KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

BIOAVAILABILITY ASSESSMENT OF SOME LOCALLY MANUFACTURED CHLOROQUINE PHOSPHATE TABLETS

PRESENTED BY

SAMUEL KWAKU PEPRAH BAIDOO

Msc. PHARMACEUTICAL ANALYSIS AND QUALITY CONTROL

DECEMBER, 2005

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THESIS

Presented by

SAMUEL KWAKU PEPRAH BAIDOO

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

(Pharmaceutical Analysis and Quality Control)

of the

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

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KUMASI, GHANA

DECEMBER, 2005

DEDICATION

This work is dedicated to God almighty, my parents and my lovely wife Kina Baidoo

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor Doctor N.N.A. Okine, for his enormous contribution in the form of advice, suggestions, encouragement and for the keen interest he showed in this project.

I am also grateful to the other senior lecturers of the department of pharmaceutical chemistry and for their contribution toward the successful completion of the work. Special mention goes to Doctor R.K Adosraku and Professor J.S.K. Ayim, Former Vice-Chancellor of Kwame Nkrumah University of Science and Technology for their regular interventions and useful suggestions which were greatly instrumental in directing the course of the work to a successful end.

To all the technicians at the department of pharmaceutical chemistry especially Mister Dankwa and Mister Ben Voure I say thank you.

My sincere thanks go to my colleagues, Azigi, Ralphilito, Kingsley, David, and Gyimah for their concern, encouragement and great sacrifice in the course of the work. God richly bless you all. To Mister Samuel Nkansah, I say thank you for your wonderful contribution. To all my friends, Sobek, FM, Marfo, Paa Yaw, Kofi, you all deserve special thanks for your help, love and concern.

To my wife, Kina Baidoo, Miss Jeaniette Abrokwah, and Maame Jane I do not even know how to express this, but, with the love and selfless sacrifice shown me in everything to see me this far only God Almighty can bless you. I am really grateful.

My final and greatest appreciation goes to the Almighty God for the strength in me.

ABSTRACT

Chloroquine used to be the first choice antimalarial drug for the treatment of malaria but has now been replaced by artesunate-amodiaquine combination due to about 30 percent treatment failures experienced in Ghana. While drug resistance can cause treatment failure, not all treatment failure is due to drug resistance. Many factors can contribute to treatment failure including; incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor or erratic absorption and misdiagnosis. Thus, to investigate whether treatment failure was due to poor drug quality and poor absorption three brands of chloroquine phosphate tablets with poor dissolution profiles were selected and *in vivo* bioavailability studies of the three brands and a reference brand were conducted in six healthy subjects in a crossover method and the amount of unchanged chloroquine phosphate in the urine voided by these subjects over a 24-hour period determined and quantified by RP-LC.

Development of new, more reliable or more sensitive method of analysis is a concern of utmost important in the field of drug analysis. In this regard a simple, specific, a sensitive and rapid method has been developed and validated for the determination of chloroquine phosphate in human urine. The assay consisted of RP-LC with UV detection at a wavelength of 333nm at ambient temperature with a mobile phase combination of methanol (99.8%): 0.1M Na H_2PO_4 buffer (pH 3): 2.5%$^{v}/_{v}$ perchloric acid (24: 75.75: 0.25). Amodiaquine hydrochloride was used as the internal standard. Total chromatographic time was approximately 11.5 minutes. The method was found to be cost- effective because the amount of solvent used per elution time was less. The limit of quantification was 0.001%$^{w}/_{v}$. The method was used to analyze some brands of

chloroquine phosphate tablets and validated using the standard titrimetric and UV methods for the analysis of chloroquine phosphate.

The products were designated as TX, TY and TZ respectively from Letap pharmaceuticals, Phyto-Riker pharmaceuticals, Dannex Ltd and product R from Astra Zeneca UK Ltd as the reference. All the products used for the work except product TZ passed the specifications described in the BP 2002.

Pharmacokinetic parameters such as the average cumulative amount of unchanged drug excreted, the time for peak urinary excretion, and the mean peak urinary excretion rate were assessed to characterize bioequivalence.

In the analysis of chloroquine phosphate in the urine of six healthy subjects an average of 8.74, 8.10, 10.38 and 10.65 percent of the administered dose for test products TX, TZ, TY and reference product R respectively were excreted in 24 hours. Only products TX and TY were bioequivalent with the reference product R with relative bioavailabilities of 82.11 and 97.52 percent respectively, that of TZ failed with relative bioavailability of 76.11 percent.

Statistical analysis also showed that there was a significant difference between the means of the time for peak excretion rate obtained for the test product TZ and the reference product R. However, there was no significant difference between the standard deviations and the means of all the pharmacokinetic parameters investigated for test products TX and TY with reference product R.

DECLARATION

I, Samuel Kwaku Peprah Baidoo, wish to declare that this thesis is my own unaided research work. It is being submitted in partial fulfilment of the requirements for the award of Master of Science Degree in Pharmaceutical Analysis and Quality Control at the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Ghana.

I further declare that this work has not been submitted anywhere else for another degree, and that the thesis is presented with the consent of my supervisor. Works by other authors, which served as sources of information, have been duly acknowledged by reference to the authors.

Signature of candidate

Signature of supervisor

Date of submission

25/02/07

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CHAPTER ONE

1.0 LITERATURE REVIEW

1.1 INTRODUCTION

Malaria has long been recognised as a major contributor to morbidity and mortality among African children and pregnant women. It threatens the entire population, undermining the health and welfare of families and endangering the survival of their children [1]. The vast majority (90%) of malaria deaths occur in Africa, south of the Sahara, where malaria also presents major obstacles to social and economic development [2]. Malaria has been estimated to cost Africa more than US$ 12 billion every year in lost GDP, even though it could be controlled for a fraction of that sum [2]. There are at least 300 million acute cases of malaria each year globally, resulting in more than a million deaths [2]. Around 90 percent of these deaths occur in Africa, mostly in young children. Malaria is Africa's leading cause of under-five mortality (20%), in areas of Africa with stable *Plasmodium falciparum* infection during pregnancy is estimated to cause as many as 10,000 maternal deaths each year, 8 to 14 percent of all low birth weight babies, and 3 to 8 percent of all infant deaths, and constitutes 10 percent of the continent's overall disease burden. It accounts for 40 percent of public health expenditure, 30 to 50 percent of in-patient admissions, and up to 50 percent of out-patient visits in areas with high malaria transmission [2].

There are several reasons why Africa bears an overwhelming proportion of the malaria burden. In sub-Saharan Africa, *Plasmodium falciparum* is the cause of most malaria infections, and this type of malaria is also the most severe and life-threatening

form of the disease. Sub-Saharan Africa is also home to the very efficient and therefore deadly female *Anopheles* mosquito, which transmits the parasite. Moreover, many countries in Africa lack the infrastructure and resources necessary to mount sustainable campaigns against malaria and as a result few have benefited from historical efforts to eradicate malaria [2]. In Ghana, malaria is endemic throughout the country and is recognised as a leading public health problem. The incidence of clinical malaria is about 3 attacks per child per year and about 7700 people get malaria everyday according to the latest statistics released by the Ghana Health Service [3]. Most of the cases are caused by *Plasmodium falciparum.* It is estimated that malaria contributes about 44 percent of all out-patient cases and about 25 percent of all deaths in children under-five in Ghana. Malaria has accounted for about 36 percent of patients admitted to hospitals for the past ten years [3]. Malaria infection during pregnancy causes maternal anaemia and placental parasitemia both of which pose substantial risks to the mother, the foetus and the new born. In pregnant women, 13.8 percent are affected and 10.6 percent go on admission with the disease. As much as 9.4 percent of pregnant women die from malaria in Ghana [3]. However, several African nations have been developing malaria control strategies to decrease malaria morbidity and mortality. These strategies include the presumptive treatment of all children with fever and selective chemoprophylaxis through a primary healthcare network. The objectives of the strategy were to prevent people from dying from malaria and to reduce suffering, social and economic cost by considering malaria control as part of sustainable national development [4]. However, the malaria mortality rate still increases each year in spite of the great effort to eradicate the condition. Malaria is one of the ten most prevalent and deadly diseases in the world [5].

Strains of *P. falciparum* resistant to chloroquine were first suspected in Thailand in 1957 and found in patients in Colombia and Thailand in 1960 [6]. Since then, resistance to this drug has spread widely and there is now high-level resistance to chloroquine in South Asia, South-East Asia, Oceania, the Amazon Basin and some coastal areas of South America. In Africa, chloroquine resistance was first documented in the United Republic of Tanzania in 1979 and has spread and intensified in the last 20 years. In most countries of East Africa and in Ethiopia more than 50 percent of patients currently experience a recurrence of parasitaemia with symptoms by day 14 after treatment. Moderate levels of resistance are found in Central and Southern Africa [6]. In West Africa, reported rates of resistance vary widely but tend to be lower than in Central and Southern Africa. Strains of *P. falciparum* remain sensitive to chloroquine in Central America north of the Panama Canal, the island of Hispaniola (Haiti and the Dominican Republic) and in El Faiyûm governorate in Egypt [6]. The increase in chloroquine resistance in East Africa has led to a rise in malaria mortality [7]. Similarly, a significant rise in malaria mortality in children under 5 years of age has been observed in Senegal in West Africa, coinciding with the emergence of chloroquine resistance in the area [8]. The incidence of severe malaria has risen with increasing chloroquine resistance in Malawi and Democratic Republic of the Congo [9]. Antimalarial drug resistance has also been implicated in the increasing frequency and severity of epidemics [10]. It is therefore important that a distinction is made between a failure to clear malarial parasitaemia or resolve clinical disease following treatment with an antimalarial drug and true antimalarial drug resistance. While drug resistance can cause treatment failure, not all

treatment failure is due to drug resistance. Many factors can contribute to treatment failure including

1. Incorrect dosing,

2. Non-compliance with duration of dosing regimen

3. Poor drug quality

4. Drug interactions

5. Poor or erratic absorption, and

6. Misdiagnosis.

Probably all of these factors, while causing treatment failure (or apparent treatment failure) in the individual, may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to suboptimal drug levels.

Antimalarial agents are drugs used for the treatment or prophylaxis of malaria. Chloroquine is one of a large series of 4-aminoquinoline compounds investigated in connection with the extensive cooperation program of antimalarial research in the United States during World War II. Although the 4-aminoquinoline derivatives had previously been described as potential antimalarials by a Russian investigator, serious attention was not paid to this chemical class until French scientists reported that 3-methyl-7-chloro-4-(4-diethylamino-1-methylbutyl-amino) quinoline was well tolerated and had high activity in human malaria [11]. Beginning in 1943, thousands of these compounds were synthesized and tested for activity in avian malaria and for toxicity in mammals; ten of the series were then examined in human volunteers with experimentally induced malaria. Of these, chloroquine proved most promising and was released for a field trial. At the end

of the Second World War, it was discovered that the chemical had been synthesized and studied under the name RESOCHINE by German scientists as early as 1934 [11]. By 1946, chloroquine had become the drug of choice for the treatment of malaria the world over [12].

Chloroquine has for several years been, and is probably still the commonest and most effective of a large number of 4-aminoquinolines investigated for antimalarial activity. It has been the most widely used drug in prophylaxis and the treatment of malaria [11, 13 14]. Under normal circumstances, therefore, there should be a high patient compliance rate of chloroquine in the treatment of malaria because of its high efficacy, safety, availability and affordability. This is however not always the case, and incomplete treatment and/ or the use of sub-standard drugs can lead to dangerous complications. Dangers associated with substandard products are widely reported and these include adverse clinical results, low efficacy, increased drug resistance, risk of mortality and loss of man hours.

Reports on bad quality drugs in international commerce are widespread. Taylor *et al* reporting on the pharmacopoeial quality of drugs in Nigerian pharmacies pointed out that between 36.5 and 48 percent of supplied drugs were actually substandard. Percentage failure in ingredient content for chloroquine phosphate tablets analyzed was found to be 94 percent [15]. A random sampling of 150 products was done by Adosraku *et al* to check compliance with the British Pharmacopoeia standards. Results obtained indicated that on the average 18.95 percent of drugs purchased in the Kumasi metropolis were substandard [16]. In another study carried out by WHO in seven selected African Countries including Ghana, on the quality of antimalarial drugs and published in 2003,

several significant problems of substandard products within the distribution chains were identified [17]. They included percentage failures in ingredient content ranging from 20 percent to 67 percent for chloroquine tablets and dissolution failures ranging from 5 percent to 29 percent for chloroquine tablets [17]. Ghana had the highest chloroquine ingredient content failure of 67 percent. The dissolution failure rates for chloroquine were generally below 10 percent except for Kenya and Ghana with 28.6 percent and 20 percent respectively [17].

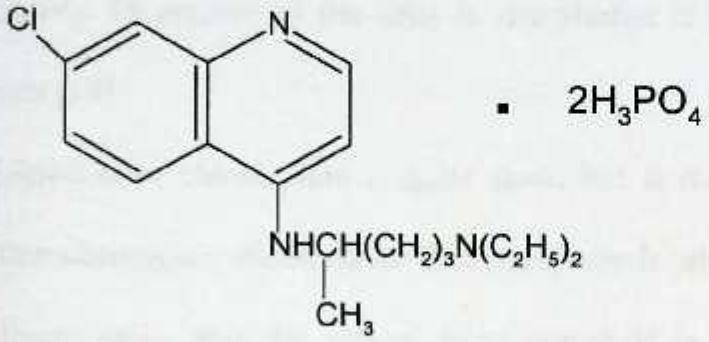
The proliferation of many brands of chloroquine on the Ghanaian market as a result of import/ trade liberalization could have resulted in the many substandard ones on the market. Chloroquine like any other drug may have the required percentage content of active ingredient when assayed, will meet pharmacopoeial standards. However, the bioavailability of the product may be poor, and variable clinical response to the same dosage form of drug products supplied by two or more drug manufacturers is well recognised [18].

It thus becomes very necessary after performing all physico-chemical compendial evaluation of a drug to further safeguard drug product quality through bioavailability assessment to ensure that the goal of every drug therapy, which depends on the rate and extent of absorption (bioavailability), is achieved. Bioavailability assessment of drugs such as chloroquine in an individual is a very important aspect of determining the therapeutic effective response in every patient.

1.2 CHLOROQUINE

1.2.1 CHEMISTRY

Chloroquine phosphate has the structural formula



Chemical Name: N^4-(7-chloro-4-quinolyl)-N^1, N^1-diethyl-1, 4-pentanediaminephosphate (1:2).

Fig 1.1: Structure of chloroquine phosphate

Properties

Chloroquine phosphate occurs as an odourless, white or almost white, bitter powder. It is freely soluble in water, very slightly soluble in alcohol, and practically insoluble in chloroform and ether. It has a plasma half-life of about 70 to 120 hours and is about 50 to 70 percent bound to protein. The plasma whole blood ratio is about 0.3 and the therapeutic plasma concentration is usually in the range of 0.02 to 0.2µg/ml [19].

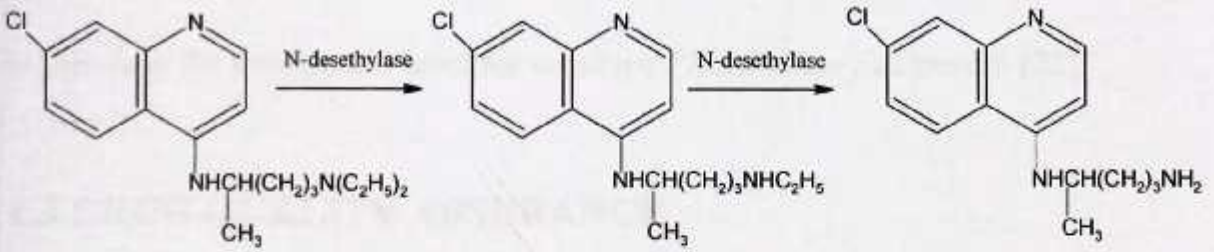
Uses

Until recently chloroquine was the drug of choice for prophylaxis and treatment of acute attack of malaria. In combination with primaquine phosphate, it is used for prophylaxis of all susceptible strains of malaria. It is useful for the treatment of hepatic amoebic infection normally combined with emetine or dehydroemetine [19].

1.2.2 ABSORPTION, METABOLISM AND EXCRETION

Chloroquine is rapidly and almost completely absorbed from the gastrointestinal tract (GIT), and only a small proportion of the administered dose is found in the stool. Approximately 55 percent of the drug in the plasma is bound to nondiffusible plasma constituents [19].

Excretion of chloroquine is quite slow, but is increased by acidification of the urine. After absorption, chloroquine is extensively localized in the tissues such as the kidney, liver, lungs and the spleen from which it is slowly released and partially biotransformed in the liver giving dealkyl metabolites [19]. Studies show that the drug accumulates in the tissues, the spleen, heart, lungs, kidney and the liver were 300 to 500 times higher than those found in the plasma [11]. It is excreted very slowly; 10 percent in the faeces and 55 percent in the urine, 60 to 70 percent as unchanged drug, 23 to 37 percent as secondary amine and about 3 percent as primary amine [20].



Chloroquine Monodesethylchloroquine Bisdesethylchloroquine

Fig 1.2: Metabolism of chloroquine to its metabolites by N-desethylase

Some of the metabolites may also be active antimalarials for example the major metabolite, monodesethylchloroquine, has antimalarial activity and reaches plasma concentrations that approximate 20 to 35 percent of those of the parent compound [11].

The urine acidification increases renal excretion appreciably. Chloroquine and its metabolites may be present in the urine for months after therapy is discontinued [19]. Following a single oral dose of 300mg to eleven subjects, peak plasma concentrations of 0.056 to 0.10μg/ml (mean 0.08) of chloroquine and 0.01 to 0.02μg/ml of monodesethylchloroquine were attained in one to six hours [20]. In another study, daily oral dosage of 300mg of chloroquine base resulted in a plasma plateau of about 125μg/L.

The kidney is the main route of elimination of chloroquine from the body and the drug is detectable in the urine one hundred and twenty days after a dose of 300mg. The maximum excretion of chloroquine occurred in the first twenty four hours and about 10 percent of the administered dose was excreted in that interval [21].

In another study, 10mg of chloroquine per kg body weight was administered in the form of tablets to children with malaria. The peak plasma concentration (about 250μg/L) was reached in two hours. The concentration reached in the plasma within the first thirty minutes after administration of an oral dose of 10mg/kg was substantially higher than the therapeutic level for sensitive *Plasmodium falciparum* [22].

1.3 DRUG QUALITY ASSURANCE

1.3.1 CONCEPT OF DRUG QUALITY AND QUALITY ASSURANCE

The concept of drug quality has evolved in the course of time. From simple checks of the final product, such as tests for appearance, colour, odour, identity, hardness, average weight or volume per unit, it has expanded into complex quality assurance systems, which span through the whole manufacturing process.

The World Health Organization (WHO) is a specialized agency of the United Nations (UN) with primary responsibility for international health matters and public

health. Since its inception in 1948, the WHO has promoted the exchange of scientific data on the subject of quality assurance in the production of drugs and medicinal products. Also, several regional or supranational organisations have been actively engaged in providing solutions to problems of achieving quality assurance of drug products. The WHO, through its Expert Committees on Specifications for Pharmaceutical Preparations, has published procedures for the organization of national control agencies for quality control, inspection and laboratory services and the requirements for general practices in the manufacture and quality control of drugs [23, 24].

1.3.2 REQUIREMENTS OF A TOTAL QUALITY ASSURANCE SYSTEM

A quality assurance system (QAS) is defined as the creation and operation of standards, procedures and management systems, which guarantee the quality of drugs. It is the system by which management can guarantee to meet their obligations to the customer and the community. QAS is thus a system for monitoring the entire process from the acquisition of a pharmaceutical raw material to a finished product for the customer. It involves a series of complex operations with checks, tests and inspections carried out at all levels and includes quality control procedures which must be built into the product during production and distribution [23]. Quality assurance therefore combines the inputs of Good Manufacturing Practice (GMP), Quality Control (QC) and Good Distribution Practice (GDP).

Variations in the quality of drug products are caused by the under listed factors which control drug product quality [23, 25]:

Men (Personnel)

Machines (Equipment and Facilities)

Materials (Active pharmaceutical ingredients, excipients, reagents, etc.)

Method (Procedure)

Milieu (Environment, Premises)

1.3.3 DRUG QUALITY ASSURANCE IN DEVELOPING COUNTRIES

The control of the quality of pharmaceutical preparations presents difficulties in many countries, especially with the increasing number of pharmaceutical substances and specialities available both internationally and locally. These difficulties are manifested more in the developing countries, where the quality assurance facilities are grossly inadequate. The most effective way of controlling the quality of imported drugs is to test representative samples in an official laboratory in the importing country. Such a national laboratory requires well-trained, experienced staff as well as adequate infrastructural facilities, analytical reagents, chemical reference standards and test organisms [23, 24].

1.3.4 WHO AND ASSURANCE OF DRUG QUALITY

Through its 'Expert Committees', the WHO has produced 'Technical Reports' on the quality control of pharmaceutical preparations in which recommended practices for the manufacture and quality control of drugs are stated. These reports stress the need for:

- National Drug Policies and National Drug Control Authorities as agencies for quality control
- Inspection and laboratory services
- Good Manufacturing Practices (GMP)
- Enforcement and coordination of regulations at national and international levels

With regard to drug quality assurance problems in many developing countries, the WHO has over the years adopted a number of measures and strategies to ensure the quality, safety, efficacy and acceptability of drugs and medicinal products moving in international commerce. The strategies include the following [24];

- Unification of quality requirements for drugs
- Setting up a code of GMP for pharmaceutical products
- Establishment of a basic test programme
- Training of quality assurance personnel.

1.4 BIOAVAILABILITY AND BIOEQUIVALENCE

1.4.1 THE CONCEPT OF BIOAVAILABILITY

Bioavailability is a pharmacokinetic term that describes the rate and extent to which the active drug ingredient is absorbed from a drug product and becomes available at the site of drug action. Since pharmacologic response is generally related to the concentration of drug at the receptor site, the availability of a drug from a dosage form is a critical element of a drug product's clinical efficacy. However, drug concentration usually cannot be readily measured directly at the site of action. Therefore, most bioavailability studies involve the determination of drug concentration in the blood or urine. This is based on the premise that the drug at the site of action is in equilibrium with the drug in the blood. It is therefore possible to obtain an indirect measure of drug response by monitoring drug levels in the blood or urine.

Thus, bioavailability is concerned with how quickly and how much of a drug appears in the blood after a specific dose is administered. The bioavailability of a drug

product often determines the therapeutic efficacy of that product since it affects the onset, intensity and duration of therapeutic response of the drug. Mathematically, bioavailability can be expressed by the following equation:

$$[AUC]_o^{\infty} = \frac{FD_o}{Cl_p} = \frac{FD_o}{kV_D} \quad \text{.....(1)}$$

Where F = fraction of dose absorbed

D_o = dose of drug administered

Cl_p = plasma clearance of the drug

k = elimination rate constant

V_D = volume of distribution

$[AUC]_o^{\infty}$ = Area under the plasma concentration time curve from t = 0 to t = ∞

AUC can be determined by numerical integration procedure, such as the trapezoidal rule method [26].

1.4.2. PURPOSE OF BIOAVAILABILITY STUDIES

Normally, all National Drug Regulatory Authorities are expected to carry out bioavailability studies on new drugs, new formulations of old drugs, as well as periodic studies on drugs already on the market to ensure that sub-standard drugs do not reach consumers. For example in the United States of America bioavailability studies are performed for both approved active drug ingredients and therapeutic moieties not yet approved for marketing by the Food and Drugs Administration (FDA). In approving a drug product for marketing, the FDA must ensure that the drug product is safe and effective for its labelled indication for use. Moreover; the drug product must meet all applicable standards of identity, strength, quality, and purity. To ensure that these standards are met the FDA requires bioavailability/pharmacokinetic studies and where

necessary bioequivalency requirements for all drug products. For unmarketed drugs, which do not have a full new drug application approved by the FDA, *in vivo* bioavailability studies must be performed on the drug formulation purposed for marketing. *In vivo* bioavailability studies are performed also for new formulations of active drug ingredients or therapeutic moieties that have full new drug application approval and are approved for marketing [26].

In Ghana *in vivo* bioavailability studies is not a requirement for all products. The Food and Drugs Board in Ghana requires bioavailability studies especially for new chemical entities, and also for products which have inadequate data on use among the black race and products just going off patents. However, *in vitro* dissolution test is presently required for all solid dosage forms in Ghana.

In summary, clinical studies are useful in determining the safety and efficacy of the drug product. Bioavailability studies are useful in defining the drug product in terms of its effect on the pharmacokinetics of the drug, whereas bioequivalence studies are useful in comparing the bioavailability of a drug from various drug products [18].

1.4.3 BIOEQUIVALENCE STUDIES

Bioequivalence of a drug product is achieved if its extent and rate of absorption are not statistically significantly different from those of the reference product when administered in the same molar dose. Thus, two medicinal products are said to be bioequivalents if they are pharmaceutical equivalents and if their bioavailabilities (rate and extent) after administration in the same molar dose are similar to such degree that their effects, with respect to both efficacy and safety, will be essentially the same [23].

In order to demonstrate that certain oral pharmaceutical products are therapeutically equivalent, an assessment of bioequivalence is the method of choice. There are *in vitro* tests and *in vivo* animal studies that can be used to estimate the bioequivalence of drug products. The dissolution rate test is the major *in vitro* test performed for drugs as an indicator of their bioavailability and, in certain cases, *in vitro* dissolution studies are sufficient to characterize formulation properties. It is in fact, an official compendial test for some drugs like Isoniazid tablets, oxytetracycline and tetracycline tablets and capsules, and tolbutamide tablets [27].

Bioequivalence studies may be required for a drug product in the following situations [23, 28]:

- When there is documented evidence of inequivalence between products with regard to their therapeutic effect or blood level concentration, for example tolbutamide or griseofulvin tablets.
- When the drug is used to treat a life-threatening or serious disease condition, and there is evidence that lack of bioequivalency would have a serious effect on the treatment of such a condition with the drug, for example warfarin, digitoxin or quinidine.
- When there is evidence that the drug product exhibits a steep dose-response curve and/or a narrow therapeutic index, or a low toxic effective plasma concentration ratio (safety margin) requiring careful dosage titration and patient monitoring, for example theophylline and digoxin tablets.
- When there is evidence of special formulation requirements due to certain physicochemical properties of the drug substance, for example suppositories.

- When reports from pharmacokinetic studies of the drug substance indicate poor absorption or instability in the gastrointestinal tract (for example erythromycin), first pass metabolism greater than 70 percent (for example paracetamol) or rapid metabolism or excretion (for example tolbutamide), or where the preparation requires rapid dissolution and absorption for effectiveness, or there is a high ratio of excipients to active ingredients.
- When a new drug product is to be introduced into the market.

1.4.4 CLINICAL SIGNIFICANCE OF BIOEQUIVALENCE STUDIES

Clinical interpretation is important in evaluating the results of a bioequivalence study. A small difference between drug products, even if statistically significant, may produce very little difference in therapeutic response. When the therapeutic objectives of the drug are considered, an equivalent clinical response should be obtained from the compared dosage forms if the plasma drug concentration remains above the minimum effective concentration for an appropriate interval and do not reach the minimum toxic concentration.

Reports by the US bioequivalence task force in 1988, considered that the differences of less than 20 percent in Area Under the Curve (AUC) and Maximum Plasma Concentration (C_{max}) between drug products are unlikely to be clinically significant in patients [29]. Normal variation is observed in medical practice and plasma drug levels may vary by more than 20 percent among individuals [29]. For the manufacture of a dosage form, specifications are set to provide uniformity of dosage forms and with proper specifications, quality control procedures should minimise product

to product variability by different manufacturers and lot to lot variability with a single manufacturer.

1.4.5 FACTORS INFLUENCING BIOAVAILABILITY

Before the therapeutic effect of an orally administered drug can be realized, the drug must be absorbed. The systemic absorption of an orally administered drug in a solid dosage form comprises three distinct steps:

1. Disintegration of the drug product [30-32].

2. Dissolution of the drug in the fluids at the absorption site [30, 33-34]

3. Transfer of drug molecule across the membrane lining of the gastrointestinal tract into the systemic circulation [26, 35-37]. Any factor that affects any of these three steps can alter the drug's bioavailability and thereby its therapeutic effects.

1.4.6 METHODS OF ASSESSING BIOAVAILABILITY

There are several direct and indirect methods of assessing bioavailability in humans. The selection of a method depends on the purpose of the study, analytical method of drug measurement, and nature of the drug product. The parameters that are useful in determining the bioavailability of a drug from a drug product include the following:

- Plasma Data [18, 26, 38, 39].
- Urine Data [18, 26, 38, 39].
- Acute Pharmacologic Effect [18, 26, 38, 39].
- Clinical Observation [18, 26, 38, 39].

Because the free or therapeutically active drug can be accurately quantified in biological fluids, plasma and urine data give the most objective information on bioavailability.

PLASMA DATA

The following are the parameters used in assessing bioavailability:

- The time of peak plasma (blood) concentration (t_{max})
- The peak plasma concentration (C_{max})
- The area under the plasma level-time curve (AUC)

The time of peak plasma (blood) concentration

The time of peak plasma concentration corresponds to the time required to reach maximum drug concentration after drug administration. At t_{max}, absorption is maximized, and the rate of drug absorption exactly equals the rate of drug elimination.

The peak plasma concentration

The peak plasma concentration represents the maximum plasma drug concentration obtained after oral administration of a drug. For many drugs a relationship is found between the pharmacologic effect of a drug and the plasma drug concentration. C_{max} provides an indication that the drug is sufficiently systemically absorbed to provide a therapeutic response. In addition, C_{max} provides warning of possible toxic levels of a drug.

The area under the plasma level-time curve

The area under the plasma level-time curve is a measurement of the extent of bioavailability of a drug. The AUC reflects the total amount of active drug that reaches the systemic circulation. The AUC is the area under the plasma level-time curve from $t = 0$ to $t = \infty$, and is equal to the amount of unchanged drug reaching the general circulation divided by the clearance.

URINE DATA

Urinary drug excretion data can be useful in estimating bioavailability. In order to obtain valid estimates, the drug must be excreted in significant quantities in the urine. The following are the parameters used in assessing bioavailability:

- The cumulative amount of drug excreted in the urine (D_u^{∞})
- The rate of drug excretion in the urine (dD_u/dt)
- The time for maximum urinary excretion (t_{max})

The cumulative amount of drug excreted in the urine

The cumulative amount of drug excreted in the urine is directly related to the total amount of drug absorbed. When the drug is essentially completely eliminated, the plasma concentration approaches zero and the maximum amount of drug excreted in the urine, D_u^{∞} is obtained. The determination of the cumulative amount of drug excreted in urine D_u^{∞}, coupled with the urinary excretion rate curves may afford a means of demonstrating the bioequivalence of drugs [23].

It can be shown that after a single dose of drug:

$D_u\infty = fFD$ ..... (2)

Where f = fraction of the drug reaching the circulation which is excreted in the urine

$D_u\infty$ = cumulative amount of unchanged drug excreted in the urine in infinite time after drug administration

F = fraction of the dose, D, absorbed

Assuming that f is constant and with the same dose, $F \alpha D_u\infty$,

$F_y/F_x = (D_u\infty)_y / (D_U\infty)_x$ = bioavailability of y relative to x.....(3)

The rate of drug excretion in the urine

Because a first-order rate process eliminates most drugs, the rate of drug excretion is dependent on the first-order elimination rate constant k and the concentration of drug in the plasma C_p. Hence, a graph comparing the rate of drug excretion with respect to time should be similar in shape to the plasma level-time curve for that drug [26].

Mathematically the relationship between the rate of drug excretion, elimination rate constant and concentration of drug in the plasma may be expressed by the following equation:

$C_p = C_o e^{-k_{el} t}$ ..... (4)

Where C_p = concentration of drug in the plasma at time t

C_o = concentration of drug in the plasma at time t = o

k_{el} = the elimination rate constant

The time for maximum urinary excretion

It is the total time required after drug administration for the drug to be absorbed and completely excreted through the kidneys. It is a useful parameter in bioequivalence studies comparing several drug products.

ACUTE PHARMACOLOGIC EFFECT

In some cases the quantitative measurement of a drug is not available or lacks sufficient accuracy and /or reproducibility. An acute pharmacologic effect such as effect on pupil diameter, heart rate, or blood pressure, can be useful as an index of drug bioavailability. The use of an acute pharmacologic effect to determine bioavailability may require demonstration of a dose-related response. Bioavailability may be determined by examination of the dose-response curve as well as the total area under the acute pharmacologic effect-time curve [26].

The onset, intensity and duration of the pharmacologic effect are dependent upon the administered dose and the pharmacokinetics of the drug. As the administered dose increases, the concentration of the drug at the receptor sites increases, and the pharmacologic response (effect) increases up to a maximum effect [26]. A plot of log dose versus response curve shoes a linear relationship at a dose range between 20 and 80 percent of the maximum response which typically includes the therapeutic dose range for many drugs. For a drug that follows a one-compartment pharmacokinetics, the volume of distribution is constant, therefore the pharmacologic response is also proportional to the log plasma drug concentration within a therapeutic range [26]. Mathematically the relationship may be expressed by the following equation:

$$E = m \log C + e \quad \ldots\ldots (5)$$

Where 'm' is the slope and 'e' is the extrapolated intercept

E is the pharmacologic response and

C is the plasma concentration

CLINICAL RESPONSE

For many years, medical practitioners have observed a lack of response (therapeutic failure), good therapeutic response, or toxicity in patients receiving similar drug products. Differences in the predicted clinical response may be due to differences in both the pharmacokinetic and pharmacodynamic behaviour of the drug among individuals. Bioequivalent drug products should have the same systemic drug bioavailability and therefore the same predictable drug response. However, variable clinical responses among individuals that are unrelated to bioavailability might be due to differences in the pharmacodynamics of the drug. Differences in pharmacodynamics, which is the relationship between the drug and the receptor site, may be due to differences in receptor sensitivity to the drug. Various factors affecting pharmacodynamic drug behaviour may include age, drug tolerance, drug interactions and unknown pathophysiologic factors [26].

1.5 DESIGN AND CONDUCT OF BIOAVAILABILITY AND BIOEQUIVALENCE STUDIES

The design of a bioavailability and/or bioequivalence study is dependent upon the drug, dosage form and study objectives [26]. It should be based on a reasonable knowledge of the pharmacodynamics and/or the pharmacokinetics of the active substance in question. The basic guiding principle in performing such studies is that no other human studies should be carried out simultaneously [26, 40].

1.5.1 DESIGN

The study should be designed in such a way that the formulation effect can be distinguished from other effects. If the number of formulations to be compared is two, a two-period, two-sequence crossover design is often considered to be the design of choice. However, under certain circumstances, provided the study design and the statistical analysis are scientifically sound, alternative well-established designs could be considered such as parallel design for very long half-life substances or replicate designs for substances with highly variable disposition. In general, single dose studies will suffice, but there are situations in which steady-state studies may be required, for example in the case of dose-or time-dependent pharmacokinetics, some modified release products.

The sampling schedule should be planned to provide an adequate estimation of C_{max} to provide a reliable estimate of the extent of absorption. This is generally achieved if the AUC derived from measurements is at least 80 percent of the AUC extrapolated to infinity. If a reliable estimate of terminal half-life is necessary it should be obtained by collecting at least three to four samples during the terminal log linear phase [40].

1.5.1.1 Selection of Subjects

The subject population for bioequivalence studies should be selected with the aim of minimizing variability and permit detection of differences between pharmaceutical products. Therefore, the studies should normally be performed with healthy volunteers. If the investigated active substance is known to have adverse effects and the pharmacological effects or risks are considered unacceptable for healthy volunteers it may be necessary to use patients instead, under suitable precautions and supervision [40].

The inclusion/exclusion criteria should be clearly stated in the protocol. Subjects could belong to the same sex. However, the risk to women of childbearing potential should be considered on an individual basis.

In general, subjects should be between 18-55 years old and of weight within the normal range according to accepted normal values for the Body Mass Index. They should be screened for suitability by means of clinical laboratory tests, an extensive review of medical history, and a comprehensive medical examination. Depending on the drug's therapeutic class and safety profile special medical investigations may have to be carried out before, during and after the completion of the study. Subjects should preferably be non-smokers and without a history of alcohol or drug abuse [40]. Bioavailability studies should be performed with enough subjects to characterize adequately the performance of the drug product under study. The number of subjects has ranged from 6-36 [41]. Generally the minimum number of subjects should not be less than 12 unless justified. Subsequent treatment should be separated by adequate wash out periods.

1.5.1.2 Standardization of the study

The test conditions should be standardized in order to minimize the variability of all factors involved except that of the products being tested. Therefore, standardization of the diet, fluid intake and exercise is recommended. Subjects should preferably be fasting at least during the night prior to administration of the products. The time of day for the ingestion should be specified and as fluid intake may profoundly influence gastric passage for oral administration forms, the volume of fluid should be constant. All meals and fluids taken after the treatment should also be standardized in regard to composition and time of administration during the sampling period.

The subjects should not take other medicines during a suitable period before and during the study and should abstain from food and drinks, which may interact with circulatory, gastrointestinal, liver or renal function (for example alcoholic or xanthine-containing beverages or certain fruit juices).

As the bioavailability of an active moiety from a dosage form could be dependent upon gastrointestinal transit times and regional blood flows, posture and physical activity may need to be standardized [40].

1.5.1.3 Sample Collection

Blood and urine samples are the most commonly used in bioequivalence studies.

Blood

Plasma or serum samples are usually used. This is because these samples contain fewer interfering substances than whole blood. This is why the vast majority of therapeutic concentrations of drugs reported in the literature are given for either plasma or serum.

Urine

It is easily obtained in reasonably large quantities. It generally contains detectable concentrations of drugs that are given in therapeutic doses. The disadvantage is that it is limited to drugs in which significant amounts of the administered doses are excreted unchanged or in which urinary excretion is the major route of elimination.

1.5.1.4 Sample Handling

Containers

Disposable containers should be used wherever possible to reduce the possibility of contamination. Liquid samples e.g. blood and urine are best placed in glass containers which are sealed with a liner that is impervious to the sample. Liners made of rubber and similar materials should be avoided since they may absorb drugs or contribute contaminants for example plasticizers to the sample. Glass may need to be silanised when low concentrations of drugs are present to avoid adsorption on the walls. The container size should be chosen such that the container is full, so that loss of volatile components or oxidation of the drug by atmospheric oxygen is reduced to a minimum.

Storage

Medicinal compounds can decompose during storage and may not be detected when the sample is analysed. The rate of decomposition may however, differ depending on the medium of existence and /or the storage conditions. Factors that must be considered in order to reduce chemical decomposition in stored samples are the following;

Light affects the decomposition of some drugs for example ergot alkaloids and phenothiazines. Urine and blood samples of such drugs should be kept in amber bottles. Hydrolysis is another major problem during storage of samples. Esterase activity in blood is high. Many compounds other than local anaesthetics are also esters and can be hydrolysed during storage of the sample at room temperature. The use of a high concentration of NaI (at least 1%) is recommended to counteract esterase activity. The hydrolysis of esters may also be slowed by reducing the pH of the sample to below 4.

Temperature also affects the stability of most compounds. It is best to store samples that will be analysed within a few days at 4°C. Those that are to be stored longer should be stored at -20°C, but only thawed once before analysis. For very long-term storage, freeze-drying should be considered, especially for samples that are used for standard solutions or quality assurance standards [42].

1.5.1.5 Chemical Analysis

The bioanalytical part of bioequivalence trials should be conducted according to the applicable principles of Good Laboratory Practice. The bioanalytical methods used to determine the active moiety and/or its biotransformation product(s) in plasma, serum, blood or urine or any other suitable matrix must be well characterized, fully validated and documented to yield reliable results that can be satisfactorily interpreted. The main objective of method validation is to demonstrate the reliability of a particular method for the quantitative determination of an analyte(s) concentration in a specific biological matrix [39]. The characteristics of a bioanalytical method essential to ensure the acceptability of the performance and the reliability of analytical results are:

- Stability of the stock solutions and of the analyte(s) in the biological matrix under processing conditions and during the entire period of storage;
- Specificity;
- Accuracy;
- Precision;
- Limit of quantification and
- Response function

All drug samples obtained for the test and reference preparations should be stored under identical conditions and assayed by the same method [38].

1.5.2 RELATIVE AND ABSOLUTE BIOAVAILABILITY

1.5.2.1 Absolute Bioavailability

The absolute bioavailability of a drug from a dosage form is the fraction (or percentage) of the administered dose which is absorbed intact into the systemic circulation. Absolute bioavailability may be measured by comparing the total amount of intact drug that reaches the systemic circulation after administration of a known dose of the dosage form via an absorption site with a total amount of intact drug that reaches the systemic circulation after administration of an equivalent dose of the drug in the form of an intravenous injection [43].

Using the plasma data

$$\text{Absolute bioavailability} = \frac{(AUC)_{abs}/D_{abs}}{(AUC)_{IV}/D_{IV}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

Where $(AUC)_{abs}$ and $(AUC)_{IV}$ are the area under the plasma concentration time curve after oral and intravenous administration respectively, D_{abs} is the size of the single dose drug administered via the absorption site, D_{IV} is the size of the drug administered as an intravenous bolus injection.

Using the Urinary excretory data

$$\text{Absolute bioavailability} = \frac{[D_U]^{\alpha}_{abs} / D_{abS}}{[D_U]^{\alpha}_{IV} / D_{IV}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Where $[D_U]^{\alpha}_{abs}$ and $[D_U]^{\alpha}_{IV}$ are the cumulative amounts of drug excreted over a specified time after oral and intravenous administration respectively.

1.5.2.2 Relative Bioavailability

Relative (apparent) bioavailability is the availability of a drug product compared to a recognised standard. The availability of the drug in the formulation is compared to the availability of the drug in a standard dose formulation [26]. This is normally determined in the case of a drug, which cannot be administered in the form of an intravenous injection.

Using the plasma data

$$\text{Relative bioavailability} = \frac{[AUC]_{test} / D_{test}}{[AUC]_{std} / D_{std}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(8)$$

Using the Urinary excretion data

$$\text{Relative bioavailability} = [D_u]_{test} / [D_u]_{std} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(9)$$

Where D_{test} and D_{std} are the sizes of the single dose of the test and standard dosage form administered respectively via the oral route.

$[AUC]_{test}$ and $[AUC]_{std}$ are the areas under the plasma concentration time curve of the test and standard dosage form respectively.

The systemic availability of a drug in the form of a tablet after oral administration rarely exceeds that obtained from an intravenous administration. In almost all cases the performance of a solution or other formulations can be evaluated by comparing their systemic availability with that of intravenous administration. However, equivalent

availability does not imply complete availability. For example Wager et al , have shown that the availability of propoxyphene is the same after oral administration of commercially available capsules and an aqueous solution but the systemic availability of propoxyphene is less than 25 percent of the administered dose because of first pass metabolism [44].

Although relative bioavailability studies are useful for characterising a formulation, absolute bioavailability is necessary to characterise the systemic performance of a drug product [32].

1.5.3 CAUSES OF LOW BIOAVAILABILITY

Low bioavailability is most common with oral dosage forms which are poorly water soluble and slowly absorbed drugs. More factors can affect bioavailability when absorption is slow or incomplete than when it is rapid and complete, so slow or incomplete absorption leads to variable therapeutic responses. Moreover, insufficient time in the gastrointestinal tract is a common cause of low bioavailability. Ingested drug is exposed to the entire gastrointestinal tract for no more than 1 or 2 days and to the epithelial membrane, time at the absorption site may be insufficient. In such cases, bioavailability tends to be highly variable as well as low. Age, sex, genetic phenotype, stress, disease or previous gastrointestinal surgery can affect drug bioavailability.

Reactions that compete with absorption can reduce bioavailability and these include complex formation, hydrolysis by gastric acid or digestive enzymes, and conjugation in the gut wall, adsorption to other drugs and metabolism by luminal microflora [45].

1.5.4 APPLICATIONS OF BIOAVAILABILITY STUDIES

Bioavailability studies are useful in establishing generic or multisource equivalence. They also establish non-equivalence of two or more drug products or formulations. In addition, they are used in determining the effect of food on the absorption of active ingredients in drug products or formulations. They are also very useful as a tool in quality assurance of drug products, in dosage regimen establishment and in correlation studies [23].

1.5.5 LIMITATIONS OF BIOAVAILABILITY STUDIES

Bioavailability studies have a number of limitations which may constitute a challenge to the quality of results obtained from bioavailability studies of drug products [23]:

- There is often a wide subject variability as well as worker to worker variation in techniques and measurements.
- Blood level testing is expensive, requires sophisticated analytical equipment and personnel as well as large number of cooperative subjects for the test. Blood sampling is rather inconvenient to the subject. Also urinary excretion study is limited to drugs in which urinary excretion is the major route of elimination.
- Studies are conducted under conditions that have little relationship to the actual clinical use. In most cases, healthy volunteers are used instead of patients suffering from the relevant sickness.
- Drugs such as, topical drugs and hormonal replacements may be difficult to be assessed through this procedure

1.5.6 DISSOLUTION STUDIES

Dissolution is the process by which a drug becomes dissolved in a solvent. Dissolution rate is an important physical characteristic of a tablet, capsule or other solid oral dosage forms. It provides an estimate of the extent to which the drug substance is released from the dosage form into a dissolution medium under standardized conditions. The dissolution test is used by the manufacturer on marketed products and also during product development. It is also used for verification of batch to batch consistency of the manufacturing process. Following approval for marketing, it is also used to check the consistency of the release characteristics of the dosage form during storage. It may also provide a useful check on a number of characteristics of a dosage form, including the particle size distribution, state of hydration, crystal form and the mechanical properties of the dosage form itself. Dissolution test may often be used to document equivalence between two multi-source products.

Dissolution test is a more predictive indicator of absorption *in vitro* than disintegration test, especially for drugs that are not problem pharmaceutical products for which it can serve as the sole documentation of equivalence. It is therefore used to discriminate between similar drug products in the prediction of equivalent drug bioavailability. Dissolution requirements are thus regarded essentially as a quality control tool to establish the release characteristics of specific solid oral dosage forms. The formulation factors which affect the rate of disintegration, also affect dissolution rate. The BP and USP have provided the procedures for dissolution rate testing and limits have been set for various tablets and capsules as specified in their individual monographs.

Several methods are available for the study of dissolution rates, the most commonly adopted is the 'rotating' basket method (USP, BP) which is now being substituted by the paddle method (USP, BP) probably because the latter is easily calibrated, simple and robust, and because the disintegration process can be monitored by direct observation.

Manufacturers carry out these tests routinely especially on poorly soluble drugs and the results plotted as percentage drug dissolved against time (in minutes). Typical specifications for the amount of active ingredient dissolved, expressed as a percentage of the labelled content, are in the range of 70 to 80 percent dissolved within 45 minutes (USP) and 70 percent within 45 minutes (BP), unless otherwise specified in the monograph. The paddle method is preferred for tablets, while the basket method is preferred for capsules and dosage forms that tend to float or disintegrate slowly [23].

1.5.7 DISSOLUTION TESTING IN LIEU OF BIOAVAILABILITY STUDIES.

There has been much discussion in the industry as to whether *in vitro* dissolution tests may substitute for an *in vivo* bioavailability study. According to USP-XXII (1990), "there is no known medically significant bioinequivalence problem with drug substances where 75 percent of the drug substance is dissolved in water at 37°C in 45 minutes using an official apparatus at the usual speed" [46].

For certain drugs, there is a strong correlation between dissolution of the drug and the bioavailability of the drug. If these drugs have no known bioavailability problems, are well absorbed systemically, are well correlated with *in vitro* dissolution, and have a large margin of safety, then arguments for not performing an *in vivo* bioequivalence study

might be valid. However, for oral solid dosage forms, an *in vivo* bioequivalence study may be required to support at least one dose strength of the product.

There are several ways of checking for *in vitro-in vivo* correlation. These include the following [26]:

- Dissolution Rate Versus Absorption Rate
- Percent of Drug Dissolved Versus Percent of Drug Absorbed
- Maximum Plasma Concentration Versus Percent of Drug Dissolved In Vitro
- Serum Drug Concentration versus Percent of Drug Dissolved.

a) Dissolution Rate versus Rate of Absorption

If dissolution of the drug is rate limiting, a faster dissolution rate may result in a faster rate of appearance of the drug in the plasma. It may be possible to establish a correlation between rates of dissolution and rate of absorption of the drug. The absorption rate is usually more difficult to determine than absorption time. Therefore, the absorption time may be used in correlating dissolution data to absorption data. In the analysis of the *in vitro-in vivo* drug correlation, rapid drug absorption may be distinguished from the slower drug absorption by observation of the absorption time for the preparation. The absorption time refers to the time for a constant amount of drug to be absorbed [26].

b) Percent of Drug Dissolved versus Percent of Drug Absorbed

If a drug is absorbed completely after dissolution, a linear correlation may be obtained by comparing the percent of the drug absorbed to the percent of drug dissolved. In choosing the dissolution method, one must consider the appropriate dissolution

medium and use a slow dissolution-stirring rate so that *in vivo* dissolution is approximated [26].

c) Maximum Plasma Concentration versus Percent of Drug Dissolved *In Vitro*

When different formulations are tested for dissolution, a poorly formulated drug will not be completely dissolved and released, resulting in lower plasma drug concentrations. The percent of drug released at any time interval will be greater for the more available drug product. When such drug products are tested *in vivo*, the peak drug serum concentration will be higher for the drug product that shows the highest percent of drug dissolved.

An example of *in vitro-in vivo* correlation for 199mg phenytoin sodium capsules has been reported. Several products were tested and a linear correlation was observed between the maximum drug concentration in the body and the percent of drug dissolved *in vitro*. It is important that any new dissolution method be carefully researched before being adopted as a method for predicting drug absorption [26].

d) Serum Drug Concentration versus Percent of Drug Dissolved

In a study on aspirin absorption, the serum concentration of aspirin was correlated to the percent of drug dissolved using an *in vitro* dissolution method. The dissolution medium was simulated gastric juice. Because aspirin is rapidly absorbed from the stomach, the dissolution of the drug is the rate limiting step, and various formulations with different dissolution rates would cause differences in the serum concentration of aspirin by minutes [26].

1.6 EXPERIMENTAL METHODS OF ANALYSIS

1.6.1 TITRIMETRY

Titrimetric assay methods are still widely used in pharmaceutical analysis because of their robustness, cheapness, and capability for high precision. In every titrimetric assay, an analyte is chemically reacted with a standard solution of a reagent of precisely known concentration or with a concentration that can be precisely determined. The amount of a standard solution required to completely react with all the sample is used to estimate the content of the sample. Colour indicators or instrumental methods are used to locate the end point of the reaction.

The accuracy and reproducibility of a titrimetric method depends on accurate weighing and solution making, the tolerance of the burette, pipette and volumetric glassware used and the ability to locate the endpoint of a reaction accurately.

NON-AQUEOUS TITRATION

Non-aqueous titration is the most common titrimetric procedure used in pharmacopoeial assays and serves a double purpose, as it is suitable for the titration of very weak acids and bases and provides a solvent in which organic compounds are soluble. The most commonly used procedure is the titration of organic bases with perchloric acid in acetic acid. Weak bases posses strong conjugate acids which hydrolyse immediately in aqueous solution. As a result titration in aqueous media does not provide reliable results. However, in certain solvents for example acetic acid the acidity or basicity of the compound being determined is enhanced.

Three main classes of compounds which may be determined by non-aqueous titration are [47, 48]:

- Weak Basic substances e.g.chloroquine, pyrimethamine
- Weak Acidic substances e.g. phenobarbitone
- Organic salts e.g. ephedrine hydrochloride

Colour indicators or instrumental methods are used to locate the end point of the reaction.

Potentiometry

Potentiometry refers to the measurement of the electromotive force (emf) of an electrochemical cell. The cell consist of an indicator electrode, with a potential of e_{ind}, which is a function of the activity (concentration) of the ion being determined and the experimental conditions, plus a reference electrode with a potential of e_{ref}, which is a known constant, and independent of the chemical composition of the solution into which the two electrodes are immersed.

The measurement of the emf of the cell is carried out with special potential-measuring instruments, known as electrometers, which draw a current of negligible (practically zero) intensity, so that the composition of the measured solution remains essentially unchanged. Special types of electrometers are pH meters, which are used for the measurement of pH, and p Ion meters which are used more generally for the measurement of activity of various ions. The emf of a cell is given by:

$$E_{cell} = E_{ind} - E_{ref} + Ej \quad \ldots\ldots (10)$$

Where Ej is the liquid–junction potential due to the different rates of mobility of ions across a liquid junction between a salt bridge and the test solution, which is kept constant as much as possible during measurements, so that it can be included in the constant term E_{ref} [49].

Electrodes

Electrodes used in potentiometry may be classified as indicator and reference electrodes. Indicator electrodes are responsive to changes in the solution whose property is being measured while the reference electrodes make up the other half-cell. Examples of indicator electrodes include glass electrode, quinhydrone electrodes and antimony electrodes. An example of reference electrode is the saturated calomel electrode. However, depending upon the type of titration, an indicator electrode for one titration could be used as a reference electrode in another titration [23].

Potentiometric determination of pH

The measurement of pH probably is the most common of all chemical measurements and is usually carried out potentiometrically with a pH meter. The measurement can be performed with a variety of electrodes, but usually the glass electrode/ calomel pair is used. Since it is impossible to construct two glass electrodes that give exactly the same pH reading when immersed in the same solution, and since the potential of a glass electrode does not remain constant for prolonged periods of time, the pH meter should be calibrated with a standard buffer prior to the pH measurement.

Potentiometric titration

Potentiometric titrations have an advantage over titrations with the use of indicator because the end point is detected electrically rather than visually. The components needed are a potentiometer, reference electrode, and an indicator electrode. In cases where the change in emf or pH at the end point is not sharp, it is more difficult to determine it precisely. In such a case, the endpoint may be determined by plotting a first

derivative curve. The calculations and plot of such a curve are facilitated by using small, constant volumes of titrant during the titration particularly near and soon after the end point [23].

1.6.2 ULTRAVIOLET-VISIBLE ABSORPTION SPECTROPHOTOMETRY

Analytical absorption spectroscopy in the ultraviolet and visible regions of the electromagnetic spectrum has been widely used in pharmaceutical and biomedical analysis for quantitative purposes. Ultraviolet–visible absorption spectrophotometry involves the measurement of ultraviolet (190-380nm) or visible (380-800nm) radiation absorbed by a substance in solution. The two empirical laws made by Lambert and Beer govern the phenomenon of absorption of light by molecules. A combination of the two laws is known as the Beer-Lambert law. It defines the absorbance of a solution of a substance as being related to the path length of the solution through which the light passes '*b*' and to its concentration '*c*'.

Mathematically, the law is represented as shown below:

$A = abc$ ..... (11)

Where, A = Absorbance of an absorbing substance

b = path length

c = concentration of solution

a = absorptivity

The name and value of '*a*' depend on the units of concentration and the path length. When 'c' is in moles per litre '*b*' is in cm, the constant is called molar absorptivity (formerly the molar extinction coefficient) and has the symbol ε.

Equation (9) then becomes:

$A = \varepsilon bc$ ..... (12)

Another form of the Beer-Lambert proportionality constant is the specific absorbance, which is the absorbance of a specified concentration in a cell of a specified path length. The most common form in pharmaceutical analysis is the A (1%, 1cm), which is the absorbance of a 1g/100ml ($1\%^{w}/_{v}$) solution in a 1cm cell. The Beer-Lambert equation therefore takes the form;

A = A (1%, 1cm) *bc* where '*c*' is in ($\%^{w}/_{v}$) and '*b*' is in cm

Occasionally, the concentration of liquids in solutions is given as ($\%^{v}/_{v}$) (e.g. in the British Pharmacopoeial assay of methyl salicylate and diethyl phthalate in surgical spirit) in which case the specific absorbance is the absorbance of a 1ml/100ml solution in a 1cm cell. A mathematical relationship between ε and A (1%, 1cm) is shown below:

ε = (A (1%, 1cm) x molecule weight)/10 ..... (13)

a) Causes of Deviation from the Beer-Lambert Law

True adherence to Beer's Law is observed only with truly monochromatic radiation. The use of radiation that is restricted to a single wavelength is seldom practical. Deviation from the Beer-Lambert law may be due to physical, chemical, or instrumental variations. Instrumental errors may be caused by slit-width effects, by stray light or by polychromatic radiation. Apparent failure of the law may be due to change in concentration resulting from solute-solute or solute-solvent interactions due to hydrogen bonding leading to association, dissolution or ionisation of the molecules [23].

b) Spectrophotometric Applications in Pharmaceutical Analysis

The majority of applications in which Spectrophotometric measurements are made rely on the compliance of the absorbing substance in solution with the Beer-Lambert law at the wavelength of measurement. It is used for both qualitative and quantitative analysis.

Identification

Most organic molecules that contain a chromophore give rise to a characteristic electronic spectrum. In addition to other physical and chemical data, this provides a method for identifying structural components in such molecules. The characteristic λ_{max} values and molar absorptivities are as well used in both qualitative (especially in the detection of impurities) and structural applications. This is because this information can sometimes differentiate between two chromophores that absorb at the same wavelength [23].

Quantitative Analysis

Absorption spectroscopy is one of the most useful and widely used techniques for quantitative analysis. It is used not only for finished pharmaceutical products (such as granules) but also for raw materials and intermediate products. Most drugs in current use contain chromophoric systems, which make them suitable for absorption spectrophotometric analysis [23].

i) ***Analysis of raw materials, intermediate and finished products***

The assay of an absorbing (or UV-active) substance may be carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength. The wavelength normally selected is a wavelength of maximum absorption (λmax) where small errors in setting the wavelength scale have little effect on the measured absorbance.

Ideally, the concentration should be adjusted to give an absorbance of approximately 0.9 around which the accuracy and precision of the measurement are optimal. The preferred method is to read the absorbance from the instrument display under non-scanning conditions, i.e. with the monochromator set at the analytical wavelength. Alternatively, the absorbance may be read from a recording of the spectrum obtained by using a recording double-beam spectrometer. The latter procedure is particularly useful for qualitative purposes and in certain assays in which absorbancies at more that one wavelength are required. The concentration of the absorbing substance is then calculated from the measured absorbance using one of the following principal procedures [23]:

Use of a standard absorptivity value:

This procedure is adopted by official compendia (e.g. British Pharmacopoeia) for stable substances such as Methyl testosterone that have reasonably broad absorption bands and which are practically unaffected by variations of instrumental parameters such as slit width and scan speed. The use of standard A (1%, 1cm) or ε *values* avoids the need to prepare a standard solution of the reference substance in order to determine its

absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

Use of a calibration graph:

In this procedure, the absorbancies of a number (typically 4-6) of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph constructed. Such calibration curves are drawn based upon the Beer-Lambert law that absorption is proportional to concentration. A plot of absorbance against concentration theoretically gives a straight line passing through the origin. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution.

ii) Analysis based on formation of (coloured) derivatives

Some drug molecules may absorb UV-vis radiations at specific wavelengths where irrelevant absorptions due to the impurities also occur. In such circumstances, it is better to prepare a derivative, which absorbs at a higher wavelength different from that of the parent compound, and compare the absorptivity with that prepared from a reference standard.

The BP and other pharmacopoeias make use of such derivatives in the analysis of drugs like steroids (use of triphenyltetrazonium chloride or 2, 4-dinitrophenylhydrazine), alkaloids (use of 4-dimethylamino benzaldehyde for ergotamine/ ergometrine alkaloids or sodium nitrite for reserpine-type alkaloids), and penicillins (use of imidazole–mercury reagent or sorbitol reagent).

1.6.3 CHROMATOGRAPHY

Chromatography is a technique used in the separation and analysis of the components of a mixture by a continuous distribution of the components between two phases one of which, the mobile phase is moving past the other, the stationary phase [20]. The two phases are chosen such that the components would have a differential preference. Components of a mixture are carried through the stationary phase by the flow of the mobile phase and the separation is based on the differences in migration rate which may be due to difference in adsorption, partition, solubility, vapour pressure, molecular size or ionic charge density [50].

1.6.3.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) has retained favour as an analytical method primarily because of its simplicity, reliability, low cost and selectivity of detection through the use of various location procedures [20]. It is one of the most widely used techniques for the separation and identification of drugs.

TLC is a method of chromatography in which a mobile phase moves by capillary action across a uniform thin layer of finely divided stationary phase (absorbent) bonded on to a plate. When a mixture of drugs is applied to the plate and developed with a mobile phase, the drugs move across the plate at different rates depending on their solubilities, pK_a values and capability of hydrogen bonding and so become separated. Although TLC is primarily a separation technique, under controlled conditions it can be used for identification and quantification.

The substances most frequently used as coating materials for TLC are silica gel, alumina and cellulose and to give stable layers, they often contain binders such as calcium sulphate and starch [23].

The choice of mobile phase for TLC, either single or mixture will depend on the compound to be separated and the stationary phase to be used. Solvent for TLC should be

- Cheap and easily obtainable in the pure form
- Stable in air or when mixed with acids or alkali
- Capable of being easily removed from the plate after chromatographic run
- Non- toxic and
- Non-reactive with the substances to be separated [20].

The technique of TLC involves a number of different stages, namely, preparing the plate, applying the sample, running the plate and locating the spots.

Preparing the Plate

Prepared TLC plates can be purchased but they may be prepared easily in the laboratory. For the silica gel G plate, a specified amount of the silica gel G is mixed with the appropriate amount of water and the mixture well stirred to remove all bubbles and the resultant mass is quickly applied in a layer of required thickness to the plates, which have been previously cleaned with acetone to remove grease. The plate is allowed to dry in air. Commercial slurry spreading equipment is available.

Application of Samples

The samples are spotted on a line drawn with a pencil parallel to and 2cm from the bottom of the plate. The line is called the origin. The sample is applied in as small a

volume as possible. The application may be done using commercial micropipette, or by a prepared capillary tube or by a calibrated syringe. When a large number of samples is to be applied the use of a template to position the spot accurately is helpful [20].

Running the Plate

The saturated developing chamber, which is normally used, consists of a glass tank, which normally has ground edges at the top to make an airtight seal with the glass lid when coated with soft paraffin. The tank is lined with filter paper on 3 sides and the mobile phase added. The tank is then allowed to equilibrate with the vapour for at least 30 minutes after which the TLC plate is placed in a vertical position in the tank so that the application line is above the level of the mobile phase. Other types of tanks are also available. Once the plate has been developed for the pre-determined distance, it is removed from the tank and the position of the solvent front quickly marked before any solvent evaporates. The solvent can then be allowed to evaporate naturally or removed with a hot air blower [20].

Location of spots [48]

The two most important techniques used to locate colourless drug substances on the thin layer chromatogram are:

- Spraying with a reagent that reacts with the substance to produce a coloured zone. Several reagents have been developed, which have varying degrees of selectivity for certain functional groups in the molecule. Dragendorff's reagent for the detection of alkaloids is among the large number of reagents commonly used.

- Examination under ultraviolet light of non-fluorescing plates and plates containing a fluorescent indicator. Any substance that absorbs at the wavelength of excitation (254nm or 365nm) quenches the fluorescence of the indicator and is observed as a dark zone on a yellow-green background. Non-fluorescing plates can also be used to distinguish between two substances that have the same R_f values but different colours in the UV at 254 or 366nm or both. If the two substances have different colours under UV, these will be the true UV colours of the substances without the aid of any fluorescent material. The sensitivity of detection is thus related to the absorptivity of the substance at the wavelength of irradiation.

R_f Values [20]

The basic chromatographic measurement of a substance is the R_f value defined as:

$R_f = (D_s/D_f)$.....(14)

Where:

D_s = distance the substance travels from the origin and

D_f = distance the solvent front travels from the origin.

R_f values vary from 0 to1. The distance travelled by the substance is measured from the centre of the spot which is easily determined if the spot is round, but with tailing spots it should be measured from the middle of the most dense area. R_f values can also be expressed in relative terms as percentages. For pharmaceutical analyses this is sometimes preferred as it enables one to distinguish more clearly between two substances with very close R_f values. For example 0.12 and 0.09, which will both be spots very close to the

point of origin and difficult to distinguish will be expressed as 09 and 12 percent respectively.

Identification of substances

The quality control specification of organic drug substances and their formulation frequently include TLC test to confirm the identity of the substance or to confirm the presence of the correct drug(s) in the formulation. Confirmation of the identity of the drug is based upon the coincidence of the R_f values of the sample spots with those of authentic reference standards in one or more solvent systems.

1.6.3.2 High Performance Liquid Chromatography (HPLC)

It is an analytical separation technique developed from a number of separation processes in which the resolving agent is packed into a narrow-diameter, long-length, high-resolution column and high inlet pressures (up to 42 kilopascals) to accelerate the separation process via the generation of more theoretical plates per unit length of column. The separation processes include ion exchange, partition (liquid-liquid) adsorption (liquid-solid), and size exclusion principles [23].

Advantages of HPLC over traditional low–pressure chromatography include the following:

- Greater sensitivity
- Ready adaptability to accurate quantitative determination
- Suitability for separating non–volatile species as well as thermally fragile ones

- Widespread applicability to substances such as amino acids, carbohydrates, proteins, drugs, pesticides, a variety of inorganic compounds that are of prime interest to industry, to many fields of science and to the public
- Reusable column for many analyses

a) Components of an HPLC system

The essential HPLC equipment consists of an eluent reservoir, a high-pressure pump, an injector for introducing the sample, stainless steel column containing the packing material, a detector and a chart recorder [46].

Mobile phase reservoir

These are made of glass or stainless steel equipped with a means of removing dissolved gases (O_2, N_2), and a means of filtering off dust and particulate matter from solvents. Millipore filters under vacuum (ultrafiltration) are normally used. Solvent must be ultra pure [23].

Pumps

These are used to force the mobile phase from the enclosed solvent reservoir to the column. The pumping system which contains corrosive resistant components must be able to provide pressure up to 42 kilopascals, pulse free output, flow rates ranging from 0.1 to 10ml/min, flow control and flow reproducibility of 0.5 percent relative or better [23]. Pumps are either 'constant–pressure' or 'constant–flow', the constant flow pumps being generally more convenient as changes in column resistance or eluent viscosity are compensated by changes in operating pressure [20].

Injection Systems

These are systems by which the test sample is introduced into the column of the HPLC. Injection ports are of two basic types:

- Those in which the sample is injected directly into the column and
- Those in which the sample is deposited before the column inlet and then swept by a valving action into the column by the mobile phase.

In the first method also called syringe injection, the injection of the sample is done by means of a syringe through a septum into the centre of the packing material. High pressure syringes that can be used at pressures up to 65.861×10^3 kilopascals allow the injection of the sample while the mobile phase is flowing. Alternatively if a low pressure syringe is used, the flow must be stopped during injection. On column injection methods are not as reproducible as the second method also called valve injection. The second and the more modern injection is based on injection valves, which allow the sample at atmospheric pressure to be transferred to the high pressure mobile phase immediately before the column inlet. With the injector in the load position, the sample is injected from a syringe through a needle port into a loop. The valve lever is then turned to the inject position and the sample is swept into the flowing mobile phase [50].

Columns

HPLC columns are made of high quality stainless steel (to cope with high pressure) containing the packing material, often at ambient temperatures but sometimes may need to be maintained at high temperatures [23]. Columns 10 to 30cm long with an internal diameter of 4.5 to 5mm and an outside diameter of 6.3mm are normally used

[20]. The choice of column material varies widely, depending on the type and nature of material to be separated. Common particle size of highly pressure-resistant micro particulate packing are 3, 5 and 10μm in diameter, and are composed of silica gel (most common), alumina or an ion-exchange resin, which provide a performance efficiency superior to that of gas chromatographic (GC) columns [23].

There are normal and bonded (reverse) phase columns. Both normal and bonded (reverse) phase columns can be used depending on the physical and chemical characteristics of the mixture to be separated. Bonded phase packing is classified as reverse-phase (most commonly used) when the bonded coating is non-polar in character and normal phase when the coating contains polar functional groups such as the cyano, diol, amino, diamino and dimethylamino groups. Bonded-phase columns are prepared from silica by reacting the surface silanol groups with an organochlorosilane or alloxysilane to give permanently bonded linkage, which is hydrolytically stable.

Octadecylsilane (ODS) bonded columns are most widely used. Most separations are carried out using either silica micro porous particle column (for non-polar compounds) or a reversed-phase C_{18} bonded phase column (for polar compounds), for which the mobile phase is relatively polar, for example water, methanol or acetonitrile [23].

Separations that employ a single solvent of constant composition are known as isocratic elution, while in gradient elution, two or more solvent systems that differ significantly in polarity are used. This enhances separation efficiency and shortens time of separation.

Detector

This device is fixed to the HPLC for the detection of the separated components in the eluate from the column. Four detectors have found widespread application. These are:

- Ultraviolet-visible detector
- The fluorescence detector
- The refractive index detector
- Electrochemical detector.

The UV-visible light absorption detector

This is based on UV-vis light absorption and is the most commonly used because of its high light sensitivity, reproducibility and ease of operation at fixed, multiple or variable wavelengths .It is usable with the gradient elution technique and can detect as low as 1ng of solute, with detection limit of 10^{-10}g/ml. The most powerful UV detectors are the diode array instruments, which provide continual monitoring of an entire spectrum.

Fluorescence detector

It is highly sensitive and selective for fluorescent compounds or derivatized fluorescent compounds. It has a detection limit of 10^{-11}g/ml.

Refractive index detector

This is the second most often used. It measures the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographic compound as it emerges from the column. It can be considered as a

universal detector as virtually all compounds cause a change in refractive index when dissolved in an eluent, but is the least sensitive; it is temperature-sensitive and cannot be used with gradient elution. It has a detector limit of 10^{-7}g/ml and it is especially valuable for compounds that do not show any UV absorption.

Electrochemical detectors

These include coulometric, amperometric, potentiometric, or polarographic detectors. They are very sensitive and selective for bioactive compounds which are electroactive and can be oxidised (for example aromatic amines, phenols, peroxides and mercaptans) or reduced (for example ketones, aldehydes, nitro and halogen containing compounds) at electrode surfaces. They are not gradient-elution compatible and have detection limit of 10^{-11}g/ml [23].

The recorder

The recorder for the HPLC is a device for obtaining a hard copy of the separating profile and it could be a simple chart recorder or an elaborate interface with a computer.

b) Systems Suitability Determinations in HPLC

Systems suitability tests are an integral part of liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done [51]. One of these tests is the calculation of theoretical plates for a column and there are two other main parameters for assessing performance; peak symmetry and the resolution between critical pairs of peak. The

reproducibility of peak retention times is also an important parameter for controlling performance [52].

Number of theoretical plates (N)

The number of theoretical plates expresses the efficiency of the column being used. The higher the number of theoretical plates the higher the efficiency in separating the components of the sample [52]. The number of theoretical plates (N) is expressed by the following equation:

$$N = \frac{5.54x^2}{LW^2_{1/2h}} \quad \text{..... (15)}$$

Where x: Retention time (minutes)

L: length of column in meters

$W_{1/2h}$: width at half height (in minutes)

N > 2000 [51].

Resolution

The more efficient a column the greater degree of resolution it will produce between closely eluting peaks. The resolution between two peaks A and B is expressed by the following equation [52]:

$$\text{Resolution } (R_s) = \frac{1.18\,(x_2 - x_1)}{W_1 + W_2} \quad \text{..... (16)}$$

$R_s > 2$ [51].

Peak asymmetry

Another situation which may lead to poor integrator performance is where peaks are trailing and thus have a high element of asymmetry. The expression used to assess this is;

Asymmetry factor (AF) = b/a ..... (17)

Where 'a' is the leading half of the peak measured at 10 percent of the peak height and 'b' is the trailing half of the peak measured at 10 percent of the peak height. This value should fall ideally, in the range 0.95 – 1.15 [52]. Poor symmetry may be caused through; loading too much sample onto the column, sample decomposition, the analyte adsorbing strongly onto active sites in the stationary phase, poor trapping of the analyte when it is loaded onto the column or too much 'dead volume' in the chromatographic system [52].

c) The Impact of pH on HPLC Method Development

HPLC method development requires optimising a wide variety of mobile phase and column parameters. The pH of the mobile phase is just one. In a reversed-phase HPLC separation of acids and bases, pH plays a crucial role in determining retention and selectivity, and in controlling the reproducibility and ruggedness of a method. The pH range often used for reversed-phase HPLC is 1 to 8 and can be divided into low pH (1 to 4) and intermediate pH (4 to 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimised. For this reason, operating at low pH is recommended. However, there may be some circumstances where operation at intermediate pH is beneficial. For example, intermediate pH may increase analyte retention and improve selectivity. In addition, if any analytes are not stable at low pH, an intermediate pH may be necessary.

To develop the most rugged and reproducible method, it is important to understand how mobile phase pH affects the analyte and the column. Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often as a result of different interactions between the column and the analytes when the ionization of these compounds changes [53].

d) Why Use a Buffered Mobile Phase?

When separating acids and bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, by definition, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. Buffers play an important role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanol. They also reduce potential ion-exchange interactions with unprotonated silanol. To be most effective, a buffer concentration range of 10 to 50mM is recommended [53].

e) Precision in HPLC

The pharmaceutical laboratory is totally dependent on a very high level of HPLC precision to meet the increasingly tighter assay specifications for drug substances. For most pharmaceutical assays under a GMP environment, peak area precision of <2.0% relative standard deviation (RSD) must be demonstrated before any sample can be analysed. This level of precision is readily achievable by modern autosamplers, which are specified typically at <0.5%, though routinely capable of precision of <0.2% RSD [54].

1.6.3.3 Calibration Methods

HPLC analysis of drugs is based on a comparison of either the height or the area of the analyte peak with that of one or more standards. This involves the use of internal or external standards.

Calibration by External Standards

An external standard is required when the work-up before injection onto the chromatograph does not involve an extraction process. In such a case, a series of concentrations of the drug substance is prepared (usually from a stock solution) and injected onto the chromatograph. A calibration graph of peak area or peak height is plotted against the concentration of the drug. The unknown concentration of the drug is run in the same system and from the value of the peak area or height; the concentration of the drug can be read from the calibration curve.

Calibration by Internal Standards

When an extraction process is involved, as in metabolic and pharmacokinetic studies, the addition of an internal standard to a sample and analytical standard solutions (solution of a pure sample of the compound to be analysed) during the extraction serves to ensure reproducibility and precision of the analytical method, as the uncertainties introduced by sample injection are avoided. The internal standard; which obviates the difficulty of introducing precisely measured quantities onto the HPLC, elutes at a position near the substance being analysed and is well resolved but cannot be converted to the analyte under the conditions of the analysis.

The choice of compounds to be used as internal standards is dictated by convenience, availability in high purity, solubility in a solvent in which the sample is readily soluble, stability (both on the shelf and in solution) and the similarity of physicochemical characteristics of the compound to the drug being analysed and hence the retention time. When the internal standard is used, the ratio of the peak area (or height) of the standard or analyte to that of the internal standard is used to prepare the calibration curve and determine the unknown concentration. This method compensates for variations in physical parameters, especially inaccuracy in pipetting and injecting micro litre volumes of samples [55].

Summary of Requirements for an Internal Standard

It must have a completely resolved peak; no interferences

It must elute close to compound(s) of interest (similar k^1 values)

It must behave equivalently to compounds of interest for analyses involving pre-treatment, derivatives formation, etc.

More than one internal standard may be required for multi-component mixtures to achieve highest precision.

It must be added at a concentration that will produce a peak-area or peak height ratio of about unity with compound(s) of interest.

It must not be present in the original sample

It must be stable; unreactive with sample components, column packing, or mobile phase

It is desirable for it to be commercially available in high purity [55].

1.7 STATISTICAL ANALYSIS

In the development of an analytical method, it is important that the method is compared statistically to an existing method. Also in order to obtain maximum information from analytical results, it is often necessary to apply statistical techniques to their interpretation. Thus a number of variables need to be obtained in order to make statistical comparison. These include the mean, standard deviation, median, mode etc. For most comparisons, however the use of the mean and standard deviation are employed.

Mean: This is the sum of all the measured properties of all the members of the sample divided by the total number (n) of all the members of the sample [56].

It is represented by

$$\bar{(x)} = \sum X_i / n \quad \text{..... (18)}$$

$$\mu = \sum X_i / N \quad \text{.....(19)}$$

Where μ is the population mean and $\bar{(x)}$ is the sample mean.

In the absence of systematic errors μ represents the true mean of the population.

The sample mean $\bar{(x)}$ gives an estimate of μ.

Often the sample mean $\bar{(x)}$ is used to replace the population mean (μ).

Standard deviation: Is one of the measures of spread of individual measurements about the mean. This can be said to be a measure of precision. Mathematically standard deviation (s) is given by

$$s = \frac{\sqrt{\sum (x - \bar{x})^2}}{(n-1)} \quad \text{.....(20)}$$

$$s_m = s / \sqrt{n} \quad \text{.....(21)}$$

Where s_m is the standard error (deviation) of the mean (SEM) and s is the standard deviation of the observations in the sample.

SEM gives an indication of the difference between the sample mean and the true population mean. It provides information about the precision and, thus, the random error associated with the mean.

An analytical method becomes accepted if it is known to be free from systematic error. This property can be verified by comparing the mean of the method to the mean obtained using a standard method. This form of testing is known as significance testing. Significance testing involves what is known as the null hypothesis. This is that there is no significant difference between the two means. This implies any differences would be due to random error.

Three statistical methods were used to check the accuracy and precision of the method used. The statistical methods used were the F-test for comparison of standard deviation and the t-test for the comparison of the mean of two sample means whereas the Analysis of Variance (ANOVA) is used for the testing of the differences between more than two means.

F-test for the comparison of standard deviation

Mathematically it is given by:

$F = S_1^2 / S_2^2$ ..... (22)

Where S_1^2 and S_2^2 are the variances of the samples

The variance is arranged such that $F \geq 1$

If F exceeds a critical value the null hypothesis is rejected.

t-test for the comparison of means of two sample means

Using the equation:

$t = \overline{x_1} - \overline{x_2} / S\sqrt{(1/n_1 + 1/n_2)}$ ..... (23)

Where $(\overline{x_1})$ and $(\overline{x_2})$ are the means of the sample analysed by the different methods. S is the pooled standard deviation. n_1 and n_2 are the sizes of the samples. The pooled standard deviation S is used if the two samples have standard deviations which are not significantly different. S can be calculated from the individual standard deviation S_1 and S_2.

S is the pooled standard deviation given by:

$$S^2 = \frac{\{(n_1-1)\, S_1^2 + (n_2-1)\, S_2^2\}}{n_1 + n_2 - 2} \quad \text{..... (24)}$$

If the calculated 't' from equation (23) is greater than a critical value at the significance level the null hypothesis is rejected, that is the two means are significantly different.

Analysis of Variance (*ANOVA) for the comparison of several means*

ANOVA can be used to separate and estimate the different causes of variation and it can be used to separate any variation which is caused by changing the controlled factor from the variation due to random error. It can therefore test whether altering the control factor leads to a significant difference between the mean values obtained. Thus considering the following samples;

sample 1	$x_{11}, x_{12}.....x_{1j}.....x_{1n}$	$\overline{x_1}$
sample 2	$x_{21}, x_{22}.....x_{2j}.....x_{2n}$	$\overline{x_2}$
sample i	x_{i1} x_{ij}	$\overline{x_i}$
sample h	x_{h1} x_{hj}.....	$\overline{x_h}$
	Overall	$\overline{x}$

The problem can be generalised to consider h samples each with n-members where xij is j's measurement of i's sample. The means of the samples are $\overline{x_1}, \overline{x_2}, \overline{xn}$ and the mean of all the values grouped together is $\overline{x}$. The null hypothesis adopted is that all the samples are drawn from a population with mean μ and variance σ_o^2. On the basis of this hypothesis the variance σ_o^2 can be estimated in two ways, one involving the variation within the samples and the other the variation between the samples.

Within sample estimate general formula:

$$\sigma_o^2 = \sum_i \sum_j (x_{ij}-x_i)^2 / h\,(n-1) \quad \ldots\ldots (25)$$

The summation over j and division by n-1 gives the variance of each sample. The summation over 'i' and division by 'h' averages these sample variances. The within sample formula is also known as the Mean square.

Formula for between sample estimates:

$$\sigma_o^2 = n\sum_i (\overline{x_i} - \overline{x})^2 / h\text{-}1 \quad \ldots\ldots (26)$$

This is also a mean square value.

If the null hypothesis is correct then the two estimates should not differ significantly. To test whether there is significant variation between the sample mean squares the F-test is used [56].

1.8 OBJECTIVES OF THE RESEARCH

The objectives of this research are to:

- Conduct a market survey on the number of brands of chloroquine phosphate tablets available in the Kumasi metropolis
- Perform dissolution/pharmacokinetic studies on all the available brands of chloroquine phosphate tablets in the Kumasi metropolis
- Select three brands of chloroquine phosphate tablets with poor dissolution profiles and conduct *in vivo* bioavailability studies of the three brands and a reference brand in six healthy subjects.
- Design an HPLC method for the direct determination of chloroquine phosphate in urine samples
- Determine the cumulative amount of chloroquine phosphate excreted periodically over 24 hours and the mean time of maximum urinary excretion
- Determine the relative bioavailability of chloroquine phosphate tablets from the three test brands
- To check the compliance of pure chloroquine phosphate powder, test chloroquine phosphate tablets and reference chloroquine phosphate tablets with standard pharmacopoeial requirements
- Compare the *in vitro* and *in vivo* studies
- Perform statistical analysis of the pharmacokinetic parameters or data.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Pure chloroquine phosphate powder

Distributor: Indukern Chemie AG

Wiesenstrasse 33, CH8952 Schlieren

Switzerland

Product code: 21996

Batch number: 0021

Date of Manufacture: Feb 2003

Date of Expiry: June 2008

Chloroquine phosphate tablets (Letaquine) –Test sample TX

Strength: 250mg

Batch Number: 0940084

Date of Manufacture: NIL

Date of Expiry: June 2007

Manufacturer: Letap Pharmaceuticals Ltd, Ghana

Chloroquine phosphate tablets –Test sample TY

Strength: 250mg

Batch Number: F08324

Date of Manufacture: NIL

Date of Expiry: July 2007

Manufacturer: Phyto-Riker Pharmaceuticals Ltd, Ghana

Chloroquine phosphate tablets (Malarex) –Test sample TZ

Strength: 250mg

Batch Number: 206402

Date of Manufacture: June 2002

Date of Expiry: June 2006

Manufacturer: Dannex Ltd

Chloroquine phosphate tablets (Avloclor) –Reference sample R

Strength: 250mg

Batch Number: BC 190

Date of Manufacture: NIL

Date of Expiry: June 2006

Manufacturer: AstraZeneca, UK Ltd

Shimadzu Libor Analytical balance AEG-220,

Kent EIL 7020 pH meter

ERWEKA GmbH Dissolution Apparatus

Electrothermal 9200 melting point apparatus

Fisher scientific isotemp programmable oven (800 series)

High performance liquid chromatography:

HPLC Pump 422 (Kontron Instrument),

Syringe loading sample injector fitted with an external 20µl loop (Model-8125, Rheodyne Inc.)

Zorbax HPLC column-Rx-C_{18} (250mm × 4.6mm)

Shimadzu CR501 Chromatopac Integrator

HPLC syringe (SGE 100µL syringe)

Gelman Acrodisc13 CR, PTFE 0.2µm

783A Programmable Absorbance Detector (Applied Biosystems)

Stuart Scientific Flask Shaker

Cecil CE 7200 UV Spectrophotometer (7000 series), Quartz cuvette

Whatman membrane filter 0.3µm, diameter 74mm

Chemicals

Hydrochloric Acid (BDH), Strong Ammonia Solution (BDH), Chloroform (FISONS), Sodium Hydroxide (BDH), Methanol (BDH), Ethanol (BDH),), Sodium Dihydrogen Orthophosphate (BDH),Orthophosphoric Acid (BDH), Glacial Acetic Acid (BDH), Caffeine (BDH), Acetanilide (BDH), Paracetamol (Weisheng Pharmaceuticals Company Limited, China), Chloramphenicol (Weisheng Pharmaceuticals Company Limited, China), Amodiaquine hydrochloride (Weisheng Pharmaceuticals Company Limited, China), Ether (BDH), Picric acid (BDH), Acetic Anhydride (BDH), Perchloric Acid (BDH), Dragendoff's reagent (BDH), Silica gel (BDH)

Table 2.1: Profile of subjects

Subjects	Sex	Age (years)	Weight (kg)	Height (m)
1	Male	29	70	1.68
2	Male	31	50	1.50
3	Male	20	64	1.76
4	Male	21	70	1.84
5	Male	29	57	1.65
6	Male	32	65	1.65

2.2 METHODOLOGY

2.2.1 COMPOUND IDENTIFICATION

2.2.1.1 UV Spectrophotometric Analysis

Pure Powder: 0.1005g of pure chloroquine phosphate powder was weighed and dissolved in 5ml of distilled water in a 100ml volumetric flask. It was then diluted serially with dilute hydrochloric acid (1 in 1000) to obtain a solution whose concentration was 10.05µg\ml. The absorbance of the solution was measured at 343nm and 329nm in a 1cm cell using dilute hydrochloric acid (1 in 1000) as the blank [46]. For results, refer to Table 3.1

Tablets: An amount of 1.3500g of powdered chloroquine phosphate tablet for test tablet TX equivalent to 1g of chloroquine phosphate was accurately weighed and dissolved in 5ml of distilled water. The solution was transferred into a 100ml volumetric flask and diluted serially with dilute hydrochloric acid (1 in 1000) to obtain a concentration of

10.0119µg\ml. The absorbance of the solution was measured at 343nm and 329nm in a 1cm cell using dilute hydrochloric acid (1 in 1000) as the blank. The same procedure was repeated for test tablets TY, TZ and the reference tablet R. [46]. For results, refer to Table 3.1.

2.2.1.2 Thin Layer Chromatography (TLC)

Pure powder: 0.1001g of the pure chloroquine powder was weighed and dissolved in 20ml of water. 10ml of the solution was pipetted and diluted to 100ml in a 100ml volumetric flask such that the concentration of the solution was 0.050%$^{w}/_{v}$ [20, 57].

Tablets: 1.3485g, 1.4626g and 1.6035g of the test powdered chloroquine phosphate tablets TX, TY, TZ and 1.3356g of the reference powdered chloroquine phosphate tablets R, each containing 1g of chloroquine phosphate were weighed and each dissolved in 20ml of water. The solutions were centrifuged for 30minutes at 400rpm and the supernatant liquid filtered through a glass fibre paper. 1ml each of the solutions were pipetted and diluted to the mark in a 100ml volumetric flask such that the concentration of the solutions were 0.050%$^{w}/_{v}$, 0.050%$^{w}/_{v}$, 0.050%$^{w}/_{v}$ for the test tablets TX, TY ,TZ and 0.050%$^{w}/_{v}$ for the reference tablet respectively [20, 57].

Development: The spots of the pure, reference and the test solutions, 2cm apart, were allowed to dry. The chromatank was filled with a mixture of methanol: strong ammonia solution (100:1.5) and allowed to equilibrate. The plate was placed in the tank and left till the solvent had travelled 11cm. It was then removed and dried. The dried plate was

viewed under U.V light, sprayed with dragendoff's reagent and then dried and the outline traced. For results, refer to Table 3.2

2.2.1.3 Melting Point Determination

Pure Powder: 0.0251g of the pure chloroquine phosphate powder was dissolved in 20ml of water and 8ml of picric acid solution added. It was then filtered and the precipitates washed with water, 96% cthanol and ether and then allowed to dry.

Tablet: 0.0337g, 0.0369g and 0.0402g of the test tablets TX, TY,TZ and 0.0334g of the reference powdered chloroquine phosphate tablets R each equivalent to 25mg of chloroquine phosphate were weighed and each dissolved in 20ml of water, filtered and 8ml of picric acid solution added to each of the filtrate. The melting points of the crystals formed were determined after washing consecutively with water, 96% of cthanol and ether [46, 57]. For results, refer to Table 3.3.

2.2.1.4 Test for Phosphates

Pure powder: 0.1g of pure chloroquine phosphate was dissolved in 10ml of distilled water. 2ml of dilute sodium hydroxide solution was then added and shaken with two quantities, each of 20ml, of chloroform. The aqueous layer was neutralised by the addition of nitric acid. To 5ml of the neutralised solution, 5ml of silver nitrate solution was added. The resulting precipitate was boiled and dissolved in 10M ammonia.

Tablets: A quantity of the powdered test tablets TX containing 0.5g of chloroquine phosphate was extracted with 25ml of water and filtered. To the filtrate was added 2.5ml

of 5M sodium hydroxide and extracted with three 10ml quantities of ether. The aqueous layer was neutralised with 2M nitric acid. To 5ml of the neutralised solution, 5ml of silver nitrate solution was added. The resulting precipitate was boiled and dissolved in 10M ammonia. The same procedure was repeated for test tablets TY, TZ and the reference tablet R [57]. For results, refer to Table 3.4.

2.2.2 WEIGHT UNIFORMITY TEST

Twenty chloroquine phosphate tablets of the test chloroquine phosphate sample TX were collectively weighed and the mean weight per tablet calculated. The tablets were then weighed individually and the percentage deviation calculated. The same procedure was followed for the other test samples TY, TZ and the reference sample respectively [48, 57]. For results, refer to Table 3.5.

2.2.3 DISSOLUTION TEST

U.V Calibration

0.04, 0.08, 0.1, 0.2 and 0.3 of 1mg/ml solution of the pure chloroquine phosphate was pipetted into a 10ml volumetric flask to prepare solutions whose concentrations were 4µg/ml, 8µg/ml, 10µg/ml, 20µg/ml and 30 µg/ml respectively. The absorbance of each solution was determined at a wavelength of 344nm using 0.1M hydrochloric acid as the blank. For results refer to Table 3.6

Tablets

900ml of the dissolution medium, free from dissolved air, was introduced into the vessel of the apparatus. The dissolution medium was then warmed to between 36.5 °C and 37.5

°C. A tablet each of test chloroquine phosphate TX was put into each of the six baskets containing 900ml of 0.1M Hydrochloric acid and then rotated at a speed of 100 revolutions per minute at a temperature of 37°C. Sampling times were 10, 30, 45, 60, 90 and 120 minutes. At the stated times when sampling was done, 10ml of samples were withdrawn from the dissolution vessel from a point half-way between the surface of the dissolution medium and the top of the rotating blade, not less than 10mm from the wall of the vessel, and filtered. 2.5ml of the filtered solution was pipetted into a 25ml volumetric flask and made up to volume with the dissolution medium. The diluted solution was then kept in sample tubes stored away from light. 10ml of dissolution medium equal to the volume of the samples withdrawn was then added to the dissolution vessel. The absorbance was measured at a wavelength of 344nm in a 1cm cell. The content of chloroquine phosphate was then calculated .The procedure was repeated for the other test tablets and the reference tablet. The complete operation was repeated five times [57]. For results, refer to Table 3.7

2.2.4 ASSAY

2.2.4.1 U V Spectrophotometric Method

Twenty chloroquine phosphate tablets of the reference and test samples were each weighed and powdered. 1.0788g of the test powdered chloroquine phosphate tablet TX equivalent to 800mg of chloroquine phosphate was weighed and 100ml of water added in a 200ml volumetric flask. The solution was shaken for 20 minutes on a mechanical shaker. Water was then added to the mark and the solution filtered. The first 50ml of the filtrate was discarded. 50ml of the clear solution was pipetted into a 250ml separating funnel and 5ml of 6M ammonium hydroxide added. The solution was agitated and

extracted with five 25ml portions of chloroform. The combined chloroform extract was washed with 10ml of water and the water extracted by washing with 10ml of chloroform. The combined chloroform extract was evaporated on a steam bath to about 10ml and 50ml of dilute hydrochloric acid (1 in 250) added. The solution was heated until the odour of chloroform was no longer perceptible. The solution was transferred into a 200ml volumetric flask and then made to the mark with dilute hydrochloric acid (1 in 1000). The solution was diluted serially with hydrochloric acid (1 in 1000) to obtain a concentration 10µg/ml. The same procedure was repeated for 1.1699g, 1.2826g and 1.0686g of the other test samples TY, TZ and the reference sample R respectively [46].

Pure Powder: 0.1000g of pure chloroquine phosphate powder was accurately weighed and dissolved in about 5ml of water and diluted quantitatively and stepwise with dilute hydrochloric acid (1 in 1000) to obtain a solution containing 10µg/ml.

The absorbance of the tablets and pure chloroquine phosphate powder were determined at the same time at a wavelength of 343nm using dilute hydrochloric acid (1 in 1000) as the blank [46].

The quantity of chloroquine in the tablets was calculated. For results, refer to Table 3.8

2.2.4.2 Titrimetric Method

Standardization of 0.1M perchloric acid

0.3500g of potassium hydrogen phthalate was weighed accurately into a 250ml conical flask. 50ml of anhydrous acetic acid was added and warmed until the salt dissolved. The resulting solution was allowed to cool and titrated with 0.1M perchloric acid using 2 drops of 0.5% $^{w}/_{v}$ acetous crystal violet as indicator .There was a colour change from blue

to blue-green at the end point. The procedure was repeated and a blank determination carried out to determine the volume of perchloric acid consumed by 50ml of the acetic acid. For results, refer to Appendix C Table 6.1 and 6.2

Pure chloroquine: 0.2004g of pure chloroquine powder was weighed and dissolved in 50ml of anhydrous acetic acid. The end point was determined potentiometrically with standardised 0.1M perchloric acid [57]. For results, refer to Table 3.9a

Tablets: Twenty tablets of chloroquine phosphate of the reference and test samples were each weighed, crushed, and powdered. 0.6742g, 0.7310g and 0.8015g of the powdered chloroquine phosphate tablets of the test samples TX, TY and TZ respectively, which are equivalent to 0.5g of chloroquine phosphate, were weighed accurately and each dissolved in 20ml of 1M NaOH solution in a separating funnel. The chloroquine phosphate was extracted using four 25ml quantities of chloroform. The chloroform extracts were combined and allowed to evaporate to a volume of about 10ml. 40ml of anhydrous acetic acid was added and stirred. The resultant solution was titrated against 0.1M perchloric acid. The end point was determined potentiometrically. This procedure was repeated for the reference tablet R [57]. For results, refer to Tables 3.9b-3.9e

2.2.5. DETERMINATION OF OPTIMUM CONDITIONS FOR HPLC ANALYSIS

Choice of stationary Phase

A non-polar stationary phase, Zorbax Rx-C_{18} (250mm x 4.6mm) was used because of the polar nature of the drug sample

Choice of mobile phase

Various Mobile phase combinations of methanol (99.8%): 0.1M sodium dihydrogen orthophosphate at varying pH (4.5-2.0): perchloric acid (2.5%$^{v}/_{v}$) were used to check for the retention time of pure chloroquine powder. The combination that gave the most satisfactory resolution and retention for pure chloroquine was methanol (99.8%): 0.1M sodium hydrogen orthophosphate (pH3): perchloric acid (2.5%$^{v}/_{v}$) in the ratio 24:75.75:0.25. Blank urine (urine without chloroquine) was also run to see if there would be any interference with the chloroquine peak. The chromatogram of the blank urine is shown in figure 3.7

Choice of internal standard

The combination, as mentioned above, with other predetermined conditions such as flow rate (1ml/min), Absorbance Unit Fraction Scale (AUFS) of 0.5, chart speed of 5mm/min at wavelength of detection of 333nm was applied to a number of compounds. These were paracetamol, caffeine, chloramphenicol, acetanilide and amodiaquine hydrochloride. 0.1%$^{w}/_{v}$ solution each of the compound was prepared and their relative resolution and retention times used to select the best compound as internal standard. Suitable concentration of the selected internal standard likely to give test sample, internal standard peak ratio close to unity was then investigated.

Selection of UV detection wavelength

Different wavelengths were tried with emphasis on the maximum wavelength of absorption of chloroquine phosphate and amodiaquine hydrochloride. Finally the detection wavelength of 333nm that ensured that the peak area ratio of chloroquine

phosphate to amodiaquine hydrochloride, the internal standard, was close to unity was selected. Using this wavelength, there was a significant detection of both solutes with no spurious peaks.

Selection of absorbance unit fraction scale (AUFS)

The AUFS was varied to obtain a quantitative detection of the drug at very low concentrations. A sensitivity setting of 0.5 was found appropriate.

Selection of chart recorder speed

This was selected so as to give resolved peaks of analytes with adequate separation on the chart. Speed chosen was 5mm/min.

Selection of flow rate

It was ensured that the value was not too high to introduce air bubbles in the mobile phase or the pump or any part of the equipment to affect the reproducibility of the results. After careful varying of values, 1.0ml/min gave significant solute retention times in the minimum amount of time and was thus used for the work.

2.2.6 HPLC CALIBRATION GRAPH

0.2ml of 1mg /ml solution of the internal standard was pipetted into seven different 10ml volumetric flask. 0.05, 0.07, 0.1, 0.15, 0.2, 0.25 and 0.3ml of 1mg/ml solution of the pure chloroquine phosphate was pipetted and added to each of 0.2ml of the internal standard in the volumetric flask to prepare solutions whose concentrations were 5µg/ml, 7µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml and 30µg/ml respectively. 20µl of each of the

solutions prepared were injected on the HPLC column. The peak area ratio was plotted against concentration. For results refer to Table 3.10b figure 3.5

2.2.7 VALIDATION OF ANALYTICAL METHOD

This was done by determining the within-run precision (repeatability) of the results of the method applied to a certain analyte concentration. This was done to provide a measure of the precision of the procedure under normal operations of laboratory conditions. The between-run precision (reproducibility) of the method was also considered. Moreover, comparison of the designed method with the standard methods was done with respect to the assay of the tablets.

2.2.7.1 Determination of within-run precision (repeatability) of analytical method.

A 0.002%$^{w}/_{v}$ solution of reference chloroquine phosphate was accurately prepared together with an internal standard such that the concentration of the internal standard in the mixture was 0.002%$^{w}/_{v}$. This solution was then filtered through the syringe filter to remove any particles before the analyte got onto the column. Samples from this homogenous batch were successively run seven times after stabilising the chromatographic system with the conditions determined. Each set was run three times and the average peak area ratio taken to represent the set. The integrator printed out the retention time for each peak whilst the peak areas were calculated. The various solutes were identified by their retention times. The peak area ratios were then used to calculate the actual concentration by interpolation from a calibration graph. These values were then tabulated and statistically analysed for the standard deviation of the method. For results refer to Table 3.11

2.2.7.2 Determination of between-run precision of analytical method.

Another set of solutions of pure chloroquine phosphate powder of similar concentrations was prepared on two different occasions and subjected to similar treatments as above. The results were then paired and statistically analysed for a potential significant difference in their standard deviations. For results refer to Table 3.12

2.2.7.3 Comparison of new method with standard non-aqueous titration method and UV method

The BP 2002 and the USP 1990 methods for the assay of chloroquine phosphate reference powder and the tablets were used as the standard tests.

The procedure is as described in the assay method.

Results obtained were tabulated and that of the new method compared with it statistically for significant or insignificant differences in their respective means and variances.

2.2.7.4 HPLC Method

20 tablets of one of the experimental test products TX were weighed and powdered. A quantity of the powder equivalent to 0.1g of chloroquine phosphate was accurately weighed and quantitatively transferred into a clean 100ml volumetric flask with 25ml of the mobile phase. It was then shaken for 20 minutes on a mechanical shaker and the solution made up to the 100ml mark. A 0.1%$^{w}/_{v}$ solution of amodiaquine hydrochloride was also prepared with the mobile phase to be used as the internal standard. Into a clean 10ml volumetric flask was pipetted 0.1 and 0.2ml respectively of the chloroquine phosphate solution and that of the internal standard and was diluted to 10ml with the mobile phase so that the final concentration of chloroquine phosphate in the mixture was

0.001% $^{w}/_{v}$ and that of the internal standard, 0.002% $^{w}/_{v}$. After filtering a portion of this solution through a syringe filter to further remove particulate matter, triplicate injections onto the column were successively done using the above-determined conditions for the elution. All chromatographic precautions were observed. The procedure was repeated for the reference chloroquine phosphate and the other test product under study. Average peak area ratios (Test sample/Internal standard) for the various samples were respectively calculated. From a calibration curve, the actual concentration of chloroquine phosphate in each of the samples was interpolated and with respect to the reference sample, the percentage content of chloroquine phosphate in each of the products was calculated. These were tabulated and statistically compared to the standard BP and USP method. For results refer to Table 3.13

2.2.8 BIOAVAILABILITY

a) Selection of subjects

Six healthy male volunteers participated in the study after being informed of the purpose, protocol and risks. They were randomly selected in such a way that, they were not interrelated in a way that may affect their pharmacokinetics. They were between 20 and 32 years of age and had body weights between 50 and 70kg. Subjects were non-smokers, not on any medication as at the time of the study, and had normal dietary habits. Subjects did not take alcohol or any caffeine-containing product for at least seven days prior to and throughout the entire study. In addition, no subject was taking any known enzyme inducing or inhibiting agent two weeks before and throughout the entire study. They gave

their consent after receiving verbal information on the study. (The signed document is available at 6.4Appendix D).

b) Selection of products

The products used in this study were the reference product Avloclor from Astra Zeneca designated as R which is an established product with documented evidence of quality, efficacy and safety. The other three products from Dannex, Phyto-Riker and Letap Pharmaceuticals designated as TZ, TY and TX respectively were selected due to their poor dissolution profiles.

c) *In vivo* study

The study was performed in a crossover fashion. The volunteers were divided into four groups. One group was given product R whiles the other three groups took product TX, TY and TZ respectively. After a twenty-one day washout period between treatments, the administration of the products was reversed so that at the end of the study, every subject in the panel had received each of the four products. Each subject fasted overnight for 10 hours prior to the experiment and food was withheld for 4 hours after dosing. An hour before the administration of a product, each subject was made to drink 400ml of water to ensure adequate hydration.

Four tablets (600mg chloroquine base) of chloroquine phosphate were given to each of the subjects with 200ml of water. Subsequently, 200ml of water was given to each of the subjects hourly until 1200ml had been consumed after four hours.

d) Urinary Excretion

Blank urine samples were obtained from each subject prior to drug administration. Urine samples were obtained by completely emptying the bladder. Quantitative urine collection was made during each of the following time points after drug administration 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20 and 24 hours. The entire volume of urine voided during a sampling time interval was collected and shaken to ensure a uniform specimen. The volume of each urine specimen sampled was measured and recorded. It was then poured into a sample container and stored at -20°C until the time of analysis

e) Analysis of Urine samples

A simple, specific, sensitive and rapid reverse-phase HPLC with UV detection method was designed and validated for the detection and quantification of free chloroquine phosphate in human urine. Dilution of urine samples was therefore necessary to ensure that concentration of analyte in urine sample fell within the linear regions of chloroquine. In most cases, a 5 in10 dilution with the mobile phase was made but in few cases, other dilutions were made. Whatever level of dilution employed, the volume of the internal standard stock solution added was such that its concentration in the final solution was 0.002%$^{w}/_{v}$. After filtering a portion of the final solution through an Acrodisc CR 13 PTFE 0.2µm membrane filter, 20µl of the filtrate was taken for chromatography. All samples were assayed thrice. A linear calibration curve was used to convert peak area ratio to concentration and the results recorded in Tables. For results refer to Tables 3.16, 6.8 - 6.30.

f) Rate of urinary drug excretion profile

Rate of urinary drug excretion was obtained by dividing the amount of drug excreted in a time interval by the change of time in that interval. The average of six subjects was taken and the value plotted against the midpoint of the collection time interval. The peak excretion rate and the time for peak excretion rate were obtained from these data. For results refer to Tables 3.17 – 3.22.

g) Cumulative Urinary Drug Excretion Profile

Cumulative Urinary Drug Excretion was plotted as a function of time to obtain the above-mentioned profile. To do this, urine samples were collected at various time periods after the administration of the drug. The amount of drug excreted in each sample after analysis was added to the amount of drug recovered in the previous urine sample. This was done for 24 hours for each subject and the average of six taken. All four chloroquine phosphate products were covered in the same manner. For results refer to Table 3.23

h) *In Vivo-In Vitro* Correlations

Correlations between time required for a given percentage of cumulative amount to be excreted and time required for the same percentage of *in vitro* dose to be dissolved was assessed. Percentage amounts used were 25, 50, 75, and 80. Excretion and dissolution times for the respective amount were generated from the equations of the respective cumulative amount excreted and dissolution curves. These equations were generated by default with Microsoft Excel. The time for drug to be excreted was plotted against time for drug to dissolve. The excretion and dissolution data were paired along product lines. Details can be found in Tables 3.24 and 3.25, figure 3.16.

i) Assessment of Bioequivalence

The pharmacokinetic variables used to characterise the bioequivalence of the products were the cumulative amount of drug excreted in 24 hours, peak urinary excretion rate and the time for peak excretion rate. These were chosen because they parallel respectively the AUC, C_{max} and t_{max} used for the same assessment with plasma data. Significant or insignificant differences in these pharmacokinetic parameters among products were assessed by means of the student's t-test for significance in means of products and some parameters by the F-test. For results refer to Tables 3.26 and 3.27.

CHAPTER THREE

3.0 RESULTS AND CALCULATIONS

3.1 COMPOUND IDENTIFICATION

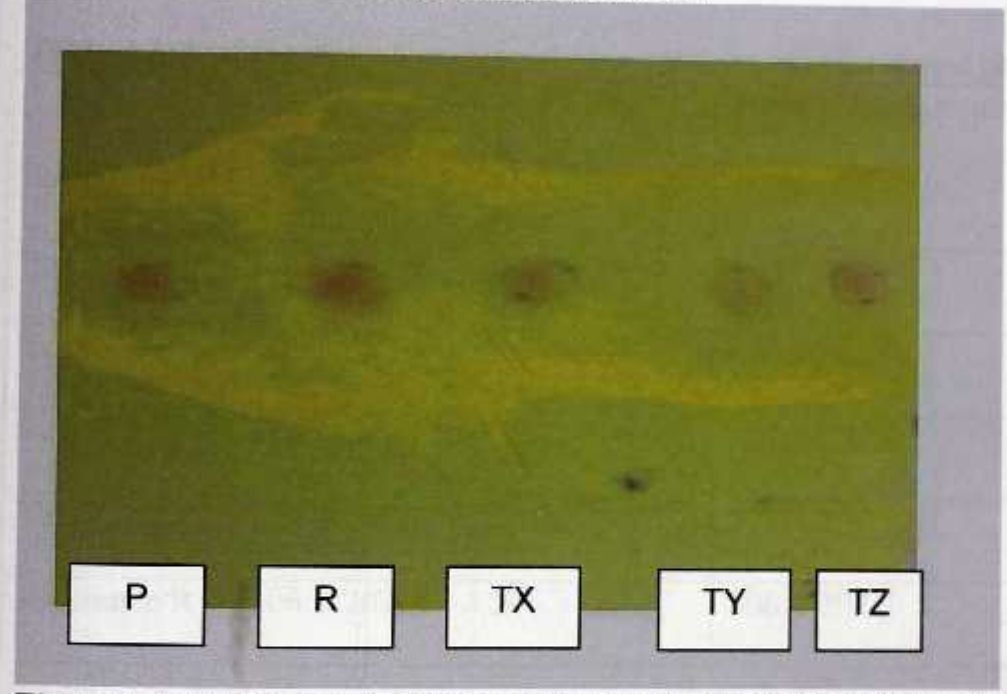
3.1.1 U.V SPECTROSCOPY

Table 3.1: UV Identification test of drug samples

Sample	Mean Absorbance		Ratio of A_{343}/A_{329}
	343nm	329nm	
Pure chloroquine powder	0.344	0.331	1.04
TX	0.344	0.329	1.05
TY	0.346	0.326	1.06
TZ	0.293	0.267	1.10
R	0.348	0.332	1.05

The ratio of the absorbance at 343nm and 329nm should be between 1.0 and 1.15 [46].

3.1.2 THIN LAYER CHROMATOGRAPHY



SOLVENT SYSTEM
Methanol: Strong ammonia
100 : 1.5

Figure 3.1: Thin layer chromatography of pure chloroquine (P) and products R, TX, TY and TZ

Table 3.2: R_f values of chloroquine phosphate

Sample	Distance moved (cm)	R_f values
Pure chloroquine phosphate powder(P)	5.3	0.482
Test tablet TX	5.4	0.491
Test tablet TY	5.3	0.482
Test tablet TZ	5.5	0.500
Reference tablet R	5.3	0.482

$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$

Distance moved by solvent front = 11cm

Distance moved by reference chloroquine phosphate tablet = 5.3cm

$R_f = \frac{5.3}{11} = 0.482$

Other R_f values were calculated similarly.

3.1.3 MELTING POINT

Table 3.3: Melting Point Range for the picrate salts of chloroquine phosphate

Sample	Melting Point Range (°C)		Mean melting point range (°C)
	1	2	
Pure powder	206.5-206.8	206.5-207.0	206.5-206.9
Test TX	206.1-207.7	206.3-208.1	206.2-207.9
Test TY	207.0-208.5	207.2-208.9	207.1-208.6
Test TZ	206.4-208.6	206.2-208.8	206.3-208.7
Reference R	206.5-207.7	206.3-207.7	206.4-207.7

3.1.4 TEST FOR PHOSPHATE

Table 3.4: Test for the presence of phosphate in the samples

Sample	Observation	Inference
TX	A yellow precipitate was produced, the colour of which was not changed on boiling and was soluble in 10M ammonia	Chloroquine phosphate present
TY	A yellow precipitate was produced, the colour of which was not changed on boiling and was soluble in 10M ammonia	Chloroquine phosphate present
TZ	A yellow precipitate was produced, the colour of which was not changed on boiling and was soluble in 10M ammonia	Chloroquine phosphate present
R	A yellow precipitate was produced, the colour of which was not changed on boiling and was soluble in 10M ammonia	Chloroquine phosphate present
Pure powder	A yellow precipitate was produced, the colour of which was not changed on boiling and was soluble in 10M ammonia	Chloroquine phosphate present

3.2 UNIFORMITY OF WEIGHT TEST

Table 3.5: Results of uniformity of weight test

Sample	Weight of 20 tablets (g)	Average weight per tablet (g)	No of tablets that deviated by more than 5%
TX	6.7418	0.3371	0
TY	7.3124	0.3656	0
TZ	8.0169	0.4008	0
R	6.6786	0.3339	0

Percentage Deviation = $\frac{X_i - \overline{X}}{\overline{X}}$ x 100%

Where X_i = weight of sample and $\overline{X}$ the mean weight

3.3 *IN VITRO* DISSOLUTION TESTING

Table 3.6: Calibration points for pure chloroquine phosphate using UV Spectrophotometer at λ_{max} of 344nm

Concentration $\%^{w}/_{v}$	Absorbance at 344nm		Mean Absorbance
	1	2	
0.0004	0.039	0.041	0.040
0.0008	0.143	0.143	0.143
0.0010	0.194	0.192	0.193
0.0020	0.500	0.501	0.5005
0.0030	0.882	0.883	0.8825

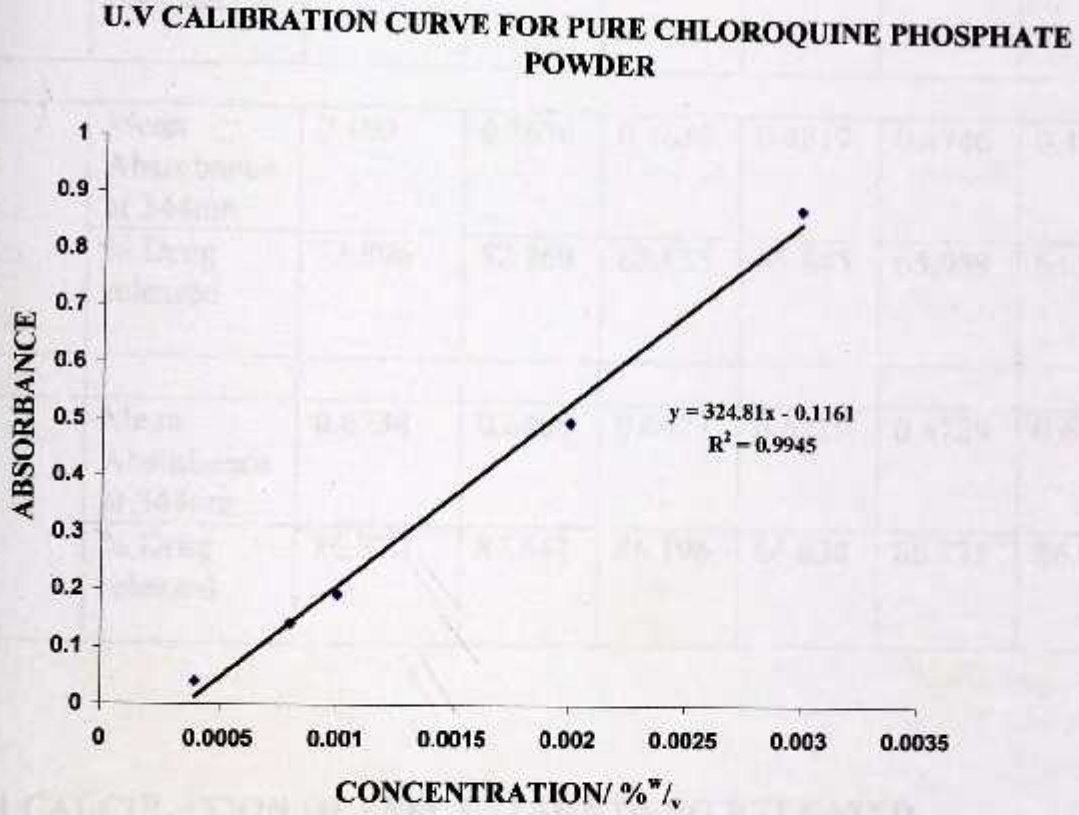


Figure 3.2: UV calibration curve for pure chloroquine phosphate powder at λ_{max} of 344nm.

The equation of the line is $y = 324.81x - 0.1161$

$R^2 = 0.9945$, correlation coefficient(r) = 0.9972

Table 3.7: UV dissolution data for product TX, TY, TZ and R

Sample	Parameter	Values					
	Time/min	10	30	45	60	90	120
TX	Mean Absorbance at 344nm	0.4528	0.5131	0.5225	0.5183	0.505	0.5235
	% Drug released	62.668	69.311	70.789	69.888	68.444	70.466
TY	Mean Absorbance at 344nm	0.0346	0.3472	0.6473	0.6697	0.5439	0.6678
	% Drug released	16.615	51.020	84.116	86.581	72.712	86.369
TZ	Mean Absorbance at 344nm	0.100	0.3630	0.4635	0.4817	0.4746	0.4733
	% Drug released	23.806	52.769	63.855	65.845	65.059	64.930
R	Mean Absorbance at 344nm	0.6738	0.6800	0.6677	0.6719	0.6729	0.6742
	% Drug released	86.870	87.543	86.196	86.638	86.735	86.889

3.3.1 CALCULATION OF PERCENTAGE DRUG RELEASED

The percentage drug released by product TX at time 45mins is being used as an example.

The mean absorbance = 0.5225

Volume of dissolution medium = 900ml

Volume of aliquot withdrawn = 10ml

Calibration curve thus converts the mean absorbance to concentration by interpolation

Thus, 0.5225 ≡ 0.0019661%$^{w}/_{v}$

Dilution factor = 10

Weight of drug in aliquot = (10 x 0.0019661 x 10)/100

= 0.0019661g

Weight of drug released into bulk dissolution medium = (900/10) x 0.0019661g

= 0.176949g

% Drug Released = Amount released /Labelled quantity) x 100

Labelled quantity of chloroquine phosphate = 0.250g

% Drug Released = (0.176949/0.250000) x 100 = 70.780%

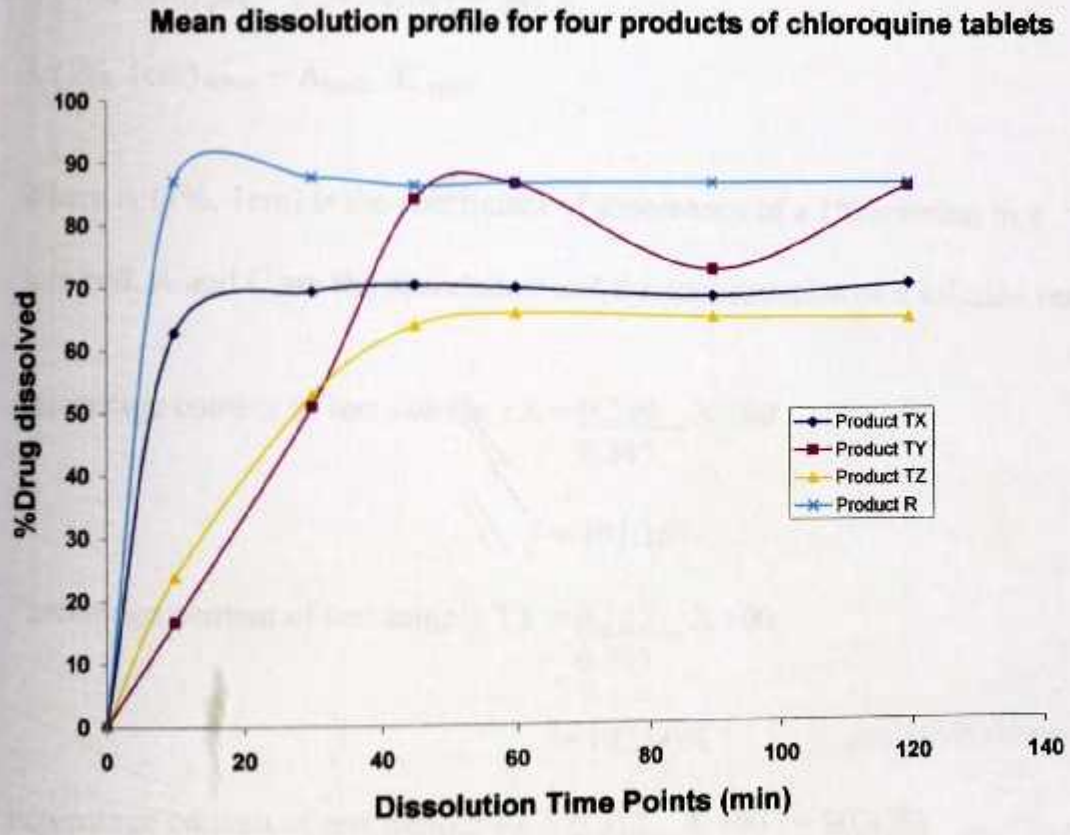


Figure 3.3: Mean dissolution profile of products TX, TY, TZ and R

3.4 ASSAY

3.4.1 UV SPECTROPHOTOMETRIC METHOD

Table 3.8: Assay of chloroquine phosphate using UV spectrophotometer

Sample	Absorbance at 343nm			Mean Absorbance
Pure powder	0.345	0.345	0.345	0.345
Test TX	0.349	0.349	0.349	0.349
Test TY	0.355	0.355	0.355	0.355
Test TZ	0.312	0.312	0.312	0.312
Reference R	0.356	0.356	0.356	0.356

From Beer Lambert's equation

A (1%, 1cm) = A/C

$A\ (1\%, 1cm)_{pure} = 0.345/0.001 = 345.0$

$A\ (1\%, 1cm)_{tablet} = A_{tablet} / C_{tablet}$

Where A (1%, 1cm) is the coefficient of absorbance of a 1% solution in a

1cm cell, A and C are the absorbance and the concentration of a solution respectively.

$$\text{Percentage content of test sample TX} = \frac{0.349}{0.345} \times 100$$

$$= 101.16\%$$

$$\text{Percentage content of test sample TY} = \frac{0.355}{0.345} \times 100$$

$$= 102.90\%$$

$$\text{Percentage content of test sample TZ} = \frac{0.312}{0.345} \times 100 = 90.43\%$$

Percentage content of reference sample R = $\frac{0.356}{0.345}$ X 100

= 103.18%

3.4.2 TITRATION

Table 3.9a: Potentiometric points for pure chloroquine powder (0.2004g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	280	0
1.0	290	10
2.0	300	10
3.0	310	10
4.0	310	0
5.0	320	10
6.0	330	10
6.2	340	50
6.4	350	50
6.6	360	50
6.8	360	0
7.0	370	50
7.1	370	0
7.2	380	100
7.3	390	100
7.4	410	200
7.5	430	200
7.6	460	300
7.7	530	700
7.8	540	100
7.9	550	100
8.0	550	0

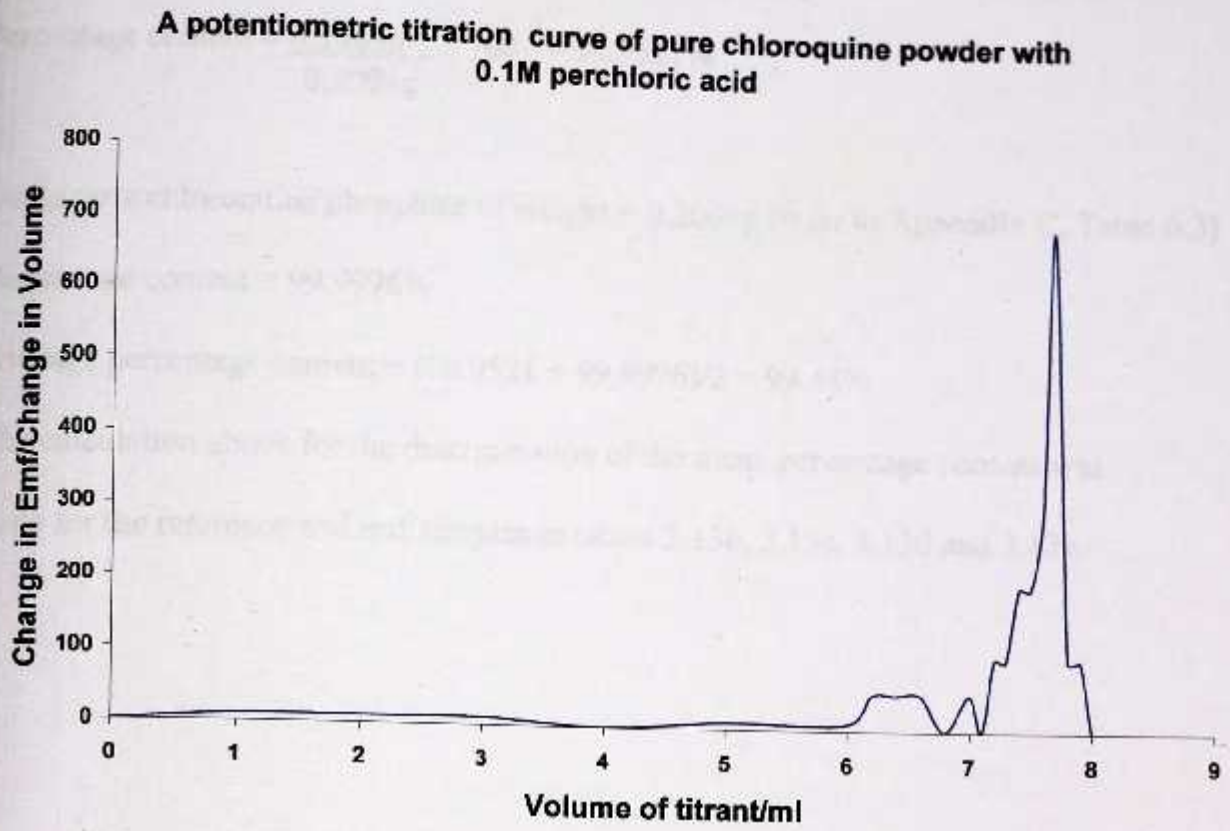


Figure 3.4a: A Potentiometric titration curve for pure chloroquine phosphate powder with 0.1M perchloric acid

From the Potentiometric titration table in Table 3.10a the endpoint was = 7.65ml

∴ Actual volume of perchloric acid used = 7.65 x factor

= 7.65 x 1.0051

= 7.6890ml

Where 1.0051 is the factor of 0.1M perchloric acid (refer to 6.1Appendix C)

But 1ml of 0.1M $HClO_4$ ≡ 0.02579g of chloroquine phosphate

∴ 7.6890ml of 0.1M $HClO_4$ ≡ 7.6890 x 0.02579 = 0.19830g of chloroquine phosphate

Amount of pure chloroquine phosphate weighed = 0.2004g

Percentage content = $\frac{0.1983g}{0.2004g} \times 100 = 98.9521\%$

Using pure chloroquine phosphate of weight = 0.2009g (refer to Appendix C, Table 6.3)

Percentage content = 99.9996%

Average percentage content = (98.9521 + 99.9996)/2 = 99.48%

The calculation above for the determination of the mean percentage content was done for the reference and test samples in tables 3.13b, 3.13c, 3.13d and 3.13e.

Table 3.9b: Potentiometric points for test sample TX (0.6742g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	250	0
2.0	250	0
4.0	250	0
6.0	260	5
8.0	260	0
10.0	260	0
12.0	270	5
14.0	280	5
15.0	290	10
16.0	300	10
17.0	310	10
18.0	320	20
18.2	330	50
18.4	340	50
18.5	350	100
18.6	370	200
18.7	450	800
18.8	490	400
18.9	500	100
19.0	510	100
20.0	530	20
21.0	540	10

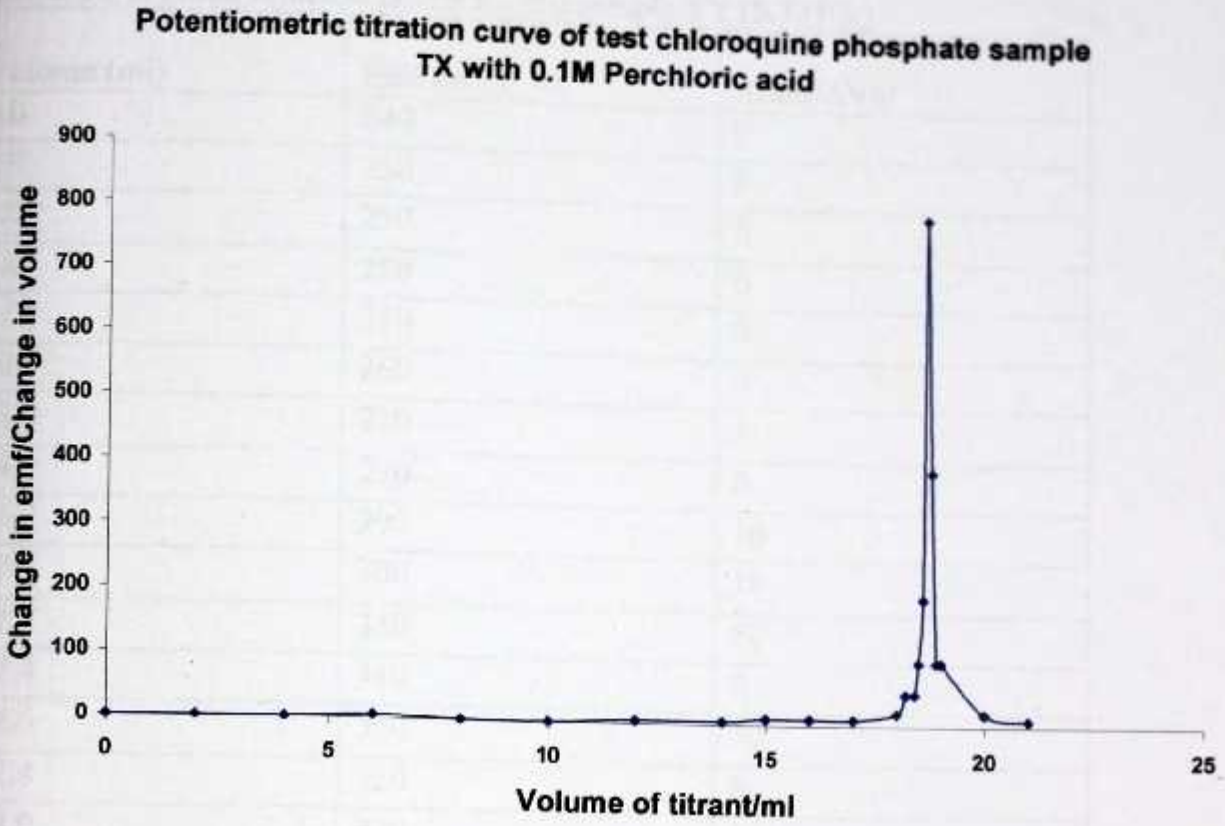


Figure 3.4b: A Potentiometric titration curve for test chloroquine phosphate sample TX with 0.1M perchloric acid

From the potentiometric titration table in Table 3.10b the end point was = 18.65ml

Percentage content = 96.69%

Using sample TX of weight = 0.6744g (refer to 6.1 Appendix C, Table 6.5)

Percentage content = 96.67%

Average percentage content = (96.69 +96.67)/2 = 96.68%

Table 3.9c: Potentiometric points for test sample TY (0.7310g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	240	0
2.0	250	5
4.0	250	0
6.0	250	0
8.0	250	0
10.0	260	5
12.0	270	5
14.0	270	0
16.0	290	10
17.0	300	10
17.2	310	50
17.4	310	0
17.6	320	50
17.8	320	0
18.0	330	50
18.1	330	0
18.2	340	100
18.3	340	0
18.4	350	100
18.5	360	100
18.6	370	100
18.7	380	100
18.8	400	200
18.9	430	300
19.0	490	600
19.5	510	40
20.0	530	40
21.0	540	10

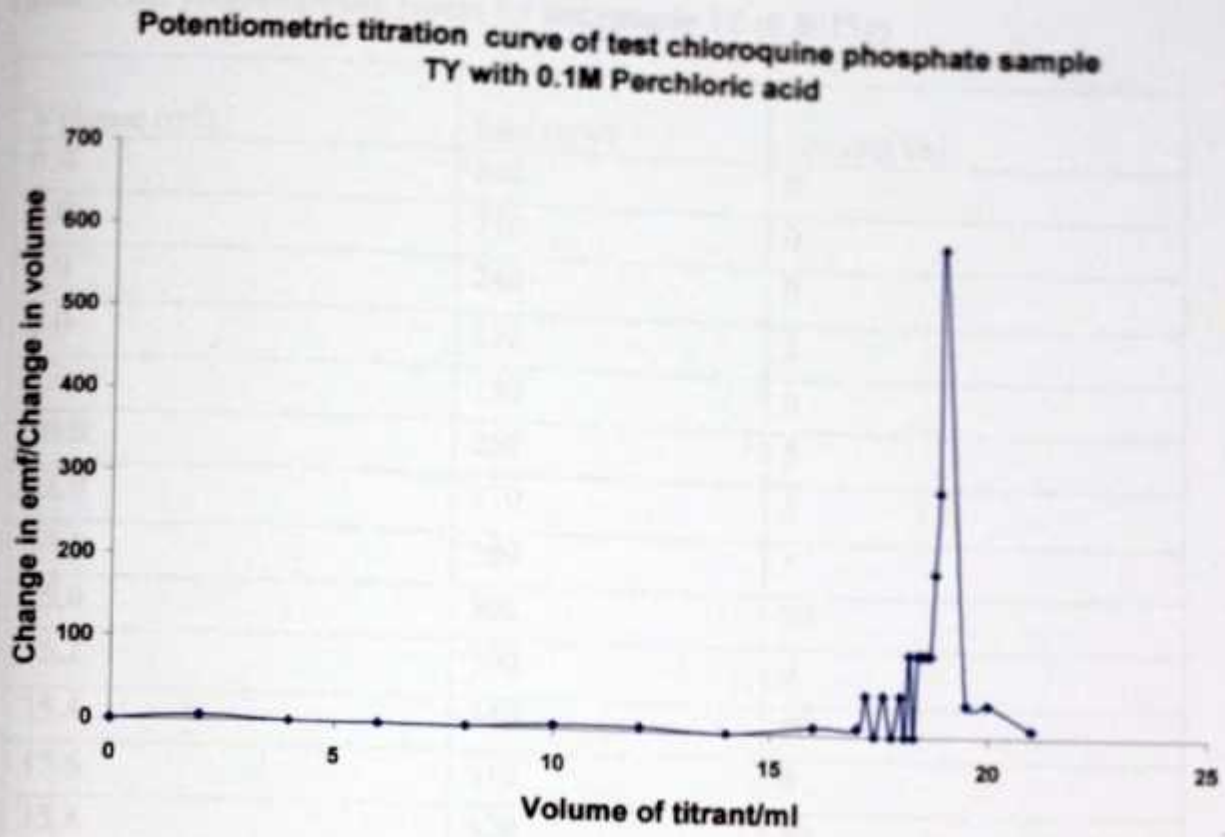


Figure 3.4c: A Potentiometric titration curve for test chloroquine phosphate sample TY with 0.1M perchloric acid

From the potentiometric titration table in Table 3.10b the end point was =18.95ml

Percentage content = 98.26%

Using sample TY of weight = 0.7312g (refer to 6.1 Appendix C, Table 6.4)

Percentage content = 98.76%

Average percentage content = (98.26 + 98.76)/2 = 98.51%

Table 3.9d: Potentiometric points for test sample TZ (0.8015g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	240	0
2.0	240	0
4.0	240	0
6.0	250	5
8.0	250	0
10.0	260	5
12.0	270	5
14.0	280	5
15.0	300	20
15.2	300	0
15.4	310	50
15.6	310	0
15.8	320	50
16.0	320	0
16.1	330	100
16.2	340	100
16.3	340	0
16.4	350	100
16.5	360	100
16.6	370	100
16.7	390	200
16.8	430	400
16.9	480	500
17.0	490	100
18.0	500	10
19.0	520	20
20.0	530	10
21.0	530	0

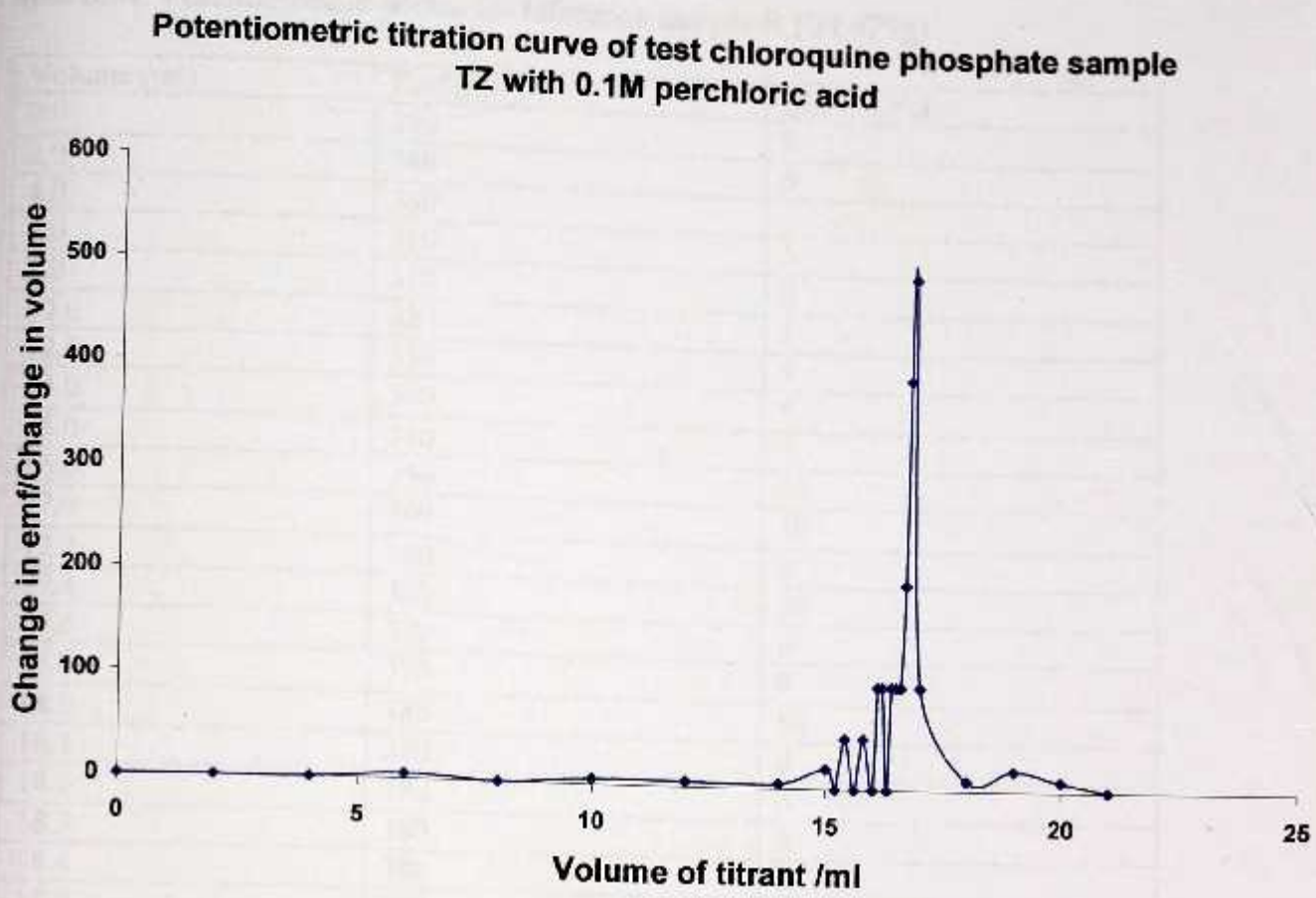


Figure 3.4d: A Potentiometric titration curve for test chloroquine phosphate sample TZ with 0.1M perchloric acid

From the potentiometric titration table in Table 3.10d the end point was = 16.85ml

Percentage content = 87.38%

Using sample R of weight = 0.8017g (refer to 6.1Appendix C Table 6.6)

Percentage content = 87.86%

Average percentage content = (87.86 + 87.38)/2 = 87.62%

Table 3.9e: Potentiometric points for reference sample R (0.6679g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	280	0
2.0	290	5
4.0	300	5
6.0	310	5
8.0	310	0
10.0	320	5
12.0	330	5
14.0	340	5
15.0	340	0
16.0	350	10
17.0	360	10
17.2	360	0
17.4	370	50
17.6	370	0
17.8	370	0
18.0	380	50
18.1	380	0
18.2	380	0
18.3	380	0
18.4	380	0
18.5	390	0
18.6	390	0
18.7	400	100
18.8	400	0
18.9	400	0
19.0	400	0
19.1	410	100
19.2	410	0
19.3	420	100
19.4	430	100
19.5	430	0
19.6	440	100
19.7	460	200
19.8	480	200
19.9	580	1000
20.0	600	200
20.1	630	300
20.2	640	100
20.3	650	100
20.4	660	100
20.5	670	100
21.0	680	20
22.0	690	10
23.0	700	10

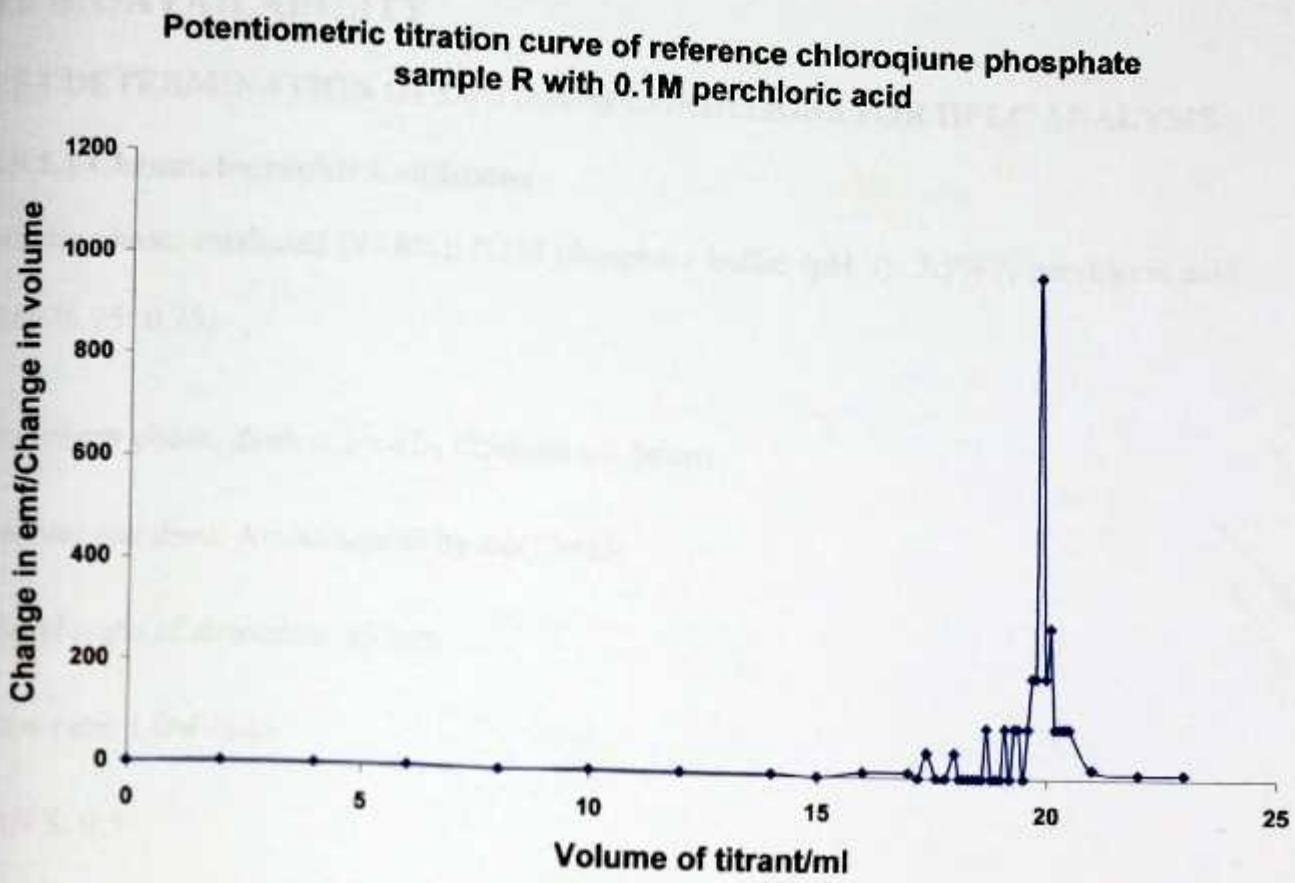


Figure 3.4e: A Potentiometric titration curve for reference chloroquine phosphate sample R with 0.1M perchloric acid

From the potentiometric titration table in Table 3.10e the end point was = 19.85ml

Percentage content = 102.89%

Using sample R of weight = 0.6680g (refer to 6.1Appendix C Table 6.7)

Percentage content = 102.87%

Average percentage content = (102.89 + 102.87)/2 = 102.88%

3.5 BIOAVAILABILITY

3.5.1 DETERMINATION OF OPTIMUM CONDITIONS FOR HPLC ANALYSIS

3.5.1.1 Chromatographic Conditions

Mobile phase: methanol (99.8%): 0.1M phosphate buffer (pH 3): 2.5%$^{v}/_{v}$ perchloric acid (24: 75.75: 0.25)

Stationary phase: Zorbax Rx-C_{18} (250mm x 4.6mm)

Internal standard: Amodiaquine hydrochloride

Wavelength of detection: 333nm

Flow rate: 1.0ml/min

AUFS: 0.5

Chart recorder speed: 5mm/min

Mean retention time of chloroquine phosphate: 9.196 ± 0.0949min

Mean retention time of amodiaquine hydrochloride: 11.36 ± 0.0584min

Temperature : Ambient

3.5.2 CALIBRATION GRAPH

Table 3.10a: HPLC calibration data for finding limits of UV detector linearity

Concentration ($\%^{w}/_{v}$)	Peak Area Ratio		Mean Peak Area Ratio
0.0005	0.1943	0.1991	0.1967
0.0007	0.460	0.2960	0.3780
0.001	0.5920	0.5936	0.5928
0.0015	0.8642	0.8642	0.8642
0.0020	1.1536	1.2334	1.1935
0.0025	1.5256	1.5492	1.5374
0.003	1.8740	1.8764	1.8752

Table 3.10b: HPLC Calibration Points for pure chloroquine phosphate powder

Concentration ($\%^{w}/_{v}$)	Peak Area Ratio		Mean Peak Area Ratio
0.0010	0.5920	0.5936	0.5928
0.0015	0.8642	0.8642	0.8642
0.0020	1.1536	1.2334	1.1935
0.0025	1.5256	1.5492	1.5374
0.003	1.8740	1.8764	1.8752

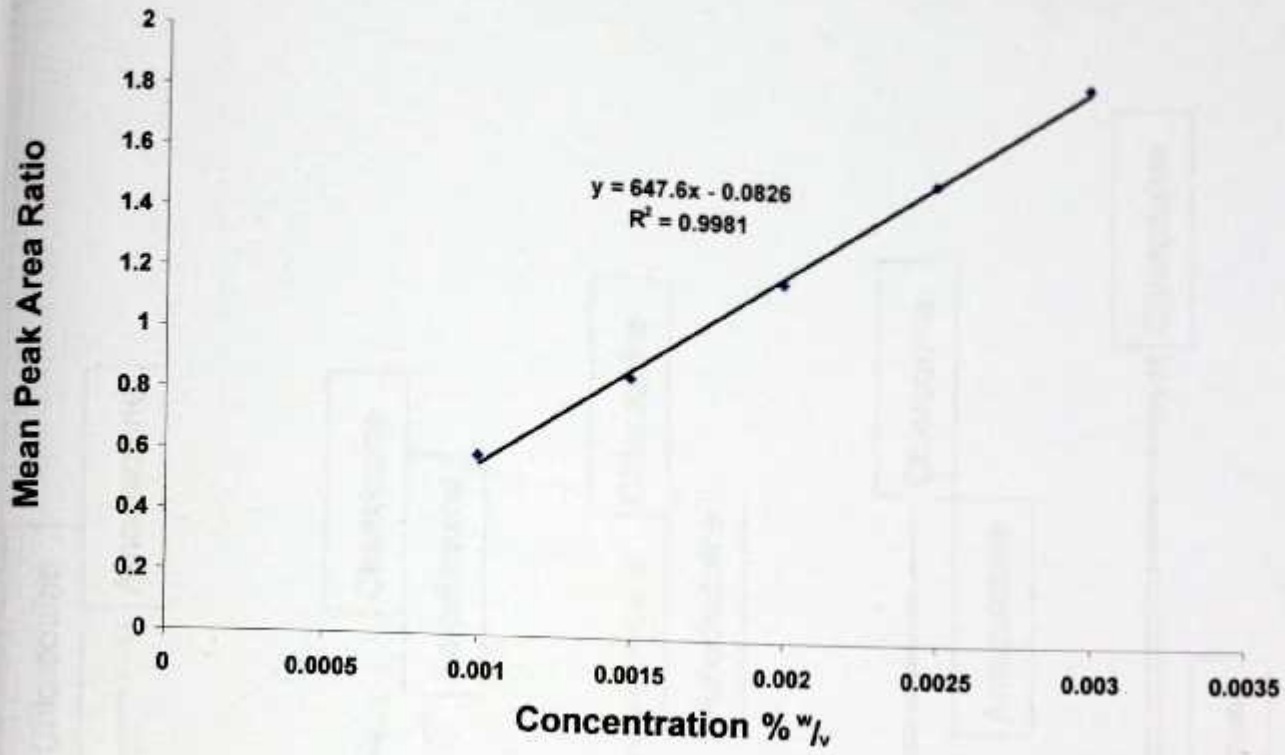


Figure 3.5: HPLC calibration curve of pure chloroquine phosphate powder

3.5.3 CHROMATOGRAMS

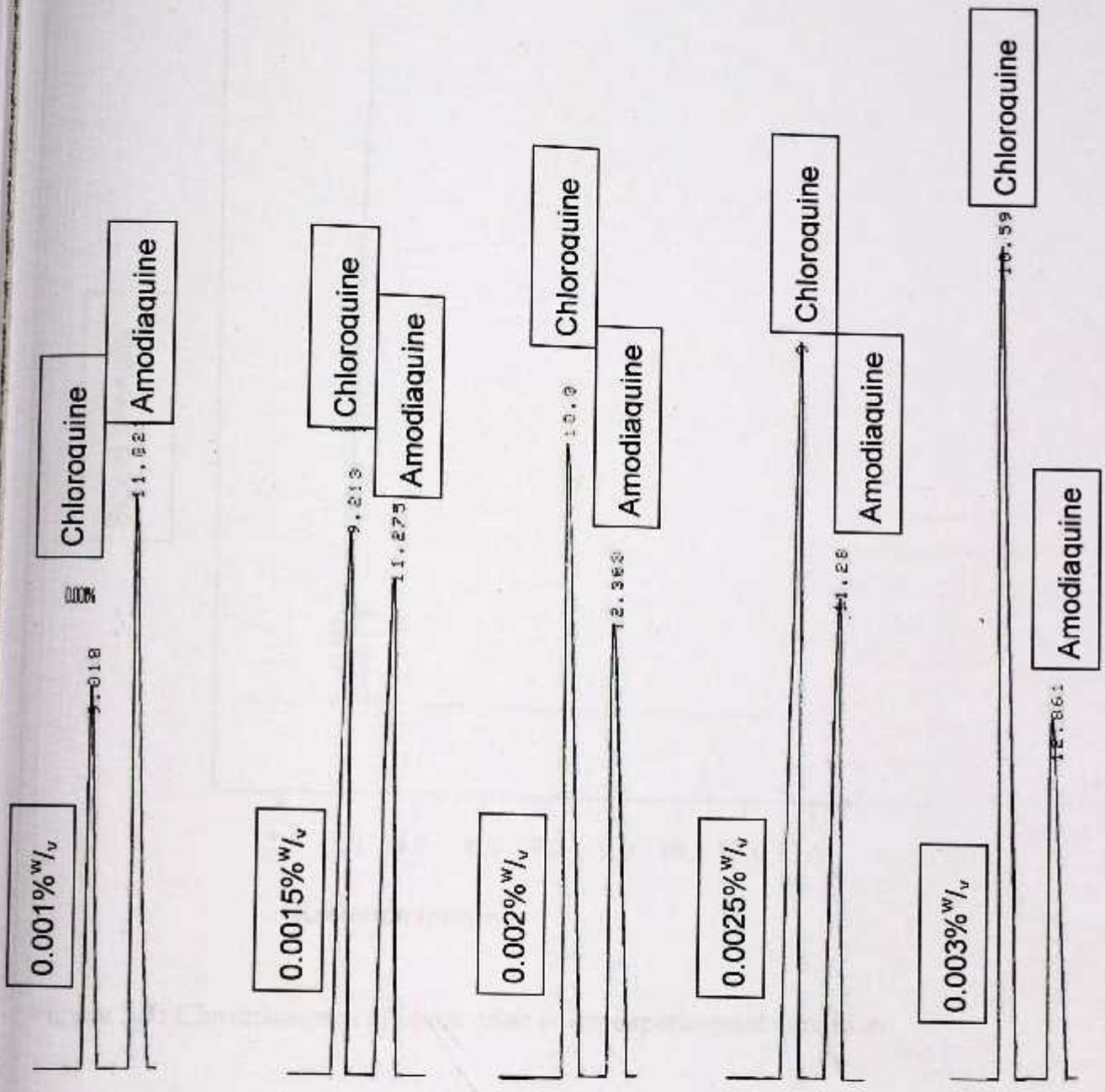


Figure 3.6: Chromatogram of pure chloroquine phosphate of increasing concentration and amodiaquine hydrochloride (internal standard).

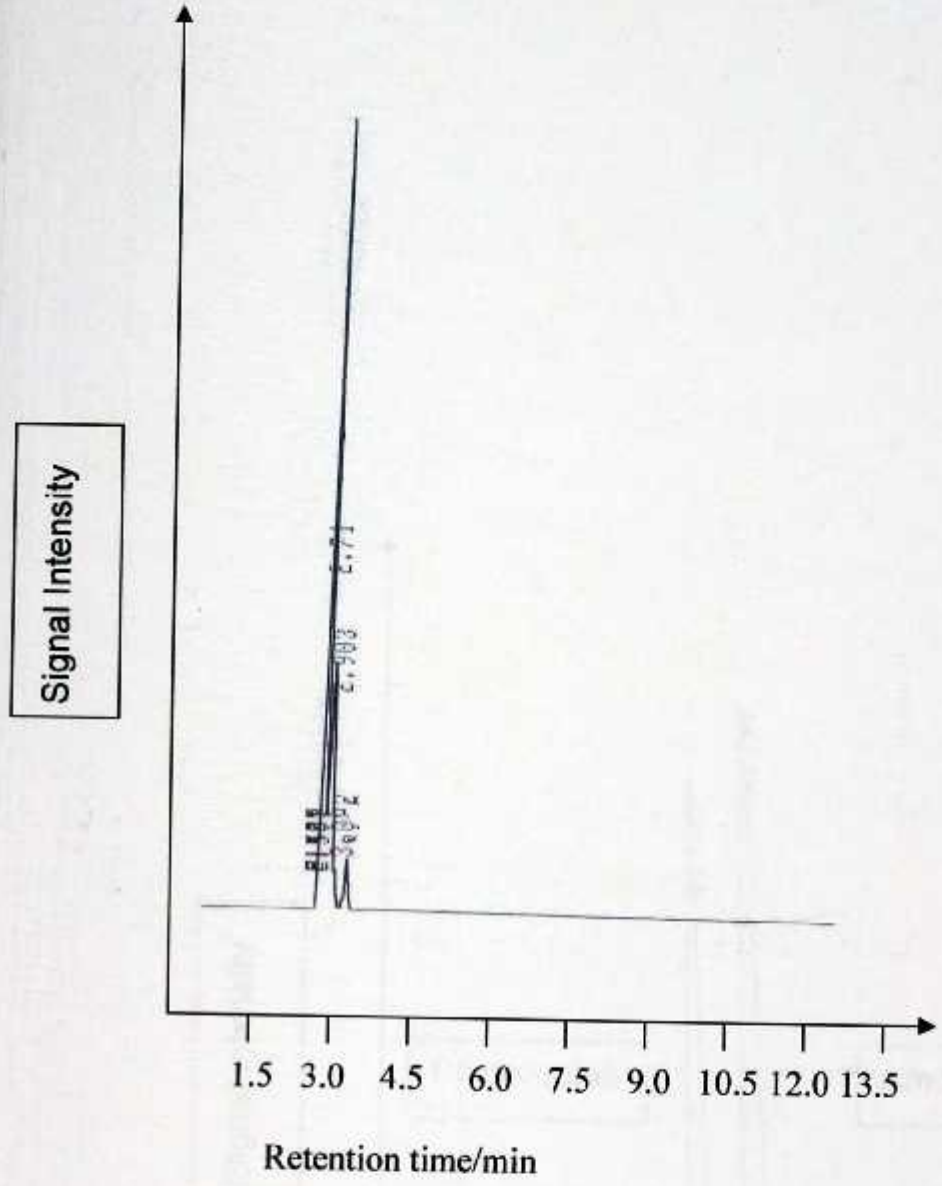


Figure 3.7: Chromatogram of blank urine under experimental conditions

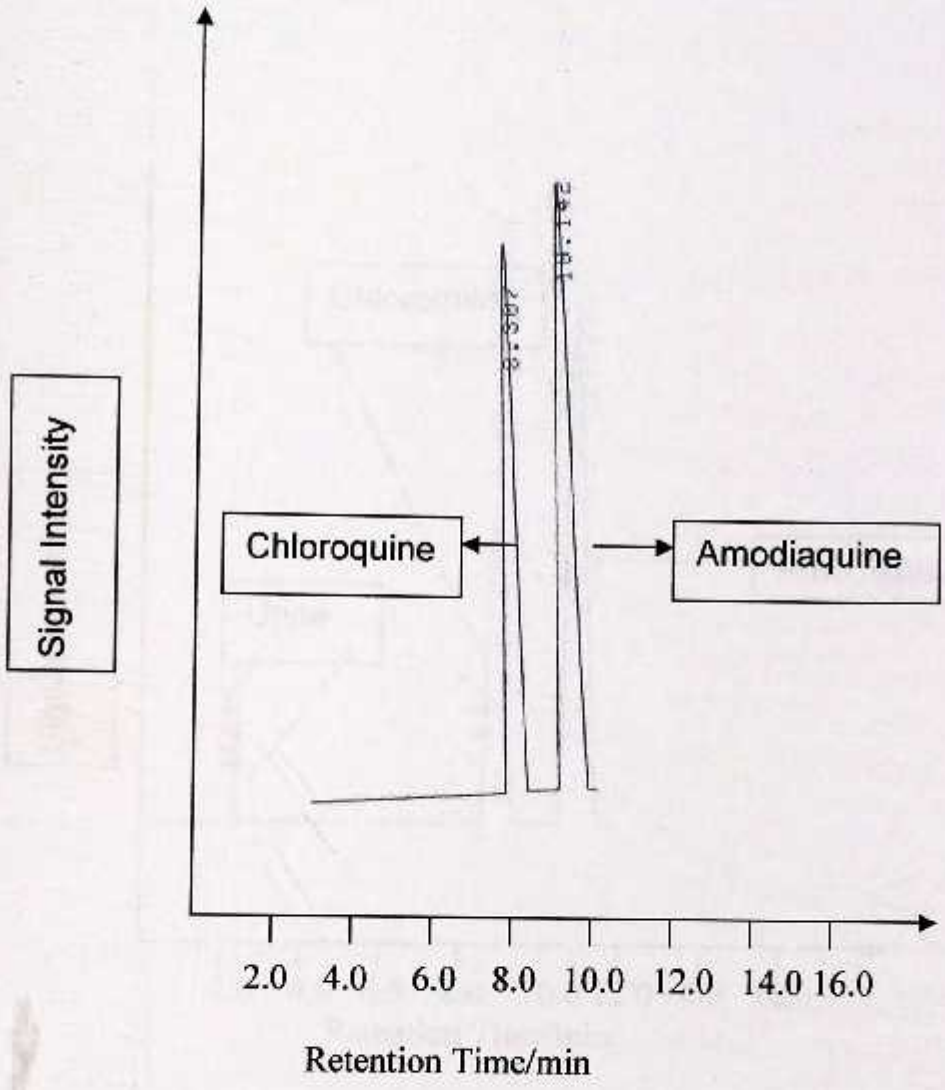


Figure 3.8: Chromatogram of pure chloroquine phosphate and amodiaquine hydrochloride (Internal standard) under experimental conditions

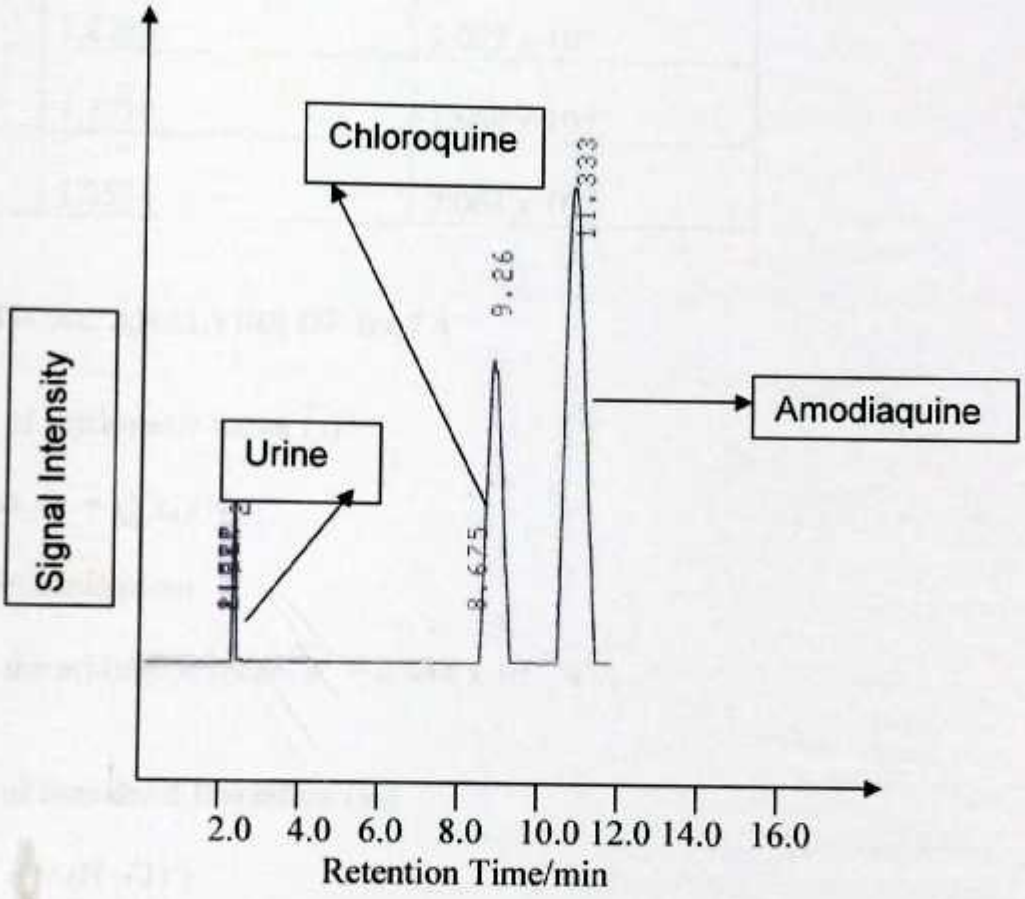


Figure 3.9: Chromatogram of unchanged chloroquine phosphate in urine and amodiaquine hydrochloride (Internal standard) under experimental conditions

3.6 VALIDATION OF ANALYTICAL METHOD

3.6.1 WITHIN-RUN PRECISION (REPEATABILITY) OF ANALYTICAL METHOD

Table 3.11: HPLC data for repeated measurement of 0.002%$^{w}/_{v}$ solution of chloroquine phosphate

Number of Run	Mean Peak Area Ratio	Concentration (%$^{w}/_{v}$)
1	1.2644	2.080×10^{-3}
2	1.2553	2.066×10^{-3}
3	1.2638	2.079×10^{-3}
4	1.2320	2.030×10^{-3}
5	1.2268	2.022×10^{-3}
6	1.1925	1.969×10^{-3}
7	1.2534	2.063×10^{-3}

3. 6.2 STATISTICAL ANALYSIS OF DATA

(a) Calculation of arithmetic mean ($\bar{x}$)

Using the relation $\bar{x} = (\sum x_i)/N$

N = number of determinations

By calculations; the arithmetic mean, $\bar{x} = 2.044 \times 10^{-3}$ %$^{w}/_{v}$

(b) Calculation of Standard Deviation (s_X)

$s_X = \sqrt{(\sum(x_i - \bar{x})^2 / (N-1))}$

By calculations, $s_X = 4.015 \times 10^{-5}$ %$^{w}/_{v}$

Relative standard deviation = $(s_X / \bar{x}) \times 100 = (4.015 \times 10^{-5} / 2.044 \times 10^{-3}) \times 100 = 1.96\%$

(c) Calculation of standard error of the mean (S.E.M.)

S.E.M = $s_X/\sqrt{N} = 4.015 \times 10^{-5}/\sqrt{7} = 1.517 \times 10^{-5}$

(d) Calculation of absolute precision of data at 95% confidence level

Absolute precision = $t_{95, v}$ (S.E.M)

Where, v = degrees of freedom = N -1

From above data, v = 6

From statistical tables $t_{95, 6} = 2.45$

Thus absolute precision = $2.45 \times 1.517 \times 10^{-5} = 3.717 \times 10^{-5}$

(e) Confidence limits of the mean (μ)

$\mu = \bar{x} \pm t_{95, v}$ (S.E.M) = $2.044 \times 10^{-3} \pm 3.717 \times 10^{-5}$ %$^{w}/_{v}$

(f) Absolute error of the mean

The calculated percentage content of reference chloroquine phosphate powder = 99.42

An accurately prepared 0.002% solution of chloroquine phosphate should therefore contain $0.9942 \times 0.002 = 1.9884 \times 10^{-3}$ %

From the experiment the value is 2.044×10^{-3} %$^{w}/_{v}$

Thus, absolute error = $2.044 \times 10^{-3} - 1.9884 \times 10^{-3} = 5.56 \times 10^{-5}$

% Relative error = $(5.56 \times 10^{-5} / 1.9884 \times 10^{-3}) \times 100 = 2.80\%$

3.6.3 BETWEEN-RUN PRECISION (REPRODUCIBILITY) OF METHOD

Table 3.12: HPLC calibration data collected on two different occasions

1ST OCCASION			2ND OCCASION	
Mean Peak Area Ratio	Mean Concentration (%w/v)		Mean Peak Area Ratio	Mean Concentration (%w/v)
0.0460	0.000199		0.0296	0.000173
0.1943	0.000428		0.1991	0.000435
0.5920	0.001042		0.5936	0.001044
0.8642	0.001462		0.8642	0.001462
1.1536	0.001909		1.2334	0.002032
1.5256	0.002484		1.5492	0.00252
1.8740	0.003021		1.8764	0.003025

3.6.3.1 Statistical analysis of between-run precision data

(a) Calculation of variance

Let s_1 and s_2 be the respective standard deviations for data collected on 1st and 2nd occasions.

By calculation, $s_1 = 0.001041$; $s_2 = 0.001061$

Variance = (standard deviation)^2

$s_1^2 = 1.0837 \times 10^{-6}$; $s_2^2 = 1.1257 \times 10^{-6}$

(b) F-Test for the comparison of standard deviations

Since it is important to know whether the data collected on the two different occasions differ significantly in their precision, a two tailed test was used for the statistical analysis. This covers either possibility.

This test considers the ratio of the two variances and it is given by;

$Fv_2, v_1 = s_2^2 / s_1^2$

The respective variances are allocated in the equation in such a way that F is always ≥ 1

v_1 and v_2 are the degrees of freedom of the numerator and denominator respectively.

The null hypothesis adopted was that the population from which the samples are taken are normal, and that the population variances are equal.

From the above values,

Calculated $F_{6,6} = 1.1257 \times 10^{-6} / 1.0837 \times 10^{-6} = 1.0388$

Critical $F_{6,6}$ for a two-tailed test at 95% confidence level = 5.820

Thus, since the calculated F value was less than the critical value, the null hypothesis was retained.

The two sets of results are said to be reproducible.

3.6.4 COMPARISON OF NEW ANALYTICAL METHOD AND STANDARD B.P AND USP METHODS

Table 3.13: Assay of four brands of chloroquine phosphate tablets by HPLC

Sample	Mean Peak Area Ratio	Concentration ($\%^w/_v$)	Mean % Content
TX	0.5741	1.014×10^{-3}	101.30
TY	0.5857	1.032×10^{-3}	103.16
TZ	0.5268	9.41×10^{-4}	94.23
R	0.5883	1.036×10^{-3}	103.50

Table 3.14: Results of assay of four products of chloroquine phosphate tablets by HPLC, UV and Non-aqueous titration methods

SAMPLE	ANALYTICAL METHOD		
	MEAN % CONTENT (HPLC)	MEAN % CONTENT (UV)	MEAN % CONTENT (NON-AQ.TITRATION)
TX	101.30	101.16	96.68
TY	103.16	102.90	98.51
TZ	94.23	90.43	87.62
R	103.50	103.19	102.88
MEAN	100.55	99.42	96.42

3.6.4.1 Statistical Analysis of Data

The mean percentage content of chloroquine phosphate tablets (B.P.2002) = 100.0

(a) Calculation of absolute error of the mean

Mean for HPLC = 100.55%

Mean for Non-aqueous titration = 96.42%

Mean for U.V method = 99.42%

Absolute error for HPLC = | 100-100.55 | = 0.55%

Absolute error for Non-aqueous titration = | 100-96.42 | = 3.58%

Absolute error for U.V method = | 100-99.42 | = 0.58%

(b) Calculation of S.E.M

S.E.M. = $s/\sqrt{N}$

Standard deviation for HPLC = 4.321

Standard deviation for Non-aqueous titration = 6.419

Standard deviation for U.V method = 6.060

S.E.M. for HPLC = $4.321/\sqrt{4} = 2.161$

S.E.M for Non-aqueous titration = $6.419/\sqrt{4} = 3.210$

S.E.M for U.V method = $6.060/\sqrt{4} = 3.03$

(c) Calculation of absolute precision at 95% confidence level

Absolute precision = $t_{95, v}$ (S.E.M)

Absolute precision for HPLC = $t_{95, 3}$ (S.E.M) = 3.18 x 2.161 = 6.8720

Absolute precision for Non-aqueous titration = 3.18 x 3.210 = 10.2078

Absolute precision for U.V method = 3.18 x 3.03 = 9.6354

(d) Test for significant difference in precision and means of analytical methods

The analysis of variance (ANOVA) test of significance was applied to verify if the differences between the means among the three methods could be explained by random error. The null hypothesis was that, no significant difference existed between the means and variances of the above. If all the four chloroquine phosphate samples were assumed to have been drawn from a population of mean μ and variance σ^2, then σ^2 can be estimated in two ways by the principle of ANOVA. These are by way of the variation within the samples and variation between the samples.

Table 3.15: Results of test of significant difference in precision and means of analytical methods

ANOVA Table	Estimate of σ^2		
	SS	df	MS
Treatment (between samples)	36.36	2	18.18
Residual (within samples)	289.8	9	32.20
Total	326.2	11	

Test of significance

If the null hypothesis was valid, the two estimates of σ^2 should not differ significantly. A one tailed F-test was used.

$F_{9,2} = 32.20/18.18 = 1.7712$

At 95 percent confidence level, the critical value of F is 19.38. Since the calculated F was less than the critical value, the null hypothesis was retained. Thus the sample means for the respective products using the three methods do not differ significantly.

3.7 *IN VIVO* DISSOLUTION OF CHLOROQUINE PHOSPHATE TABLETS

Table 3.16: HPLC Analysis of excreted chloroquine phosphate in Urine (Test sample TX)

Subject T1X

Time/hr	Vol. of Urine (ml)	Mean Peak Area Ratio	Conc. (%$^{w}/_{v}$)	Amount of drug excreted (mg)	dDu/dt	Cumulative amount (mg)
1	176.0	0.4834	0.000874	3.0779	3.0779	3.0779
2	116.0	0.3901	0.000730	8.4634	8.4634	11.5413
3	222.0	0.6576	0.001143	5.0758	5.0758	16.6171
4	55.0	0.6576	0.001143	6.2876	6.2876	22.9047
5	33.0	1.0041	0.001678	5.5361	5.5361	28.4408
6	25.0	0.8914	0.001504	5.752	5.752	34.1928
7	23.0	0.6880	0.001190	5.4758	5.4758	39.6686
8	23.0	0.5378	0.000958	4.4050	4.4050	44.0736
10	64.0	0.4465	0.000817	10.4550	5.2275	54.5286
12	52.0	0.5644	0.000999	10.3917	5.1959	64.9203
15	104.2	0.4290	0.000790	16.4719	5.4906	81.3922
20	437.0	0.2788	0.000558	24.4021	4.8804	105.7943
24	194.0	0.7373	0.001266	12.2841	3.0710	118.0784

Mean Peak Area Ratio = $\frac{\text{Peak Area of chloroquine (analyte)}}{\text{Peak Area of amodiaquine (internal standard)}}$

From the calibration curve in figure the equation of the line is $Y = 647.6X - 0.0826$

$$X = \frac{Y + 0.0826}{647.6}$$

Where Y is the mean peak area ratio and X the concentration (%$^{w}/_{v}$) of the excreted chloroquine phosphate.

Calculation of amount of drug excreted

Amount of drug excreted = concentration (%$^{w}/_{v}$) x volume of urine x dilution factor

All other values under the amount of drug excreted in the tables in Appendix T and R were similarly generated.

Table 3.17: Calculation of excretion rate over 24 hours for subject T1X

Time/hr	Amount Excreted (Du)	Change in time (dt)	dDu/dt	Midpoint of Time (hr)
0	0.0	0	0.0	0.0
4	22.9047	4	5.7262	2.0
8	21.1689	4	5.2922	6.0
15	37.3162	7	5.3309	11.5
24	36.6862	9	4.0762	19.5

Mid point of time = the sum of the limit of the interval/2

Table 3.18: Rate of change of amount of chloroquine phosphate excreted over 24 hours for test sample TX

Mid point of time (hr)	The Rate of Change of amount of chloroquine phosphate excreted for test sample TX (dDu/dt)					
	T1X	T2X	T3X	T4X	T5X	T6X
0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	5.7262	2.4463	3.5675	3.2756	0.5830	3.1217
6.0	5.2922	2.5235	5.5206	3.936	2.2609	4.6390
11.5	5.3309	1.8108	5.3548	6.9862	3.6064	3.7892
19.5	4.0762	1.8769	2.2030	3.6660	3.7460	2.7516

The excretion rate for subjects T2X to T6X were similarly generated from Table

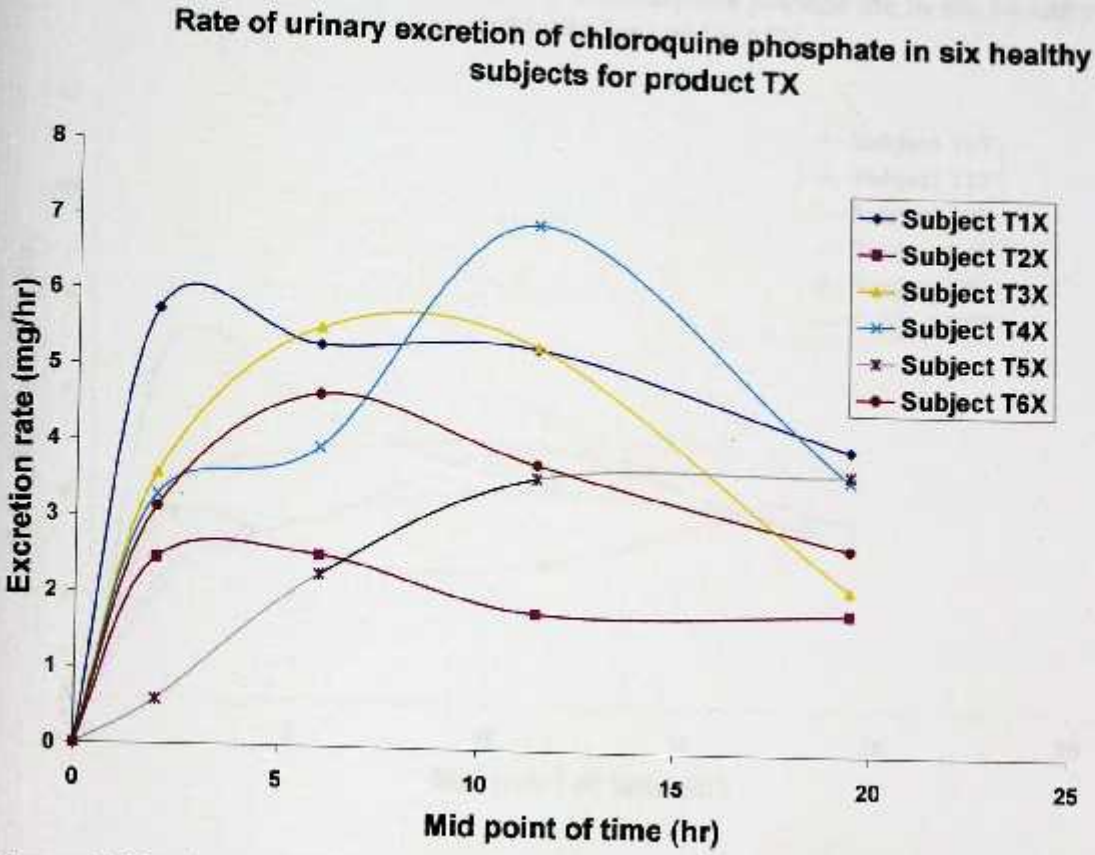


Figure 3.10: A graph of rate of change of total amount of chloroquine phosphate excreted (mg) versus mid point of time (hr) for test sample TX

Table 3.19: Rate of change of amount of chloroquine phosphate excreted over 24 hours for test sample TY

Mid point of time (hr)	The Rate of Change of amount of chloroquine phosphate excreted for test sample TY (dDu/dt)					
	T1Y	T2Y	T3Y	T4Y	T5Y	T6Y
0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	7.0667	3.6737	3.7466	2.2694	2.5544	4.1168
6.0	5.5808	2.7620	9.8605	3.6902	3.7221	4.9715
11.5	4.3495	2.7883	5.7105	4.2373	5.0960	5.0806
19.5	3.7565	4.5917	5.0832	3.5427	3.7668	3.2779

The excretion rate for subjects T1Y to T6Y were similarly generated from Table

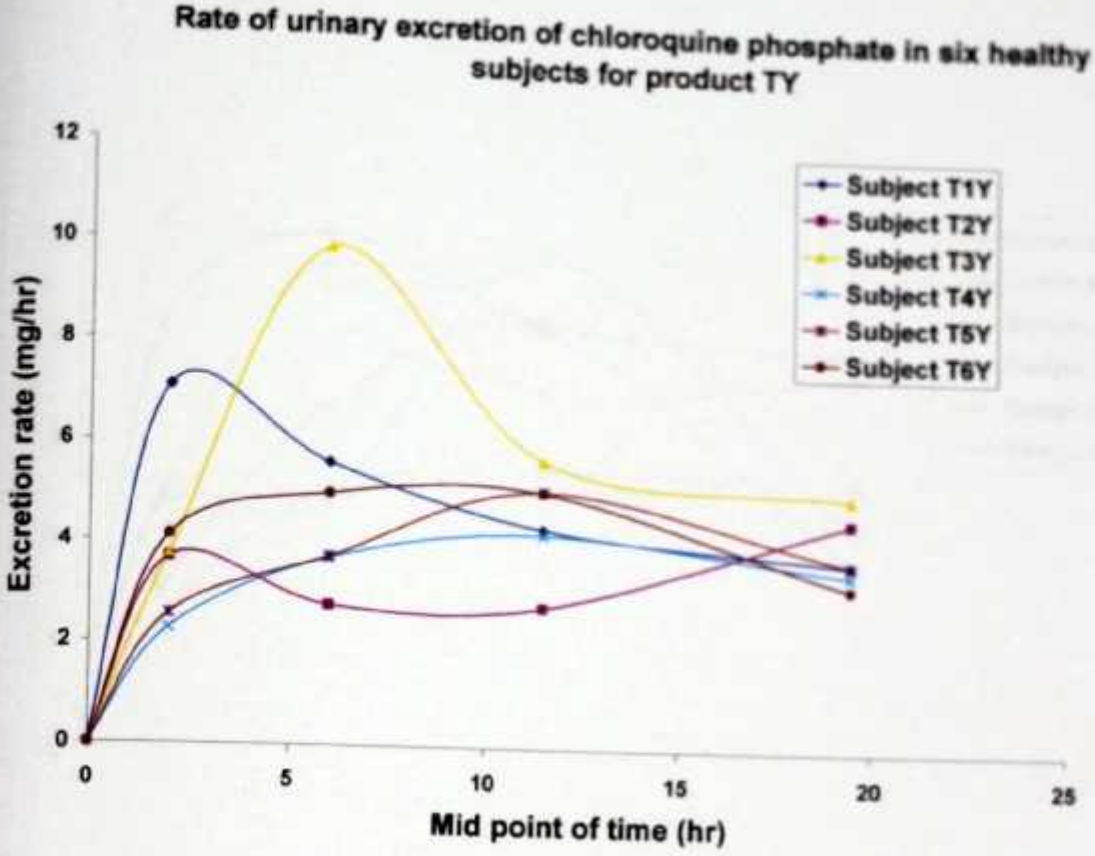


Figure 3.11: A graph of rate of change of total amount of chloroquine phosphate excreted (mg) versus mid point of time (hr) for test sample TY

Table 3.20: Rate of change of amount of chloroquine phosphate excreted over 24 hours for test sample TZ

Mid point of time (hr)	The Rate of Change of amount of chloroquine phosphate excreted for reference sample R (dDu/dt)					
	T1Z	T2Z	T3Z	T4Z	T5Z	T6Z
0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	2.4527	1.1997	5.1619	2.3448	4.1089	1.5184
6.0	4.7933	2.5447	4.5325	2.3948	5.0759	3.3554
11.5	3.5643	2.7729	5.0067	4.8991	4.2345	4.1680
19.5	3.7768	2.6612	2.8056	2.1776	4.0108	1.8790

The excretion rate for subjects T1Z to T6Z were similarly generated from Table

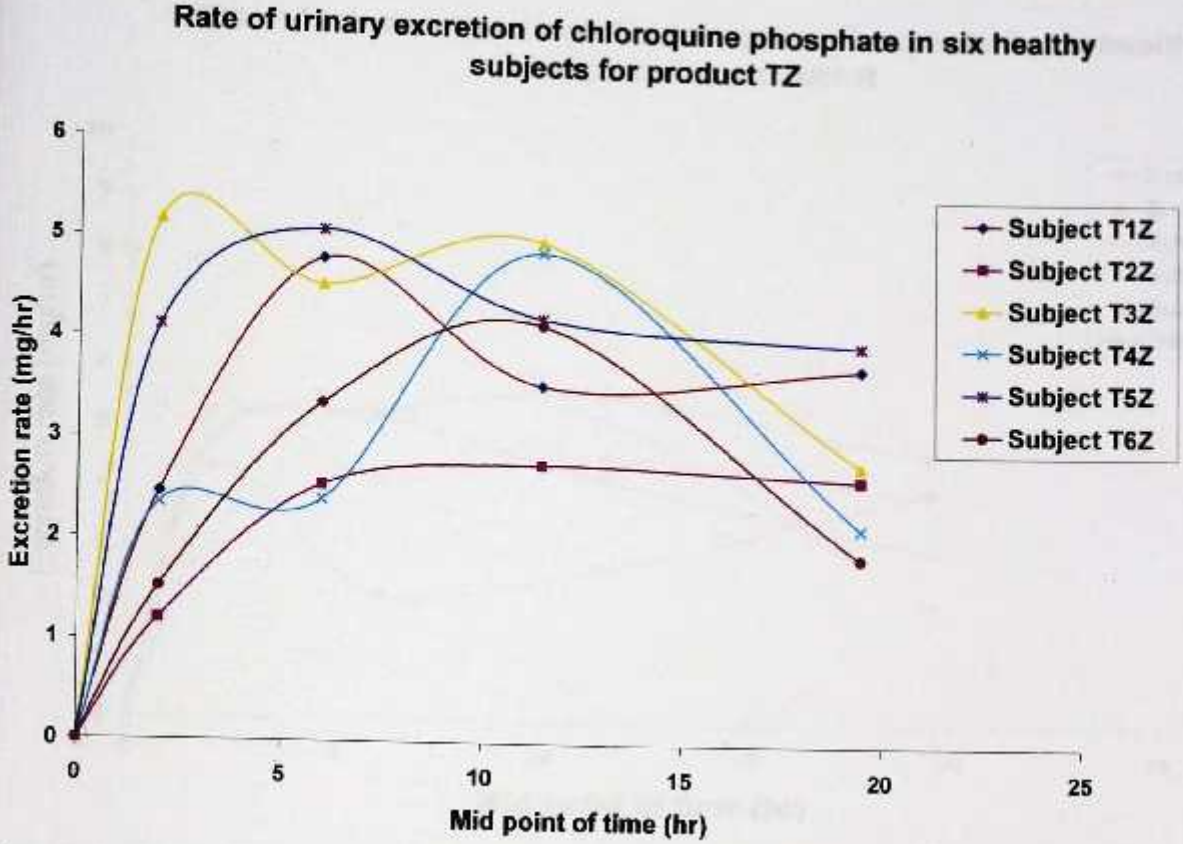


Figure 3.12: A graph of rate of change of total amount of chloroquine phosphate excreted (mg) versus mid point of time (hr) for test sample TZ

Table 3.21: Rate of change of amount of chloroquine phosphate excreted over 24 hours for reference sample R

Mid point of time (hr)	The Rate of Change of amount of chloroquine phosphate excreted for reference sample R (dDu/dt)					
	R1	R2	R3	R4	R5	R6
0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	5.2719	4.2993	5.1704	3.0338	4.7329	4.7635
6.0	4.1221	2.1655	8.9388	5.2026	5.1776	5.3238
11.5	4.6032	2.6531	6.5055	5.2359	4.1905	5.6175
19.5	3.9567	4.0356	4.9569	2.0902	3.018	4.6556

The excretion rate for subjects R1 to R6 were similarly generated from Table

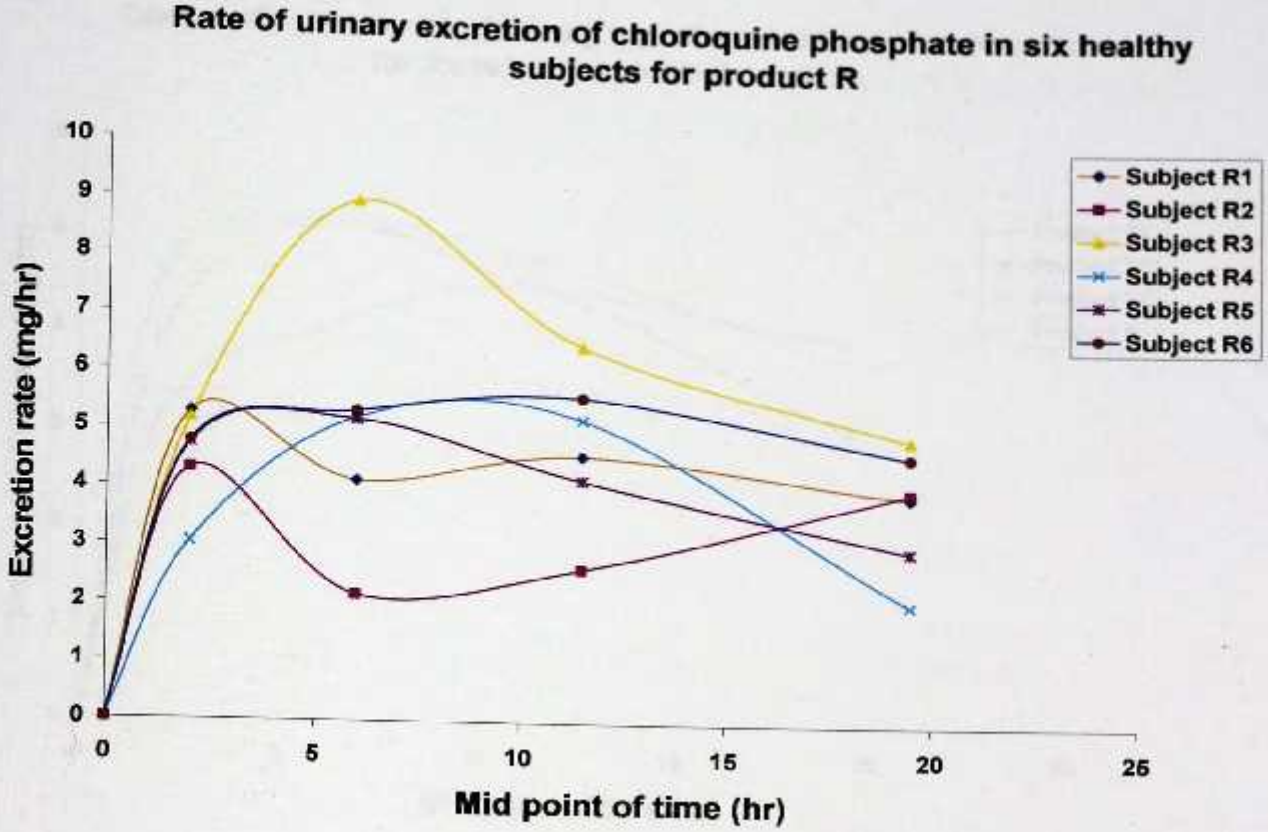


Figure 3.13: A graph of rate of change of total amount of chloroquine phosphate excreted (mg) versus mid point of time (hr) for reference sample R

Table 3.22: Mean rate of change of amount excreted over 24 hours

Mid point of time (hr)	0.0	2.0	6.0	11.5	19.5
Mean rate of excretion (dDu/dt) (Test sample TX)	0.0	3.1201	4.0287	4.4797	3.0533
Mean rate of excretion (dDu/dt) (Test sample TY)	0.0	3.9046	5.0979	4.5437	4.0031
Mean rate of excretion (dDu/dt) (Test sample TZ)	0.0	2.7977	3.7828	4.1076	2.8852
Mean rate of excretion (dDu/dt) (Reference sample R)	0.0	4.5453	5.1551	4.801	3.7855

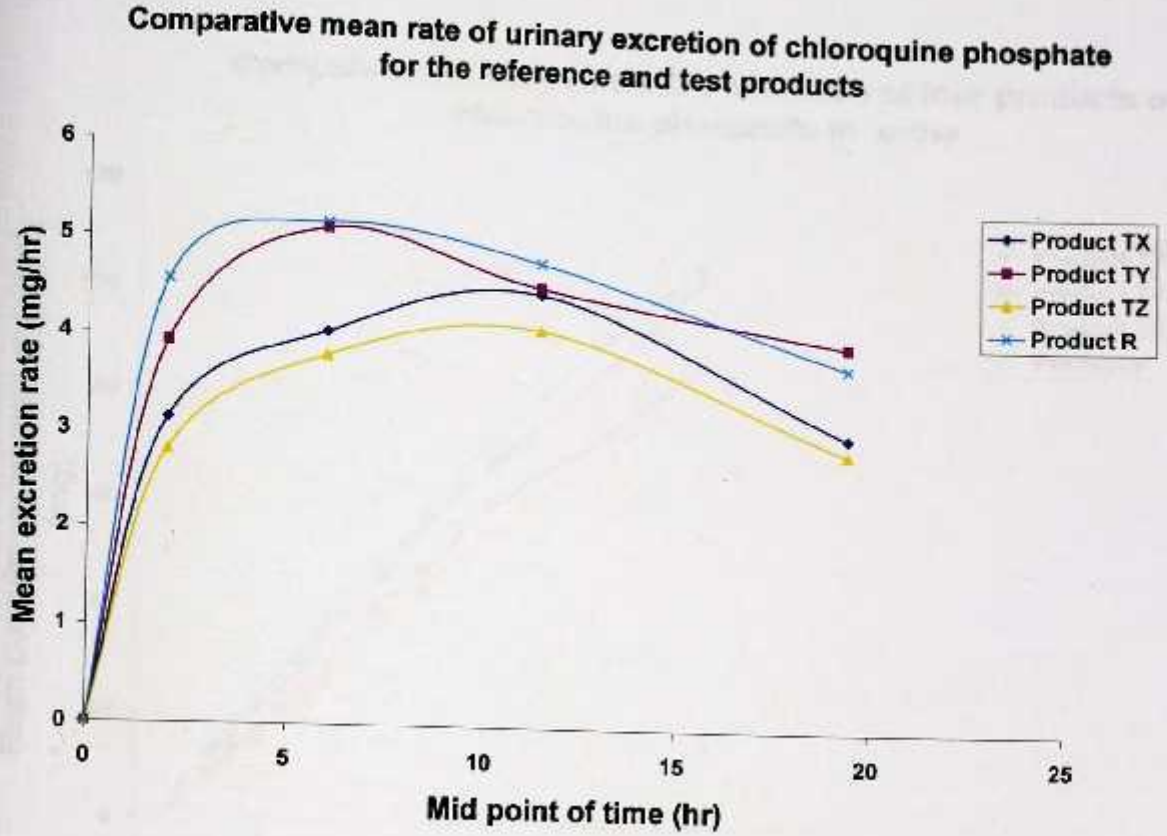


Figure 3.14: A graph showing the mean amount of chloroquine phosphate excreted in 24 hours for the reference and test samples

Table 3.23: Mean cumulative amount of chloroquine phosphate excreted for the four products

Time (hr)	Mean cumulative amount excreted (mg)			
	Products			
	TY	TX	TZ	R
0	0	0	0	0
1	0.3861	0.513	0.1672	0.8924
2	6.4404	4.0154	2.6622	6.1858
3	11.457	8.3666	6.7879	12.273
4	15.618	12.48	11.191	18.181
5	20.326	16.996	14.428	22.525
6	24.325	20.55	18.061	26.695
7	28.995	24.63	21.778	32.44
8	36.01	28.595	26.322	38.801
10	43.887	40.003	36.562	47.765
12	55.327	47.124	43.764	56.789
15	67.816	59.953	55.075	72.408
20	86.584	74.262	71.44	91.799
24	103.84	87.433	81.042	106.48

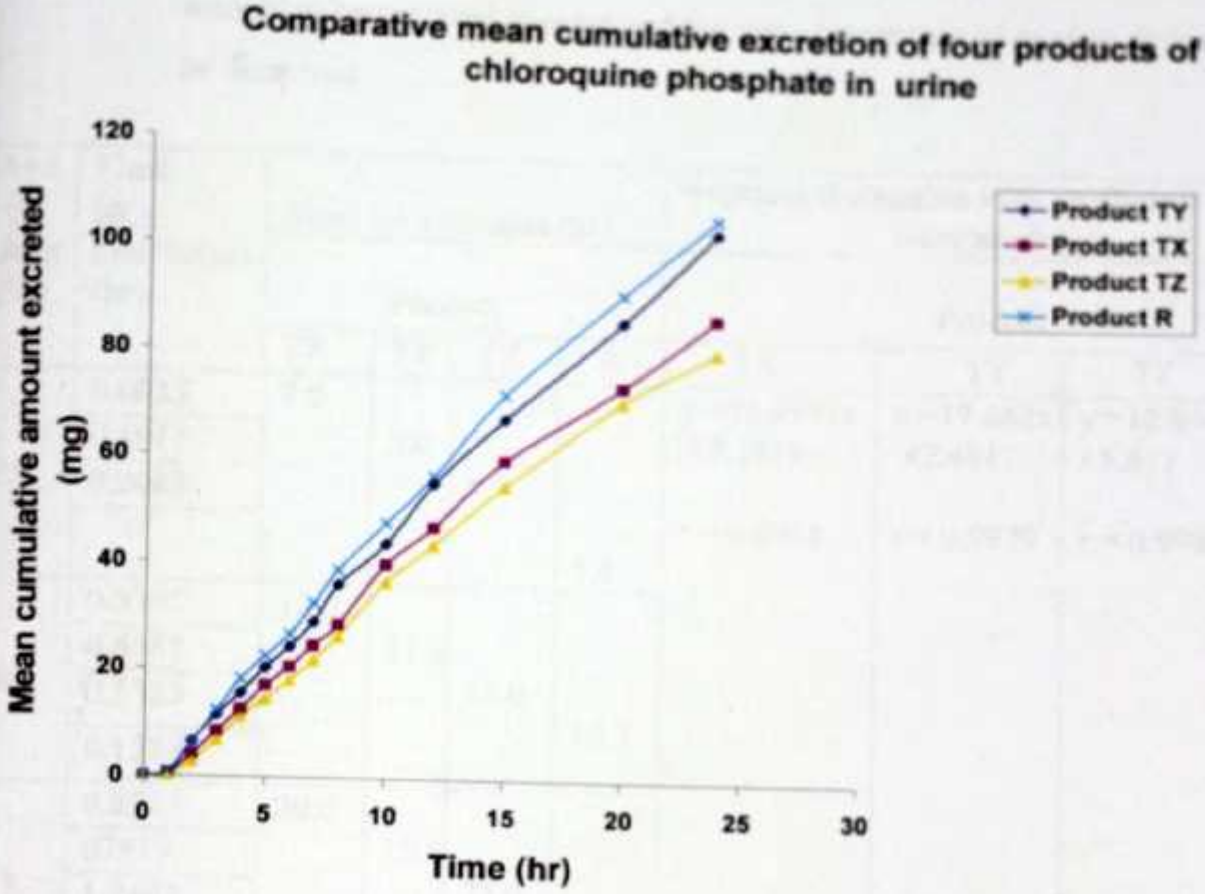


Figure 3.15: Comparative mean cumulative excretion of four products of chloroquine phosphate in urine

3.7.1 *IN VITRO-IN VIVO* CORRELATION ASSESSMENT

Table 3.24: Equations for cumulative amount excreted and dissolution curves of products TX, TY, TZ and R

PRODUCT	EQUATION	
	Dissolution curve	Cumulative amount excreted curve
TX	$y = -0.0093x^2 + 1.4373x + 24.347$	$y = -0.0188x^2 + 4.911x - 3.038$
TY	$y = -0.012x^2 + 2.0861x + 0.8716$	$y = -0.0249x^2 + 4.4328x - 3.7573$
TZ	$y = -0.009x^2 + 1.6428x + 5.9549$	$y = -0.0195x^2 + 4.1022x - 4.1744$
R	$y = -0.0112x^2 + 1.718x + 33.847$	$y = -0.0344x^2 + 5.4304x - 3.2405$

Table 3.25: Correlation between times required for a given percentage of cumulative amount to be excreted in urine and the same percentage of *in vitro* dose to be dissolved

Amt of drug (%)	Time for dissolution (hr)	Time for excretion (hr)				Regression equation with coefficient of correlation (r)			
		Product				Product			
		TX	TY	TZ	R	TX	TY	TZ	R
25	0.0083	7.0	5.6	6.0	5.1	y =13.9991x +7.1419 r = 0.9968	y =17.842x +2.4917 r = 0.9979	y =12.844x +5.677 r = 0.9988	y =16.641x +7.6644 r =1
	0.1917								
	0.2083								
	-								
50	0.3300	12.0	11.2	13.0	10.3				
	0.4583								
	0.5333								
	0.1583								
75	0.8833	20.0	16.5	22.0	15.7				
	07917								
	1.2667								
	0.4833								
80	1.075	21.7	18.3	23.7	17.1				
	0.8917								
	1.4167								
	0.5667								

Respective dissolution profiles of products were compared with corresponding cumulative amount excreted profiles.

Data was grouped so as to have a common dissolution time axis for all products.

Data and equations were generated by default with Microsoft Excel using Table 3.24 and figure 3.16

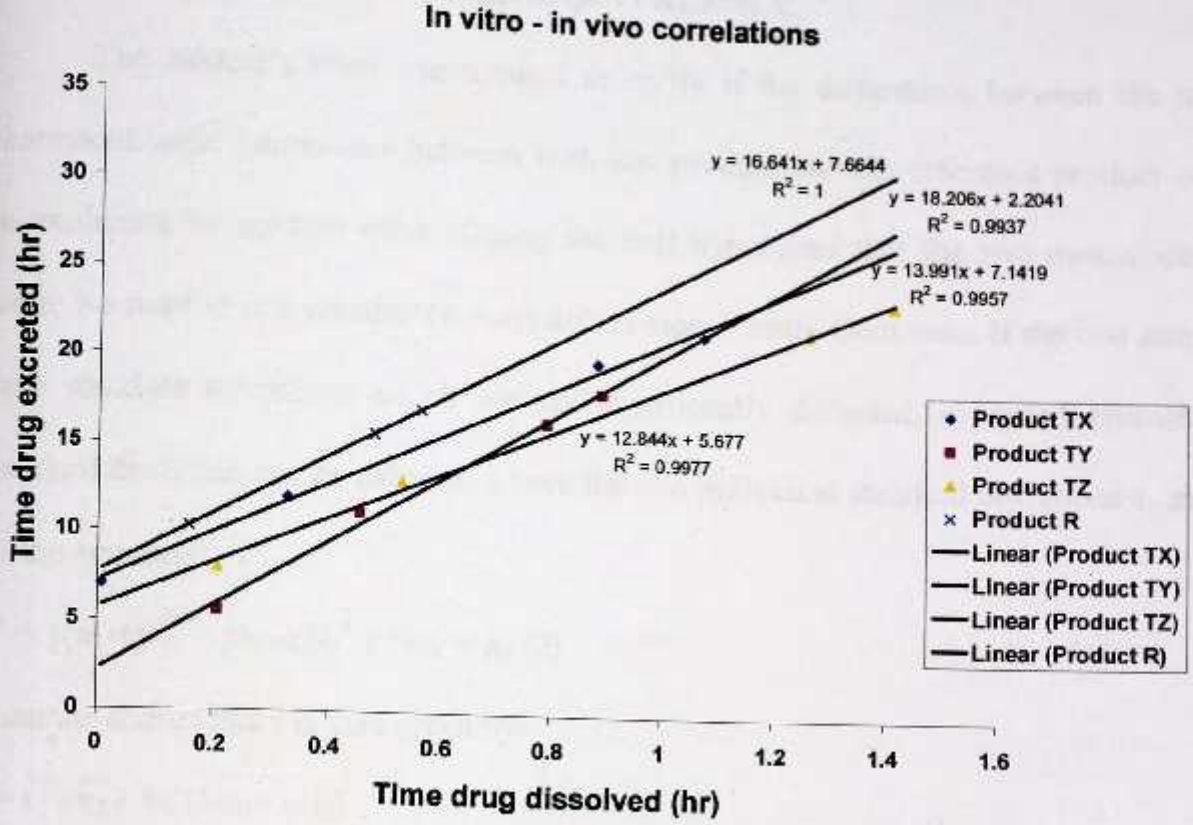


Figure 3.16: *In vivo-in vitro* correlation plot

3.7.2 STATISTICAL EVALUATION OF BIOEQUIVALENCE OF PRODUCTS TX, TY, TZ AND R

Assessment of bioequivalence of products TX, TY, TZ and R

Table 3.26: Pharmacokinetic parameters of product TX, TY, TZ and R for the assessment of bioequivalence. Data has been reported as mean ± standard deviation

Parameter	Products			
	TX	TY	TZ	R
Mean cumulative amount excreted (mg)	87.43±25.65	103.8±20.82	81.04±18.19	106.5±24.22
Mean peak excretion rate (mg/hr)	7.73±2.68	10.56±3.22	6.63 ±1.72	9.11±2.96
Mean time for peak excretion rate (hr)	7.8±4.9	5.8±4.1	8.8±3.0	4.8± 2.8

3.7.3 STATISTICAL TEST OF BIOEQUIVALENCE

The student's t-test was applied to verify if the differences between the mean pharmacokinetic parameters between each test product and the reference product could be explained by random error. Taking the null hypothesis that the two means are the same, we need to test whether $(\overline{x_1} - \overline{x_2})$ differs significantly from zero. If the two samples have standard deviations which are not significantly different, a pooled estimate of standard deviation can be calculated from the two individual standard deviations s_1 and s_2 by the equation:

$$S^2 = \{(n_1-1)s_1^2 + (n_2-1)s_2^2\} / (n_1 + n_2 - 2)$$

It can be shown that t is then given by:

$$t = \overline{x_1} - \overline{x_2} / S\sqrt{(1/n_1 + 1/n_2)}$$

F-test

F- test considers the ratio of the two sample variances; that is,

$$F = s_1^2/s_2^2$$

s_1^2 and s_2^2 being allocated in the equation so that F is always ≥ 1. The null hypothesis adopted is that the population from which the samples are taken is normal, and that the population variances are equal. If the null hypothesis is true then the variances ratio should be close to 1. Differences from 1 occur because of random variation but if the difference is too great it can no longer be attributed to this cause.

Using products TX and R as an example, the variances for products TX and R for cumulated amount of chloroquine phosphate excreted are respectively 7.1663 and 8.7557

$$F_{5,5} = 8.7557/ 7.1663 = 1.2218$$

These samples were each administered to six subjects so the number of degrees of freedom in each case is 5, as indicated by the subscripts.

$F_{Critical}$ (two-tailed) = 7.146

The confidence level was 95 percent. Since the experimental F value was less than the critical, the null hypothesis is retained. Since the two standard deviations are not different a pooled standard deviation is calculated.

S = 2.8215

$T_{calculated}$ = 0.8470

There are 10 degrees of freedom, so the critical value of t (p = 0.05) is 2.23. Since the experimental t value is less than the critical value of t, the difference between the cumulated amounts of chloroquine phosphate excreted from products TX and R is insignificant at the 95 percent level and the null hypothesis is retained.

The other pharmacokinetic parameters were taken through the same procedure and the results obtained tabulated below.

Table 3.27: Results of statistical tests of bioequivalence of products TX, TY, TZ with R

Pharmacokinetic parameter	$F_{5,5}$ experimental	$F_{5,5}$ critical	Comment on H_O	$t_{experimental}$	$t_{critical}$	Comment on H_O
Product TX vrs. R						
Cumulative amount excreted (mg)	1.119	7.146	Valid	1.323	2.23	Valid
Peak excretion rate (mg/hr)	1.221	7.146	Valid	0.8470	2.23	Valid
Time for peak excretion rate (hr)	3.060	7.146	Valid	1.309	2.23	Valid
Product TY vrs R						
Cumulative amount excreted (mg)	1.353	7.146	Valid	0.2020	2.23	Valid
Peak excretion rate (mg/hr)	1.186	7.146	Valid	0.8169	2.23	Valid
Time for peak excretion rate (hr)	2.185	7.146	Valid	0.4926	2.23	Valid
Product TZ vrs R						
Cumulative amount excreted (mg)	1.774	7.146	Valid	2.057	2.23	Valid
Peak excretion rate (mg/hr)	2.953	7.146	Valid	1.771	2.23	Valid
Time for peak excretion rate (hr)	1.155	7.146	Valid	2.395	2.23	Not valid

H_o represents the null hypothesis

The null hypothesis adopted is that the population from which the samples are taken is normal, and that the population variances are equal.

3.7.4: CALCULATION OF RELATIVE BIOAVAILABILITY OF TESTED PRODUCTS

The relative bioavailability of a test product is given by the ratio percentage of mean cumulative amount of test chloroquine phosphate excreted to the reference. It can be shown that after a single dose of a drug;

$D_{U^{\infty}} = f\,FD$

Where; f = fraction of the drug reaching the circulation which is excreted in the urine

$D_{U^{\infty}}$ = cumulative amount of unchanged drug excreted in the urine in infinite time after drug administration

F = fraction of the dose, D, absorbed

Assuming that f is constant and with the same dose, $F \alpha D_{U^{\infty}}$,

$F_y/F_x = (D_{U^{\infty}})_y / (D_{U^{\infty}})_x$ = bioavailability of y relative to x

Hence, Relative Bioavailability of Product TX = (87.433/106.48) x 100

= 82.11%

Relative Bioavailability of Product TY = (103.84/106.48) x100

= 97.52%

Relative Bioavailability of Product TZ = (81.042/106.48) x100

= 76.11%

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

4.1.1 COMPOUND IDENTIFICATION

4.1.1.1 UV Spectroscopy

The absorbance ratio of chloroquine should fall within a range of 1 to 1.15 when the ratio of absorbance at 343nm to that at 329nm is taken [46]. The pure, reference and test samples TX, TY and TZ had values of 1.04, 1.05, 1.05, 1.06 and 1.10 respectively. These values obtained fell within the specified range. The samples are then said to have passed the identity test as stated.

4.1.1.2 Thin Layer Chromatography

The presence of chloroquine in the reference and test samples was confirmed by comparing their R_f values with that of the pure chloroquine sample. The TLC showed approximately the same R_f. values for both the pure powder and the ones extracted from the tablets of the reference and test samples and thus confirmed the identity of the active principles in the tablets. The presence of the one band of the same shape and colour on the chromatoplates respectively for each of the spotted samples after development was an indication of the absence of significant degradation in the products. This thus supports the homogeneity and the purity of the samples under review (Refer to Table 3.2 and Figure 3.1).

4.1.1.3 Melting Point

Extracted chloroquine phosphate has a melting point range of 206°C to 209°C [46]. The mean melting point range values obtained for all the samples were within the above limits. The melting range for both the extracted chloroquine from the formulated products and the reference powder were sharp indicating a high level of purity. The narrowest range was registered by product R (206.4-207.7°C) whiles the largest range was indicated by product TZ (206.3-208.7°C). However, that of product R was the closest to the reference powder (206.5-206.9°C) (Refer Table 3.3). The above information gives an indication of the high probability of the samples being chloroquine and also shows the level of purity of the samples.

4.1.1.4 Test for the presence of Phosphate in the samples

The test for the presence of phosphate further confirmed the presence of chloroquine phosphate in all the samples as well as the pure powder (Refer Table 3.4).

4.1.2 UNIFORMITY OF WEIGHT TEST

For tablets of average weight 250mg or more, not more than two of the individual weights should deviate from the average weight by more than the ±5 percent and none should deviate by more than twice the percentage cited above [57]. The results shown in Table 3.5 indicated that none of the 20 tablets weighed deviated by more than ±5 percent. The samples therefore passed the weight uniformity test.

4.1.3 *IN VITRO* DISSOLUTION TESTING

Dissolution test *in vitro* measures the rate and extent of dissolution of a drug product in an aqueous medium in the presence of one or more excipients. In the gastrointestinal tract, a drug is considered to be dissolving in an aqueous medium [26]. Drug product dissolution in aqueous medium therefore becomes an important condition for systemic absorption. It was in respect of this requirement that the release characteristics of the drugs were investigated before the *in vivo* testing. Thus since dissolution tests are used as quality control to measure variability between drug products which may be reflected by *in-vivo* performance, the *in vitro* test may be a quick method of ensuring *in vivo* performance. Going by the monograph requirement of BP 2002, for each of the six tablets tested for a product at 45 minutes, the amount of active ingredient in solution should not be less than 70 percent of the prescribed amount [57].

With the exception of product TZ all the six tablets for products TX, TY and R tested passed this requirement with respective mean percent drug dissolved of 70.789, 84.116, and 86.196 whiles that of product TZ was 63.855 percent (Refer Table 3.7). With reference to figure 3.3, it is realised that the dissolution profiles of all the tested products were different at all time points. The USP-XXII (1990) states a tolerance of not less than 75 percent of labelled amount of chloroquine phosphate dissolved in 45 minutes [46].

Using the limit of the USP as a benchmark, product TY and R could be said to have the same release characteristics and hence bioequivalent. Products TX and TZ would have failed the dissolution test. However, three products TY, TZ and R whose labels indicated compliance with BP specifications, satisfied those specifications. TX had no pharmacopoeial specification on its label. Products TY and R can be said to have met

the dissolution requirements of both the BP and USP. For the purpose of this work poor dissolution profile products were to be subjected to further *in vivo* bioavailability studies. However, product TY was also subjected to this test even though it satisfied the BP dissolution requirements, because of its initial slow release profile before 45 minutes, which is an indication of poor formulation. Product TX, which had neither BP nor USP pharmacopoeial label claims, marginally passed the BP requirement but failed the USP requirement. It was therefore also subjected to bioavailability studies. Product TZ which is believed to be the most widely used product for the treatment of malaria in Ghana was not released on time, which means that the product would not be bio-available even if the right dose is taken.

The clear differences in profile as indicated by all the products could not be explained by such factors as physicochemical characteristics of the drug, dosage form, dissolution medium, temperature and the level of agitation, because they were the same for all the products. Likely reasons for the difference could therefore be the selection of excipients for the formulation and the manufacturing technique as the choice of an excipient and manufacturing technique becomes the preserve of the manufacturer. Since all the products came from different manufacturers, such an observation is a common occurrence. In effect it could be observed that not all the products were well formulated because not all the products passed the BP 2002 dissolution requirements.

4.1.4 ASSAY

Chloroquine phosphate powder BP contains not less than 98.5 percent and not more than 101.1 percent of the label claim calculated with reference to the anhydrous substance. Similarly chloroquine phosphate tablets should contain 92.5 to 107.5 percent

of the stated or prescribed amount or label claim [57]. The reference powder met the BP 2002 monograph requirement with a mean content of 99.48 percent. It could be seen from the results obtained in Table 3.14 that the percentage content of chloroquine phosphate obtained in both the titrimetric and UV spectrophotometric methods for products TX, TY and R were within the specified range given in the official compendia, hence they passed the assay test. Product TZ did not comply with the BP 2002 monograph requirement with a mean content of 87.62 and 90.43 percent obtained in the titrimetric and UV spectrophotometric method respectively.

The failure in active ingredient content in the quality control test on product TZ was further confirmed by the UV identification test for the samples. The same concentrations of the samples were used for the UV measurements however the mean absorbance values for TZ were so significantly lower than the absorbance values of the other samples including the pure chloroquine powder even though the A_{343}/A_{329} was between 1.0 and 1.15 for all the samples. Therapeutic failure with the use of product TZ may occur due to pharmaceutical failure resulting from poor quality of product TZ.

Using the designed and validated HPLC assay method, it gave percentage contents of 101.30, 103.16, 94.23, and 103.50 percent respectively for products TX, TY, TZ and R. This was so because it was observed that the standard deviation, the relative standard deviation, and the standard error of the mean obtained for chloroquine using the UV and the trimetric methods were higher than those from the HPLC. This indicates that the HPLC had lower margin of error compared with the UV and titrimetric methods. Thus, the HPLC method was selective, accurate, precise, and reproducible and eliminated

the extraction process used in the titrimetric and UV methods, which are associated with high error as some of the active ingredient, might be lost during the extraction processes.

4.1.5 DESIGN OF HPLC METHOD OF ANALYSIS

Development of new, more reliable or more sensitive methods of analysis is a concern of utmost importance in the field of drug analysis. This is basically because in a drug quality assurance system, products are either released or rejected on the basis of very good analytical results. However, any new method must be proven to be reliable in the sense that it performs the intended purpose as adequately and consistently as designed. This would help provide the confidence that any data realised during routine analysis with it would be meaningful and acceptable.

A method for the analysis of amodiaquine, chloroquine and their monodesethyl metabolites in biological samples using a mobile phase consisting of methanol: phosphate buffer (0.1M): perchloric acid and C_{18} bonded stationary phase has been previously reported [58]. Thus an attempt was made to design a method based on the above mobile phase. The selection of methanol: phosphate buffer: perchloric acid was based on the fact that buffered mobile phase, resists changes in pH so that the analyte and silica will be consistently ionized to maintain consistent retention and selectivity resulting in reproducible chromatography. Perchloric acid was added to modify the mobile phase since it has the ability to precipitate any urine protein that might interfere. Various mobile phase combinations of methanol (99.8%): 0.1M sodium dihydrogen orthophosphate at varying pH (4.5-2.0): perchloric acid (2.5% $^{v}/_{v}$) were used to check for the retention times of pure chloroquine phosphate powder and amodiaquine hydrochloride. The combination that gave the most satisfactory resolution and retention time for pure chloroquine

phosphate and amodiaquine hydrochloride was methanol: 0.1M sodium hydrogen orthophosphate (pH 3): perchloric acid (2.5% $^{v}/_{v}$) in the ratio 24:75.75:0.25. Hence, the use of the mobile phase reported in 3.5.1.1. The absorbance of the drugs was monitored at 333 nm and no endogenous compound interfered at this wavelength.

The reliability of the method for the evaluation of the quality of pharmaceutical products had to be investigated by seeing as essential the following analytical performance parameters: accuracy, precision, selectivity, sensitivity, linearity and limits of detection.

Applying the procedure repeatedly to separate identical samples drawn from the same homogenous stock of concentration (nominal, 0.002%$^{w}/_{v}$), the confidence limits of the mean was found as $2.044 \times 10^{-3} \pm 3.717 \times 10^{-5}$ %$^{w}/_{v}$ at 95 percent confidence level (Refer subsection 3.6.2e). This yielded a relative error of 2.80 percent (Subsection 3.6.2f) and a precision in terms of RSD of 1.96 percent (Subsection 3.6.2b). With respect to the fact that only HPLC analysis with modern autosamplers under a good manufacturing practice yields a precision value in terms of RSD of less than 2 percent [54], the value obtained for this procedure using a valve injector has a good potential to produce a reliable data for quality evaluations. From Table 3.11 and subsection 3.6.2f, it is realised that the deviations of the observations from the true mean of 1.9884×10^{-3} %$^{w}/_{v}$ were either positive or negative. This probably suggests that the errors in the measurements are random.

A calibration curve was employed in the estimation of sample concentration [23, 55]. The equation of the line obtained was $y = 647.6x - 0.0826$ with a correlation coefficient of 0.9990. This equation conforms to the basic equation of a straight line. The

correlation coefficient as stated suggests that there was a high degree of correlation between the peak area ratios and concentration within the range of analysis, 0.001-0.003%w/v.

Under the set of experimental conditions, the lowest level concentration of analyte that was detected but not necessarily quantitated was 0.0005%w/v. This was because from Table 3.10a, it was realised the signal for analyte concentration of 0.001%w/v was approximately twice that of 0.002%w/v. However, that of 0.0005%w/v was far less than half the value for 0.001%w/v. Respectively, signals for solutions of concentration 0.0005, 0.001 and 0.002%w/v are as follows; 0.1967, 0.5928 and 1.1935. This therefore set the limit of quantification of the procedure at 0.001%w/v. The upper limit of quantification was also set at 0.003%w/v because there was no proportional increase in signal on increasing analyte concentration from 0.003%w/v.

One other important parameter of an analytical method is its selectivity. From figures 3.7-3.9, it was shown that the method was selective for the quantitative detection of chloroquine in the solution of either the reference powder or unchanged form in a biological sample such as urine.

The between-run precision of the procedure was investigated to assess the likely variations in results from day to day. This was because the method was going to be applied to a set of samples over a period of time which could run into weeks. It was therefore necessary to ascertain if the results obtained at different times of the analysis could be reliable and comparable. From Table 3.12 and subsection 3.6.3.1, there was no significant difference between the variances compared at a confidence level of 95 percent for a two-tailed F-test. Hence, the method can be said to be reproducible.

Evidence of correlation between the new method and the official BP 2002 and USP XXII 1990 methods for the assay of chloroquine phosphate tablets was also investigated. Using the Analysis of variance at 95 percent confidence level, no significant difference was found between the variances and means of the HPLC, UV and the titrimetric methods (Refer subsection 3.6.4.1d). This means that within certain limits all the three methods have equal precision. However, the calculation of the absolute precision of the three methods at the same confidence level indicate that within limits of experimental error, the HPLC method has a better precision than the UV which in turn has a better precision than the titrimetric method as they respectively have values of 6.872, 9.6354 and 10.2078 (Refer subsection 3.6.4.1c).

Assessing the absolute error of the mean for the three methods, the HPLC results were found to be more accurate than those from the UV method which also were more accurate than those from the titrimetric method as they have respective values of 0.55, 0.58 and 3.58 percent (Refer subsection 3.6.4.1a).

4.1.6 *IN VIVO* DISSOLUTION TESTING

The bioavailability of a drug may be reproducible among fasted individuals [59]. This is because the nature of the diet may affect the plasma drug level through variable absorption in the presence of food or a change in the metabolic clearance of the drug [59]. Hence, the study was carried out in fasted individuals. The study was carried out over 24 hours to allow enough time for removal of adequate amount of the drug from the body.

Pharmacokinetically, renal filtration and excretion of a drug depend on its concentration in the blood such that, the pattern of drug excretion in the urine is affected

by the rate and extent of drug absorption. If the renal clearance is assumed to be constant, it can be said that the urinary excretion rate is proportional to plasma concentration and plotting urinary excretion rate against time is like plotting plasma concentration against time. In that case, the cumulative amount of drug excreted in urine, peak urinary excretion rate and the time for peak excretion used in assessing the urine data become analogous to the AUC, C_{max} and the t_{max} respectively, which are parameters reflective of the systemic exposure of the drug. This therefore explains the basis of the urinary pharmacokinetic variables for the assessment of bioavailability and bioequivalence in this study.

The mean amounts of chloroquine excreted were 87.43 ± 25.65mg, 81.04 ± 18.19mg, 103.8 ± 20.82mg and 106.5 ± 24.22mg for products TX, TZ, TY, and R respectively (Refer Table 3.26). Walker *et al* in a similar study reported that the total excretion of chloroquine in the first 24 hours after oral drug administration was 83 ± 18mg [60]. The high standard deviations obtained in the current study are quite normal as they are in agreement with that already reported. Individual variations in the amount of chloroquine phosphate excreted were expected. The variations may be attributed to age, weight difference and genetic make-up of the subjects [32]. As indicated by the mean cumulative amount excreted, there was a clear difference in performance between the test products TZ and the reference product R. This observation suggests that *in vivo* performance may be predicted by *in vitro* studies as this product was not released on time. Thus, it is expected that all things being equal, a drug that has passed an official dissolution requirement *in vitro* should have a satisfactory systemic performance.

The mean time for peak excretion rate for product TX, TY, TZ and R were 7.8 ± 4.5hr, 5.8 ± 4.1hr, 8.8 ± 3.0hr, 4.8 ± 2.8hr respectively. The F- test for the comparison of standard deviations shows that there was no significant difference between the variances of the mean time for peak excretion rate for all the test products and the reference product at 95 percent confidence level (Refer Table 3.27). The student t-test was employed to check for the significant difference between the means of the time for peak excretion rate for the test product and the reference product at 95 percent confidence level. It showed that there was no significant difference between the means of the time for peak excretion rate obtained for the test products TX, TY and the reference product R. However, there was a significant difference between the means of the time for peak excretion rate obtained for the test product TZ and the reference product R (Refer Table 3.27). This observation further shows similarity in the results of the *in vitro* test where it was realized that product TZ was not released on time.

The mean peak excretion rates were 7.73 ± 2.68mg/hr, 10.56 ± 3.22mg/hr, 6.63 ± 1.72mg/hr, and 9.11 ± 2.96mg/hr for products TX, TY, TZ and R respectively. The results of the F-test showed no significant difference between the variances of the test products and that of the reference product. The t-test also showed no significant difference between the means of the peak excretion rate at 95 percent confidence level.

Analysis of chloroquine phosphate in urine revealed that 8.74, 8.10, 10.38 and 10.65 percent of the administered dose for test products TX, TZ, TY and reference product R respectively were excreted in 24 hours. Gustafsson L.L., in a similar study stated that 10 percent of the administered dose in his study was excreted in 24 hours [21].

The results from the present study are closely in agreement with what has already been reported.

Multisource ("generic") pharmaceutical products such as chloroquine phosphate need to conform to the same standards of quality, efficacy and safety as required of the originator's product. Specifically, the multisource product should be therapeutically equivalent and interchangeable with the innovator's product.

Thus, to safeguard standards with respect to quality, safety and efficacy, the World Health Organization (WHO) stipulates in the guideline registration requirement to establish interchangeability that the acceptance range for bioequivalence should be between 80 to 125 percent [61]. Hence, with reference to product R, the relative bioavailability of products TX, TY and TZ were respectively 82.11, 97.52 and 76.11 percent (Refer subsection 3.7.4). This result clearly shows that product TZ has failed the WHO standard and therefore is not bioequivalent with the reference product. Products TX and TY however, passed the WHO standard.

From literature, a bioequivalent product should produce no significant difference in all pharmacokinetic parameters tested [26]. These were the cumulative amount of unchanged drug excreted, peak urinary excretion rate and the time for peak excretion rate. Subjecting these parameters to statistical analysis (Table 3.27), it was realised that there was a significant difference between the means of the time for peak excretion rate obtained for the test product TZ and the reference product R at 95 percent confidence level and this further confirms the fact that product TZ and R are not therapeutically equivalent or interchangeable. Because of the poor quality and low bioavailability of product TZ it will lead to drug under-dosage which can promote the development of

resistance. Products TX and TY can be said to be bioequivalent with reference product R with regard to the tested parameters as no significant difference at 95 percent confidence level was found among the tested parameters when the F-test and the t-test were employed.

4.1.7 *IN VITRO-IN VIVO* CORRELATIONS

There has been much discussion between the pharmaceutical manufacturers and the Food and Drugs Administration on whether *in vitro* dissolution tests may substitute for an *in vivo* bioavailability study [26]. According to USP XXII (1990), there is no known medically significant bioequivalent problem with drug substances where 75 percent of the drug substance is dissolved in water at 37°C using either the paddle or basket method at the usual speed. This implies that *in vivo* dissolution results can be predicted by *in vitro* studies. Not all the drugs tested under this study had mean percent drug dissolved greater than 75 percent in 45 minutes when their respective dissolution profiles were examined. The values were 70.79, 86.12, 63.86 and 86.20 percent respectively for products TX, TY, TZ and R (Refer Table 3.7). After the *in vivo* assessment only product TZ was found not to be bioequivalent, making the results of this study not entirely consistent with that of the USP in using dissolution data to predict bioavailability.

However, in the present study *in vitro-in vivo* correlations were found when dissolution characteristics of the drug products were individually considered. Linear correlation was found between the time taken for a given percentage of the *in vitro* dose of the drug to dissolve and that for the same percentage of the cumulative amount to be excreted. Percentages considered for each drug product were 25, 50, 75 and 80 (Refer

Table 3.25). A good linear correlation was obtained for each product between *in vitro* and *in vivo*. The coefficient of correlation for products TX, TY, TZ and R are 0.9968, 0.9979, 0.9988 and 1 respectively (Refer Table 3.25). As can be seen from figure 3.16 and the coefficients of regression in Table 3.25 the *in vitro-in vivo* correlation plot presents a scatter whose accuracy could be adequate for predictable purposes. Hence, the dissolution rate of the products used for this study correlates with the rate of absorption of the drug into the blood.

4.2 CONCLUSIONS

The following conclusions have been drawn from this study

- The content analysis of the reference product and the three locally manufactured products showed that the specified amounts were present in products R, TX and TY but not in product TZ, however the *in vitro* dissolution tests revealed possible formulation problems with products TY and TZ.
- Chloroquine phosphate in a biological sample such as urine can be analysed accurately and precisely by HPLC with a mobile phase consisting of methanol (99.8%): phosphate buffer (0.1M, pH 3): perchloric acid (2.5%$^{v}/_{v}$) (24: 75.75: 0.25) on a C_{18} column at a wavelength of 333nm using amodiaquine hydrochloride as the internal standard.
- In the analysis of chloroquine phosphate in the urine of six healthy subjects an average of 8.74, 8.10, 10.38 and 10.65 percent of the administered dose for test

products TX, TZ, TY and reference product R respectively were excreted in 24 hours

- Only products TX and TY are bioequivalent with the reference product R with relative bioavailabilities of 82.11, and 97.52 percent respectively that of TZ failed with relative bioavailability of 76.11 percent.
- Statistical analysis also showed that there was a significant difference between the means of the time for peak excretion rate obtained for the test product TZ and the reference product R whiles there were no significant differences between the standard deviations and the means of all the pharmacokinetic parameters investigated for test products TX and TY with reference product R.
- The above observations support the view that the suspected treatment failure associated with chloroquine therapy may not necessarily be due to resistance but rather formulation problems.
- *In vivo* dissolution results correlates with the *in vitro* dissolution test.

4.3 RECOMMENDATIONS

- This study should be carried out by other research centres in the country.

  All generic chloroquine phosphate products on the Ghanaian market should be analyzed for bioequivalence to confirm or otherwise whether treatment failure with chloroquine is as a result of poor quality drugs or drug resistance.

- Post-market surveillance on the new Artemisinin-based combination therapy should be conducted in all research centres to avert possible formulation problems in the near future.
- More funds should be made available by pharmaceutical companies for such laudable research.
- Manufacturers should be supported by Food and Drugs Board to improve GMP compliance.

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6.0 APPENDICES

6.1 APPENDIX C: DATA ON POTENTIOMETRIC TITRATION

Table 6.1: Standardisation of 0.1M $HClO_4$ (perchloric acid) using potassium hydrogen phthalate (KHP)

	Weight of potassium hydrogen phthalate (W)	
Volume (ml)	W1 = 0.3509g	W2 = 0.3510g
Final volume	17.10	35.30
Initial volume	0.00	18.00
Titre volume	17.10	17.30

Table 6.2: Blank titration of acetic acid with perchloric acid

Volume (ml)	1	2
Final volume	0.10	0.20
Initial volume	0.00	0.10
Titre volume	0.10	0.10

Average weight of potassium hydrogen phthalate = $\frac{0.3509 + 0.3510}{2}$

= 0.35095g

Average titre = $\frac{(17.10 + 17.30)}{2} - 0.1\text{ml}$ = 17.10ml

But 0.02042g of KHP ≡ 1ml of $HClO_4$

∴ 0.35095g = $\frac{0.35095}{0.02042}$ = 17.187ml of $HClO_4$

∴ Factor of $HClO_4$ = $\frac{17.187}{17.10}$ = 1.005

Table 6.3: Potentiometric points for pure chloroquine powder (0.2009g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	280	0
1.0	290	10
2.0	300	10
3.0	310	10
4.0	320	10
5.0	330	10
6.0	340	10
6.2	350	50
6.4	360	50
6.6	360	0
6.8	370	50
7.0	380	50
7.1	390	100
7.2	390	0
7.3	400	100
7.4	410	100
7.5	420	100
7.6	440	200
7.7	460	200
7.8	550	900
7.9	570	200
8.0	580	100

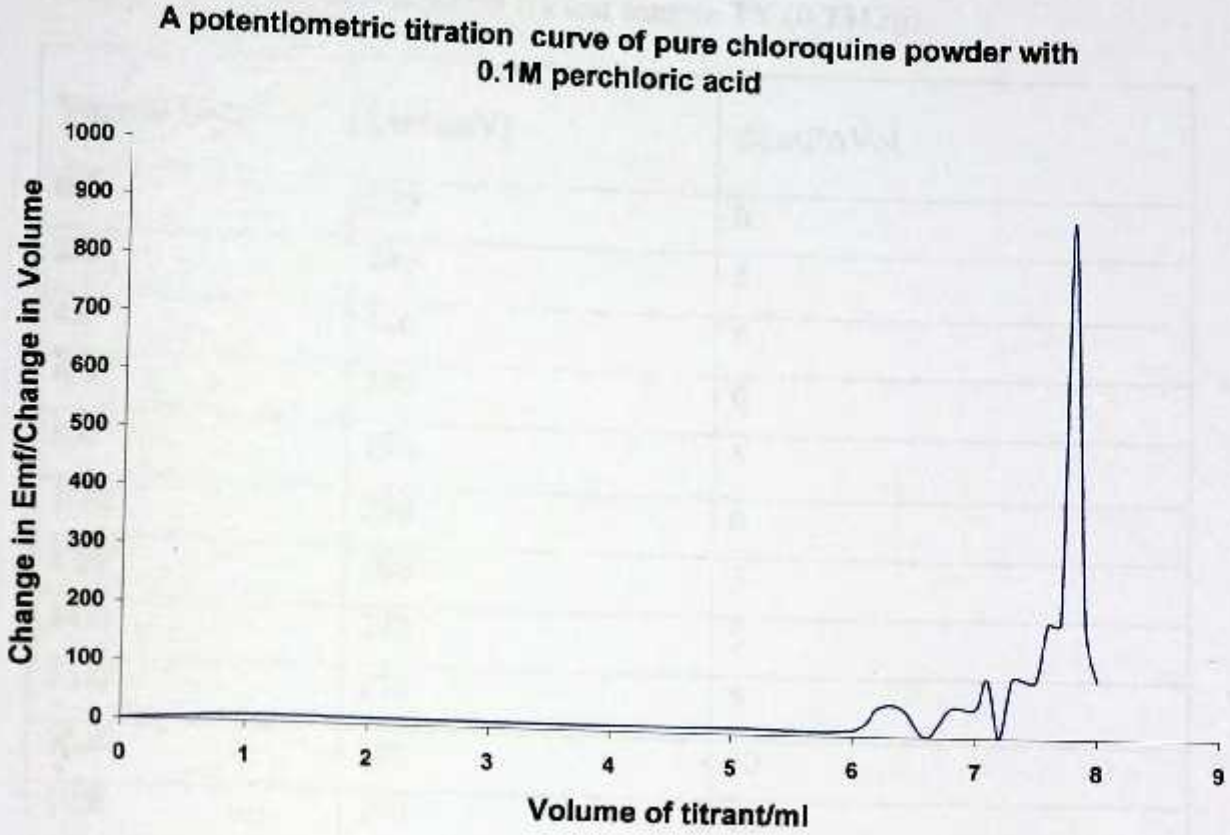

Figure 6.1: A potentiometric titration curve for pure chloroquine phosphate powder with 0.1M perchloric acid

Table 6.4.: Potentiometric points for test sample TY (0.7312g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	230	0
2.0	240	5
4.0	240	0
6.0	240	0
8.0	250	5
10.0	250	0
12.0	260	5
14.0	270	5
15.0	270	5
16.0	280	10
17.0	290	10
18.0	320	30
18.2	330	50
18.4	340	50
18.5	350	100
18.6	360	100
18.7	380	200
18.8	390	100
18.9	400	100
19.0	450	500
19.1	530	800
19.2	540	100
19.6	550	25
20.0	560	25
21.0	570	10

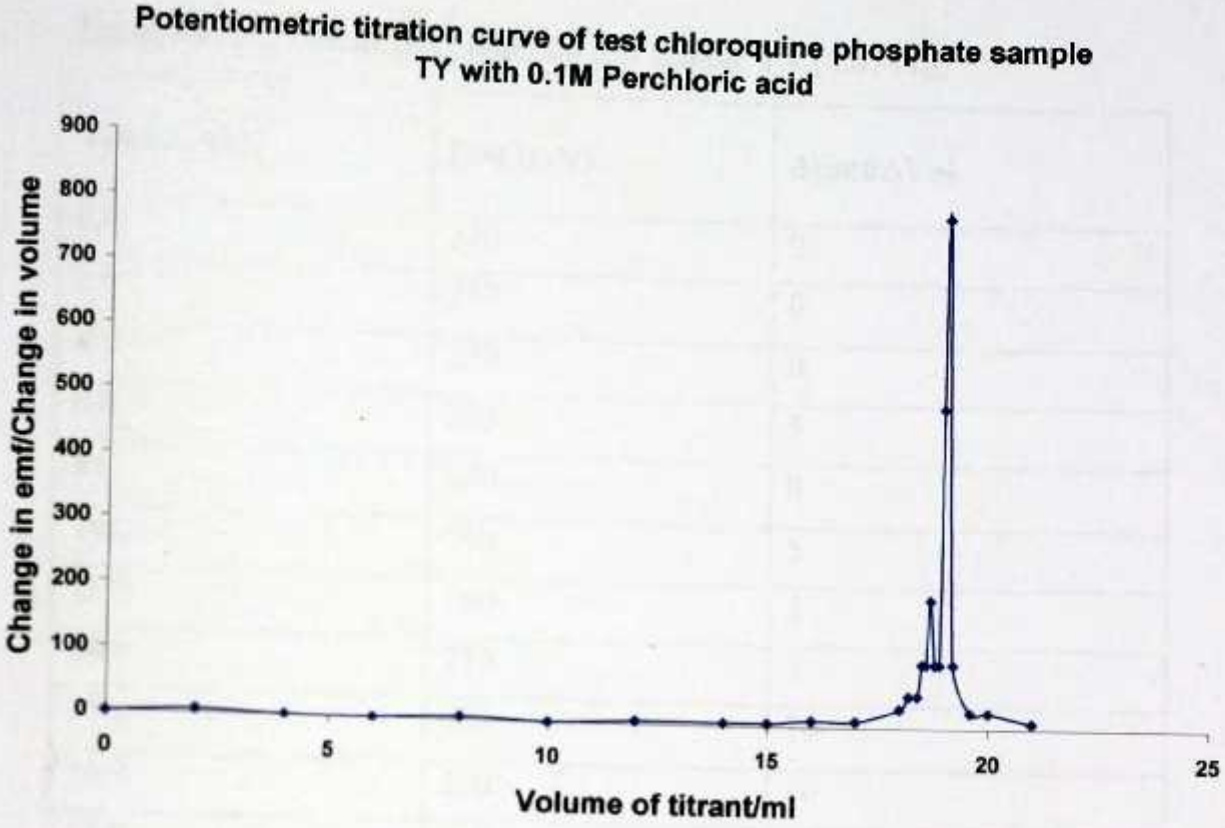

Figure 6.2: A potentiometric titration curve for test chloroquine phosphate sample TY with 0.1M perchloric acid

Table 6.5: Potentiometric points for test sample TX (0.6744g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	230	0
2.0	230	0
4.0	230	0
6.0	240	5
8.0	240	0
10.0	250	5
12.0	260	5
14.0	270	5
15.0	280	10
16.0	290	10
17.0	310	20
18.0	330	20
18.2	340	50
18.4	350	50
18.5	360	100
18.6	390	300
18.7	450	600
18.8	490	400
18.9	490	0
19.0	500	100
20.0	520	20
21.0	530	10

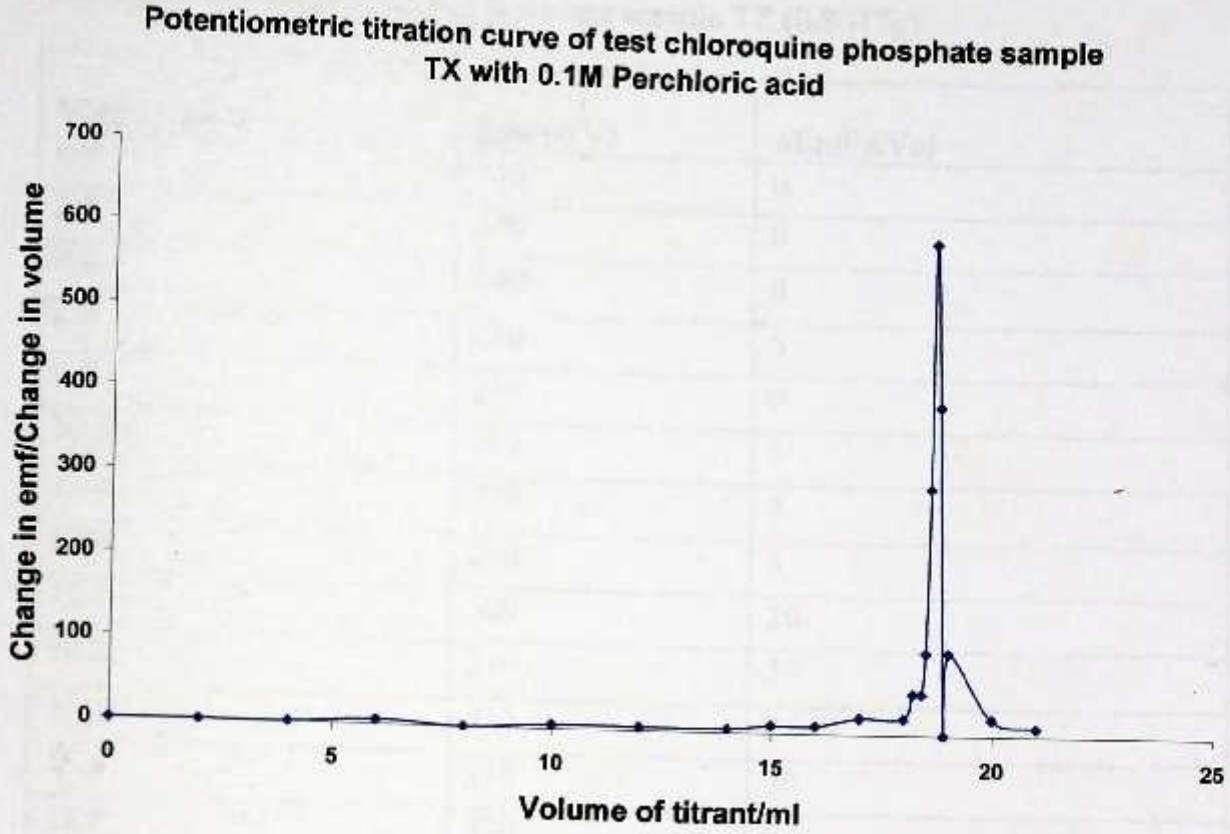

Figure 6.3: A potentiometric titration curve for test chloroquine phosphate sample TX with 0.1M perchloric acid

Table 6.6: Potentiometric points for test sample TZ (0.8017g)

Volume (ml)	Emf (mV)	ΔEmf/ΔVol
0.0	240	0
2.0	240	0
4.0	240	0
6.0	250	5
8.0	250	0
10.0	260	5
12.0	270	5
14.0	280	5
15.0	300	20
15.2	310	50
15.4	310	0
15.6	320	50
15.8	320	0
16.0	330	50
16.1	340	100
16.2	350	100
16.3	350	0
16.4	360	100
16.5	370	100
16.6	380	100
16.7	390	100
16.8	410	200
16.9	450	400
17.0	510	600
17.1	520	100
17.5	530	25
18.0	530	0
19.0	530	0
20.0	530	0
21.0	540	10