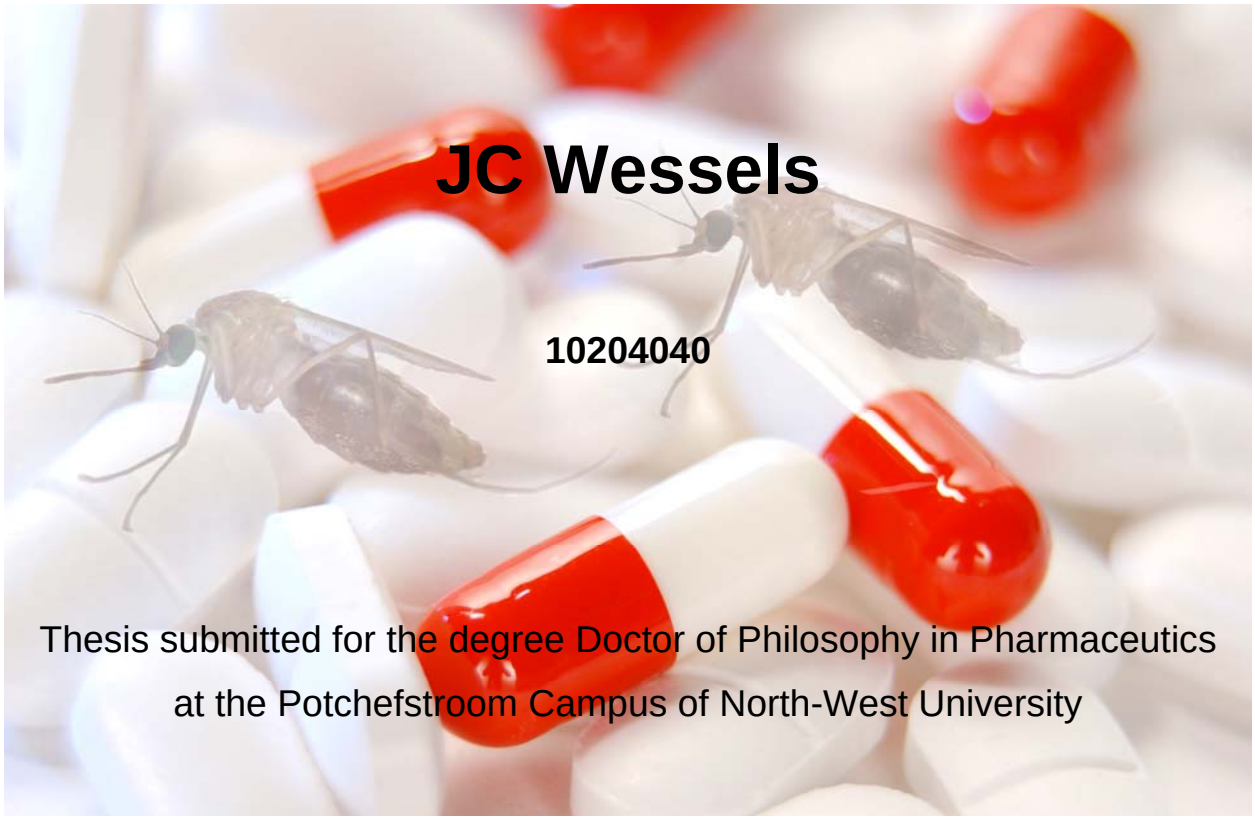
International Pharmacopoeia Monographs: Antimalarial Dosage Forms



JC Wessels

10204040

Thesis submitted for the degree Doctor of Philosophy in Pharmaceutics at the Potchefstroom Campus of North-West University

Promoter: Prof. T.G. Dekker

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This thesis is dedicated to Bernard, Lizae & Jurgen

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ABSTRACT

Keywords: Malaria, amodiaquine, sulfadoxine, pyrimethamine, mefloquine, monograph, specifications.

Malaria is a disease affecting millions of people in 109 malarious countries and territories, causing approximately one million deaths annually. In 2004 one of the parasites causing human malaria, *Plasmodium falciparum*, was among the leading global causes of death from a single infectious agent, especially in Africa (WHO, 2008:23).

Treatment of this disease with single active pharmaceutical ingredients has led to the emergence of resistant *P. falciparum* parasites, resulting in the most severe form of this illness. Alarmingly, the poor quality of commercially available antimalarial products, especially in Africa, has increasingly been reported as a major cause of resistance to antimalarials. In Pakistan it was found that a *P. falciparum* epidemic that initially was attributed to drug resistance, was actually caused by substandard sulfadoxine/pyrimethamine products, causing a 50 times higher incidence of malaria in these areas than elsewhere (Leslie *et a*l., 2009:1758). Other results indicated that up to 10% of sulfadoxine/pyrimethamine tablets, sampled in six African countries, failed the assay test, whilst up to 40% failed the USP dissolution test. Furthermore, the World Health Organization (WHO) reported that 20 – 90% of products failed quality requirements during 1999 and 2000 in seven African countries (WHO, 2003:263).

Cases like these have raised the awareness of the vast number of inferior products that are being distributed. The subsequent need for establishing mechanisms to pro-actively detect substandard medicines, specifically antimalarials, easily and effectively had indirectly led to the origin of this study, long before it was formally undertaken.

Testing monographs for pharmaceutical products are developed to formalise, or standardise, the regulation of pharmaceutical dosage forms. Problems have, however, been reported with regards to the inadequacy of existing antimalarial monographs in assuring quality medicines, fit for their intended use.

The WHO had requested the Research Institute for Industrial Pharmacy, incorporating the Centre for Quality Assurance of Medicines (RIIP®/CENQAM®), both operating at the Potchefstroom Campus of the North-West University, to develop monographs for three immediate-release antimalaria dosage forms, namely amodiaquine tablets, sulfadoxine/pyrimethamine fixed-dose combination tablets and mefloquine tablets. The undertaking of these projects, to develop specifications for the quality control of these pharmaceutical products, formed the object of this research study.

Data had been accumulated since 2000, as a result of continuous requests by the WHO to help solve problems that had been experienced with analytical test methods, especially from manufacturers. These requests either led to the refinement of existing methods, or to the development of new ones. The success with which these outcomes were implemented worldwide, finally led to the decision to publish these research findings under the umbrella of this project.

The proud product is a comprehensive package of tests for three commercial antimalarial products, the outcomes of which are hoped to contribute towards the combat against resistance formation to these important disease fighters.

UITTREKSEL

Sleutelwoorde: Malaria, amodiakien, sulfadoksien, pirimetamien, meflokien, monograaf, spesifikasies.

Malaria, 'n siekte wat miljoene mense in 109 lande en gebiede affekteer, is die oorsaak van ongeveer een miljoen sterftes jaarliks. *Plasmodium falciparum* is een van die parasiete wat malaria in mense veroorsaak en was in 2004 een van die hoofoorsake van sterftes weens infeksie met 'n enkele infektiewe organisme, waarvan die meeste in Afrika rapporteer is (WHO, 2008:23).

Behandeling van malaria met enkele aktiewe farmaseutiese bestanddele het tot die ontwikkeling van weerstandige *P. falciparum* parasiete gelei, wat gevolglik tot die ernstigste vorm van hierdie siekte aanleiding gegee het. Dit is onrusbarend dat die swak kwaliteit van kommersieel beskikbare antimalaria produkte, veral in Afrika, al hoe meer as 'n hoofoorsaak van weerstand teen antimalaria geneesmiddels vermeld word. In 'n studie wat deur Leslie *et al*. (2009:1758) in Pakistan uitgevoer is, is bevind dat 'n malaria-epidemie, wat aanvanklik aan weerstandigheid toegeskryf is, inderdaad deur substandaard sulfadoksien/pirimetamien-tablette veroorsaak is. Dit het tot gevolg gehad dat die voorkoms van malaria in Pakistan 50 keer hoër as in enige ander gebied was. Verdere resultate het ook getoon dat soveel as 10% van sulfadoksien/pirimetamien-tablette, wat in ses Afrika-lande versamel is, nie aan die spesifikasies vir die gehaltetoets voldoen het nie en dat 40% van hierdie tablette nie aan die USP se vereistes vir dissolusie voldoen het nie. Voorts het studies, wat in 1999 en 2000 deur die Wêreldgesondheidsorganisasie (WGO) in sewe Afrika-lande geloods is, daarop gedui dat 20 – 90% van die produkte wat getoets is, nie aan die nodige kwaliteitvereistes voldoen het nie (WHO, 2003:263).

Gevalle soos hierdie het 'n bewustheid oor die groot aantal substandaard medisyne wat in omloop is, begin skep. Die gevolglike ontstaan van die behoefte om meganismes daar te stel, ten einde op 'n pro-aktiewe wyse substandaard medisyne, spesifiek antimalaria produkte, maklik en effektief te identifiseer, het indirek tot die ontstaan van hierdie studie gelei, lank voor dit formeel onderneem is.

Toetsmonograwe vir farmaseutiese produkte word ontwikkel om die regulering van farmaseutiese doseervorme te standaardiseer. Probleme rakende die ondoeltreffendheid van bestaande antimalaria monograwe, om kwaliteit antimalaria geneesmiddels te verseker, is egter oor die jare gerapporteer.

Die Navorsingsinstituut vir Industriële Farmasie, waarby die Sentrum vir Kwaliteitsversekering van Medisyne (RIIP®/CENQAM®) ingelyf is, wat vanaf die Potchefstroomse Kampus van die Noordwes-Universiteit aktief is, is deur die WGO genader om behulpsaam te wees met die ontwikkeling van monograwe vir drie onmiddellik-vrystellende antimalaria doseervorms, spesifiek vir amodiakientablette, sulfadoksien/pirimetamien-tablette en meflokientablette. Die doel van hierdie studie was dus om spesifikasies vir die kwaliteitsbeheer van hierdie farmaseutiese produkte te ontwikkel.

Die betrokkenheid van die RIIP®/CENQAM® sedert die jaar 2000 by die WGO om oplossings te vind vir probleme met analitiese metodes ervaar, veral met metodes afkomstig vanaf vervaardigers, het ’n nuttige databasis daargestel. Sodanige versoeke deur die WGO het óf tot die verfyning van bestaande metodes, óf tot die ontwikkeling van totaal nuwe metodes gelei. Die sukses waarmee hierdie resultate wêreldwyd toegepas is, het uiteindelik tot die besluit aanleiding gegee om hierdie navorsingsuitkomste as deel van hierdie studie te publiseer.

Die trotse produk is ’n omvattende stel toetse vir drie kommersieel beskikbare antimalaria produkte, wat ten doel het om ’n bydrae tot die stryd teen die ontstaan van weerstandigheid teen hierdie belangrike geneesmiddels te maak.

LIST OF ABBREVIATIONS

ACT - Artemisinin-based combination therapy

API - Active Pharmaceutical Ingredient

BCS - Biopharmaceutics Classification System

BP - British Pharmacopoeia

EDL – Essential Drug List

EML – Essential Medicine List

EMLC – Essential Medicine List for Children

EMP – Essential Medicines and Pharmaceutical Policies

EP - European Pharmacopoeia

GMP - Good Manufacturing Practices

HIV - Human immunodeficiency virus

HPLC – High Performance Liquid Chromatography

HCl – Hydrochloride/hydrochloric acid

ICH – International Conference on Harmonisation

IPT - Intermittent preventive treatment

IR - Infrared

LOD – Limit of Detection

LOQ – Limit of Quantitation

NaOH – Sodium hydroxide

Ph.Int. – The International Pharmacopoeia

QC – Quality Control

RIIP®/CENQAM® - Research Institute for Industrial Pharmacy® incorporating the Centre for Quality Assurance of Medicines®

rpm – Rotations per minute

%RSD – Percentage Relative Standard Deviation

SP – Sulfadoxine/Pyrimethamine

TLC – Thin Layer Chromatography

TDI – Total daily intake

USFDA – US Food and Drug Administration

USP - United States Pharmacopeia

UV - Ultraviolet

WGO – Wêreld Gesondheidsorganisasie

WHO – World Health Organization

OBJECTIVES

The International Pharmacopoeia (Ph.Int.) comprises a collection of quality specifications for pharmaceutical substances, i.e. active pharmaceutical ingredients (APIs) and excipients, as well as dosage forms, together with supporting general methods of analysis. These specifications and analytical methods serve as source material for reference, or adaptation, by any WHO Member State wishing to establish pharmaceutical requirements (WHO, 2010d). The selection of monographs for inclusion in The International Pharmacopoeia recognises the needs of specific disease programmes and the essential medicines being nominated under these programmes, and is based primarily on those substances included in the current WHO Model Lists of Essential Medicines (WHO, 2010c).

The process of monograph development has been set out in an official document by the WHO (2004).

The objective of this study was to develop analytical methods to be utilised in Ph.Int. monographs for specific products as indicated below. As part of the study, methods from manufacturers (supplied by the WHO), from existing pharmacopoeial monographs and published in literature would be evaluated for their suitability.

A. The methods that required development were:

I. Amodiaquine tablets

a) Method to determine the contents of the API in the dosage form, or assay method; and

b) Method to determine the amount of API that is dissolved during dissolution testing.

II. Sulfadoxine/Pyrimethamine tablets

a) Method(s) to determine the contents of the APIs in the dosage form, or assay method(s);

b) Method to determine the amount of APIs that is dissolved during dissolution testing; and

c) Method to quantify the related substances in dosage forms.

III. Mefloquine tablets

a) Identification tests;

b) Method to determine the content of the API in the dosage form, or assay method;

c) Method(s) to determine the amount of the API that is dissolved during dissolution testing; and

d) Method to quantify the related substances in dosage forms.

B. For the dissolution testing, it was also necessary to consider the following:

i. The type of dissolution medium;

ii. The volume of dissolution medium; and

iii. The acceptance criterion for the dissolution test.

Development studies were to be undertaken to establish practical, affordable and robust analytical methods. As soon as a set of parameters were established for each method, these methods would be validated according to the International Conference on Harmonisation guidelines (ICH, 2005) in order to verify the performance of each method.

The goal of this study thus was to develop suitable analytical methods as a package to be included as part of the relevant product monographs in The International Pharmacopoeia and therefore supporting the fight against malaria.

CHAPTER 1
BACKGROUND

1.1 INTRODUCTION

Malaria is a disease, affecting millions of people annually. Worldwide, three billion people are at risk of infection in 109 malarious countries and territories, with about 250 million cases being reported annually and approximately one million of deaths. In 2004, *P. falciparum* was among the leading global causes of death from a single infectious agent, especially in Africa (WHO, 2008:23).

Unfortunately, treatment of this disease with single APIs has led to the emergence of resistant *P. falciparum* parasites, resulting in the most severe form of this illness. Therapy should consequently include the combination of two or more APIs, having independent modes of action and molecular targets, hence resulting in synergistic or additive effects (Aweeka & German, 2008:92).

Resistance to antimalarials has, however, also been linked to poor quality products that are available on the market, which is the focus of this study. The quality of commercially available antimalarial products has been questioned for a number of years now. In 1999 (WHO, 2002:5), the WHO launched a pilot study to sample antimalarial products in six African countries. Three types of products were included in the study: chloroquine syrup, chloroquine tablets and sulfadoxine/pyrimethamine tablets.

The results were published in a summary report in 2002, indicating that up to 10% of sulfadoxine/pyrimethamine tablets failed the assay test, whilst up to 40% failed the dissolution test of the USP. Invariably, the poor dissolution results have been attributed to the poor dissolution of pyrimethamine (WHO, 2002:16).

This has led to an awareness of the vast number of inferior products that are being distributed, especially in Africa, resulting in various papers being published on this

issue (Amin *et al*., 2005:559; Amin & Kokwaro, 2007:429; Atemnkeng *et al.,* 2007:123; Hebron *et al*., 2005:575; Minzi *et al*., 2003a:117).

It is of crucial importance, therefore, that practical, affordable and reliable analytical test methods are being developed to test these products, before distribution to members of the public, thus assuring quality medicines, fit for their intended use.

1.2 PHARMACEUTICAL PRODUCTS

1.2.1 QUALITY ASSURANCE

Medicines are unique, in that virtually no other product is consumed by the public in such utter trust – trust that it will not cause them more harm than the illness it is meant to cure. Such trust can only be assured, if it has been adequately tested during development and manufacture. Testing should give assurance that side effects have been established and that the drug is efficacious. Hence, when given to patients, it must have been appropriately manufactured, tested and packaged to assure that it: (i) is the correct product, (ii) is of the correct strength, (iii) has not degraded, (iv) is free from harmful impurities and micro-organisms, (v) has not been contaminated, (vi) is correctly labelled, and (vii) is properly packaged in a suitable container (Moores, 2003:3).

To ensure the quality of medicinal products, quality measures must be built in at each stage of the manufacturing process, and not merely tested in the final product. Any variable that may affect the quality of the final product, must be controlled. Batch to batch consistency should be maintained, by reducing variability of all supporting processes, sub-processes and procedures. End-product testing then is just a final check of the quality of the product. This testing is used in conjunction with the written records, which demonstrate that all critical factors have been controlled, as the supporting documentation allows the product to be released for use (Moores, 2003:3).

As global pharmaceutical regulatory requirements have become more alike, due to deliberate harmonisation efforts, analytical methods for global products must be able to meet global regulatory requirements (Chan & Jensen, 2004:7). Ideally, a method

being developed and validated in South Africa, for example, should not require revalidation elsewhere in the world.

1.2.2 COUNTERFEIT MEDICINES

The United States Food and Drug Administration (USFDA) estimates that counterfeits constitute more than 10% of the global medicines market, in both developed and developing countries (Akunyili & Nnani, 2004:186).

A counterfeit medicine is defined as "one, which is deliberately and fraudulently mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products, and counterfeit products may include products with the correct ingredients, or with the wrong ingredients, without active ingredients, with insufficient active ingredients, or with fake packaging" (WHO, 2010a).

The risk of counterfeit medicines is multidimensional. The health related risks of counterfeit medicines arise, because they are inherently defective. They may be placebos, they may contain toxic materials, or they may be contaminated, because they are not produced under good manufacturing practices (GMP), and perhaps outside of any form of regulation whatsoever. Use of counterfeit medicines may result in treatment failure consequent to lack of effect, which in turn could result in worsening of the disease condition, deformity and death. Counterfeit medicines could introduce new adverse drug reactions, dangerous interactions, or intensify already known ones (Akunyili & Nnani, 2004:187).

In a study conducted in Pakistan it was found that a *P. falciparum* epidemic, that initially was attributed to drug resistance, was actually caused by substandard sulfadoxine/pyrimethamine products. The incidence of malaria in the area where these substandard products were distributed, was 50 times higher than in other areas (Leslie *et al*., 2009:1758).

In a survey, done by the World Health Organisation (WHO), on the quality of antimalarials in seven African countries during the period 1999 - 2000, it was revealed that 20 – 90% of the products failed quality testing (WHO, 2003:263).

Cases like these emphasise the need for establishing mechanisms to pro-actively detect substandard products easily and effectively.

1.2.3 ANALYSES OF PHARMACEUTICAL PRODUCTS

A formulation is required to deliver the API to its targeted site. Formulation development is a complex process, involving the physiochemical characterisation of the API, identifying compatible excipients, developing a reliable manufacturing process, and thorough analytical characterisation of the dosage form. The goal of any formulation development is to ensure that each batch being manufactured, meets the specifications for identity, strength, quality and purity (Patel & LoBrutto, 2007:679).

International Conference on Harmonisation guideline Q6A (ICH, 1999) defines specification as follows: "A list of tests, references to analytical procedures, and appropriate acceptance criteria, which are numerical limits, ranges, or other criteria, for the tests described. It establishes the set of criteria to which a drug substance, or drug product, should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the drug substance and/or drug product, when tested according to the listed analytical procedures, will meet the listed acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities."

According to the Eurachem working group (Eurachem, 1998:1), six principles in analytical practice were identified, which, when grouped together, are considered to constitute best practice. Four of the six principles are directly related to the analytical measurement process, namely:

i. Analytical measurement should be made to satisfy an agreed requirement;

ii. Analytical measurement should be made, using methods and equipment, which have been tested to ensure that they are fit for purpose;

iii. There should be a regular independent assessment of the technical performance of a laboratory; and

iv. Analytical measurements made in one location, should be consistent with those made elsewhere.

1.2.4 MONOGRAPH DEVELOPMENT

The need to formalise, or standardise, the regulation of pharmaceutical dosage forms, has led to the development of testing monographs for these products. Various compilations of these monographs are currently available and include the International Pharmacopoeia (Ph.Int.), United States Pharmacopeia (USP), British Pharmacopoeia (BP) and the European Pharmacopoeia (EP).

The development of specifications for the quality control of pharmaceutical products entails a number of predetermined steps. The International Pharmacopoeia describes the process of monograph development as follows (WHO, 2004):

Step 1: *Identification of specific pharmaceutical products for which Quality Control (QC) specifications need to be developed, confirmation by all WHO parties concerned (including Department of Essential Medicines and Pharmaceutical Policies (EMP), specific disease programmes and the Prequalification Programme).*

Step 2: *Provision of contact details from manufacturers of the above products in collaboration with all parties concerned.*

Step 3: *Contact manufacturers for provision of QC specifications and samples.*

Step 4: *Identify and contact QC laboratories for collaboration in the project (2-3 laboratories depending on how many pharmaceutical products have been identified in step 1). Contract for laboratory work.*

Step 5: *Prepare the contract for drafting the specifications and undertaking the necessary laboratory work.*

Step 6: *Search for information on QC specifications available in the public domain.*

Step 7: *Laboratory testing, development and validation of Quality Control Specifications.*

Step 8: *Support WHO Collaborating Centre in the establishment of International Chemical Reference Substances.*

Step 9: *Follow the consultative process, mailing of draft specifications to Expert Panel and specialists.*

Step 10: *Discussion of comments with contract laboratories, WHO Collaborating Centres and additional laboratory testing to verify and/or validate specifications.*

Step 11: *Consultation to discuss the comments and test results received as feedback.*

Step 12: *Recirculation for comments.*

Step 13: *As step 10.*

Step 14: *Present the drafts to the WHO Expert Committee on Specifications for Pharmaceutical Preparations for possible formal adoption. If not adopted repeat steps 11 to 13 as often as necessary. If adopted proceed to step 15.*

Step 15: *Incorporate all changes agreed during the discussion leading to adoption together with any editorial points.*

Step 16: *Where necessary, also take account of any further comments that may still be received due to comment deadlines for recirculated texts (Step 12 and beyond) falling shortly after the meeting**.***

Step 17: *In all cases, confirm the amended text by correspondence with the relevant experts and/or contract laboratory before making it available on the WHO Medicines website.*

Step 18: *Make "final texts" available on the Medicines website to provide users such as PQ assessors and manufacturers with the approved specifications in advance of the next publication date.*

The WHO requested the Research Institute for Industrial Pharmacy®, incorporating the Centre for Quality Assurance of Medicines® (RIIP®/CENQAM®), both operating at the Potchefstroom Campus of the North-West University, to develop monographs for three immediate-release, antimalaria dosage forms, i.e. amodiaquine tablets,

sulfadoxine/pyrimethamine tablets and mefloquine tablets. The undertaking of these projects formed the object of this research study.

1.2.5 METHOD DEVELOPMENT

1.2.5.1 Identification testing

Identification testing should establish the identity of the API and should be able to discriminate between compounds of closely related structure, likely to be present. Identification tests should be specific to the API, e.g. infrared (IR) spectroscopy (ICH, 1999).

Identification, solely by a single chromatographic retention time, is not being regarded as specific. However, the use of two chromatographic procedures, where the separation is based on different principles, or a combination of tests into a single procedure, is generally acceptable (ICH, 1999).

1.2.5.2 Assay testing

A specific, stability indicating procedure should be included to determine the content of the API for all new products (ICH, 1999).

In many cases, it is possible to employ the same procedure for both assay and quantitation of impurities (ICH, 1999).

1.2.5.3 Dissolution testing

Even though dissolution testing has evolved into a powerful tool for characterising the quality of oral pharmaceutical products, detailed guidelines for the development of dissolution tests still do not exist to date (WHO, 2006:378) (this being the most current and valid source in this regard).

Dissolution testing, included in pharmacopoeial monographs, primarily serves as a quality control test. In developing these tests, the Biopharmaceutics Classification System (BCS) is used as guideline. The BCS is based on aqueous solubility and intestinal permeability of the API. An API is considered highly soluble when the highest dose recommended by WHO, or highest dose strength available on the market as an oral solid dosage form, is soluble in 250 ml, or less, of aqueous media

over the pH range of 1.2 - 6.8. The pH solubility profile of the API should be determined at 37 ±1ºC in aqueous media (WHO, 2006:378).

Based upon their dissolution properties, immediate-release dosage forms can be categorised as having 'very rapid', 'rapid', or 'not rapid' dissolution characteristics. A product is considered to be very rapidly dissolving, when no less than 85% of the labelled amount of the API dissolves in 15 minutes, using a paddle apparatus at 75 rotations per minute (rpm), or a basket apparatus at 100 rpm, in a volume of 900 ml, or less, in each of pH 1.2 HCl solution, pH 4.5 acetate buffer and pH 6.8 phosphate buffer. A product is considered to be rapidly dissolving, when no less than 85% of the labelled amount of the API dissolves in 30 minutes (WHO, 2006:380).

Single point measurements are normally considered suitable for immediate-release dosage forms (ICH, 1999).

For determining the amount of API having dissolved during the dissolution testing, analytical techniques, such as ultraviolet (UV) spectroscopy and high performance liquid chromatography (HPLC), are usually used.

1.2.5.4 Related substances

The related substances found in an API, may originate during the synthesis steps, from the original starting materials/intermediates, or from impurities from the starting materials that react in the downstream chemistry. All of these are known as synthesis by-products. When a given API is utilised to manufacture a pharmaceutical product, the degradation products found in the dosage form must be identified, characterised, and/or qualified, according to ICH guidelines (Patel & LoBrutto, 2007:687). The relationship between synthesis by-products, degradation products, and related substances, is that related substances contain the sum of synthesis by-products (originating from chemical synthesis and not changing with time and varying conditions) and degradation products (increase with time and vary under different storage conditions). Sometimes, the synthesis by-products of the API can also be degradation products of the API (Patel & LoBrutto, 2007:687).

The analytical technique employed to detect and quantify related substances, should be validated to demonstrate specificity for the specified and unspecified degradation

products. The quantitation limit for the analytical procedure should be not more than (≤) the reporting threshold (ICH, 2006).

Related substance levels can be measured by a variety of techniques. The techniques used most often are:

i. Comparing an analytical response for a related substance to that of an appropriate reference standard; and

ii. Comparing an analytical response for a related substance to that of the response of the API itself (ICH, 2006).

Where the API is used to estimate the levels of related substances, it sometimes happens that the response factors of the related substances are not close to that of the API. In such cases, a correction factor may be used (ICH, 2006).

The level at which related substances, present in dosage forms, should be qualified[1], identified, or reported, depends on the maximum daily dose of the API (table 1.1).

1.2.6 METHOD VALIDATION

Method validation is usually considered to be very closely tied to method development. Indeed, it is often impossible to determine exactly where method development finishes and validation begins. Many of the method performance parameters that are associated with method validation are in fact usually evaluated, at least approximately, as part of method development (Eurachem, 1998:7).

For an analytical result to be fit for its intended purpose, it must be sufficiently reliable that any decision being based upon it, should be made with confidence. The method performance must hence be validated, and the uncertainty on the result, at a given level of confidence, estimated (Eurachem, 1998:8).

[1] **Qualification**: The process of acquiring and evaluating data that establishes the biological safety of an individual degradation product, or a given degradation profile, at the level(s) specified (ICH, 2006).

Table 1.1: Thresholds for related substances in new drug products (ICH, 2006)

REPORTING THRESHOLDS	
MAXIMUM DAILY DOSE *	**THRESHOLD #, $**
≤ 1 g	0.1%
> 1 g	0.05%
IDENTIFICATION THRESHOLDS	
MAXIMUM DAILY DOSE *	**THRESHOLD #, $**
< 1 mg	1.0% or 5 µg TDI, whichever is lower
1 mg – 10 mg	0.5% or 20 µg TDI, whichever is lower
> 10 mg – 2 g	0.2% or 2 mg TDI, whichever is lower
> 2 g	0.10%
QUALIFICATION THRESHOLDS	
MAXIMUM DAILY DOSE *	**THRESHOLD #, $**
< 10 mg	1.0% or 50 µg TDI, whichever is lower
10 mg – 100 mg	0.5% or 200 µg TDI, whichever is lower
> 10 mg – 2 g	0.2% or 3 mg TDI, whichever is lower
> 2 g	0.15%

* - The amount of drug substance administered per day
\# - Thresholds for degradation products are expressed either as a percentage of the drug substance, or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation is usually toxic.
$ - Higher thresholds should be scientifically justified.

A method should be validated when it is necessary to verify that its performance parameters are adequate for use for a particular analytical problem. For example:

i. New method developed for a particular problem;

ii. Established method being revised to incorporate improvements, or extended to a new problem;

iii. When quality control indicates that an established method is changing with time;

iv. Established method being used in a different laboratory, or by different analysts, or on different instrumentation; and

v. To demonstrate the equivalence between two methods, e.g. a new method and a standard method (Eurachem, 1998:5).

1.2.7 METHOD PARAMETERS

The WHO technical report series 937 (WHO, 2006:138) indicates that the following characteristics should be considered during the validation of an analytical method:

i. Specificity;

ii. Linearity;

iii. Range;

iv. Accuracy;

v. Precision;

vi. Detection limit;

vii. Quantitation limit; and

viii. Robustness.

The definitions, as given in this technical report (WHO, 2006:138), are presented below.

Accuracy: "*The degree of agreement of test results with the true value, or the closeness of the results obtained by the procedure to the true value*". It should be established across the specified range of the analytical procedure.

Precision: "*The degree of agreement among individual results*". Three different techniques exist for determining precision, namely repeatability, intermediate precision/within-laboratory variations, and reproducibility/between-laboratory precision.

Robustness: "*The ability of the procedure to provide analytical results of acceptable accuracy and precision under a variety of conditions*". Factors that can have an effect during chromatography include:

i. Stability of the standard and test samples;

ii. Different columns;

iii. Variations of pH of a mobile phase;

iv. Variations in mobile phase composition; and

v. Flow rate.

Linearity: "*The ability to produce results that are directly proportional to the concentration of the analyte in the samples*". A minimum of five concentrations should be used.

Range: "*An expression of the highest and lowest levels of analyte that have been demonstrated to be determinable for the product*". The specified range is normally derived from linearity studies.

Specificity: "*The ability to measure unequivocally the desired analyte in the presence of other components*".

Detection Limit: "*The smallest quantity of an analyte that can be detected and not necessarily determined in a quantitative fashion*". Approaches may include instrumental and non-instrumental procedures, such as:

i. Visual inspection;

ii. Signal to noise ratio;

iii. Standard deviation of the response and the slope;

iv. Standard deviation of the blank; and

v. Calibration curve.

The following equation can be used to calculate the detection limit:

$$\frac{3.3\sigma}{S}, \quad \text{where}$$

σ = the residual standard deviation of the standard curve, and

S = slope of the calibration curve (ICH, 2005).

Quantitation limit: "*The lowest concentration of an analyte in a sample that may be determined with acceptable accuracy and precision*". The same approaches as for detection limit can be used.

The following equation can be used to calculate the quantitation limit:

$$\frac{10\sigma}{S}, \quad \text{where}$$

σ = the residual standard deviation of the standard curve, and

S = slope of the calibration curve (ICH, 2005).

1.3 MALARIA

1.3.1 INTRODUCTION

The Merck Manual (Beers & Berkow, 1999:239) defines malaria as "Infection with any of four different species of *Plasmodia*, causing periodic paroxysms of chills, fever and sweating, anaemia and splenomegaly".

The parasites causing human malaria are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale*, of which *P. falciparum* is the most deadly (WHO, 2009).

The life cycle of *Plasmodium* is complex, comprising a sexual phase (sporogony) in the mosquito (vector) and an asexual phase (schizogony) in man (Sweetman, 2009).

Fever, headache, chills and vomiting, the common first symptoms, usually appear 10 to 20 days after infection by *P. vivax*, 12 to 14 days for *P. falciparum* and about one month for *P. malariae* (Beers & Berkow, 1999:241). Vomiting occurs in about 20% of patients and mild diarrhoea in less than 5%. As the infection progresses, the spleen enlarges and the patient may develop anaemia and thrombocytopenia (Sweetman, 2009).

1.3.2 ANTIMALARIAL MEDICINES

Antimalarial medicines can be classified by the stage of the parasitic cycle they affect, or by the chemical group to which they belong (Sweetman, 2009). A summary of the chemical groups is given in table 1.2.

The different mechanisms of action of antimalarial drugs sometimes allow the use of combinations of antimalarials to improve efficacy. Such combinations are usually designed to potentiate each other, or they may be complementary. The main reason

for using combinations, is to attempt the delay of the development of resistance (Sweetman, 2009).

Table 1.2: Chemical classification of antimalarial medicines (Sweetman, 2009)

CHEMICAL GROUP	EXAMPLE OF APIs IN GROUP
4-methanolquinolines	Cinchona alkaloids (quinine) Mefloquine
4-aminoquinolines	Chloroquine Hydroxychloroquine Amodiaquine
8-aminoquinolines	Primaquine Tafenoquine
Biguanides	Proguanil Chlorproguanil
Diaminopyrimidines	Pyrimethamine
Dichlorobenzylidines	Lumefantrine
Hydroxynaphthoquinones	Atovaquone
9-phenanthrenemethanols	Halofantrine
Sesquiterpene lactones	Artemisinin
Sulfonamides	Sulfadoxine Sulfametopyrazine
Tetracyclines	Doxycycline Tetracycline
Lincosamide	Clindamycin
Sulfones	Dapsone

1.3.3 TREATMENT OF MALARIA

According to the World Malaria Report of 2008 (WHO, 2008:25), the following rules should be applied in the diagnosis and treatment of malaria:

i. The treatment of malaria infections should be based on a laboratory confirmed diagnosis, with the exception of children under five years of age in areas of high transmission, in whom treatment may be provided on the basis of a clinical diagnosis.
ii. All uncomplicated *P. falciparum* infections should be treated with an artemisinin-based combination therapy (ACT). In central America, the only

remaining region where *P. falciparum* is sensitive to chloroquine, the change to ACT should be made when chloroquine failure rates reach 10%.

P. vivax should be treated with chloroquine and primaquine, except where *P. vivax* is resistant to chloroquine, then it should be treated with ACT and primaquine.

iii. Four ACTs are currently recommended for use, i.e. artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloquine and artesunate/sulfadoxine/ pyrimethamine.[2]

The choice of the ACT should be based on the efficacy of the partner medicine in the country, or area of intended deployment.

iv. Patients, suffering from severe malaria presenting at the peripheral levels of the health system, should be provided prereferral treatment with quinine, or artemisinins, and transferred to a health facility where full parenteral treatment and supportive care can be given.

v. Severe malaria should be treated parenterally with either an artemisinin derivative (artesunate is preferred in areas of low to moderate transmission), or quinine, until the patient can swallow, when a complete course of ACT must be administered.

vi. In areas of high transmission, intermittent preventive treatment (IPT) with sulfadoxine/pyrimethamine (SP) should be administered to pregnant women, at least twice during the second and third trimesters of pregnancy, and three times in the case of HIV positive pregnant women. The effectiveness of IPT should be monitored in light of increasing SP resistance.

1.4 ANTIMALARIALS FOR MONOGRAPH DEVELOPMENT

The WHO (WHO, 2010b) identified eight monographs for finished products that required development for inclusion in the General Pharmacopoeial Information (Ph.Int.). They are:

i. Mefloquine tablets;

ii. Amodiaquine tablets;

[2] The recommended ACTs could be in fixed-dose formulated form, or in the form of co-blistered solid dosage forms.

iii. Quinine bisulfate tablets;

iv. Sulfadoxine and pyrimethamine tablets;

v. Artemether and lumefantrine tablets;

vi. Artemether and lumefantrine suspension;

vii. Chloroquine sulfate oral solution; and

viii. Quinine sulfate tablets.

Tests for three of these monographs have been developed during this study, i.e. amodiaquine tablets, sulfadoxine and pyrimethamine combination tablets, and mefloquine tablets. The properties of their respective APIs are discussed next.

1.4.1 AMODIAQUINE HYDROCHLORIDE

1.4.1.1 Introduction

Amodiaquine is a 4-aminoquinoline antimalarial, with an action similar to that of chloroquine. It is as effective as chloroquine against chloroquine-sensitive strains of *P*. *falciparum* and it is also effective against some chloroquine-resistant strains. However, resistance to amodiaquine has developed and there may be partial cross-resistance between amodiaquine and chloroquine (Sweetman, 2008). A dose of 153 mg amodiaquine (200 mg amodiaquine HCl), should be used in combination with 50 mg artesunate. For the treatment of *P.vivax, P. ovale* and *P. malariae*, it may be used alone (EML, 2009; EMLC, 2009).

1.4.1.2 General pharmacopoeial information (Ph.Int., 2008)

Chemical name: 4-[(7-chloro-4-quinolyl)amino]-α-(diethylamino)-*o*-cresol dihydrochloride dihydrate; 4-[(7-chloro-4-quinolinyl)amino]-2-[(diethylamino)-methyl]phenol dihydrochloride dihydrate

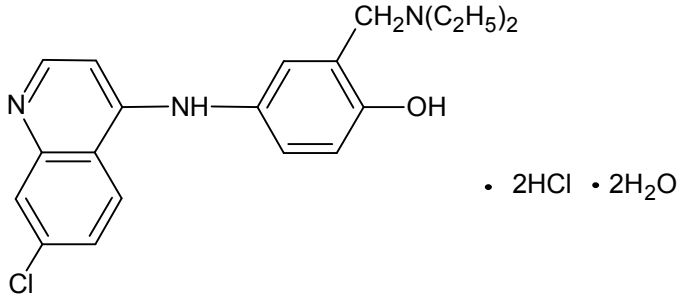
Molecular formula: $C_{20}H_{22}ClN_3O,2HCl,2H_2O$

Description: A yellow, odourless, crystalline powder

Solubility: Soluble in about 22 parts of water; sparingly soluble in ethanol (~750 g/l) TS; practically insoluble in ether R

Relative molecular mass: 464.8

Molecular structure:



Storage conditions: Amodiaquine HCl should be kept in a tightly closed container.

1.4.1.3 Pharmacological properties

Amodiaquine has shown to inhibit haemoglobin proteolysis and to interfere with the accumulation of an insoluble polymer, haemozoin, by forming a complex with ferriprotoporphyrin IX. This API also inhibits the glutathione-dependent destruction of ferriprotoporphyrin IX, resulting in the accumulation of this toxic peptide (Aweeka & German, 2008:91).

Amodiaquine is administered orally as the hydrochloride, but doses are expressed in terms of the equivalent amodiaquine base. Amodiaquine HCl 260 mg is equivalent to 200 mg of amodiaquine base.[3] For the treatment of *falciparum* malaria and uncomplicated chloroquine-resistant *vivax* malaria, a total dose of 30 mg/kg is administered over 3 days (10 mg/kg daily for 3 days) (Sweetman, 2008).

1.4.2 SULFADOXINE

1.4.2.1 Introduction

Sulfadoxine is a long acting sulfonamide that has been used in the treatment of various infections (Sweetman, 2009). It has a structure similar to p-aminobenzoic acid (Aweeka & German, 2008:94).

[3] The proposed monograph for Amodiaquine tablets will also indicate that 153 mg of amodiaquine is equivalent to approximately 200 mg of amodiaquine HCl.

1.4.2.2 General pharmacopoeial information (Ph.Int., 2008)

Chemical name: N^1-(5,6-Dimethoxy-4-pyrimidinyl)sulfanilamide; 4-amino-*N*-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide

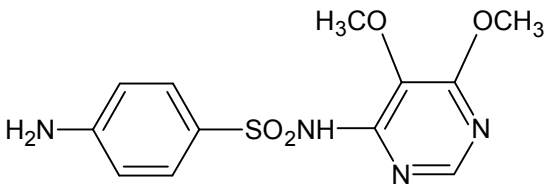
Molecular formula: $C_{12}H_{14}N_4O_4S$

Description: A white or creamy-white, odourless, crystalline powder

Solubility: Very slightly soluble in water; slightly soluble in ethanol (~750 g/l) TS and methanol R; practically insoluble in ether R

Relative molecular mass: 310.3

Molecular structure:



Storage conditions: Sulfadoxine should be kept in a well-closed container, protected from light.

1.4.2.3 Pharmacological properties

Sulfadoxine is a competitive inhibitor of dihydropropeteroate synthase (Aweeka & German, 2008:94). It interferes with the synthesis of nucleic acids in sensitive micro-organisms, by blocking the conversion of p-aminobenzoic acid into the coenzyme, dihydrofolic acid, a reduced form of folic acid. Its action is primarily bacteriostatic. It has a broad spectrum of action, but due to widespread resistance, its usefulness has greatly been reduced (Sweetman, 2009).

It is administered in a fixed-dose combination of 20 parts of sulfadoxine with 1 part of pyrimethamine in the treatment of *falciparum* malaria, being resistant to other therapies, usually after a course of quinine (Sweetman, 2009).

The usual oral dose is 1.5 g of sulfadoxine with 75 mg of pyrimethamine as a single dose – this should not be repeated for at least 7 days. Oral doses for children are:

- 5 - 10 kg body weight: 250 mg sulfadoxine with 12.5 mg pyrimethamine;
- 11 - 20 kg body weight: 500 mg sulfadoxine with 25 mg pyrimethamine;
- 21 - 30 kg body weight: 750 mg sulfadoxine with 37.5 mg pyrimethamine; and
- 31 - 45 kg body weight: 1 g sulfadoxine with 50 mg pyrimethamine (Sweetman, 2009).

1.4.3 PYRIMETHAMINE

1.4.3.1 Introduction

Pyrimethamine is a diaminopyrimidine antimalarial, used with a sulfonamide in the treatment of malaria and toxoplasmosis (Sweetman, 2009).

1.4.3.2 General pharmacopoeial information (Ph.Int., 2008)

Chemical name: 2,4–Diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine; 5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine

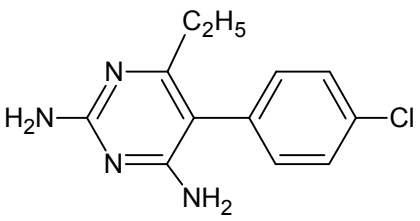
Molecular formula: $C_{12}H_{13}ClN_4$

Description: A white, odourless, crystalline powder

Solubility: Practically insoluble in water; slightly soluble in ethanol (~750 g/l) TS and in acetone R

Relative molecular mass: 248.7

Molecular structure:



Storage conditions: Pyrimethamine should be kept in a well-closed container, protected from light.

1.4.3.3 Pharmacological properties

Pyrimethamine exerts its antimalarial activity, by inhibiting plasmodial dihydrofolate reductase, thus indirectly blocking the synthesis of nucleic acids in the malaria parasite. It is active against pre-erythtocytic forms and it is also a slowly acting blood schizontocide (Sweetman, 2009).

Due to the development of plasmodial resistance, pyrimethamine is no longer being used alone in the treatment of malaria (Sweetman, 2009).

For the treatment of uncomplicated malaria, pyrimethamine is administered orally with sulfadoxine, in a fixed dose ratio of 1:20 (refer to 1.4.2.3) (Sweetman, 2009).

1.4.4 MEFLOQUINE HYDROCHLORIDE

1.4.4.1 Introduction

Mefloquine is a 4-methanolquinoline antimalarial, related to quinine (Sweetman, 2009).

1.4.4.2 General pharmacopoeial information (Ph.Int., 2008)

Chemical name: DL-*erythro*-α-2-piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol monohydrochloride; (*R*,S**)-(±)-α-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol monohydrochloride

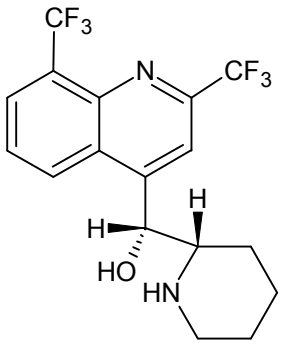
Molecular formula: $C_{17}H_{16}F_6N_2O,HCl$

Description: A white to slightly yellow, crystalline powder

Solubility: Very slightly soluble in water; freely soluble in methanol R; soluble in ethanol (~750 g/l) TS, sparingly soluble in dichloromethane R

Relative molecular mass: 414.8

Molecular structure:



• HCl and enantiomer

Storage conditions: Mefloquine HCl should be kept in a tightly closed container, protected from light.

1.4.4.3 Pharmacological properties

Mefloquine is a blood schizontocide that is effective against all forms of malaria, including chloroquine-, or multidrug resistant strains of *P. falciparum*, although some strains are naturally resistant to mefloquine (Sweetman, 2009).

Mefloquine is administered orally as the hydrochloride. For the treatment of malaria, mefloquine base 20 - 25 mg/kg (maximum of 1.5 g) as a single dose, or preferably in 2 or 3 divided doses, 6 - 8 hours apart, is recommended. For the prophylaxis of malaria, a dose of mefloquine base 250 mg, once weekly in adults and children over 45 kg, is recommended. The prophylactic dose for children is:

- 5-19 kg body weight: one quarter of the adult dose;
- 20-30 kg body weight: half of the adult dose; and
- 31-45 kg body weight: three quarters of the adult dose (Sweetman, 2009).

Prophylaxis should be started 1 - 3 weeks before exposure and continued for 4 weeks, after leaving the malaria area (Sweetman, 2009).

1.5 CONCLUSION

In this chapter, the risk that malaria poses worldwide, was introduced. The importance of effective, affordable testing methods, to ensure the safety and efficacy

of quality medicines to the public, was discussed. Due to a backlog of monographs for priority diseases, this study was undertaken to assist in the WHO's request for developing and establishing trustworthy test monographs for three commercial, antimalarial products, namely amodiaquine tablets, sulfadoxine/pyrimethamine combination tablets and mefloquine tablets. The general pharmacopoeial information as given in the Ph.Int., as well as the pharmacological properties of the four APIs that are present in the finished products, were presented.

During this study, new and existing analytical methods for identification, assay testing, dissolution testing and related substances testing were developed, evaluated and validated for four antimalarial APIs in these three commercial products, for submission to the WHO for possible inclusion in the Ph.Int., as dosage form monographs. The results of these studies are presented and discussed in the following chapters.

CHAPTER 2

MONOGRAPH
AMODIAQUINE TABLETS[1]

2.1 INTRODUCTION

According to Olliaro & Mussano (2003), controversy on the efficacy of amodiaquine caused it to be deleted from the World Health Organization (WHO) Essential Drug List (EDL) in 1979 and to be reinstated in the same year. In 1988 it was deleted from WHO EDL again, only to be reinstated again in 1996.

According to the WHO Model List of Essential Medicines (EML) and the WHO Model List of Essential Medicines for Children (EMLC), a dose of 153 mg amodiaquine (equivalent to 200 mg amodiaquine HCl), should be used in combination with 50 mg artesunate. For the treatment of *P. vivax, P. ovale* and *P. malariae*, it may be used alone (EML, 2009; EMLC, 2009).

The innovator product, Flavoquine®, manufactured by Roche Pharmaceuticals, is labelled as containing 153 mg amodiaquine per tablet. Generic products available on the market, with higher active contents per tablet, are labelled as either 300 mg or 600 mg amodiaquine per tablet (table 2.12).

2.2 AIM

For the purposes of this study, analytical methods will be developed for the determination of:

i. The active pharmaceutical ingredient (API), i.e. amodiaquine HCl, also called the assay method, and

[1] Amodiaquine tablets contain amodiaquine hydrochloride as the active

ii. The percentage API being dissolved during dissolution testing. Dissolution acceptance criteria will also be set, based on results obtained for commercially available tablets.

Other tests currently included in the final monograph of amodiaquine tablets (Annexure A), were not part of this investigation.

2.3 ANALYTICAL METHODS

A monograph for amodiaquine hydrochloride tablets is published in the United States Pharmacopeia (USP, 2008; USP, 2010). The monograph includes testing for identification, dissolution, uniformity of dosage units and assay.

The assay method being described in the 2008 USP for amodiaquine HCl, involves a lengthy liquid-liquid extraction procedure with chloroform and diluted hydrochloric acid, before the final solution is analysed spectrophotometrically (USP, 2008). The assay method was later revised to a more simple method, involving direct extraction from the tablet powder. Samples are analysed on the HPLC, utilising buffer and methanol as mobile phase on a C_{18} column, containing 5 µm packing (USP, 2010).

The dissolution test is performed in 900 ml water with paddles rotating at 50 revolutions per minute (rpm). A Q-value of 75% is specified after 30 minutes. The samples are analysed spectrophotometrically at 342 nm (USP, 2008). The dissolution method remained unchanged in the later edition (USP, 2010).

Rao *et al.* (1982:777) and Sanghi *et al.* (1990:333) describe similar methods of analyses, involving the treatment of amodiaquine HCl with periodate to yield a chromogen that can be determined spectrophotometrically at about 442 nm.

A number of high performance liquid chromatographic (HPLC) methods for the determination of amodiaquine in blood samples appear in literature (e.g. Minzi *et al.,* 2003b:475; Bell *et al.,* 2007:233).

HPLC methods for the determination of amodiaquine in dosage forms are described by Sanghi *et al.* (1990:333) and by Phadke *et al.* (2008:1003). Sanghi *et al.* (1990:334) also published a method to simultaneously determine amodiaquine,

primaquine and chloroquine on a C_{18} column. The method by Phadke *et al.* (2008:1005) was developed for amodiaquine and artesunate combination products.

2.4 ASSAY

2.4.1 REFERENCE METHOD

The WHO supplied a reference method for use in this study that had been obtained from a manufacturer. The parameters were as follows:

Column: 150 x 4.6 mm, C_{18}

Mobile phase: **Solvent A** (380 ml) : **Solvent B** (620 ml)

Solvent A: Transfer 1.36 g of KH_2PO_4 into a 1000 ml volumetric flask, add about 600 ml of water[2], then add 1.4 ml of triethylamine. Mix and fill up to volume with water. Adjust pH to 9.0 with a 1M KOH solution.

Solvent B: Methanol

Injection volume: 20 µl

Detection wavelength: 254 nm

Flow rate: 1 ml/min

This reference method was evaluated and used for the development of a refined assay method and submitted for possible inclusion in the Ph.Int. monograph. The monograph (Annexure A) was adopted during the 44th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (12-16 October 2009, Geneva).

2.4.2 DEVELOPMENT STUDIES

Since the development studies had originally been started on a different brand HPLC system, the retention times of the peaks may differ between the various studies as described in this section.

[2] Purified water prepared by reverse osmosis with a resistivity of at least 18 MOhm.

2.4.2.1 Resolution solution

A resolution test is considered important for any pharmacopoeial assay method. Since the reference method did not include a resolution test, it had to be developed.

Four APIs, namely chloroquine sulfate, chloroquine phosphate, primaquine HCl and mefloquine HCl were identified as possible substances in setting a resolution test, based on these APIs being readily available, also as commercial materials, and because of their similar chemical structures.

Primaquine eluted very close to the solvent peak group at approximately 3 minutes, relative to amodiaquine at approximately 12 minutes, whilst interferences from the solvent peak group were detected. A representative chromatogram is given in figure 2.1.

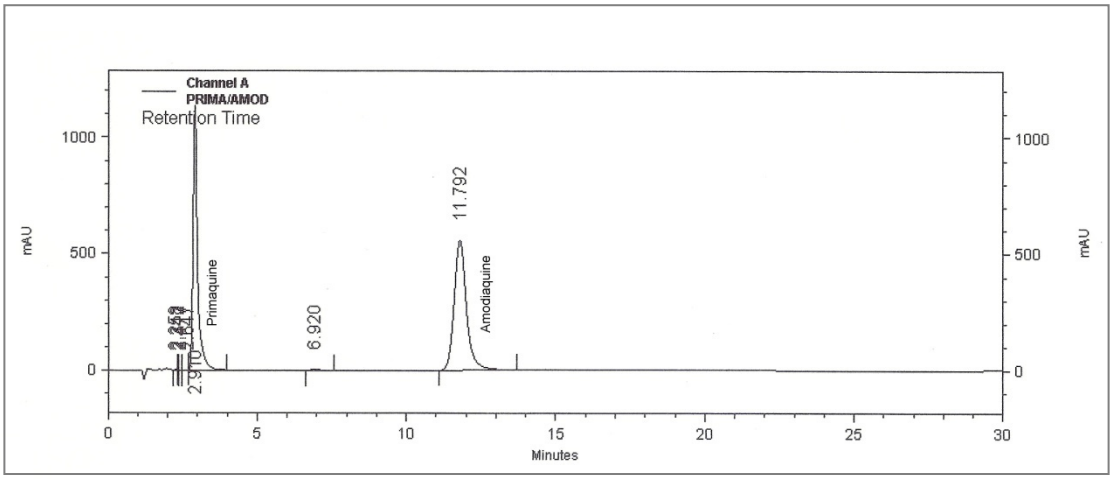


Figure 2.1: Example of a chromatogram of primaquine HCl and amodiaquine HCl.

Mefloquine eluted later than amodiaquine HCl at approximately 28 minutes, relative to amodiaquine at approximately 11 minutes. Apart from a very long run time, the amodiaquine peak shape was unacceptable. A representative chromatogram is given in figure 2.2.

Chloroquine sulfate proved to be the substance of choice, since it eluted earlier than amodiaquine HCl at approximately 11 minutes, relative to amodiaquine at approximately 17 minutes (relative retention time = 0.63). The peaks of the two APIs were acceptable and comparable with respect to shape and size. A representative chromatogram is given in figure 2.3.

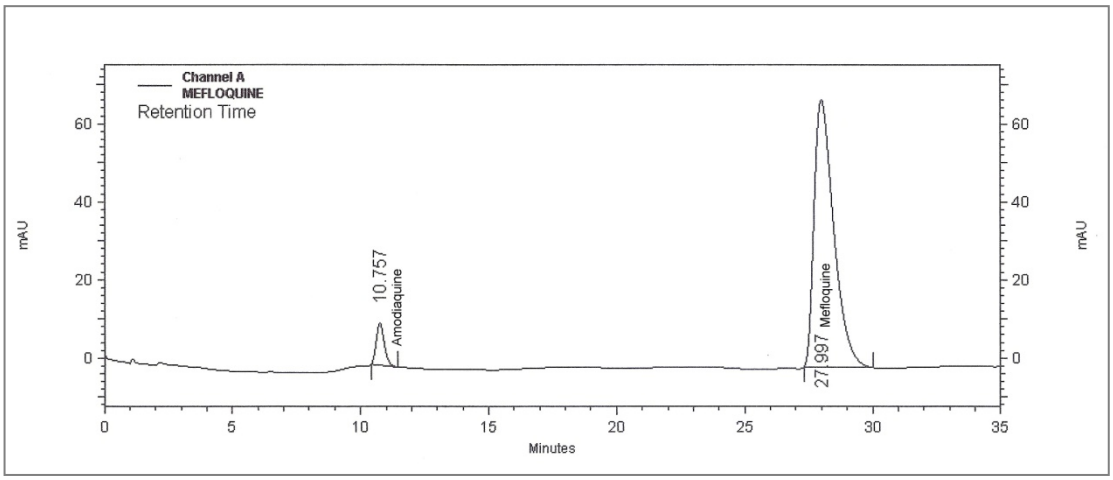


Figure 2.2: Example of a chromatogram of mefloquine HCl and amodiaquine HCl.

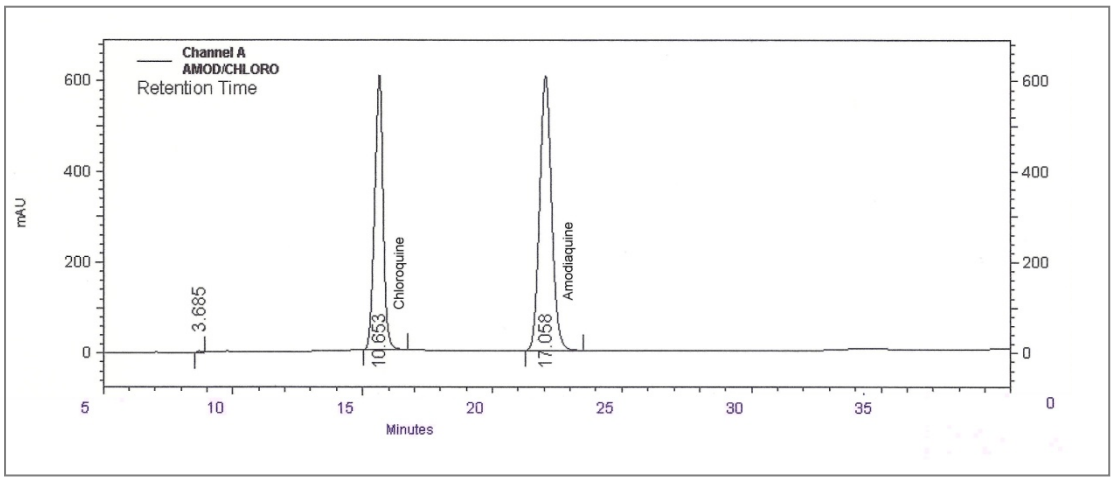


Figure 2.3: Example of a chromatogram of chloroquine sulfate and amodiaquine HCl.

As was expected from the mobile phase being buffered at pH 9.0, test results with chloroquine phosphate showed that it could be used as an alternative for chloroquine sulfate, as both eluted at the same time.

The chloroquine sulfate was selected for the resolution test, with the resolution factor between the chloroquine sulfate and amodiaquine HCl peaks being set at not less than 3.5.

The resolution solution was prepared as follows:

i. Weigh approximately 25 mg each of chloroquine sulfate and amodiaquine HCl and transfer into a 50 ml volumetric flask with about 40 ml of water.

ii. Sonicate for 5 minutes.

iii. Fill up to volume with water.

iv. Dilute 3 ml of the solution to 10 ml and fill up to volume with water to obtain a concentration of approximately 150 µg/ml for both APIs.

2.4.2.2 Solvent

Four possible solvents were tested for use in the preparation of the test, reference and resolution solutions, i.e. (A) the mobile phase, (B) a mixture of 50:50 water and methanol, (C) 0.1M hydrochloric acid (HCl), and (D) water.

A. Mobile phase

Initially the mobile phase was considered as the solvent of choice, since it had been used as solvent in the reference method. However, after standing for a period of approximately 24 hours, a precipitate was noticed in the reference solution. A sample was filtered and the precipitate dissolved in methanol and analysed on the HPLC, using the reference method. The retention time of the peak was the same as that of the original sample and the reference, thus indicating that the precipitate was in fact amodiaquine. It thus appeared that, at the concentration being used in the sample and reference solutions, the free base of the API was inadequately soluble in mobile phase to remain in solution.

B. 50% Water : Methanol

A 50:50 water and methanol mixture caused fronting of the chloroquine sulfate peak and was accordingly considered unacceptable as a solvent (figure 2.4). This solvent did not show any significant influence on the peak shape of the amodiaquine.

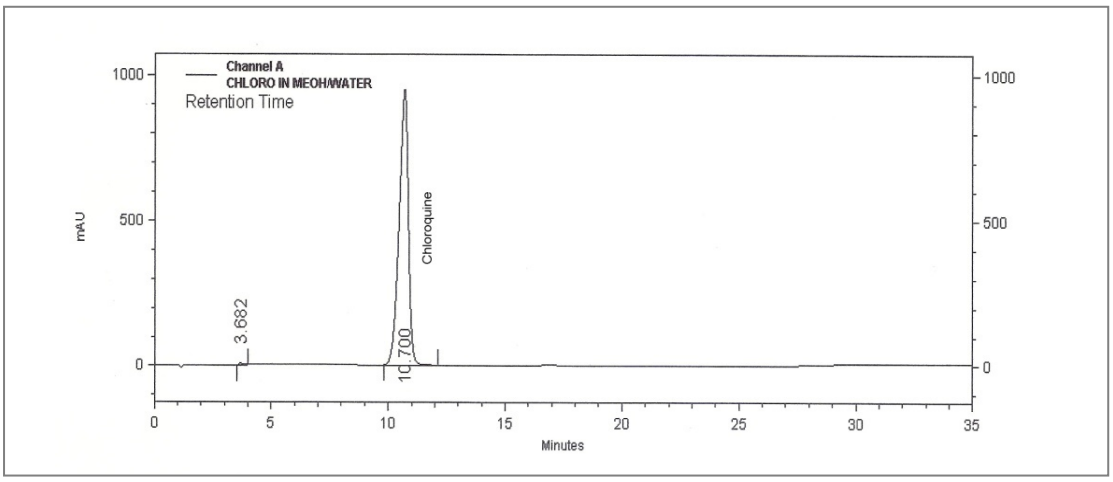


Figure 2.4: Example of a chromatogram of chloroquine in a 50:50 mixture of methanol and water.

C. 0.1M HCl

This solvent caused interfering solvent peaks and was considered unacceptable as a solvent (figure 2.5). The peak group eluted at about 8 minutes.

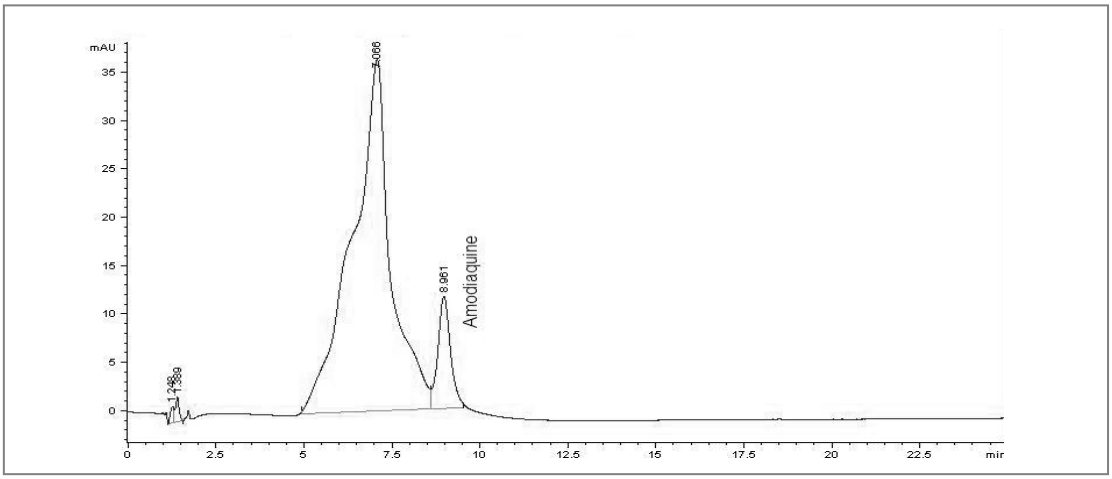


Figure 2.5: Example of a chromatogram of amodiaquine HCl in 0.1M HCl.

D. Water

Water did not cause any interference during analysis (figure 2.6). In addition, the solubility of amodiaquine HCl (one part in 22 parts) and chloroquine sulfate (freely soluble) in water (WHO, 2008), convincingly made water the solvent of choice.

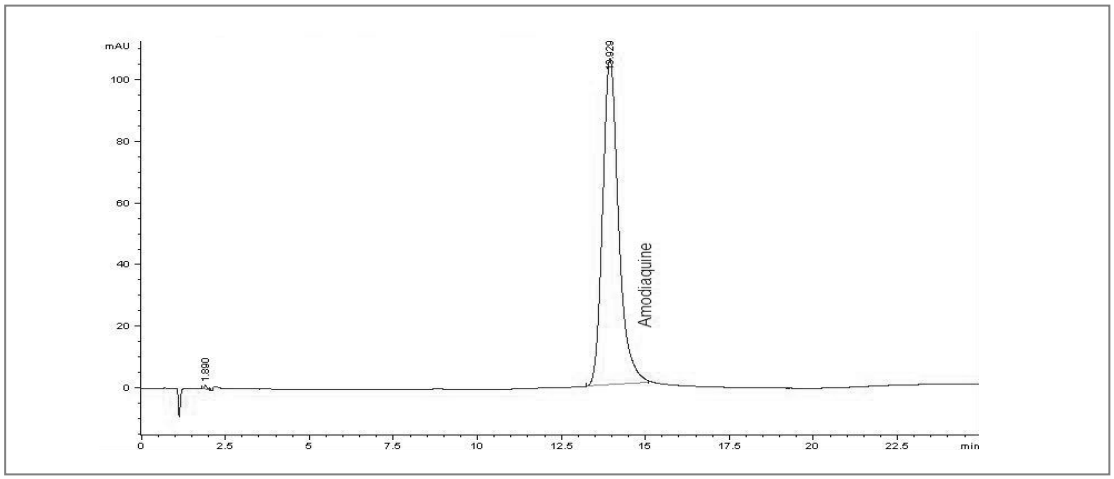


Figure 2.6: Example of a chromatogram of amodiaquine HCl in water.

The reference solution was thus prepared as follows:

i. Accurately weigh approximately 25 mg of amodiaquine HCl and quantitatively transfer into a 50 ml volumetric flask with 40 ml of water.

ii. Sonicate for 5 minutes.

iii. Fill up to volume with water (stock solution).

iv. Dilute 3 ml of the stock solution to 10 ml with water and fill up to volume with water to obtain a concentration of approximately 150 µg/ml amodiaquine HCl.

2.4.2.3 Flow rate

Flow rates of 1 ml/min and 1.5 ml/min were evaluated. A flow rate of 1 ml/min resulted in a retention time of approximately 17 minutes for amodiaquine HCl, whilst a flow rate of 1.5 ml/min produced a retention time of approximately 13 minutes. The shortened analyses time at 1.5 ml/min was preferable.

2.4.2.4 Detection wavelength

Literature (Moffat *et al.,* 2004:631) indicates peaks of maximum absorbance for amodiaquine HCl in aqueous acid at 237 nm, and in aqueous alkali at 273 nm and 287 nm (figure 2.7).

In this study a USP amodiaquine HCl reference standard was dissolved separately in 0.1M HCl, in 0.1M sodium hydroxide and in water. The ultraviolet (UV) spectra of the three solutions were recorded (figure 2.8).

The UV spectrum (figure 2.8) of amodiaquine HCl in water exhibited peaks of maximum absorbance at approximately 224 nm and 345 nm.

It was also decided to investigate absorbance at a wavelength of 254 nm, as specified in the reference method. In order to determine the wavelength of choice, linearity studies were performed at 224 nm and 254 nm. Results of the linearity studies are presented in tables 2.1 – 2.2 and are represented graphically in figures 2.9 and 2.10, respectively.

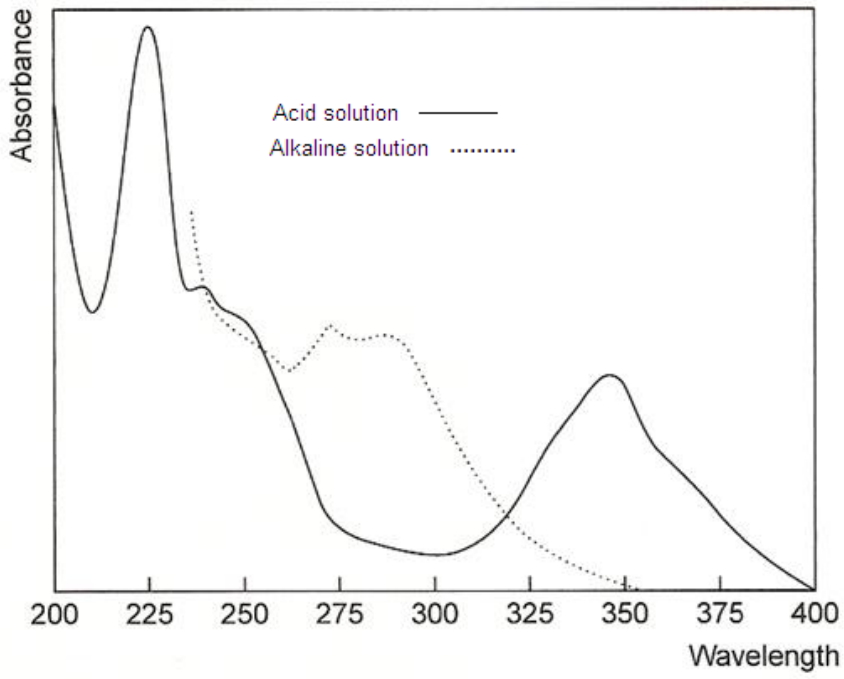


Figure 2.7: UV spectra of amodiaquine HCl in acidic and alkaline solvents (Moffat *et al.*, 2004:631).

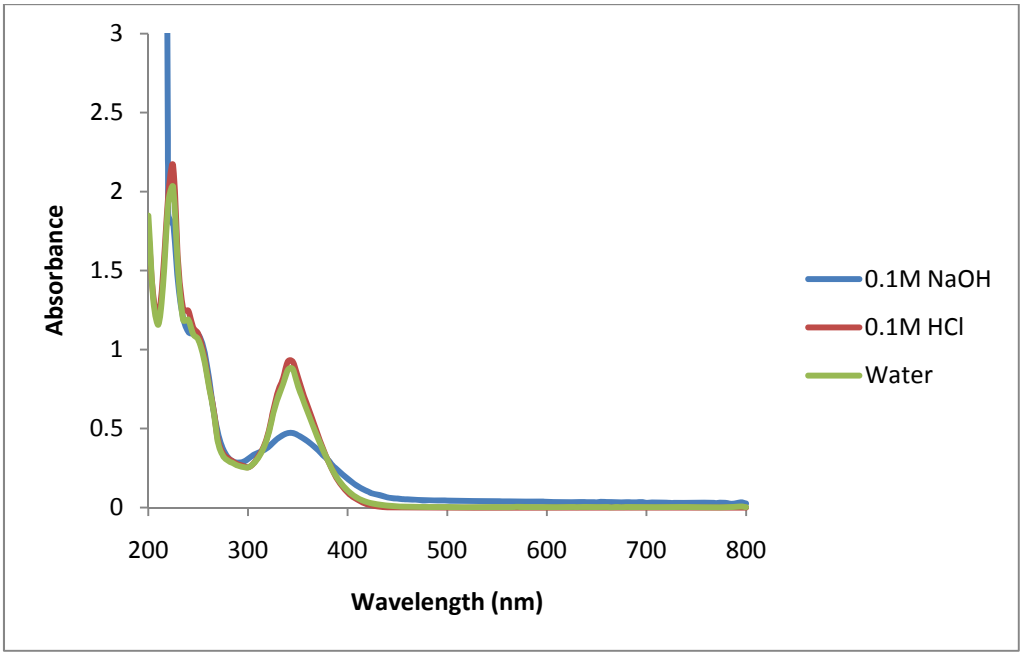


Figure 2.8: UV spectra of amodiaquine HCl in various solvents (obtained in-house).

Table 2.1: Peak areas of linear regression graph of amodiaquine at 224 nm

CONCENTRATION (µg/ml amodiaquine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
58.6	51.0	4003 4012 3997	4003	0.19
78.1	68.0	6144 6139 6148	6144	0.08
117.1	102.0	9104 9110 9086 9102 9110	9201	0.11
130.1	113.3	10322 10312 10340	10325	0.14
156.2	136.0	12309 12336 12308	12318	0.13

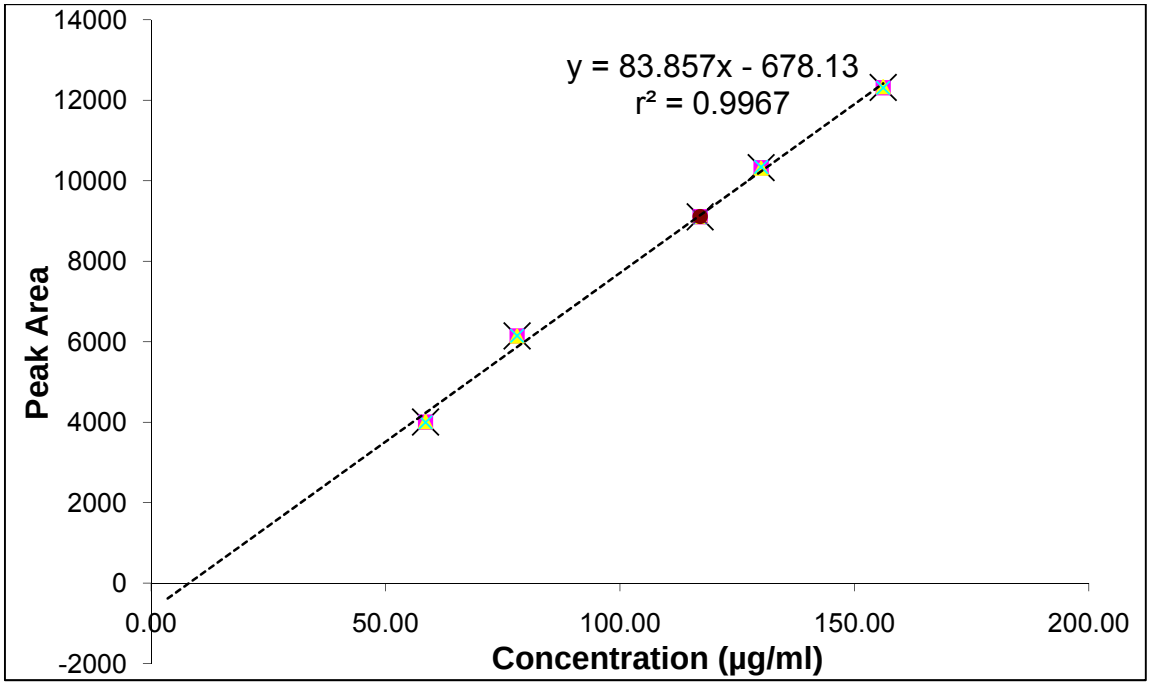


Figure 2.9: Linear regression graph of amodiaquine at 224 nm.

Table 2.2: Peak areas of linear regression graph of amodiaquine at 254 nm

CONCENTRATION (µg/ml amodiaquine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
58.6	51.0	1524 1523 1520	1522	0.14
78.1	68.0	2352 2347 2345	2349	0.17
117.1	102.0	3484 3488 3482 3486 3489	3486	0.07
130.1	113.3	3958 3955 3960	3958	0.07
156.2	136.0	4729 4737 4737	4734	0.10

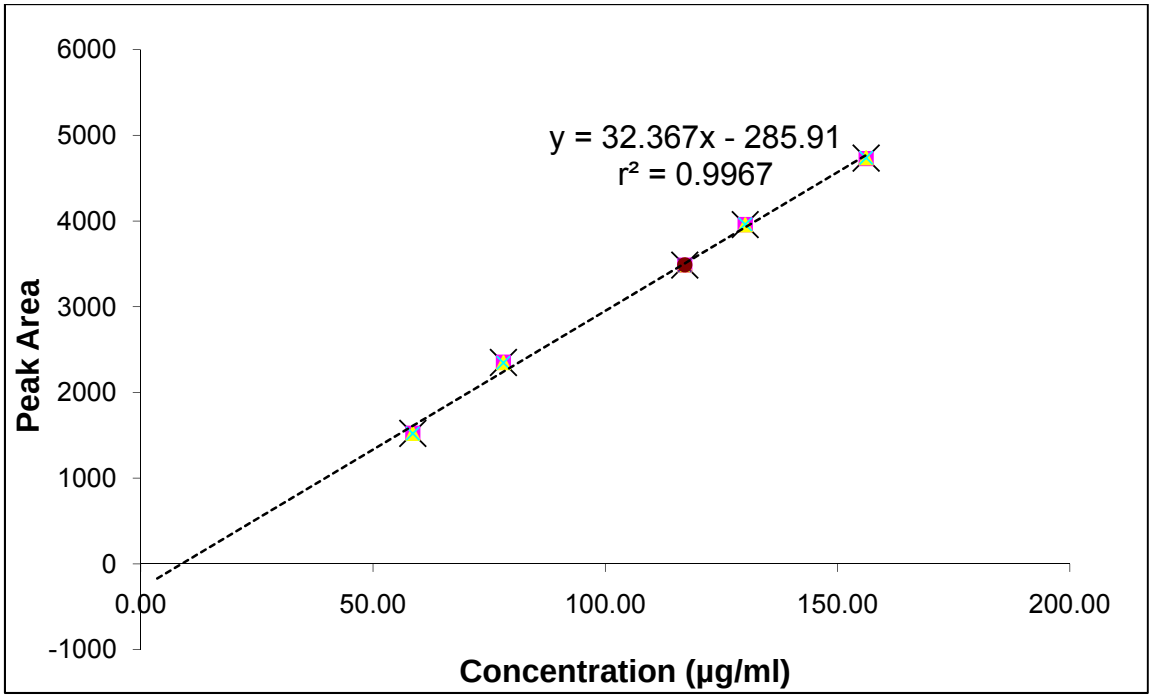


Figure 2.10: Linear regression graph of amodiaquine at 254 nm.

Results indicated that the peak areas obtained at 224 nm were approximately 2.6 times larger than those obtained at 254 nm. The %RSD for all concentrations at both wavelengths was excellent, with all values being below 0.2. These results thus indicated that both 224 nm and 254 nm were suitable detection wavelengths for analyses. It was decided to analyse at 254 nm, in accordance with the reference method, and in order to minimise the possibility of interferences from excipients and solvents.

2.4.2.5 Stability of reference solution

A. Storage and stress conditions

A 150 µg/ml reference solution was prepared in water, in 0.1M hydrochloric acid (HCl) and in a 10% peroxide solution. Samples were kept at room temperature (20 – 25ºC). Samples of each solution were analysed over a 17-hour period. The percentage difference, with reference to the initial peak area obtained, was calculated for each interval.

The solution prepared in 0.1M HCl exhibited very poor chromatography and the results could not be used (refer 2.4.2.2).

The results for the water and 10% peroxide solutions are shown in table 2.3.

Table 2.3: Stability of the amodiaquine reference solution in water and 10% peroxide solution over a period of 17 hours

	WATER		10% PEROXIDE	
TIME (hours)	**Peak area**	**% Difference**	**Peak area**	**% Difference**
Initial	3129.1	-	2177.9	-
6	3130.7	0.05	2051.5	-5.81
7.5	3137.0	0.25	2012.4	-7.60
9	3122.6	-0.21	1985.2	-8.85
10	3117.6	-0.37	1971.1	-9.49
12	3112.2	-0.54	1942.2	-10.82
13	3115.2	-0.44	1920.0	-11.84
14	3122.6	-0.21	1897.8	-12.86
16	3113.1	-0.51	1880.8	-13.64
17	3113.6	-0.50	1861.9	-14.51

The results showed that a 10% reduction in peak area occurred after 12 hours in the 10% peroxide solution. The API was thus sensitive towards oxidative conditions. No significant reduction in peak area occurred in the solution prepared in water. Extrapolation of the data being obtained in water, indicates that a reduction in peak area of 1.7% should be obtained after 48 hours if the rate of degradation was assumed to be linear.

A second test was performed to confirm the stability of the reference solution being prepared in water. A reference solution was prepared and analysed to determine the initial peak area. A sample of the same solution was analysed after 24 hours and 48 hours. The results indicated that a reduction in peak area of only 0.77% was obtained after 48 hours.

These results confirmed that the reference solution, when prepared in water, was stable for at least 48 hours.

B. Resolution solution

A resolution solution containing approximately 150 µg/ml chloroquine sulfate and amodiaquine HCl was prepared, as described in 2.4.2.1, and analysed over a period of 2 days. The results are shown in table 2.4.

Table 2.4: Stability of the resolution solution over a period of two days

	CHLOROQUINE		AMODIAQUINE		RESOLUTION FACTOR
TIME (days)	Peak area	% Difference	Peak area	% Difference	
Initial	1091.4	-	1272.3	-	5.9
Day 1	1078.4	-1.19	1267.4	-0.39	6.6
Day 2	1090.8	-0.05	1290.4	1.42	6.4

The results indicate that the resolution solution was stable for at least 48 hours, with no significant differences detected in the peak areas, nor the resolution factors between the peaks.

2.4.3 VALIDATION OF ANALYTICAL METHOD

Based on the development studies, the following parameters were selected for the assay of amodiaquine HCl in amodiaquine tablets:

Column: 150 x 4.6 mm, C_{18}, 5 µm (Phenomenex Gemini, or equivalent)

Mobile phase: **Solvent A** (380 ml) : **Solvent B** (620 ml).
Solvent A: Transfer 1.36 g of KH_2PO_4 into a 1000 ml volumetric flask, add about 600 ml of water, then add 1.4 ml of triethylamine. Mix and fill up to volume with water. Adjust pH to 9.0 with a 1M KOH solution.
Solvent B: Methanol

Injection volume: 20 µl

Temperature: Ambient (20 - 25ºC)

Flow rate: 1.5 ml/min

Detection wavelength: 254 nm

2.4.3.1 Equipment used in the validation studies

The equipment used for the HPLC analyses included:

- An Agilent Technologies® 1200 series HPLC system, with Chemstation® Software Revision A.10.02.
- Binary pump - Firmware Revision A.05.11
- Diode array detector - Firmware Revision A.05.11
- Column thermostat - Firmware Revision A.05.11
- Thermostatted autosampler - Firmware Revision A.05.11

2.4.3.2 Preparation of solutions

A. Resolution solution

i. Weigh approximately 25 mg of chloroquine sulfate and amodiaquine HCl and transfer into a 50 ml volumetric flask with about 40 ml water.

ii. Sonicate for 5 minutes.

iii. Fill up to volume with water.

iv. Dilute 3 ml of the solution to 10 ml and fill up to volume with water to obtain concentrations of approximately 150 µg/ml for both APIs (150 µg/ml amodiaquine HCl ≡ 115 µg/ml amodiaquine).

B. Reference solution

i. Accurately weigh approximately 25 mg amodiaquine HCl and quantitatively transfer into a 50 ml volumetric flask with about 40 ml water.

ii. Sonicate for 5 minutes.

iii. Fill up to volume with water (stock solution).

iv. Dilute 3 ml of the solution to 10 ml and fill up to volume with water to obtain a concentration of approximately 150 µg/ml amodiaquine HCl (≡115 µg/ml amodiaquine).

C. Sample solution

i. Weigh and powder 20 tablets.

ii. Accurately weigh tablet powder equivalent to 115 mg amodiaquine and quantitatively transfer into a 100 ml volumetric flask with about 70 ml water.

iii. Sonicate for 15 minutes.

iv. Fill up to volume with water (stock solution).

v. Filter a portion of the solution through a 0.45 µm filter, discarding the first 10 ml.

vi. Dilute the filtrate tenfold with water to obtain a concentration of approximately 115 µg/ml amodiaquine.

2.4.3.3 Validation parameters

The parameters as indicated in table 2.5 were evaluated during the validation study.

Table 2.5: Validation parameters and acceptance criteria required for assay validation studies (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA
Specificity	No interferences detected from solvents, nor excipients
Linearity	$r^2 \geq 0.99$
Range	80 – 120% of 100% theoretical concentration
Repeatability	%RSD ≤ 2 for 5 injections from same solution
Recovery	98.0 – 102.0%
Robustness	Investigate the effect of small, but deliberate changes to the method

A. Specificity

Solutions containing chloroquine sulfate and amodiaquine HCl were prepared in water to have a known concentration of about 150 µg/ml. These solutions and a solvent sample (water) were injected separately in order to determine retention times and possible interferences. Chloroquine sulfate eluted at approximately 9 minutes and amodiaquine HCl at approximately 14 minutes (figure 2.11).

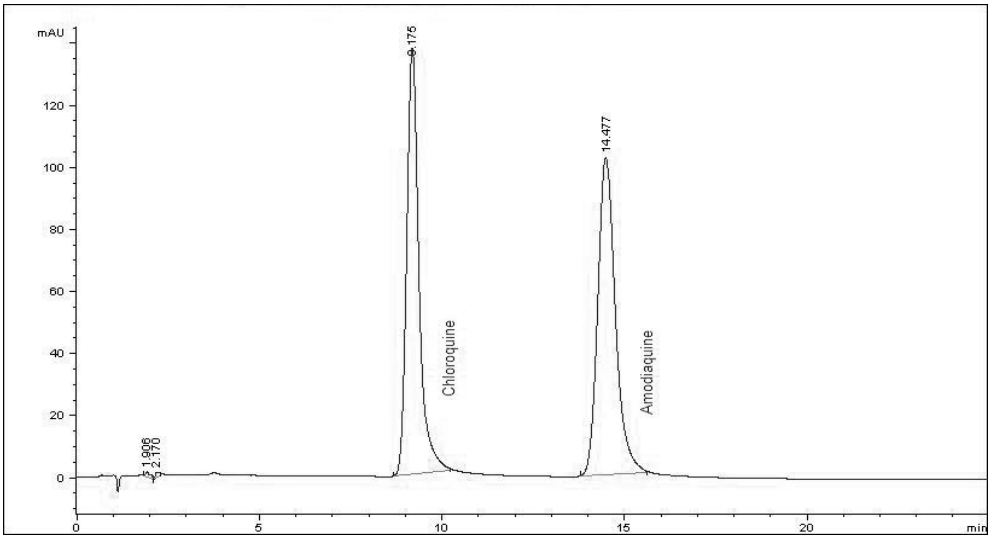


Figure 2.11: Example of a chromatogram of chloroquine sulfate and amodiaquine HCl in water.

Water as solvent caused no interference with the amodiaquine peak (figures 2.12 and 2.6), neither were interferences seen when amodiaquine was dissolved in the 10% peroxide solution (figure 2.13). The retention time of amodiaquine shifted from approximately 14 minutes (in water) to about 8 minutes in the 10% peroxide solution.

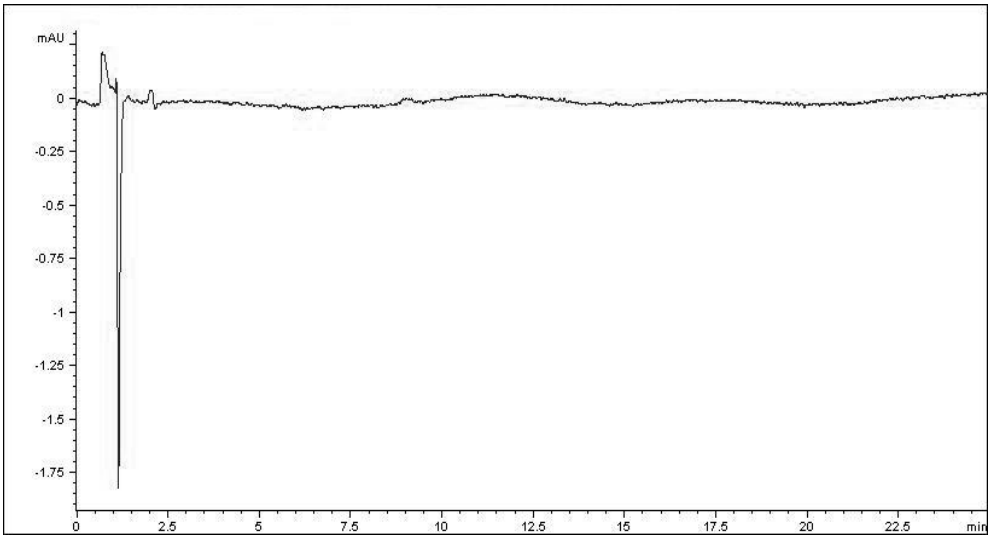


Figure 2.12: Example of a chromatogram of the solvent (water).

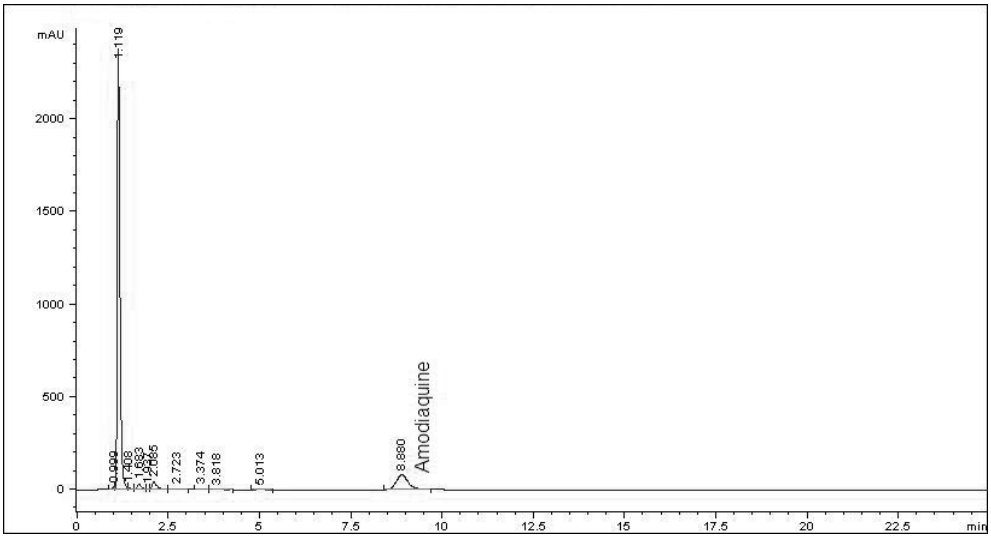


Figure 2.13: Example of a chromatogram of amodiaquine reference solution in a 10% peroxide solution.

The purity of the amodiaquine peak was evaluated by means of the diode array detector (figure 2.14).

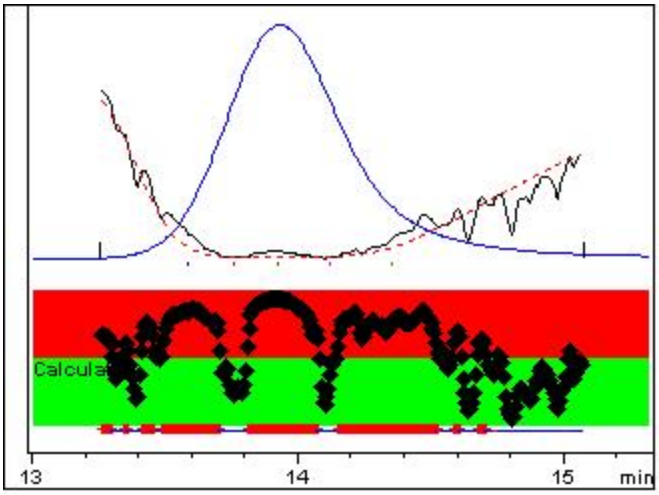


Figure 2.14: Peak purity profile of the amodiaquine peak in water.

B. Linearity and range

The 100% theoretical concentration was taken as 115 µg/ml amodiaquine, targeting the concentration of the sample solution at a 100% label claim. Five different amodiaquine HCl reference solutions, covering the range of 50 - 136% of the 100% theoretical concentration, were prepared by diluting the reference stock solution (refer 2.4.3.2) with water (table 2.6).

Results are given in table 2.2 and are graphically represented in figure 2.10.

Table 2.6: Dilutions of amodiaquine HCl reference stock solution used in assay linearity studies

SOLUTION	DILUTION	TARGET CONCENTRATION (µg/ml)
1	3 ml to 20 ml	75
2	3 ml to 15 ml	100
3	3 ml to 10 ml	150
4	5 ml to 15 ml	167
5	4 ml to 10 ml	200

A regression analyses was performed on the results obtained for the linearity studies.

The r^2 value was 0.9967, with an overall uncertainty of 3.4 µg/ml. A linear graph was hence produced in the concentration range of 58.6 – 156.2 µg/ml. The equation of the line is:

$$y = 32.367x – 285.91$$

C. Repeatability

Reference solution 3 (representing the 100% concentration), as prepared for the linearity study, was used in the repeatability study, and was injected 5 times.

The %RSD of the injections was 0.07 (table 2.2), thus indicative of excellent repeatability.

D. Recovery

A recovery solution, containing approximately 115 µg/ml amodiaquine was prepared similarly to the reference solution (refer 2.4.3.2) and analysed. The peak areas obtained were used to calculate the concentration of the solution by means of the equation given in 2.4.3.3 B. The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

A recovery of 102.0% was obtained. A recovery of 98.0 – 102.0% is deemed acceptable. The method thus complied with the requirements.

E. Robustness

For the purpose of reproducibility, different types of columns were tested and the mobile phase ratio adjusted. Commercial products were analysed to ascertain the suitability of the method for product analyses.

i. Columns

Three different types of C_{18}, 150 x 4.6 mm, 5 µm columns were used to determine the effect of different brands of columns on the resolution factor between the chloroquine and amodiaquine peaks (table 2.7).

Table 2.7: Resolution factors of chloroquine and amodiaquine peaks using different brands of columns

BRAND NAME	RESOLUTION FACTOR
Phenomenex Luna	4.2
Zorbax Eclipse	3.9
Phenomenex Gemini	7.8

From the results it can be seen that the brand of the column influenced the resolution between the peaks. It was decided to set a resolution factor of ≥ 3.5 as acceptance value.

It should be noted that some column brands would be unsuitable for prolonged use, as the pH of the mobile phase is 9.0.

ii. Change in mobile phase composition

The mobile phase ratio was adjusted in order to determine its effect on the time of elution of the individual peaks, as well as on the resolution between the peaks in the

resolution solution (refer 2.4.3.2). A Phenomenex Gemini column was used for this study. The ratios used are indicated in table 2.8.

The results are shown in table 2.9. These results represent the average of five injections.

Table 2.8: Ratios of mobile phases used in robustness studies

% ABSOLUTE CHANGE (in terms of Solvent A)	SOLVENT A VOLUME (ml) Buffer	SOLVENT B VOLUME (ml) Methanol
-10	340	660
-5	360	640
0	380	620
+5	400	600
+10	420	580

Table 2.9: Effect of a change in mobile phase composition on retention time, tailing and resolution factor of chloroquine and amodiaquine peaks

	CHLOROQUINE		AMODIAQUINE		
MOBILE PHASE RATIO	Retention time (min)	Tailing	Retention time (min)	Tailing	Resolution factor
340**A**:660**B**	5.1	1.3	6.3	1.3	2.9
360**A**:640**B**	5.5	1.4	7.3	1.4	3.5
380**A**:620**B**	5.8	1.5	8.2	1.5	3.9
400**A**:600**B**	8.5	2.0	13.3	1.6	4.0
420**A**:580**B**	10.9	1.8	17.9	1.5	4.7

Mobile phases containing 400 ml or more of Solvent A (buffer) caused the retention times of both chloroquine and amodiaquine to increase significantly. Larger volumes of buffer (≥ 400 ml) influenced the tailing factor of chloroquine negatively, whilst the amodiaquine peak was affected insignificantly.

Mobile phases containing ≤ 380 ml of Solvent A had no significant effect on the retention time of the chloroquine peak, but caused a decrease in amodiaquine retention time. Since the volume of Solvent A influenced the retention time of

amodiaquine more than that of chloroquine, the reduction of solvent A had a negative influence on the resolution between the peaks (table 2.9).

iii. Mobile phase pH

The pH of the mobile phase was adjusted by ± 0.2 pH units, from pH 9.0 to 8.8 and 9.2 respectively, in order to determine the effect on the retention times, tailing factors and resolution factor between peaks of the resolution solution (refer 2.4.3.2). The results are given in table 2.10.

Table 2.10: Effect of a change in mobile phase pH on retention time, tailing and resolution factor of chloroquine and amodiaquine peaks

	CHLOROQUINE		AMODIAQUINE		
pH	**Retention time (min)**	**Tailing**	**Retention time (min)**	**Tailing**	**Resolution factor**
9.2	6.9	1.9	9.4	1.5	2.8
9.0	5.8	1.5	8.2	1.5	3.9
8.8	6.6	1.8	9.1	1.5	2.9

Increasing and decreasing of the pH from 9.0 by 0.2 units resulted in longer retention times for both chloroquine and amodiaquine peaks, with the larger relative influence on the first eluting chloroquine sulfate peak, hence decreasing the resolution factor of the two peaks by a factor of approximately one in both cases, relative to the original pH. The pH of the mobile phase can thus be considered very important for successful analyses.

iv. Product analyses/assays

After successful validation of the analytical method, it was used to perform assay analyses of five commercial product batches of amodiaquine tablets. These assays were performed in 2008. The product details are given in table 2.11.

Sample solutions were prepared in triplicate, according to the method described in 2.4.3.2, and each result is the average of the three determinations. The %RSD was calculated for the three determinations.

Three products were analysed by a second analyst to verify the reproducibility/intermediate precision of the method.

Table 2.11: Details of commercial amodiaquine tablets used in assay analyses

PRODUCT NAME	MANUFACTURER	LABEL CLAIM (amodiaquine/tablet)	BATCH NO	EXPIRY DATE
Flavoquine	Roche Pharmaceuticals	153 mg	418	10/2009
Camosunate Junior	Danpong-Adams Pharmaceutical Industry (Ghana)	300 mg	0507019	7/2008
Camosunate Plus	Danpong-Adams Pharmaceutical Industry (Ghana)	600 mg	0510073	10/2008
Artekamoc	XS Laboratories, (India)	300 mg	E962	8/2008
Gsunate Kit	GVS Lab (India)	600 mg	G1-01	1/2009

Results of the assay analyses are given in table 2.12. Representative chromatograms of all products are shown in figures 2.15 – 2.19.

Table 2.12: Assay results of commercial amodiaquine tablets

PRODUCT	ASSAY VALUE		%RSD
	% Assay	mg amodiaquine/tablet	
Flavoquine	98.5	150.8	0.40
Camosunate Junior	97.9	293.8	0.79
Camosunate Plus	98.1	588.7	0.21
Artekamoc	99.6	298.7	1.07
Gsunate Kit	94.4	566.3	1.26

From the results obtained it is clear that the method was suitable for performing assays on the amodiaquine products. The %RSD for all products was below 1.3%, indicating that the method of preparation of the samples did not give rise to significant differences in outcomes.

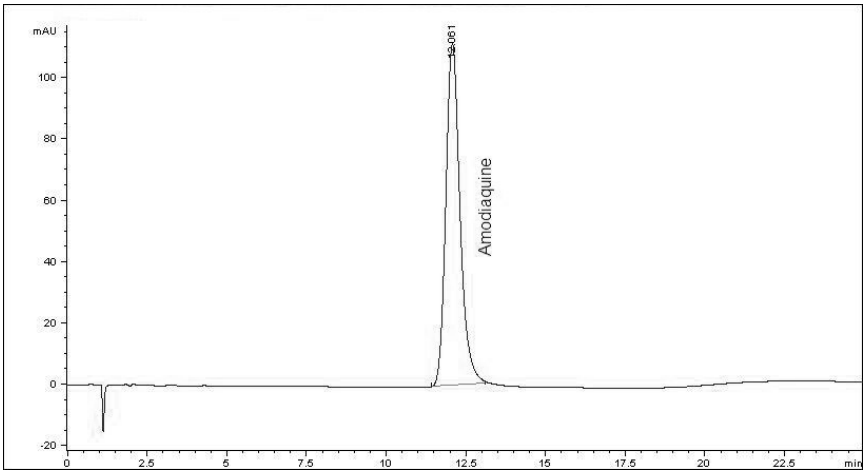


Figure 2.15: Example of a chromatogram obtained for Flavoquine tablets.

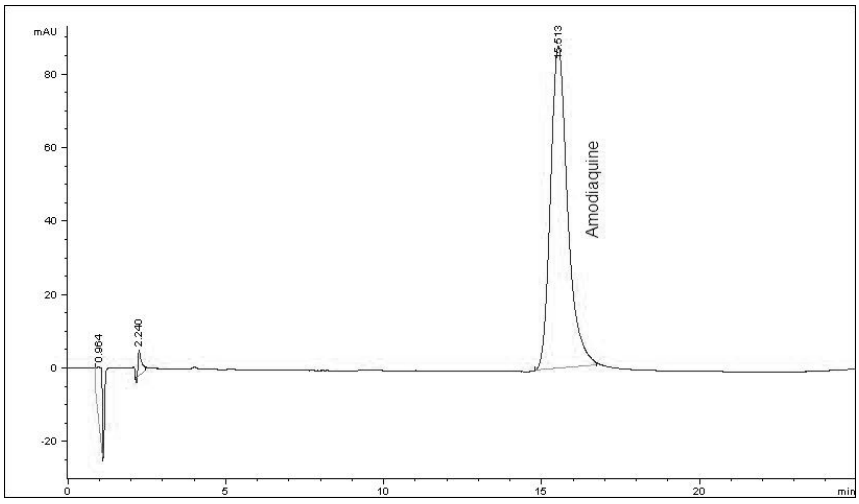


Figure 2.16: Example of a chromatogram obtained for Camosunate Junior tablets.

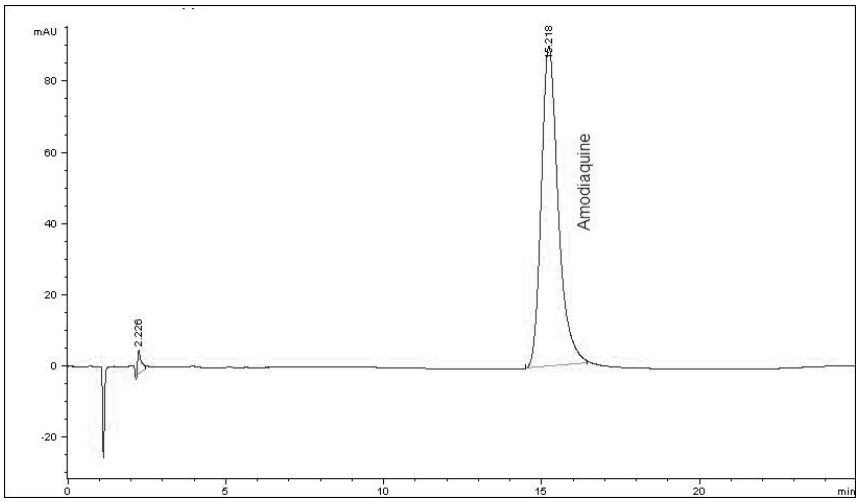


Figure 2.17: Example of a chromatogram obtained for Camosunate Plus tablets.

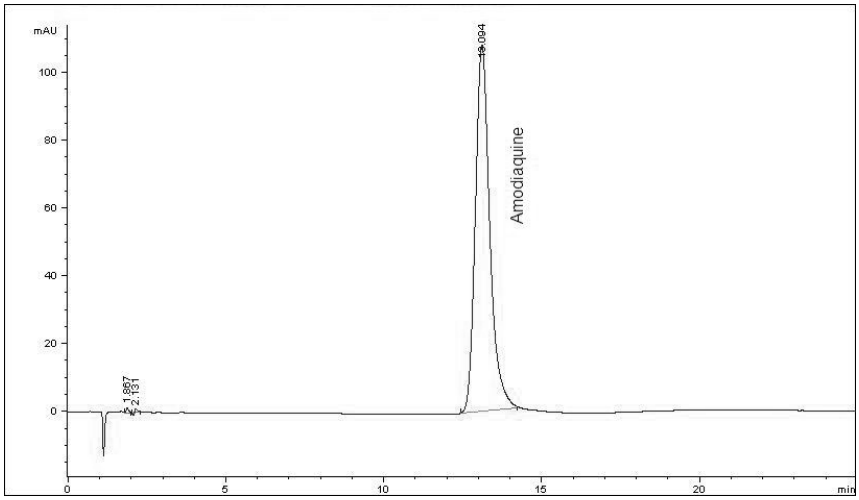


Figure 2.18: Example of a chromatogram obtained for Artekamoc tablets.

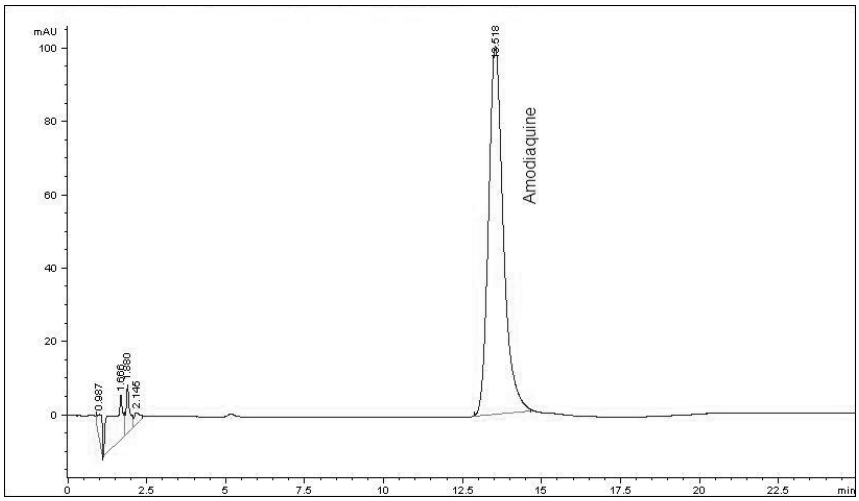


Figure 2.19: Example of a chromatogram obtained for Gsunate Kit tablets.

The results of the subsequent reproducibility studies are summarised in table 2.13.

Table 2.13: Comparative assay results of three commercial amodiaquine tablets for two analysts

PRODUCT	ASSAY VALUE		% DIFFERENCE
	% Assay 1	% Assay 2	
Flavoquine	98.5	100.8	2.30
Artekamoc	99.6	100.1	0.50
Gsunate Kit	94.4	95.0	0.60

The reproducibility of the results for the same products was acceptable, with differences suitably ranging between 0.50 and 2.30%.

2.4.4 SUMMARY OF VALIDATION RESULTS

The method for assay analyses was successfully validated and satisfactory results were obtained during assay testing of commercial amodiaquine tablets. A summary of the results is given in table 2.14.

Table 2.14: Summary of test results of assay validation studies performed on amodiaquine

PARAMETER	RESULTS OBTAINED
Specificity	No interferences were detected from solvents or other related substances
Linearity	r^2 = 0.9967
Range	58.6 – 156.2 µg/ml (50 – 136%)
Repeatability	%RSD = 0.07
Recovery	102.0%
Robustness	
Column	Equivalent columns are suitable
Mobile phase ratio	-5% - +10% change resulted in acceptable results
pH of mobile phase	Change in pH resulted in unacceptable peak resolution
Product analyses	%RSD < 1.3 for 5 commercial products

2.5 DISSOLUTION TESTING

2.5.1 INTRODUCTION

As discussed in 2.3, the USP (USP, 2010) prescribes 900 ml water as the dissolution medium for amodiaquine. According to the Biopharmaceutics Classification System (BCS), amodiaquine HCl is classified as a highly soluble API for a dose of 153 mg (WHO, 2006:403). Precipitation of amodiaquine was noted in solvents with higher pH values (refer 2.4.2.2). To avoid such precipitation of the amodiaquine during dissolution, 0.1M HCl was the only dissolution medium considered for this study.

2.5.2 DEVELOPMENT STUDIES

2.5.2.1 Dissolution test

The proposed dissolution testing parameters were as follows:

Apparatus:	2 (Paddles)
Dissolution medium:	500 ml 0.1M HCl
Rotation speed:	75 rpm
Temperature:	37 ±0.5ºC
Acceptance criterion:	80% in 30 minutes
Withdrawal times (min):	10, 15, 20, 30 and 45 for profile studies
Wavelength of detection (UV):	342 nm

Except for the dissolution medium (preferably pH 6.8 buffer), these parameters are generally used in the Ph.Int. for solid oral dosage forms containing BCS class 1 and 3 APIs (WHO, 2006:403).

Carry out the test as described under “Dissolution test for solid oral dosage forms” (Ph.Int., 2008).

2.5.2.2 Reference solution

i. Accurately weigh approximately 30 mg amodiaquine HCl and quantitatively transfer into a 50 ml volumetric flask with about 40 ml 0.1M HCl (dissolution medium).
ii. Sonicate for 5 minutes.
iii. Fill up to volume with 0.1M HCl (stock solution).
iv. Dilute 2 ml to 50 ml and fill up to volume with 0.1M HCl to obtain a concentration of approximately 24 µg/ml amodiaquine HCl (≡ 18 µg/ml amodiaquine).

2.5.2.3 Sample solution

i. Withdraw a 10 ml sample of the medium, at the appropriate time, through an in-line filter (0.45 µm).

ii. Allow the filtered sample to cool to room temperature.

iii. Suitably dilute with dissolution medium to obtain a concentration of approximately 20 µg/ml of amodiaquine, based on complete dissolution.

2.5.3 METHOD VALIDATION

The parameters as indicated in table 2.15 were evaluated during the validation study.

Table 2.15: Validation parameters and acceptance criteria required for dissolution validation studies (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA
Specificity	No interferences detected from solvents
Linearity	$r^2 \geq 0.99$
Range	± 30% of acceptance criteria
Repeatability	%RSD ≤ 2 for 5 determinations from same solution
Recovery	95.0 – 105.0%
Robustness	Investigate the capability of the method to obtain acceptable analytical results

2.5.3.1 Specificity

A solution of amodiaquine HCl in 0.1M HCl showed peaks of maximum absorbance at 225 nm and 342 nm (refer to 2.4.2.4). Due to less interferences being expected at 342 nm, this wavelength was chosen.

2.5.3.2 Linearity and range

The 100% theoretical concentration was taken as approximately 20 µg/ml, hence targeting the concentration that would result in the most acceptable and reliable absorbance reading on the spectrophotometer. Absorbance readings of 0.5 – 1.2 were preferred.

Five different amodiaquine HCl reference solutions, covering the range 52 - 156% of the 100% theoretical concentration, were prepared by diluting the stock solution (2.5.2), as described in table 2.16, with 0.1M HCl (dissolution medium).

Table 2.16: Dilutions of amodiaquine HCl reference stock solution used in dissolution linearity studies

SOLUTION	DILUTION	TARGET CONCENTRATION (µg/ml amodiaquine)
1	1 ml to 50 ml	9
2	3 ml to 100 ml	14
3	2 ml to 50 ml	18
4	1 ml to 20 ml	23
5	3 ml to 50 ml	28

Results are given in table 2.17 and graphically represented in figure 2.20.

Table 2.17: Absorbance values for dissolution linear regression graph of amodiaquine at 342 nm

ACTUAL CONCENTRATION (µg/ml amodiaquine)	% RANGE	ABSORBANCE	AVERAGE ABSORBANCE	%RSD
10.4	51.9	0.505 0.506 0.506	0.506	0.10
15.6	77.9	0.771 0.769 0.770	0.770	0.10
20.8	103.8	1.025 1.024 1.025 1.024 1.023	1.024	0.06
26.0	129.8	1.282 1.282 1.283	1.282	0.05
31.1	155.7	1.522 1.523 1.523	1.523	0.04

A regression analyses was performed on the results obtained during linearity studies.

The r^2 value was 0.9998, with an overall uncertainty of 0.08 µg/ml. A linear graph was thus produced in the concentration range of 10.4 - 31.1 µg/ml. The equation of the regression line is:

$$y = 0.0491x + 0.0025$$

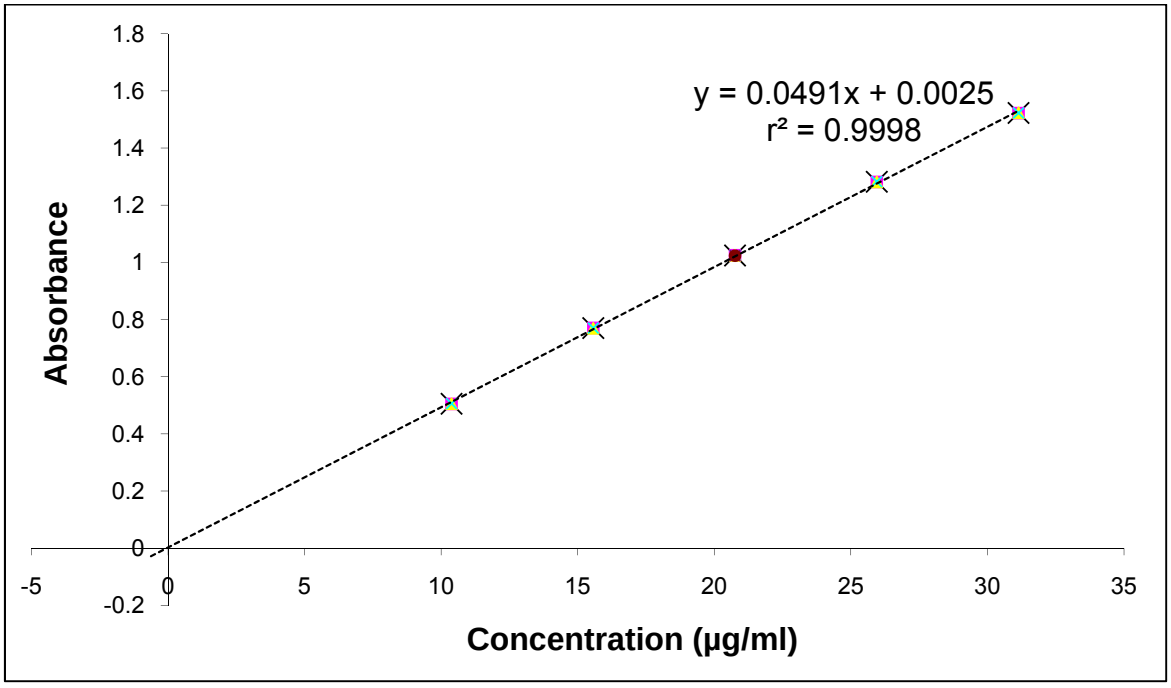


Figure 2.20: Linear regression graph of amodiaquine at 342 nm (UV).

2.5.3.3 Repeatability

Reference solution 3 prepared for the linearity study was used for repeatability studies (table 2.16) and was analysed 5 times.

The %RSD for the determinations was 0.06 (table 2.17), thus indicative of excellent repeatability obtained.

2.5.3.4 Recovery

A recovery solution, containing approximately 20 µg/ml of amodiaquine, was prepared similarly to the reference solution (refer 2.5.2) and analysed spectrophotometrically. The absorbance results obtained, were used to calculate the

concentration of the solution by means of the equation provided in 2.5.3.2. The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

A recovery of 100.6% was obtained. Since a recovery of 95.0 – 105.0% is deemed acceptable, the method complied with the requirements.

2.5.3.5 Robustness

Multipoint dissolution testing was performed on 4 commercial batches of amodiaquine tablets, in order to evaluate the method. At the time of assessment, no products with higher dosage (300 mg and 600 mg) could be sourced. Most of the products used for the assay analyses (2.4.3.3 E.iv.) had expired since, and valid products had to be sourced for performing the dissolution testing. The product details are given in table 2.18.

Table 2.18: Details of commercial amodiaquine tablets used in dissolution testing

PRODUCT NAME	MANUFACTURER	LABEL CLAIM (amodiaquine/tablet)	BATCH NO	EXPIRY DATE
Flavoquine	Roche Pharmaceuticals	153mg	418	10/2009
Falcimon Kit	Cipla (India)	153mg	G84425	11/2009
Arunate-AQ tablets	Strides Arcolab Ltd (India)	153mg	7204870	10/2009
Dart Child	Swiss Pharma NIG Ltd (Nigeria)	153mg	L27108	6/2010

The dissolution testing parameters were as follows:

Apparatus: 2 (Paddles)

Dissolution medium: 500 ml 0.1M HCl

Rotation speed: 75 rpm

Temperature: 37 ±0.5ºC

Acceptance criterion: 80% in 30 minutes

Withdrawal times (min): 10, 15, 20, 30 and 45 for profile studies

Wavelength of detection (UV): 342 nm

Samples were suitably diluted with dissolution medium to a concentration of approximately 20 µg/ml of amodiaquine, assuming complete dissolution.

The average results and %RSD of six tablets per product are summarised in table 2.19. The dissolution profiles are given in figure 2.21.

Table 2.19: Percentage dissolution of commercial amodiaquine tablets

	% DISSOLUTION (%RSD)			
TIME (min)	**Flavoquine**	**Arunate-AQ**	**Dart Child**	**Falcimon Kit**
10	90 (0.83)	79 (0.85)	77 (0.84)	91 (0.37)
15	94 (1.11)	84 (1.11)	85 (1.06)	94 (1.41)
20	95 (1.00)	88 (1.06)	86 (0.93)	103 (0.72)
30	96 (0.69)	89 (0.67)	87 (0.83)	107 (0.24)
45	96 (0.19)	91 (0.89)	93 (1.26)	109 (0.53)

An average percentage dissolution of more than 80% was obtained after 15 minutes of dissolution for all the products.

The results obtained indicated that the proposed method was acceptable for dissolution testing of amodiaquine tablets. A Q-value of not less than 80% at 30 minutes was chosen as the acceptance criterion. This was in agreement with current practice of the Ph.Int. for BCS highly soluble APIs.

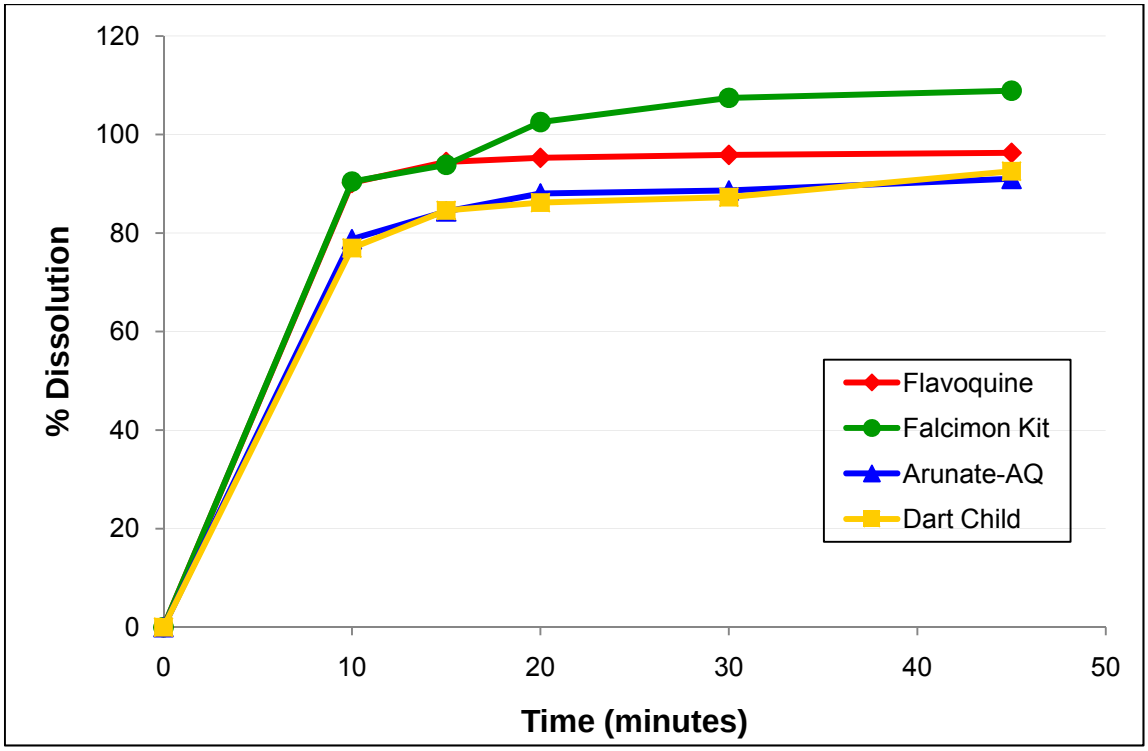


Figure 2.21: Dissolution profiles of commercial amodiaquine tablets in 0.1M HCl.

2.5.4 SUMMARY OF VALIDATION RESULTS

The method for dissolution analyses was successfully validated and satisfactory results were obtained during the analyses of dissolution samples of commercial amodiaquine products. The results are summarised in table 2.20.

Table 2.20: Summary of results obtained during dissolution validation studies performed on amodiaquine

PARAMETER	RESULTS OBTAINED
Specificity	No interferences detected from solvents
Linearity	r^2 = 0.9998
Range	10.4 – 31.1 µg/ml (52 – 156%)
Repeatability	%RSD = 0.06 (5 determinations)
Robustness Product analyses	Dissolution > 80% at 15 minutes for 4 commercial products
Recovery	100.6%

2.6 CONCLUSION

The WHO supplied a reference method that had been obtained from a manufacturer, for the assay analyses of amodiaquine tablets for its evaluation and improvement during this study. Development studies were first performed to evaluate the suitability of this method. After the development studies, a set of parameters were proposed and accepted for validation purposes. The validation parameters included specificity, linearity, range, repeatability, robustness and recovery. All parameters were tested and complied with requirements. The final method submitted for possible inclusion in the Ph.Int. differed from the reference method, with regards to the preparation of the test and reference solutions. Water was used as the solvent, since the mobile phase as specified in the reference method, resulted in precipitation of amodiaquine.

Another media than water or pH 6.8 phosphate buffer for the purpose of dissolution testing had to considered due to the precipitation that was noticed during assay studies. 0.1M HCl was assessed and proved a suitable dissolution medium. A criterion of not less than 80% in 30 minutes was proposed as acceptance value. A spectrophotometric analytical method for analysing the dissolution test samples was validated. The validation parameters included specificity, linearity, range, repeatability and recovery. All the parameters were tested and complied with requirements.

Based on the satisfactory outcomes of this study, a final monograph for amodiaquine tablets was compiled and submitted to the WHO for possible inclusion in the Ph.Int. monograph. After consultations this monograph (Annexure A) has been adopted during the 44th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (12-16 October 2009, Geneva).

CHAPTER 3

MONOGRAPH SULFADOXINE/PYRIMETHAMINE TABLETS

3.1 INTRODUCTION

In its commitment to making available to pharmaceutical manufacturers quality control methods that would pro-actively assist them in identifying and preventing the distribution of inferior drugs, the WHO requested the refinement of existing analytical methods for commercial sulfadoxine/pyrimethamine combination tablets. This part of the study especially addresses the widespread problem of inadequate dissolution, specifically the poor release of pyrimethamine from combination antimalarial tablets.

3.2 AIM

For the purpose of this study, suitable analytical methods were adapted for sulfadoxine/pyrimethamine combination tablets for:

i. The determination of the content of the APIs (assays);

ii. The percentages of APIs being dissolved during dissolution; and

iii. The determination of related substances for these APIs.

The identification tests that are included in the final monograph (Annexure B), were not part of this investigation.

3.3 ANALYTICAL METHODS

A monograph for sulfadoxine/pyrimethamine tablets is included in the current United States Pharmacopeia (USP, 2009a). This monograph details the testing for identification, dissolution, uniformity of dosage units and assay.

The assay method, as described in the latest USP, utilises a mixture of 0.1% phosphoric acid and acetonitrile as mobile phase on a phenyl column (packing L11). The wavelength of detection is 230 nm (USP, 2009a). Until the end of 2008 a

different method had been described. A mixture of 1% glacial acetic acid and acetonitrile was used as mobile phase on a C_{18} column. The wavelength of detection was 254 nm. The use of an internal standard, namely phenacetin, was also required (USP, 2008). The reason for having changed the method is unknown, but it may have been due to numerous reports regarding the USP method that had to be adapted to deliver acceptable results (Atemnkeng *et al.,* 2007:125; Hebron *et al*., 2005:577; Minzi *et al*., 2003a:118). The current USP method was not considered as it was published after the conclusion of this study. Boca *et al*. (2005:461) published a method for the determination of sulfadoxine/pyrimethamine in very low concentrations for the purpose of a cleaning validation of manufacturing equipment.

The current USP dissolution test is performed in 900 ml pH 6.8 phosphate buffer, with paddles rotating at 75 rpm. A Q-value of 60% is specified for both sulfadoxine and pyrimethamine after 30 minutes. The same analytical technique as for the assay is used for the analyses of the dissolution samples (USP, 2009a).

3.4 ASSAY

3.4.1 REFERENCE METHODS

The WHO provided a method from a manufacturer, as reference for use in this study (Method 1). The parameters were as follows:

Column: C_{18}, 5 μm, 250 x 4.6 mm

Mobile phase: **Solvent A** (34 ml) : **Solvent B** (5 ml) : **Solvent C** (61 ml)

Solvent A: Acetonitrile

Solvent B: Methanol

Solvent C: 0.04M potassium dihydrogen phosphate solution, pH adjusted to 2.5 with diluted phosphoric acid.

Injection volume: 20 µl

Detection wavelength: 227 nm

Flow rate: 1 ml/min

Using the 2008 USP method as reference, Method 2 was also developed during this study for further evaluation. The parameters were as follows:

Column: C_{18}, 5 μm, 250 x 4.6 mm

Mobile phase: **Solvent A** (800 ml) : **Solvent B** (200 ml)

Solvent A: Transfer about 600 ml of water into a 1,000 ml volumetric flask, add 10 ml of glacial acetic acid and 0.5 ml of triethylamine. Mix and fill up to volume with water. Adjust pH to 4.2 with 10M NaOH solution.

Solvent B: Acetonitrile

Injection volume: 20 µl

Detection wavelength: 254 nm

Flow rate: 2 ml/min

3.4.2 DEVELOPMENT STUDIES PERFORMED ON ASSAY AND DISSOLUTION METHODS

3.4.2.1 Detection wavelength

Moffat *et al.* (2004:1578) indicate peaks of maximum absorbance for sulfadoxine in aqueous acid at 264 nm, and in aqueous alkali at 272 nm (figure 3.1). As indicated in figure 3.2, one peak of maximum absorbance for pyrimethamine in aqueous acid is found at 272 nm, whilst in aqueous alkali at 286 nm (Moffat *et al.,* 2004:1578).

Samples of sulfadoxine and of pyrimethamine were prepared and injected onto the HPLC. Spectra of these samples showed a peak of maximum absorbance at 275 nm, instead of at 272 nm for both APIs.

Both methods, as described in 3.4.1, were used to analyse samples of sulfadoxine and pyrimethamine separately and in combination, at 227 nm, 254 nm and 275 nm.

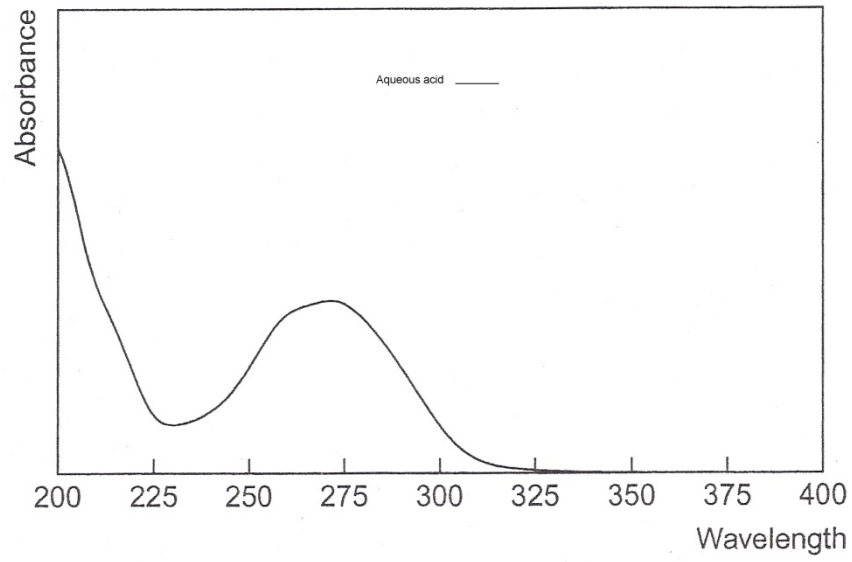


Figure 3.1: UV spectrum of sulfadoxine in aqueous acid (Moffat *et al*., 2004:1578)**.**

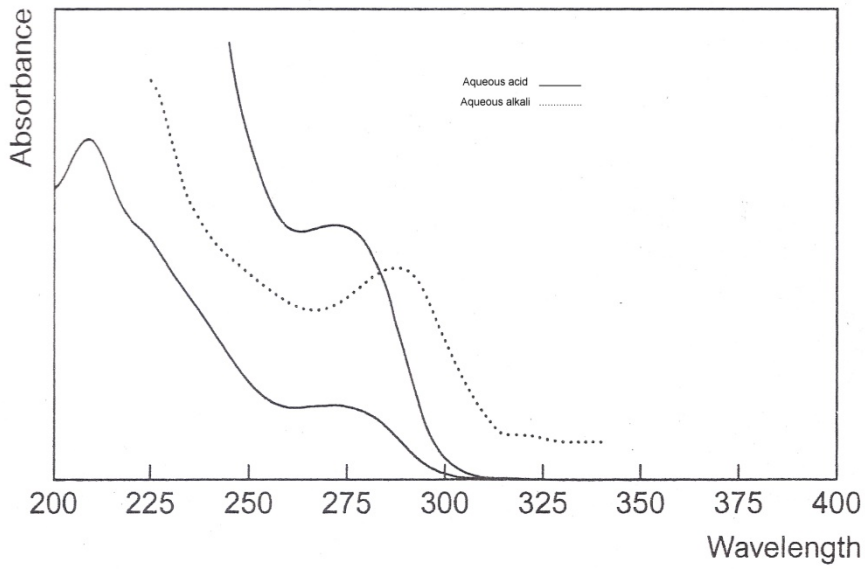


Figure 3.2: UV spectra of pyrimethamine in various solvents (Moffat *et al*., 2004:1511)**.**

For pyrimethamine, the largest peak areas were present at 227 nm, and the smallest at 275 nm (figures 3.3 and 3.4). For sulfadoxine the smallest peak area existed at 227 nm, whilst comparable peak areas were obtained at 254 nm and 275 nm (figures 3.5 and 3.6). Since the concentration ratio among the two APIs is large (S:P =

500:25), it would have been ideal if a wavelength of detection could be used where the largest concentration would result in a smaller peak and *vice versa*.

The wavelength of detection was thus chosen as 227 nm, since the ratio of these peak areas is more suitable for quality chromatography.

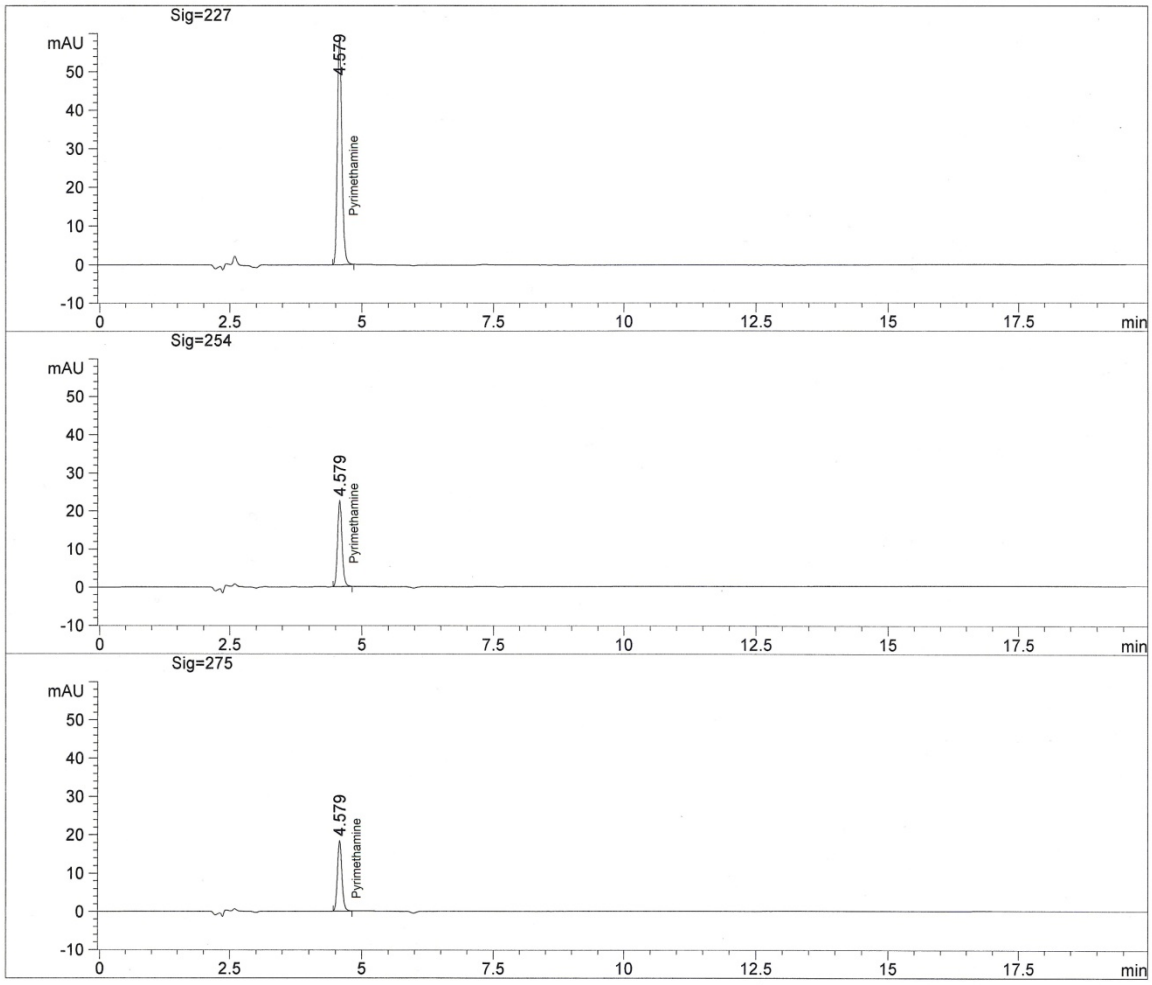


Figure 3.3: Example of chromatograms of pyrimethamine at 227 nm (top), 254 nm (middle) and 275 nm (bottom), using Method 1.

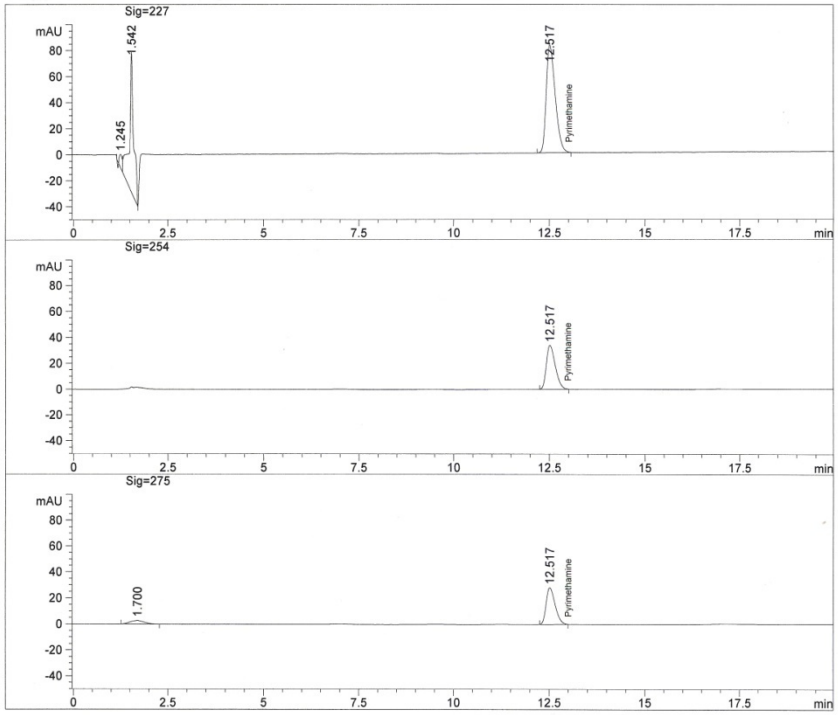


Figure 3.4: Example of chromatograms of pyrimethamine at 227 nm (top), 254 nm (middle) and 275 nm (bottom), using Method 2.

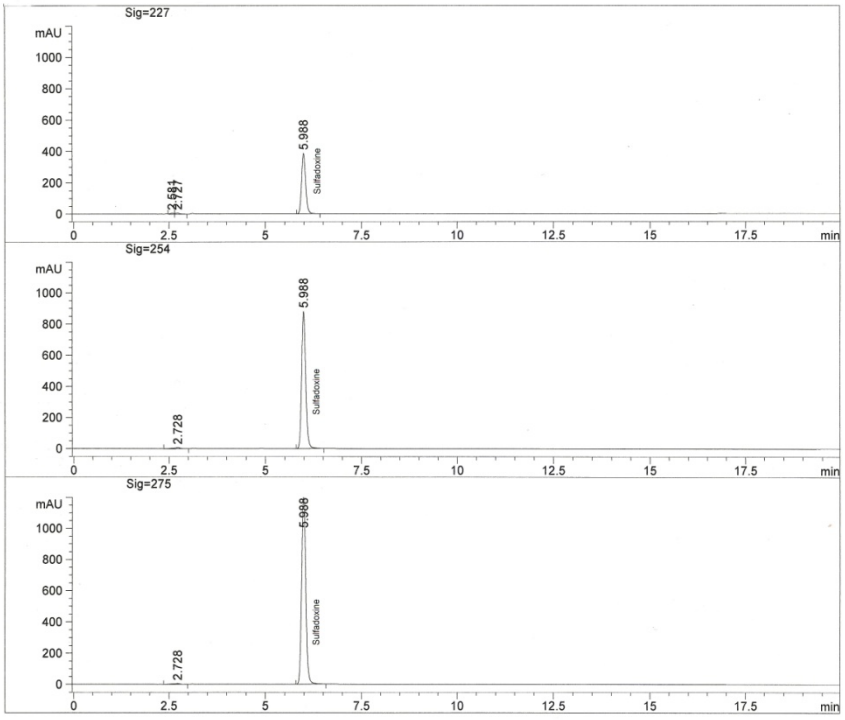


Figure 3.5: Example of chromatograms of sulfadoxine at 227 nm (top), 254 nm (middle) and 275 nm (bottom), using Method 1.

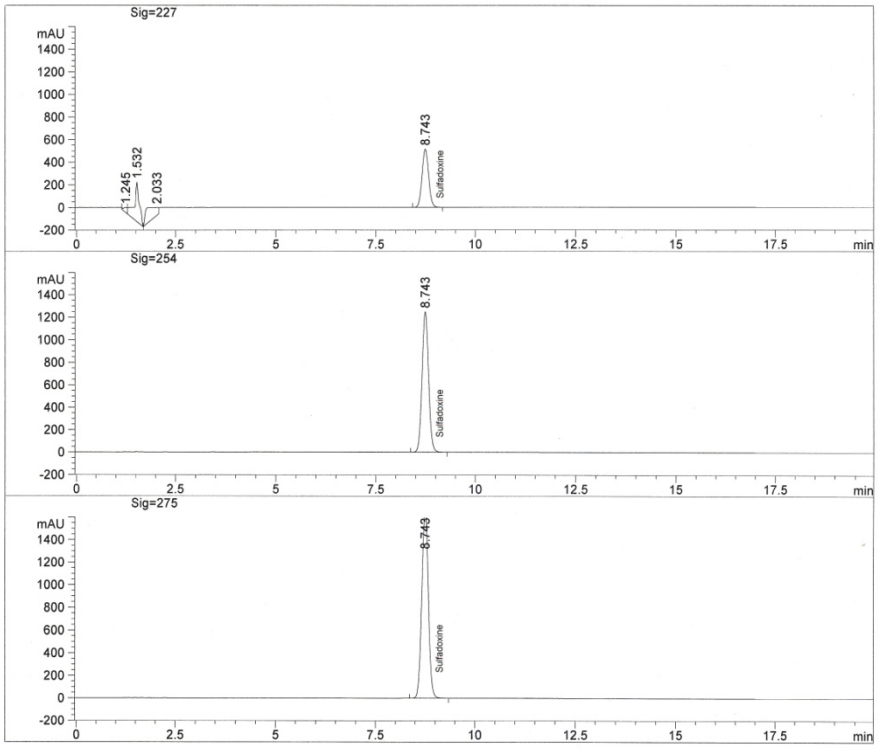


Figure 3.6: Example of chromatograms of sulfadoxine at 227 nm (top), 254 nm (middle) and 275 nm (bottom), using Method 2.

3.4.2.2 Peak separation

Figures 3.3 - 3.6 indicate that the sulfadoxine and pyrimethamine peaks were well separated for both methods. With Method 1, pyrimethamine eluted before sulfadoxine (relative retention time = 0.77), whereas with Method 2 it eluted thereafter (relative retention time = 1.42).

3.4.2.3 Source of reference standards

The WHO supplied the necessary reference standards for use in these development studies.

During development, extra peaks were detected for sulfadoxine, when a commercially available reference material was used as secondary standard (figures 3.7 & 3.8).

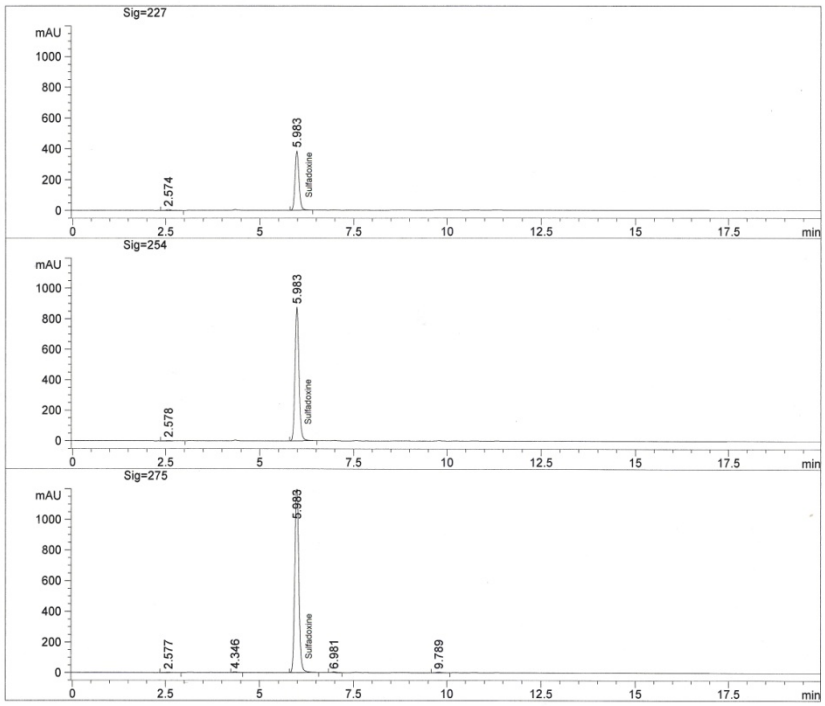


Figure 3.7: Example of chromatograms of a commercially available sulfadoxine reference material at 227 nm (top), 254 nm (middle) and 275 nm (bottom), using Method 1.

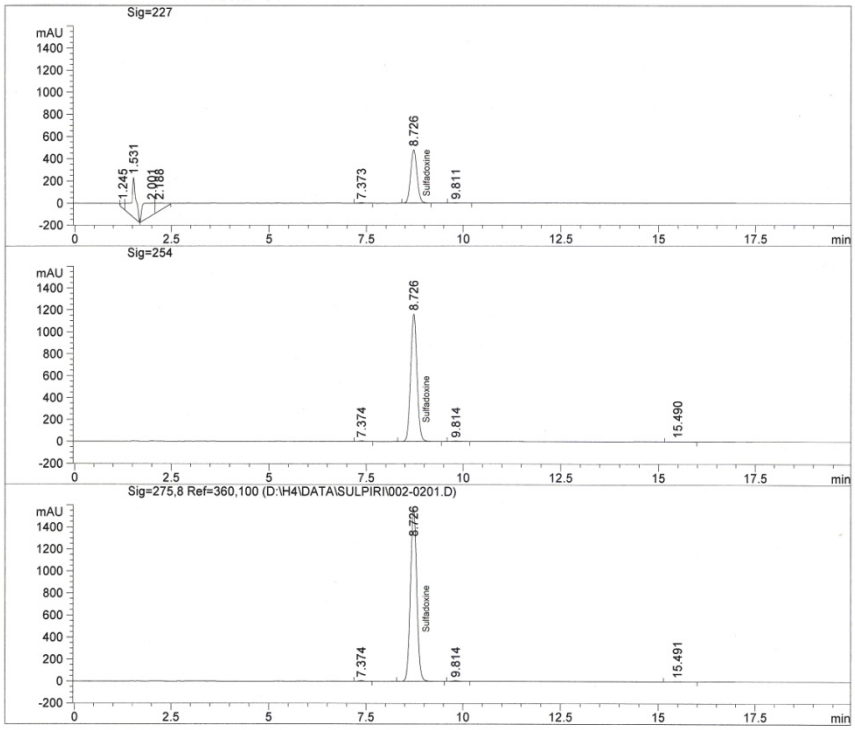


Figure 3.8: Example of chromatograms of a commercially available sulfadoxine reference material at 227 nm (top), 254 nm (middle) and 275 nm (bottom), using Method 2.

With Method 1, three impurity peaks were detected at 275 nm, whereas with Method 2, peaks were detected at all three wavelengths. Three impurity peaks each were detected at 254 nm and 275 nm, whereas at 227 nm only two peaks were detected.

Hebron *et al*. (2005:579) also described a peak of unknown origin for sulfadoxine/pyrimethamine. Although the peak could not be identified, they indicated that the peak was an impurity related to sulfadoxine.

During the assay analyses for this study, an unknown peak was also detected in some of the commercial products (3.4.3.3 E iv).

3.4.2.4 Stability of reference solution

For the purpose of stress studies, Method 2 was used at a detection wavelength of 227 nm.

A. Storage conditions

Separate stock solutions of sulfadoxine and pyrimethamine were prepared, using a mixture of acetonitrile and the mobile phase as solvent. Dilutions were prepared in mobile phase, with final concentrations of 500 μg/ml for sulfadoxine and 25 μg/ml for pyrimethamine. A sample of the reference solution was injected over a period of four days to determine the stability of the two APIs, when kept on the bench.

The results are given in table 3.1. The percentage difference over time was calculated in relation to the initial peak area.

The results indicated that no significant differences were detected in the peak areas for both APIs, thus indicating that the reference solution was stable for at least 4 days.

B. Stress conditions

Separate reference solutions were prepared in 0.1M NaOH, 0.1M hydrochloric acid, a 50% mixture of methanol and water and a 5% peroxide solution. A sample of each solution was analysed over a period of four days.

Table 3.1: Stability data of sulfadoxine and pyrimethamine reference solutions over a period of four days (111 hours)

	SULFADOXINE		PYRIMETHAMINE	
TIME (hours)	**Peak area**	**% Difference**	**Peak area**	**% Difference**
Initial	5739	-	1321	-
7	5732	-0.12	1327	0.45
12	5740	0.02	1316	-0.37
23	5741	0.03	1319	-0.15
32	5717	-0.38	1316	-0.37
48	5719	-0.35	1316	-0.38
59	5708	-0.55	1312	-0.68
70	5704	-0.62	1319	-0.16
90	5675	-0.71	1332	0.87
100	5678	-1.06	1333	0.90
111	5680	-1.02	1339	1.35

The 0.1M NaOH solution exhibited a split peak for sulfadoxine, with the results being represented as the sum of the two peaks. The results are given in tables 3.2 and 3.3. Examples of chromatograms for sulfadoxine in the various solvents are given in figures 3.9 – 3.12.

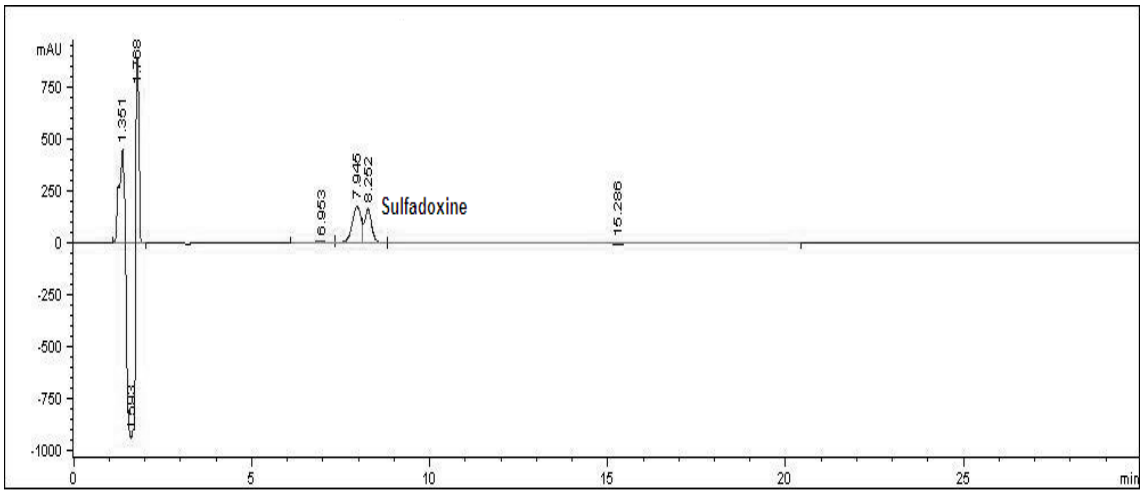


Figure 3.9: Example of a chromatogram of sulfadoxine when dissolved in 0.1M NaOH.

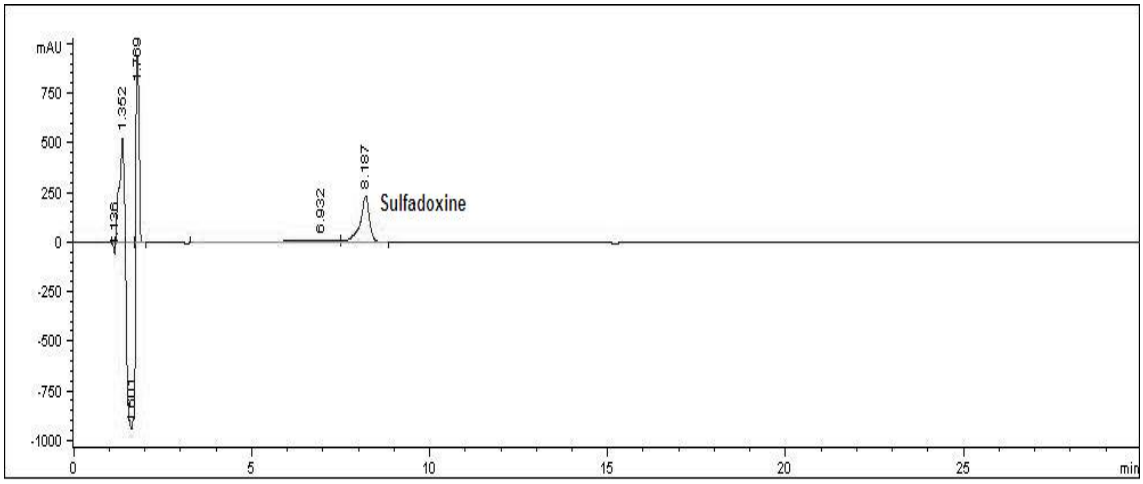


Figure 3.10: Example of a chromatogram of sulfadoxine when dissolved in 0.1M HCl.

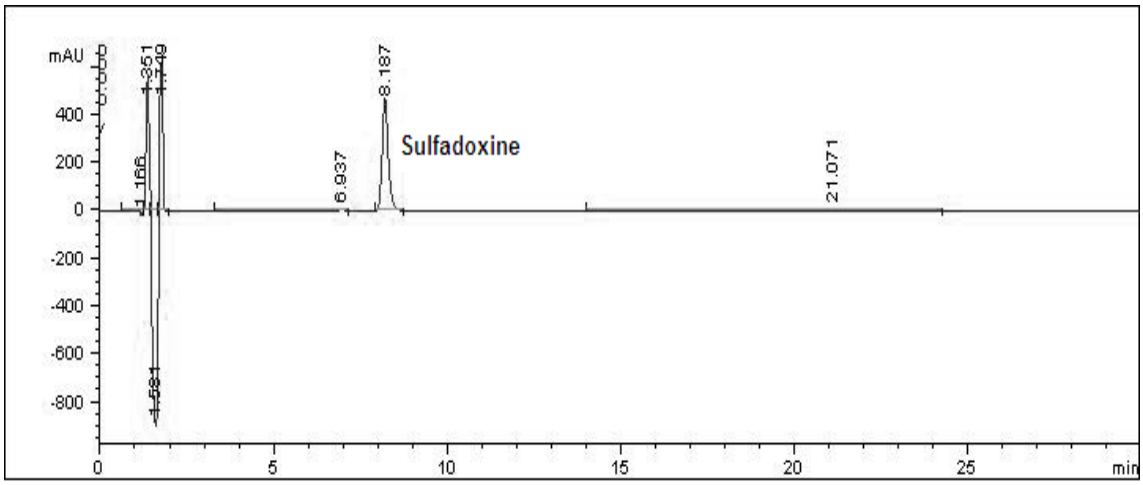


Figure 3.11: Example of a chromatogram of sulfadoxine when dissolved in 50% water:methanol.

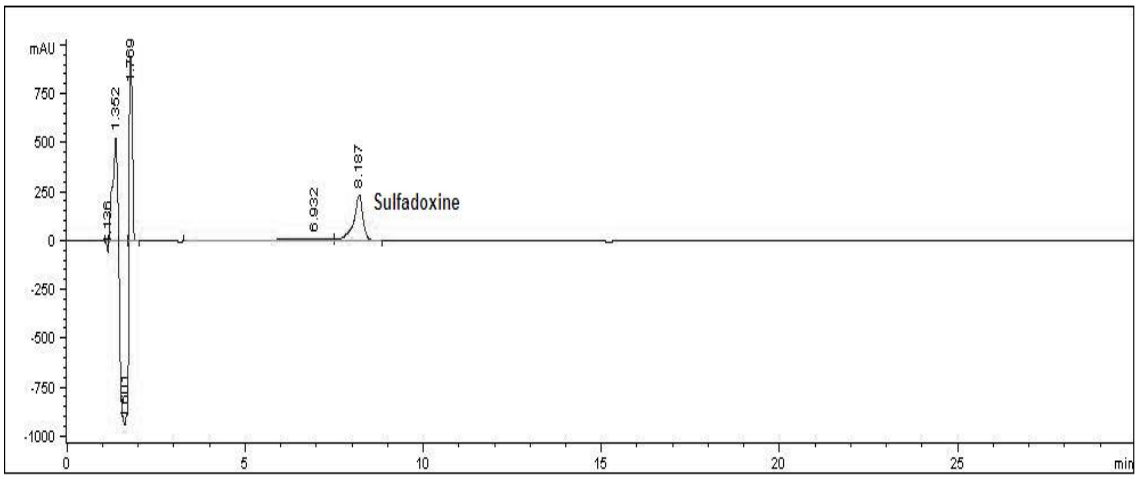


Figure 3.12: Example of a chromatogram of sulfadoxine when dissolved in 5% peroxide.

Table 3.2: Stability data of sulfadoxine reference solutions during stress conditions over a period of four days (111 hours)

	0.1M NaOH		0.1M HCl	
TIME (hours)	**Sum of peak areas**	**% Difference**	**Peak area**	**% Difference**
Initial	5464	-	4377	-
12	5467	0.06	4244	-3.04
23	5444	-0.36	4216	-3.68
32	5405	-1.08	4375	-0.03
48	5367	-1.78	4600	5.11
59	5457	-0.13	4413	0.83
70	5419	-0.82	4349	-0.63
90	5391	-1.34	4377	0.01
100	5381	-1.50	4308	-1.56
111	5345	-2.17	4529	3.48
	5% Peroxide		**50% Methanol:Water**	
TIME (hours)	**Peak area**	**% Difference**	**Peak area**	**% Difference**
Initial	4475	-	5739	-
12	4410	-1.45	5741	0.02
23	4423	-1.15	5741	0.03
32	4268	-4.63	5717	-0.38
48	4351	-2.76	5719	-0.35
59	4255	-4.92	5708	-0.55
70	4213	-5.02	5704	-0.62
90	4222	-5.65	5698	-0.71
100	4058	-9.31	5678	-1.06
111	4065	-9.16	5681	-1.02

Table 3.3: Stability data of pyrimethamine reference solutions during stress conditions over a period of four days (111 hours)

	0.1M NaOH		0.1M HCl	
TIME (hours)	**Peak area**	**% Difference**	**Peak area**	**% Difference**
Initial	1322	-	1335	-
12	1324	0.18	1299	-2.75
23	1328	0.46	1295	-3.01
32	1320	-0.16	1283	-3.90
48	1327	0.36	1272	-4.76
59	1329	0.55	1314	-1.63
70	1320	-0.11	1303	-2.44
90	1326	0.32	1316	-1.45
100	1360	2.87	1386	3.76
111	1350	2.13	1400	4.87
	5% Peroxide		**50% Methanol:Water**	
TIME (hours)	**Peak area**	**% Difference**	**Peak area**	**% Difference**
Initial	1287	-	1315	-
12	1253	-2.61	1313	-0.03
23	1240	-3.66	1309	-0.39
32	1229	-4.50	1312	-0.14
48	1180	-8.28	1308	-0.43
59	1169	-9.14	1310	-0.27
70	1168	-9.23	1304	-0.72
90	1168	-9.20	1309	-0.38
100	1168	-9.18	1319	0.25
111	1160	-9.85	1312	-0.12

The inconsistency in data of sulfadoxine and pyrimethamine in 0.1M HCl was most probably due to the poor chromatography of the samples (figure 3.10).

Sulfadoxine proved to be stable in a 5% peroxide solution for at least 23 hours, whilst for pyrimethamine, a more than 2% difference was detected already after 12 hours. After 32 hours a percentage difference of more than 2% was reported for sulfadoxine. Both sulfadoxine and pyrimehamine thus showed degradation, due to

oxidation. Oxidative degradation was the only significant trend being observed during these stress studies.

For pyrimethamine in 0.1M NaOH, a difference, exceeding 2%, was detected only after 100 hours.

In the 50% mixture of methanol and water, no significant signs of degradation were observed for either sulfadoxine or pyrimethamine, even after 111 hours.

C. Temperature stress

Stock solutions of both sulfadoxine and pyrimethamine were prepared in a mixture of acetonitrile and mobile phase. The reference solutions were then diluted in 1M HCl and 1M NaOH each and kept at 37ºC for 24 hours.

The results are presented in tables 3.4 and 3.5.

Table 3.4: Results of sulfadoxine and pyrimethamine reference solution in 1M HCl after 24 hours at 37ºC

	1M HCl			
	SULFADOXINE		PYRIMETHAMINE	
TIME (hours)	Peak area	% Difference	Peak area	% Difference
Initial	5648	-	1292	-
24	5570	-1.39	1292	0.00

Table 3.5: Results of sulfadoxine and pyrimethamine reference solution in 1M NaOH after 24 hours at 37ºC

	1M NaOH			
	SULFADOXINE		PYRIMETHAMINE	
TIME (hours)	Sum of peak areas	% Difference	Peak area	% Difference
Initial	5850	-	1301	-
24	5631	-3.73	1245	-4.30

For both APIs, no significant changes were observed when their stock solutions were diluted with 1M HCl.

When sulfadoxine was diluted in 1M NaOH, two peaks were detected, with the results in table 3.5 representing the sum of the peak areas. These chromatograms showed a peak eluting before the solvent peak group (figure 3.9), which for the purposes of this study was not investigated further. The sum of the peak areas decreased by 3.7% after 24 hours, representing the sum of peak area one that decreased by 5.2% over the 24 hours and peak area two that increased by 1.5%.

The pyrimethamine peak area showed a decrease of 4.3% over a period of 24 hours when diluted with 1M NaOH.

Sulfadoxine and pyrimethamine were both thus unstable in NaOH solution.

3.4.2.5 Conclusion

A summary of advantages and disadvantages of both methods are given in table 3.6.

Table 3.6: Summary of the advantages and disadvantages of analytical reference Methods 1 & 2

	ADVANTAGE	DISADVANTAGE
METHOD 1	i. Short run times ii. Good peak separation iii. Wavelength of detection more suitable	i. Buffer in mobile phase ii. pH of mobile phase very low iii. Possible interferences of unknown impurities with pyrimethamine peak
METHOD 2	i. No buffer in mobile phase ii. pH not too low iii. Detection of unknown impurities iv. Good peak separation	i. Longer run times ii. Wavelength of detection unsuitable

The major attributing factors for not choosing Method 1 were:

i. The buffer in the mobile phase; and

ii. The very low pH (2.5) of the mobile phase.

The detection wavelength of Method 1 of 227 nm, however, was more suitable than the 254 nm of Method 2. It was decided that the benefits of the mobile phase of Method 2 outweighed the disadvantage of the longer runtime. Method 2 was thus

selected for validation purposes of assay and dissolution analyses of sulfadoxine/pyrimethamine tablets with detection at 227 nm.

3.4.3 VALIDATION OF ANALYTICAL METHOD FOR ASSAY

The following parameters were derived from the development studies for the purpose of validation:

Column:	250 x 4.6mm, C_{18}, 5 µm (Phenomenex Luna)
Mobile phase:	**Solvent A** (800 ml) : **Solvent B** (200 ml)
	Solvent A: Transfer about 600 ml of water into a 1,000 ml volumetric flask, add 10 ml of glacial acetic acid and 0.5 ml of triethylamine. Adjust pH to 4.2 with 10M NaOH solution. Mix and fill up to volume with water.
	Solvent B: Acetonitrile
Test solvent:	Stock solutions are prepared using acetonitrile (about 35% of the stock solution volume) in which to dissolve the APIs. Stock solutions are made up to volume with mobile phase. Dilutions are prepared using mobile phase.
Injection volume:	20 µl
Temperature:	Ambient
Flow rate:	2 ml/min
Detection:	227 nm

3.4.3.1 Equipment used in the validation studies

The equipment used for the HPLC analyses included:

- Agilent Technologies® 1200 series HPLC system with Chemstation® Software Revision A.10.02
- Binary pump - Firmware Revision A.05.11
- Diode array detector - Firmware Revision A.05.11
- Column thermostat - Firmware Revison A.05.11

- Thermostatted autosampler - Firmware Revision A.05.11.

3.4.3.2 Preparation of solutions

A. Reference solution

i. Accurately weigh approximately 25 mg of sulfadoxine and quantitatively transfer it into a 25 ml volumetric flask with 10 ml of acetonitrile. Sonicate for 10 minutes. (stock solution S).

ii. Accurately weigh approximately 25 mg of pyrimethamine and quantitatively transfer it into a 100 ml volumetric flask with 35 ml acetonitrile. Sonicate for 10 minutes. (stock solution P).

iii. Alllow both solutions to cool to room temperature.

iv. Fill both solutions up to volume with mobile phase.

v. Transfer 10 ml of stock solution S and 2 ml of stock solution P into a 20 ml volumetric flask.

vi. Fill up to volume with mobile phase to obtain a solution with concentrations of approximately 500 µg/ml of sulfadoxine and 25 µg/ml of pyrimethamine.

B. Sample solution

i. Weigh and powder 20 sulfadoxine/pyrimethamine tablets.

ii. Accurately weigh tablet powder equivalent to 500 mg sulfadoxine and quantitatively transfer it into a 200 ml volumetric flask, using about 70 ml of acetonitrile.

iii. Sonicate for 10 minutes and allow to cool to room temperature.

iv. Fill up to volume with mobile phase.

v. Filter a portion of the solution through a 0.45 µm filter, discarding the first 10 ml.

vi. Dilute 5 ml of the filtrate in a 25 ml volumetric flask with mobile phase to obtain a concentration of approximately 500 µg/ml of sulfadoxine and 25 µg/ml of pyrimethamine.

3.4.3.3 Validation parameters

The parameters, as indicated in table 3.7, were evaluated during the subsequent validation study.

Table 3.7: Validation parameters and acceptance criteria required for assay validation studies (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA
Specificity	No interferences detected from solvents or excipients
Linearity	$r^2 \geq 0.99$
Range	80 – 120% of declared content
Repeatability	%RSD ≤ 2 for 5 injections from same solution
Recovery	98.0 – 102.0%
Robustness	Investigate the effect of small, but deliberate changes to the method

A. Specificity

Reference solutions of sulfadoxine and pyrimethamine were prepared in solvent (mobile phase) to have known concentrations of about 500 µg/ml and 25 µg/ml, respectively. Samples of these solutions were injected onto the HPLC in order to determine retention time and possible interferences (figures 3.13 - 3.15). The purity of the peaks was evaluated by means of the diode array detector (figures 3.16 and 3.17). Sample solution chromatograms (figures 3.21 – 3.24) indicated that no interferences were evident from tablet excipients.

No interfering peaks were detected during these stress studies (refer 3.4.2.4).

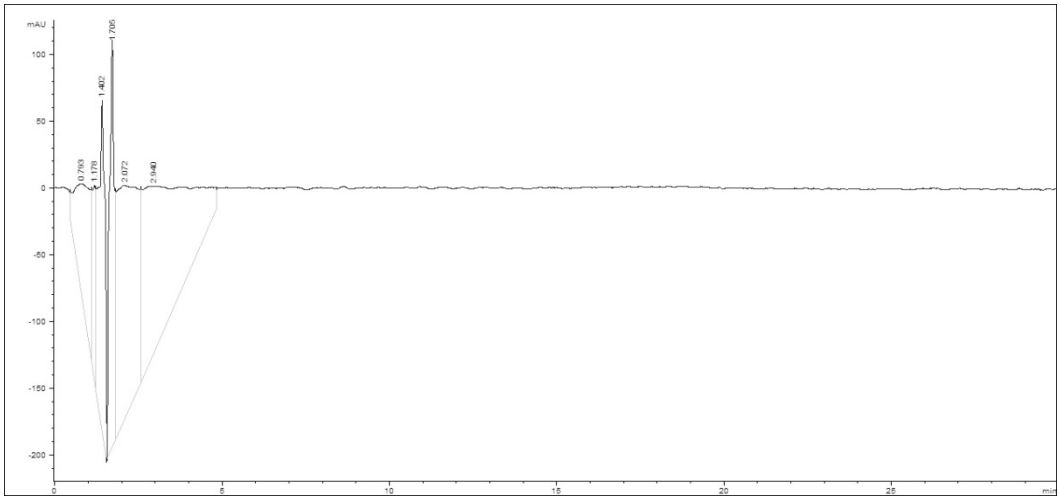


Figure 3.13: Example of a chromatogram of the solvent (mobile phase).

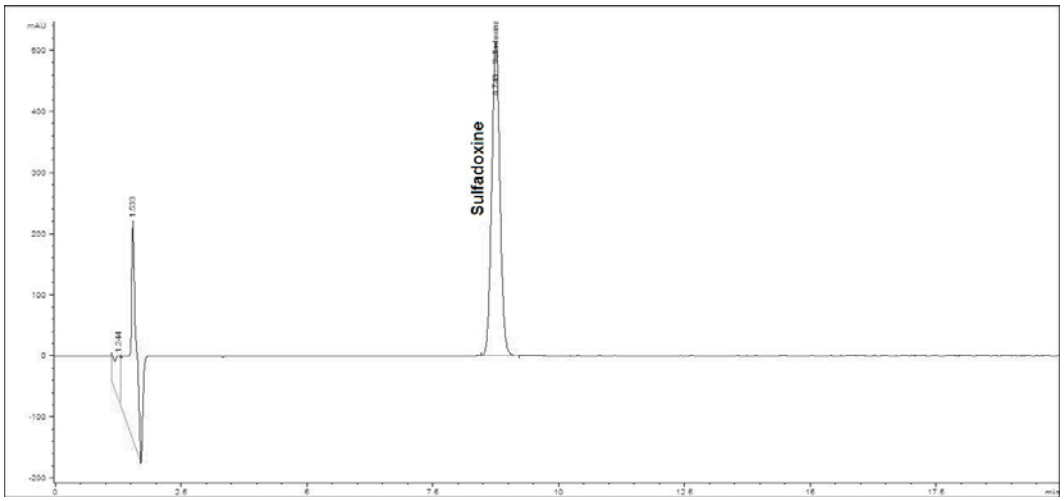


Figure 3.14: Example of a chromatogram of sulfadoxine in mobile phase.

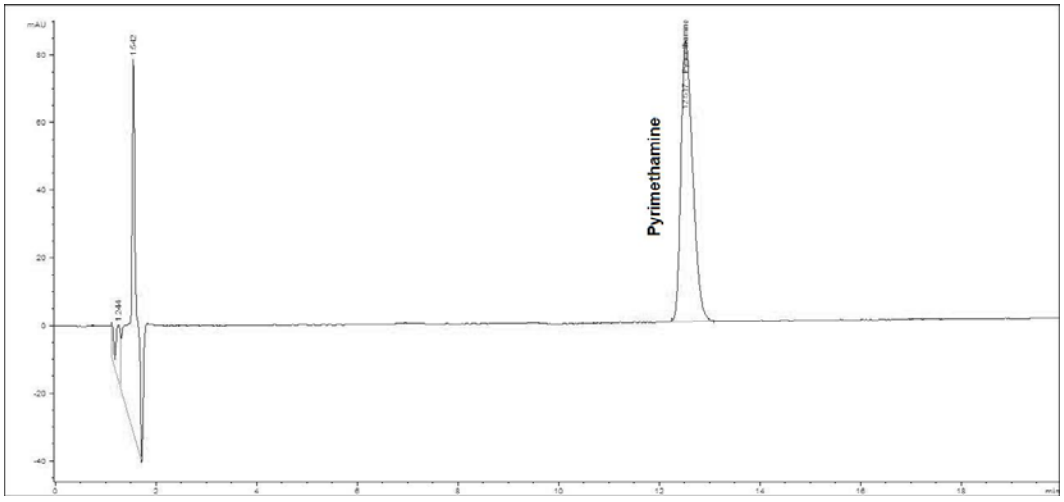


Figure 3.15: Example of a chromatogram of pyrimethamine in mobile phase.

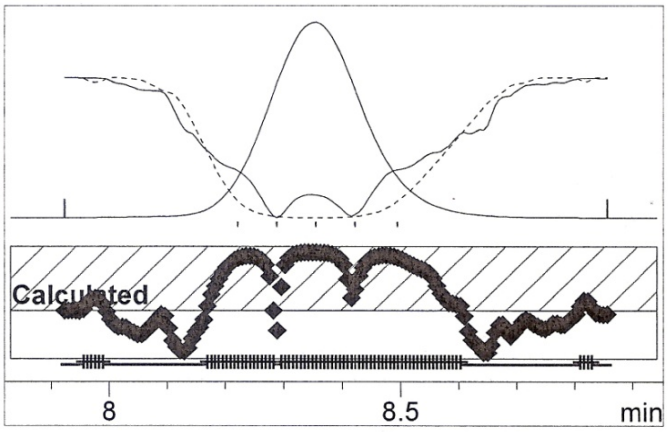


Figure 3.16: Peak purity profile of sulfadoxine reference solution.

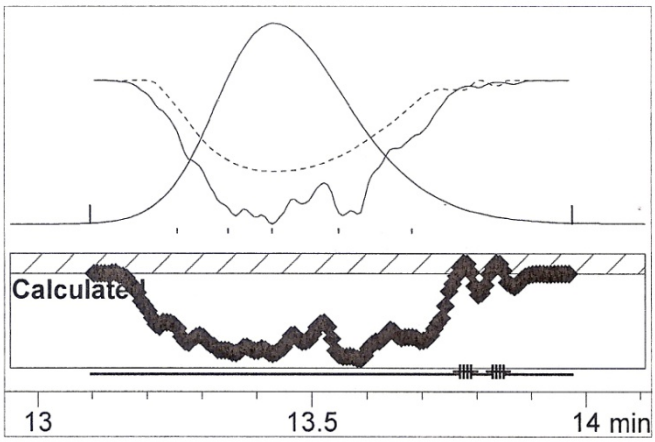


Figure 3.17: Peak purity profile of pyrimethamine reference solution.

B. Linearity and range

Sulfadoxine

The 100% theoretical concentration was taken as 500 µg/ml, targeting the concentration of the sample solution at a 100% label claim. Five different reference solutions, covering the range of 50 - 150% of the 100% theoretical concentration, were prepared by diluting a stock solution of sulfadoxine (1000 µg/ml) with mobile phase (table 3.8).

Table 3.8: Dilutions of sulfadoxine and pyrimethamine reference stock solutions used in assay linearity studies

	DILUTION		FINAL VOLUME	TARGET CONCENTRATION (µg/ml)	
SOLUTION	S	P		S	P
1	5 ml	1 ml	20 ml	250	12.5
2	10 ml	2 ml	25 ml	400	20
3	10 ml	2 ml	20 ml	500	25
4	10 ml	2 ml	15 ml	667	33
5	15 ml	3 ml	20 ml	750	37.5

Results are summarised in table 3.9 and are graphically represented in figure 3.18.

The r^2 value was 0.9999 with an overall uncertainty of 7.5 µg/ml. A linear graph was thus produced in the concentration range of 250.7 - 752.5 µg/ml. The equation of the line is:

$$y = 11.126x + 66.398$$

Pyrimethamine

The 100% theoretical concentration was taken as 25 µg/ml, targeting the concentration of the sample solution at a 100% label claim. Five different reference solutions, covering the range of 50 - 150% of the 100% theoretical concentration, were prepared by diluting a stock solution of pyrimethamine (250 µg/ml) with mobile phase (table 3.8).

Results are summarised in table 3.10 and are graphically represented in figure 3.19.

Table 3.9: Peak areas of linear regression graph of sulfadoxine

CONCENTRATION (µg/ml sulfadoxine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
250.7	49.6	2827.4 2828.4	2827.9	0.03
401.1	80.2	4540.5 4545.1	4542.8	0.07
501.4	100.3	5688.0 5682.1 5682.3 5671.1 5684.2	5681.5	0.11
668.6	133.7	7505.2 7505.5	7503.4	0.00
752.2	150.4	8414.3 8409.1	8411.7	0.04

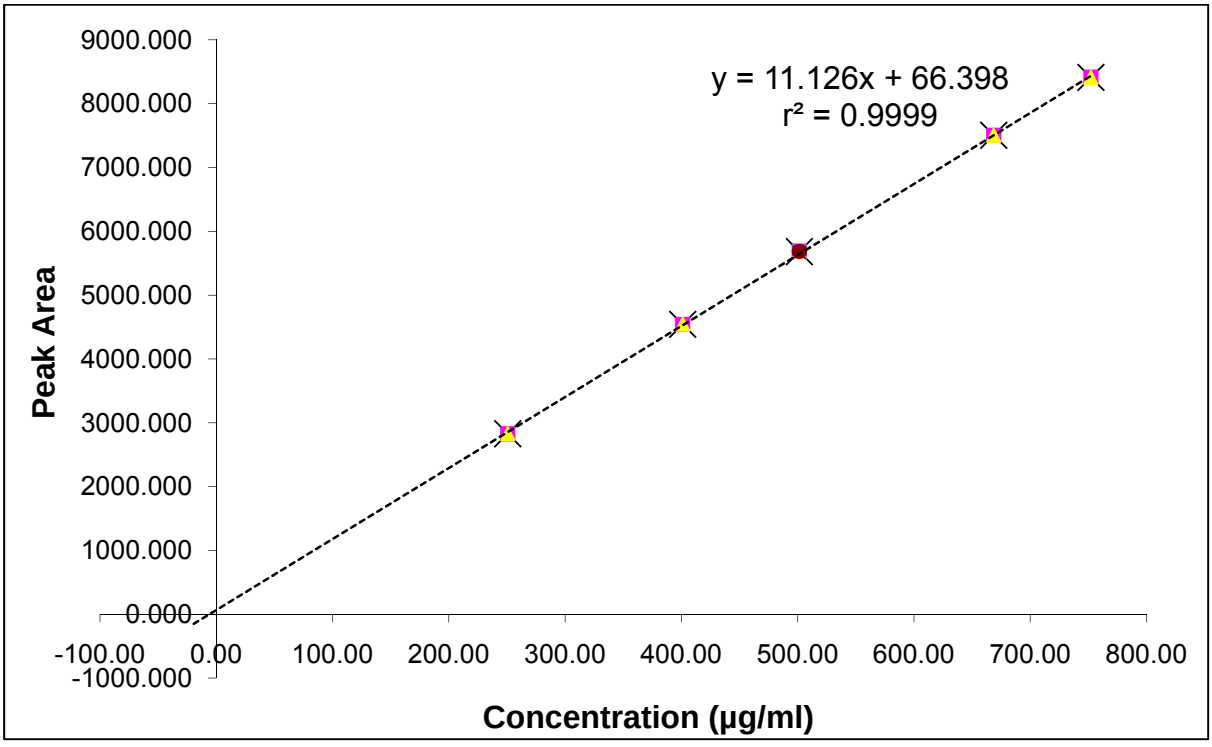


Figure 3.18: Linear regression graph of sulfadoxine for assay testing.

Table 3.10: Peak areas of linear regression graph of pyrimethamine

CONCENTRATION (µg/ml pyrimethamine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
12.7	50.8	662.6 666.1	664.3	0.40
20.3	81.3	1061.5 1049.2	1055.3	0.82
25.4	101.6	1319.3 1303.5 1303.3 1305.5 1307.5	1307.8	0.51
33.9	135.5	1758.3 1726.5	1742.4	1.30
38.1	152.5	1941.8 1947.1	1944.5	0.20

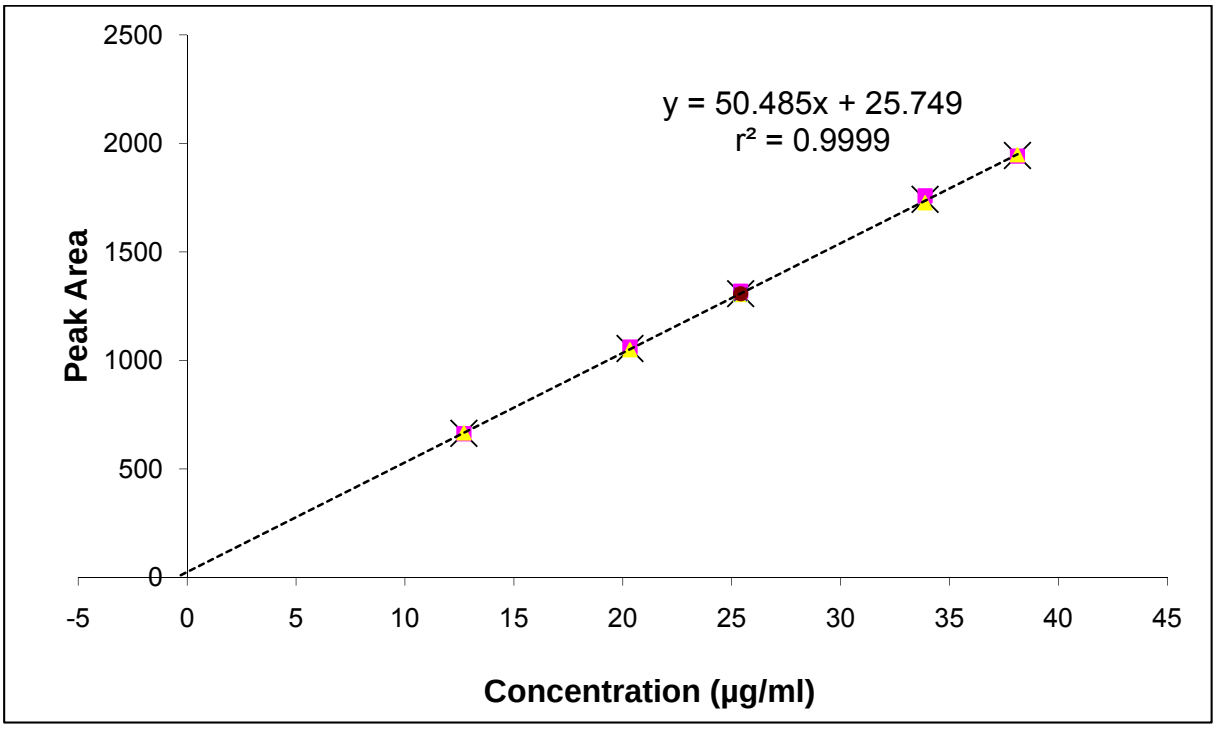


Figure 3.19: Linear regression graph of pyrimethamine for assay testing.

The r^2 value was 0.9999, with an overall uncertainty of 0.57 µg/ml. A linear graph was thus produced in the concentration range of 12.7 - 38.1 µg/ml. The equation of the line is:

$$y = 50.4858x + 25.749$$

C. Repeatability

Reference solution three (representing the 100% concentration), being prepared for the linearity study, was used in the repeatability study and was injected five times.

The %RSD for the sulfadoxine injections was 0.11 (table 3.9), and for pyrimethamine 0.51 (table 3.10), thus indicative of excellent repeatabilities being obtained.

D. Recovery

A solution, spiked with approximately 500 µg/ml of sulfadoxine and 25 µg/ml of pyrimethamine, was analysed on HPLC. The resulting peak areas were used to calculate the concentration of the solution by means of the equations given in 3.4.3.3 B. The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

A recovery of 99.1% for sulfadoxine and 99.2% for pyrimethamine was obtained. A recovery of 98.0 – 102.0% is deemed acceptable and the method thus complied with the requirements.

E. Robustness

For the purpose of reproducibility, different types of columns were tested and the ratio of the mobile phase was adjusted. The spiked solution as described in 3.4.3.3 D was used in these tests.

i. Columns

Three different types of C_{18}, 250 x 4.6 mm, 5 µm columns were used to determine the effect of different brands of columns on the tailing, retention time and resolution between the sulfadoxine and pyrimethamine peaks (table 3.11).

Table 3.11: Peak properties of sulfadoxine and pyrimethamine using different brands of columns (C_{18}, 250 x 4.6 mm, 5 µm)

	SULFADOXINE		PYRIMETHAMINE		
BRAND NAME	**Retention time (min)**	**Tailing**	**Retention time (min)**	**Tailing**	**Resolution factor**
Phenomenex Luna	8.2	1.3	13.1	1.9	11.5
Macherey Nagel	5.0	1.1	11.4	1.8	17.4
Waters Symmetry	6.0	1.1	8.7	1.3	8.4

The results indicated that, although different types of columns influenced the retention times differently and therefore the resolution between the two API peaks, all three these columns were suitable for use. The three columns showed acceptable resolution between the two API peaks, and the tailing of both APIs was smaller than the accepted value of 2.0 for these columns.

The analyses were also done on a C_{18}, 250 x 4.6 mm, 10 µm column. The results indicated that a 10 µm column was unsuitable for the analyses of sulfadoxine/pyrimethamine, since the peaks eluted very early (approximately 2.8 and 5.2 minutes for sulfadoxine and pyrimethamine, respectively), with poor peak shapes (figure 20). The resolution factor between the peaks was approximately 3.0.

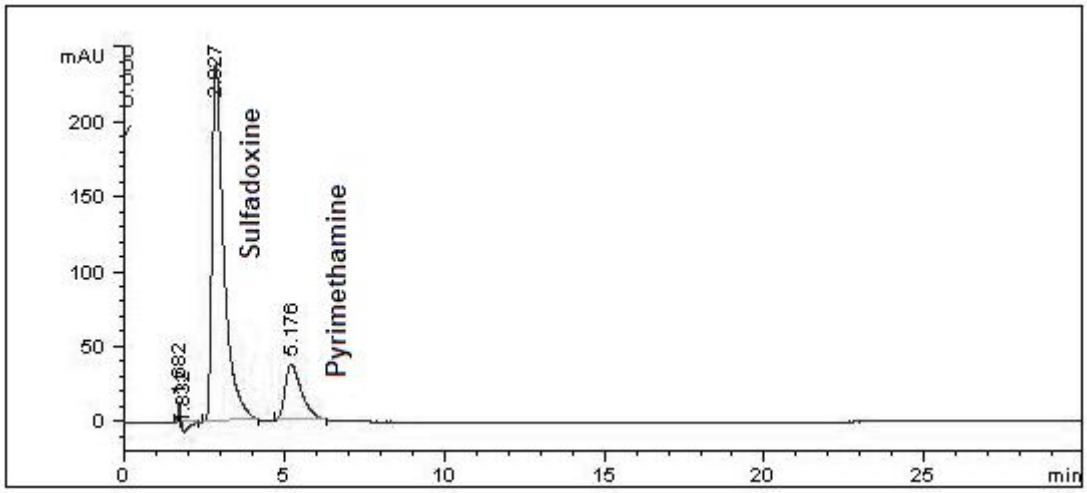


Figure 3.20: Example of a chromatogram of reference solution using a 250 x 4.6 mm, 10 µm, C_{18} column.

ii. Change in mobile phase composition

The ratio of mobile phase was adjusted in order to determine the effect on the elution time of the individual peaks, as well as the resolution between the peaks in the reference solutions. The ratios being used are shown in table 3.12.

Table 3.12: Mobile phase compositions used in robustness studies

% ABSOLUTE CHANGE (with respect to Solvent B)	SOLVENT A VOLUME (ml)	SOLVENT B VOLUME (ml)
-10	820	180
-5	810	190
0	800	200
+5	790	210
+10	780	220

Results are given in table 3.13. The results represent the average of five injections.

Table 3.13: Results of a change in mobile phase composition on retention time, tailing and resolution factor of sulfadoxine and pyrimethamine peaks

	SULFADOXINE		PYRIMETHAMINE		
MOBILE PHASE RATIO	Retention time (min)	Tailing	Retention time (min)	Tailing	Resolution factor
820**A**:180**B**	10.4	1.3	18.9	1.7	15.4
810**A**:190**B**	9.0	1.3	17.8	1.8	14.7
800**A**:200**B**	8.6	1.3	13.9	1.8	12.3
790**A**:210**B**	7.7	1.3	11.7	1.8	10.6
780**A**:220**B**	No results obtained due to high back pressure				

The results indicated that a larger volume of Solvent A (a 5% decrease in Solvent B), influenced the retention time of pyrimethamine more than that of sulfadoxine. The retention time of the pyrimethamine peak increased with about four minutes, from 13.9 minutes to 17.8. Contrary, the sulfadoxine peak eluted only 0.4 minutes later. Resolution between the API peaks decreased as a higher percentage of solvent B was used in the mobile phase.

None of the ratios had a significant influence on the tailing of either of the peaks.

No results were obtained for the 780A:220B ratio, as the back pressure on the column was too high, causing system failure.

iii. Mobile phase pH

The pH of the mobile phase was adjusted by ± 0.2 pH units, from pH 4.2 to 4.0 and 4.4, in order to determine the effect on the retention times, tailing factors and resolution factor of the peaks of the reference solution.

The results are given in table 3.14.

Table 3.14: Results of a change in pH of mobile phase on retention time, tailing and resolution factor of sulfadoxine and pyrimethamine peaks

	SULFADOXINE		PYRIMETHAMINE		
pH	Retention time (min)	Tailing	Retention time (min)	Tailing	Resolution factor
4.4	8.1	1.2	13.3	1.6	13.2
4.2	8.6	1.3	13.9	1.3	12.3
4.0	8.0	1.3	12.0	1.9	7.9

The only significant difference between pH 4.2 and 4.4, was the higher tailing factor of the pyrimethamine peak at pH 4.4. However, a decrease in pH from 4.2 to 4.0 significantly increased the tailing factor of the pyrimethamine peak, whilst its retention time decreased by about 2 minutes, from 13.9 min to 12 min. It was thus recommended that a pH of 4.2 – 4.4 should be maintained for the mobile phase.

iv. Product analyses/assays

After successful validation of the analytical method, it was used to perform assay analyses of four commercial batches of sulfadoxine/pyrimethamine tablets. Two products were assayed by another analyst in order to ascertain the reproducibility of the assay procedure. All products contained 500 mg of sulfadoxine and 25 mg of pyrimethamine per tablet. The details of the products are given in table 3.15.

Table 3.15: Details of commercial sulfadoxine/pyrimethamine products used in assay analyses

PRODUCT NAME	MANUFACTURER	BATCH NO	EXPIRY DATE
Fansidar	Roche Products	Z6283	3/2011
Tansidar	Interchem Pharma	TST5022	11/2007
Shelys sulfadoxine/pyrimethamine	Shelys Pharmaceuticals	415	9/2007
Sulphadar	Shelys Pharmaceuticals	6025	3/2010

Sample solutions were prepared as described in 3.4.3.2.

Samples were prepared in triplicate and each result is the average of the three. The %RSD was calculated for the three samples.

The assay results are summarised in table 3.16. The results of the reproducibility studies are shown in table 3.17. Representative chromatograms of the products are shown in figures 3.21 – 3.24.

Table 3.16: Assay results of commercial sulfadoxine/pyrimethamine tablets

PRODUCT	% ASSAY (%RSD)	
	Sulfadoxine	Pyrimethamine
Fansidar	102.1 (0.44)	98.1 (0.65)
Tansidar	97.8 (0.26)	94.1 (1.44)
Shelys SP	94.0 (1.73)	93.0 (0.51)
Sulphadar	104.0 (0.65)	85.2 (0.83)

Table 3.17: Comparative assay results of two commercial sulfadoxine/ pyrimethamine tablets for two analysts

PRODUCT	SULFADOXINE			PYRIMETHAMINE		
	Assay 1	Assay 2	% Diff	Assay 1	Assay 2	% Diff
Fansidar	102.1	97.6	-4.4	98.1	95.6	-2.5
Sulphadar	104.0	103.3	-0.67	85.2	85.4	0.23

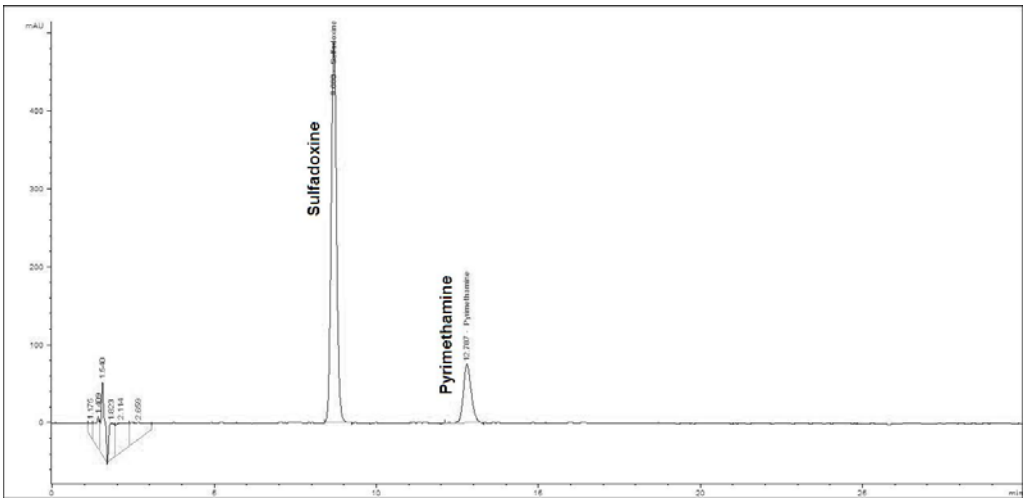


Figure 3.21: Example of a chromatogram for Fansidar tablets.

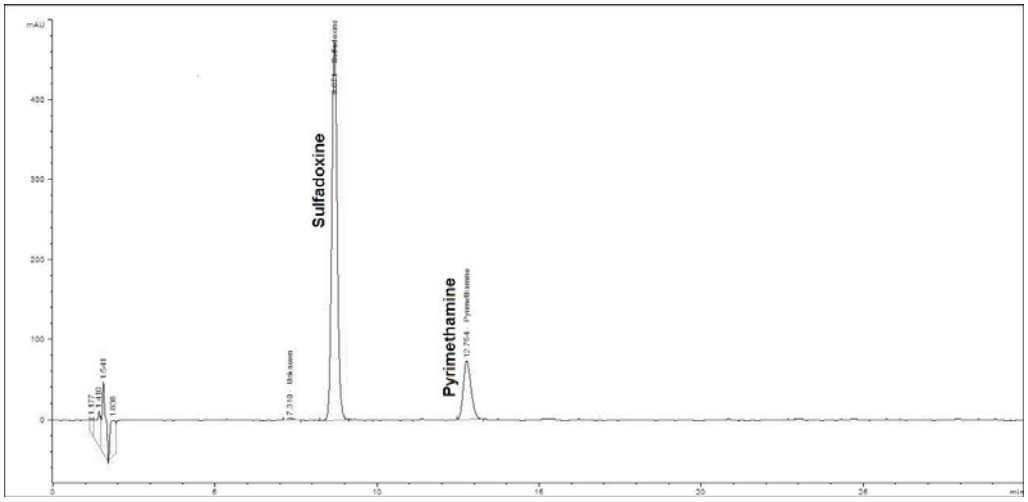


Figure 3.22: Example of a chromatogram for Tansidar tablets.

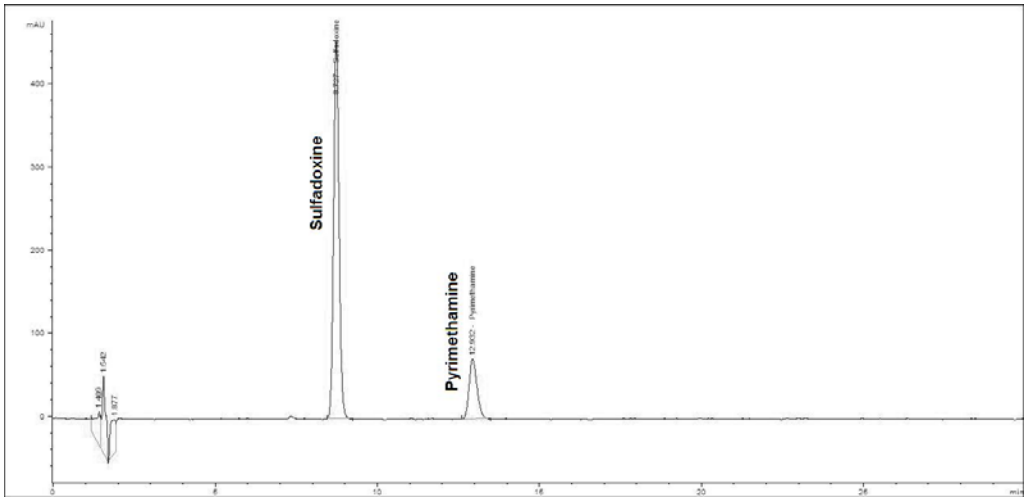


Figure 3.23: Example of a chromatogram for Shelys sulfadoxine/ pyrimethamine tablets.

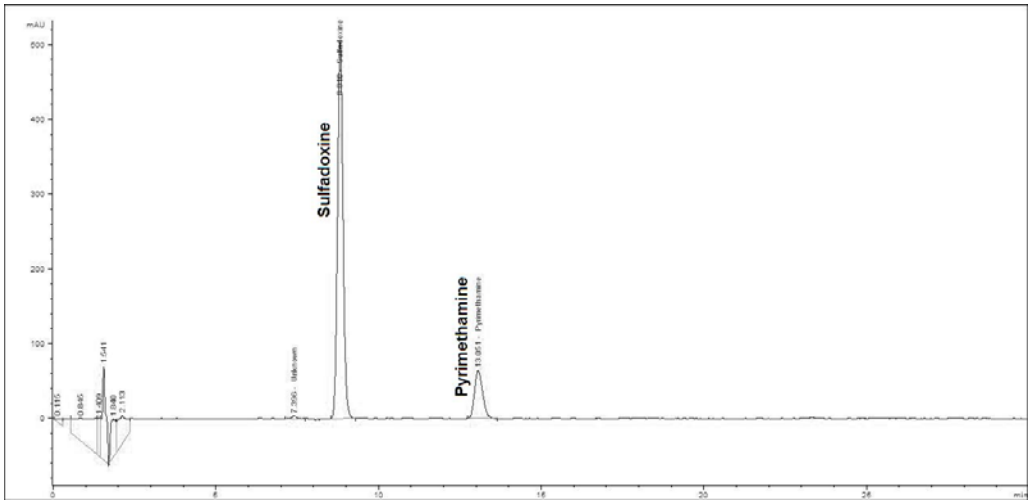


Figure 3.24: Example of a chromatogram for Sulphadar tablets.

The results indicated that the method was suitable for performing assay analyses of the sulfadoxine/pyrimethamine products. The %RSD for all products (triplicate samples) was below 2.0%, indicating that the method of preparation of the samples did not give rise to significant differences in outcomes. Regarding reproducibility of the test results of the Fansidar tablets, the independent second assay results were lower than expected. Upon investigation, it was established that during preparation, the initial sonification of the stock solutions was omitted, most likely resulting in the poor repeatability and inaccurate results. The independent assay of the Sulphadar was subsequently performed, with specific attention to sonification as per the method, and acceptable results, with %RSDs lower than 1% for both APIs, were obtained.

3.4.4 SUMMARY OF VALIDATION RESULTS

The method for assay analyses was successfully validated and satisfactory results were obtained during assay testing of commercial sulfadoxine/pyrimethamine products. A summary of the results are given in table 3.18.

Table 3.18: Summary of results obtained during assay validation of sulfadoxine and pyrimethamine

	RESULTS OBTAINED	
PARAMETER	**Sulfadoxine**	**Pyrimethamine**
Specificity	No interferences were detected from solvents, excipients nor other related substances during stress testing.	
Linearity	r^2 = 0.9999	r^2 = 0.9999
Range	248.1 – 752.5 µg/ml (50 – 150%)	12.7 – 38.1 µg/ml (51 – 153%)
Repeatability	%RSD = 0.11	%RSD = 0.51
Recovery	99.1%	99.2%
Robustness		
Column	Equivalent columns are suitable	
Mobile phase ratio	-10% to +5% change (with respect to solvent B) resulted in acceptable results	
pH of mobile phase	pH = 4.2 to 4.4 resulted in acceptable results	
Product analyses	%RSD < 2.0 for 4 commercial products	

3.5 DISSOLUTION

3.5.1 DEVELOPMENT STUDIES

As was discussed earlier, many quality control failures of sulfadoxine/pyrimethamine tablets can be attributed to the poor dissolution performance of the products. The USP monograph (USP, 2009a) for pyrimethamine tablets prescribes 0.1M HCl as the dissolution medium, whilst the medium for the sulfadoxine/pyrimethamine combination tablets is indicated as pH 6.8 phosphate buffer. The question was raised as to the feasibility of also using 0.1M HCl as the dissolution medium for the combination products.

In a study done by Badenhorst (2007:23), alternative dissolution media for the combination products were investigated. The conclusion was made that 0.1M HCl should be the dissolution medium of choice.

3.5.1.1 Solubility study

The solubility of pyrimethamine in dilute HCl is known to be about 5 g/l (Loutfy & Aboul-Enein, 1983:465), whilst that of sulfadoxine had to be determined experimentally.

Five samples each of an over saturated sulfadoxine suspension were prepared in 0.1M HCl, phosphate buffer pH 6.8 and acetate buffer pH 4.5. The samples were rotated in a water bath for 24 hours at 37ºC. The samples were then filtered, suitably diluted where necessary, and the concentrations were determined by means of the validated method described in 3.4.3.

The results are presented in table 3.19.

Table 3.19: Solubility data for sulfadoxine in 0.1M HCl, pH 4.5 buffer and pH 6.8 buffer

SOLVENT	SOLUBILITY (mg/ml)	SOLUBILITY (mg/250 ml)
0.1M HCl	1.23	307.5
pH 4.5 buffer	0.29	72.5
pH 6.8 buffer	1.36	340

From the results it was concluded that the pH 6.8 phosphate buffer could indeed be substituted with 0.1M HCl as dissolution medium, since the solubility of sulfadoxine in these solvents was comparable. The solubility data indicated that sulfadoxine is of BCS low solubility (<500 mg/250 ml), therefore a volume of 1,000 ml was selected for the dissolution medium. Buffer pH 4.5 is unsuitable, due to the low solubility, and therefore the studies were done in 0.1M HCl and buffer pH 6.8.

3.5.1.2 Disintegration studies

Since disintegration of a product may be the limiting factor during dissolution testing, the disintegration of the five products, that were used during evaluation of the different dissolution media, was also determined. The details of the products are given in table 3.20.

Table 3.20: Details of commercial sulfadoxine/pyrimethamine products used in disintegration and dissolution analyses

PRODUCT NAME	MANUFACTURER	BATCH NO	EXPIRY DATE
Fansidar	Roche Products	Z6283	3/2011
Tansidar	Interchem Pharma	TST5022	11/2007
Sulphadar	Shelys Pharmaceuticals	6025	3/2010
		8004	2/2012
Malostat	Intas Pharmaceuticals	H002	1/2010

The disintegration study was done as prescribed by the general monograph for disintegration of the Ph.Int., using water as medium (Ph.Int., 2008). The expired product, Tansidar, was included in this study on purpose, in order to verify the correlation between disintegration and dissolution.

The results are presented in table 3.21.

Table 3.21: Results of disintegration studies performed on various sulfadoxine/pyrimethamine commercial products

	MINUTES'SECONDS				
TABLET	**Fansidar**	**Tansidar**	**Sulphadar (Batch 6025)**	**Sulphadar (Batch 8004)**	**Malostat**
1	1'32	>30	7'11	4'08	3'54
2	1'46	>30	7'19	6'10	4'35
3	1'54	>30	7'27	8'23	4'47
4	1'54	>30	7'33	9'25	4'57
5	1'57	>30	7'39	11'59	5'07
6	2'07	>30	7'47	14'19	5'13

The results indicated that the disintegration times varied from as little as two minutes up to almost 15 minutes for those products that disintegrated within the allowed 30 minutes. Tansidar tablets did not disintegrate completely, even after 30 minutes. The one batch of Sulphadar showed very high within-batch variances, whilst inter batch variances also occurred.

3.5.1.3 Dissolution medium

Dissolution tests were performed on the five commercial product batches (refer table 3.20) in both pH 6.8 phosphate buffer and 0.1M HCl, each having a volume of 1,000 ml.

The other dissolution testing parameters were as follows:

Apparatus: 2 (Paddles)

Rotation speed: 75 rpm

Withdrawal times (min): 10, 15, 20, 30 and 45

All dissolution samples were analysed by the same analytical method as described for the assay.

The results of the five products are summarised in tables 3.22 – 3.31 and are graphically represented in figures 3.25 – 3.29.

Table 3.22: Percentage dissolution of Fansidar tablets in 0.1M HCl as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	70	80	87	93	98	94	96	97	97	98
2	65	81	87	95	99	93	95	94	97	97
3	68	83	90	95	100	97	96	95	96	97
4	67	83	90	97	101	93	93	98	95	96
5	59	78	87	95	101	81	92	95	96	97
6	66	82	90	97	102	95	95	95	95	95
AVG	66	81	88	95	100	92	94	95	96	97
%RSD	5.4	2.4	1.7	1.4	1.5	6.2	2.0	1.4	0.98	0.89

Table 3.23: Percentage dissolution of Fansidar tablets in phosphate buffer pH 6.8 as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	62	76	87	97	101	45	49	75	74	82
2	76	85	93	101	102	61	66	73	84	87
3	78	88	94	100	104	44	60	68	85	84
4	79	88	94	99	105	57	61	69	82	88
5	75	89	91	96	100	50	70	70	82	87
6	66	82	91	99	106	33	59	68	82	91
AVG	73	85	92	99	103	49	61	71	82	86
%RSD	9.5	5.6	2.8	2.1	2.5	20.7	11.9	3.8	4.9	3.6

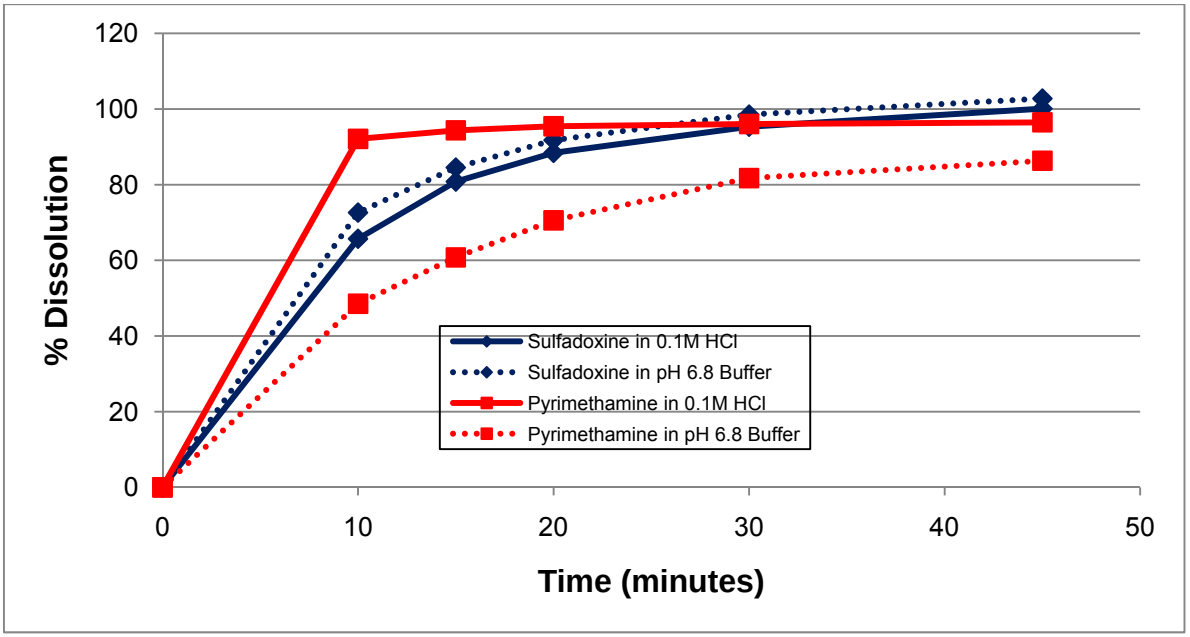


Figure 3.25: Dissolution profiles of Fansidar tablets in 0.1M HCl and phosphate buffer pH 6.8.

Table 3.24: Percentage dissolution of Malostat tablets in 0.1M HCl as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	80	89	92	96	101	86	86	89	92	95
2	89	97	98	101	103	92	95	97	97	96
3	92	102	104	107	109	98	101	98	101	103
4	83	97	99	101	103	89	93	95	97	96
5	72	83	94	99	104	74	84	95	93	94
6	89	98	99	101	104	93	95	98	96	96
AVG	84	94	98	101	104	89	92	95	96	97
%RSD	8.9	7.3	4.3	3.6	2.7	9.5	6.8	3.5	3.4	3.3

Table 3.25: Percentage dissolution of Malostat tablets in phosphate buffer pH 6.8 as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	98	102	101	103	101	86	94	93	94	94
2	94	101	102	102	102	83	94	95	96	94
3	92	99	101	100	100	82	91	92	94	92
4	84	98	101	102	101	74	92	93	93	93
5	90	101	103	102	102	82	93	101	95	94
6	89	103	104	104	103	80	97	96	96	96
AVG	91	101	102	102	102	81	93	95	95	94
%RSD	5.1	1.8	1.2	1.4	1.2	5.2	2.2	3.5	1.3	1.3

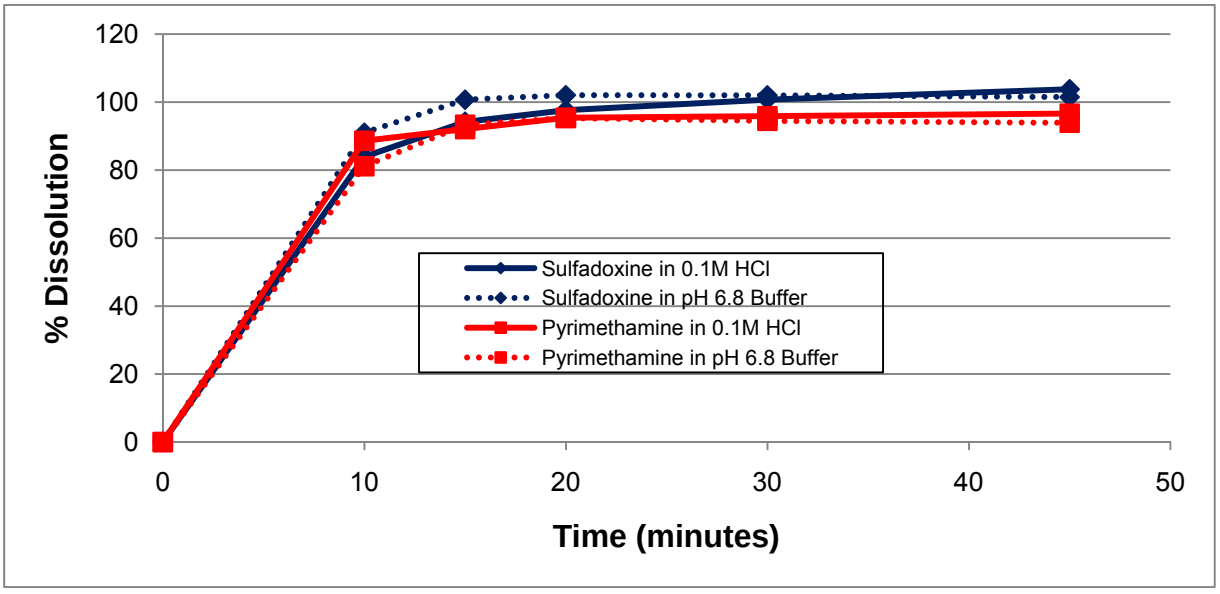


Figure 3.26: Dissolution profiles of Malostat tablets in 0.1M HCl and phosphate buffer pH 6.8.

Table 3.26: Percentage dissolution of Sulphadar (batch 6025) tablets in 0.1M HCl as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	74	85	88	92	95	79	84	87	92	91
2	76	85	89	93	95	82	89	86	91	90
3	72	82	87	90	93	73	82	86	90	90
4	70	84	88	92	94	75	81	86	87	90
5	63	81	87	92	94	76	82	88	90	91
6	73	85	89	93	95	78	83	86	87	88
AVG	71	84	88	92	95	77	84	87	89	90
%RSD	6.5	2.1	1.0	1.1	0.92	4.1	3.7	0.93	2.3	1.1

Table 3.27: Percentage dissolution of Sulphadar (batch 6025) tablets in a phosphate buffer pH 6.8 as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	71	91	93	92	93	53	75	70	73	75
2	59	89	102	104	104	45	70	81	84	83
3	88	101	103	104	104	70	82	86	85	88
4	79	103	107	108	108	60	83	86	87	92
5	85	102	104	104	105	67	82	83	85	83
6	77	102	107	108	108	61	81	84	89	87
AVG	77	98	102	103	104	59	79	82	84	85
%RSD	13.5	6.6	5.1	5.6	5.2	15.3	6.5	7.1	6.5	6.8

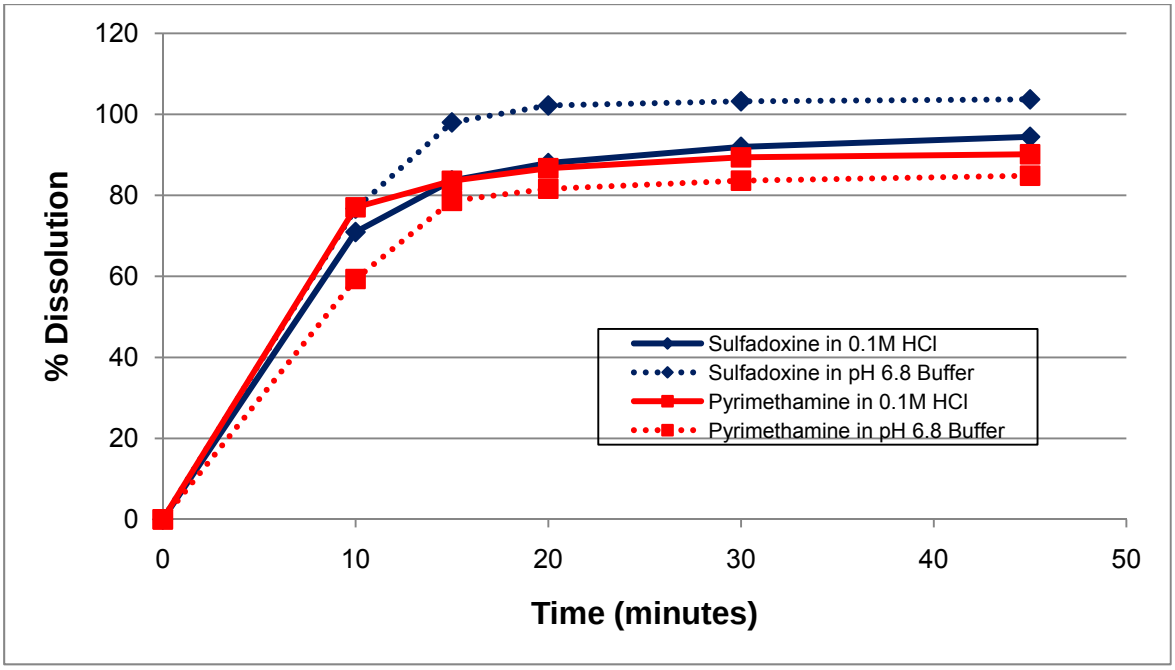


Figure 3.27: Dissolution profiles of Sulphadar (batch 6025) tablets in 0.1M HCl and phosphate buffer pH 6.8.

Table 3.28: Percentage dissolution of Sulphadar (batch 8004) tablets in 0.1M HCl as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	43	53	59	65	72	78	82	87	90	90
2	44	54	60	67	73	78	86	85	93	94
3	45	54	60	66	73	78	85	91	91	91
4	45	54	58	65	72	77	82	87	88	90
5	42	51	57	64	71	77	83	86	92	94
6	45	54	59	66	73	77	83	87	90	93
AVG	44	53	59	65	72	78	83	87	91	92
%RSD	2.6	2.1	1.9	1.5	1.1	0.62	1.8	2.4	1.6	1.9

Table 3.29: Percentage dissolution of Sulphadar (batch 8004) tablets in phosphate buffer pH 6.8 as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	48	60	68	76	84	26	34	43	53	64
2	48	58	65	73	81	26	35	42	52	62
3	50	61	67	75	82	29	36	44	54	65
4	46	58	65	73	80	27	36	43	53	64
5	30	50	64	77	86	17	29	38	54	64
6	49	60	66	73	82	29	38	43	54	66
AVG	45	58	66	75	83	26	35	42	53	64
%RSD	16.5	7.0	2.1	2.3	2.4	18.0	8.8	4.6	1.8	2.0

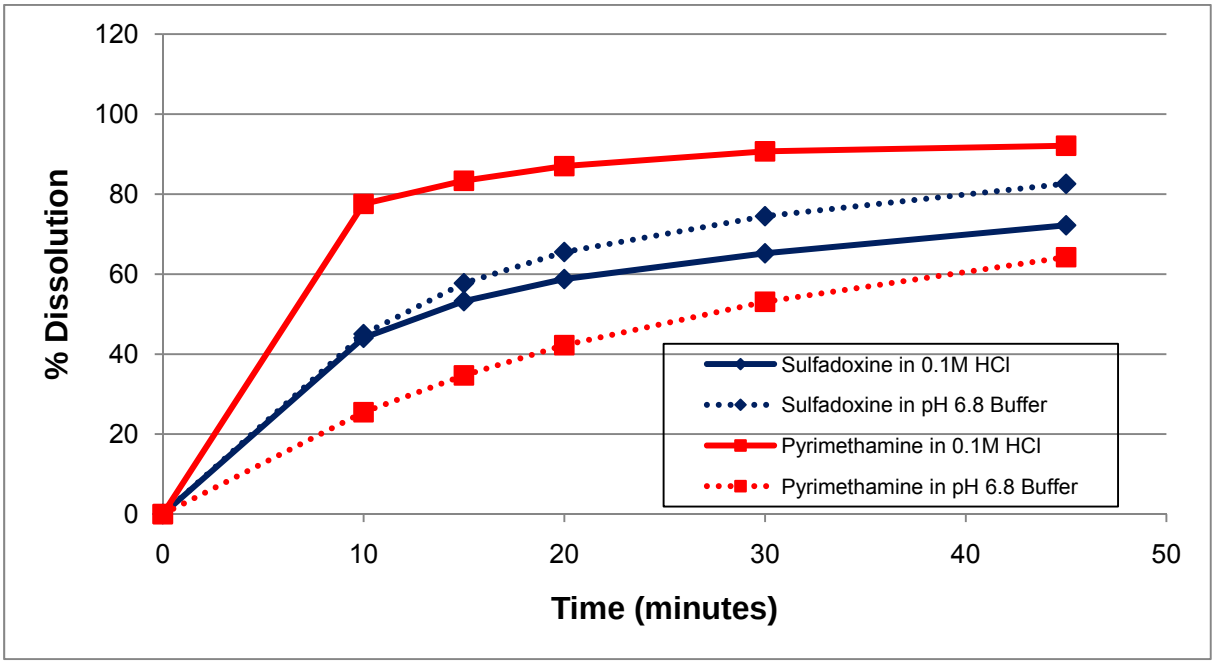


Figure 3.28: Dissolution profiles of Sulphadar (batch 8004) tablets in 0.1M HCl and phosphate buffer pH 6.8.

Table 3.30: Percentage dissolution of Tansidar tablets in 0.1M HCl as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	2	4	5	11	21	12	19	21	32	45
2	2	4	7	13	23	14	19	24	35	46
3	6	10	14	22	31	20	25	34	46	57
4	2	4	6	12	19	14	16	22	32	43
5	2	3	5	10	17	13	16	23	33	45
6	3	4	7	12	23	14	22	26	36	52
AVG	3	5	7	13	22	15	20	25	35	48
%RSD	60.7	54.5	42.7	33.7	22.3	19.6	16.9	18.0	14.7	10.9

Table 3.31: Percentage dissolution of Tansidar tablets in phosphate buffer pH 6.8 as dissolution medium

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	9	15	23	35	44	3	0	5	9	10
2	9	15	21	34	43	4	7	5	10	14
3	4	8	15	27	34	3	4	5	6	9
4	13	18	23	30	36	0	4	6	9	12
5	10	14	20	30	37	3	3	6	9	10
6	9	14	20	28	33	4	3	4	8	9
AVG	9	14	20	30	38	3	3	5	9	11
%RSD	32.6	22.5	15.0	10.4	12.2	51.7	65.6	16.6	16.5	16.5

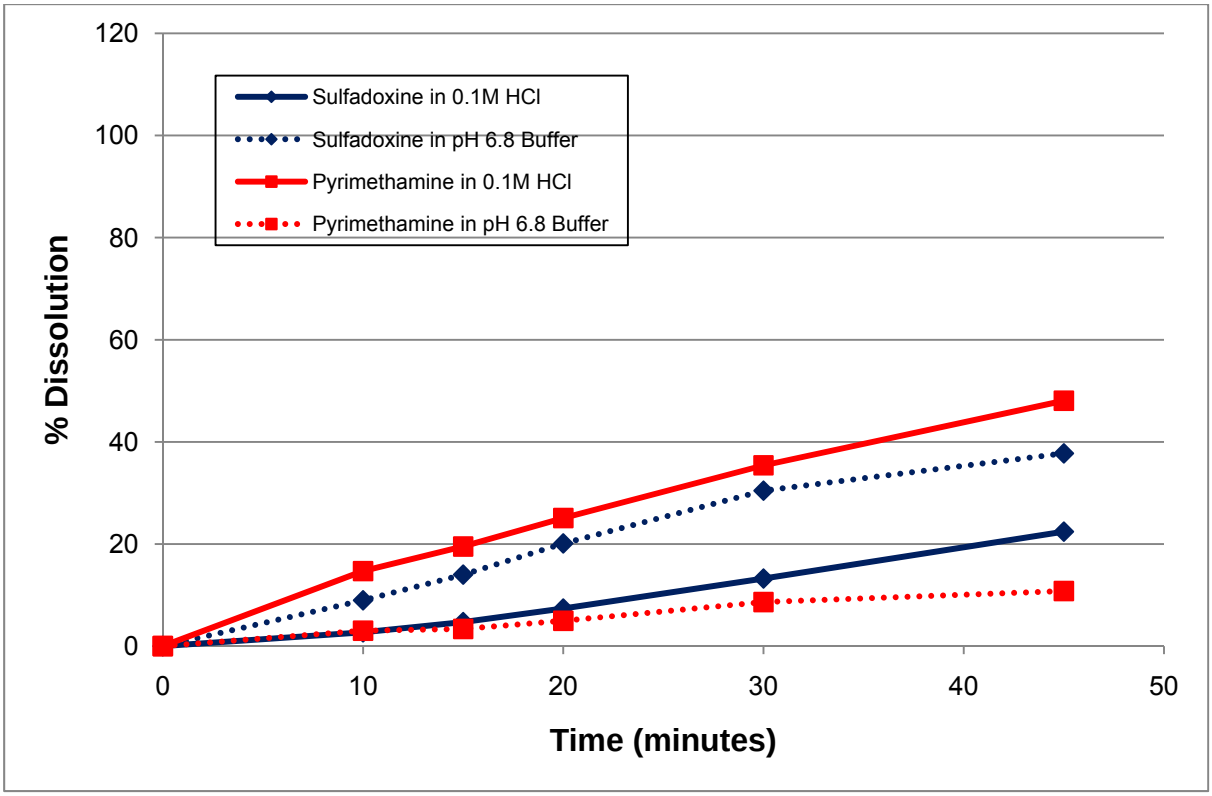


Figure 3.29: Dissolution profiles of Tansidar tablets in 0.1M HCl and phosphate buffer pH 6.8.

The dissolution results indicated that although 0.1M HCl as dissolution medium, resulted in better dissolution for especially pyrimethamine in many cases, it still was discriminatory to such an extent that poor quality products will not comply with the dissolution criterion. However, the 0.1M HCl dissolution medium successfully increased the acceptance criterion to 80% in 30 minutes for both APIs.

The correlation between the disintegration and dissolution results were of particular importance for the testing of sulfadoxine/pyrimethamine products. Tansidar and Sulphadar (batch 8004) both had poor disintegration, as well as dissolution results. The dissolution profiles were indicative of poor disintegration, as the process of dissolution clearly was incomplete at 30 minutes.

The profiles of Fansidar, it being the innovator product, were of particular importance for setting acceptance criteria. Figure 3.25 shows that an acceptance criterion of 80% at 30 minutes was acceptable for both APIs. Sulphadar (batch 6025) passed the requirement, whilst Sulphadar (batch 8004) and Tansidar failed. From the results it was concluded that those tablets with a disintegration time of less than 10 minutes, should comply with the acceptance criterion of 80% in 30 minutes.

Leslie *et al*. (2009:1757) reported that the generic products being used in the treatment of patients did not meet USP dissolution criteria of 60% in 30 minutes. If disintegration studies had been performed during quality control procedures, the prevailing poor manufacturing practices would have been exposed pro-actively.

Disintegration studies offer a cheap, quick and a very good indication of what to expect during dissolution testing.

3.5.1.4 Repeatability of results

The dissolution tests for Fansidar were repeated in the two dissolution media in order to determine repeatability.

The results for these dissolution tests are summarised in tables 3.32 and 3.33 and are graphically represented in figures 3.30 and 3.31.

Table 3.32: Percentage dissolution of Fansidar tablets in 0.1M HCl as dissolution medium (Test 2)

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	65	78	85	92	96	94	96.	96.	97	96
2	61	80	91	101	110	96	103	106	110	111
3	71	84	90	96	101	93	97	96	99	96
4	70	81	88	95	98	93	93	93	95	95
5	50	69	79	87	93	71	85	91	93	93
6	57	72	81	89	95	84	91	95	94	94
AVG	62	77	85	93	99	88	94	96	98	97
%RSD	12.6	7.4	5.9	5.5	6.1	10.6	6.4	5.4	6.5	6.9

Table 3.33: Percentage dissolution of Fansidar tablets in phosphate buffer pH 6.8 as dissolution medium (Test 2)

	% DISSOLUTION									
	SULFADOXINE					PYRIMETHAMINE				
TABLET	10 min	15 min	20 min	30 min	45 min	10 min	15 min	20 min	30 min	45 min
1	77	89	94	99	100	51	64	71	80	86
2	70	88	96	101	103	47	62	70	82	87
3	80	94	99	102	104	54	67	74	82	89
4	78	90	96	102	104	54	66	72	82	89
5	81	92	97	101	103	54	66	72	81	87
6	71	89	96	100	102	47	62	71	80	86
AVG	76	90	96	101	103	51	65	72	81	87
%RSD	6.1	2.4	1.5	1.1	1.4	6.6	3.1	1.8	1.5	1.7

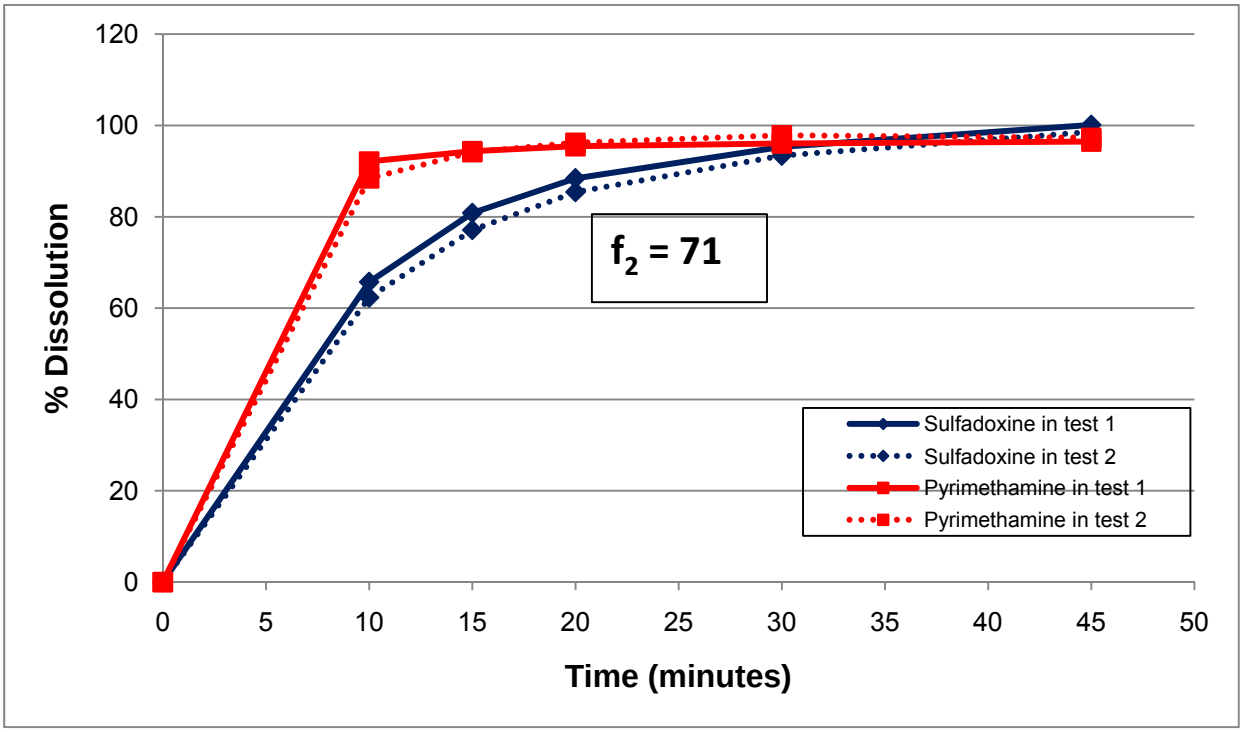


Figure 3.30: Dissolution profiles of Fansidar tablets in 0.1M HCl.

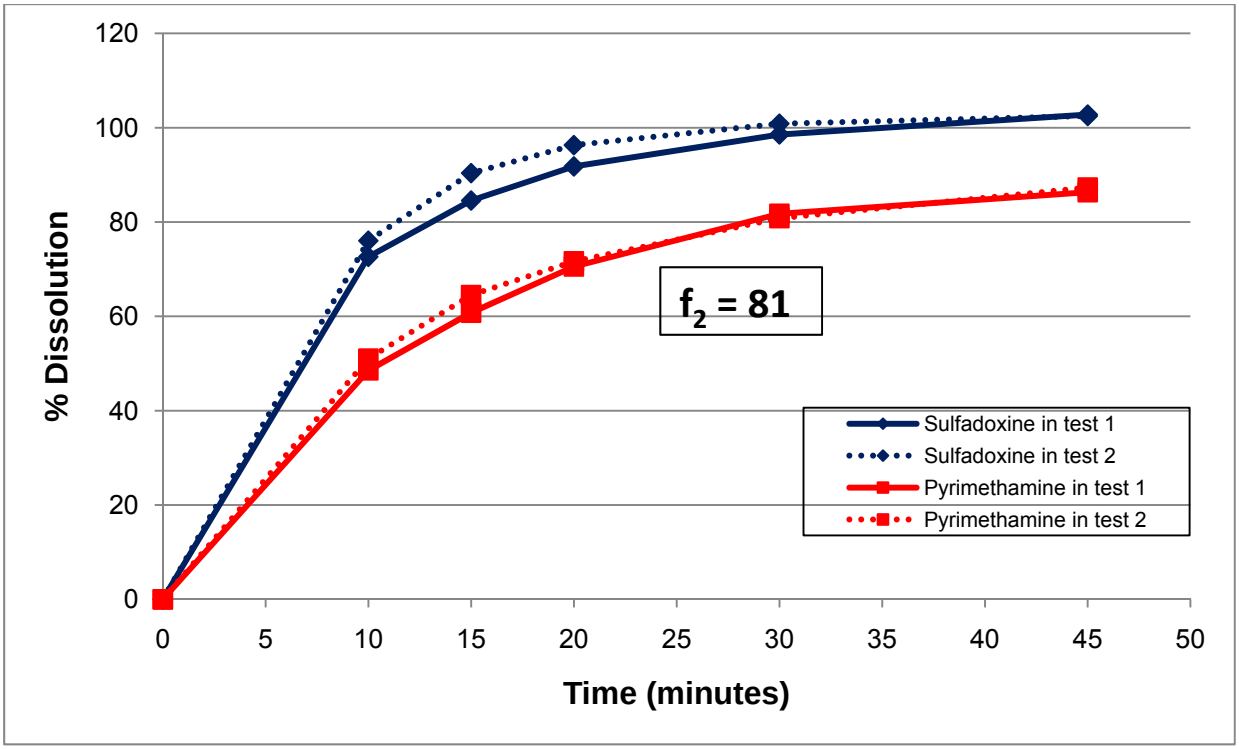


Figure 3.31: Dissolution profiles of Fansidar tablets in phosphate buffer pH 6.8.

The results demonstrated good repeatability of the dissolution tests in both media. Both sulfadoxine in pH 6.8 phosphate buffer and pyrimethamine in 0.1M HCl had dissolved 85% within 15 minutes. The similarity factors of pyrimethamine in pH 6.8 phosphate buffer and sulfadoxine in 0.1M HCl were more than 50, indicating that the profiles were similar.

3.5.1.5 Stability of samples

The 30 minute samples of dissolution test 1 of Fansidar for both media were kept for at least 24 hours on the bench and analysed again. The results are given in table 3.34.

The results showed no significant changes in the percentage dissolution of both APIs after 24 hours on the bench, indicating that the samples were stable for at least 24 hours after completion of the dissolution test.

Table 3.34: Percentage dissolution of Fansidar tablets directly after dissolution testing and 24 hours later

	% DISSOLUTION							
	0.1M HCl				pH 6.8 Buffer			
	Sulfadoxine		Pyrimethamine		Sulfadoxine		Pyrimethamine	
TABLET	Initial	24 hours	Initial	24 hours	Initial	24 hours	Initial	24 hours
1	93	90	97	100	97	98	74	81
2	95	92	97	100	101	100	84	85
3	95	93	96	101	100	100	85	83
4	97	95	95	101	99	101	82	82
5	95	93	96	103	96	98	82	86
6	97	97	95	98	99	100	82	83
AVG	95	93	96	100	99	99	82	83
%RSD	1.4	2.7	0.98	1.6	2.1	1.4	4.9	2.3

3.5.1.6 Conclusion

From the outcomes of these development studies, the following parameters were chosen for the dissolution testing of sulfadoxine/pyrimethamine tablets:

Apparatus: 2 (Paddles)

Rotation speed: 75 rpm

Medium: 1,000 ml 0.1M HCl

Withdrawal time: 30 minutes

Acceptance criteria: Not less than 80% according to Ph.Int. definition for both APIs.

The samples are analysed using the same analytical method as described for the assay.

This study thus confirmed the conclusion being reached by Badenhorst (2007:23), namely that 0.1M HCl is more suitable as dissolution medium for the fixed dose combination of sulfadoxine/pyrimethamine tablets.

3.5.2 VALIDATION OF ANALYTICAL METHOD FOR DISSOLUTION

The same method, as described in 3.4.3, was used in the analyses of the dissolution samples, except that the reference solution dilutions were made with dissolution medium. A complete validation was thus unnecessary.

3.5.2.1 Preparation of sample solution

i. At 30 minutes withdraw a 5 ml sample from the medium through an in-line filter (0.45 µm).

ii. Allow the filtered sample to cool to room temperature.

3.5.2.2 Validation parameters

The parameters as indicated in table 3.35 were evaluated during the validation study.

Table 3.35: Validation parameters and acceptance criteria required for dissolution validation studies (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA
Linearity	$r^2 \geq 0.99$
Range	±30% of specified range
Repeatability	%RSD ≤ 2 for 5 determinations from same solution
Recovery	95.0 – 105.0%

A. Linearity and range

Sulfadoxine

The 100% theoretical concentration was taken as 500 µg/ml, targeting the concentration of the sample, assuming complete dissolution. Five different reference solutions, covering the range of 49.7 – 149.0% of the 100% theoretical

concentration, were prepared by diluting the stock solution (refer 3.4.3.2) with dissolution medium (table 3.8).

The results are given in table 3.36 and are graphically represented in figure 3.32.

The r^2 value was 0.9999, with an overall uncertainty of 1.8 µg/ml. A linear graph was thus produced in the concentration range of 248.3 - 745.0 µg/ml. The equation of the line is:

$$y = 10.933x + 126.09$$

Pyrimethamine

The 100% theoretical concentration was taken as 25 µg/ml, targeting the concentration of the sample, assuming complete dissolution. Five different reference solutions, covering the range of 49.5 - 148.5% of the 100% theoretical concentration, were prepared by diluting the stock solutions (refer 3.4.3.2) with dissolution medium (table 3.8).

Table 3.36: Peak areas of linear regression graph of sulfadoxine in 0.1M HCl

CONCENTRATION (µg/ml sulfadoxine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
248.3	49.7	2817.5 2831.1	2824.3	0.34
397.4	79.5	4478.7 4490.1	4484.4	0.18
496.7	99.3	5562.7 5562.4 5561.6 5543.0 5572.7	5560.5	0.19
662.2	132.5	7412.4 7383.2	7397.8	0.28
745.0	149.0	8253.3 8225.6	8239.4	0.24

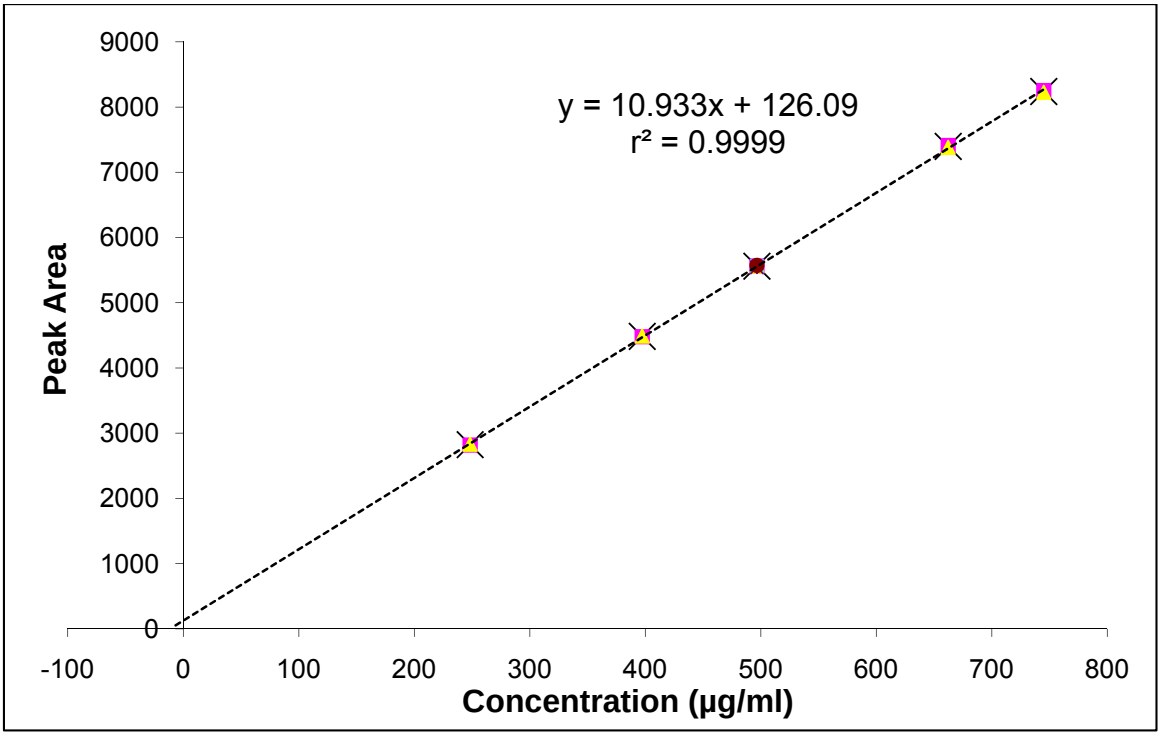


Figure 3.32: Linear regression graph of sulfadoxine in 0.1M HCl.

The results are given in table 3.37 and are graphically represented in figure 3.33.

Table 3.37: Peak areas of linear regression graph of pyrimethamine in 0.1M HCl

CONCENTRATION (µg/ml pyrimethamine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
12.4	49.5	703.5 682.9	693.2	2.10
19.8	79.2	1085.2 1078.1	1081.7	0.46
24.8	99.0	1341.6 1340.2 1387.5 1343.1 1333.1	1349.1	1.61
33.0	132.0	1786.3 1797.5	1791.9	0.44
37.1	148.5	2000.6 2023.9	2012.2	0.82

The r^2 value was 1.0, with an overall uncertainty of 0.04 µg/ml. A linear curve was thus produced in the concentration range of 12.4 - 37.1 µg/ml. The equation of the line is:

$$y = 53.392x + 29.015$$

B. Repeatability

Reference solution three, representing the 100% concentration, as prepared for the linearity study, was used for the repeatability study and was injected five times.

The %RSD of sulfadoxine was 0.19 (table 3.36) and that of pyrimethamine 1.6% (table 3.37), thus indicative of excellent repeatability being obtained.

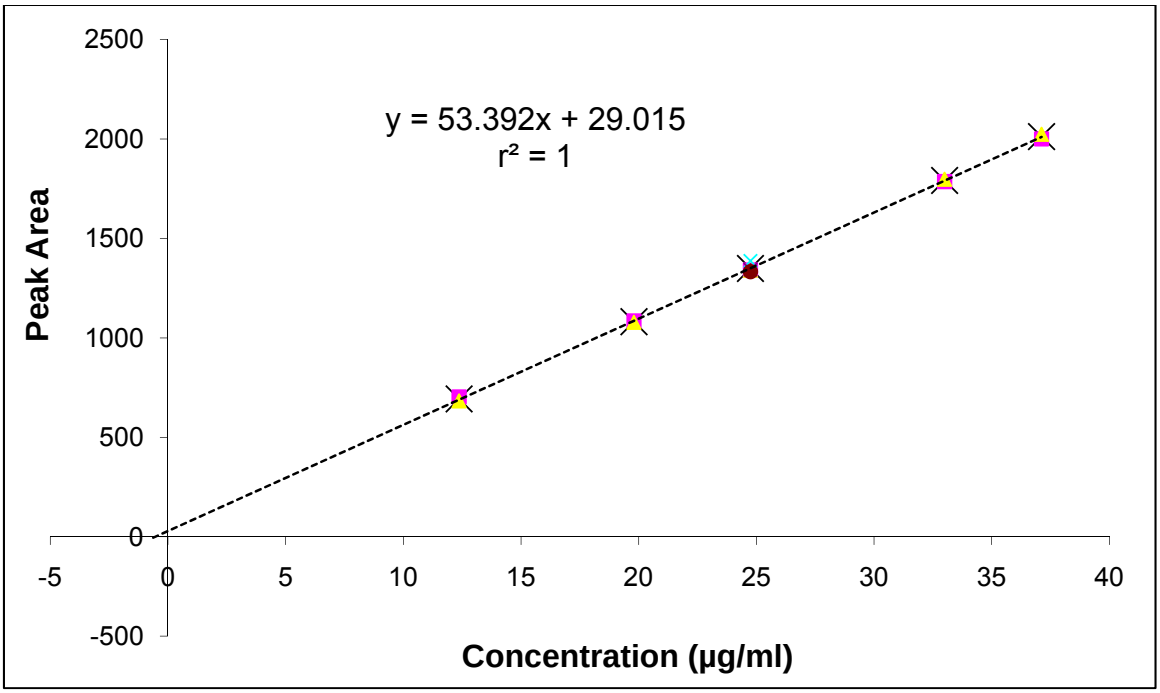


Figure 3.33: Linear regression graph of pyrimethamine in 0.1M HCl.

C. Recovery

A solution, spiked with approximately 500 µg/ml of sulfadoxine and 25 µg/ml of pyrimethamine, was injected onto the HPLC. The peak areas obtained were used to calculate the concentration of the solution by means of the respective linear equations being given in 3.6.2 A. The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

A recovery of 98.9% for sulfadoxine and 100.2% for pyrimethamine was obtained. A recovery of 95.0 – 105.0% is deemed acceptable and the method thus complied with the requirements.

3.5.2.3 Summary of validation results

The method for dissolution analyses was successfully validated and acceptable results were obtained when used in the analyses of dissolution samples of commercial sulfadoxine/pyrimethamine tablets (refer 3.5.3). A summary of the results are given in table 3.38.

Table 3.38: Summary of the results obtained during dissolution validation studies on sulfadoxine and pyrimethamine

	RESULTS OBTAINED	
PARAMETER	**Sulfadoxine**	**Pyrimethamine**
Linearity	r^2 = 0.9999	r^2 = 1.0
Range	248.3 – 745.0 µg/ml (50 – 149%)	12.4 – 37.1 µg/ml (50 – 149%)
Repeatability	%RSD = 0.19	%RSD = 1.61
Recovery	98.9%	100.2%

Since all of the tested parameters complied with the requirements for method validation, the method was deemed acceptable for analyses of dissolution samples in sulfadoxine/pyrimethamine combination tablets.

3.6 RELATED SUBSTANCES

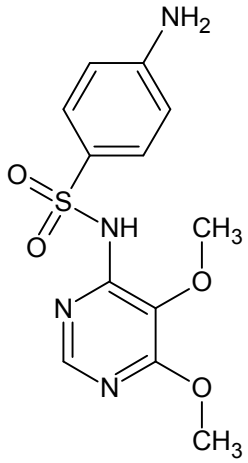
According to Kapoor (1988:582), sulfadoxine is a stable compound. Auterhoff and Schmidt (1974:1582) describe the pyrolysis and hydrolysis of sulfadoxine. The product of pyrolysis is 4-amino-5,6-dimethoxy-pyrimidine, whereas the products of acid hydrolysis are 4-aminobenzenesulfonic acid (sulfanilic acid), 4-amino-5,6-dimethoxy-pyrimidine and its mono demethylated analogue. No degradation processes are reported for pyrimethamine (Loutfy & Aboul-Enein, 1983:464).

It was agreed with WHO that in the case of sulfadoxine/pyrimethamine tablets that the related substances testing for pyrimethamine shall not be included in this study due to the large API ratio (500:25) – refer to monograph in Annexure B.

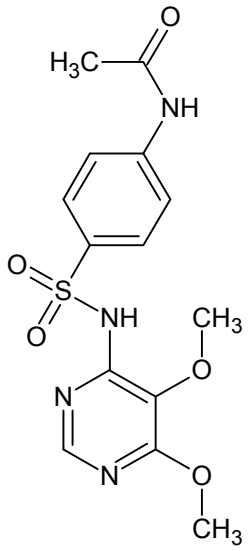
3.6.1 DEVELOPMENT STUDIES

The WHO supplied a manufacturer's method for use as reference in determining the related substances of sulfadoxine in sulfadoxine/pyrimethamine combination products during this study. The WHO also provided three related substances for use in the development of a suitable method:

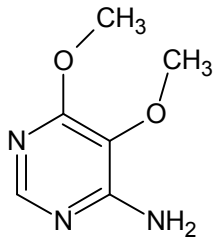
N1-(6-hydroxy-5-methoxy-4-pyrimidinyl)sulfanilamide (**Impurity B**)



4-(p-acetamide-benzenesulfonamide)-5,6-dimethoxy-pyrimidine (**Impurity C**)



4-amino-5,6-dimethoxy-pyrimidine (**Impurity D**)



It was decided to include sulfanilic acid and sulfanilamide (**Impurity A**) in the development study, since these are known related substances of sulfadoxine (Auterhoff & Schmidt, 1974:1582).

The same method as for the assay was used as reference method (refer 3.4.3), except for the injection volume of 100 µl, instead of 20 µl.

3.6.1.1 Mobile phase composition

The mobile phase for the assay (3.4.3) resulted in poor resolution of Impurities A, B and D. The mobile phase composition was thus adjusted in a number of ways, by substituting a portion of the acetonitrile (Solvent B) with methanol (Solvent C), in varying ratios.

An increase in methanol volume significantly influenced the elution time of the pyrimethamine peak. More than three percent of methanol in the mobile phase resulted in a very long retention time and poor chromatography of the pyrimethamine peak.

The percentage of the acetic acid component (Solvent A) was adjusted as well. The optimum percentage of solvent A was determined as being 85%. An increase in solvent A resulted in inadequately long retention times, whilst smaller volumes resulted in poor resolution of the peaks of impurities A (sulfanilamide), B and D.

The optimum mobile phase composition was determined as being Solvent A (850 ml) : Solvent B (120 ml) : Solvent C (30 ml). A representative chromatogram is shown in figure 3.34.

For all of the mobile phase combinations tested, sulfanilic acid eluted before the solvent peak group. It was thus decided to exclude sulfanilic acid from further

studies. The LD_{50} of sulfanilic acid in rats is reported as 12,300 mg/kg, indicating that the toxicity of this substance, when ingested, is very low, allowing for it to be eliminated as a related substance (ScienceLab.com, 2005).

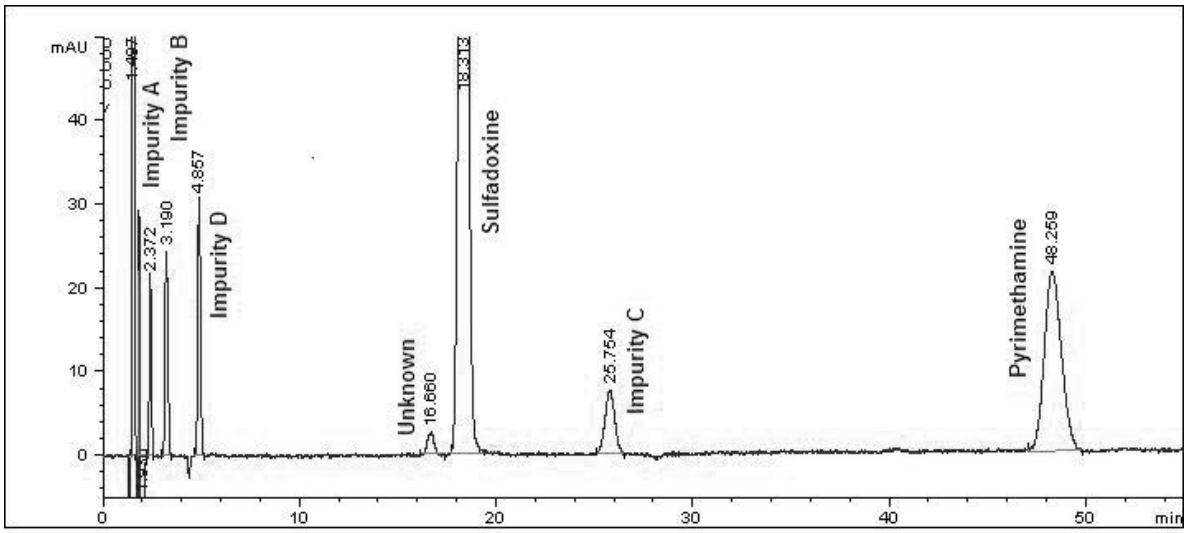


Figure 3.34: Example of a chromatogram of sulfadoxine related substances.

3.6.1.2 Detection wavelength

Since all of the related substances that were to be included in the method were related to sulfadoxine, it was decided to use the optimum wavelength of detection for sulfadoxine, i.e. approximately 272 nm (refer to 3.4.2.1). A sample of sulfadoxine, dissolved in mobile phase, was scanned spectrophotometrically and the optimum wavelength was detected as 270 nm.

3.6.1.3 Solvents

Acetonitrile, methanol and mobile phase were evaluated as solvents for sample dilution. Acetonitrile resulted in poor chromatography (figure 3.35), whilst methanol caused poor chromatography of the pyrimethamine peak. Since the pyrimethamine peak was not analysed, and as methanol did not affect the sulfadoxine, nor the related substances peaks, it was a suitable solvent. The relative retention times of the peaks were the same among all of the solvents. Both methanol and mobile

phase were thus suitable solvents for these samples, with the mobile phase finally being selected for use in this study (figure 3.34).

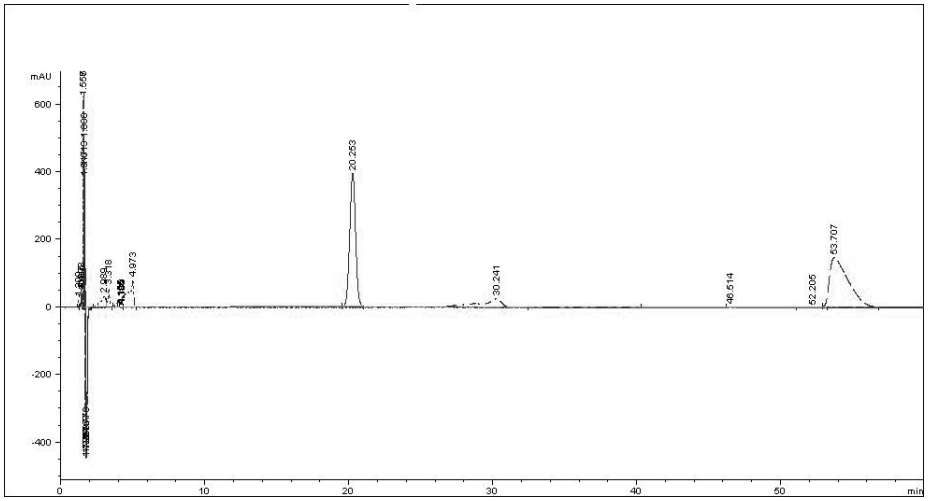


Figure 3.35: Example of a chromatogram of sulfadoxine related substances when dissolved in acetonitrile.

3.6.1.4 Concentration levels of sulfadoxine

The maximum daily dose of sulfadoxine is 1.5 g (refer 1.4.2.3). The reporting threshold is 0.05% (≡ 0.75 mg), the identification threshold is 2 mg (0.13% of 1.5 g), whilst the qualification threshold is 3 mg (0.2% of 1.5 g) (refer table 1.1).

In order to determine the capability of the analytical method to detect levels as low as 0.05% of a sulfadoxine solution, the limits of detection and quantitation of sulfadoxine were determined. This percentage (0.05%) is equal to 1 µg/ml sulfadoxine if a 2000 µg/ml solution is used as reference. Reference solutions with the following concentrations were thus prepared: 0.2 µg/ml (0.01%), 0.5 µg/ml (0.03%), 1 µg/ml (0.05%), 2 µg/ml (0.1%), 5 µg/ml (0.25%), 10 µg/ml (0.5%) and 20 µg/ml (1.0%). All reference solutions were injected five times each and the %RSD was calculated for each set of samples. The results are given in table 3.39.

Table 3.39: Determination of limits of detection and quantitation of sulfadoxine as required for the related substances test

CONCENTRATION (µg/ml sulfadoxine)	% IMPURITY RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
20.2	1.00	3758.6 3751.5 4745.2 3743.8 3759.0	3751.6	0.19
10.1	0.50	1836.2 1839.0 1835.4 1834.6 1833.4	1835.7	0.11
5.0	0.25	911.9 912.0 912.0 912.0 911.5	911.9	0.03
2.0	0.10	367.4 367.2 366.9 367.1 367.7	367.3	0.08
1.0	0.05	176.8 176.8 176.0 177.0 176.0	176.5	0.28
0.50	0.03	87.6 89.1 88.6 88.8 88.8	88.6	0.65
0.20	0.01	33.1 30.3 35.5 30.6 34.2	32.7	6.95

The results indicated that the method was capable of detecting very low concentrations (≥ 0.2 µg/ml) of sulfadoxine. The %RSD of all concentrations above 0.5 µg/ml (including 0.5 µg/ml), was less than 1.0. The repeatability of the 0.2 µg/ml solution (0.01%) was inadequate (%RSD = 7) and the limit of quantitation (LOQ) was hence set at 0.5 µg/ml (0.03%). The limit of detection (LOD) was set at 0.2 µg/ml (0.01%).

The plotted results (figure 3.36) produced a straight line, with the r^2 value being 0.9999 for the seven concentrations. The equation of the line is:

$$y = 185.65x – 12.951$$

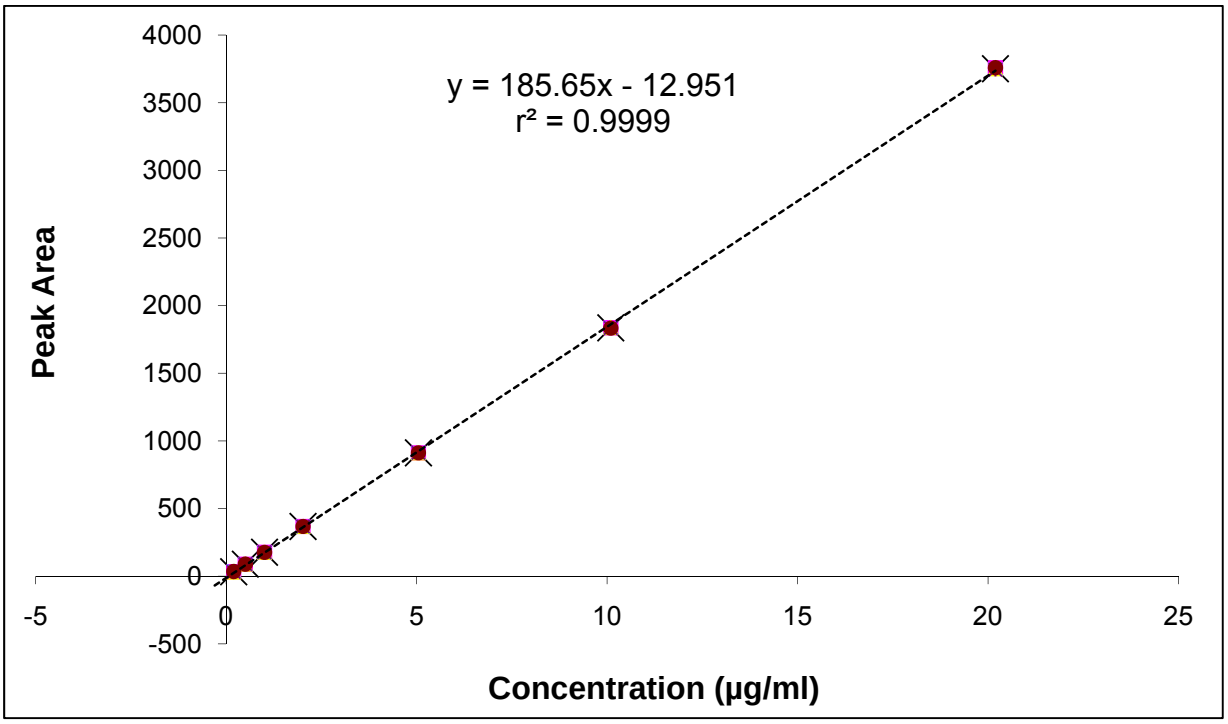


Figure 3.36: Linear regression graph of sulfadoxine as required for related substances testing.

3.6.1.5 Resolution solution

In order to verify the suitability of the system for analyses of the related substances, resolution testing was required. During the stress studies (refer 3.4.2.4), no peaks that were considered suitable for a resolution test were detected, and other substances had to be considered. Since sulfadoxine is closely related to

sulfamethoxazole, it was the first active of choice. It proved to be a good choice as the two peaks eluted within two minutes from each other, sulfadoxine at about 19 – 20 minutes, and sulfamethoxazole at about 21 – 22 minutes. A representative chromatogram is shown in figure 3.37.

The resolution solution was injected on three different types of C_{18}, 250 x 4.6 mm, 5 µm HPLC columns, in order to verify robustness. Results are given in table 3.40.

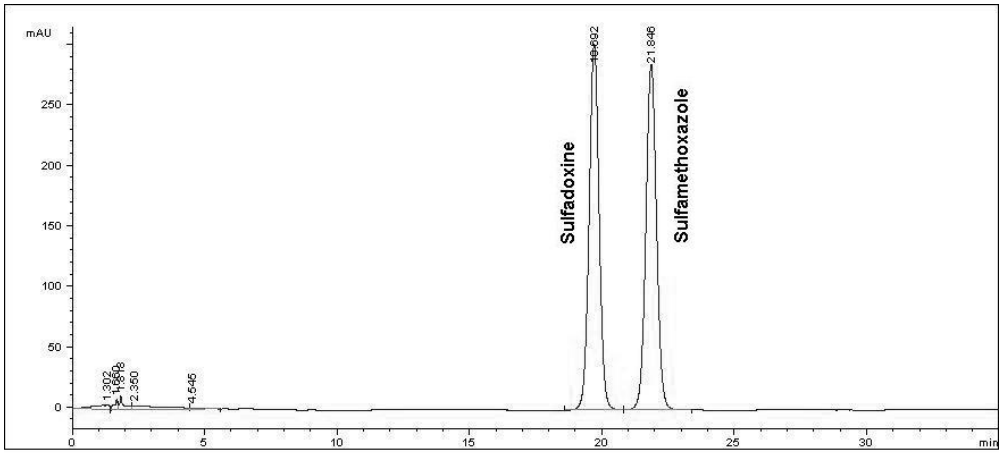


Figure 3.37: Example of a chromatogram of the resolution solution of sulfadoxine related substances.

The results indicated that, despite large differences in the retention times of the two APIs among the different columns, the resolution between the peaks remained the same. The same sample was injected onto a C_{18}, 250 x 4.6 mm, 10 µm column. The results showed that this column was unsuitable, as there was no resolution between the two peaks.

The results presented a resolution factor of 3.0, or higher, between the three different columns. A minimum requirement of 2.0 ($\geq$ 2.0) was set for the resolution factor.

Table 3:40: Peak properties obtained for the resolution solution using different brands of C_{18}, 250 x 4.6 mm, 5 µm columns

	SULFADOXINE		SULFAMETHOXAZOLE		
BRAND NAME	**Retention time (min)**	**Tailing**	**Retention time (min)**	**Tailing**	**Resolution factor**
Phenomenex Luna	19.7	1.0	21.8	1.0	3.1
Waters Symmetry	19.1	1.1	21.6	1.1	3.2
Supelco Discovery	11.3	1.0	12.6	1.1	3.1

3.6.1.6 Conclusion

The method proved to be suitable for determining the four related substances in the presence of the two APIs, sulfadoxine and pyrimethamine, and it was subsequently validated for the four related substances.

3.6.2 VALIDATION OF ANALYTICAL METHOD FOR RELATED SUBSTANCES

The development studies indicated that the following parameters were the best options for validation purposes:

Column: 250 x 4.6 mm, C_{18}, 5 µm (Phenomenex Luna was used for the validation)

Mobile phase: **Solvent A** (850 ml) : **Solvent B** (120 ml) : **Solvent C** (30 ml).

Solvent A: Transfer about 600 ml of water into a 1,000 ml volumetric flask, add 10 ml of glacial acetic acid and 0.5 ml of triethylamine. Mix and fill up to volume with water. Adjust pH to 4.2 with 10M NaOH solution.

Solvent B: Acetonitrile

Solvent C: Methanol

Injection volume: 100 µl

Temperature: Ambient

Flow rate: 2 ml/min

Detection: 270 nm

3.6.2.1 Equipment used during validation studies

The equipment used for the HPLC method validation of related substances included:

- An Agilent Technologies® 1200 series HPLC system with Chemstation® Software Revision A.10.02
- Binary pump - Firmware Revision A.05.11
- Diode array detector - Firmware Revision A.05.11
- Column thermostat - Firmware Revison A.05.11
- Thermostatted autosampler - Firmware Revision A.05.11.

3.6.2.2 Preparation of solutions

A. Reference solutions

All solutions were prepared similarly.

i. Accurately weigh approximately 10 mg each of Impurities B, C and D, and 20 mg of Impurity A. Transfer all impurities into a 50 ml volumetric flask with about 30 ml of a 50% water:methanol mixture.
ii. Sonicate for 10 minutes and allow to cool to room temperature.
iii. Fill up to volume with a 50% water:methanol mixture (stock solution).
iv. Prepare the required reference solutions by diluting the appropriate solutions with mobile phase, as indicated in table 3.41.

Table 3.41: Preparation of related substances reference solutions for linearity studies

		TARGET CONCENTRATION (µg/ml)	
SOLUTION	**DILUTION**	**Impurities B, C & D**	**Impurity A**
1	1 ml stock solution to 10 ml	20 (1%)	40 (2%)
2	1 ml stock solution to 20 ml	10 (0.5%)	20 (1%)
3	1 ml stock solution to 50 ml	4 (0.2%)	8 (0.4%)
4	1 ml stock solution to 100 ml	2 (0.1%)	4 (0.2%)
5	1 ml solution 2 to 10 ml	1 (0.05%)	2 (0.1%)
6	1 ml solution 3 to 10 ml	0.4 (0.02%)	0.8 (0.04%)
7	1 ml solution 4 to 10 ml	0.2 (0.01%)	0.4 (0.02%)
The percentage value in brackets indicate the % in relation to the API sample concentration			

B. Sample solution

i. Transfer tablet powder equivalent to 200 mg of sulfadoxine into a 100 ml volumetric flask and add 35 ml of acetonitrile.

ii. Sonicate for 10 minutes.

iii. Allow to cool to room temperature.

iv. Fill up to volume with mobile phase (Sample solution 1).

v. Filter a portion of the solution through a 0.45 µm filter, discarding the first 10 ml.

vi. Dilute 1 ml of sample solution 1 to 200 ml with mobile phase (Sample solution 2).

C. Resolution solution

i. Use the sulfadoxine stock solution as prepared for the assay (3.4.3.2 A).

ii. Accurately weigh approximately 25 mg of sulfamethoxazole and transfer into a 50 ml volumetric flask with 10 ml of acetonitrile.

iii. Sonicate for 10 minutes.

iv. Allow to cool to room temperature.

v. Fill up to volume with mobile phase.

vi. Transfer 2 ml of each solution into a 20 ml volumetric flask.

vii. Fill up to volume with mobile phase to obtain a solution with concentrations of approximately 100 µg/ml of sulfadoxine and 50 µg/ml of sulfamethoxazole.

3.6.2.3 Validation parameters

The parameters as indicated in table 3.42 were evaluated during the validation study.

Table 3.42: Validation parameters and acceptance criteria for related substances validation studies (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA
Specificity	No interferences detected from solvents, nor excipients
Linearity	$r^2 \geq 0.99$
Range	LOQ to 120% of 100% theoretical concentration
Repeatability	%RSD ≤ 5 for 5 injections from same solution
Recovery	90.0 – 110.0%
Limit of detection	Peak signal/noise ratio ≥ 3:1
Limit of quantitation	Peak signal/noise ratio ≥ 10:1 and %RSD ≤ 10
Robustness	Investigate the effect of small, but deliberate changes to the method

A. Specificity

Individual solutions of sulfadoxine, pyrimethamine and the four related substances were prepared in a mixture of 50% methanol:water, and subsequently diluted with mobile phase. Samples were injected separately in order to determine retention time and possible interferences.

A mixture of the APIs and related substances were prepared and injected - the order of elution is given in table 3.43. The relative retention times were calculated with reference to sulfadoxine. A representative chromatogram is given in figure 3.34.

Table 3.43: Order of retention and relative retention times of sulfadoxine related substances

COMPONENT	RETENTION TIME (min)	RELATIVE RETENTION TIME
Impurity A	2.4	0.13
Impurity B	3.2	0.18
Impurity D	4.9	0.27
Sulfadoxine	18	1.0
Impurity C	26	1.4
Pyrimethamine	48	2.7

B. Linearity and range

Taking the qualification level of related substances as reference, the 100% theoretical concentration was determined as 4 µg/ml for Impurity A (0.2% of a 2,000 µg/ml sulfadoxine solution). Since the toxicology of Impurities B, C and D were unknown, it was decided to reduce the 100% theoretical concentration to 2 µg/ml for validation purposes (0.1% of a 2,000 µg/ml sulfadoxine solution).

Seven different reference solutions, covering a wide range of the target value, were prepared for each of the four related substances (table 3.41). Samples of these solutions were injected onto the HPLC and the results were used to determine the parameters for the linear equation for each of the related substances.

Table 3.44: Concentrations and ranges of solutions used in related substances linearity studies

SUBSTANCE	100% CONC* (µg/ml)	TARGET VALUE (%)	CONC* RANGE (µg/ml)	% TARGET RANGE**
Impurity A	4	0.2	0.42 – 42.3	0.02 – 2.11
Impurity B	2	0.1	0.21 – 20.1	0.01 – 1.01
Impurity C	2	0.1	0.21 – 20.7	0.01 – 1.03
Impurity D	2	0.1	0.22 – 21.6	0.01 – 1.08
* CONC = CONCENTRATION ** WITH REFERENCE TO SULFADOXINE				

The results of the individual related substances are summarised in tables 3.45 -3.48 and are graphically represented in figures 3.38 – 3.41.

Table 3.45: Peak areas of linear regression graph of sulfanilamide (Impurity A)

ACTUAL CONCENTRATION (µg/ml Impurity A)	% IMPURITY RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.42	0.02	48.0 48.3 47.5	47.9	0.80
1.06	0.05	112.2 116.8 112.2	113.7	2.33
2.11	0.11	216.5 217.8 217.8	217.4	0.34
4.23	0.21	449.2 447.0 446.1 444.7 443.7	446.1	0.48
8.46	0.42	852.5 850.7 850.6	851.3	0.13
21.14	1.06	2190.9 2187.2 2191.0	2189.7	0.10
42.28	2.11	4424.8 4380.7 4358.7	4388.1	0.77
LINEAR EQUATION: y = 103.73x – 1.8063				
r^2 = 1.0				
* With reference to sulfadoxine				

Table 3.46: Peak areas of linear regression graph of N1-(6-hydroxy-5-methoxy-4-pyrimidinyl)sulfanilamide (Impurity B)

ACTUAL CONCENTRATION (µg/ml Impurity B)	% IMPURITY RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.20	0.01	38.3 38.3 38.3	38.3	0.09
0.50	0.03	94.6 97.1 94.3	95.3	1.57
1.01	0.05	185.8 183.6 184.2	184.5	0.63
2.01	0.10	386.9 384.7 382.9 382.9 381.8	383.8	0.52
4.02	0.20	742.9 740.8 740.7	741.5	0.17
10.06	0.50	1919.9 1923.4 1921.2	1921.5	0.09
20.12	1.01	3798.4 3800.6 3746.3	3781.8	0.81
LINEAR EQUATION: y = 188.45x – 0.0595				
r^2 = 0.9999				
* With reference to sulfadoxine				

Table 3.47: Peak areas of linear regression graph of 4-(p-acetamide-benzenesulfonamide)-5,6-dimethoxy-pyrimidine (Impurity C)

ACTUAL CONCENTRATION (µg/ml Impurity C)	% IMPURITY RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.21	0.01	41.8 43.3 41.0	42.0	2.8
0.52	0.03	98.9 99.6 98.7	99.0	0.50
1.04	0.05	197.1 200.3 198.5	198.6	0.82
2.07	0.10	408.8 402.6 402.4 402.6 403.4	404.0	0.67
4.15	0.20	817.4 814.8 811.3	814.5	0.38
10.37	0.50	2058.6 2146.2 2059.0	2087.9	2.42
20.74	1.01	4110.6 4125.7 4091.8	4109.4	0.41
LINEAR EQUATION: y = 198.83 – 2.5621				
r^2 = 0.9999				
* With reference to sulfadoxine				

Table 3.48: Peak areas of linear regression graph of 4-amino-5,6-dimethoxy-pyrimidine (Impurity D)

ACTUAL CONCENTRATION (µg/ml Impurity D)	% IMPURITY RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.22	0.01	38.9 37.4 37.4	37.9	2.33
0.54	0.03	93.0 94.5 92.0	93.2	1.32
1.08	0.05	185.5 181.4 181.4	182.8	1.29
2.16	0.11	368.8 366.4 365.4 367.3 364.7	366.5	0.43
4.31	0.22	744.6 744.4 737.6	742.2	0.53
10.78	0.54	1839.2 1837.3 1836.7	1837.8	0.07
21.56	1.08	3674.4 3659.5 3648.7	3660.9	0.35
LINEAR EQUATION: y = 169.81x + 2.8316				
r^2 = 1.0				
* With reference to sulfadoxine				

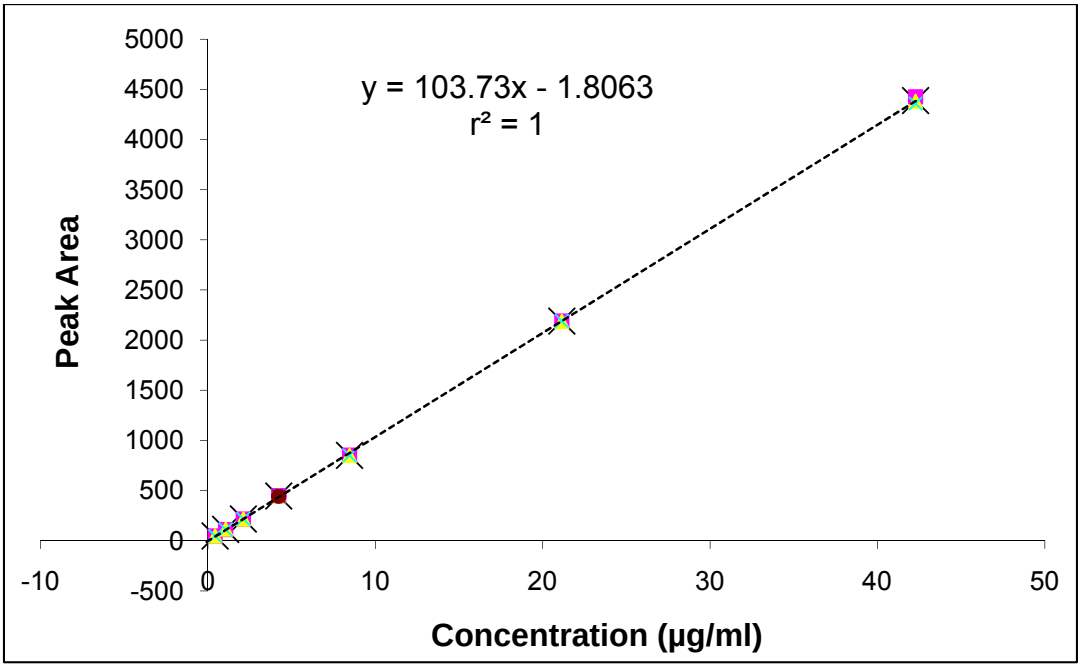


Figure 3.38: Linear regression graph of sulfanilamide (Impurity A).

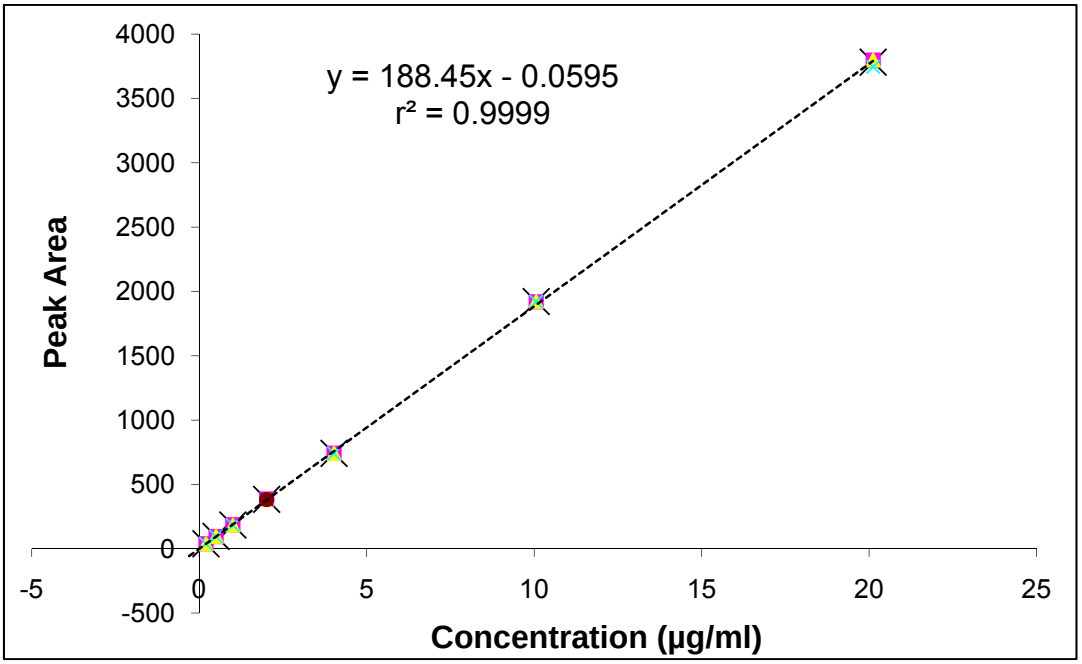


Figure 3.39: Linear regression graph of N1-(6-hydroxy-5-methoxy-4-pyrimidinyl)sulfanilamide (Impurity B).

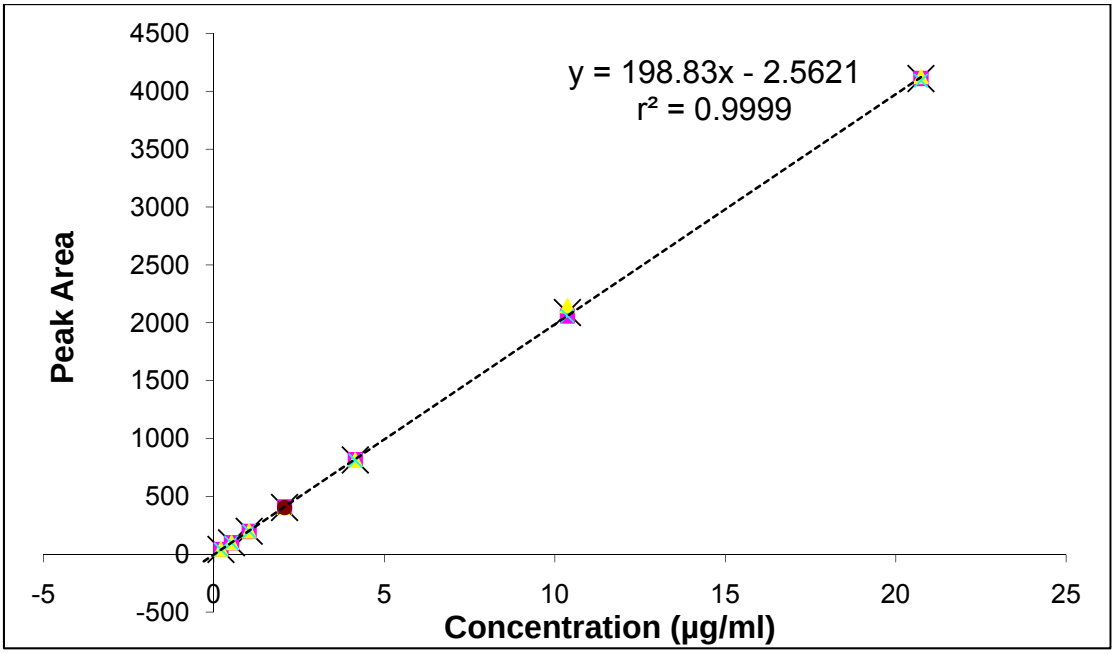


Figure 3.40: Linear regression graph of 4-(p-acetamide-benzenesulfonamide)-5,6-dimethoxy-pyrimidine (Impurity C).

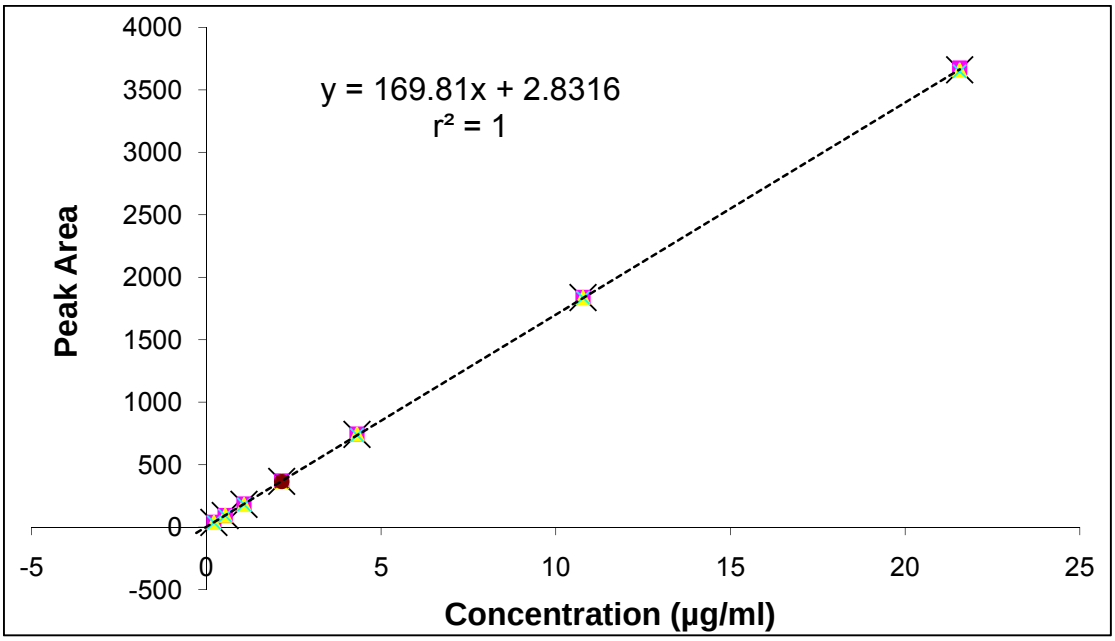


Figure 3.41: Linear regression graph of 4-amino-5,6-dimethoxy-pyrimidine (Impurity D).

C. Repeatability

The fourth reference solution (table 3.41) prepared for the linearity study was used in the repeatability study. It was injected five times. The %RSDs of all four the related substances (tables 3.45 – 3.48) were lower than 1.0, indicating that excellent repeatability was obtained.

D. Limit of detection (LOD)/Limit of quantitation (LOQ)

Since the method was developed for the purpose of determining the related substances, the LOD and LOQ were determined analytically. It was also calculated by means of the equations given in 1.2.7.

Sulfanilamide (Impurity A)

The results for the analytical determination indicated that a %RSD smaller than 5.0 was obtained for all the concentrations being prepared for the linearity study. The LOD and LOQ were set at 0.42 µg/ml (0.02%). The calculated value for LOD was 0.36 µg/ml and for LOQ it was 1.2 µg/ml.

N1-(6-hydroxy-5-methoxy-4-pyrimidinyl)sulfanilamide (Impurity B)

The results for the analytical determination indicated that an acceptable %RSD of less than 5.0 was obtained for all the concentrations in the linear graph. Analytically the LOD and LOQ were the same, i.e. 0.20 µg/ml (0.01%). The calculated values were: LOD = 0.23 µg/ml and LOQ = 0.78 µg/ml.

4-(p-acetamide-benzenesulfonamide)-5,6-dimethoxy-pyrimidine (Impurity C)

The results for the analytical determination indicated that an acceptable %RSD of less than 5.0 was obtained for all the concentrations in the linear graph. The LOQ was analytically established at 0.5 µg/ml (0.03%) and the LOD at 0.21 µg/ml (0.01%). The calculated values were: LOD = 0.22 µg/ml and LOQ = 0.72 µg/ml.

4-amino-5,6-dimethoxy-pyrimidine (Impurity D)

The results for the analytical determination indicated that an acceptable %RSD of less than 5.0 was obtained for all the concentrations in the linear graph. The LOQ

was analytically determined at 0.54 µg/ml (0.03%) and the LOD at 0.22 µg/ml (0.01%). The calculated values were: LOD = 0.08 µg/ml and LOQ = 0.26 µg/ml.

E. Recovery

A solution, spiked with approximately 2 µg/ml of Impurity A and 1 µg/ml each of Impurities B, C and D, was prepared and injected onto the HPLC. The peak areas were used to calculate the concentration of the solution by means of the linear equation, as determined for each related substance. The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

The results are given in table 3.49.

Table 3.49: Recovery results of the four related substances

RELATED SUBSTANCE	% RECOVERY
Impurity A	101.1
Impurity B	98.2
Impurity C	99.3
Impurity D	97.5

A recovery of 90 - 110% is deemed acceptable and the method thus complied with the requirements.

F. Robustness

i. Different types of columns

Three different types of C_{18}, 250 x 4.6 mm, 5 µm columns were used to determine the effect of different brands of columns on the resolution between the related substances. The Supelco column that was used during the resolution solution robustness study (3.6.1.5) unfortunately was unavailable for use in this specific investigation.

Results are shown in table 3.50. The resolution factor was calculated for each peak with respect to its preceding peak. The order of resolution was Impurity A, Impurity B, Impurity D, sulfadoxine and Impurity C. Since Impurity A was the first peak to elute, a resolution factor did not apply.

Table 3.50: Peak properties and resolution factors of related substances using different brands of columns, C_{18}, 250 x 4.6 mm, 5 µm

	IMPURITY A		
BRAND NAME	**Retention time (min)**	**Tailing**	**Resolution factor**
Phenomenex Luna	2.3	1.4	-
Waters Symmetry	2.0	1.3	-
µBondapak	2.6	1.3	-
	IMPURITY B		
BRAND NAME	**Retention time (min)**	**Tailing**	**Resolution factor**
Phenomenex Luna	3.2	1.3	3.4
Waters Symmetry	3.2	1.0	3.6
µBondapak	3.9	1.0	3.9
	IMPURITY C		
BRAND NAME	**Retention time (min)**	**Tailing**	**Resolution factor**
Phenomenex Luna	25.8	1.0	9.5
Waters Symmetry	21.1	0.9	9.1
µBondapak	21.2	1.3	7.4
	IMPURITY D		
BRAND NAME	**Retention time (min)**	**Tailing**	**Resolution factor**
Phenomenex Luna	4.9	1.2	6.1
Waters Symmetry	4.1	1.2	5.5
µBondapak	4.7	1.3	4.5

The results indicated that the different brands of columns had an insignificant effect on the retention time and/or tailing of any of the related substances peaks. The resolution factors of Impurities C and D were noticeably, but insignificantly, different on the µBondapak column, but the peaks were still well resolved.

ii. Mobile phase composition

The influence of mobile phase composition on the peak properties of the related substances was discussed under 3.6.1.1.

iii. Product analyses

After successful validation of the analytical method, it was used to perform analyses of four commercial batches of sulfadoxine/pyrimethamine tablets. The details of the products are given in table 3.51.

Table 3.51: Details of commercial sulfadoxine/pyrimethamine products used for the related substance evaluation

PRODUCT NAME	MANUFACTURER	BATCH NO	EXPIRY DATE
Fansidar	Roche Products	Z6283	3/2011
Fansidar	Roche Products	Z4819	2/2010
Sulphadar	Shelys Pharmaceuticals	6025	3/2010
Orodar	Elys Chemical Industries Ltd	6E70	4/2010

Samples were prepared as follows:

a. Accurately weigh tablet powder equivalent to 200 mg of sulfadoxine and transfer into a 100 ml volumetric flask.

b. Add about 35 ml of acetonitrile and sonicate for 10 minutes.

c. Fill up to volume with mobile phase (solution 1). Filter a portion (0.45 µm filter), discarding the first 10 ml.

d. Using the filtrate, dilute 1 ml to 200 ml with mobile phase (solution 2).

Samples were prepared in duplicate. Representative chromatograms are shown in figures 3.42 – 3.45. As the chromatograms were resized for the purpose of clarity, the pyrimethamine peak is not shown on the chromatograms. All peaks with a level lower than 0.05% were disregarded. The results are summarised in table 3.52.

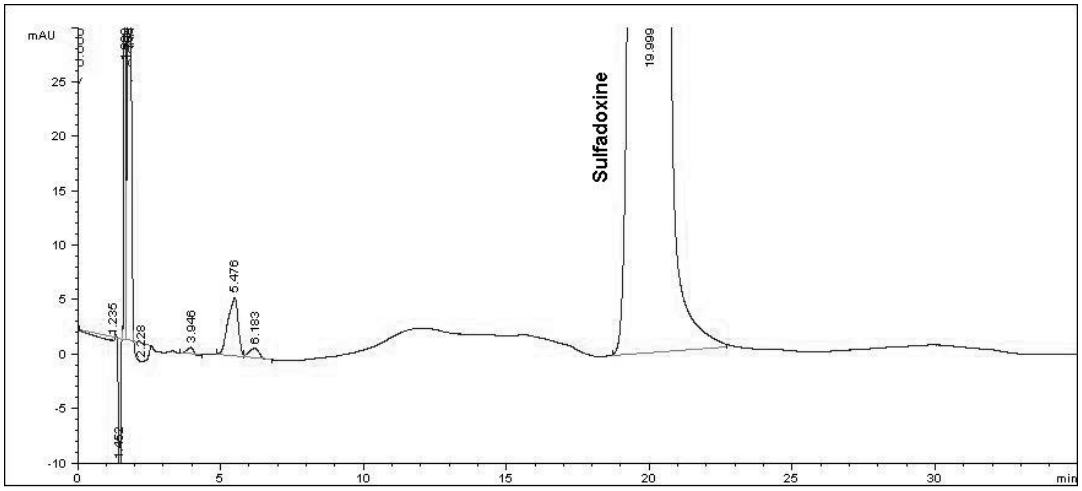


Figure 3.42: Example of a chromatogram of related substances for Fansidar (batch Z6283) tablets.

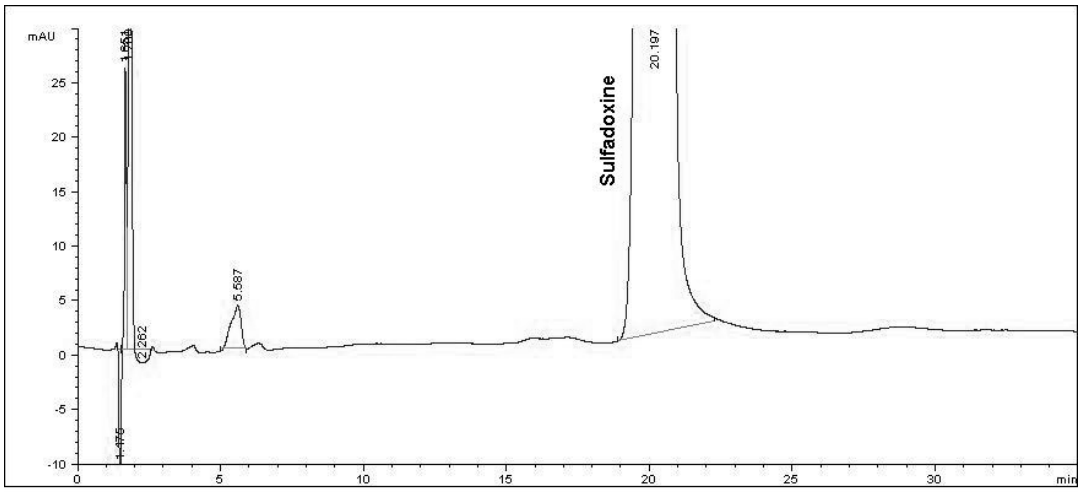


Figure 3.43: Example of a chromatogram of related substances for Fansidar (batch Z4819) tablets.

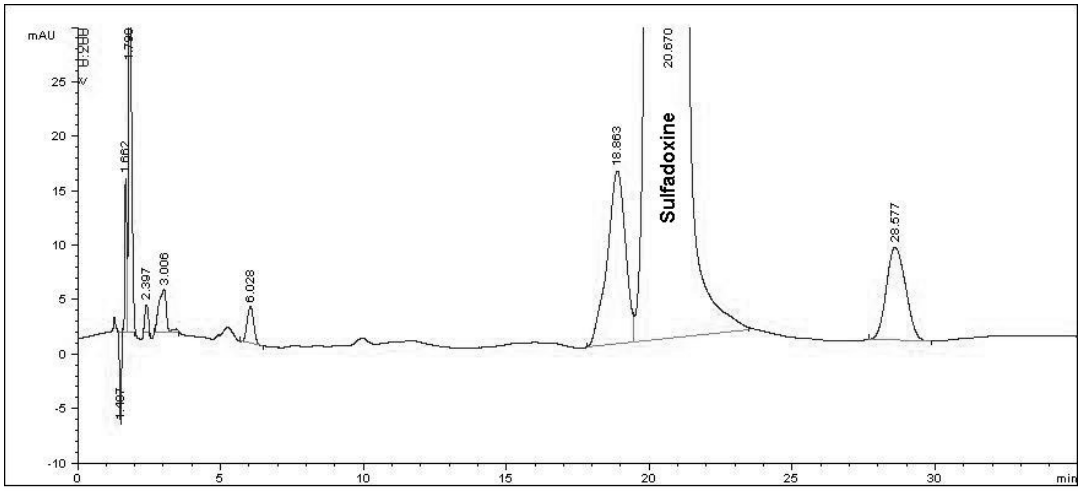


Figure 3.44: Example of a chromatogram of related substances for Sulphadar tablets.

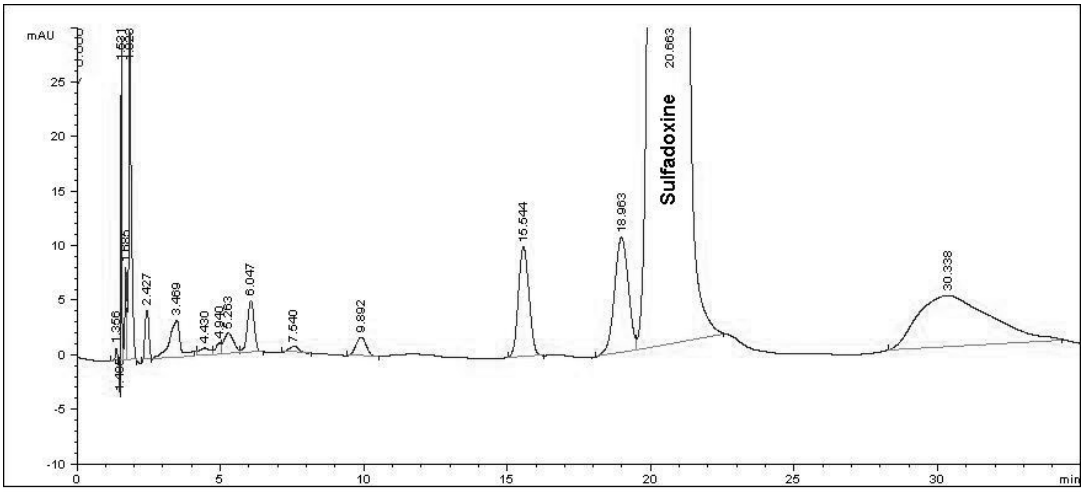


Figure 3.45: Example of a chromatogram of related substances for Orodar tablets.

The results indicated that the method was capable of separating a number of related substances peaks. None of the peaks were more than 0.5% and none of the totals were more than 1.0%. Only one related substance peak was larger than 0.2%. In line with the API monograph for sulfadoxine in the Ph.Int. the limit for individual peaks was set as 0.5% and for the total as 1.0%.

3.6.2.4 Summary of validation results

The analytical method was capable of separating known related substances of sulfadoxine. The method for related substance analyses was successfully validated and acceptable results were obtained when used in the analyses of assay samples of commercial sulfadoxine/pyrimethamine products. A summary of the results are given in table 3.53.

Since all of the determined parameters complied with the requirements for method validation, the method was deemed acceptable for use in analysing samples for the purpose of determining related substances in sulfadoxine/pyrimethamine combination tablets.

Table 3.52: Results of related substances testing on four commercial batches of sulfadoxine/pyrimethamine tablets

PRODUCT	RETENTION TIME (min)	RELATIVE RETENTION TIME	RESULT (%*)
Fansidar (Z6283)	3.9 5.5 6.2	0.20 (Unknown) 0.27 (Impurity D) 0.31(Unknown)	Disregard 0.08 Disregard **Total: 0.08**
Fansidar (Z4819)	5.6	0.28 (Impurity D)	0.06 **Total: 0.06**
Sulphadar	2.4 3.0 6.0 18.9 28.6	0.12 (Unknown) 0.15 (Unknown) 0.29 (Unknown) 0.91 (Unknown) 1.4 (Impurity C)	Disregard Disregard Disregard 0.25 0.20 **Total: 0.45**
Orodar	2.4 3.5 4.4 4.9 5.3 6.0 7.5 9.9 15.5 19.0 30.3	0.12 (Impurity A) 0.17 (Impurity B) 0.22 (Unknown) 0.24 (Unknown) 0.25 (Unknown) 0.29 (Unknown) 0.36 (Unknown) 0.48 (Unknown) 0.75 (Unknown) 0.92 (Unknown) 1.5 (Unknown)	0.05 Disregard Disregard Disregard Disregard 0.05 Disregard Disregard 0.14 0.19 0.22 **Total: 0.65**
* All peaks with a level lower than 0.05% were disregarded			

Table 3.53: Summary of results obtained during related substances validation studies

	RESULTS OBTAINED			
PARAMETER	**Impurity A**	**Impurity B**	**Impurity C**	**Impurity D**
Linearity (r^2)	1.0	0.9999	0.9999	1.0
Range (µg/ml) (%)*	0.42 - 42.28 0.02 – 2.11	0.20 - 20.12 0.01 – 1.01	0.21 - 20.74 0.01 – 1.03	0.22 – 21.56 0.01 – 108.0
Repeatability (%RSD)	0.48	0.52	0.67	0.43
Limit of detection (µg/ml)/% range	0.42 (0.02%*)	0.20 (0.01%*)	0.21 (0.01%*)	0.22 (0.01%*)
Limit of quantitation (µg/ml)/% range	1.06 (0.05%*)	0.20 (0.01%*)	0.50 (0.03%*)	0.54 (0.03%*)
Recovery (%)	101.1	98.2	99.3	97.5
* Calculated with respect to sulfadoxine				

3.7 CONCLUSION

In this part of the study, two initial methods were developed and evaluated for assay of commercial sulfadoxine/pyrimethamine combination tablets. The WHO supplied a method from a manufacturer as reference for assay analyses (Method 1). Method 2 was developed by using the 2008 USP method as reference. Development studies were performed to evaluate the suitability of these HPLC methods and to propose a final set of optimal assay parameters for validation purposes.

Validation parameters included specificity, linearity, range, repeatability, robustness and recovery. All validation studies were concluded successfully.

Widespread reports of the poor dissolution of commercial sulfadoxine/pyrimethamine tablets, especially of pyrimethamine, led to the investigation of an alternative dissolution medium than the pH 6.8 phosphate buffer, as prescribed by the USP. Since the USP prescribes 0.1M HCl as dissolution medium for pyrimethamine tablets, comparative solubility studies for sulfadoxine were performed in 0.1M HCl and pH 6.8 buffer. Both solvents produced solubilities in the same order, indicating that 0.1M HCl should be considered as an alternative to pH 6.8 phosphate buffer.

The correlation between the disintegration of commercial sulfadoxine/pyrimethamine tablets and their dissolution was also investigated. The outcomes showed a positive correlation, suggesting that disintegration indeed offers an affordable and easy screening method that manufacturers can use as preliminary quality control procedure for pro-actively identifying production failures, or inferior products.

It was further demonstrated that 0.1M HCl would be the better choice for a dissolution medium. The dissolution study outcomes required an adjustment of the acceptance criteria from 60% (as prevailing in th USP monograph) to 80% dissolved in 30 minutes for both APIs.

The same analytical method that was successfully validated for assay analyses, was proven suitable for analysis of dissolution test samples.

It was further necessary to develop a method for the determination of the related substances of sulfadoxine in finished products. The validated assay method was adapted for determining five probable related substances. Three substances were recommended by the WHO, whereas sulfanilamide and sulfanilic acid were added for evaluation during the development studies. During these studies, however, sulfanilic acid was excluded from the set of related substances as the proposed analytical method was found to be unsuitable to detect sulfanilic acid.

Development studies indicated that the proposed analytical method was suitable for determining the four related substances in the presence of the two APIs in sulfadoxine/pyrimethamine combination tablets.

The development studies in respect of the analysis of related substances in sulfadoxine/pyrimethamine combination tablets, resulted in the proposal of a final set of parameters for validation purposes. These parameters included specificity, linearity, range, repeatability, robustness, limit of detection, limit of quantitation and recovery. All of these validation studies were concluded successfully.

Based on the satisfactory outcomes of this study, a final monograph for sulfadoxine/pyrimethamine tablets was compiled and submitted to the WHO. After consultations a final draft for adoption of this monograph (Annexure B) has been accepted during the 45th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (18-22 October 2010, Geneva).

CHAPTER 4

MONOGRAPH MEFLOQUINE TABLETS[1]

4.1 INTRODUCTION

Mefloquine has been widely used as an alternative to regimens using quinine for the treatment of chloroquine -, or multidrug resistant falciparum malaria. It is also used with an artemisinin derivative in multiresistance malaria (Sweetman, 2009).

As there is no parenteral formulation of mefloquine currently available, it can only be used in patients who can take oral medication and it is therefore unsuitable for sole treatment in severe infections (Sweetman, 2009).

It had been hoped that mefloquine could be reserved for the treatment of malaria, but increasing drug resistance to chemoprophylactic regimens has led to it being widely used for malaria prophylaxis. WHO recommends that mefloquine should be used where there is a high risk of falciparum malaria and drug resistance, or a moderate to low risk but with high drug resistance (Sweetman, 2009).

In this part of the study, the essential analytical tests for mefloquine tablets were developed on request by the WHO, since no monograph currently exists for mefloquine tablets. This part of the study endeavoured the development of quality tests that take into account the specific characteristics of mefloquine tablets, to prevent manufacturing problems from going unnoticed, as has been the case with other antimalarial production due to inadequate monograph tests.

4.2 AIM

For the purpose of this study, analytical methods were developed for mefloquine tablets for:

[1] Mefloquine tablets contain mefloquine hydrochloride as active

i. The identification of the API, i.e. mefloquine HCl;

ii. The determination of the API (assay);

iii. The percentage of active being dissolved during dissolution testing; and

iv. The determination of the related substances.

4.3 ANALYTICAL METHODS

No monograph for mefloquine tablets is currently included in the USP, the BP, nor in the Ph.Int. A monograph for the API, mefloquine HCl, however, is included in the Ph.Int. (2008).

A monograph exists as a USP, non-U.S., Standards Guideline that includes tests for identification, assay, dissolution and impurities. Two identification tests are listed, namely UV absorption and thin-layer chromatography (TLC). The assay is determined spectrophotometrically. The dissolution test is performed in 900 ml of simulated gastric fluid at a paddle speed of 100 rpm and the samples are also analysed spectrophotometrically. The related substances are detected by means of the same TLC method as described for the identification of the API (USP, 2009b).

Rao and Murthy (2002:960) describe a spectrophotometric method for the determination of mefloquine HCl in dosage forms, dissolved either in methanol or 0.1M hydrochloric acid.

Gaudiano *et al*. (2006:133) describe a HPLC method for the purpose of detecting counterfeit medicines. The method is capable of detecting chloroquine, quinine and mefloquine.

4.4 IDENTIFICATION

4.4.1 REFERENCE METHODS

4.4.1.1 Method 1

A manufacturer method, as supplied by the WHO, includes the following TLC method for identifying mefloquine in dosage forms. It is indicated that this method could also be used to test for unspecified related substances:

Layer: Silica gel 60 F_{254}

Mobile phase: Toluene (70) : Ethanol 95% (30) : Ammonia 25% (2)

Test solution: Mechanically shake tablet powder, equivalent to 750 mg of mefloquine HCl, with 5.0 ml of methanol for 10 min, and centrifuge. Apply the supernatant solution.

Reference solution 1: Dissolve 150 mg of mefloquine HCl reference standard in 1.0 ml of methanol.

Reference solution 2: Dilute 0.10 ml of reference solution 1 to 100 ml with methanol (0.1% - 150 µg/ml).

Reference solution 3: Dilute 0.20 ml of reference solution 1 to 100 ml with methanol (0.2% - 300 µg/ml).

Application: Apply 10 µl each of the sample and reference solutions, and dry in a current of cold air for 3 minutes.

Detection: Dry the plate in a current of warm air for 5 minutes and view under shortwave UV light (254 nm).

A similar method, except for the concentrations of the reference and test solutions, is described by Lim (1985:173), and the USP, non-U.S., Standards Guideline (USP, 2009b). Importantly, Lim (1985:173) also indicates that the plate may be stained with iodine vapour if a UV light is unavailable.

4.4.1.2 Method 2

In the API monograph for mefloquine HCl (Ph.Int., 2008), the following method is described for detecting related substances and for the identity test. It should be noted that this is an expensive method, due to the chemicals required for preparing the spray agent.

Layer: Silica gel F_{254}

Test solution 1: 8 mg/ml mefloquine HCl in methanol.

Test solution 2: 1.6 mg/ml mefloquine HCl in methanol.

Reference solution 1: 1.6 mg/ml mefloquine HCl in methanol.

Reference solution 2: 0.04 mg/ml mefloquine HCl in methanol (0.5%).

Application: Apply 5 µl each of the samples and reference solutions.

Mobile phase: Glacial acetic acid (10) : methanol (10) : dichloromethane (80)

Detection: Dry the plate in a current of warm air for 15 minutes and spray with a freshly prepared mixture of 1 volume of sulfuric acid (1760 g/l) TS and 40 volumes of potassium iodoplatinate TS. Then spray again with hydrogen peroxide solution (330 g/l) TS and examine the chromatogram in daylight.

4.4.2 EVALUATION OF METHODS

Both methods, as summarised in 4.4.1, were evaluated.

4.4.2.1 Method 1

Plates were run in both saturated and non-saturated chambers. In the saturated chamber an R_f value of 0.29 was obtained, whilst the R_f value was 0.55 in the non-saturated chamber.

The spots corresponding to the 0.1% and 0.2% solutions were hardly visible under the UV light. The spot corresponding to the 100% solution (reference solution 1) was very large.

An attempt was made to stain the plate with iodine vapours, as described by Lim (1985:173). The principal spot was clearly detected after this staining, but the spots corresponding to the 0.1% and 0.2% solutions did not stain.

Since the identification method, as described in the USP, non-U.S., Standards Guideline (USP, 2009b) was similar, except for the test and reference concentrations, test samples and reference solutions were also prepared according to this method. The test and reference concentrations were about 10 mg/ml as described in the method USP (2009b). The plate was also stained with iodine vapours (figure 4.1). The spots of the test and reference solutions corresponded.

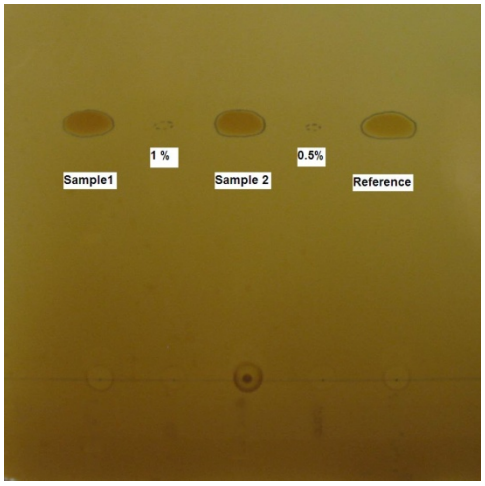


Figure 4.1: Photograph of TLC plate obtained with 10 mg/ml reference and test solutions and stained with iodine vapours after development.

4.4.2.2 Method 2

Plates were run in both saturated and non-saturated chambers. In the saturated chamber an R_f value of 0.58 was obtained, whilst the R_f value was 0.68 in the non-saturated chamber.

All spots were clearly visible under UV light. Reference solutions of 10, 20, 40, 60, 80 and 100 µg/ml concentrations were prepared and 5 µl of each was spotted. All concentrations were easily detected.

Because the method was successful without spraying, the plates were not sprayed as an alternative method of detection, since these chemicals are fairly expensive.

An attempt was made to stain the developed plates with iodine vapours, as per Method 1, but it was unsuccessful. It is suspected that the presence of glacial acetic acid in the mobile phase caused the mefloquine to be present in the salt form (mefloquine HCL) and could thus not react with the iodine vapours.

4.4.3 CONCLUSION

Both methods were suitable for the purpose of an identification test. For inclusion in the draft monograph, it was decided that Method 1 is the better choice, since it had the alternative of plate staining with iodine vapours in the absence of a UV light. The concentrations of the test and reference solutions were too high, however, and it was

decided to use the 10 mg/ml concentrations, as described in the USP, non-U.S., Standards Guideline (USP, 2009b).

From the above outcomes, the following method was proposed for the purpose of identification testing:

Layer:	Silica gel 60 F_{254}
Mobile phase:	Toluene (70) : Ethanol 95% (30) : Ammonia 25% (2)
Test solution:	Transfer tablet powder, equivalent to 250 mg of mefloquine (274 mg mefloquine HCl), into a 25 ml volumetric flask. Add about 10 ml of methanol, sonicate with intermittent swirling to obtain a uniform dispersion. Dilute with methanol to volume, mix and filter. Use the filtrate.
Reference solution:	11 mg/ml mefloquine HCl (≡ 10 mg/ml mefloquine) in methanol.
Application:	Apply 10 µl each of the test and reference solutions and dry in a current of cold air for 3 minutes.
Detection:	Dry the plate in a current of warm air for 5 minutes and view under shortwave UV light (254 nm). Alternatively the plate can be stained by means of iodine vapours. Evaluate the plate immediately after staining.
Acceptance criteria:	The principal spot obtained with the test solution corresponds in position, appearance and intensity to that obtained with the reference solution.

4.5 DISSOLUTION

4.5.1 REFERENCE METHODS

The method supplied by the WHO for the dissolution of mefloquine tablets prescribed the following parameters:

Apparatus: Paddle

Medium: 900 ml simulated gastric fluid

Rotation speed: 75 rpm

Sampling time: 30 minutes

Criteria: Not less than 75% of mefloquine dissolved after 30 minutes

Detection: Spectrophotometrically at 283 nm.

The USP, non-U.S., Standards Guideline (USP, 2009b) prescribes the following parameters:

Apparatus: Paddle

Medium: 900 ml simulated gastric fluid

Rotation speed: 100 rpm

Sampling time: 60 minutes

Criteria: Not less than 80% of mefloquine dissolved after 60 minutes

Detection: Spectrophotometrically at 283 nm.

4.5.2 DEVELOPMENT STUDIES

4.5.2.1 Medium volume

Both methods prescribe 900 ml of simulated gastric fluid. It was decided to use 900 ml of 0.1M HCl as the initial test medium and paddles rotating at 75 rpm, it being the preferred paddle speed according to The International Pharmacopoeia.

Samples were withdrawn at 10, 15, 20, 30 and 45 minutes. According to Lim (1985:160), a concentration of about 50 µg/ml mefloquine HCl (45.6 µg/ml mefloquine) will result in an absorbance reading of 1.0. The withdrawn samples were thus diluted with dissolution medium to obtain a theoretical concentration of 45 µg/ml of mefloquine, assuming 100% dissolution. All the samples were analysed spectrophotometrically at 283 nm to obtain the concentration of mefloquine that had dissolved in each individual sample.

Various mefloquine containing products were analysed during these development studies. Details of the products are given in table 4.1. Lariam tablets were used as reference product for the dissolution testing. The Artequin™ tablets are co-blister products, containing artesunate (100 or 200 mg/tablet) and mefloquine (250 mg/tablet) tablets. At the time of analyses all of these products were within their respective expiry dates (October – November 2009).

Table 4.1: Details of commercial mefloquine tablets used in dissolution testing

PRODUCT NAME	MANUFACTURER	LABEL CLAIM (mefloquine/tablet)	BATCH NO	EXPIRY DATE
Lariam	Roche Products	250 mg	Z8328	8/2013
Mefliam	Cipla-Medpro	250 mg	G8T168	8/2011
Artequin™-300/750*	Mepha	250 mg	0790022	10/2009
Artequin™-600/1500**	Mepha	250 mg	0790020	01/2010
Artequin™-600/1500**	Mepha	250 mg	0790021	01/2010

* Co-blister products containing 3 x 100 mg artesunate tablets and 3 x 250 mg mefloquine tablets
** Co-blister products containing multiple 3 x 200 mg artesunate tablets and 6 x 250 mg mefloquine tablets

The dissolution results of the Lariam tablets are given in table 4.2 and are graphically represented in figure 4.2.

Table 4.2: Dissolution results of Lariam tablets in 900 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	78	85	90	93	97
2	78	86	89	92	98
3	79	86	90	92	95
4	79	86	89	92	96
5	78	86	89	92	96
6	80	86	89	92	97
AVG	79	86	89	92	96
%RSD	1.2	0.60	0.51	0.42	1.2

Since 500 ml of dissolution medium is preferred by Ph.Int., the test was also done in 500 ml 0.1M HCl and in 500 ml pH 6.8 phosphate buffer. The results are summarised in tables 4.3 and 4.4 and are graphically represented in figure 4.2.

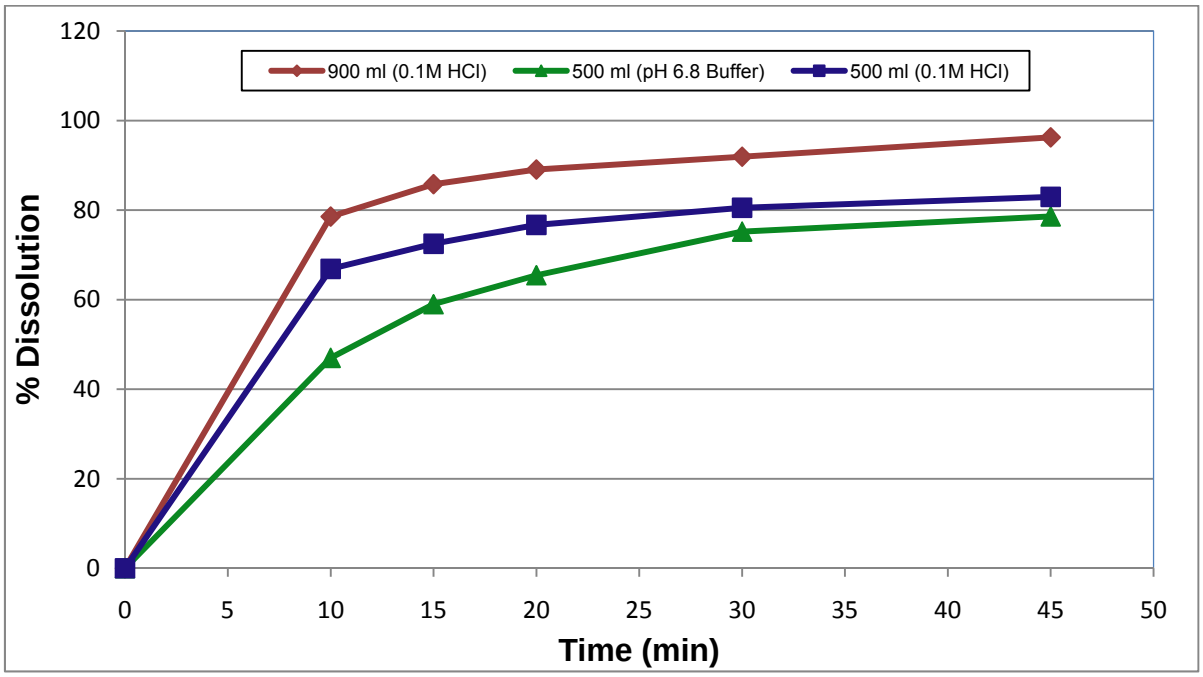


Figure 4.2: Comparative dissolution profiles of Lariam tablets in 0.1M HCl (500 ml and 900 ml) and in 500 ml phosphate buffer pH 6.8.

Table 4.3: Dissolution results of Lariam tablets in 500 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	69	73	77	81	83
2	66	72	76	81	83
3	68	73	77	80	83
4	65	72	76	80	83
5	67	73	77	80	83
6	67	73	77	80	83
AVG	67	73	77	81	83
%RSD	2.2	0.32	0.86	0.54	0.40

Table 4.4: Dissolution results of Lariam tablets in 500 ml phosphate buffer pH 6.8

	% DISSOLUTION IN pH 6.8 PHOSPHATE BUFFER				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	44	57	63	75	78
2	47	58	66	79	79
3	46	60	65	73	78
4	50	61	67	80	79
5	48	60	66	73	78
6	47	59	66	72	80
AVG	47.0	59	66	75	79
%RSD	4.2	2.7	2.1	4.7	1.1

The dissolution results were significantly lower in the 500 ml media than in the 900 ml 0.1M HCl medium. In the 500 ml 0.1M HCl, the dissolution was lower by approximately 10%, whereas in the 500 ml pH 6.8 buffer, it was approximately 15% lower. However, despite the lower percentage of dissolution in the 500 ml 0.1M HCl, 80% of the mefloquine had dissolved in 30 minutes for all six the tablets.

The lower percentage dissolution may have been attributed to saturation in the smaller volumes. Lim (1985:166) states that mefloquine HCl is slightly soluble in

0.1M HCl. This suggests that 250mg mefloquine HCl should dissolve in 25 – 250 ml of 0.1M HCl. The BP (2009) and Ph.Int. (2008), however, indicate the solubility of mefloquine HCl in water as very slightly soluble, meaning that 250 – 2,500 ml of water is required to dissolve 250 mg of the API. Hence, the solubility of mefloquine in 0.1M HCl probably was the limiting step in the dissolution in 500 ml media.

It was subsequently decided to test the viability of a 500 ml medium on other commercial products as well (refer table 4.1).

The dissolution results are given in tables 4.5 – 4.8 and are graphically represented in figure 4.3.

Comparable dissolution results were obtained for all the product batches, i.e. ≥ 80% of the active dissolved in 30 minutes. The lowest percentage dissolution of one tablet was 84% in 30 minutes. However, not a single 100% level of dissolution was reached, as the highest dissolution was 92% after 45 minutes. There was no significant increase in dissolution from 30 minutes to 45 minutes, with the largest increase being approximately 4%.

Table 4.5: Dissolution results of Mefliam tablets in 500 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	76	81	84	88	88
2	76	81	84	88	88
3	75	81	84	87	88
4	72	78	82	87	88
5	76	80	84	88	88
6	76	81	83	87	88
AVG	75	80	83	87	88
%RSD	2.4	1.8	0.96	0.64	0.30

Table 4.6: Dissolution results of Artequin™-300/750 tablets in 500 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	68	77	81	86	91
2	70	79	83	88	91
3	70	79	83	88	90
4	70	77	81	84	89
5	70	78	82	85	89
6	69	78	84	88	91
AVG	70	78	82	86	90
%RSD	1.3	0.95	1.5	1.7	1.1

Table 4.7: Dissolution results of Artequin™-600/1500 tablets (batch 0790020) in 500 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	72	79	85	89	91
2	71	79	84	88	91
3	72	81	86	88	91
4	73	79	84	89	90
5	73	80	84	89	91
6	71	80	84	88	88
AVG	72	80	84	88	90
%RSD	1.2	1.2	0.96	0.56	1.1

Table 4.8: Dissolution results of Artequin™-600/1500 tablets (batch 0790021) in 500 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	70	78	84	89	91
2	71	80	84	89	91
3	70	80	85	89	91
4	69	80	84	90	91
5	69	80	86	89	92
6	69	80	84	90	92
AVG	70	80	84	89	91
%RSD	1.1	0.78	1.1	0.80	0.39

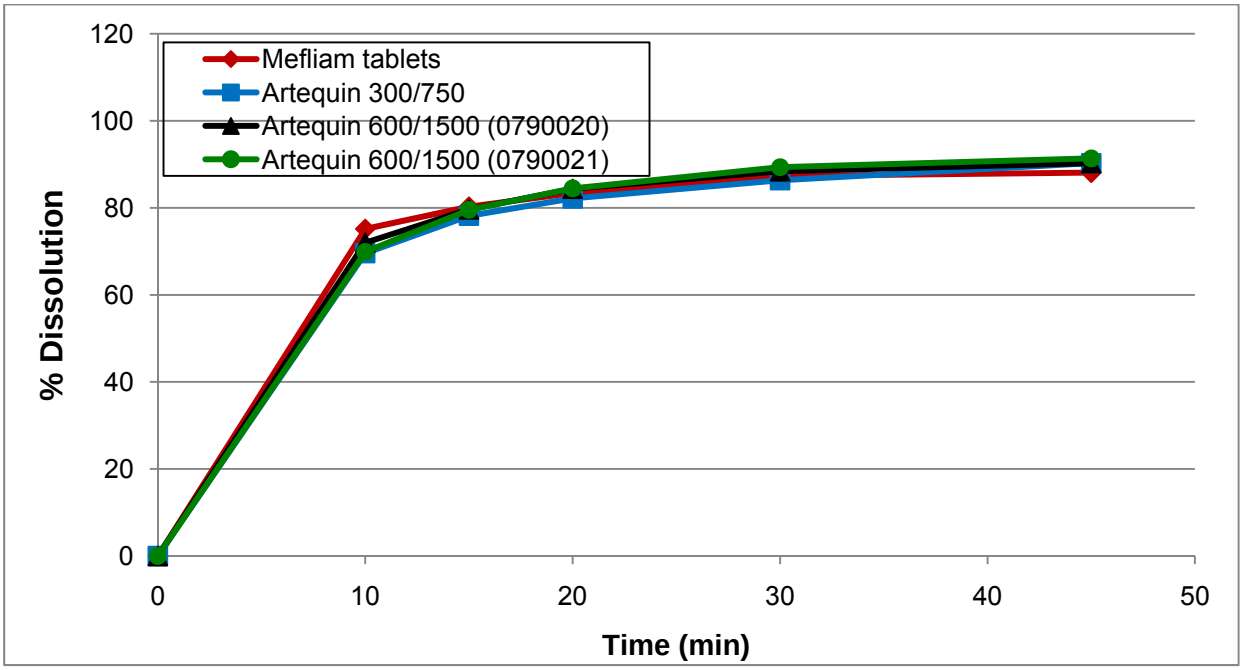


Figure 4.3: Dissolution profiles of various mefloquine tablets in 500 ml 0.1M HCl.

Chapter 1092 of the USP (2010) states that: "*Generally, when developing a dissolution procedure, one goal is to have sink conditions, defined as the volume of medium at least three times that required in order to form a saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide*

sink conditions may be acceptable if it is shown to be more discriminating or otherwise appropriately justified."

Taking the above into consideration, the obtained results confirmed that a 500 ml dissolution medium most probably was unsuitable for commercial mefloquine products.

The dissolution test for Mefliam tablets was performed using 900 ml of 0.1M HCl to confirm the assumption that the volume of the dissolution medium was the limiting factor in preventing a 100% dissolution level from being achieved. Results are presented in table 4.9 and are graphically represented in figure 4.4.

Table 4.9: Dissolution results of Mefliam tablets in 900 ml 0.1M HCl

	% DISSOLUTION IN 0.1M HCl				
TABLET	**10 min**	**15 min**	**20 min**	**30 min**	**45 min**
1	100	99	104	104	104
2	101	102	104	106	105
3	100	104	104	106	105
4	97	103	102	101	102
5	102	100	105	106	106
6	102	105	105	105	106
AVG	100	102	104	105	105
%RSD	2.0	2.2	1.3	1.9	1.6

The results indicated that a complete dissolution was reached for mefloquine in Mefliam tablets in 900 ml of dissolution medium, compared to 87% in 500 ml after 30 minutes. It was thus concluded that the volume of dissolution medium should be 900 ml.

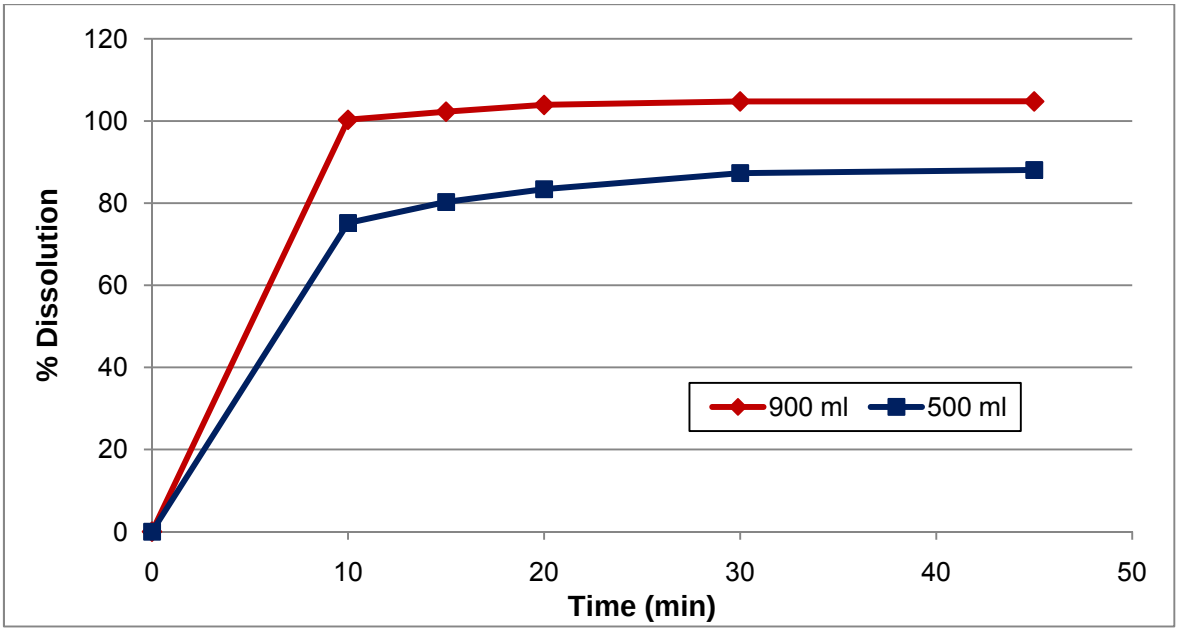


Figure 4.4: Comparative dissolution profiles of Mefliam tablets in 0.1M HCl (500 ml and 900 ml).

4.5.2.2 Summary

Based on the previous outcomes, the following parameters were proposed for dissolution testing of commercial mefloquine tablets:

Apparatus: Paddle

Medium: 900 ml 0.1M HCl

Rotation speed: 75 rpm

Sampling time: 30 minutes

Criteria: Not less than 80% mefloquine dissolved after 30 minutes

Detection: Spectrophotometrically at 283 nm.

If approved by the WHO, this dissolution test shall be carried out as described under "Dissolution test for oral solid oral dosage forms" (Ph.Int., 2008). The reference and test solutions needed for the dissolution test, should be prepared as follows:

Reference solution

i. Accurately weigh approximately 25 mg mefloquine HCl (≈ 22.8 mg mefloquine) and quantitatively transfer into a 100 ml volumetric flask with about 20 ml of methanol.

ii. Sonicate for 5 minutes and allow to cool to room temperature.

iii. Fill up to volume with methanol (stock solution).

iv. Dilute 2 ml of stock solution to 10 ml with 0.1M HCl to obtain a concentration of approximately 50 µg/ml mefloquine HCl (≈ 45.6 µg/ml mefloquine).

Sample solution

i. At 30 minutes withdraw a 10 ml sample from the medium through an in-line filter (0.45 µm).

ii. Allow the filtered sample to cool to room temperature.

iii. Suitably dilute with dissolution medium to obtain a concentration of approximately 45 µg/ml mefloquine (4 ml to 25 ml dilution is suitable), based on complete dissolution.

4.5.3 METHOD VALIDATION

The parameters as indicated in table 4.10 were evaluated during the validation of the analytical method.

Table 4.10: Dissolution validation parameters and acceptance criteria (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA
Specificity	No interferences detected from solvents
Linearity	$r^2 \geq 0.99$
Range	± 30% of acceptance criteria
Repeatability	%RSD ≤ 2 for 5 determinations from same solution
Recovery	95.0 – 105.0%

4.5.3.1 Specificity

A solution of mefloquine HCl in 0.1M HCl showed a peak of maximum absorbance at 283 nm (figure 4.5). A spectrum of 0.1M HCl did not indicate any interference at the wavelength of maximum absorbance of mefloquine, i.e. 283 nm.

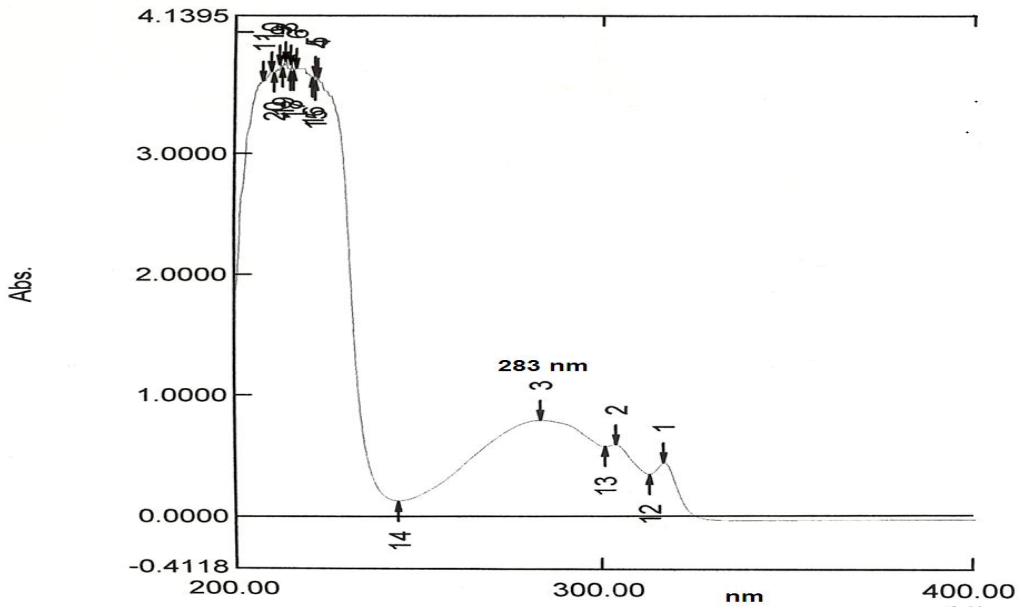


Figure 4.5: UV spectrum of mefloquine HCl in 0.1M HCl (obtained in-house).

4.5.3.2 Linearity and range

The 100% theoretical concentration was taken as 45 µg/ml mefloquine, targeting the sample concentration that would result in the most acceptable and reliable absorbance reading on the spectrophotometer. Absorbance readings of 0.5 – 1.2 were preferred.

Five different mefloquine HCl reference solutions, covering the range of 20 – 150% of a 45 µg/ml mefloquine reference sample (representing the target concentration of a suitably diluted dissolution test sample), were prepared by diluting the reference stock solution (refer 4.5.2.2) with 0.1M HCl (dissolution medium), as summarised in table 4.11.

Table 4.11: Dilutions of mefloquine HCl reference stock solution used in dissolution linearity studies

SOLUTION	DILUTION	TARGET CONCENTRATION (µg/ml mefloquine)
1	2 ml to 50 ml	9.1 µg/ml
2	2 ml to 20 ml	22.8 µg/ml
3	4 ml to 25 ml	36.5 µg/ml
4	2 ml to 10 ml	45.6 µg/ml
5	3 ml to 10 ml	68.4 µg/ml

The results are given in table 4.12 and are graphically represented in figure 4.6.

A regression analyses was performed on the results obtained for the linearity studies.

The r^2 value was 0.9993, with an overall uncertainty of 0.37 µg/ml. A linear graph was thus produced in the concentration range of 8.9 – 67.1 µg/ml. The equation of the regression line is:

$$y = 0.0156x – 0.1125$$

Table 4.12: Absorbance values for dissolution linear regression graph of mefloquine at 283 nm

ACTUAL CONCENTRATION (µg/ml mefloquine)	% RANGE	ABSORBANCE	AVERAGE ABSORBANCE	%RSD
8.9	19.9	0.2509 0.2514	0.251	0.12
22.4	49.7	0.4583 0.4586	0.458	0.03
35.8	79.5	0.6617 0.6619	0.662	0.02
44.7	99.4	0.8244 0.8241 0.8241 0.8223 0.8212	0.823	0.17
67.1	149.1	1.1506 1.1500	1.150	0.03

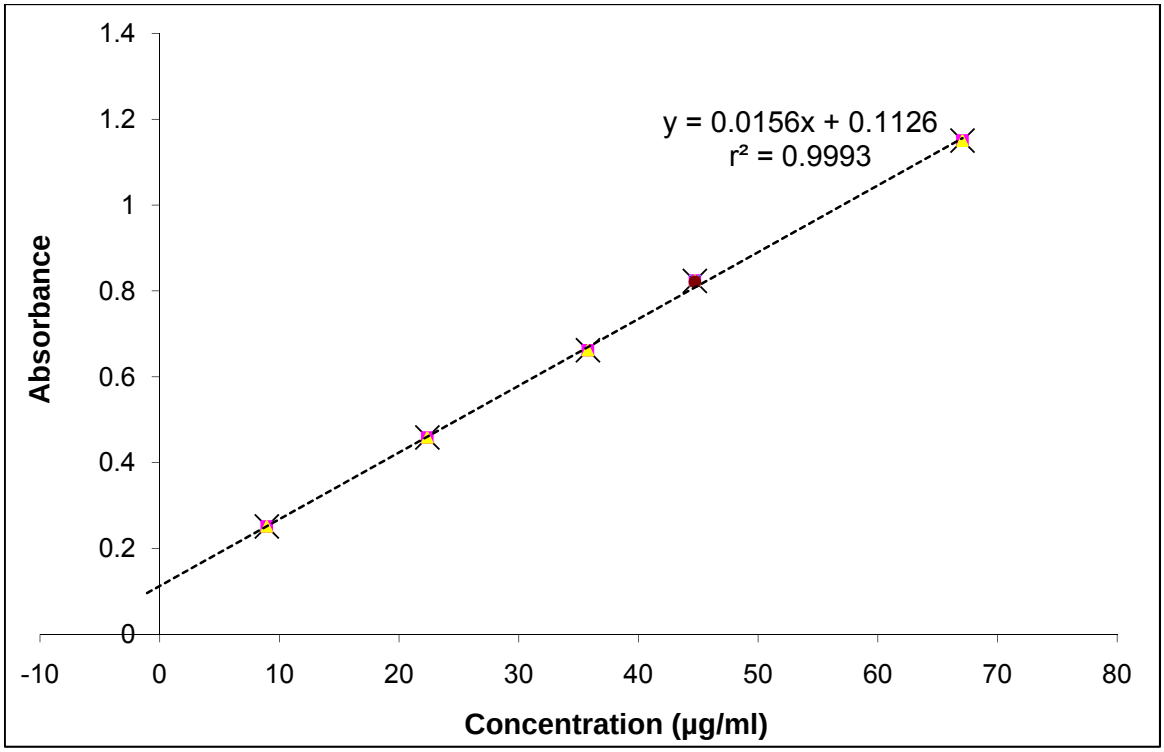


Figure 4.6: Linear regression graph of mefloquine at 283 nm (UV).

4.5.3.3 Repeatability

Reference solution 4, representing the target concentration, as was prepared for the linearity study (table 4.11), was used for the repeatability study and was analysed five times.

The %RSD for the determinations was 0.17 (table 4.12), thus indicating that excellent repeatability was obtained.

4.5.3.4 Recovery

A solution, spiked with approximately 45 µg/ml of mefloquine, was prepared and analysed spectrophotometrically. The absorbance result that was obtained was used to calculate the concentration of the solution by means of the equation given in 4.5.3.2.

The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

A recovery of 101.7% was obtained. A recovery of 95.0 – 105.0% is deemed acceptable and the method thus complied with the requirements.

4.5.4 SUMMARY OF VALIDATION RESULTS

The dissolution method was successfully validated and acceptable results were obtained when used for the dissolution of commercial product samples. A summary of the results are given in table 4.13.

Table 4.13: Summary of results obtained during dissolution validation studies on mefloquine

PARAMETER	RESULTS OBTAINED
Specificity	No interferences were detected from solvents
Linearity	r^2 = 0.9993
Range	8.9 – 67.1 µg/ml (19.9 – 149.1%)
Repeatability	%RSD = 0.17 (5 determinations)
Recovery	101.7%

4.6 ASSAY & RELATED SUBSTANCES

4.6.1 REFERENCE METHODS

4.6.1.1 Method 1

The WHO supplied a method from a manufacturer for use as reference in this study. The parameters were as follows:

Column: 250 x 4.0 mm, Licrospher 100 CN, 5 µm

Mobile phase A: 60% citric acid solution : 40% acetonitrile

Mobile phase B: 40% citric acid solution : 60% acetonitrile

Run the two mobile phases on a gradient system.

Citric acid solution: Dissolve 21.0 g of citric acid monohydrate in about 800 ml water[2], adjust to pH 5.5 with 2M of NaOH solution and fill up to volume with water.

Injection volume: 20 µl

Detection wavelength: 284 nm

Flow rate: 1.2 ml/min

4.6.1.2 Method 2

The methods, as described in both the USP (USP, 2009a) and the BP/EP (BP, 2009) for the API related substances, are the same. The parameters were as follows:

Column: 250 x 4.0 mm, C_{18}, 5 µm

Mobile phase: Dissolve 1 g of tetraheptylammonium bromide in a one litre mixture of a 1.5 g/l solution of sodium hydrogen sulphate:acetonitrile:methanol (2:2:1).

Injection volume: 20 µl

Detection wavelength: 280 nm

Flow rate: 0.8 ml/min

4.6.1.3 Method 3

A method, as published by Bergqvist *et al.* (1991: 169), was also considered. The parameters were as follows:

Column: 250 x 4.0 mm, C_{18}, 5 µm

Mobile phase: Acetonitrile : 0.1M phosphate buffer (48:52) adjusted to pH 3.5 with diluted phosphoric acid.

Injection volume: 20 µl

Detection wavelength: 229 nm

[2] Purified water prepared by reverse osmosis with a resistivity of at least 18 mOhm.

Flow rate: 0.5 ml/min

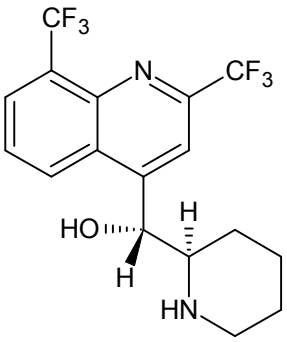
The above methods were considered and used for the development of the final method for assay and the determination of related substances, to be submitted for possible inclusion in the Ph.Int. monograph (Appendix C).

4.6.2 CHOICE OF A METHOD

The primary aim was to develop a method that would be suitable for the assay and related substances testing. Three related substances (A, B & C) were included in the development studies. The related substances are described in Methods 1 and 2. Impurities B and C were supplied by the WHO and Impurity A was sourced.

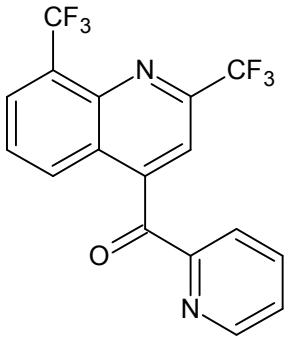
The molecular structures of the impurities are given below.

(RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl][(2RS)-piperidin-2-yl]methanol *(threo*-mefloquine) (Impurity A)

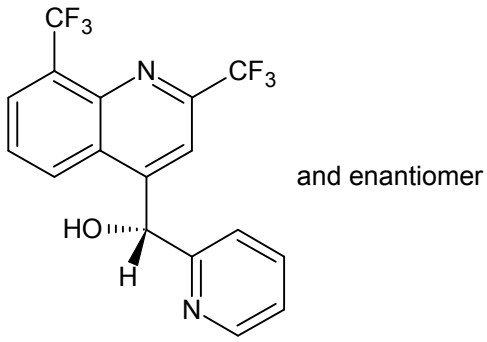


and enantiomer

[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanone (Impurity B)



(RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanol (Impurity C)



and enantiomer

4.6.2.1 Method 1

This method was not evaluated, due to the column being expensive and of a specialised nature.

4.6.2.2 Method 2 evaluation

Acceptable results were obtained with this method. A representative chromatogram is shown in figure 4.7.

4.6.2.3 Method 3 evaluation

The original method generated poor resolution between the mefloquine and *threo*-mefloquine peaks. Adjustments were made to the mobile phase in an attempt to improve the resolution. A portion of the acetonitrile was replaced by methanol, which improved the resolution to a value more than two. The percentage of organic content was further increased to reduce the retention times of impurities B and C to less than 30 minutes. The final mobile phase being developed comprised of 220 parts of methanol, 380 parts of acetonitrile and 400 parts of 0.1M phosphate buffer. A representative chromatogram is shown in figure 4.8.

The chromatograms indicated that acceptable results were obtained with both Methods 2 and 3. Because the stop time with Method 3 was shorter than that of Method 2, and its mobile phase simpler and more affordable to prepare, it was decided to further develop the adapted Method 3.

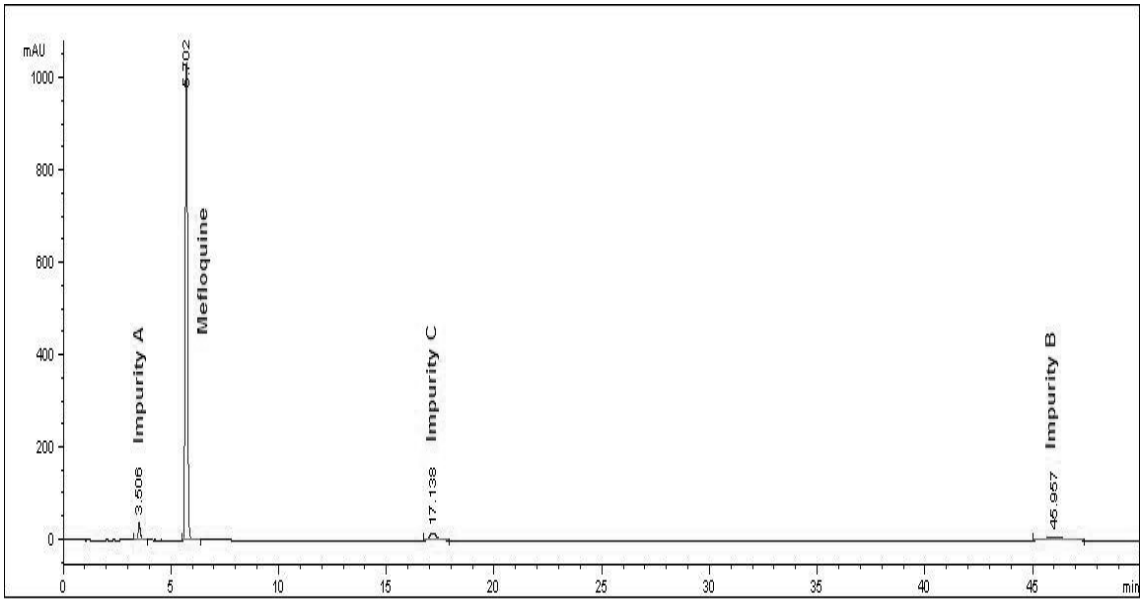


Figure 4.7: Example of chromatogram of a mixture of mefloquine and related substances obtained with Method 2.

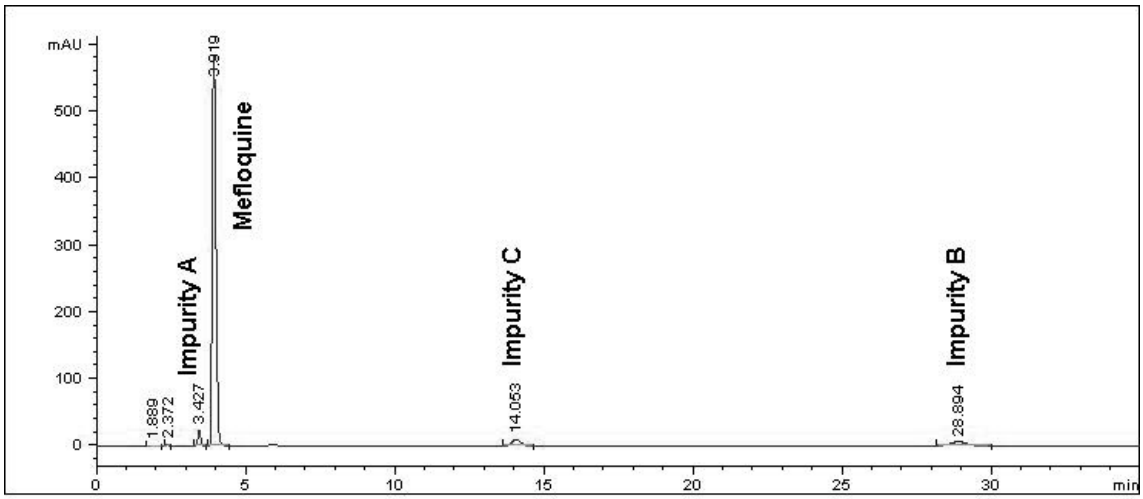


Figure 4.8: Example of chromatogram of a mixture of mefloquine and related substances obtained with an adapted Method 3.

4.6.3 DEVELOPMENT STUDIES

4.6.3.1 Detection wavelength

Solutions of the three related substances were prepared in mobile phase and spectra were generated for all. All three solutions exhibited maximum peak absorbances in the range of 280 – 283 nm (figure 4.9). The chosen wavelength of detection was thus 280 nm.

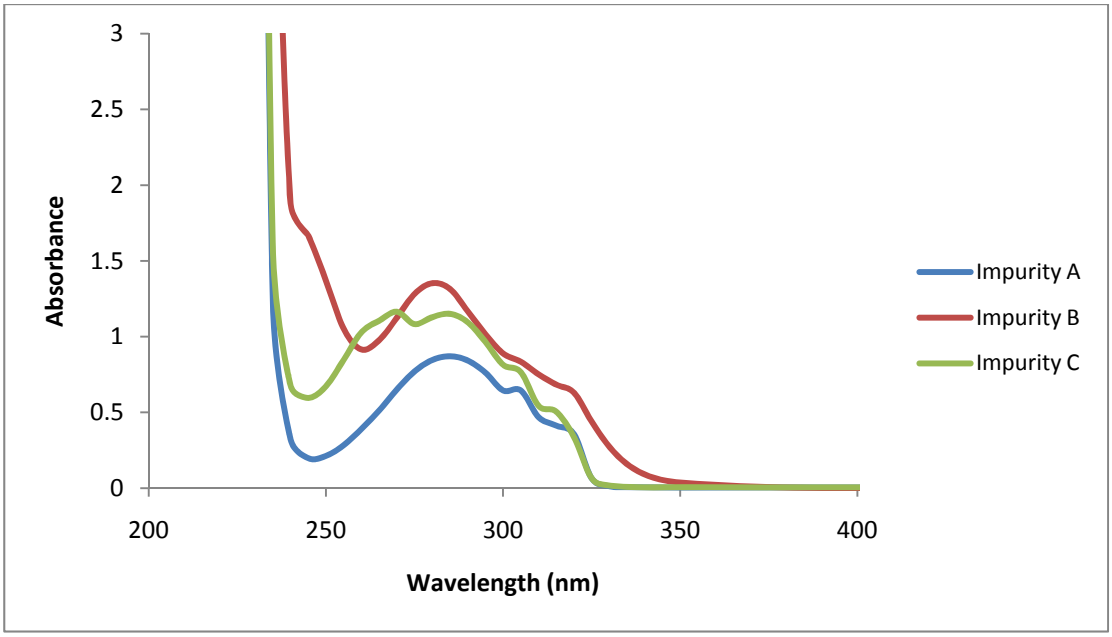


Figure 4.9: UV spectra of mefloquine Impurities A, B and C when dissolved in mobile phase.

4.6.3.2 Solvent

Water, methanol, mobile phase and acetonitrile were considered as possible solvents for the preparation of the test, reference and resolution solutions.

A. Water

As the solubility of mefloquine HCl in water is very low, it was not considered as a solvent.

B. Acetonitrile

The solubility of mefloquine HCl in acetonitrile is low. Also, since the mobile phase contained a high percentage of buffer, it was decided not to use acetonitrile, in order to prevent possible precipitation of the buffer.

C. Methanol

The solubility of mefloquine HCl in methanol is high. To prevent the possible precipitation of the buffer from the mobile phase, methanol was not considered as the solvent.

D. Mobile phase (refer 4.6.2.3)

The solubility of mefloquine HCl in mobile phase is acceptable, and the chromatograms had no solvent peaks. Mobile phase was therefore the solvent of choice. A representative chromatogram of mefloquine in mobile phase is shown in figure 4.10.

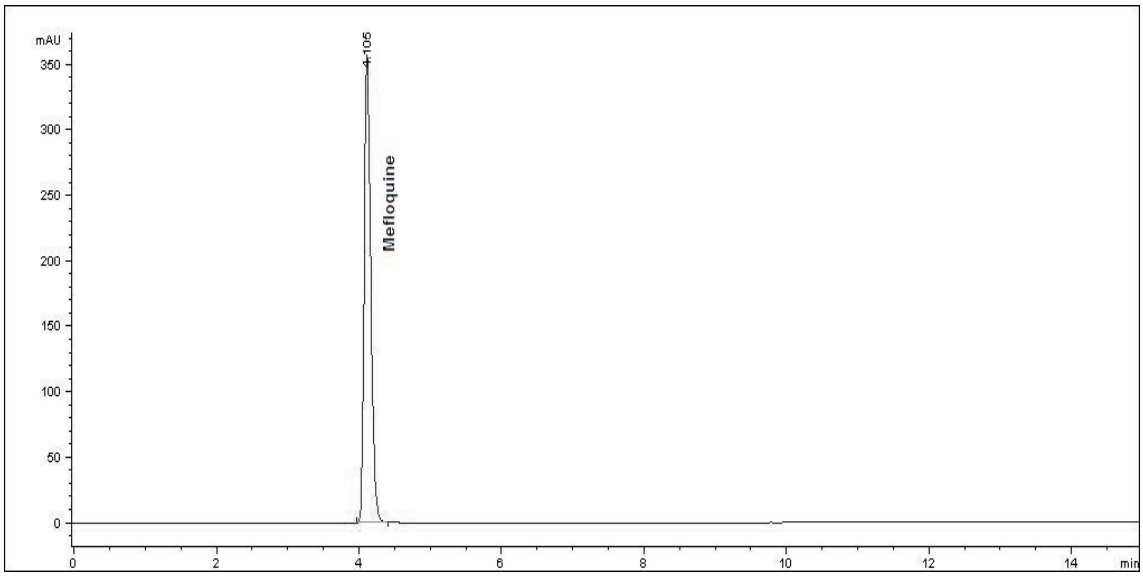


Figure 4.10: Example of chromatogram of mefloquine HCl with mobile phase as solvent.

The reference solution was thus prepared as follows:

i. Accurately weigh approximately 22 mg of mefloquine HCl and quantitatively transfer into a 50 ml volumetric flask with 40 ml of mobile phase.

ii. Sonicate for 5 minutes and allow to cool to room temperature.

iii. Fill up to volume with mobile phase.

iv. Dilute 5 ml to 10 ml with mobile phase to obtain a concentration of approximately 220 µg/ml of mefloquine HCl (≡ 200 µg/ml mefloquine).

4.6.3.3 Resolution solution

The reference method did not include a resolution test, an important consideration of the pharmacopoeial assay and related substances method(s). Of the three related substances being evaluated, it was not feasible to use *threo*-mefloquine (impurity A),

due to it being very expensive and difficult to source. The other two related substances differ too much from the API with respect to retention times to be considered for the resolution test. A resolution test thus had to be developed considering other APIs.

Six APIs, namely chloroquine phosphate, primaquine phosphate, sulfadoxine, pyrimethamine, quinine sulfate and quinidine sulfate were identified as possible candidates in setting a resolution test, based upon these APIs being readily available, also as commercial materials.

Pyrimethamine, quinine sulfate, quinidine sulfate and chloroquine phosphate eluted very close to the solvent peak group, causing interferences by the solvent peak group on the API peaks. Primaquine phosphate and sulfadoxine eluted without any interference after two minutes, making both very suitable for use in the resolution solution. Sulfadoxine was chosen, as it is easy to obtain commercially and at a good price. A representative chromatogram is given in figure 4.11.

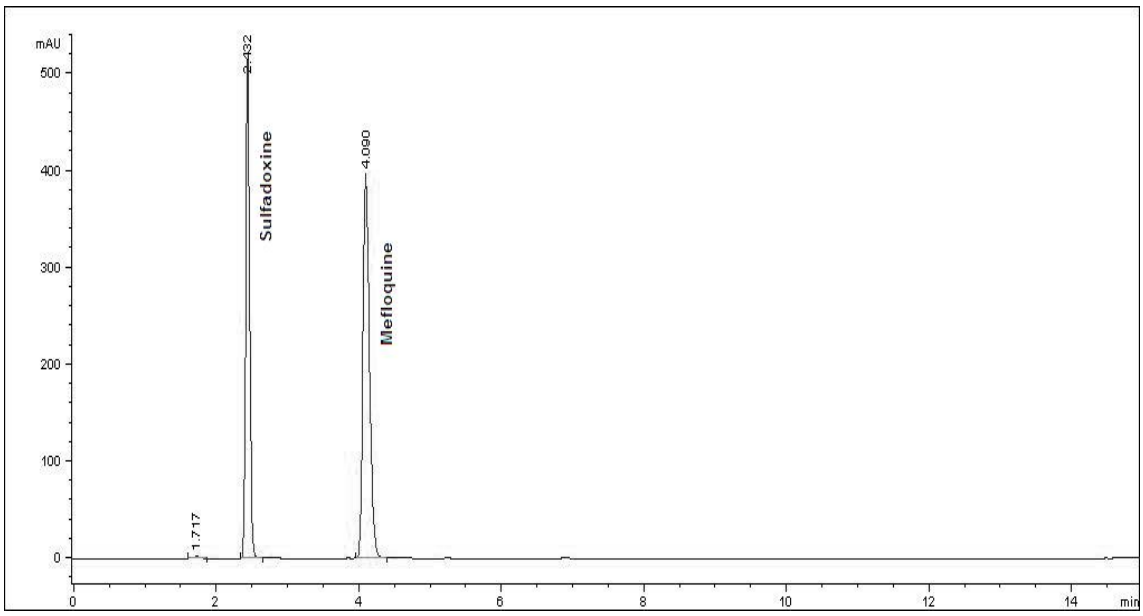


Figure 4.11: Example of chromatogram of sulfadoxine and mefloquine HCl.

The resolution factor of the sulfadoxine and mefloquine peaks was set at not less than 5.0 ($\geq$ 5.0).

The resolution solution was prepared as follows:

i. Weigh approximately 20 mg of sulfadoxine and 22 mg of mefloquine HCl and transfer into separate 50 ml volumetric flasks each with about 40 ml of mobile phase.

ii. Sonicate for 10 minutes and allow to cool to room temperature.

iii. Fill up to volume with mobile phase.

iv. Transfer 5 ml of the mefloquine solution and 1 ml of the sulfadoxine solution into a 10 ml volumetric flask and fill up to volume with mobile phase to obtain concentrations of approximately 200 µg/ml for mefloquine and 40 µg/ml for sulfadoxine.

4.6.3.4 Flow rate

Flow rates of 1 ml/min, 1.2 ml/min and 1.5 ml/min were evaluated. Results for the resolution solution and for the solution containing a mixture of mefloquine and the three related substances are given in tables 4.14 - 4.16. The relative retention times are with reference to the mefloquine peak.

Table 4.14: Retention times and relative retention times of the resolution solution employing different flow rates

	RETENTION TIME (minutes)		Relative retention time of sulfadoxine
FLOW RATE (ml/min)	Sulfadoxine	Mefloquine	
1.0	3.5	5.8	0.6
1.2	3.0	4.9	0.6
1.5	2.4	3.9	0.6

Table 4.15: Retention times of the related substances solution employing different flow rates

	RETENTION TIME (minutes)			
FLOW RATE (ml/min)	**Impurity A**	**Mefloquine**	**Impurity C**	**Impurity B**
1.0	5.1	5.9	20.1	43.5
1.2	4.3	4.9	17.3	35.4
1.5	3.4	3.9	14.1	28.9

Table 4.16: Relative retention times of the related substances solution employing different flow rates as calculated from table 4.15

	RELATIVE RETENTION TIME			
FLOW RATE (ml/min)	**Impurity A**	**Mefloquine**	**Impurity C**	**Impurity B**
1.0	0.9	1.0	3.4	7.4
1.2	0.9	1.0	3.5	7.2
1.5	0.9	1.0	3.6	7.4

The results indicated that an increase in the flow rate from 1 ml/min to 1.5 ml/min resulted in a lower retention time of the last eluting Impurity B peak, from about 44 minutes to about 29 minutes. The relative retention times were the same among all the flow rates, thus indicating that resolution among the peaks was not negatively influenced. In order to save on analyses costs, the shortest run time and thus a flow rate of 1.5 ml/min was chosen as the most appropriate.

4.6.3.5 Storage and stress conditions

A. Reference solution

Mefloquine solutions were prepared in mobile phase (reference solution), 0.1M hydrochloric acid (HCl), 1M HCl, 1M NaOH and in a 5% peroxide solution each, and kept at room temperature (20 – 25ºC). A sample of the solution prepared in mobile phase was also kept at 37ºC for 24 hours. Samples of each solution were assayed over a period of 24 hours. The percentage difference, with reference to the initial

peak area obtained, was calculated at each interval. The results are shown in table 4.17.

Table 4.17: Stability data of mefloquine in various media

CONDITION	PEAK AREA (INITIAL)	PEAK AREA (12 HOURS)	% DIFFERENCE	PEAK AREA (24 HOURS)	% DIFFERENCE
Bench*	1584.9	1584.4	0.0	1579.7	-0.3
37ºC**	1590.8	1545.8	-2.8	1528.1	-3.9
0.1M HCl	1573.7	1554.8	-1.2	1534.3	-2.5
5% H_2O_2	1657.9	1631.6	-1.6	1620.2	-2.3
1M HCl	1636.7	1631.8	-0.3	1644.7	0.5
1M NaOH	1543.6	1593.8	3.3	1578.3	2.2

* Bench = sample prepared in mobile phase kept at room temperature for 24 hours
** 37ºC = sample prepared in mobile phase and kept at 37ºC for 24 hours

After 24 hours the chromatograms showed instabilities in the baseline for the 5% peroxide, the 1M HCl and the 1M NaOH solutions. The increase in peak areas of the 1M HCl and 1M NaOH solutions could have been attributed to the poor chromatography that caused difficulties during integration of the peaks.

For the API in 0.1M HCl and in 5% peroxide, a decrease in peak area of more than 2% was seen after 24 hours, indicating that mefloquine was unstable under these conditions.

The reference solution kept at room temperature showed no significant decrease in API peak area after 24 hours. However when kept at 37°C a decrease of 2.8% was already seen after 12 hours. Thus, the reference solution can be regarded stable for analytical purposes at room temperature, but should be protected from heat.

B. Resolution solution

A resolution solution containing approximately 200 µg/ml of mefloquine and 40 µg/ml of sulfadoxine was prepared as described in 4.6.3.3, and analysed over a period of three days. The results are given in table 4.18.

Table 4.18: Stability of the resolution solution over a period of three days

	SULFADOXINE		MEFLOQUINE		RESOLUTION FACTOR
TIME	Peak area	% Difference	Peak area	% Difference	
Day 0	2015.8	-	2498.1	-	14.2
Day 1	2015.9	0.0	2494.3	-0.2	14.8
Day 3	2020.0	0.2	2501.1	0.1	12.5

These results indicated that the resolution solution was stable for at least 24 hours, with no significant differences being detected in the peak areas, nor the resolution factors of the peaks. Even after 72 hours, no significant difference was detected in the peak areas, whereas the resolution factor decreased from 14.2 to 12.5. The lower resolution factor was however insignificant, as the requirement was set at 5.0 ($\geq$ 5.0). These results confirmed the results being obtained for the reference solution (4.6.3.5 A).

C. Test solutions

Two commercial products (table 4.1) were analysed, utilising the proposed method (4.6.4). The test solutions were left on the bench for three days and analysed again. The peak areas obtained were compared to those of the freshly prepared test solutions. Results are given in table 4.19.

Table 4.19: Stability of the test solutions over a period of three days kept at room temperature (20 - 25ºC)

LARIAM			MEFLIAM		
Peak Area Day 0	Peak Area Day 3	% Difference	Peak Area Day 0	Peak Area Day 3	% Difference
2152.8	2165.1	0.57	2190.4	2182.1	-0.38
2172.8	2180.3	0.34	2230.2	2248.3	0.81
2155.5	2150.4	-0.24	2247.3	2268.9	0.96

The results indicated that the test solutions were stable for at least 72 hours, with no significant differences detected in the peak areas.

4.6.4 VALIDATION OF ANALYTICAL METHOD

Based on the preceding development studies, the following parameters were selected for the assay of mefloquine in mefloquine tablets and determination of related substances:

Column: 250 x 4.0 mm, C_{18}, 5 µm (Phenomenex Luna is suitable)

Mobile phase: Methanol (220 ml) : Acetonitrile (380 ml) : 0.1M phosphate buffer (400 ml) (buffer is adjusted to pH 3.5 with 10% phosphoric acid before preparing mobile phase).

Injection volume: 20 µl

Detection wavelength: 280 nm

Flow rate: 1.5 ml/min

4.6.4.1 Equipment used in the validation studies

The equipment used for the HPLC analyses included:

- Agilent Technologies® 1200 series HPLC system with Chemstation® Software Revision A.10.02 for HPLC analyses.
- Binary pump - Firmware Revision A.05.11
- Diode array detector - Firmware Revision A.05.11
- Column thermostat - Firmware Revision A.05.11
- Thermostatted autosampler - Firmware Revision A.05.11.

4.6.4.2 Preparation of solutions

A. Resolution solution

i. Weigh approximately 20 mg of sulfadoxine and 22 mg of mefloquine HCl and transfer into separate 50 ml volumetric flasks with about 40 ml of mobile phase.

ii. Sonicate for 10 minutes and allow to cool to room temperature.

iii. Fill up to volume with mobile phase.

iv. Transfer 5 ml of the mefloquine solution and 1 ml of the sulfadoxine solution into a 10 ml volumetric flask and fill up to volume with mobile phase to obtain

concentrations of approximately 200 µg/ml of mefloquine and 40 µg/ml of sulfadoxine.

B. Reference solution

i. Accurately weigh approximately 22 mg of mefloquine HCl and quantitatively transfer into a 50 ml volumetric flask with 40 ml of mobile phase.

ii. Sonicate for 5 minutes and allow to cool to room temperature.

iii. Fill up to volume with mobile phase.

iv. Dilute 5 ml to 10 ml with mobile phase to obtain a concentration of approximately 220 µg/ml of mefloquine HCl (≡ 200 µg/ml mefloquine).

C. Test solution for assay

i. Weigh and powder 20 tablets.

ii. Accurately weigh tablet powder, equivalent to about 200 mg of mefloquine, and quantitatively transfer into a 100 ml volumetric flask with about 70 ml of mobile phase.

iii. Sonicate for 10 minutes and allow to cool to room temperature.

iv. Fill up to volume with mobile phase (stock solution).

v. Filter a portion of the solution through a 0.45 µm filter, discarding the first 10 ml.

vi. Dilute the filtrate tenfold with mobile phase to obtain a concentration of approximately 200 µg/ml mefloquine.

D. Test and reference solution for related substances

i. Use a filtered portion of the stock solution (4.6.4.2 C) for assay (2000 µg/ml mefloquine). This is the test solution for the determination of the related substances.

ii. Dilute the filtrate of the stock solution for assay 500 times to obtain a concentration of approximately 4 µg/ml of mefloquine (0.2%). This serves as the reference solution for the determination of the related substances.

4.6.5 VALIDATION PARAMETERS

The parameters, as indicated in table 4.20, were evaluated during the assay and related substances validation testing.

Table 4.20: Assay and related substances validation parameters and acceptance criteria (LoBrutto & Patel, 2007:461; USP, 2009a)

PARAMETER	ACCEPTANCE CRITERIA ASSAY	ACCEPTANCE CRITERIA RELATED SUBSTANCES
Specificity	No interferences detected from solvents	
Linearity	$r^2 \geq 0.99$	
Range	80 – 120% of 100% theoretical concentration	LOQ: 120% of 100% theoretical concentration
Repeatability	%RSD ≤ 2 for 5 injections from same solution	%RSD ≤ 5 for 5 injections from same solution
Recovery	98.0 – 102.0%	90.0 – 110.0%
Robustness	Investigate the effect of small, but deliberate changes to the method	

4.6.5.1 Specificity

Individual solutions, containing sulfadoxine, Impurity A, Impurity B, Impurity C and mefloquine HCl, were prepared with mobile phase. The solutions and a solvent sample (mobile phase – figure 4.12) were injected separately in order to determine retention times and possible peak interferences. For the resolution solution, sulfadoxine eluted at about 2.5 minutes (relative retention 0.6) and mefloquine at about 3.9 minutes (refer to 4.6.3.3). The related substances solution peaks eluted in the following order: Impurity A at about 3.4 minutes, mefloquine at about 3.9 minutes, Impurity C at about 14.1 minutes and Impurity B at about 28.9 minutes (refer to figure 4.8 for a chromatogram of the mixture).

No interferences with the mefloquine peak were detected from the solvent (figure 4.12), or during the stress studies (4.6.3.5).

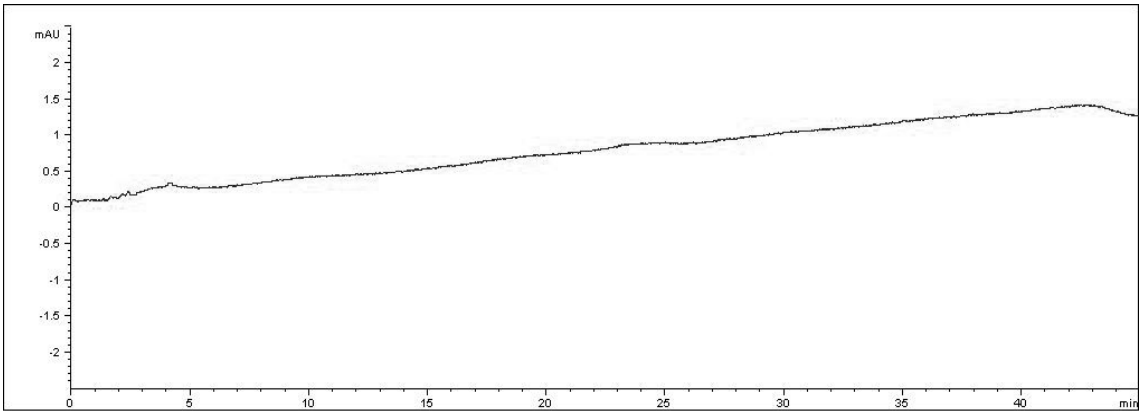


Figure 4.12: Example of chromatogram of the mefloquine reference solution solvent (mobile phase).

The purity of the mefloquine peak was confirmed by means of the diode array detector (figure 4.13).

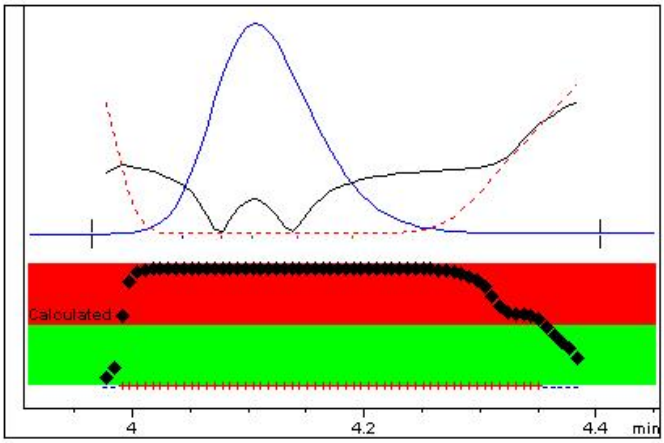


Figure 4.13: Peak purity results of the mefloquine peak.

4.6.5.2 Linearity and range

i. Assay

The 100% theoretical concentration was taken as 200 µg/ml mefloquine, targeting the concentration of the sample solution at 100% label claim. Five different mefloquine reference solutions, covering the range of 51 - 136% of the 100% theoretical concentration, were prepared by diluting a solution of 50 mg/ml mefloquine in mobile phase as indicated in table 4.21. The solvent was mobile phase.

Table 4.21: Dilutions of a 50 mg/ml mefloquine solution used for assay linearity studies

SOLUTION	DILUTION	TARGET CONCENTRATION (µg/ml mefloquine)
1	2 ml to 20 ml	100
2	3 ml to 20 ml	150
3	2 ml to 10 ml	200
4	6 ml to 25 ml	240
5	4 ml to 15 ml	267

Results are given in table 4.22 and are graphically represented in figure 4.14.

A regression analyses was performed on the linearity results obtained.

The r^2 value was 0.9998, with an overall uncertainty of 0.78 µg/ml. A linear graph was thus produced in the concentration range of 51.0 – 135.9 µg/ml. The equation of the line is:

$$y = 9.6912x + 4.3213$$

Table 4.22: Peak areas of assay linear regression graph of mefloquine

ACTUAL CONCENTRATION (µg/ml mefloquine)	% RANGE	PEAK AREAS	AVERAGE PEAK AREA	%RSD
101.9	51.0	990.9 990.1	990.5	0.06
152.9	76.4	1481.0 1479.4	1480.2	0.08
203.8	101.9	1997.3 1999.1 1998.3 1997.7 1986.4	1995.8	0.26
244.6	122.3	2373.3 2365.8	2369.6	0.22
271.8	135.9	2641.0 2628.8	2634.9	0.33

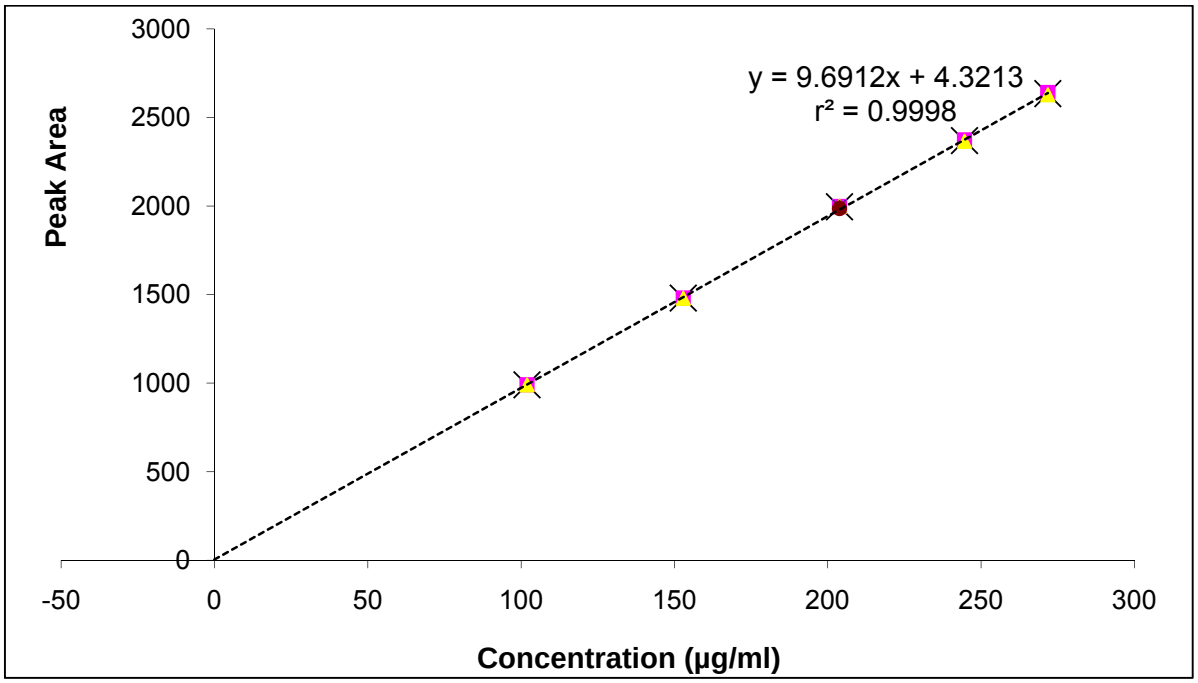


Figure 4.14: Linear regression graph for mefloquine assay.

ii. Mefloquine for related substances

For the purpose of analysing the related substances, the 100% theoretical concentration of the reference solution was taken as 4 µg/ml mefloquine, targeting the 0.02% concentration of mefloquine in the 2,000 µg/ml test solution. Ten different mefloquine reference solutions, covering the range 0.01 – 0.50% of the 2000 µg/ml solution, were prepared by diluting a 100 µg/ml mefloquine solution with mobile phase (table 4.23).

Table 4.23: Dilutions of a 100 µg/ml stock solution used in related substances linearity studies

SOLUTION	DILUTION	TARGET CONCENTRATION (µg/ml mefloquine)	% TARGET RANGE (Relative to API in test solution)
1	1 ml to 10 ml	10	0.50
2	1 ml to 15 ml	6.7	0.34
3	1 ml to 20 ml	5	0.25
4	1 ml to 25 ml	4	0.20
5	1 ml to 50 ml	2	0.10
6	1 ml solution 1 to 10 ml	1	0.05
7	1 ml solution 2 to 10 ml	0.67	0.034
8	1 ml solution 3 to 10 ml	0.5	0.025
9	1 ml solution 4 to 10 ml	0.4	0.02
10	1 ml solution 5 to 10 ml	0.2	0.01

No peak was detected for the 0.2 µg/ml (0.01%) solution. A regression analyses was performed on the linearity results obtained. Results are given in table 4.24 and are graphically represented in figure 4.15.

The r^2 value was 0.9999, with an overall uncertainty of 0.03 µg/ml. A linear graph was thus produced in the concentration range of 10.3 – 254.8 µg/ml. The equation of the line is:

$$y = 9.7938x - 2.0773$$

Table 4.24: Peak areas of linear regression graph for mefloquine reference solutions in the related substances test

ACTUAL CONCENTRATION (µg/ml mefloquine)	% RANGE*	PEAK AREAS		AVERAGE PEAK AREA	%RSD
0.41	0.02	2.1 1.9 1.9	1.9 1.8 1.7	1.86	7.18
0.51	0.025	2.6 2.7 2.7	2.9 3.0 2.7	2.78	5.56
0.68	0.034	4.5 4.5 4.6	4.7 4.5 4.8	4.59	2.90
1.02	0.05	7.9 8.2 7.9	7.9 7.8 7.8	7.93	1.50
2.04	0.10	17.7 17.7 17.8	17.9 17.8 17.9	17.79	0.55
4.08	0.20	37.6 38.1 37.8	37.4 37.7	37.72	0.59
5.10	0.26	48.2 47.8 47.7	47.8 48.5	47.99	0.69
6.79	0.34	64.3 65.0 65.8	65.7 65.8	65.31	0.99
10.19	0.51	97.0 97.7 96.9	96.8 97.3	97.15	0.40
* With respect to the strength of mefloquine in related substances test solution					

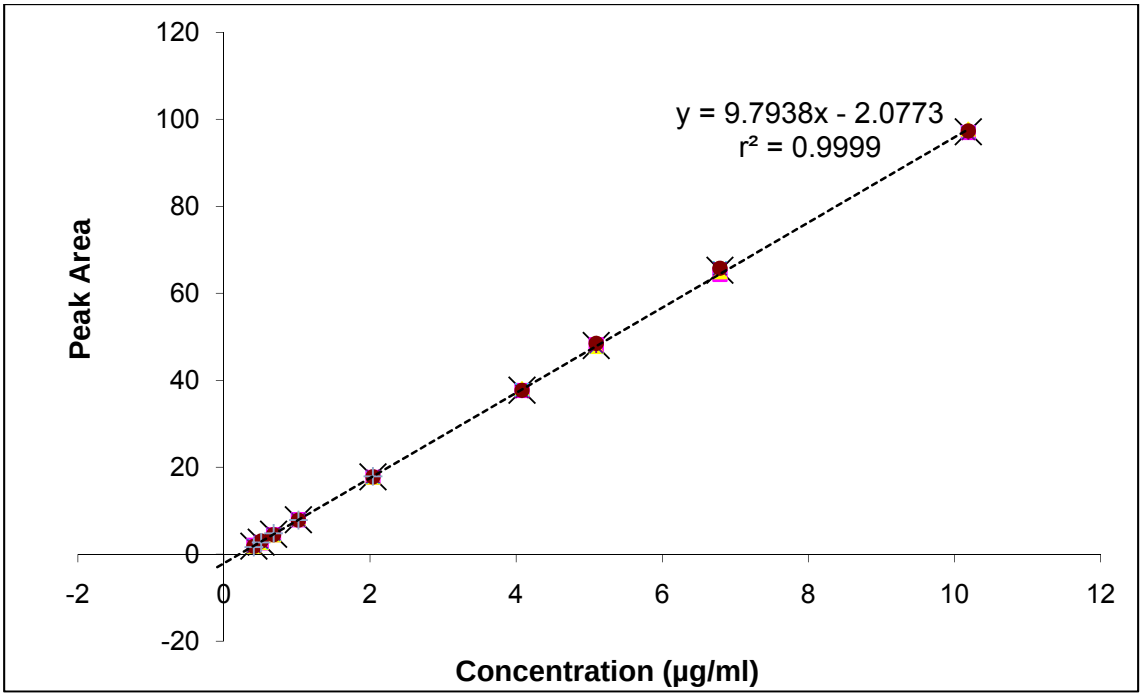


Figure 4.15: Linear regression graph for the mefloquine peak for related substances test.

iii. Related substances

The level of the related substances in mefloquine tablets was set at 0.2%, which is the identification and qualification threshold of the ICH guideline Q3B (R2) (ICH, 2006). Thus, for the purpose of analysing the related substances, the 100% theoretical concentration was taken as 4 µg/ml for each of the related substances, targeting the 0.2% concentration of the mefloquine test solution at 2,000 µg/ml. Ten different solutions, containing the three related substances, were prepared by diluting a 100 µg/ml solution of each with mobile phase (table 4.23).

The results of the individual related substances are given in tables 4.25 – 4.27 and are graphically represented in figures 4.16 – 4.18.

Table 4.25: Peak areas of linear regression graph for Impurity A

ACTUAL CONCENTRATION (µg/ml Impurity A)	% RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.21	0.01	2.3 2.2 2.2 2.2 2.2 2.5	2.3	5.34
0.43	0.02	4.6 4.2 4.2 4.5 4.4 4.4	4.4	3.65
0.53	0.03	5.2 5.3 5.3 5.4 5.6 5.4	5.4	2.55
0.71	0.04	7.1 6.9 6.8 7.1 6.9 7.1	7.0	1.90
1.06	0.05	10.6 10.4 10.5 10.6 10.8 10.7	10.6	1.33
2.13	0.1	21.8 21.8 21.8 21.6 21.7 21.7	21.7	0.38
4.26	0.2	42.1 43.9 43.4 42.5 43.3	43.0	1.72
5.32	0.3	52.8 52.7	52.7	0.06
7.09	0.4	71.4 73.4	72.4	1.96
10.64	0.5	104.7 105.1	104.9	0.28
LINEAR EQUATION: y = 9.9283x + 0.2587				
r^2 = 0.9996				
* With respect to the strength of mefloquine in related substances test solution				

Table 4.26: Peak areas for linear regression graph for Impurity B

ACTUAL CONCENTRATION (µg/ml Impurity B)	% RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.43	0.02	8.5 8.1 7.9 7.3 8.8 7.7	8.1	6.75
0.53	0.03	9.3 9.5 10.6 10.3 9.5 8.5	9.6	7.88
0.70	0.04	12.8 13.4 12.6 13.5 12.8 12.7	13.0	2.95
1.07	0.05	18.3 18.5 18.3 18.3 18.3 18.5	18.4	0.56
2.14	0.1	36.9 37.1 37.3 37.4 36.6 36.2	36.9	1.23
4.28	0.2	74.3 75.9 77.0 76.9 76.8	76.2	1.49
5.35	0.3	94.7 93.0	93.9	1.28
7.10	0.4	121.8 123.7	122.8	1.09
10.70	0.5	181.5 178.9	180.2	1.02
LINEAR EQUATION: y = 16.942x + 1.297				
r^2 = 0.9994				
* With respect to the strength of mefloquine in related substances test solution				

Table 4.27: Peak areas for linear regression graph for Impurity C

ACTUAL CONCENTRATION (µg/ml Impurity C)	% RANGE*	PEAK AREAS	AVERAGE PEAK AREA	%RSD
0.20	0.01	3.0 3.0 2.4 2.7 3.0 2.9	2.8	8.55
0.40	0.02	5.8 5.7 5.0 5.5 5.8 5.3	5.5	5.78
0.50	0.03	6.9 7.5 6.7 7.1 7.2 7.1	7.1	3.83
0.67	0.04	8.8 8.8 8.6 8.8 8.7 8.5	8.7	1.45
1.03	0.05	14.0 13.4 13.3 13.6 13.6 13.5	13.6	1.79
2.01	0.1	26.3 25.7 25.9 25.8 26.0 25.2	25.8	1.42
4.01	0.2	54.6 54.3 54.0 54.6 53.7	54.2	0.72
5.02	0.3	67.4 67.4	67.4	0.00
6.70	0.4	87.2 88.7	88.0	1.21
10.30	0.5	133.3 131.1	132.2	1.18
LINEAR EQUATION: y = 12.941x + 0.6219				
r^2 = 0.9994				
* With respect to the strength of mefloquine in related substances test solution				

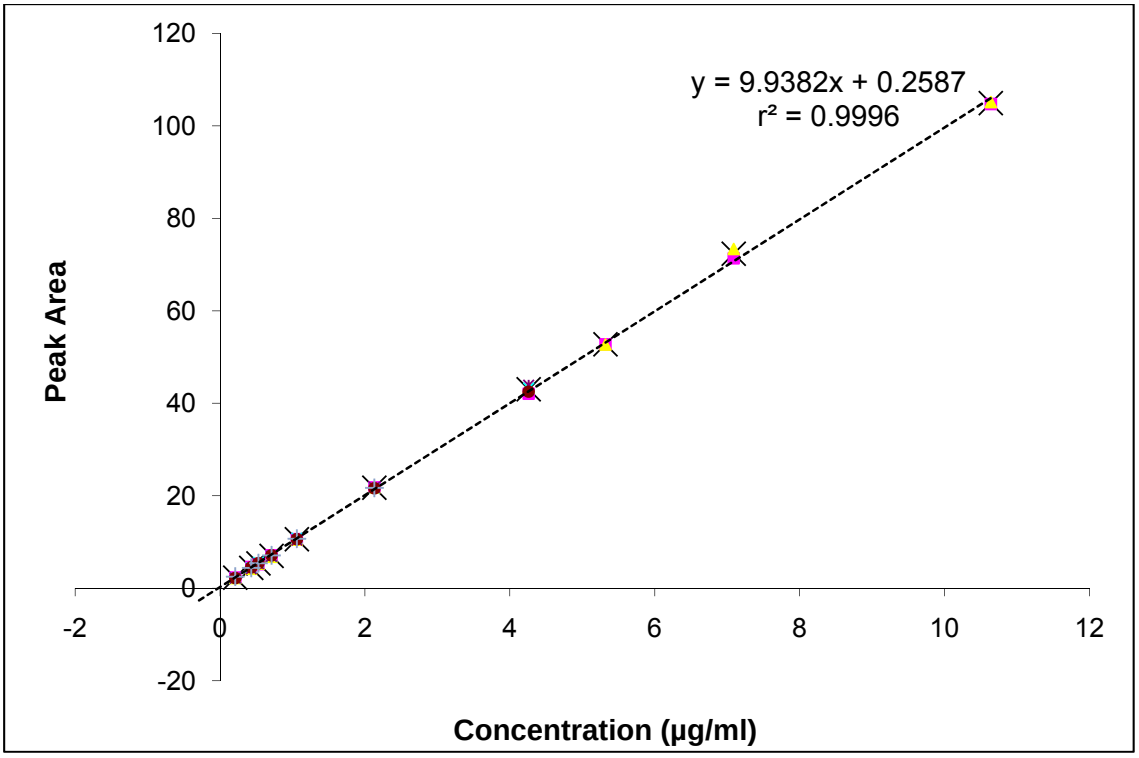


Figure 4.16: Linear regression graph for Impurity A.

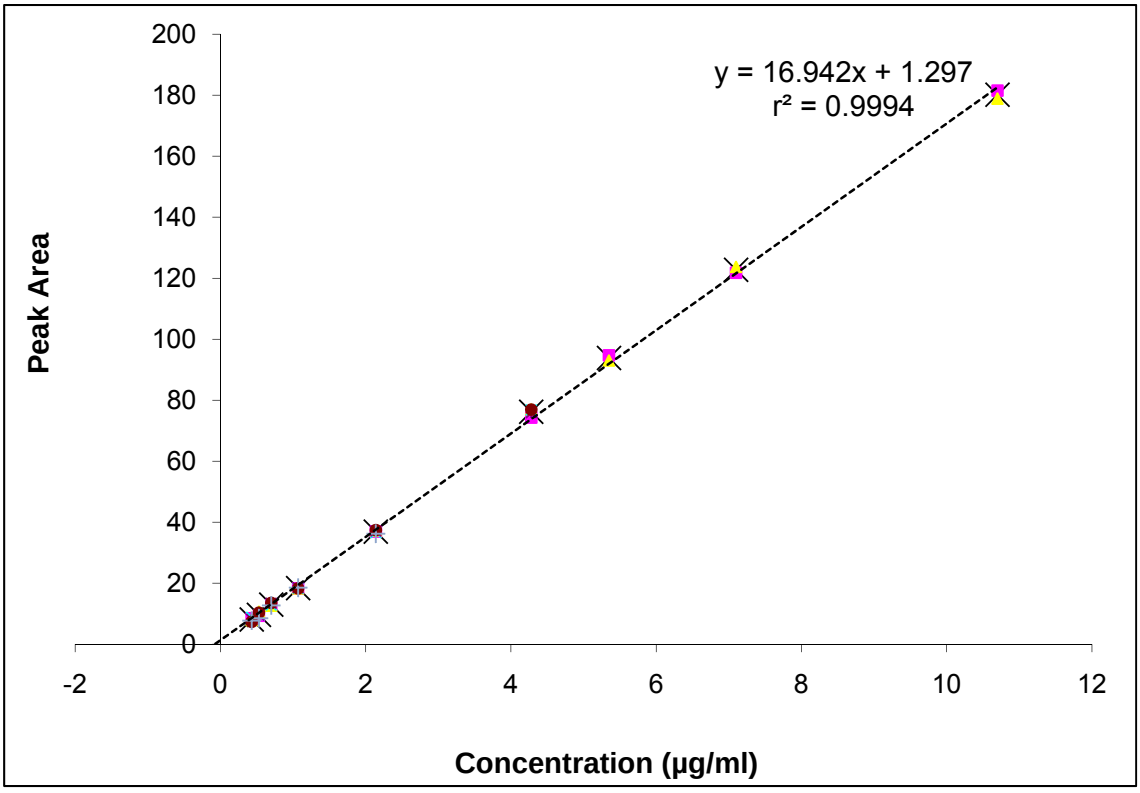


Figure 4.17: Linear regression graph for Impurity B.

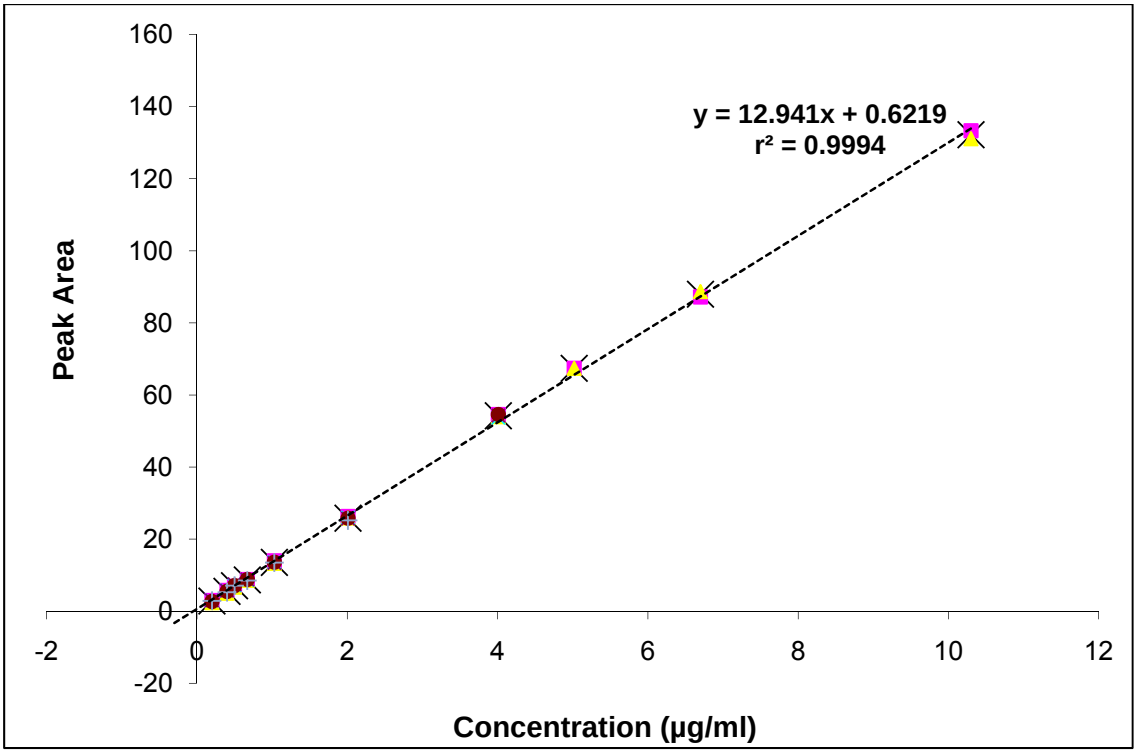


Figure 4.18: Linear regression graph for Impurity C.

4.6.5.3 Repeatability

The solutions representing the 100% target concentration, as prepared for the linearity studies, were used for the repeatability studies. Samples were injected five times. The results are summarised in table 4.28.

Table 4.28: Repeatability results of mefloquine and related substances

NAME	100% TARGET CONCENTRATION	%RSD
Mefloquine for assay	203.84 µg/ml	0.26
Mefloquine for related substances	4.08 µg/ml	0.59
Impurity A	4.26 µg/ml	1.72
Impurity B	4.28 µg/ml	1.49
Impurity C	4.01 µg/ml	0.72

The results indicated that all %RSDs were below the requirement of 2% for the assay test, and below 5% for the related substances test.

4.6.5.4 Limit of detection (LOD)/Limit of quantification (LOQ) for related substances test

Since the method was developed for the purpose of the determination of related substances, the LOD and LOQ were determined analytically. These were also calculated by means of the equations given in 1.2.7.

Mefloquine test solution

The analytical results indicated that a %RSD of more than 5.0 was obtained (5.6%) for a concentration of 0.51 µg/ml of mefloquine (table 4.24). The LOQ was thus set at 0.68 µg/ml (0.034%), where repeatable results were acceptable (%RSD = 2.90). The LOD was 0.41 µg/ml (0.02%), as no peaks could be detected for the 0.2 µg/ml solution. The calculated value of the LOD was 0.13 µg/ml (0.01%), and for the LOQ 0.42 µg/ml (0.02%).

Impurity A reference solution

The analytical results indicated that a %RSD of more than 5.0 was obtained (5.34%) for a concentration of 0.21 µg/ml (0.01%) of Impurity A (table 4.25). The LOQ was thus set at 0.43 µg/ml (0.02%), where repeatable results were acceptable (%RSD = 3.65). The LOD was set at 0.21 µg/ml (0.01%). The calculated value of the LOD was 0.23 µg/ml (0.01%), and for the LOQ 0.75 µg/ml (0.038%).

Impurity B reference solution

The results of the analytical determination indicated that a %RSD of more than 5.0 was obtained (7.9%) for a concentration of 0.53 µg/ml of Impurity B (table 4.26). The LOQ was thus set at 0.70 µg/ml (0.04%), where repeatable results were acceptable (%RSD = 2.95). The LOD was 0.43 µg/ml (0.02%), as no peaks could be detected for the 0.2 µg/ml solution. The calculated value of the LOD was 0.29 µg/ml (0.015%), and for the LOQ 0.96 µg/ml (0.05%).

Impurity C reference solution

The results of the analytical determination indicated that a %RSD of more than 5.0 was obtained (5.8%) for a concentration of 0.4 µg/ml of Impurity C (table 4.27). The LOQ was thus set at 0.50 µg/ml (0.03%), where repeatable results were acceptable

(%RSD = 3.83). The LOD was set at 0.20 µg/ml (0.01%). The calculated value of the LOD was 0.16 µg/ml (0.01%), and for the LOQ 0.54 µg/ml (0.027%).

4.6.5.5 Recovery

Solutions spiked with approximately the same concentration as the 0.2% concentration for the related substances linearity study (table 4.23) and the 200 µg/ml (100%) concentration for the assay (table 4.21) were prepared and injected onto the HPLC. The peak areas that were obtained were used to calculate the concentration of the solution by means of the applicable linear equations as had been determined for each related substance and for mefloquine (4.6.5.2). The percentage recovery was then calculated by means of the following equation:

$$\frac{Concentration\ obtained}{Theoretical\ concentration}\ x\ 100$$

The results are given in table 4.29.

Table 4.29: Recovery results of mefloquine and three related substances

NAME	% RECOVERY
Mefloquine	99.1
Mefloquine for related substances	97.9
Impurity A	102.6
Impurity B	98.5
Impurity C	97.5

A recovery of 90.0 – 110.0% is deemed acceptable for related substances and 98.0 – 102.0% for assay. The method thus complied with the requirements.

4.6.5.6 Robustness

For the purpose of evaluating the robustness of the assay/related substances method, different types of columns were tested and the ratio of the mobile phase was adjusted. Commercial products were analysed to ascertain the suitability of the method for product analyses.

i. Columns

Four different types of C_{18}, 250 x 4.6 mm columns varying in particle sizes were used to determine the effect of different brands of columns on the resolution factor between of the Impurity A and mefloquine peaks (table 4.30). These two substances were selected since they elute closer to each other than sulfadoxine and mefloquine, and since it was important to verify that these two peaks remain separated.

Table 4.30: Resolution factors between Impurity A and mefloquine peaks using different brands of columns

BRAND NAME	RELATIVE RETENTION TIME OF IMPURITY A	RESOLUTION FACTOR
Waters Symmetry, 5 µm	0.87	2.5
Phenomenex Luna, 5 µm	0.87	2.5
Macherey-Nagel, Licrospher, 4 µm	0.90	1.9
Waters µBondapak, 10 µm	0.91	1.7

The results show that the different brands of columns influenced the resolution factor. Although none of these resolution factors were unacceptable, the best results were obtained with 5 µm columns. A resolution factor higher than 1.5 is acceptable, but it should preferably be more than two (USP, 2010).

ii. Change in mobile phase composition

The mobile phase composition was adjusted in order to determine the effect on the elution time of the individual peaks, as well as on the resolution between the peaks in the related substances solution. A Phenomenex Luna column as described in table 4.30 was used in this study. The ratios being used are shown in table 4.31. It is important to note that the total volume of the organic phase could not exceed 600 ml in a 1,000 ml mixture, as it caused the buffer to precipitate.

Table 4.31: Mobile phase compositions used in robustness studies

Mobile phase number	Solvent A volume (ml) Methanol	Solvent B volume (ml) Acetonitrile	Solvent B volume (ml) Buffer
1	120	360	520
2	200	400	400
3*	220	380	400
4	250	350	400
* - Proposed composition			

Results are given in table 4.32.

Table 4.32: Results of a change in mobile phase composition on retention time of mefloquine and related substances

	RETENTION TIME (minutes)				
MOBILE PHASE	Impurity A	Mefloquine	Impurity B	Impurity C	Resolution factor*
1	8.8	9.9	> 60	39.0	4.8
2	4.3	4.6	28.1	17.6	2.8
3	3.3	3.8	31.8	14.8	3.3
4	5.4	6.4	59.6	28.3	4.7
* Resolution factor between Impurity A and mefloquine					

Mobile phases 1 and 4, containing larger volumes of buffer and methanol respectively, had a negative effect on the retention time of especially Impurity B. It eluted at about 60 minutes which is not acceptable. Mobile phase 2 and 3 resulted in very similar retention times, but a lower resolution factor between Impurity A and mefloquine was obtained. Mobile phase 3 was therefore mobile phase of choice.

It was concluded that a ratio of about 1:2 for methanol:acetonitrile was important to obtain a practically acceptable retention time for Impurity B.

iii. Buffer pH

The pH of the buffer was adjusted from 3.5, as in the proposed method, to 3.3 and 3.7 in order to determine the influence on the respective retention times. Results are presented in tables 4.33 – 4.35.

Table 4.33: Retention times and relative retention times of the resolution solution employing varying buffer pH

	RETENTION TIME (minutes)		RELATIVE RETENTION TIME*
BUFFER pH	Sulfadoxine	Mefloquine	
3.3	2.4	3.9	0.6
3.5	2.4	3.9	0.6
3.7	2.3	3.9	0.6
* - Relative retention time of sulfadoxine with respect to mefloquine			

Table 4.34: Retention times of the related substances and resolution factor of Impurity A and mefloquine, employing varying buffer pH

	RETENTION TIME (minutes)				RESOLUTION FACTOR*
BUFFER pH	Impurity A	Mefloquine	Impurity C	Impurity B	
3.3	3.4	3.9	13.7	28.3	2.4
3.5	3.4	3.9	14.1	28.9	2.4
3.7	3.4	3.9	13.5	27.9	2.5
* - Resolution factor between mefloquine and Impurity A					

Table 4.35: Relative retention times of the related substances solution employing varying buffer pH

	RELATIVE RETENTION TIME*			
BUFFER pH	Impurity A	Mefloquine	Impurity C	Impurity B
3.3	0.9	1.0	3.5	7.3
3.5	0.9	1.0	3.6	7.4
3.7	0.9	1.0	3.5	7.2
* - With respect to mefloquine				

The results indicate that the buffer pH did not significantly influence the chromatography and a pH range of 3.3 to 3.7 was thus acceptable for the assay and related substances test.

iv. Product analyses/assay

After successful validation of the analytical method, it was used to perform assay analyses on two batches of commercial mefloquine tablets. Both products contained 250 mg of mefloquine per tablet. The details of the products are given in table 4.1.

Test solutions were prepared in triplicate, according to the method as described in 4.6.4.2, and the results (table 4.36) were the average of the three. The %RSD was calculated for the triplicate analyses.

Both assays were repeated by another analyst, in order to verify the reproducibility/intermediate precision of the method.

The assay results are given in table 4.36. Representative chromatograms are shown in figures 4.19 – 4.20.

Table 4.36: Assay results of commercial mefloquine tablets

PRODUCT	ASSAY VALUE		%RSD
	% Assay	mg mefloquine/tablet	
Lariam	98.0	244.9	0.44
Mefliam	100.7	251.7	1.3

The results indicate that the method is suitable for performing assays on commercial mefloquine products. The %RSD for triplicate samples of these products is 0.44 and 1.3, indicating that the method of preparation of the samples did not give rise to significant differences in results.

The results of the assays performed by another analyst are given in table 4.37.

Table 4.37: Comparative assay results of two commercial mefloquine tablets for two analysts

PRODUCT	ASSAY VALUE		% DIFFERENCE
	% Analyst 1	% Analyst 2	
Lariam	98.0	96.1	1.9
Mefliam	100.7	99.1	1.6

The reproducibility of the results for the same products is acceptable, with differences of less than 2%.

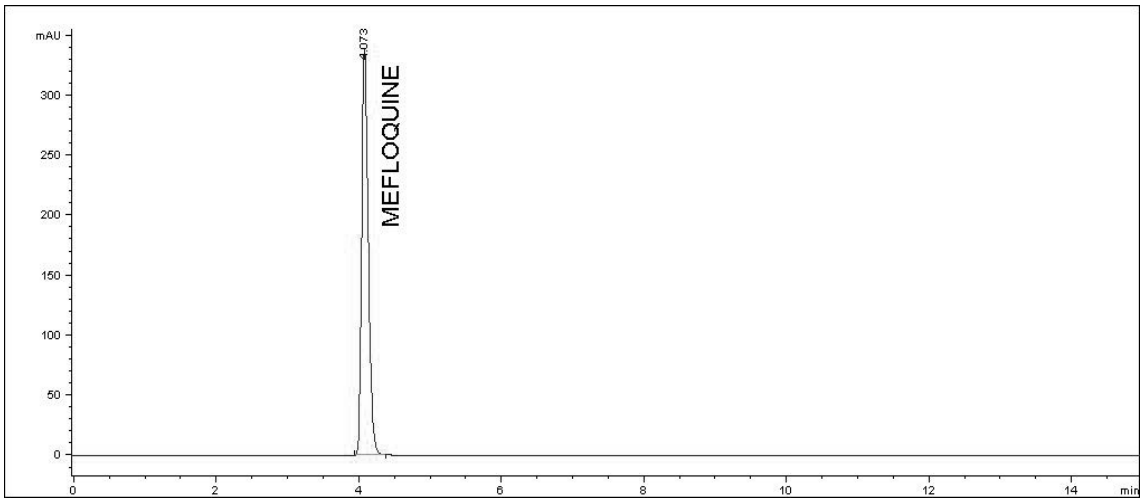


Figure 4.19: Example of chromatogram for Lariam tablets.

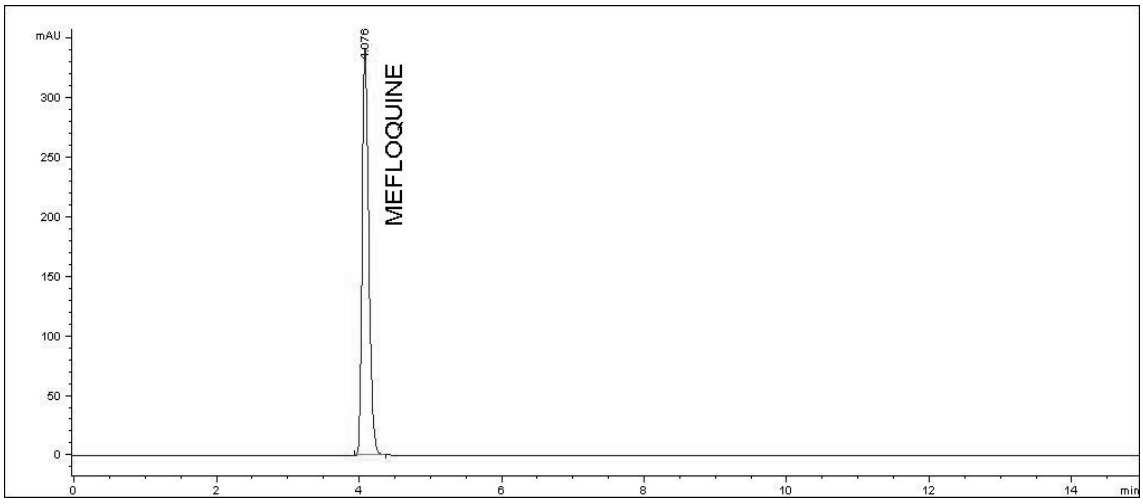


Figure 4.20: Example of chromatogram for Mefliam tablets.

v. Product analyses for related substances

After successful validation of the analytical method, it was used to perform related substances analyses on five batches of commercial mefloquine tablets. All products contained 250 mg mefloquine per tablet. The details of the products are given in table 4.1.

Test solutions were prepared in duplicate, according to the method summarised in 4.6.4.2, and the results were the average of the two. All analyses were run for 60 minutes. None of the chromatograms showed any peaks eluting later than 8 minutes. The chromatographs are shown in figures 4.21 – 4.25.

For all the products tested, the area of none of the peaks in the test solution, other than the mefloquine peak, was more than 0.5 times the area of the mefloquine peak in the reference solution. Thus, no related substance was present at a level higher than 0.1%.

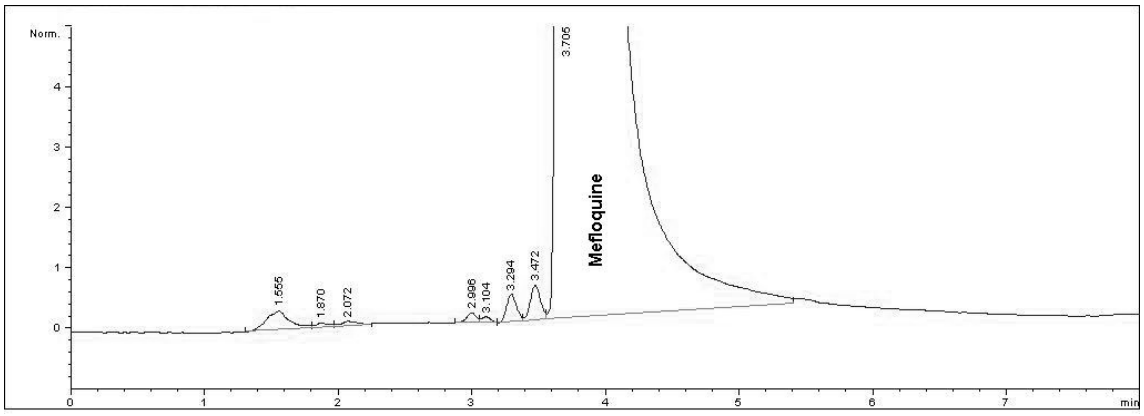


Figure 4.21: Example of chromatogram of related substances for Lariam tablets.

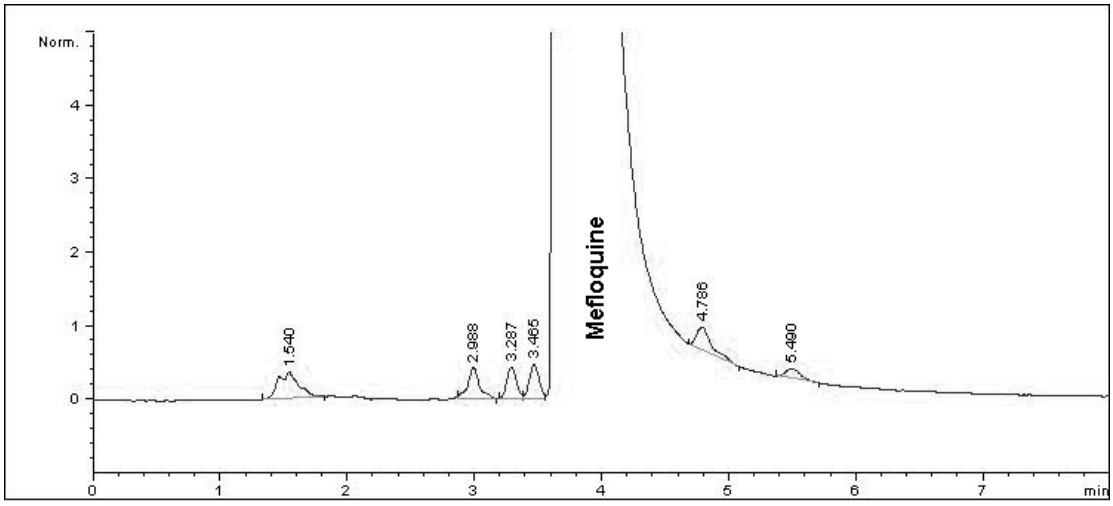


Figure 4.22: Example of chromatogram of related substances for Mefliam tablets.

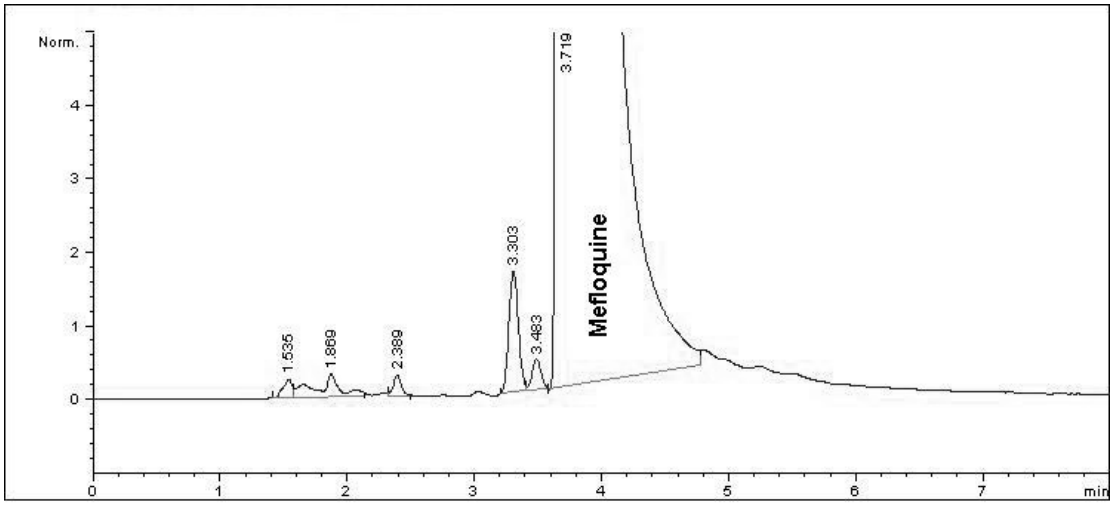


Figure 4.23: Example of chromatogram of related substances for Artequin™-300/750 tablets.

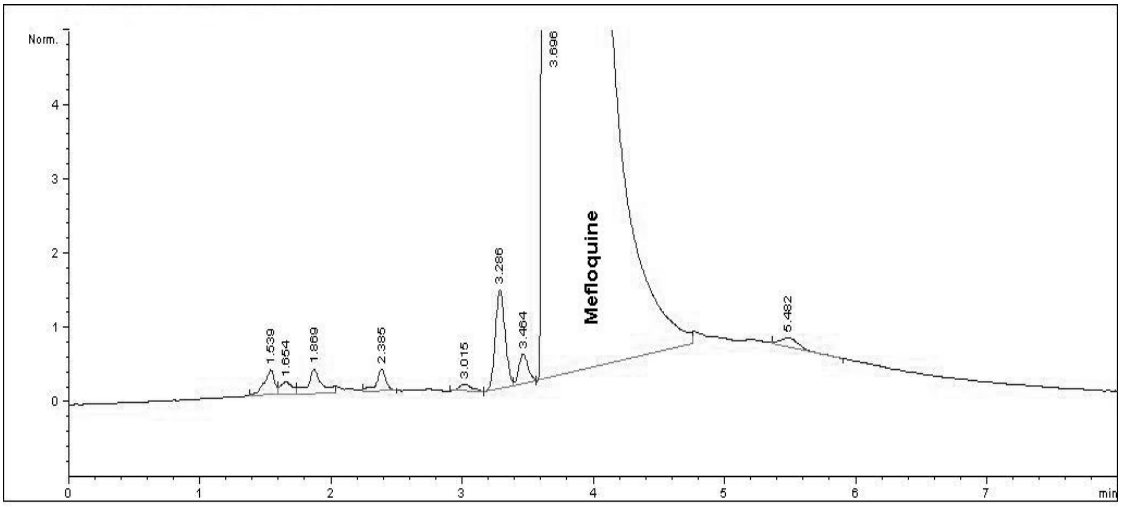


Figure 4.24: Example of chromatogram of related substances for Artequin™-600/1500 tablets (batch 0790020).

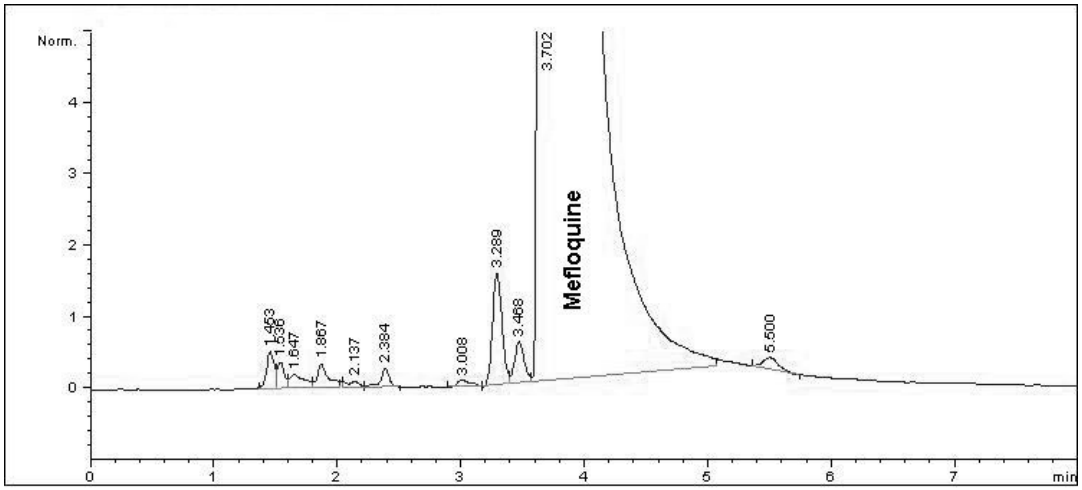


Figure 4.25: Example of chromatogram of related substances for Artequin™-600/1500 tablets (batch 0790021).

From the data obtained for the five commercial batches the limit for related substances in mefloquine tablets could be set at:

- not more than 0.2% for any individual unspecified related substance, namely the threshold level for 250 mg daily dose according to ICH Q3B (R2) (ICH, 2006);
- the sum of all related substances not more than 0.5%; and
- the disregard level at 0.1%, according to ICH Q3B (R2) for 250 mg daily dose (ICH, 2006).

4.6.6 SUMMARY OF VALIDATION RESULTS

The analytical method is capable of separating known related substances of mefloquine HCl in different commercial mefloquine tablets.

The method for assay and related substance analyses was successfully validated and acceptable results were obtained when used for the analyses of samples of commercial products. A summary of the results are given in table 4.38 for the assay and in table 4.39 for the related substances tests.

The acceptance criteria for related substances could be set for mefloquine tablets. The related substances criteria are similar to those of mefloquine hydrochloride BP (BP, 2009), but tighter than the acceptance criteria of mefloquine hydrochloride

Ph.Int. (Ph.Int., 2008). The mefloquine API monograph of the Ph.Int. utilises a TLC test for related substances and needs to be updated.

Table 4.38: Summary of results obtained during assay validation of mefloquine

PARAMETER	RESULTS OBTAINED
Specificity	No interferences detected from solvents, nor related substances
Linearity	r^2 = 0.9998
Range	101.9 – 271.8 µg/ml (51.0 – 135.9%)
Repeatability	%RSD = 0.26 (5 determinations)
Robustness	
Columns	Equivalent columns acceptable
Mobile phase	Ratio of organic:buffer = 60:40; Ratio of methanol:acetonitrile ≅ 1:2
pH of buffer	pH 3.3 to 3.7 acceptable
Product analyses	Acceptable results for 2 commercial products
Recovery	99.1%

Table 4.39: Summary of results obtained during related substances validation

	RESULTS OBTAINED			
PARAMETER	**Mefloquine**	**Impurity A**	**Impurity B**	**Impurity C**
Linearity (r^2)	0.9999	0.9996	0.9996	0.9994
Range (µg/ml) (%)	0.41 – 10.19 0.02 – 0.51	0.21 – 10.64 0.01 – 0.5	0.43 - 10.70 0.02 – 0.5	0.20 – 10.3 0.01 – 0.5
Repeatability (%RSD)	0.59	1.72	1.49	0.72
Limit of detection (µg/ml)/% range	0.41 (0.02*)	0.21 (0.01*)	0.43 (0.02*)	0.20 (0.01*)
Limit of quantitation (µg/ml)/% range	0.68 (0.034*)	0.43 (0.02*)	0.70 (0.04*)	0.50 (0.03*)
Recovery (%)	97.9	102.6	98.5	97.5
* All % ranges are given with respect to the mefloquine test solution				

Since all of the determined parameters complied with the requirements for method validation, the method was deemed acceptable for use in the analyses of samples for the purpose of determining the content of the mefloquine HCl, as well as the related substances in mefloquine tablets.

4.7 CONCLUSION

Analytical methods for identification (ID), dissolution testing, assay and related substances had to be developed and/or evaluated.

Specifications were obtained from a manufacturer and were supplied by the World Health Organization (WHO) as reference for ID, assay, dissolution and related substances tests of mefloquine tablets.

Methods published in literature and monographs from the BP/EP and USP were also considered.

For the ID test a method was proposed where detection by means of UV or with iodine vapours was feasible.

For the dissolution testing a volume of 500 ml 0.1M HCl was considered but the results indicated that 900 ml was more suitable. Acceptance criteria were established following multipoint dissolution, with UV detection.

A method for the purpose of assay is published in literature and was considered (Bergqvist *et al.,* 1991:169). Methods for related substances of mefloquine are published in the USP and BP/EP - the parameters for the two pharmacopoeial methods are the same. Based on these methods a new method was developed whereby the assay and determination of related substances could be done simultaneously. After the development studies, parameters for the assay and related substances test was proposed for validation purposes.

The parameters included for the validation were specificity, linearity, range, repeatability, robustness and recovery. For related substances LOD and LOQ were also determined. The validation studies were concluded successfully.

Based on the satisfactory outcomes of this study, a final monograph for mefloquine tablets was compiled and submitted to the WHO. After consultations a final draft for adoption of this monograph (Annexure C) has been accepted during the 45th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (18-22 October 2010, Geneva).

SUMMARY

Malaria is a disease leading to approximately one million deaths annually. In 2004, *P. falciparum* was among the leading causes of death worldwide from a single infectious agent, especially in Africa (WHO, 2008:23).

Because of the emergence of resistant *P. falciparum* parasites, therapy should include the combination of two or more APIs with independent modes of action and molecular targets, resulting in synergistic or additive effects (Aweeka & German, 2008:92).

The quality of the products is furthermore of the utmost importance since poor quality products contribute to the development of resistance to treatment.

Analytical methods for the testing of amodiaquine tablets (assay and dissolution testing), sulfadoxine/pyrimethamine tablets (assay, dissolution and related substances testing) and mefloquine tablets (identification, assay, dissolution and related substances testing) had to be developed, validated and evaluated for inclusion in the Ph.Int. dosage form monographs.

Methods and/or specifications obtained from manufacturers and supplied by the WHO, and monographs published in pharmacopoeia such as the USP and BP/EP were considered as reference methods.

Amodiaquine tablets

The manufacturer's method supplied by the WHO was evaluated and found to be suitable for the assay testing of amodiaquine tablets. Two adjustments were made to the original method. To shorten the stop time the flow rate was increased from 1.0 ml/min to 1.5 ml/min. During the development studies for the assay testing of amodiaquine tablets it was found that solvents with higher pH-values caused precipitation of amodiaquine and the solvent was changed to water for reference and test solutions. This was a very important consideration in the choice of an appropriate dissolution medium. Dilute HCl (0.1M) was evaluated and found to be suitable as dissolution medium. A criterion of not less than 80% amodiaquine dissolved in 30 minutes was proposed as acceptance value. A spectrophotometric

analytical method for the purposes of analysing the dissolution test samples was validated. For the purposes of the assay testing a HPLC method was developed and validated.

Based on the satisfactory outcomes of this study, a final monograph for amodiaquine tablets was compiled and submitted to the WHO for possible inclusion in the Ph.Int. monograph. After consultations this monograph (Annexure A) has been adopted during the 44th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (12-16 October 2009, Geneva).

Sulfadoxine/pyrimethamine tablets

An adapted version of the USP (2008) analytical method for assay testing of sulfadoxine/pyrimethamine tablets was developed, since problems with peak interferences were experienced with the original method. The mobile phase composition was thus adjusted. The detection wavelength was also changed to 227 nm since it favoured the smaller of the two API peaks, i.e. pyrimethamine. The method was validated and found suitable for commercial product analyses.

Poor dissolution performance reported in literature for especially the pyrimethamine lead to the investigation of an alternative dissolution medium to the pH 6.8 phosphate buffer prescribed by the USP. The USP prescribes 0.1M HCl as the dissolution medium for pyrimethamine tablets. Solubility studies for sulfadoxine indicated that 0.1M HCl and pH 6.8 buffer resulted in solubilities of the same order, indicating that 0.1M HCl could be considered as an alternative dissolution medium.

The influence of the disintegration of the tablets was also investigated on the outcome of the dissolution tests.

Results indicated that a correlation exists between disintegration of the tablets and the percentage active dissolved during dissolution testing. It was also shown that 0.1M HCl is more suitable as dissolution medium. The acceptance criterion for the dissolution was set at 80% dissolved in 30 minutes for both APIs, following multipoint dissolution studies on commercial tablets. The same analytical method that was developed for the assay test was used to analyse the dissolution test samples.

The same method that was used for the assay was adapted for determining likely related substances of sulfadoxine. Three substances were described in the

manufacturer's method, and sulfanilamide and sulfanilic acid, as known related substances of sulfadoxine (Auterhoff & Schmidt, 1974:1582) were also included for the development studies.

Results indicated that the proposed analytical method was suitable for determining N1-(6-hydroxy-5-methoxy-4-pyrimidinyl)sulfanilamide, 4-(p-acetamide-benzene-sulfonamide)-5,6-dimethoxy-pyrimidine, 4-amino-5,6-dimethoxy-pyrimidine and sulfanilamide in the presence of the two APIs. In line with the API monograph for sulfadoxine in the Ph.Int. the limit for individual related substances was set as 0.5% and for the total as 1.0%.

Based on the satisfactory outcomes of this study, a final monograph for sulfadoxine/pyrimethamine tablets was compiled and submitted to the WHO. After consultations a final draft for adoption of this monograph (Annexure B) has been accepted during the 45th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (18-22 October 2010, Geneva).

Mefloquine tablets

For the identification (ID) test a TLC method was developed where the spots can be detected by means of UV or with iodine vapours.

For the dissolution testing a volume of 500 ml 0.1M HCl was considered but the results indicated that sink conditions were not reached with 500 ml medium. Further multipoint studies indicated 900 ml was more suitable. Following multipoint dissolution studies on commercial products, the acceptance criterion was set at not less than 80% mefloquine dissolved in 30 minutes. The samples were analysed spectrophotometrically.

Development studies were performed to establish one analytical method suitable for the simultaneous analysis of the API and related substances. Three related substances, namely (RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl][(2RS)-piperidin-2-yl]methanol *(threo*-mefloquine), [2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanone and (RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanol were included in the development studies. The method was suitable for determining mefloquine in the presence of the three related substances. Following the analyses

of commercial products, the acceptance criteria were set at not more than 0.2% for any individual unspecified related substance; the sum of all related substances not more than 0.5%; and the disregard level at 0.1%.

Based on the satisfactory outcomes of this study, a final monograph for mefloquine tablets was compiled and submitted to the WHO. After consultations a final draft for adoption of this monograph (Annexure C) has been accepted during the 45th meeting of WHO's Expert committee on specifications for pharmaceutical preparations (18-22 October 2010, Geneva).

The outcomes of this study were thus a contribution to the endeavours of the WHO to combat resistance against existing antimalarial treatments caused by substandard medication, available especially in Africa. By establishing accurate and robust analytical test methods, the quality control of commercial products can be governed in the same manner worldwide.

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ANNEXURE A

AMODIAQUINE TABLETS

World Health Organization

AMODIAQUINE TABLETS:

Final text for addition to *The International Pharmacopoeia* (December 2009)

This monograph was adopted at the Forty-fourth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2009 for addition to the 4th Edition of the International Pharmacopoeia

Category. Antimalarial.

Storage. Amodiaquine tablets should be kept in a well-closed container.

Labelling. The designation of the container of Amodiaquine tablets should state that the active ingredient is in the hydrochloride form and the quantity should be indicated in terms of the equivalent amount of amodiaquine.

Additional information. Strengths in the current WHO Model list of essential medicines: 153 mg and 200 mg of amodiaquine. Strengths in the current WHO Model list of essential medicines for children: 153 mg and 200 mg of amodiaquine.

153 mg of amodiaquine is approximately equivalent to 200 mg of amodiaquine hydrochloride; 200 mg of amodiaquine is approximately equivalent to 260 mg of amodiaquine hydrochloride.

Requirements

Comply with the monograph for "Tablets".

Definition. Amodiaquine tablets contain Amodiaquine hydrochloride. They contain not less than 90.0% and not more than 110.0% of the amount of amodiaquine ($C_{20}H_{22}ClN_3O$) stated on the label.

Identity tests

- . Either test A and E or any two of tests B, C and D together with test E may be applied.

A. Shake a quantity of powdered tablets containing the equivalent of about 50 mg of amodiaquine with 20 ml of water R and transfer to a separating funnel. Add 1 ml of ammonia (~260 g/l) TS and 25 ml of dichloromethane R and shake well. Let the layers separate and filter the dichloromethane extract through glass-fibre paper or a cotton plug previously washed and moistened with dichloromethane R. Evaporate the dichloromethane and dry the residue at 105°C for one hour. Carry out the examination as described under 1.7 Spectrophotometry in the infrared region. The infrared

absorption spectrum is concordant with the spectrum obtained from amodiaquine hydrochloride RS, treated in the same way as the test substance, or with the *reference spectrum* of amodiaquine.

B. Carry out the test as described under 1.14.1 Thin-layer chromatography. Prepare a solution of chloroform saturated with ammonia by shaking chloroform R with ammonia (~260 g/l) TS and separate the chloroform layer (solution Chl). Use silica gel R5 as the coating substance and a mixture of 9 volumes of solution Chl, and 1 volume of dehydrated ethanol R as the mobile phase. Apply separately to the plate 10 µl of each of the following two solutions in solution Chl. For solution (A) shake vigorously a quantity of the powdered tablets containing the equivalent of about 0.15 g of amodiaquine with 10 ml of solution Chl for 2 minutes in a glass-stoppered test-tube, filter through a 0.45-µm filter and use the filtrate. For solution (B) shake vigorously 20 mg of amodiaquine hydrochloride RS per ml of solution Chl for 2 minutes in a glass stoppered test-tube, allow the precipitate formed to settle and use the clear supernatant. After removing the plate from the chromatographic chamber, allow it to dry in air or in a current of cool air and examine the chromatogram in daylight .

The principal spot obtained with solution A corresponds in position, appearance and intensity to that obtained with solution B.

C. To a quantity of the powdered tablets containing the equivalent of about 30 mg of amodiaquine, add 100 ml of hydrochloric acid (~4 g/l) TS, shake, and filter. Dilute 5 ml of the filtrate to 100 ml with the same solvent. The absorption spectrum (1.6) of the resulting solution,, when observed between 300 nm and 400 nm, exhibits one maximum at about 342 nm.

D. See the test described under Assay. The retention time of the principal peak in the chromatogram obtained with solution (1) is similar to that in the chromatogram obtained with solution (2).

E To a quantity of powdered tablets containing the equivalent of about 0.15 g of amodiaquine add 10 ml of water R, shake well, and filter. The filtrate yields reaction B described under 2.1 General identification tests as characteristic of chlorides.

Dissolution

Carry out the test as described under 5.5 Dissolution test for solid oral dosage forms, using as the dissolution medium, 500 ml of hydrochloric acid (~4 g/l) TS and rotating the paddle at 75 revolutions per minute. At 30 minutes withdraw a sample of 10 ml of the medium through an in-line filter. Measure the absorbance (1.6) of a 1-cm layer of the filtered sample, suitably diluted if necessary, at the maximum at about 342 nm. At the same time measure the absorbance at the maximum at about 342 nm of a suitable solution of amodiaquine hydrochloride RS in hydrochloric acid (~4 g/l) TS using hydrochloric acid (~4 g/l) TS as the blank. Each mg of amodiaquine hydrochloride ($C_{20}H_{22}ClN_3O,2HCl,2H_2O$) is equivalent to 0.7656 mg of amodiaquine ($C_{20}H_{22}ClN_3O$).

For each of the six tablets tested, calculate the total amount of amodiaquine ($C_{20}H_{22}ClN_3O$) in the medium. The amount in solution for each tablet is not less than 80% of the amount declared on the label. If the amount obtained for one of the six tablets is less than 80%, repeat

the test using a further six tablets; the average amount for all 12 tablets tested is not less than 75% and the amount obtained for no tablet is less than 60%.

Related substances
Carry out the test as described under 1.14.1 Thin-layer chromatography. Prepare a solution of chloroform saturated with ammonia by shaking chloroform R with ammonia (~260 g/l) TS and separate the chloroform layer (solution Chl). Use silica gel R6 as the coating substance and a mixture of 9 volumes of solution Chl and 1 volume of dehydrated ethanol R as the mobile phase. Apply separately to the plate 10 µl of each of the following two solutions in solution Chl. For solution (A) shake a quantity of the powdered tablets containing the equivalent of 0.15 g of amodiaquine with 10 ml of solution Chl vigorously for 2 minutes in a glass-stoppered test-tube, filter through a 0.45-µm filter and use the filtrate. For solution (B) dilute 1.0 ml of solution A to 200 ml with solution Chl. After removing the plate from the chromatographic chamber, allow it to dry in air or in a current of cool air and examine the chromatogram in ultraviolet light (254 nm).

Any spot obtained with solution A, other than the principal spot, is not more intense than that obtained with solution B (0.5%).

Assay
Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (15 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm).[1]

As the mobile phase, use a mixture of 62 volumes of methanol R and 38 volumes of buffer pH 9.0 prepared as follows: dilute a mixture of 100 ml of potassium dihydrogen phosphate (13.6 g/l) TS and 1.4 ml of triethylamine R to 900 ml, adjust the pH to 9.0 by addition of potassium hydroxide (~55 g/l) TS and dilute to 1000 ml.

Prepare the following solutions in water R. For solution (1), weigh and powder 20 tablets. To a quantity of the powder containing the equivalent of about 115 mg of amodiaquine, accurately weighed, add 70 ml of water R and sonicate for about 15 minutes. Dilute to 100 ml. Filter a portion of this solution through a 0.45-µm filter, discarding the first few ml of the filtrate. Dilute 5 ml of the filtrate to 50 ml. For solution (2), use 0.15 mg of amodiaquine hydrochloride RS per ml. For solution (3) use 0.15 mg of amodiaquine hydrochloride RS and 0.15 mg of chloroquine sulfate R per ml.

Operate with a flow rate of 1.5 ml per minute. As a detector, use an ultraviolet spectrophotometer set at a wavelength of about 254 nm.

Inject 20 µl of solution (3). The assay is not valid unless the resolution between the two principal peaks is at least 3.5.

Inject alternately 20 µl each of solutions (1) and (2).

Measure the areas of the peak responses in the chromatograms obtained with solutions (1) and (2) and calculate the content of amodiaquine ($C_{20}H_{22}ClN_3O$) in the tablets, using the declared content of amodiaquine hydrochloride ($C_{20}H_{22}ClN_3O,2HCl,2H_2O$) in amodiaquine

[1] Luna® was found suitable.

hydrochloride RS. Each mg of $C_{20}H_{22}ClN_3O,2HCL,2H_2O$ is equivalent to 0.7656 mg of $C_{20}H_{22}ClN_3O$.

***

New reagents to be added to Ph.Int.

Hydrochloric acid (~4 g/l) TS

Dilute 10 ml of hydrochloric acid (~420 g/l) TS with sufficient water to produce 1000 ml (approximately 0.1 mol/l).

Potassium hydroxide (~55 g/l) TS

A solution of potassium hydroxide R containing about 55 g/l of KOH (approximately 1 mol/l).

***

ANNEXURE B

SULFADOXINE/PYRIMETHAMINE TABLETS

Working document QAS/07.218/Rev.2
September 2010
RESTRICTED

World Health Organization

SULFADOXINE AND PYRIMETHAMINE TABLETS

Revised draft proposal for *The International Pharmacopoeia*

(September 2010)

REVISED DRAFT FOR COMMENT

This document was provided by a quality control expert and was discussed at the recent WHO consultation on specifications for medicines and quality control laboratory issues Previous comments received have been incorporated into this revised draft. Should you have any comments, please send these to Dr S. Kopp, Manager, Medicines Quality Assurance Programme, Quality Assurance and Safety: Medicines, World Health Organization, 1211 Geneva 27, Switzerland; fax: (+41 22) 791 4730 or e-mails: kopps@who.int with a copy to Ms C. Mendy mendyc@who.int by 14 October 2010.

In order to speed up the process for receipt of documents, please let us have your e-mail address (to bonnyw@who.int) which we will add to our electronic mailing list for monographs.

SCHEDULE FOR THE ADOPTION PROCESS OF DOCUMENT QAS/07.218

International Pharmacopoeia monograph on Sulfadoxine and Pyrimethamine tablets

	Date
Preparation of first draft by laboratory	February 2007 –May 2007
First draft mailed out for comments	June 2007
Any comments received reviewed in Consultation on Specifications for Medicines and Quality Control Laboratory Issues	27-29 June 2007
Second draft mailed out for comments	August 2007
Review of second round of comments with WHO Expert panel	September 2007
Presentation to WHO Expert Committee on Specifications for Pharmaceutical Preparations	15-19 October 2007
Revised draft sent by collaborating laboratory	February 2010
Revision mailed out for comments	March 2010
Collation of comments received	April-May 2010
Discussion of revised monograph at consultation on specifications for medicines and quality control laboratory issues	10-12 May 2010
Revised draft monograph mailed out for comments	July 2010
Collation of comments	August 2010
Comments discussed during video-/teleconference on specifications for medicines	25 August 2010
Revised draft mailed out for comments	September 2010
Presentation to WHO Expert Committee on Specifications for Pharmaceutical Preparations for possible adoption.	18-22 October 2010
Any further action as required	…

SULFADOXINE AND PYRIMETHAMINE TABLETS: Revised draft proposal for *The International Pharmacopoeia* (September 2010)

Category. Antimalarial.

Storage. Sulfadoxine and pyrimethamine tablets should be kept in a well-closed container, protected from light.

Additional information. Strength in the current WHO Model list of essential medicines: 500 mg sulfadoxine and 25 mg pyrimethamine.

Strength in the current WHO Model list of essential medicines for children: 500 mg sulfadoxine and 25 mg pyrimethamine.

Requirements

Comply with the monograph for "Tablets".

Definition. Sulfadoxine and pyrimethamine tablets contain Sulfadoxine and Pyrimethamine. They contain not less than 90.0% and not more than 110.0% of the amounts of sulfadoxine ($C_{12}H_{14}N_4O_4S$) and pyrimethamine ($C_{12}H_{13}ClN_4$) stated on the label.

Identity tests

A. Carry out test A.1 or, where UV detection is not available, test A.2.

A.1 Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R6 as the coating substance and a mixture of 75 volumes of ethyl acetate R, 25 volumes of methanol R and 1 volume of glacial acetic acid R as the mobile phase. Apply separately to the plate 10 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets containing about 100 mg of Sulfadoxine for 5 minutes with 20 ml, filter, and use the filtrate. For solution (B) use 5 mg of sulfadoxine RS and 0.25 mg of pyrimethamine RS per ml. After removing the plate from the chromatographic chamber, allow it to dry in a current of air and examine the chromatogram in ultraviolet light (254 nm).

The two principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

A.2 Carry out the test as described under 1.14.1 Thin-layer chromatography, using the conditions described above under test A.1 but using silica gel R5 as the coating substance. Dip the plate in modified Dragendorff reagent TS. Examine the chromatogram in daylight.

The two principal spots obtained with solution A correspond in position, appearance, and intensity to those obtained with solution B (the spot due to pyrimethamine is faintly visible).

B. See the test described under Assay. The retention times of the two principal peaks in the chromatogram obtained with solution (1) are similar to those in the chromatogram obtained with solution (4).

Dissolution

Carry out the test as described under 5.5 Dissolution test for solid oral dosage forms, using as the dissolution medium, 1000 ml of hydrochloric acid (~4 g/l) TS, and rotating the paddle at 75 revolutions per minute. At 30 minutes withdraw a sample of about 5 ml of the medium through an in-line filter and use the filtrate. Determine the content of sulfadoxine ($C_{12}H_{14}N_4O_4S$) and pyrimethamine ($C_{12}H_{13}ClN_4$) in the filtrate according to the method as described under Assay and preparing solution (4) under Assay as follows: dilute 10.0 ml of solution (2) and 2.0 ml of solution (3) to 20.0 ml with hydrochloric acid (~4 g/l) TS.

For each of the six tablets, calculate the total amount of sulfadoxine ($C_{12}H_{14}N_4O_4S$) and pyrimethamine ($C_{12}H_{13}ClN_4$), in the medium from the results obtained. For both substances, the amount in solution for each tablet is not less than 80% of the amount declared on the label. For either substance, if the amount obtained for one of the six tablets is less than 80%, repeat the test using a further six tablets; the average amount for all 12 tablets tested is not less than 75% and no tablet contains less than 60%.

Sulfadoxine-related substances

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1].

As the mobile phase, use a solution prepared as follows: dissolve 10 ml of glacial acetic acid R and 0.5 ml of triethylamine R in about 800 ml of water R, dilute to 1000 ml and adjust the pH to 4.2 by adding sodium hydroxide (~400 g/l) TS. Mix 850 ml of this solution with 120 ml of acetonitrile R and 30 ml of methanol R.

For solution (1), weigh and powder 20 tablets. Transfer a quantity of the powder containing about 200 mg of Sulfadoxine into a 100-ml volumetric flask. Add 35 ml of acetonitrile R and sonicate for about 10 minutes. Allow to cool to room temperature and make up to volume with mobile phase. Filter a portion of this solution through a 0.45-µm filter, discarding the first few ml of the filtrate. For solution (2) dilute 1 ml of solution (1) to 200 ml with the mobile phase.

For solution (3) prepare a solution containing about 1 mg of sulfadoxine RS and about 0.5 mg of sulfamethoxazole R per ml in acetonitrile R. Dilute 10 ml of this solution to 100 ml with the mobile phase.

Operate with a flow rate of 2 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 270 nm.

[1] Phenomenex Luna® is suitable.

Inject separately 100 µl each of solutions (1), (2) and (3). Record the chromatograms for about 3.5 times the retention time of sulfadoxine (to ensure that pyrimethamine is eluted).

In the chromatogram obtained with solution (1), the following impurity peaks, if present, are eluted at the following relative retention with reference to sulfadoxine (retention time about 18 minutes): impurity A (sulfanilamide) about 0.1, impurity B about 0.2, impurity D about 0.3, impurity C about 1.4. A peak due to pyrimethamine has a relative retention of about 2.7. The test is not valid unless in the chromatogram obtained with solution (3), the resolution between the peaks due to sulfadoxine and to sulfamethoxazole (with relative retention of about 1.1 with reference to sulfadoxine) is at least 2.

In the chromatogram obtained with solution (1) the area of any peak, other than the peaks due to sulfadoxine and to pyrimethamine, is not greater than the area of the peak due to sulfadoxine in the chromatogram obtained with solution (2) (0.5%). The sum of the areas of all peaks, other than the peaks due to sulfadoxine and pyrimethamine, is not greater than twice the area of the principal peak in the chromatogram obtained with solution (4) (1.0%). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with solution (2) (0.05%).

*[**Note from Secretariat**: in accordance with WHO's guideline on the development of fixed-dose combinations dosage forms (least stable API controlled), and considering the ratio between the two APIs in the formulation which is 1 to 20, a test for related substances is only proposed for sulfadoxine.]*

Assay

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm).[2]

As the mobile phase, use a solution prepared as follows: dissolve 10 ml of glacial acetic acid R and 0.5 ml of triethylamine R in about 800 ml of water R, dilute to 1000 ml and adjust the pH to 4.2 by adding sodium hydroxide (~400 g/l) TS. Mix 800 ml of this solution with 200 ml of acetonitrile R.

For solution (1) weigh and powder 20 tablets and transfer a quantity of the powder containing about 0.50 g of Sulfadoxine, accurately weighed, into a 200-ml volumetric flask. Add about 70 ml of acetonitrile R and sonicate for 10 minutes. Allow to cool to room temperature, make up to volume using the mobile phase and sonicate for 10 minutes. Dilute 5 ml of this solution to 25 ml with mobile phase and filter a portion of this solution through a 0.45-µm filter, discarding the first few ml of the filtered solution. For solution (2), transfer 25 mg of sulfadoxine RS, accurately weighed, to about 10 ml of acetonitrile R, sonicate until dissolved and dilute to 25.0 ml with the mobile phase. For solution (3), transfer 25 mg of pyrimethamine RS, accurately weighed, to about 35 ml of acetonitrile R, sonicate until dissolved and dilute to 100.0 ml with the mobile phase. For solution (4) dilute 10.0 ml of solution (2) and 2.0 ml of solution (3) to 20.0 ml with the mobile phase.

Operate with a flow rate of 2 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 227 nm.

Inject 20 µl of solution (4). The assay is not valid unless the resolution between the peaks due to sulfadoxine and to pyrimethamine, eluting in this order, is at least 5. The run time for the analyses is not less than 25 minutes.

[2] Phenomenex Luna® is suitable.

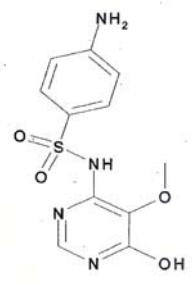
Inject alternately 20 µl each of solutions (1) and (4).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (4), and calculate the content of sulfadoxine ($C_{12}H_{14}N_4O_4S$) and pyrimethamine ($C_{12}H_{13}ClN_4$) in the tablets.

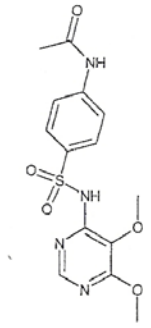
Impurities

The following list of known and potential impurities that have been shown to be controlled by the tests in this monograph is given for information.

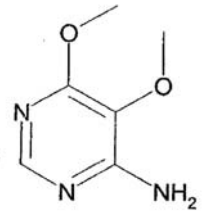
A. sulfanilamide



B. *N*1-(6-hydroxy-5-methoxy-4-pyrimidinyl) sulfanilamide



C. 4-(*p*-acetamido-benzolsulfonamido)-5,6-dimethoxy-pyrimidine



D. 4-Amino-5,6-dimethoxy-pyrimidine.

*[**Note from Secretariat**: structures and chemical names for related substances to be confirmed.]*

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New reagent to be added to Ph.Int.

Sulfamethoxazole R. *N'*-(5-Methyl-3-isoxazolyl)sulfanilamide: 4-amino-*N*-(5-methyl-3-isoxazolyl)benzenesulfonamide; $C_{10}H_{11}N_3O_3S$

A commercialy available reagent of suitable grade.

Description. A white or yellowish white, crystalline powder.

Solubility. Very slightly soluble in water; soluble in 50 parts of ethanol (~750 g/l) TS and in 3 parts of acetone R.

Hydrochloric acid (~4 g/l) TS.

Dilute 10 ml of hydrochloric acid (~420 g/l) TS with sufficient water to produce 1000 ml (approximately 0.1 mol/l).

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ANNEXURE C

MEFLOQUINE TABLETS

Working document QAS/10.370/Rev.1
September 2010
RESTRICTED

World Health Organization

MEFLOQUINE TABLETS

Draft proposal for *The International Pharmacopoeia*

(September 2010)

REVISED DRAFT FOR COMMENT

This document was provided by a quality control expert and was discussed at the recent WHO consultation on specifications for medicines and quality control laboratory issues Previous comments received have been incorporated into this revised draft. Should you have any comments, please send these to Dr S. Kopp, Manager, Medicines Quality Assurance Programme, Quality Assurance and Safety: Medicines, World Health Organization, 1211 Geneva 27, Switzerland; fax: (+41 22) 791 4730 or e-mails: kopps@who.int with a copy to Ms C. Mendy mendyc@who.int by 11 October 2010.

During the past few years we have moved more towards an electronic system for sending out our draft monographs for comment, for convenience and in order to speed up the process. If you do not already receive our documents electronically, please let us have your e-mail address (to bonnyw@who.int) which we will add to our electronic mailing list.

SCHEDULE FOR THE ADOPTION PROCESS OF DOCUMENT QAS/10.370

International Pharmacopoeia monograph on Mefloquine tablets

	Date
Preparation of first draft by laboratory	September 2009–May 2010
Discussion at consultation on specifications for medicines and quality control laboratory issues	10-12 May 2010
Draft monograph mailed out for comments	July 2010
Collation of comments	August 2010
Comments discussed during video-/teleconference on specifications for medicines	25 August 2010
Revised draft mailed out for comments	September 2010
Presentation to WHO Expert Committee on Specifications for Pharmaceutical Preparations	18-22 October 2010
Further action as necessary	

MEFLOQUINE TABLETS
Draft proposal for *The International Pharmacopoeia*
(September 2010)

Category. Antimalarial.

Storage. Mefloquine tablets should be kept in a well-closed container, protected from light.

Labelling. The designation of the container of Mefloquine tablets should state that the active ingredient is in the hydrochloride form and the quantity should be indicated in terms of the equivalent amount of mefloquine.

Additional information. Strength in the current WHO Model list of essential medicines: 250 mg. Strength in the current WHO Model list of essential medicines for children: 250 mg.

Requirements

Comply with the monograph for "Tablets".

Definition. Mefloquine tablets contain Mefloquine hydrochloride. They contain not less than 90.0% and not more than 110.0% of the amount of mefloquine ($C_{17}H_{16}F_6N_2O$) stated on the label.

Identity tests

- Any two of tests A, B or C may be applied together with test D.

A. Carry out test A.1 or, where UV detection is not available, test A.2.

A.1 Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R6 as the coating substance and a mixture of 70 volumes of toluene R, 30 volumes of ethanol R and 2 volumes of 25% ammonia solution R as the mobile phase. Apply separately to the plate 10 µl of each of the following two solutions in methanol R. For solution (A) sonicate, with intermittent shaking, a quantity of the powdered tablets containing the equivalent of about 250 mg of mefloquine for 5 minutes with 25 ml, filter, and use the filtrate. For solution (B) use 10 mg of mefloquine RS per ml. After removing the plate from the chromatographic chamber, allow it to dry in a current of air and examine the chromatogram in ultraviolet light (254 nm).

The principal spot obtained with solution A corresponds in position, appearance and intensity to that obtained with solution B.

A.2 Carry out the test as described under 1.14.1 Thin-layer chromatography, using the conditions described above under test A.1 but using silica gel R5 as the coating substance. Stain the plate with iodine vapours. Examine the chromatogram in daylight.

The principal spot obtained with solution A corresponds in position, appearance, and intensity to that obtained with solution B.

B. See the test described under Assay. The retention time of the principal peak in the chromatogram obtained with solution (1) is similar to that in the chromatogram obtained with solution (2).

C. To a quantity of the powdered tablets containing the equivalent of 50 mg of mefloquine add 100 ml of methanol R, shake and filter. Dilute 5 ml of the filtrate to 50 ml with the same solvent. The absorption spectrum (1.6) of the resulting solution, when observed between 250 nm and 290 nm, exhibits one maximum at about 283 nm.

D To a quantity of powdered tablets containing the equivalent of about 0.5 g of mefloquine add 10 ml of water R, sonicate for 10 minutes and filter. The filtrate yields reaction B described under 2.1 General identification tests as characteristic of chlorides.

Dissolution. Carry out the test as described under 5.5 Dissolution test for solid oral dosage forms, using as the dissolution medium, 900 ml of hydrochloric acid (~4 g/l) TS and rotating the paddle at 75 revolutions per minute. At 30 minutes withdraw a sample of 10 ml of the medium through an in-line filter. Measure the absorbance (1.6) of a 1-cm layer of the filtered sample, suitably diluted if necessary, at the maximum at about 283 nm. At the same time measure the absorbance at the maximum at about 283 nm of a suitable solution of mefloquine hydrochloride RS, initially dissolved in methanol R and then diluted in 0.1 mol/l hydrochloric acid, using hydrochloric acid (~4 g/l) TS as the blank. Each mg of mefloquine hydrochloride ($C_{17}H_{16}F_6N_2O,HCl$) is equivalent to 0.912 mg of mefloquine ($C_{17}H_{16}F_6N_2O$).

For each of the six tablets tested, calculate the total amount of mefloquine ($C_{17}H_{16}F_6N_2O$) in the medium. The amount in solution for each tablet is not less than 80% of the amount declared on the label. If the amount obtained for one of the six tablets is less than 80%, repeat the test using a further six tablets; the average amount for all 12 tablets tested is not less than 75% and no tablet contains less than 60%.

Related substances

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using the conditions described under Assay.

Use solutions (1) and (4) as described under Assay. For solution (5) transfer 1 ml of solution (1) as prepared for the assay, to a 50-ml volumetric flask and make up to volume with the mobile phase. Dilute 2 ml of this solution to 20 ml with the mobile phase.

Inject 20 µl of solution (4). The test is not valid unless the resolution between the two principal peaks is at least 5.

Inject separately 20 µl each of solutions (1) and (5). Record the chromatograms for about 10 times the retention time of mefloquine.

In the chromatogram obtained with solution (1) the following impurities, if present, are eluted at the following relative retention with reference to mefloquine (retention time about 3.9 minutes): impurity A about 0.9, impurity C about 3.6 and impurity B about 7.4.

In the chromatogram obtained with solution (1) the area of any peak, other than the peak due to mefloquine, is not greater than the area of the principal peak in the chromatogram obtained with solution (5) (0.2%). The sum of the areas of all peaks, other than the peak due to mefloquine, is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with solution (5) (0.5%). Disregard any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with solution (5) (0.1%).

*[**Note from Secretariat.** The limit of 0.2% for individual related substances in this test is tighter than the limit of 0.5% in the current Ph.Int. monograph for mefloquine hydrochloride API, as determined by TLC. It is therefore intended to revise accordingly the Related substances test of the API monograph (list of impurities, limits).]*

Assay

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm).[1]

As the mobile phase, use a mixture of 22 volumes of methanol R, 38 volumes of acetonitrile R and 40 volumes of buffer pH 3.5 prepared as follows: dissolve 13.6 g potassium dihydrogen phosphate in about 900 ml of water R, adjust the pH to 3.5 by addition of 10% phosphoric acid and dilute to 1000 ml.

Prepare the following solutions in mobile phase. For solution (1), weigh and powder 20 tablets. Transfer a quantity of the powder containing the equivalent of about 200 mg of mefloquine, accurately weighed, into a 100-ml volumetric flask. Add 70 ml of mobile phase and sonicate for about 10 minutes. Allow to cool to room temperature and make up to volume with mobile phase. Filter a portion of this solution through a 0.45-µm filter, discarding the first few ml of the filtrate. For solution (2) dilute 5 ml of solution (1) to 50 ml with mobile phase. For solution (3), use 0.22 mg of mefloquine hydrochloride RS per ml. For solution (4) use about 0.22 mg of mefloquine hydrochloride RS and about 0.04 mg of sulfadoxine R per ml.

Operate with a flow rate of 1.5 ml per minute. As a detector, use an ultraviolet spectrophotometer set at a wavelength of about 283 nm.

Inject 20 µl of solution (4). The assay is not valid unless the resolution between the two principal peaks is at least 5.

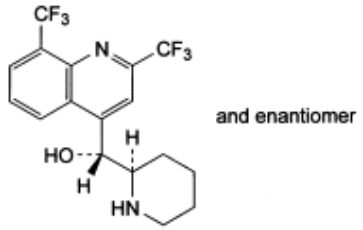
Inject separately 20 µl each of solutions (2) and (3).

Measure the areas of the peaks responses obtained in the chromatograms from solutions (2) and (3) and calculate the content of mefloquine ($C_{17}H_{16}F_6N_2O$) in the tablets, using the declared content of mefloquine hydrochloride ($C_{17}H_{16}F_6N_2O,HCl$) in mefloquine hydrochloride RS. Each mg of mefloquine hydrochloride ($C_{17}H_{16}F_6N_2O,HCl$) is equivalent to 0.912 mg of mefloquine ($C_{17}H_{16}F_6N_2O$).

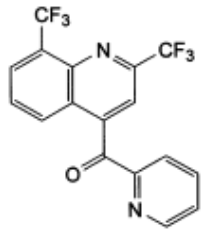
[1] Luna® was found suitable.

Impurities

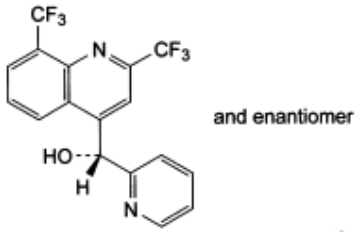
The following list of known and potential impurities that have been shown to be controlled by the tests in this monograph is given for information.



A.(*RS*)-[2,8-bis(trifluoromethyl)quinolin-4-yl][(2*RS*)-piperidin-2-yl]methanol *(threo*-mefloquine)



B.(*RS*)-[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl]methanone



C.(*RS*)-[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl]methanol

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New reagents to be added to Ph.Int.

Hydrochloric acid (~4 g/l) TS

Dilute 10 ml of hydrochloric acid (~420 g/l) TS with sufficient water to produce 1000 ml (approximately 0.1 mol/l).

Sulfadoxine R. N^1-(5,6-Dimethoxy-4-pyrimidinyl)sulfanilamide; 4-amino-*N*-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide; $C_{12}H_{14}N_4O_4S$

A commercialy available reagent of suitable grade.

Description. A white or creamy white, crystalline powder.

Solubility. Very slightly soluble in water; slightly soluble in ethanol (~750 g/l) TS and in methanol R; practically insoluble in ether R.

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