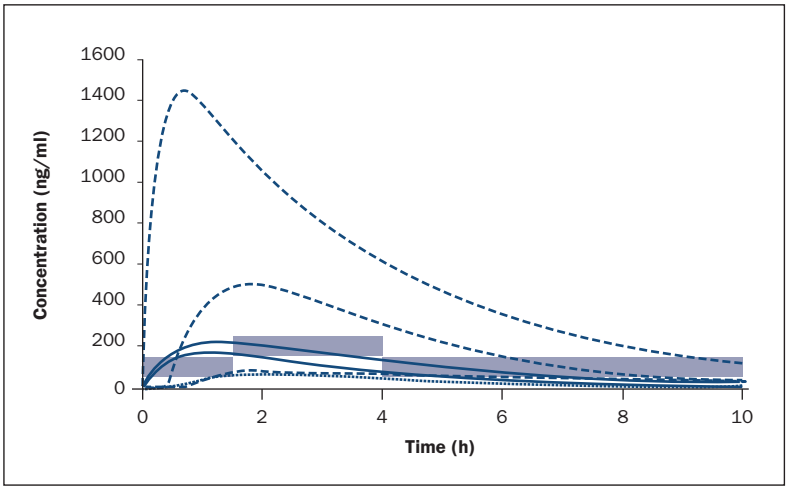
Dihydroartemisinin *(239, 241-242)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Artemisinins (oral administration)

Suggested sampling windows for population pharmacokinetics studies:
0–1.5 h, 1.5–4 h, 4–10 h
Additional windows can be sampled to capture additional doses.

Figure 3.4. Pharmacokinetics of artemisinins after a standard single oral dose



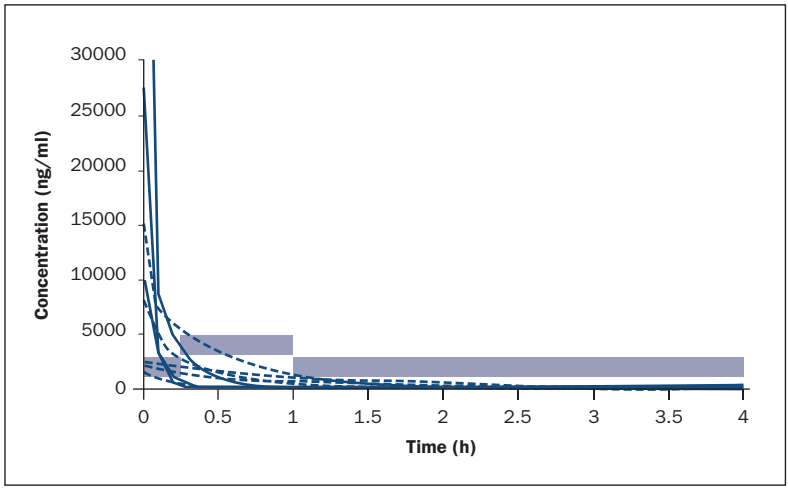
Artemisinin *(352)*
Artemether *(298)*
Dihydroartemisinin *(246-247, 298, 351)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Artesunate (intravenous administration)

Suggested sampling windows for population pharmacokinetics studies:
0–0.25 h, 0.25–1 h, 1–4 h
Additional windows can be used to capture additional doses.

Figure 3.5. Pharmacokinetics of artesunate and dihydroartemisinin after a single intravenous dose of artesunate



Artesunate *(238, 240, 353-355)*

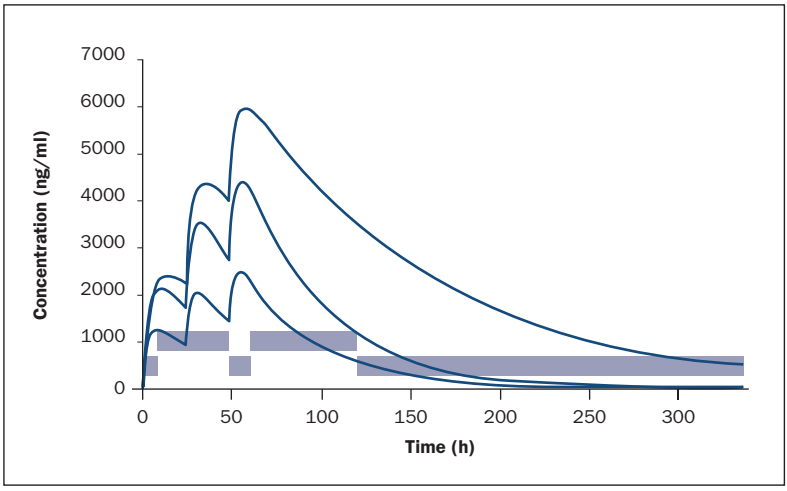
Dihydroartemisinin *(238, 240, 353-355)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Atovaquone and proguanil

Suggested sampling windows for population pharmacokinetics studies:
0–8 h, 8–48 h, 48–60 h, 60–120 h, 5–14 days (atovaquone)
0–6 h, 6–48 h, 48–56 h, 56–96 h, 3–7 days (proguanil and cycloguanil)
0–6 h, 6–48 h, 48–60 h, 60–120 h, 5–14 days (atovaquone, proguanil and cycloguanil)

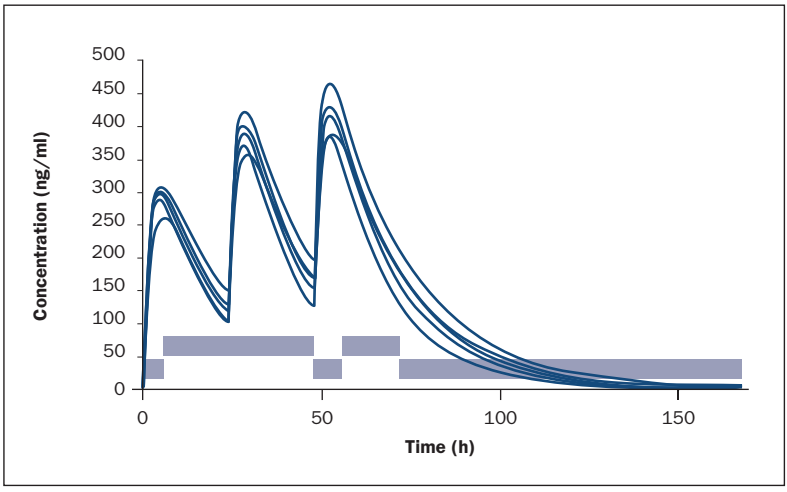
Figure 3.6. Pharmacokinetics of atovaquone after a standard oral treatment



Atovaquone *(256-257)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Figure 3.7. Pharmacokinetics of proguanil after a standard oral treatment



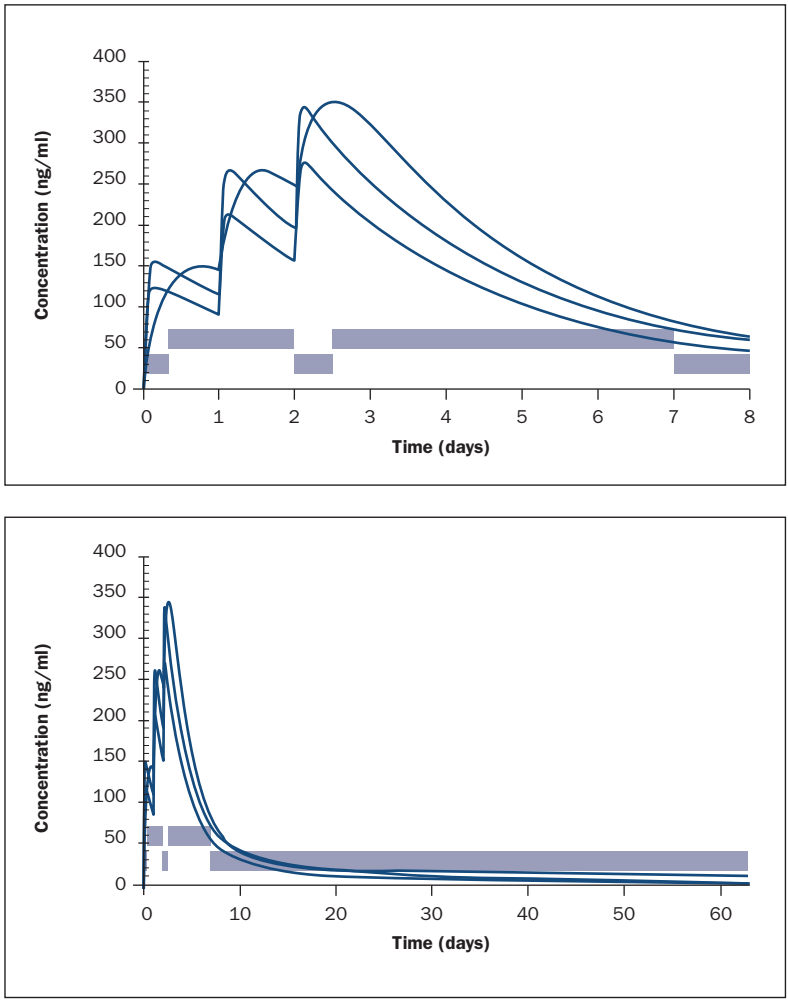
Proguanil *(255, 257)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Chloroquine and desethylchloroquine

Suggested sampling windows for population pharmacokinetics studies (for both chloroquine and desethychloroquine):
0–8 h, 8–48 h, 48–60 h, 60–168 h, 7–63 days

Figure 3.8. Pharmacokinetics of chloroquine after a standard oral treatment



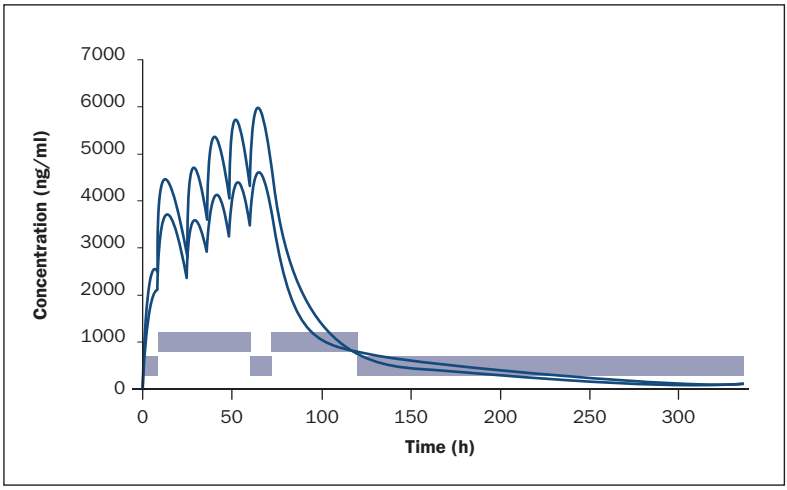
Chloroquine *(278, 281)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Lumefantrine

Suggested sampling windows for population pharmacokinetics studies:
0–8 h, 8–60 h, 60–72 h, 72–120 h, 5–14 days

Figure 3.9. Pharmacokinetics of lumefantrine and desbutyl-lumefantrine after a standard oral treatment



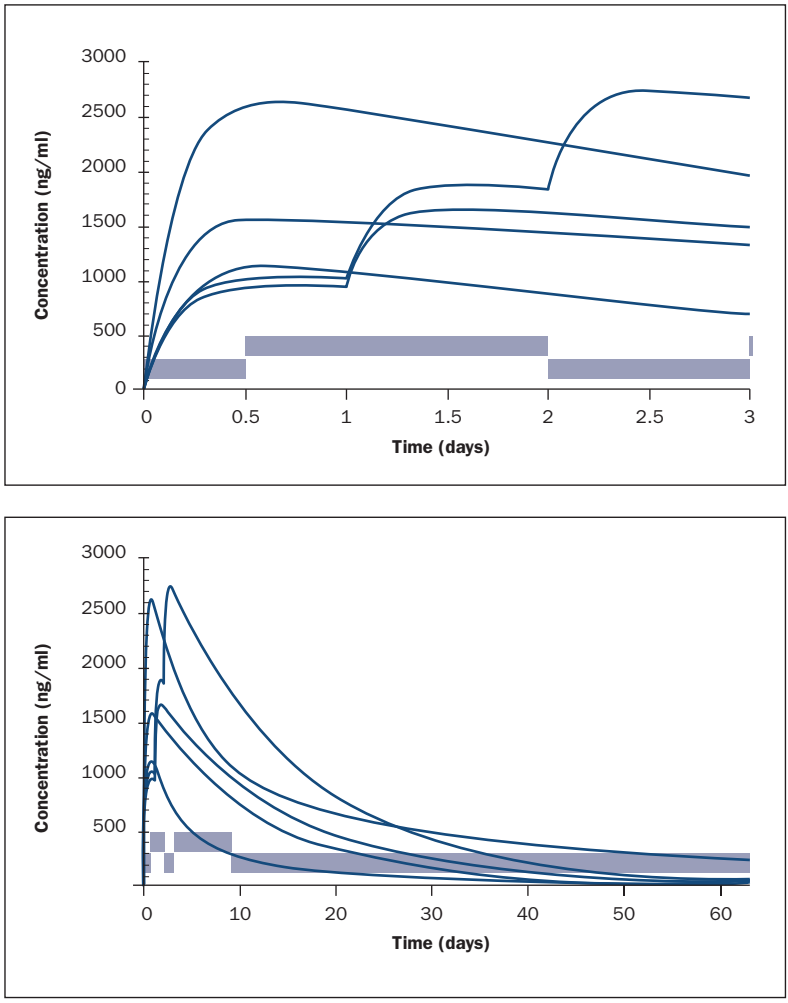
Lumefantrine *(270-271)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Mefloquine

Suggested sampling windows for population pharmacokinetics studies:
0–24 h, 24–168 h, 7–63 days (single administration of mefloquine)
0–24 h, 24–48 h, 48–192 h, 8–63 days (split administration of mefloquine at 0 and 24 h)
0–12 h, 12–48 h, 48–72 h, 72–216 h, 9–63 days (repeated administration of mefloquine at 0, 24 and 48 h)

Figure 3.10. Pharmacokinetics of mefloquine after a standard oral treatment



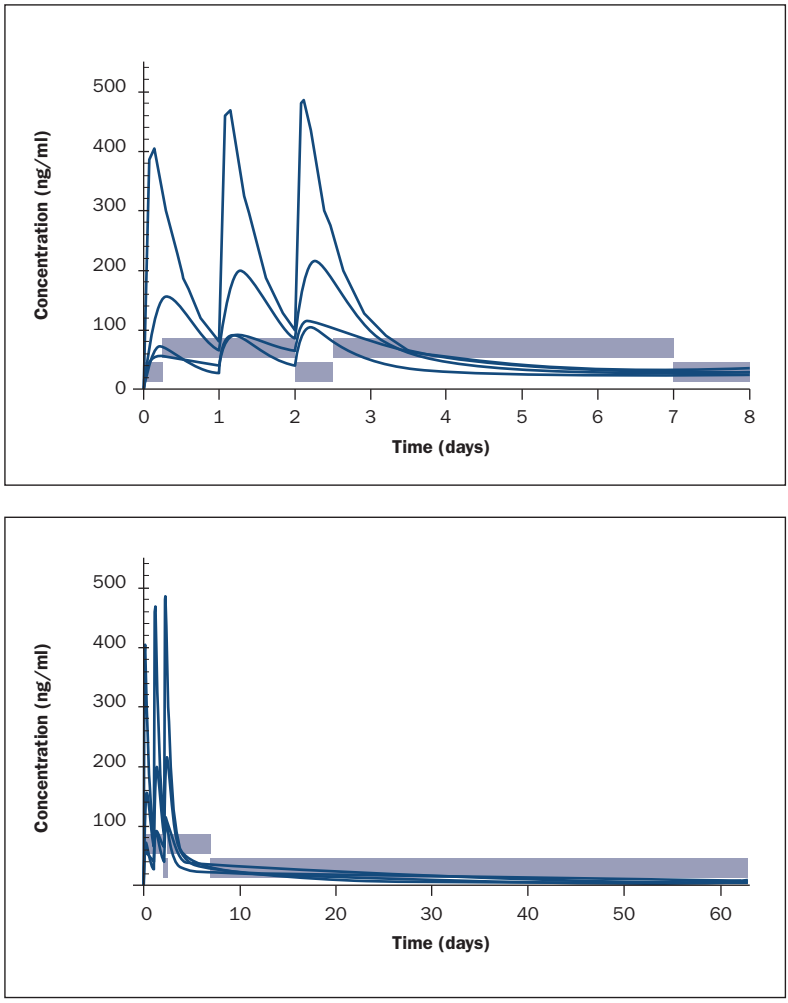
Mefloquine *(283-285)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Piperaquine

Suggested sampling windows for population pharmacokinetics studies:
0–6 h, 6–48 h, 48–60 h, 60–168 h, 7–63 days

Figure 3.11. Pharmacokinetics of piperaquine after a standard oral treatment



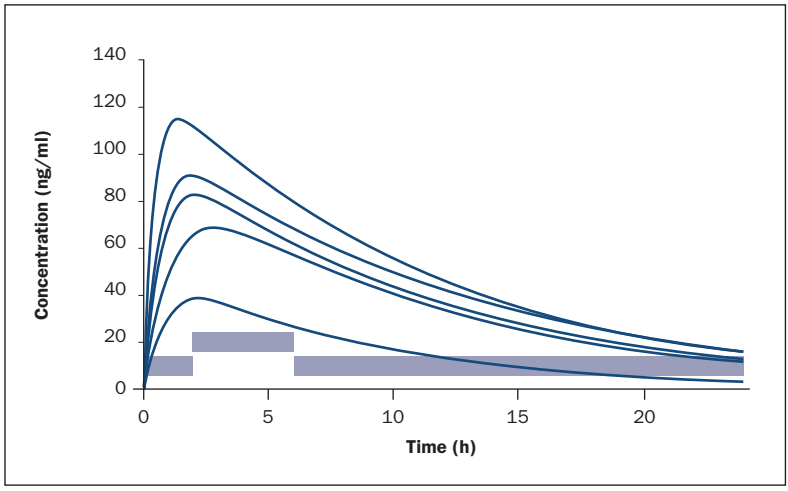
Piperaquine *(290-292)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Primaquine

Suggested sampling windows for population pharmacokinetics studies:
0–2 h, 2–6 h, 6–24 h, 24–48 h, 48–72 h
Two additional windows at 24–48 h and 48–72 h to cover the main metabolite, carboxyprimaquine

Figure 3.12. Pharmacokinetics of primaquine after a standard oral treatment



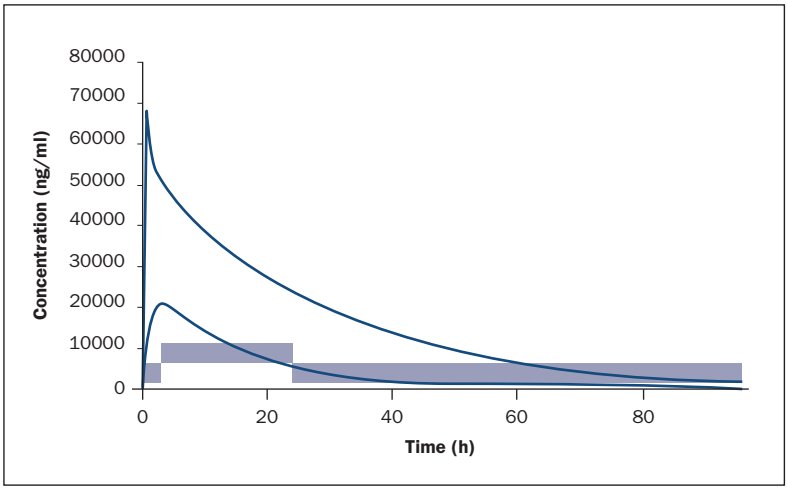
Primaquine *(251-253)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Quinine

Suggested sampling windows for population pharmacokinetics studies:
0–3 h, 3–24 h, 24–96 h (oral administration)
0–1 h, 1–24 h, 24–96 h (intramuscular administration)
Additional windows can be used to capture additional doses.

Figure 3.13. Pharmacokinetics of quinine after a single oral (8.3 mg/kg) or intramuscular (20 mg/kg) dose



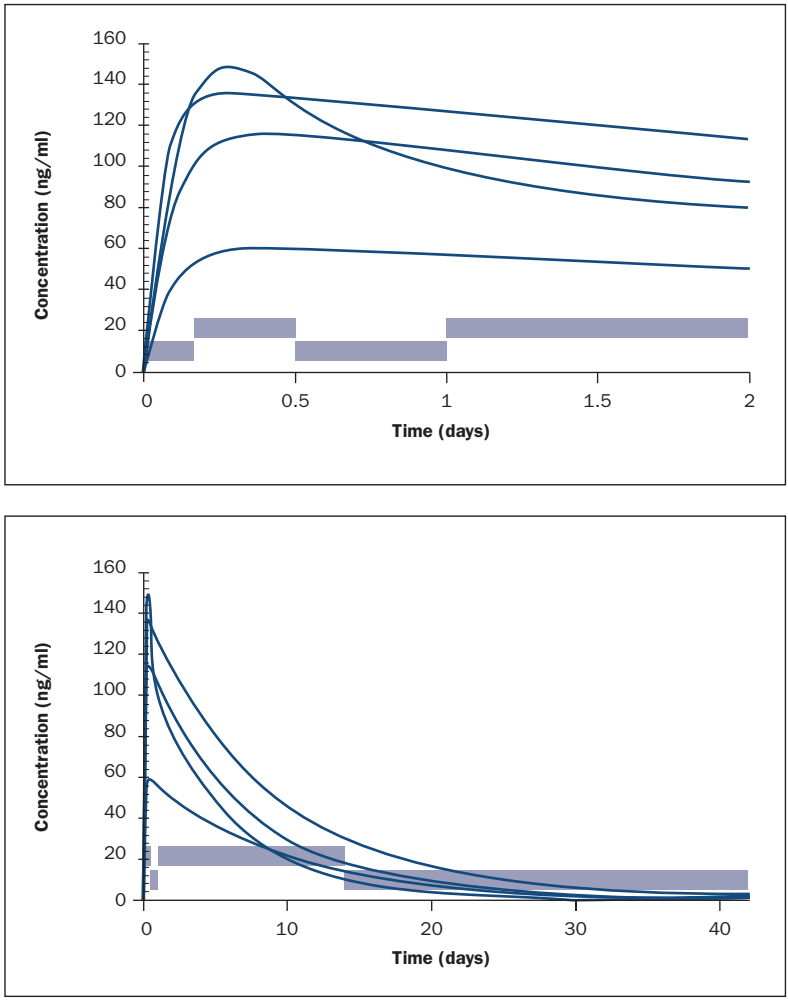
Quinine *(259-260)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Sulfadoxine–pyrimethamine

Suggested sampling windows for population pharmacokinetic studies:
0–4 h, 4–12 h, 12–24 h, 1–14 days, 14–42 days (sulfadoxine) (Figure 3.14)
0–4 h, 4–12 h, 12–24 h, 1–14 days, 14–42 days (pyrimethamine) (Figure 3.15)
Alternative reduced windows for sulfadoxine and pyrimethamine: 0–8 h, 8–36 h, 1.5–42 days

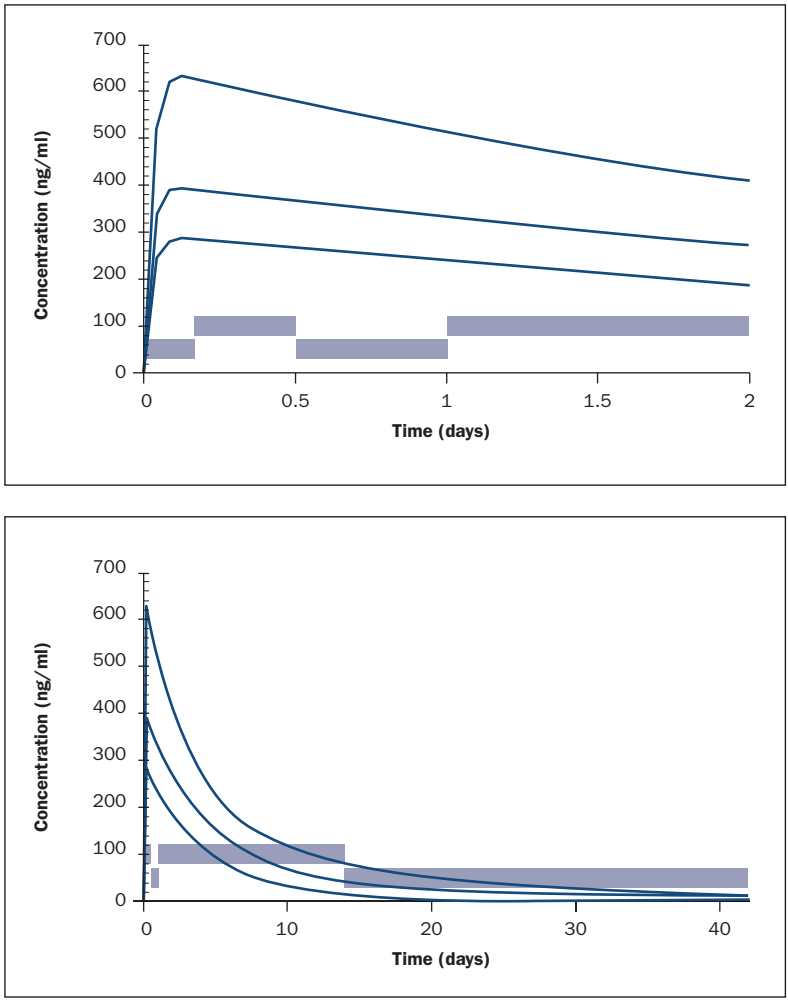
Figure 3.14. Pharmacokinetics of sulfadoxine after a single oral dose



Sulfadoxine *(276-278)*

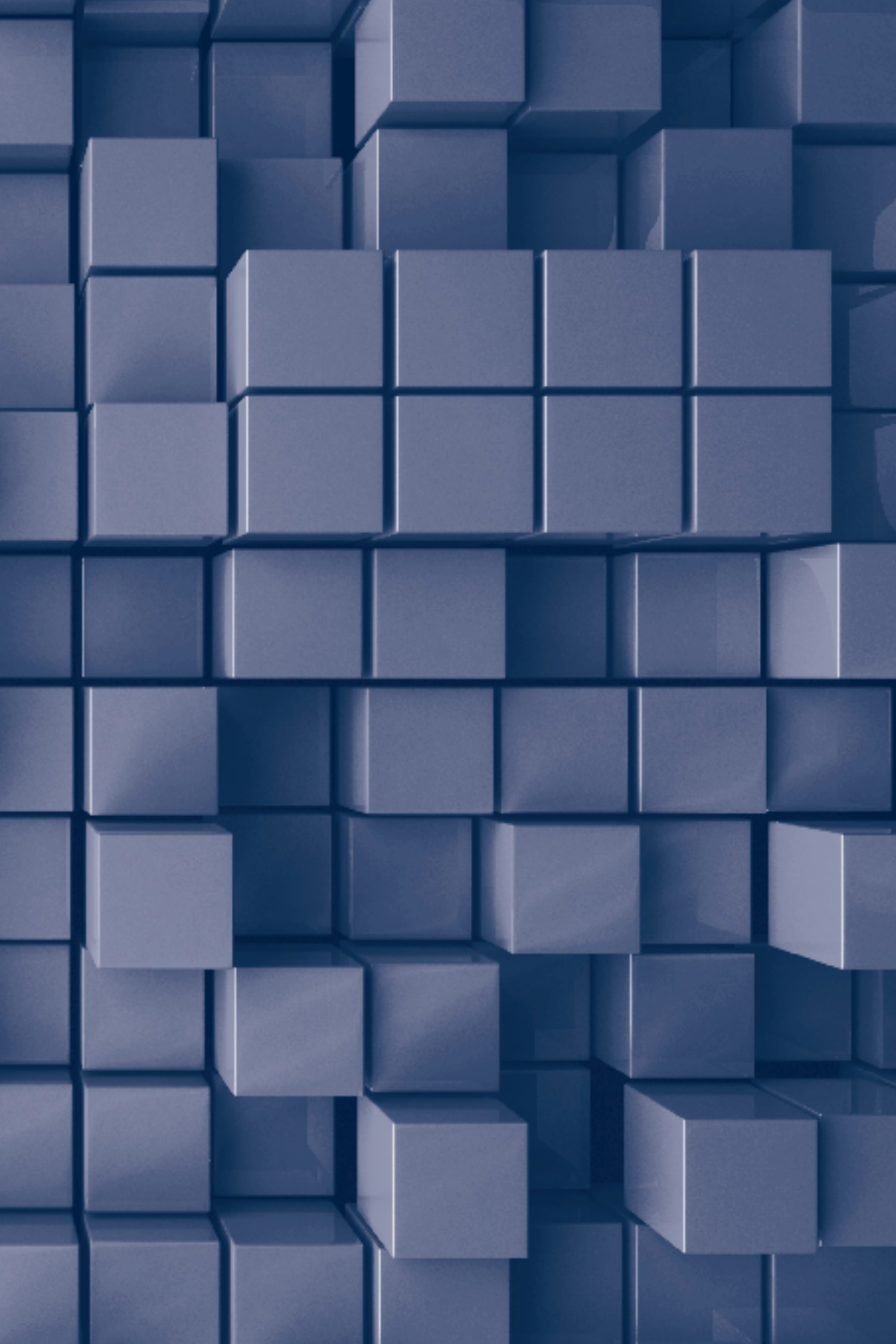
Blue blocks are suggested sampling windows for population pharmacokinetic studies.

Figure 3.15. Pharmacokinetics of pyrimethamine after a single oral dose



Pyrimethamine *(276-277)*

Blue blocks are suggested sampling windows for population pharmacokinetic studies.



Chapter 4. Validation strategies

4.1 VALIDATION ACCORDING TO INTERNATIONAL GUIDELINES

Once a bioanalytical method has been developed, it must be validated properly before it can be used in routine analysis. A rigorous validation minimizes the chance that a method will fail in routine analysis and confirms its acceptability for its intended purpose. Generally accepted recommendations for analytical method validation can be found in guidelines from the United States Food and Drug Administration *(357)* and the International Conference on Harmonisation *(358-359)*. Validation ensures that the performance of the method and guidelines include the fundamental parameters of accuracy, precision, selectivity, sensitivity, reproducibility, recovery and stability *(357)*. The aim of this chapter is to discuss various aspects of analytical validation and suggest suitable validation strategies for academic research laboratories. If analyses are conducted for product registration purposes, it is critical that the appropriate national and international regulatory guidance be followed closely and unconditionally.

Before a method is validated, the analytical system must be working correctly and the reference standard compounds should be well characterized. It is important to control the chain of 'custody' of samples during validation and for implementation of the methods used to analyse clinical samples. This can be done with a simple paper-based system, but many laboratories are moving to laboratory information management systems or combinations of paper, electronic files and bar code systems. A laboratory should document sample tracking, from receipt through analysis to disposal. Regulatory agencies often target sample handling during audits.

This chapter focuses on method validation. Table 4.1 lists the terms and definitions used by the United States Food and Drug Administration *(357)* in validating methods.

Table 4.1. Validation terms and definitions used by the United States Food and Drug Administration

Term	Definition
Accuracy	Degree of closeness of the observed value to the nominal or known true value under prescribed conditions; sometimes referred to as 'trueness', measured from the percentage nominal concentration
Analyte	The chemical moiety being measured, which can be an intact drug, a biomolecule or its derivative, a metabolite or a degradation product in a biological matrix
Analytical run (or batch)	A complete set of analytical and study samples extracted with the appropriate number of standards and quality controls for their evaluation
Biological matrix	A discrete material of biological origin that can be sampled and processed in a reproducible manner; e.g. blood, serum, plasma, urine, faeces, saliva, sputum and various discrete tissues
Blank	A sample of a biological matrix to which no analytes have been added, which is used to assess the specificity of the bioanalytical method
Calibration standard	A biological matrix to which a known amount of analyte has been added or 'spiked'; used to construct calibration curves, from which the concentrations of analytes in quality controls and in unknown study samples are determined
Carryover	Effect that a previous sample might have on subsequent sample(s), usually due to retention of extraneous compounds on the analytical column
Cross-validation	Comparison of two bioanalytical methods
Full validation	Establishment of all the validation parameters for sample analysis with the bioanalytical method for each analyte
Incurred sample re-analysis	Retesting of unknown samples to demonstrate that a bioanalytical method is reproducible from one occasion to another
Internal standard	Test compound(s) (e.g. structurally similar analogue, stable labelled compound) added to all analysed samples at a known, constant concentration to facilitate quantification of the target analyte(s)
Limit of detection	Lowest concentration of an analyte that can be reliably differentiated from background noise by the bioanalytical procedure; usually estimated as a signal-to-noise ratio of 3:1
Lower limit of quantification	Lowest amount of an analyte in a sample that can be determined quantitatively with suitable precision and accuracy; usually the concentration of the lowest calibration standard
Matrix effect	Direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample; particularly relevant to LC–MS analysis
Method	All procedures used in sample analysis
Partial validation	Modifications of validated bioanalytical methods that do not necessarily require full revalidation

Term	Definition
Precision	The closeness of agreement (degree of scatter) among a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions; described by calculating the percentage coefficient of variation
Processed	Final extract (before instrumental analysis) of a sample that has been subjected to various manipulations (e.g. extraction, dilution, concentration)
Quality control sample	Spiked sample used to monitor the performance of a bioanalytical method and to accept or reject the results for the unknown samples analysed in a particular batch
Quantification range	Range of concentrations, including the upper limit of quantification and lower limit of quantification that can be reliably and reproducibly quantified with accuracy and precision by use of a concentration–response relation
Recovery	Extraction efficiency of an analytical process, reported as a percentage of a known amount of analyte carried through the sample extraction and processing steps of the method
Reproducibility	Agreement between two laboratories; also represents the precision of the method under the same operating conditions over a short time
Robustness	Capacity of a method to remain unaffected by small, deliberate variations in method parameters
Ruggedness	According to the United States Pharmacopeia, the degree of reproducibility of results obtained under a variety of conditions, expressed as relative standard deviation
Sample	A generic term encompassing controls, blanks, unknowns and processed samples
Selectivity	Ability of the bioanalytical method to measure and differentiate analytes in the presence of the components that may be expected to be present, such as metabolites, impurities, degradation products and matrix components
Stability	Chemical stability of an analyte in a given matrix under specific conditions for a given time interval
Standard curve	Relation between experimental response value and analytical concentration (also called a ‘calibration curve’)
System suitability	Determination of instrument performance (e.g. sensitivity and chromatographic retention) by analysis of a reference sample before running the analytical batch
Unknown	The biological sample being analysed
Upper limit of quantification	Largest amount of an analyte in a sample that can be determined quantitatively with precision and accuracy

LC–MS, liquid chromatography–tandem mass spectrometry. From reference *(357)*.

Before method validation is conducted, the hardware (instrumentation) used in bioanalysis must have been verified. Installation, operational and performance qualifications should be validated:

- **Installation qualification:** Before an instrument is installed, all the information pertinent to its proper installation, operation and maintenance should be reviewed. Site requirements and the receipt of all the required components must be confirmed. The equipment is then physically installed, and all serial numbers are recorded. Documentation of the installation, including who performed it, should be archived.
- **Operational qualification:** Operational qualification ensures that the modules of the system operate according to the defined specifications for accuracy, linearity and precision. The process may be as simple as verifying the module self-diagnostic routines, or it may be more specific, including running tests to verify, for example, detector wavelength accuracy, flow rate or injector precision.
- **Performance qualification:** In this step, the performance of the system is verified under actual running conditions over the anticipated working range. In practice, however, operational and performance qualification are usually done together, particularly for tests for linearity and precision (repeatability), which can be conducted more easily at system level. For the performance qualification of HPLC, a well-characterized analyte mixture, column and mobile phase should be used.

4.2 TYPES OF METHOD VALIDATION

A new method that has been developed must be validated thoroughly before it is used for sample analysis. A full validation should be carried out, and all validation parameters should be assessed. Changes might be introduced later, such as the type of chromatography or changing from UV detection to MS detection. The altered method should be subjected to revalidation, but this time a partial validation will suffice, covering only the aspects of the assay likely to change because of the alterations made. The three basic types of validation that might be used before starting routine analysis are full validation, partial validation and cross-validation.

4.2.1 Full validation

This type of validation comprises all the aspects listed in various regulatory guidelines, such as accuracy, precision, stability, recovery, linearity, ruggedness, robustness, specificity and selectivity. Recently, other parameters have been proposed as equally important and mandatory for a full validation, including dilution integrity for analysing samples above the limit of quantification, carryover, incurred sample re-analysis and matrix effects *(360-363)*. Matrix effects are an especially important parameter in validating 'hyphenated' techniques, such as LC–MS *(357)*.

The guidelines of the International Conference on Harmonisation cover accuracy, precision, specificity, detection limit, quantification limit, linearity and range *(358-359)*. International guidelines are vague with regard to the experimental design of a validation study, and the details are largely decided

by laboratories themselves. The content of a validation report is, however, specific; regulatory agencies insist on full, complete validation of new assays. Full validation of a parameter is not needed if it has been validated previously (e.g. stability in a biological matrix when a method is changed to include another analytical technique).

4.2.2 Partial validation

Partial validation may be performed if small changes are made to a method, such as extending or reducing the calibration range, changing the detection technique, instrument or software platform, changing the species for the matrix (e.g. rat plasma to mouse plasma) or changing the matrix within a species (e.g. human plasma to human urine) *(357)*. Partial validation can consist of as little as determining the accuracy and precision of one assay or as much as a nearly full validation. As it is difficult to determine how extensive a partial validation should be, many laboratories conduct a full validation when in doubt. It can be difficult to determine whether a method should be partially revalidated. A change of anticoagulant from EDTA to heparin, for instance, would usually require some revalidation, as the two anticoagulants act in different ways. Currently, there is no consensus on whether changing the counter-ion, such as sodium to lithium, would require revalidation experiments. Some agencies issue noncompliance letters and request validation when a counter-ion is changed *(364)*.

4.2.3 Cross-validation

Cross-validation is suitable if a validated method is transferred from one laboratory to another, when two or more bioanalytical methods are used to generate data in the same study or different studies, or if a method is to be used with another instrument. Cross-validation would be conducted, for instance, when a validated bioanalytical method serves as the reference and is compared with a revised method. Comparisons should be made both ways. When samples from a single study are analysed at more than one site or in more than one laboratory, cross-validation with spiked matrix standards and patient samples should be conducted at each site or laboratory in order to establish inter-laboratory reliability. This is commonly referred to as an 'inter-laboratory comparison'. Cross-validation should also be considered when data generated with different analytical techniques (e.g. LC–MS/MS vs enzyme-linked immunosorbent assay) in different studies are included in a regulatory submission.

The extent and nature of the validation also depends on the purpose of the method. The criteria for accepting a qualitative (or semi-quantitative) method are different from those for a quantitative method *(365)*. Many studies have been published on how best to interpret the international guidelines *(360-363, 366-410)*. Most authors suggest pre-validation of a method and, if the pre-validation is successful, a full validation. The pre-validation step includes selection of a regression model for the calibration curve, establishing the limit of quantification and the limit of detection and assessing the recovery. The formal validation includes an evaluation of all the main parameters, such

as precision, accuracy, linearity and recovery, and confirms that the method performs satisfactorily. Several references provide good, practical overviews of what is required for a typical bioanalytical method validation *(360, 363, 410)*. The main validation parameters—accuracy, precision, linearity, range, selectivity, sensitivity, reproducibility, recovery and stability—are discussed briefly below.

4.3 STANDARD REFERENCE MATERIAL

A reference standard is a sample of a pure constituent of an antimalarial drug or its metabolite, which is used to prepare calibration and quality control standards. Calibration standards prepared by spiking a control matrix with a solution containing the analyte(s) of interest are used in all bioanalytical assays, regardless of the method of analysis. Hence, an adequately characterized reference standard must be available for the preparation of solutions.

A certificate of analysis or chemical data sheet for the reference standard should be requested from the manufacturer, with the following details:

- source of material: commercial, synthesized in-house or donated;
- chemical formula or chemical structure;
- batch or lot number;
- percentage purity: any adjustment calculation to be included in the preparation of standards and controls;
- expiry date;
- chemical properties, including molecular mass of the base compound, molecular mass of the salt and solubility characteristics; and
- storage requirements, including light sensitivity, temperature sensitivity and deliquescence.

The certificate of analysis for reference materials should contain information on traceability in order to satisfy regulatory requirements. Information on free base vs base–salt formulations should appear on the certificate and should be taken into account when preparing stock solutions. Records of the source and batch or lot number, expiration date, certificate of analysis when available or internally or externally generated evidence of identity and purity should be kept for each reference standard *(357)*. For an internal standard, a specific certificate is not necessary, but the absence of interference between the internal standard and the analyte should be established. The concentration of the internal standard should be shown not to interfere with the LLOQ of the analyte by more than 20% (i.e. a blank matrix plus internal standard should produce a peak lower than 0.2 × LLOQ). The standard response should not be affected by high analyte concentrations.

Suppliers of reference standards include: the British Pharmacopoeia (http://www.bpclab.co.uk), the European Pharmacopoeia (http://www.pheur.org/site/page_627.php), LGC standards (http://lgcstandards.com/home/home_en.aspx), the United States Pharmacopoeia http://www.usp.org/referenceStandards/catalog.html) and the WorldWide Antimalarial Resistance Network reference standard programme (http://www.wwarn.org/research/tools/qaqc/reference-standards).

4.4 PREPARATION OF STANDARDS AND QUALITY CONTROLS

The reference standard (e.g. 0.5–10 mg) is weighed out on a calibrated analytical balance. For weighing small amounts of material, it is important to establish that the balance is appropriate for the amount being weighed (i.e. analytical or micro-analytical balance). The amount weighed should be corrected for mass differences, depending on whether the standard is a salt, has bonded water or is a free base, taking into account its reported percentage purity. A suitable solvent is selected to dissolve the reference standard, producing a stock solution, usually at a concentration of 0.25–5 mg base/ml. Working solutions are then prepared from the stock solution. In order to verify accuracy, separate weightings of the reference standard may be used to produce calibration standards and quality controls. This is usually done to control reproducibility between weightings before analysis.

Calibration standards and quality controls are prepared by spiking aliquots of blank matrix with working solutions. The calibration curve is constructed by selecting concentrations ranging from the LLOQ to greater than the highest expected maximum drug concentration (e.g. maximum observed concentration +50%) in the biological matrix, defined as the upper limit of quantification (ULOQ). A minimum of six (ideally eight to ten) non-zero calibration points are included in the calibration curve. A double blank (containing no reference standard or internal standard) and a blank (containing the internal standard but no analyte) should be processed at the same time as the calibration curve. These blank samples are used to check each run for interference and carryover, but the results are never incorporated into the regression calculation of the calibration curve. Quality controls are prepared at low, medium and high concentrations, the low being within three times the LLOQ, the high being around 80% of the ULOQ and the medium concentration being midway between the low and high concentrations. Preferably, the concentration of the quality control should not be equivalent to a point on the calibration curve.

Calibration standards and quality controls should be prepared to mimic the clinical study samples as closely as possible. Therefore, they should contain as low a proportion of working solution as is feasible. Many laboratories set a maximum limit at 5% or less of the total volume of a standard or quality control. It is especially important to keep the amount of working solution low when the drug is dissolved in an organic solvent such as acetonitrile, methanol or ethanol. The organic solvent will precipitate a small amount of protein when added to plasma and will haemolyse RBCs in blood, which can sometimes degrade the analytes *(160)*. Approaches in which the standards and quality controls are prepared on the day of analysis by mixing equal volumes of biological matrix and working solution are strongly discouraged. Occasionally, it can be difficult to find an authentic blank matrix (i.e. analyte-free, interference-free), or it may not be ethical to obtain large quantities of blank matrix (capillary blood or cerebrospinal fluid). These situations call for use of artificially prepared solutions with approximately the same salt and protein content and pH. It is debatable whether it is better to prepare the

standards and quality controls in bulk and freeze aliquots or to prepare them fresh. Both techniques have advantages and disadvantages, but laboratories tend to choose one approach and apply it consistently.

4.5 ACCURACY AND PRECISION

Accuracy defines the difference between the measured and the true value. It is estimated by replicate analyses of samples of known concentration. Accuracy is often expressed as the percentage deviation between the mean measured value and the nominal value. It is calculated as:

$$\text{Accuracy (\%)} = [(\text{mean} - \text{nominal})/\text{nominal}] \times 100$$

The mean value should be within 15% of the nominal value, except at the LLOQ, where it should be within 20% *(357)*.

Precision describes the distribution or variation of the results produced by multiple analyses of a single homogeneous sample. The coefficient of variation of all quality control samples analysed during a study is used to evaluate the precision of the analytical method. The coefficient of variation (%) is calculated as:

$$\text{Coefficient of variation (\%)} = \text{standard deviation}/\text{mean} \times 100$$

Precision is subdivided into intra-assay precision (also called 'within-series' or 'within-day' precision) and inter-assay precision (also called 'between-series' or 'day-to-day' precision). Accuracy and intra- and inter-assay precision should be evaluated by analysing replicate quality control samples (five or more replicates) at three concentrations that cover the entire concentration range. The quality control samples may be at the same concentration as the calibration curve standards, but they must be prepared separately. It is also important to evaluate the accuracy and precision at both limits (LLOQ and ULOQ) and to evaluate dilution integrity. As sample concentrations may exceed the ULOQ, a test for sample dilution accuracy should be performed with a blank matrix during validation. A quality control sample that is usually 2–5 × the ULOQ is diluted with blank matrix to bring the concentration to within the calibration range and then analysed. Dilution integrity should be ascertained on at least one day of the validation *(360)*.

The samples used to determine precision and accuracy must be independent of the calibration curve samples; if standards from the calibration curve are used, they influence the regression and thereby affect their own accuracy. A popular approach for evaluating precision is to use experimental design and analysis of variance calculations *(360, 397-398)*, which provide estimates of within-batch, between-batch and total precision. An approach regularly used by industry is to analyse five replicates of each quality control sample for 4 days. An alternative method for calculating accuracy and precision is the 'accuracy profile' *(370, 383-385, 389-390)*, in which estimates of both accuracy and precision are used to construct the 'beta-expectation tolerance interval', which is the profile of accuracy over the entire calibration range.

4.6 LINEARITY AND RANGE

The linearity of a method is characterized by its ability to describe the relationship between response y (peak height or peak area or ratios) and concentration x. At least six calibration standards prepared in biological matrix should be used to construct the calibration curve and to evaluate linearity *(357)*. The calibration standards can be prepared individually or by serial dilution, both methods having advantages and disadvantages.

The simplest way to fit a calibration curve to the data points (x and y) is by ordinary linear regression with a least-squares calculation. This approach requires, however, that all the data have a constant absolute variance (i.e. homoscedastic data). This is rarely the case in bioanalytical assays, which usually cover a broad concentration range (typically a 100- to 1000-fold increase between the LLOQ and the ULOQ), and the absolute variance usually increases with increasing concentration (i.e. heteroscedastic data). If ordinary linear regression is applied to heteroscedastic data, accuracy and precision will be impaired at the lower end of the concentration range. A regression model more complex than ordinary linear regression should therefore be used in all bioanalytical assays. The most popular approach is weighted linear regression with $1/x$ or $1/x^2$. Data transformations such as log–log are used less frequently but can be useful, especially for a small curvature in the data *(371)*. Quadratic regression is often used when the detection techniques suffer from saturation, such as some MS assays with a large concentration range. The highest quality control should be selected close to the ULOQ when quadratic regression is used, as a given change in instrument response will have a greater effect on the back-calculated concentrations in the upper part of the curve than on those in the lower portion, because of the polynomial function of the equation. Singtoroj et al. *(371)* described an experimental approach to finding the optimal regression model for validation of bioanalytical assays.

The regression model and linearity should not be evaluated simply with an acceptance criterion for the correlation coefficient (r^2) *(407, 410)*. The correlation coefficient alone is not adequate to demonstrate linearity, as r^2 values above 0.99 can be achieved even when the data show signs of curvature *(410)*. Instead, back-calculated values and deviations from nominal values for each calibration standard should be used to confirm the linearity and calibration model. The back-calculated concentration for each standard is calculated in the same manner as for an unknown sample (i.e. using the response and the regression equation for the calibration curve), and for all the standards should be within 85–115% of their nominal value, except at the LLOQ, where it should fall within 80–120%. The final range of the method is the concentration interval for which accuracy, precision and linearity have been validated *(357, 360, 363)*. Quantifying concentrations above and below these limits is not generally accepted.

4.7 SELECTIVITY

In validation, it is important to demonstrate that the method is selective. The terms 'selectivity' and 'specificity' are not interchangeable *(369)*. A selective method can generate a pure signal for the compound of interest in the presence of possible interference, while a specific method will produce a response only for the compound of interest and nothing else. For instance, typical bioanalytical methods with LC–UV are not specific but rely on selective sample preparation and selective chromatography to attain different degrees of selectivity. Specific analytical methods are rare, but use of MS/MS detection can sometimes enhance method selectivity to such an extent that it comes close to being specific.

Even if a pure signal is generated, however, quantification may still be severely and erroneously affected by the presence of undetected co-eluting compounds. The intensity of the signal could be affected by enhancement or suppression or what are typically called 'matrix effects'. A method can be useless if the effects of potential interference are not fully evaluated. Selectivity with respect to interference from biological fluids should be evaluated by processing blank samples from six independent sources of the same matrix. Selectivity with respect to metabolites, degradation products and anticipated concomitant medication should also be tested. It is equally important to evaluate the influence of the internal standard on the analyte(s) and vice versa. A blank sample spiked with the internal standard should generate a signal less than 20% of the LLOQ for the analyte(s). This can be an issue when stable isotope-labelled internal standards or chemical analogue internal standards with lower purities are used. When the internal standard produces a signal for the analyte, the concentration of the internal standard should be carefully selected relative to the LLOQ of the assay in order to limit interference. A ULOQ sample without internal standard should generate less than a predetermined (typically less than 2–5% of the working concentration) signal for the internal standard.

4.8 SENSITIVITY

The sensitivity of the method is defined by the LLOQ and the limit of detection. The limit of detection is defined as the lowest concentration that can be distinguished from background noise, which is usually set as the concentration that produces a signal-to-noise ratio > 3:1. At the LLOQ, the relative standard deviation (i.e. precision) should be lower than 20% and the accuracy within 20% of the nominal value *(357)*. Again, the samples used to determine the accuracy and precision at the LLOQ must be independent of the calibration curve samples. As previously indicated, use of the LLOQ standards from the calibration curve will influence the regression and thereby affect their own accuracy.

4.9 RECOVERY

Recovery is an important parameter in all validation studies. Extraction recovery is calculated by comparing the peak area or height obtained with the extracted sample containing a known amount of the analyte with the peak area or height from a direct injection of a solution containing the same

amount of the analyte. Although there is usually no clear criterion for acceptance during validation, besides consistent recovery over the concentration range, recovery rates > 85% are preferred. If the recovery is low or variable, it is essential that an appropriate internal standard be included in the analysis. The recovery of a compound or internal standard must be consistent and independent of variation in the biological matrix, such as variations in protein binding *(25)*. All bioanalytical methods should include at least one internal standard and ideally one per analyte. This is the most appropriate way of detecting variations in the recovery of a compound between participants (e.g. recovery could be reduced due to increased α-1 acid glycoprotein during acute infection) and artificially prepared standards and quality controls.

4.10 STABILITY STUDIES

The stability of the analyte, as well as the internal standard, under various conditions is an important parameter in bioanalytical method validation. Analyte stability in a biological fluid is a function of the storage conditions, the chemical properties of the analyte, the matrix and the storage container. Stability should be established at all steps in the sample chain (i.e. from withdrawal of the sample until the sample has generated a response in the detector). The guidelines of the United States Food and Drug Administration *(357)* recommend that studies be designed to evaluate the stability of the analyte(s) during sample collection and handling, freeze and thaw cycles, short-term (bench top, room temperature) and long-term (frozen at the intended storage temperature) storage, in stock solution and in sample extracts for re-analysis *(357)*. Studies of the long-term and short-term stability of an analyte in biological matrix are performed to ensure that clinical samples can be stored under various conditions without compromising their integrity. The extent of stability studies included in a validation depends on the field of application of the method and the information that is already available. For example, viruses such as HIV can be inactivated by heating at 56 °C for 30 min *(411)*. If this procedure is to be applied to study samples, it is necessary to validate that the analyte of interest is stable after heating.

Workshops of the American Association of Pharmaceutical Scientists and the United States Food and Drug Administration *(372)* indicate that these assessments are best performed by preparing sets of samples at a minimum of two concentrations: 3 × the LLOQ and 0.80 × the ULOQ. The samples are prepared by adding small volumes of concentrated stock analyte solutions to a volume of blank matrix. The volume of stock solution should not be more than 5% of the volume of blank matrix. After preparation, aliquots of the stability sample pools are placed in individual tubes, similar to those used in clinical studies. A set of freshly prepared stability samples must be measured before the samples are stored under different conditions, so that the accuracy of the preparation of the stability samples can be determined. The initial analysis should take place within 24 h of stability sample preparation. The stability samples must be quantified against freshly spiked matrix standards. The mean concentrations derived from the freshly prepared stability samples should be within 5–7% of the nominal concentrations, with a precision of ≤ 15%. Failure

to obtain results within these parameters may indicate that the stability samples were prepared incorrectly, or it may indicate analyte instability. If a second batch of samples fails again to meet these criteria, methods to improve stability, such as storage at a lower temperature, should be considered.

4.10.1 Freeze-and-thaw stability

A demonstration that an analyte is stable after multiple freeze–thaw cycles is useful in case of failed analytical runs or sample concentrations reporting above the ULOQ (i.e. initial results are outside the range of the standard curve). Analyte stability should be determined after at least three freeze–thaw cycles. Replicate (minimum, three) stability samples at low and high concentrations should be stored at their intended storage temperature for 24 h and thawed unassisted at room temperature. When the samples are completely thawed, they should be refrozen for at least 12 h under the same conditions. The freeze–thaw cycle should be repeated two more times, and the samples should be analysed on the third cycle with a set of samples subjected to only one freeze–thaw cycle. To establish stability for chromatographic assays, the mean results for both sets of samples should be within 15% of the nominal concentrations, with a precision ≤ 15%. A greater difference might indicate potential freeze–thaw instability if the number of freeze–thaw cycles is increased; an alteration of storage and/or thawing conditions should be considered in such cases.

4.10.2 Short-term temperature stability

Short-term stability (also known as 'process' or 'bench-top' stability) is evaluated to confirm that the analyte does not degrade during preparation or extraction of study samples at room temperature before analysis. The time chosen for testing should be in line with the expected time that samples will be kept at room temperature *(357)*. Three aliquots at each of the low and high concentrations are removed from frozen storage and allowed to remain on the bench-top for the time for which stability is to be assessed (typically 4–48 h). At the end of this period, an additional set of stability samples is removed from the freezer. Once the second set of samples has thawed, a set of fresh matrix standards is prepared, and the two sets of stability samples are analysed against the matrix standards. Stability is indicated for chromatographic assays if the difference in the analysed results for the two sets of samples (i.e. those maintained on the bench-top for the assessment period and those extracted immediately after thawing) is < 15% and the quantified results are within 15% of the nominal values.

4.10.3 Long-term stability

Long-term storage stability is assessed in order to confirm the stability of the analyte in the test matrix over at least the length of time between sample collection and sample analysis. Long-term storage stability is assessed by repeated analyses of at least three aliquots of samples at low and high concentrations taken over the assessment time frame. It is critical that quantification of stability samples be made against freshly spiked standards. The time allowed to elapse between assessments may vary. The first few assessments

are usually made daily (as part of the short-term stability assessments); once stability has been demonstrated during the initial assessments, the time between assessments can be extended to weekly, then monthly or even less frequently. The concentrations of all the samples should be compared with the mean of back-calculated values for the standards at the appropriate concentrations from the first day (day 0) of long-term stability testing. To establish long-term stability for chromatographic assays, the samples should be within 15% of the nominal sample concentrations, with a precision of ≤ 15%.

4.10.4 Stock solution stability

Information on the stability of stock solutions serves to validate the period over which the solutions will be used. The stability of a stock solution is demonstrated by preparing a fresh solution of the reference material and comparing the absolute response of the fresh solution with that of the stored solution. The stability of stock solutions of the analyte and the internal standard should be evaluated at room temperature for at least 6 h. If the laboratory intends to store the solutions for additional testing, they should be stored at 4 °C and, for thermolabile drugs, at –80 °C for as long as they are intended to be used. The recommended acceptable difference between the absolute responses of fresh stock solutions and aged stock solutions is within 5%.

4.10.5 Post-preparative stability

The stability of processed samples, including that of samples in the autosampler and the eluate after liquid–liquid or solid-phase extraction, should be determined. The stability of the analyte should be assessed over the anticipated run time of an entire batch. The concentrations should be determined from the original calibration standard curve or re-injections of the same calibration standards.

Autosampler or post-extraction stability is assessed to determine whether processed samples can be re-injected if the initial analysis is unexpectedly interrupted, such as due to instrument failure. One method of assessing the stability of extracts for re-analysis is to prepare and extract a set of matrix standards and stability samples (a minimum of three replicates at low and high concentrations). After the initial analysis, the processed samples (matrix standards and stability samples) are left in the autosampler usually for 24–72 h. The samples are then re-analysed, and the results are calculated from both the standard curve derived from the initial analysis of the standards as well as that from the re-analysed standards. The absence of a difference in stability samples containing low and high concentrations (i.e. values within 15%) between these two sets of results indicates that the processed samples can be re-injected within the assessment period, without re-injection of matrix standards. If the difference in stability sample concentrations between the results is > 15%, the results of the initial analysis of the quality control samples, as calculated from the initial analysis of the matrix standards, should be compared with the results of the re-injected quality control samples, as calculated from the re-injected matrix standards. Agreement between these results (i.e. quality control values within 15% of nominal

concentration) based on the re-injected matrix standards indicates that an entire run sequence (matrix standards, samples and quality control samples) should be re-injected if an instrument failure occurs during a run. If neither of these conditions is met, it can be concluded that processed samples cannot be re-analysed and that samples must be re-extracted.

4.10.6 On-instrument stability

Matrix standard samples, quality control samples and study samples are generally analysed in a serial rather than a parallel manner. Thus, standards are analysed at different times from the study samples. For this reason, it is necessary to assess the stability of processed samples in the instrument over the anticipated run time of sample batches. The assessment can be done by comparing the results for quality control samples analysed at the end of the run with those analysed at the beginning. On the assumption that study samples are bracketed by quality control samples during their analysis, data are generated with each batch of samples to demonstrate on-instrument stability.

4.11 CARRYOVER

It is important to verify that a blank sample produces no signal during selectivity testing. It is equally important to validate that carryover throughout the analysis is characterized and minimized. A well-established criterion used in the pharmaceutical industry is that the carryover for the highest standard (i.e. the ULOQ) should be less than 20% of a LLOQ standard *(360, 364)*. If there is carryover in the assay, procedures should be in place to deal with the problem, such as placing blanks after samples expected to have a high concentration. Then, a sample-by-sample assessment should be made of the potential effect of carryover from the previous sample. It is debatable what contribution should be allowed from the previous sample during a sample-by-sample assessment, however, and there are no clear guidelines. Whatever strategy a laboratory chooses, it should be covered by a standard operating procedure and justified by data.

4.12 HAEMOLYSIS AND LIPAEMIA

There is general consensus that haemolysed samples should be evaluated during validation of a bioanalytical method *(364)*. The extent and exact procedure differ between laboratories, but a typical approach is to create a haemolysed sample by adding about 2% blood to plasma. When these samples are used to test for matrix effects by post-column infusion or post-extraction spike in MS, both freshly prepared samples and samples that have been deep-frozen at –80 °C for 1 or 2 days before testing should be used to make sure that the RBCs are lysed. When these samples are used to test for accuracy and precision, the drug should be added to fresh samples, which are later deep-frozen at –80 °C before testing. Hughes et al. *(412)* showed that haemolysed samples sometimes generate variable and unpredictable matrix effects in MS assays, making it impossible to quantify the analyte. Lindegardh et al. *(160)* showed that artesunate and dihydroartemisinin were severely degraded in haemolytic samples during analysis with a validated method. There is less information on the preparation of lipaemic samples and what

should be tested during validation *(364)*; however, such testing will probably be a requirement of regulatory agencies in the future.

4.13 VALIDATION ASPECTS OF MASS SPECTROMETRIC ASSAYS

Hyphenated techniques such as LC–MS seldom suffer from problems of selectivity, as they generate a signal only for the compound of interest. LC–MS/MS methods (selected reaction monitoring) tend to be more selective than single LC–MS methods (single-ion monitoring). The weakness of both LC–MS and LC–MS/MS is matrix effects. The presence of undetected, co-eluting compounds in the matrix may affect the response of the analytes, by either enhancing or suppressing the signal. Both scenarios lead to incorrect quantification unless an internal standard is used that is affected in exactly the same way as the analytes (i.e. the drug–internal standard response is unchanged).

The commonest interfaces, electrospray ionization and atmospheric pressure chemical ionization, are both susceptible to matrix effects, and evidence indicates that the ionization effects depend on the system *(413)*. Ionization suppression or enhancement can theoretically occur in either the solution or the gas phase. The most likely explanation for suppression in electrospray is a change in the properties of the spray droplet solution *(414)*. Co-eluting compounds change the efficiency of droplet formation or droplet evaporation or compete for charges, resulting in a change in the number of charged analyte ions in the gas phase that ultimately reaches the detector.

A method is more susceptible to matrix effects when the sample preparation is non-selective (e.g. protein precipitation) and chromatography is poor (e.g. ballistic gradients). Phospholipids are a well-known cause of matrix effects. Little et al. *(3)* and Xia et al. *(415)* demonstrated two efficient strategies for monitoring the presence of phospholipids during method development.

In many current LC–MS and LC–MS/MS assays, stable isotope-labelled internal standards are used in order to circumvent these problems. These standards are designed to behave in exactly the same way as the analyte (i.e. co-elute) but can be separated within the MS by their heavier isotope labels. Typical labels are H_2 (deuterium), N_{15} and C_{13}; for most small drug molecules, the internal standard is ideally 4 mass units heavier than the drug. A stable isotope-labelled internal standard with less mass difference usually suffers from an isotope contamination effect when the concentration of the analyte is high, thus restricting the calibration range. What this means in practice is that a high analyte concentration will produce a signal (100%) for the most abundant isotope (e.g. M = 300 *m/z*) but will also produce a signal for M + 1 = 301 *m/z*, M + 2 = 302 *m/z* and sometimes even for M + 3 = 303 *m/z*. The signal for these traces will depend on the atomic composition of the compound. In theory, a stable isotope-labelled internal standard will co-elute with the unlabelled analyte and thus compensate for changes in ionization efficiency; however, it is well known that deuterated stable isotope-labelled standards are often partly separated due to a small change in lipophilicity

when exchanging hydrogen for deuterium. The effect depends on the number of labels, the size of the molecule, the efficiency of the column, the retention mechanism and the retention time of the compounds. Wang et al. *(416)* and Lindegardh et al. *(129)* recently showed that even stable isotope-labelled internal standards occasionally fail to compensate for matrix effects. This happens when the analyte co-elutes in a region with a steep gradient of severe ion suppression. Van Eeckhaut et al. *(375)* thoroughly reviewed matrix effects in LC–MS-based methods. The two most popular methods for assessing matrix effects are post-column infusion and spiking blank matrix post-extraction *(417-418)*.

4.14 INCURRED SAMPLE RE-ANALYSIS

Validation of a bioanalytical assay requires the preparation of quality control samples by spiking the matrix (e.g. plasma) with a working solution. As a result, quality control samples do not necessarily mimic study samples drawn from people treated with the drug ('incurred samples'). Audits by the United States Food and Drug Administration during the past few years have shown that incurred sample re-analysis (ISR) sometimes yields dramatically different results, even when a validated assay is used. In order to identify these cases, a limited number of incurred samples should be re-analysed systematically as part of assay validation *(360, 362-363)*. Incurred samples are re-analysed not only to confirm the reproducibility of the method but also to identify issues in the stability of matrix standards.

At a minimum, ISR should be conducted for the first study of human samples, either in a new matrix or when studying new groups of patients (e.g. with hepatic failure or renal impairment), or for drug–drug interaction studies. In addition, ISR should always be performed when conducting bioequivalence studies *(362-363)*. In fact, ISR should be conducted for a small subset of samples as part of the routine analysis of all studies. If the ISR results suggest that the method is reproducible, the initial result should be reported as the 'final' value. If the method is found to be reproducible but extreme results are observed in a few samples, the samples should be investigated to determine whether they have anything in common (e.g. all from the same patient, all peak concentrations, haemolysis, day 0 vs last day of blood collection) and to establish the cause of the problem. Failed ISR must lead to an investigation, and the bioanalytical portion of the study should be temporarily halted until the investigation is completed and follow-up action (a 'corrective action request') is taken. It is unclear what follow-up action is required if most of the re-analyses agree with the original result while others differ substantially. The laboratory should design a standard operating procedure for ISR, and ISR should be performed on individual samples, not pooled samples (unless the sample volume is insufficient).

For ISR, it is better to select a few samples from each person in a large population rather than to determine an entire PK profile for two or three individuals. The samples should include those close to the maximum concentration and near the end of the terminal elimination phase. ISR should be conducted as

early as possible in a study, with the same number of replicates of each sample as were used to run the study. There is no clear consensus on the selection of samples for ISR, but 20–50 samples or 10% of the total number of samples per study appears to be in accordance with current recommendations *(362, 419-420)*. Acceptance criteria have not been established by international regulatory agencies, but the most widely used criterion is that either two thirds of all replicates should be within 20% of the mean of the values, or two thirds of all replicates should be within 20% of the original values. The first approach allows sample pairs to differ by 30%, while the second requires a difference of < 20% between sample pairs. Other approaches for setting acceptance criteria may be equally acceptable but should be set a priori. Samples with an original result of ≤ 3 × the LLOQ might be excluded from the incurred sample evaluations. ISR should be repeated after any major change in a method. These acceptance criteria hold for most assays based on quality control acceptance criteria of ≤ 15% from nominal values.

4.15 DRIED BLOOD SPOTS

The collection of blood samples on paper, known as DBS, is an established technique for screening for inherent metabolism disorders. Its application for therapeutic drug monitoring and PK studies of antimalarial drugs has been widely reported *(12-13, 39, 56, 63, 66, 78, 96, 112, 130, 179, 214, 216, 228, 421)*. Validation of DBS methods, however, requires more thought. Validating DBS methods to current regulatory standards to make sure that they deliver the same quality of data as methods with the usual blood and venous matrices is a challenge. Collection of blood samples as DBS for PK studies offers a number of advantages over conventional plasma sampling. The blood volume required for DBS samples is small (≤ 100 µl), while > 0.5 ml of blood is usually taken for conventional plasma analyses, making DBS a particularly suitable approach for collecting blood samples from children. In addition, it offers the advantage of less invasive sampling (i.e. finger- or heel-prick rather than the conventional venous cannula), facilitating recruitment for clinical studies. The simpler matrix preparation, ease of transfer (no refrigerated centrifugation to produce plasma) and ease of storage and shipment to analytical laboratories (no requirement for freezers and dry ice) are further benefits. Transport and storage of samples are further simplified by the antimicrobial properties of DBS samples, removing the requirement for special biohazard arrangements.

The many logistical and practical advantages of DBS collection over conventional venous sampling are potentially offset by errors related to capillary sampling, volume, drying and the filter paper used. The quality of the DBS and the sampling paper are important factors, which must be thoroughly assessed. For each application, the benefits of DBS methods must be weighed against potential errors in sample collection (e.g. expression of interstitial tissue fluid), sample processing (e.g. homogeneity of the DBS) and drug distribution (e.g. some drugs have complicated venous–capillary and blood–plasma concentration relations). Improved standardization, quality assurance, basic research and assay development are required before the DBS

technique can replace traditional venous sampling without excessive sacrifice of accuracy and precision.

4.16 RECOMMENDATIONS

It is recommended that all analytical assays be completely validated. Although this may not seem appropriate for small research studies, the data they produce may not be comparable with those of studies conducted elsewhere if the assay is not properly tested at all levels. Each subsection (e.g. accuracy and precision) should clearly indicate whether the validation met the a priori established criteria. Table 4.2 outlines the minimum testing recommendations for full validation of a bioanalytical method.

Table 4.2. Minimum experimental recommendations for full validation of an analytical method

Method characteristic	Samples required	Acceptance parameter	Acceptance criteria
Linearity	Six non-zero calibration standards, blank and a double blank sample; at least one per analytical batch	Linearity	Simplest regression that describes the relationship accurately
		Accuracy	85–115% nominal concentration
		Precision	Within 15% CV
Sensitivity (LLOQ)	Five samples spiked to the LLOQ	Accuracy	80–120% nominal concentration
		Precision	Within 20% CV
Sensitivity (LOD)	An estimate of the concentration of the analyte that generates a signal:noise ratio of 3:1	–	Signal:noise ≥ 3:1
Accuracy and precision (within-batch)	Five samples at each quality control level (low, medium and high)	Accuracy	85–115% nominal concentration
		Precision	Within 15% CV
Accuracy and precision (between-batch)	Five samples at each quality control level (low, medium and high)	Accuracy	85–115% nominal concentration
		Precision	Within 15% CV
Recovery	Three replicates at each quality control level, compared with 'post-extraction spiked' samples (see 'Matrix effects' in section 4.13)	Recovery	Consistent at each concentration
Freeze–thaw stability	Three replicates over three cycles at low and high quality control levels	Stability	85–115% nominal concentration
Short-term stability	Three replicates kept at ambient temperature for at least 4 h at low and high quality control levels	Stability	85–115% nominal concentration
Long-term stability	Three replicates kept over the total sample storage period at low and high quality control levels	Stability	To support sample storage before analysis
Stock solution stability	Replicate assessment of stock standard solution kept at ambient temperature for at least 6 h against fresh samples	Stability	85–115% nominal concentration
Post-preparative (extracted sample or autosampler) stability	Replicate injections of a homogeneous sample over an extended period (length of a typical analytical batch); may be used to validate re-injection	Stability	85–115% nominal concentration

Method characteristic	Samples required	Acceptance parameter	Acceptance criteria
Matrix effect	Post-column infusion, checking for apparent ion suppression or enhancement	Selectivity	No apparent effect at the retention time of the analyte and incurred sample
	Replicate analysis of low and high quality control samples spiked into extracted blank matrix samples of different sources *(417)*, compared with standards in mobile phase	Selectivity	Within 15% CV
Analyte–internal standard interference	ULOQ sample without internal standard	Selectivity	< 10% average internal standard peak area
Internal standard–analyte interference	Blank spiked with internal standard (blank)	Selectivity	< 20% analyte LLOQ
Blank response	Blank sample without internal standard (double blank)	Selectivity	< 20% analyte LLOQ
Carryover	Blanks and double blanks placed after the highest calibration standards in each run	Carryover	< 20% of the LLOQ
Incurred sample re-analysis	Suggested 10% of total number of samples from different patients, clustered around C_{max} and the terminal phase	Reproducibility	Preferably, two thirds of repeats should be within 20% of the original value *(362)*
Robustness	Various other factors; may include anticoagulant used, haemolysis and lipaemia	–	Assessed on a case-by-case basis
Ruggedness	How well the assay withstands typical laboratory variation, such as analysts, instruments, reagents	–	Assessed on a case-by-case basis

CV, coefficient of variation; LLOQ, lowest limit of quantification; LOD, lowest limit of detection; ULOQ, upper limit of quantification. From reference *(357)*.

General and specific standard operating procedures and good record-keeping are an essential part of a validated analytical method. It is recommended that the following standard operating procedures be available in the laboratory:

- acceptance and rejection criteria for calibration standards;
- acceptance and rejection criteria for calibration curves;
- acceptance and rejection criteria for quality control samples and assay runs;
- acceptance criteria for reported values when unknown samples are assayed in duplicate;
- sample code designations, including clinical or preclinical and bioassay sample codes;
- assignment of clinical or preclinical samples to assay batches;
- sample collection, processing and storage;
- re-analysis of samples; and
- reintegration of samples.

The data generated during development of a bioanalytical method and details of preparation of the quality control and calibrator should be documented and available for data audit and inspection. Before a validation study is initiated, a well-planned protocol should be written and reviewed for scientific soundness and completeness by qualified people. The protocol should describe the procedure in detail and should include predefined acceptance criteria and statistical methods. After approval in an appropriate, independent quality control review, the protocol should be implemented in a timely manner.

After implementation of the validation protocol, all the data must be analysed and the results, conclusions and deviations presented in an official validation summary report. Provided the predefined acceptance criteria are met and the deviations (if any) do not affect the scientific interpretation of the data, the method can be considered valid.

The recommended minimum content of a validation report comprises sections on: analyte identification; method description; preparation of stock solutions; preparation of calibration standards; preparation of quality control standards; determination of within-batch accuracy and precision; determination of between-batch accuracy and precision; summary of combined quality control results for the validation batches; stability assessments of the stock solutions, after short-term storage, after freezing and thawing in biological matrix, on the bench-top and on-instrument after preparation; specificity; sensitivity; recovery; testing for matrix effects; and a discussion, including a statement about the success of the validation.

Each subsection (e.g. accuracy and precision) should clearly indicate whether the validation met the criteria established a priori.

4.17 RATIONALE

The minimum requirements for validation of an assay depend on the situation. Is the laboratory obliged to adhere to good laboratory practice? Is the laboratory accredited, and is it obliged to adhere to specific guidelines? Is the laboratory based in an unregulated environment and simply trying to implement as many safeguards as possible to ensure the quality and accuracy of data on the PK of a drug analysis? In the first two cases, the laboratory is obliged to adhere fully to the requirements stipulated by a regulatory agency. In the last case, it can be difficult to decide which guidelines to fulfil and how to do it.

For all bioanalytical methods, the key is to evaluate accuracy and precision thoroughly. This can be done by testing several replicates throughout the calibration range over several days. It is also important to evaluate the LLOQ properly. This is the part of the calibration range that differs the most in inter-laboratory comparisons and which strongly affects measurements of PK. Any laboratory in which MS techniques are used must include a proper assessment of matrix effects, the factor that can render a MS method useless. Detailed, clear, systematic documentation of all procedures and experiments is critical. The laboratory should be able to refer to the documents subsequently and show in detail what was done and by whom. The feasibility of comparing data between studies and worldwide is largely determined by whether the data were derived with a properly validated assay.

The main purpose of method validation is to raise confidence in the results generated by the method. At a minimum, the appropriate regulatory guidelines must be followed.

Chapter 5. Implementation of methods for the analysis of clinical samples

Implementation of a method for analysing clinical samples involves more than just using a validated method. The purpose of this chapter is to explain the importance of continuous 'revalidation' or monitoring of the method during routine sample analysis, ensuring that the performance characteristics established during validation are maintained during routine use. The guidelines of both the United States Food and Drug Administration *(357)* and the International Conference on Harmonisation *(358-359)* for assay validation include a requirement for implementation of validated methods.

5.1 ACCEPTANCE CRITERIA IN ROUTINE ANALYSIS

Calibration standards are processed with each analytical batch in order to quantify the concentrations of analyte in the unknown samples in that batch. Calibration curves can be placed anywhere in the batch, with single or duplicate standards at each concentration. Quality control samples are designed to monitor the analytical run and are made up according to the rules and guidelines discussed in Chapter 4. Quality controls should be spaced appropriately throughout the run in order to identify the effect of assay drift over the course of the run. All bioanalytical methods should incorporate at least one internal standard—ideally one internal standard per analyte or analyte class. For instance, an amine analyte could be metabolized to a carboxylic acid; as these two chemical forms have different physicochemical properties, there be an internal standard for each. An internal standard is the only means of detecting method variations for individual samples or subject series. Table 5.1 gives an example of analytical batch acceptance criteria for < 100 unknown samples.

Table 5.1. Analytical batch acceptance criteria, with the minimum eight-point calibration curve and a batch of < 100 samples as an example

Sample	Accuracy	Precision	N	Inclusion criteria
Double blank	Signal < 20% LLOQ	–	1	Must be accepted
Blank with internal standard	Signal < 20% LLOQ	–	1	Must be accepted
Calibration standard (LLOQ)	80–120%	< 20%	Single or duplicate	Must be accepted
Calibration standards	85–115%	< 15%	Single or duplicate	4/6 minimum
Calibration standard (ULOQ)	85–115%	< 15%	Single or duplicate	Must be accepted
Quality controls (low, medium and high)	85–115%	< 15%	6 (minimum)	4/6 with no duplicate samples at any of the 3 levels outside the acceptance criteria
Internal standard	Fluctuations > 30% of the mean should be investigated, as they may indicate matrix effects or a problem with the batch			

LLOQ, lowest limit of quantification; ULOQ, upper limit of quantification.

5.1.1 Calibration curves

Individual point values calculated from the regression curve should not deviate from the nominal value by more than 15%, with the exception of the LLOQ standard, for which a deviation of 20% is acceptable. At least two thirds of all non-zero standards on a calibration curve should meet the above requirements. The outlying points can be omitted from the regression calculation of the calibration curve; however, omitting the lowest or highest point will reduce the calibration range. If less than 75% of the points (or less than six of eight) on the regression curve meet the acceptance criteria, the calibration curve is not acceptable and the batch should be rejected. It is recommended that at least eight to ten standards (minimum, six) be used for the calibration curve in order to minimize the number of failed batches. No extrapolation from the calibration curve is allowed; therefore, the range of the calibration curve will be truncated if either the LLOQ or the ULOQ is eliminated. Use of duplicate samples at the lower and upper limits of quantification will minimize the risk of having to reduce the calibration range. Failed batches can be re-injected, provided that this is done for a scientific reason (e.g. instrument failure during the run). Re-injection of a batch is acceptable, provided that the stability of the samples is demonstrated. Should the re-injection not return an acceptable result, the batch must be re-analysed.

5.1.2 Quality controls

For analytical batches containing fewer than 100 unknown samples, quality controls should be prepared and analysed in duplicate at each level (low, medium and high). For analytical batches containing > 100 unknown samples, a minimum of 5% of the batch should be quality control samples. It is not acceptable to re-inject quality controls during analytical batch testing instead of performing multiple extractions. The purpose of quality control is to monitor all the analytical processes, including the extraction process.

Regulatory requirements stipulate that 'quality controls are placed throughout the run in order to be able to detect assay drift' *(357)*. Variation in the quality controls might indicate a problem with the assay during the run.

The quality controls should be within 85–115% of their nominal concentration. If, during testing of quality controls in duplicate, more than two values for samples at different concentrations (low, medium or high) fall outside the above acceptance criteria, the batch is unacceptable and must be re-run. Furthermore, if both quality control results for a single concentration are outside the acceptance criteria, the batch must be re-run.

5.1.3 Unknown samples

Chromatograms are reviewed for retention time, peak shape, the presence of interfering peaks, baseline stability and a lower-than-expected internal standard area ('short draw'). If any of these chromatographic parameters appears to have an adverse affect on the sample chromatogram, the result should be rejected and the sample re-analysed.

Certain instrument software allows for reintegration of peaks. Chromatograms can be reintegrated at the discretion of the analyst, but the audit trail must be preserved. Modification of peak integrations should be avoided whenever possible. It is better to optimize automated integration rather than perform repeated reintegrations. An authorized individual should perform a reintegration on the basis of clear guidelines or documented procedures, and there should be a clear reason for a reintegration. The original records should never be overwritten. The laboratory must have a specific standard operating procedure in place stating how reintegration is performed and who is authorized to perform it.

No concentration below the LLOQ should be reported. Rather than reporting a value, the concentration is reported as 'below the limit of quantification'. The actual cut-off reporting limit (e.g. 80% of the LLOQ or the LLOQ) can be determined by the laboratory, but extrapolating beyond the LLOQ can have consequences for PK, depending on the sensitivity of the assay, the error associated with the measurement and how many consecutive values have been reported as below the limit of quantification.

Clinical samples that show a value greater than the highest point on the calibration curve (the ULOQ) should be diluted with blank matrix and

re-analysed. Concentrations above the ULOQ should be reported only if there are logistical reasons (e.g. limited sample volume) for not conducting a re-analysis with a dilution and scientific evidence to support the decision (e.g. re-analysis of diluted samples in previous studies gave equivalent concentrations).

Depending on the method used, the response of the internal standard in each batch should be within 70–130% of the mean for that batch. Obvious spikes and dips and large fluctuations should be investigated, as they may indicate problems with the method. Owing to the complexity of incurred sample matrices, variable internal standard responses are quite common when a validated method is used to analyse incurred samples. To maintain the integrity of a study, it is therefore important to monitor variations in the response of the internal standard during bioanalysis and to rapidly identify the causes of any variations observed.

5.1.4 System check samples

The use of 'system check' or 'system suitability' samples to monitor an analytical batch is becoming commonplace *(422)*. These samples are used to assess instrument stability during the analysis of a batch of samples. System check samples are different from quality controls as they are not linked to the extraction process and are not quantified. These samples are used specifically to monitor the stability of the instrument during an analytical run. They can consist of repeated injections of a working solution.

The sample can consist of mid-range extracted samples that have been pooled to form a homogeneous solution, which is then split into separate samples and injected at the beginning and end of an analytical batch. A system check sample is often injected three or more times before injection of the first batch of samples. The injections are assessed before the analytical batch is run to ensure that the instrument has reached an equilibrated state before the first sample is injected. This exercise can be repeated at the end of the run, after the last sample in the batch has been analysed. Depending on the run size, injections could also be made in the middle of the run.

After the run has been completed, the injections of the system check sample (analyte peak area or analyte:internal standard peak area ratio) are reviewed and trends evaluated. These could be informative if, for instance, the extracted quality control samples show a trend and fail at the end of the batch.

System checks are not officially required by the United States Food and Drug Administration, nor can they replace the usual run acceptance criteria *(363)*. Nevertheless, it is advisable to conduct some type of system check before the start of any bioanalytical experiment, to demonstrate that the analytical system performs properly. The results of system suitability testing ensure that the total analytical system is sufficiently sensitive, specific and reproducible.

5.2 TIMING OF SAMPLE RE-ANALYSIS

All samples in a batch should be re-analysed when a batch fails the acceptance criteria for either the calibration curve or the quality control. Selected samples should be re-analysed:

- When they show values greater than the highest point on the calibration curve, they are then diluted with blank biological matrix and re-extracted. The dilution procedure should be validated;
- A clear error with the sample is identified, which could be attributed to instrument failure (e.g. unexpected shut down) or possible extraction errors;
- Chromatograms indicate that the sample was adversely affected during processing, e.g. missing internal standard peak, lower-than-expected internal standard peak, interfering peaks due to contamination or endogenous material, unstable baseline or major shift in retention time.

It is advisable to consider the re-injection of a failed run before authorizing re-analysis. In certain instances, such as a lower-than-expected internal standard, it is recommended that the sample be re-analysed both as a naive sample and after dilution (1:5 or more) to identify matrix effects in the original sample. Re-analysis of samples on the basis of inconsistent PK should be examined closely and strictly controlled. A standard operating procedure must be in place defining the conditions under which a sample should be re-analysed and the person responsible for authorizing re-analysis. All the data should be preserved; records must be kept of the original result, the reason for re-analysis, the repeat results (usually in duplicate) and the final accepted result.

5.3 CORRECTIVE ACTION REQUESTS

A 'corrective action request' is required in the event of non-conformity, deviation from a method or altered performance of reference standards, consumables or equipment used in drug analysis. A corrective action request contains a statement of the problem and instigates prompt corrective action to rectify the problem. These requests also identify recurring problems and modifications to prevent such recurrence. They can be generated by anyone associated with drug analysis, including auditors and sponsors.

The corrective actions must be immediate and must trigger an automatic review by the head of the facility or a nominated person responsible for the corrective action request. A written action plan is required to:

- establish a system to allow and promote a rapid response to deviations from a critical limit;
- correct and eliminate the cause of the deviation and restore analysis;
- identify the affected consumable or equipment;
- maintain accurate documentation and records; and
- determine the appropriate action to prevent or minimize recurrence of the problem and any effect the problem might have had on previous analyses.

Each corrective action must be documented on an appropriate form. At a minimum, corrective action requests must include:

- identification of the consumable or equipment (description, code or lot number);
- description of the deviation;
- date of occurrence;
- an in-depth analysis of the possible cause of the problem;
- corrective action taken and process adjustments made to prevent recurrence of the problem;
- name of the person(s) who raised the corrective action request and who will follow it up;
- review and acknowledgement by the unit head or study director; and
- results of the evaluation and corrective action.

5.4 BENEFIT OF EXTERNAL QUALITY ASSURANCE SCHEMES

The purpose of an external quality assurance scheme is to indicate the presence of systemic errors intrinsic to an analytical process that would not be picked up by in-house quality control or quality assurance procedures (e.g. reference standards not at stated purity or weighing errors). External quality assurance schemes are also referred to as 'inter-laboratory comparisons' or 'proficiency testing schemes'. They are used in the cross-validation of an assay and may be critical to the validation of a new laboratory assay. External quality assurance facilitates the pooling of results from more than one laboratory and comparisons of results from different laboratories that use:

- the same methods and instrumentation;
- the same methods but different instrumentation;
- different methods but the same instrumentation; or
- different methods and different instrumentation.

In external quality assurance schemes for drugs in blood, plasma or serum, samples are usually sent to participating laboratories monthly, quarterly or every 6 months. Ideally, each analyte is sent in a range of concentrations, including the highest and lowest concentrations found in the samples. This allows each laboratory to test the limits of its assay. The samples can be spiked laboratory samples or patient samples. Inclusion of a blank sample allows control for a proficiency testing programme, so that laboratories can further check their methods for endogenous interference. Laboratories that find no interference from blank samples are assured of method specificity, while laboratories that find interference are made aware that the interference is in the sample matrix and could affect the accuracy of their results.

The target value can be a value derived from the consensus value of the participating laboratories (for large numbers of participants) or the nominal or 'spiked' value (for schemes with few participants). The participating laboratories are asked to treat all the external quality assurance samples as normal unknown samples. The scheme provides dates by which results are required, to allow batch analysis of all results. The results permit the participating laboratories to assess how close their results are to the target value

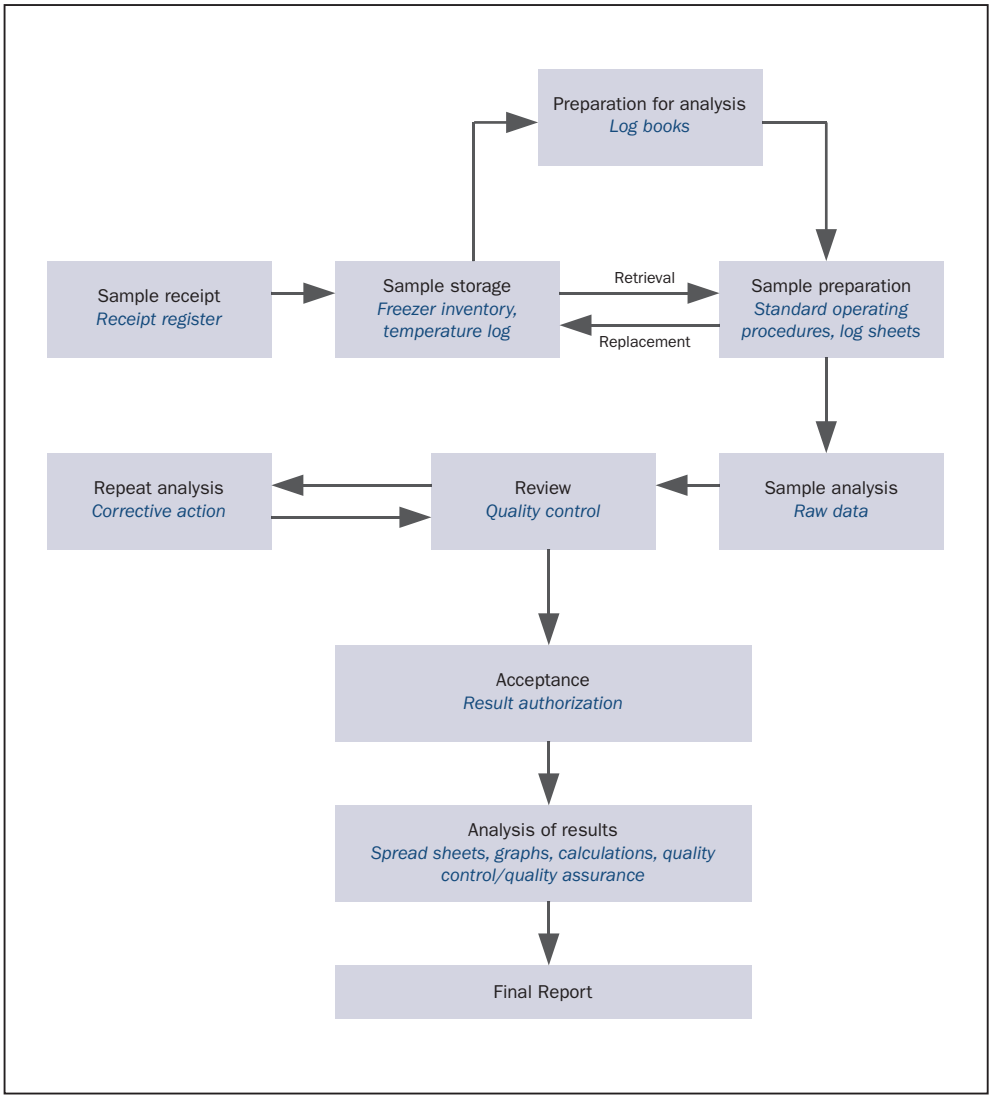
and how well they perform against the median value for all participants. Analysis of the results also allows comparison of median values obtained for the same samples with different methods, provided that the scheme is large enough to allow such stratification.

In therapeutic drug monitoring, quality assurance schemes are valuable for laboratories that perform a particular assay continuously, as they give a good indication of the performance of the assay over time and in relation to the performance of other laboratories and methods. When an assay is performed sporadically, use of a quality assurance scheme is less straightforward. In these cases, an inter-laboratory comparison is more useful, although its success depends on finding comparable laboratories that conduct similar assays and are willing to cooperate. When it is difficult to adhere to an external quality assurance scheme (e.g. sporadic assay performance), externally prepared quality controls can be useful, and commercially available quality controls are helpful in eliminating inter-laboratory differences in the accuracy of preparation of standard curves and quality controls. For laboratories that prepare their own standard curves and quality controls, commercial calibrators and controls are valuable for assessing the accuracy of in-house procedures. Use of the same externally prepared calibrator or controls by a group of laboratories might be more effective than participation in an external quality assurance programme for eliminating differences in results due to inaccuracies in weighing out standards. Reference standards are often available only in small amounts, and an appropriately sensitive analytical balance is required for weighing out small amounts in the production of standards. Microbalances are not always available in poorly financed laboratories.

5.5 BIOANALYTICAL RECORD-KEEPING AND REPORTS

An 'audit trail' is a chronological sequence of paper or electronic records, each of which contains evidence directly pertaining to and resulting from execution of a procedure or process. The audit trail is a fundamental part of the operation of any laboratory providing clinical or study results, and questions that might arise during review of a study can be addressed only by the maintenance of such a trail. Various quality guidelines state that laboratories should retain the original raw data, with sufficient supplementary information to maintain a complete audit trail, which may include records of who performed the test and authorized and released the results, forms, contracts, worksheets, work books, check sheets, work notes, control graphs, external and internal test reports and calibration certificates *(423)*. A copy of each report or result issued must be filed with the data. Figure 5.1 shows a flow chart of drug analysis processes and documentation.

Figure 5.1. Flow chart of drug analysis processes and documentation



All retained data should be legible and should be preserved, so it may be reviewed at any time by sponsors, regulatory authorities or auditors. Corrections should never obscure the original data, so that the audit trail is complete. Data recorded on heat-sensitive paper should be copied before archiving. Scanning of data sheets to ensure an electronic record should be encouraged.

5.6 EXAMPLES OF BIOANALYTICAL STUDY RECORDS

5.6.1 Sample receipt register

A biological sample register lists all study samples that are received by the facility responsible for drug analysis. The sample register must include all information on the receipt and storage of the sample to prove that the integrity of the sample was preserved.

5.6.2 Matrix register

A biological matrix register lists the blank biological samples required for preparation of calibration curves. The register should include all the information necessary for tracing the source of the matrix and for proving that it was stored correctly.

5.6.3 Laboratory notebooks

Laboratory notebooks must be retained and filed appropriately to preserve analytical data. Details of the preparation of stock solutions, reagents, reference standards, calibration curves and quality controls should be recorded in log books or similar supports and should always be signed by a responsible person and checked and signed by another.

5.6.4 Corrective action reports

These are important for the audit trail of a study as they show the procedure used to identify errors and the subsequent means taken to correct the errors. The report should include the name of the person who identified the problem, the person who corrected the problem and a management representative who authorized the report.

5.6.5 Raw analytical data

Raw analytical data (on calibration, quality control and batch samples) must be retained and never overwritten. Raw data can be retained electronically, provided that it is feasible to retrieve it, even after several years.

5.6.6 Supplementary reports (accumulated data)

Laboratory spreadsheets, interim reports, statistical analyses and graphs and quality control and quality assurance records must be stored. Analysis of supplementary data is usually required for study reports.

Additional documentation that might be required to validate analytical data includes:

- **a reference standard register designed to** retain vital information on the reference standards used to quantify unknown samples. This information should be retained and updated with new information, such as an extended expiry date. The objective of this register is to prove that the integrity of the reference standard was ensured by proper use and storage.
- **instrument maintenance and calibration records:** Proper maintenance of instrumentation is essential for obtaining accurate results. Some laboratories check instruments routinely before analysing samples from a study (Instrument maintenance is discussed in section 5.9).

- **standard operating procedures, including methods:** In order to recreate a study during an audit, the standard operating procedures that were in place at the time of the study must be retained.
- **temperature monitoring charts (e.g. for freezers and refrigerators):** These data are required to prove that the integrity of the samples, quality controls and calibrators was not compromised during their storage at the laboratory.
- **validation data and reports:** These should be retained for as long as the study data are retained or for as long as the analytical method is in use. They include all raw data, validation protocols, validation reports and any graphs, calculations or spreadsheets generated during validation.

5.7 FINAL ANALYTICAL REPORTS

The records listed above should be stored appropriately, in an archive-type location, and should be readily available for any audit of the study. If required, a comprehensive final report should be produced. The different regulatory agencies require the final report in various formats. Headings may include: a preface, table of contents, summary, background, materials and methods, results and discussion, chromatograms, method validation and concentrations in the samples.

The **preface** should include the name and address of the facility that carried out the analysis, the facility study number, the accreditation status of the test facility for drug analysis, the date of the analysis, the signatures of the author of the report and the person who approved it, and the location and time for storage of the records.

The **background** should consist of a brief outline of the source of the samples, the protocol title and the ethics committee that approved the study in which the samples were derived, with its approval number.

The **materials and methods** should include:

- responsibilities (name of sponsor, name of person(s) who carried out the analysis, data evaluation and quality assurance);
- the analytical method used;
- sources of reference compounds, consumables and equipment for the analysis;
- method for preparing standard solutions for the calibration curve and quality control samples;
- description of samples for analysis;
- chromatographic conditions;
- sample preparation;
- software packages and version numbers used to acquire and process raw data;
- calculation of results;
- data evaluation (definitions of precision, accuracy, lower limit of quantification, confidence interval); and
- internal audit procedures.

The **chromatograms** should be representative of blank biological samples, spiked blank standards, quality control samples and subject samples.

The **method validation** should include linearity, reproducibility and accuracy estimates.

5.8 DATA ARCHIVING

All laboratory data (results, general quality and technical records, quality records, equipment records) should be stored in an archive-type environment. Regional regulations may apply on the length of time data must be stored. Electronic data should be backed up on a non-rewritable CD or DVD or on tape and stored in a location separate from the primary computer on which the data were collected. The archive should be located separately from the laboratory, and access should be limited to named individuals. An archive should ideally be equipped to prevent untimely deterioration or destruction of its contents (e.g. gas fire-suppression systems for paper-based archives, light protection).

5.9 EQUIPMENT VERIFICATION

All major analytical equipment should be calibrated, serviced and properly maintained to ensure accurate, reproducible results. Registers or service histories should be maintained for every piece of equipment used in the analytical process. All equipment should be checked periodically to ensure that it is functioning optimally, and the result must be recorded appropriately.

LC systems must be preventatively maintained in accordance with the manufacturer's specifications. The components to be checked include pump back pressure, flow rate, detector source for mass spectrometers and the precision and accuracy of results for direct injections of reference standards.

Likewise, measuring equipment, such as pH meters, analytical balances, adjustable research pipettes and thermometers (including gauges on storage units), should be verified against national standards to ensure that the readings obtained in the laboratory are correct.

5.10 RECOMMENDATIONS

A standard curve (single or duplicate points) should be generated with each analytical run and used to quantify the concentrations in the unknown samples in that run. Each standard curve should contain eight to ten non-zero points. A duplicate standard curve is recommended so that if one critical point, such as the LLOQ or ULOQ, fails, a successful duplicate point obviates truncation of the calibration range.

It is strongly advised that estimates should not be made of concentrations in unknown samples beyond the range of the standard curve. If a concentration exceeds the ULOQ, the sample should be diluted and re-analysed. The process must be tested during validation.

Reporting of concentrations below the LLOQ is also strongly discouraged; such concentrations should be reported as 'below the limit of quantification'. Although clinical investigators and pharmacometricians might prefer to receive data rather than 'below the limit of quantification' substitutions, these concentrations cannot be determined accurately in the laboratory and cannot therefore be reported.

Quality controls are used to accept or reject a run and are analysed in duplicate at three concentrations. The values for at least four of six controls must fall within 85–115% of their nominal concentrations. Two of six controls (not at the same concentration) may fall outside these limits.

Re-analysis should be performed only according to clearly defined criteria. Problems such as sample processing errors, quality control or calibration failures, high concentrations or instrument failure may necessitate re-analysis. It is not recommended that re-analyses be based on PK theory without justification.

5.11 RATIONALE

All bioanalytical laboratories are responsible for providing high-quality data and for proving to all clients that the data can be used with the utmost confidence. Having checks in place not only gives confidence to all those who work with the data but will ultimately save money and time by obviating re-analysis of unknown samples. As in any area of science, measurement techniques will continue to improve; therefore many of the recommendations made here are likely to change.

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