Pharmacokinetic Studies of Bulaquine, A Potent Antirelapse Antimalarial Agent

THESIS
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By
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Under the Supervision
of **Dr. R.C. Gupta**

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DEDICATED
TO
MY FAMILY

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE
PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled, "Pharmacokinetic Studies of Bulaquine, A Potent Antirelapse Antimalarial Agent" and submitted by **Nitin Mehrotra** ID. No. **2002PHXF022** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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(Nitin Mehrotra)

CONTENTS

ABBREVIATIONS USED

ACN	- acetonitrile
ADME	- absorption, distribution, metabolism, excretion
AIDS	- acquired immune deficiency syndrome
ANOVA	- analysis of variance
AS	- analytical standard
ASF	- analytical standard for feces
AUC	- area under the curve
3-BBQ	- 3 bromo bulaquine
BCS	- biopharmaceutics classification system
BMP	- blank monkey plasma
BRP	- blank rat plasma
BRbP	- blank rabbit plasma
BQ	- bulaquine
BRU	- blank rat urine
C	- concentration
CAD	- collision activated dissociation
C_{max}	- maximum concentration
C_p	- concentration in plasma
CDRI	- Central Drug Research Institute
CE	- collision energy
Cl	- clearance
CS	- calibration standards
CV	- coefficient of variation
CWIS	- combined working stock solutions of both IS
C_{wb}	- Concentration in whole blood
CYP	- cytochrome
DALYs	- disability-adjusted life years
DMOA	- dimethyl octylamine
DNA	- deoxy ribonucleic acid
DHFR	- dihydro folate reductase
DPBS	- dulbecco's phosphate buffered saline
ESI	-electro spray ionization
f-t	- freeze-thaw

F	- bioavailability (fraction absorbed)
g	- gram
GIT	- gastrointestinal tract
G6PD	-glucose 6-phosphate dehydrogenase
h	- hour
H	- high
H_C	- hematocrit value
HPLC	- high performance liquid chromatography
HTS	- high throughput screening
i.d.	- inner diameter
IPA	- iso propyl alcohal
IS	- internal standard
i.v.	- intravenous
kg	- kilogram
$K_{wb/p}$	- partition coefficient of whole blood to plasma
L	- low
LC	- liquid chromatography
LC-MS-MS	- liquid chromatography tandem mass spectrometry
LOQ	- limit of quantitation
LOD	- limit of detection
Log P	- octanol-water partition coefficient
M	- medium
MS	- mass spectrometry
min	- minute
mg	- milligram
µg	-microgram
ml	- milliliter
mM	- millimolar
MRM	- multiple reaction monitoring
MRT	- mean residence time
MWS	- mixed working stock
NCE	- new chemical entity
NOEL	- no observed effect level
ng	- nanogram

PABA	- Para amino benzoic acid
PD	- pharmacodynamic
PG	- propylene glycol
pH	- negative logarithm of hydrogen ion concentration
PK	- pharmacokinetic
pk_a	- negative logarithm of dissociation rate constant
p.o.	- per oral
PQ	- primaquine
3-BPQ	- 3 bromo primaquine
q.s.	- quantity suffecient
QC	- quality control
QCH	- quality control high
QCL	- quality control low
QCM	- quality control medium
RBC	- red blood cell
RP	- reversed phase
RSD	- relative standard deviation
SD	- standard deviation
SEM	- Standard error of mean
sec	- second
SGF	- simulated gastric fluid
SS	- stock solution
SIR	- selective ion recording
t	- time
$t_{1/2}$	- half life
t_{max}	- time of maximum concentration
UV	- ultra violet
UV/VIS	- ultra violet/ visible
V_d	- volume of distribution
v/v	- volume by volume
WS	- working stock
WSF	- working stock for feces

List of Tables

List of Figures

CHAPTER 1

Review of Literature

SECTION 1

Pharmacokinetics in Drug Development and Discovery

1.1.1 Pharmacokinetics and its Importance

It has been estimated that the current average cost to research and develop a new drug entity is as high as $ 670 million. Of equal importance is the fact that this discovery and development process on an average requires 12 years for completion [1]. One of the major factors contributing to this exorbitantly high cost and time requirements are mainly due to the fact that many drug candidates fail at multiple points along with their discovery and development track. Some might even make in to the market, only to be withdrawn following an initial launch. Often the withdrawals are for safety reasons, such as unforeseen occurrences of adverse reactions or cross-reactions that were only likely to become apparent with large-scale use or abuse, of the compound in populations of different ethnic groups [2]. Most of the withdrawals are due to improper pharmacokinetic (PK) characteristic of the drug candidate. Therefore, in drug discovery and development, it is of particular interest to estimate the PK behavior of drug candidates as early as possible, to select the most promising compounds for further development [3]. Pharmacokinetics is the study of the time course of absorption, distribution, metabolism and elimination (ADME) of the drugs in biological system and helps to understand the relationship between pharmacological and toxicological effects and concentration of a drug and its metabolites in the body fluid. Pharmacokinetics and toxicology traditionally had a major role during discovery. When studies revealed that poor PK properties caused development attrition, organization implemented vigorous testing during candidate selection to ensure that compound with poor PK properties did not advance.

The systematic application of pharmacokinetics can, therefore, considerably reduce the cost and time involved in new drug development. Today PK and metabolism are among the most highly interactive disciplines in the pharmaceutical research and development and intimately involve in the design of new chemical entities. The phases of PK are briefly divided in pre-clinical and clinical pharmacokinetics. The pre/non clinical pharmacokinetics done in suitable animals models provides information whether the drug is poorly absorbed to yield sub therapeutic levels or experience presystemic biotransformation, providing the guidance in terms of structure/formulation modification of new chemical entities resulting in the selection of best compound. It also provides the basis for the design of clinical trials. Clinical pharmacokinetics initiated during phase I and II helps in the optimization of therapy in terms of activity/toxicity. It is during the phase I clinical

trials that the first use of a novel drug candidate takes place in humans, once the proposed trial is deemed to be safe and independent local ethical approval has been secured. Phase I trials usually involve healthy volunteers, and focus on developing a safety profile for a candidate drug, as well as gaining ADME and pharmacology data. The starting dose is normally 1-10% of the no observed effect level (NOEL) seen in the most sensitive animal species, or a dose below that is expected to be pharmacologically active. If the results of phase I studies are satisfactory, the drug moves into phase II trials using 100-300 patients. Regulatory agencies set out the minimum animal data normally expected before phase II studies commence, but are usually flexible where there is a sound scientific justification. As the drug proceeds into phases II and III, long term studies of toxicity and follow up and/or additional safety pharmacology in animals also continue. The typical development process for a new drug is illustrated in Figure 1.1.1 [4].

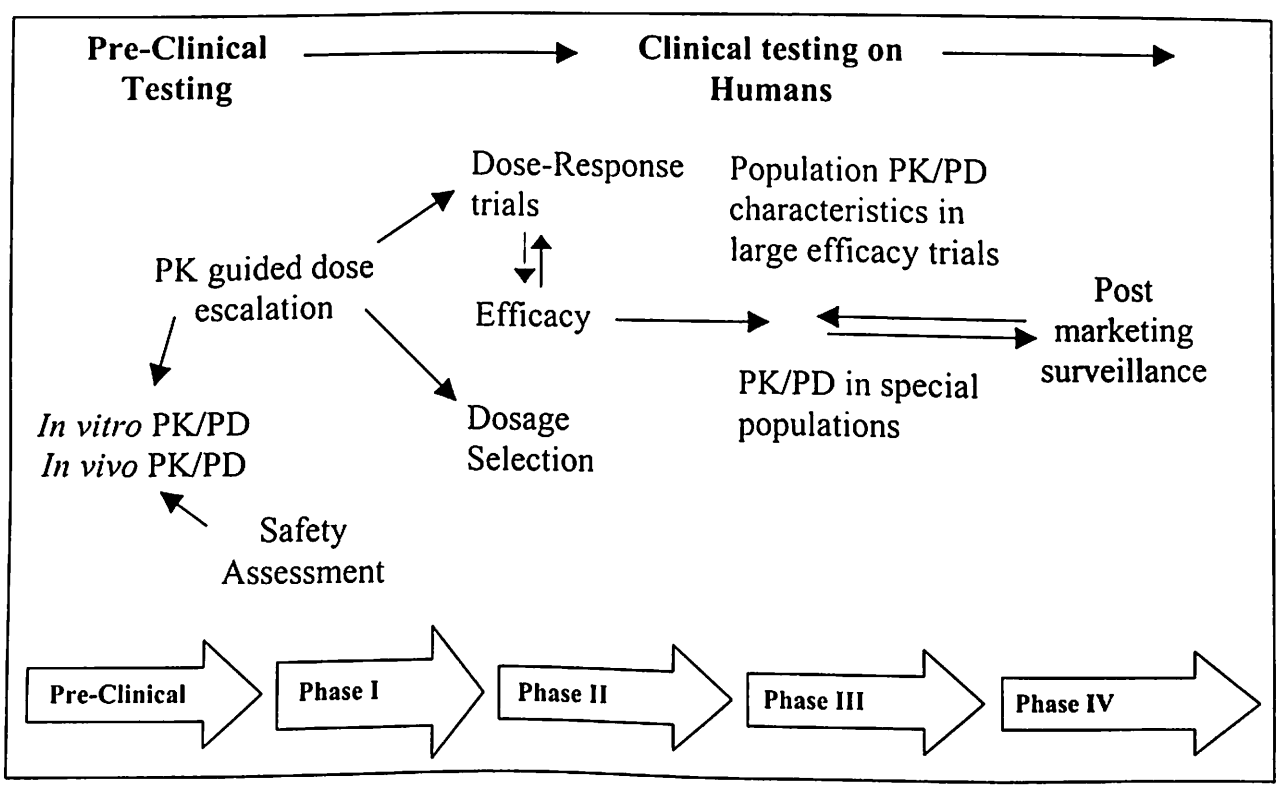

Figure 1.1.1. Stages of new drug development

In nutshell, the events following drug administration can be divided into two phases: a pharmacokinetic phase, in which the adjustable elements of dose, dosage form, frequency and route of administration are related to drug level-time relationships, and a pharmacodynamic phase, in which the concentration of drug at

the site of action is related to the magnitude of the effects produced [4]. Magnitude of both the desired actions and toxicity are functions of the drug concentrations at the site(s) of action. Highlighting the importance of pharmacokinetics in drug development and therapy, it has been reported earlier that 'what the body does to the drug is equally important as what the drug does to the body' [5]. Awareness of the benefits of understanding pharmacokinetics and concentration-response relationships has led to the extensive application of such information by the pharmaceutical industry to drug design, selection and development. The knowledge of PK is important at every stage of drug development

The ultimate goal of pharmacokinetics is to give patients maximal benefit of the drug by close and accurate measurement of the drug and/or its metabolite in different biological matrices thus offering better drug management and patient care. The use of pharmacokinetics for better patient care includes:

- Individualization of patient dose and dosing regimen
- Assessment of the bioavailability and bioequivalence of the drug by the proposed routes.
- Aid in determining the mechanisms of drug-drug interactions and their avoidance.
- Prediction of pharmacokinetics in man, from results obtained in animals.
- Identification of optimum methods to accelerate drug elimination from the body in cases of toxicity and/or over dosage.
- Identification of active metabolites of drugs and quantification of their role in producing the overall response following drug administration.

1.1.2 Various Strategies in Pharmacokinetics

The new drug discovery and development process involves a series of developmental and evaluative steps to be carried out before getting approval from regulatory authorities for marketing. The process of drug discovery can be divided into a number of distinct phases: 'hit generation', 'lead generation' and 'lead optimization'.

The goal of the 'hit generation' step is to screen large compound libraries to find molecules with a specific biological response, while at the lead generation step, medicinal chemists use a variety of empirical and semi-empirical structure-activity relationships to modify the chemical structure of a compound to improve the *in vitro*

activity of the hits [6]. However, good *in vitro* activity cannot be considered as the sole descriptor for desirable *in vivo* activity unless a drug has acceptable bioavailability and duration of action. The lead optimization phase typically includes iterative ADME screening paradigms to improve upon the degree of potency, which has already been achieved. These processes are highly subjective and vary depending on the characteristic features shown by the candidate molecule.

Pharmacokinetics at pre-clinical level is basically of two types: *in vitro* and *in vivo*. Studies of drug databases showed that successful drug candidates tend to have 'drug like properties'. Druglikeness, when viewed at the *in vivo* level, is thought of in term of PK and safety. These complex *in vivo* properties result from an interaction of physiochemical and structural properties, such as solubility, permeability and stability, which are studied *in vitro*. These properties are, in turn, dictated by fundamental molecular properties, such as molecular weight, hydrogen bonding and polarity, which are studied *in silico*. As a result of the importance of properties, a new strategy emerged: testing the 'drug-like' properties of compounds during early discovery using high-throughput property methods *in silico*, *in vitro* and *in vivo* (often termed 'pharmaceutical profiling). By studying the properties of drug candidates, especially PK related properties along with pharmacodynamic efficacy, during discovery, increased efficiency and success should result.

1.1.2.1 *In vivo* Strategies

In vivo PK is about administering the candidate to the intact animal and then determination of its various parameters using different approaches. *In vivo* exposure of PK involve the collection of plasma or tissue sample at a certain time point after dosing, followed by measurement of compound concentration. Simple, rapid throughput sample preparation method such as acetonitrile precipitation, liquid liquid extraction or solid phase extraction are used in conjunction with analytical techniques such as HPLC-UV, LC-MS or LC-MS-MS. There is no universal model for the principles of optimizing leads for ADME. Main aim is to have an oral delivery of a drug with sufficient ensuing longevity in the body to produce appropriate efficacy. Implied within this is the need for compounds to be absorbed across the intestinal mucosa, to survive potential 'first pass' metabolism when they encounter the liver via the portal supply, then to continue to avoid systemic clearance, whether metabolic or renal, until useful efficacy has been achieved. An *in vivo* pharmacokinetic profile is

the synthesis of all of these processes, and much of the past optimization processes have depended upon *in vivo* kinetic studies to select molecules. These studies though very important, however are time-consuming, low-throughput because of the need for analytical method development, and costly in both compound requirement and operator time. Recent developments in improving the user-friendliness and data analysis software of mass spectrometry have shown great potential in maximizing the throughput of these *in vivo* studies via the introduction of so called 'cassette', or 'n-in-l' dosing protocols [7-9]. These new methods have delivered significant productivity gains over old methods. Thus, multiple compounds, typically five or ten at a time, can be combined in a single dose and administered to single animal. Plasma or serum samples containing the mixtures of components are then separated and analysed rapidly on mass spectrometers. Although, these methods have delivered significant productivity gains over the traditional low-throughput kinetic studies, they are not without complication. In particular, the risk of interaction between the members of a cassette is quite significant if high doses, which could saturate the metabolizing enzymes are used. This is likely to be a major problem with oral administration, when concentrations of drug reaching the liver through the portal system are at their highest. Greater success is likely after intravenous dosing, as concentrations reaching the liver will be much lower because of the distribution of the drug. Another complicating factor is the effect of any potent inhibitor of the metabolizing enzyme being included in the cassette. This could potentially block the metabolism of other members, resulting in erroneous kinetic profiles. If used judiciously as an initial screen, however, cassette dosing is a major adjunct to compound screening.

Thus, exposure study with selected compounds provide an overview of series PK performance. If PK is poor, root cause can be diagnosed, so that the structural modification can be made to improve PK. Less expensive *in vitro* can then be used to monitor future compounds in the series [10]. These studies contribute to animal model validation, dosing level selection and the need for formulation can be determined. Exposure in surrogate species is verified before dosing expensive or time consuming animal model. PK samples from animal activity studies are used to correlate plasma concentration with pharmacology.

1.1.2.2 *In vitro* Strategies

In vitro studies, by their nature, also offer a major problem in developing information that will aid drug design. It is often difficult to dissect out data that relates to specific kinetic factors. Thus, for example, it is not easy to derive direct relationships between the structures of molecules and the extent of absorption across the gastro-intestinal tract or blood brain barrier *in vivo,* because a number of complicating factors such as clearance, or protein binding, also contribute to the kinetic profile observed. Thus, *in vitro* cannot always be directly correlated to *in vivo* situation. However, they do provide useful information about the drug candidates in terms of its metabolic stability and absorption properties.

1.1.2.2.1 Absorption

Compound activity at intracellular targets or good absorption after oral dosing requires lipid membrane permeation. Permeability *in vivo* is a complex phenomenon, involving several possible mechanisms: passive diffusion, paracellular, active transport and efflux. Artursson [11] and Mandagere [12] have estimated that the predominant mechanism of gastrointestinal absorption for most commercial drugs is passive diffusion. Therefore, compound selection and optimization for passive diffusion is an effective approach.

Most models of absorption study involve the use of cultured, immortalised cells, which are generally intestinal in nature for obvious reasons [13-16]; however, it has been demonstrated that there is a little difference in compound ranking and correlation with absorption *in vivo* between a human intestinal cell line, Caco-2, and a dog kidney cell line, the Madin-Derby Canine kidney cell line (MDCK), with respect to passive diffusion of relatively lipophilic molecules. This data is important in the context of high-throughput. Caco-2 cells suffer the major disadvantage that they take about three weeks to grow to confluence before they can be used. This compares with about 3 days for the MDCK line, which, as a consequence imparts more flexibility in screening strategy, although recent developments in preparation of Caco-2 cells have reduced this differential. Whichever cell line is used, the constraints in the currently accepted methodology surrounding preparation and use of these cells prevent them for being classed as truly high-throughput screens for absorption, although some throughput gains have been made. The predominant factor that regulates the passage of drugs across the gastro-intestinal mucosa, and indeed any other cell barrier, is their

ability to passage through the lipid cell membranes. One way of solving the low-throughput inherent in isolated cell methods, is to build synthetic lipid model surrogates of mucosal membranes and several examples have been tested against data obtained from cell lines [17, 18]. The ideal output from all of these models is a set of data that can be converted into a mathematical model that predicts permeation based on the physicochemical properties of an actual, or a planned series of compounds.

1.1.2.2.2 Metabolism

Many discovery compounds exhibit low bioavailability because of high rates of metabolism. Drug metabolism by its nature is a multifactorial process and there are usually multiple pathways involved. These are 'phase I' cytochrome P450 oxidation (e.g. hydroxylation, dealkylation) of 'phase II' conjugation (e.g. glucuronidation). Metabolism reduces the circulating drug concentration and increases elimination. Plasma and cells also contain enzymes that convert drugs (e.g. esterase). Metabolic stability assays are conducted with liver microsomes [19], S9 fraction [12, 20], hepatocytes and plasma [21]. Microsomes contain the cytochrome P450 oxidizing enzymes and some phase II conjugating enzymes (e.g. UDP-glucuronosyl transfeases). The S9 fraction is a cruder preparation than microsomes and contains the microsomal enzymes, as well as additional metabolizing enzymes. Hepatocytes contain all the liver metabolizing enzymes that are found *in vivo*. Microsomes are easiest and cheapest to use, but increased detail can be obtained with the S9 fraction and hepatocytes. Plasma contains other types of enzymes found in blood, such as esterases and amidases. Stability incubations are automated using laboratory robots. The metabolic stability assay requires high sensitivity (<1μM) and selectivity (from incubation matrix interferences) that can only be achieved using LC-MS-MS. Metabolic stability results are greatly affected by solvent concentration, sample concentration and microsomal preparation. The data can be misleading with improper methodology. It is important to remember that metabolism rate and mechanisms vary among animal species. Thus, rodent metabolism studies are most useful in early discovery to assist interpretation of rodent pharmacology studies, provide alerts of liabilities and assist structural modification. Human metabolism studies become more important as the project moves toward development and require increased safety precautions.

1.1.3 Key Pharmacokinetic Parameters: Theory and Rationale

1.1.3.1 Bioavailability

The bioavailability of a drug is defined as the fraction of the ingested or administered dose that is available to the systemic circulation. Thus, both absorption and elimination process rules the bioavailability of a given drug. The significance of oral route of delivery or oral bioavailability of a drug goes beyond the simple issue of preference or convenience in most cases, a new therapeutic entity cannot become a profitable product if it is not given orally [22].

As a general rule, oral bioavailability can be estimated as:

$$F = F_a \times F_g \times F_h \times F_l$$

Where F refers to systemic oral bioavailability, F_a is the fraction absorbed across intestinal wall and $F_g \times F_h \times F_l$ is the product of the fractions escaping clearance by the GIT, liver and lung. The product of the fraction available after gut and liver extraction ($F_g \times F_h$) after oral administration primarily determines the oral clearance of a drug, though the contribution of lung clearance need to be evaluated.

1.1.3.2 Clearance

The best estimate for drug elimination can be obtained by determining total clearance (Cl) of the drug. Organ clearance is defined as the volume of blood that must be cleared of a drug in unit time in order to account for the rate of drug elimination. Thus, clearance is the ratio of elimination rate of the drug to the drug concentration in the blood entering the organ. The total clearance is calculated as the sum of all individual organ clearances of the drug.

Assuming liver and/or kidneys as major organs involved in drug elimination prediction of hepatic and renal clearances are considered most important in predicting PK characteristics of new chemical entities (NCEs).

1.1.3.3 Volume of distribution

The volume of distribution (V_d) is a measure of the extent of drug distribution and is determined by the binding of the drug in plasma as well as tissues. Because it is assumed that only the unbound drug can diffuse across membranes, it is implicit that the distribution to tissues is affected by plasma protein binding. It is also important to understand that because of significant tissue binding for most drugs, the

apparent volume of distribution far exceeds the total body water. As such, the volume of distribution is a proportionality constant relating the drug concentration in blood or plasma to the amount of drug in the body.

$$V_d = V_b + V_e \times (f_b/f_t)$$

Where V_d is the volume of distribution, V_b is the blood volume, V_e is the extra vascular tissue space volume, f_b is the unbound fraction in blood and f_t is the unbound fraction in tissues.

1.1.3.4 Elimination half life

The half-life ($t_{1/2}$) of a drug is related to its apparent V_d and Cl as per the following equation:

$$t_{1/2} = 0.693 \times (V_d /Cl)$$

Thus, the half-life of any drug is a function of blood and tissue binding of the drug as well as its total clearance and is a derived parameter from Cl and V_d. For drugs with high clearance, the half-life is relatively independent of changes in intrinsic clearance, whereas for drugs with low clearance, increase in intrinsic clearance result in decreased half-life.

1.1.4 New Trends in ADME

Testing the safety and efficacy of a successful human medicine involves many laboratory animals, which can some times be subjected to considerable suffering and distress. Ethical and legislative imperatives relating to animal experimentation need to be considered in the development of pharmaceuticals. On these lines, the regulatory authorities of UK and European countries have taken pioneering steps to enact the concept of 3 Rs, refinement, reduction and replacement, of animal procedures wherever possible, and this legislation applies fully to the development and assessment of novel medicines.

Over the last decade, there has been considerable progress with applying *in vitro* and *in silico* methods to both drug efficacy and safety studies. These include the increased emphasis placed on predictive descriptors of ADME properties and novel approaches like early micro-dose drug studies in human volunteers.

Since early drug discovery efforts are to evaluate whether the NCE has the ability to be absorbed and become bioavailable, emphasis on predicting oral

bioavailability rapidly and early is critical. As reliable estimation of oral bioavailability requires *in vivo* experimentation, surrogate parameters for oral bioavailability are estimated from a battery of *in silico, in vitro* or *in situ* experiments. Retrospective studies of marketed drugs have shown that physiochemical properties including partition coefficient, molecular weight, aqueous solubility and conformational flexibility influence the oral bioavailability of drugs [23], though oral bioavailability is influenced by several other factors including intestinal and liver metabolism, biliary and other efflux pump mediated excretion and conditions in the gastrointestinal tract (GIT) [24, 25]. Attempts to correlate physiochemical properties and physiological factors in drug absorption has recently resulted in the proposition of Biopharmaceutics Classification System (BCS) for drugs that successfully correlates *in vitro* dissolution and *in vivo* bioavailability [26].

The most widely used methods for estimating intestinal permeability of molecules to predict *in vivo* drug absorption are rat *in situ* single pass intestinal perfusion, rat everted sac experiments, and *in vitro* human colon adenocarcinoma cell lines (Caco-2) [27-31]. However, the recent trend is towards the use of immobilized artificial membrane (IAM) based techniques including IAM powered HPLC columns and parallel artificial membrane permeation assay (PAMPA) [32, 33]. The IAM chromatography offers the advantages of experimental simplicity and good correlations between IAM k' values and Caco-2 permeability. These techniques are compatible with HTS methodologies, wherein a large number of drug candidates or NCEs are evaluated in short span of time. A caveat of the IAM and PAMPA approaches is to remember that they will under estimate the absorption of compounds subject to active or para cellular transport *in vivo* and over estimate the absorption of compounds subject to efflux pump transport [34].

The prediction of human renal clearance has been quite successful using interspecies allometric scaling approaches [35-37]; however, despite numerous attempts, prediction of hepatic clearance by interspecies scaling approaches has been less successful [38]. Recent advances have made available several models for the prediction of hepatic clearance using liver microsomes, isolated hepatocytes, liver S9 fractions, recombinant CYP iso-enzymes, liver slices and *in situ* gastrointestinal/liver single pass perfusion preparations. All these approaches are reasonably predictive of hepatic clearance when liver metabolism is the predominant contributor to total clearance.

Prediction of distribution parameter and half-life, which plays an important role in dosing frequency are normally carried out by allometric scaling approaches [39], though research have attempted alternate approaches with limited success.

Thus, the use of *in vitro* approaches for predicting ADME characteristics of NCEs have enabled rapid evaluation of their pharmacokinetics allowing more bioavailable compounds to move in to development from discovery stage.

1.1.5 Role of Mass Spectrometry in Pharmacokinetics

LC-MS-MS has been found to be most suitable analytical technique for PK studies due to its high selectivity, specificity and sensitivity. Mass spectrometry has played a very important role in the structural characterization and quantification of drug metabolites [40]. Development of the atmosphere pressure ion sources, such as electrospray and atmospheric pressure chemical ionization has added liquid chromatography-mass spectrometry (LC-MS) to the list of present day's bioanalytical techniques, which have become indispensable for rapid and sensitive determination of pharmaceuticals in biological fluids. LC-MS-MS analysis offers high specificity and speed, good precision and accuracy, a wide dynamic range, high sensitivity and applicability to almost every type of compound. The high specificity of MS-MS eliminates most of the problems relating to the presence of endogenous components.

Thus, LC-MS-MS helps in generating PK profile of almost all type of compounds very rapidly and with its use one can achieve answers to the following:

- Selection of compounds with favorable PK credentials.
- Provide directions for the synthesis of metabolically stable analogues.
- Provide the understanding of the physical and biological effects of the parent drug.
- Provide the elimination pathways of the drug.
- Provide the information for the formation of reactive intermediates.
- Provide the information on the formation of active metabolites.

In the drug discovery program, success rate increasingly relies on the ability to rapidly identify quality molecules that possess the desired attributes of bioavailability, chemical tractability, selectivity and potency. Today, the key tools for rapidly generating potent and selective compounds are combinatorial chemistry and functional genomics. Turning a chemical lead into a marketable drug requires a

balance of potency, safety and pharmacokinetics. Pre-clinical drug development teams are struggling to deal with this increased rate owing to the slow throughput of the pharmacokineticists and drug metabolism experts. Method development focuses on discovering a way to make the analyte, and perhaps a key metabolite or two, elute from the column in the 'empty regions' of the chromatograms. Many hours are being spent in testing sample clean-up methods, and exploring the effects of slight changes in pH, gradient composition, and packaging material on the retention times of the analytes and the interfering peaks. Thus, there is a need to increase the throughput of the *in vivo* pharmacokinetic optimization studies, to aid in selection and to add value to the development candidates.

LC-MS-MS is such a technology that can provide a high throughput of bioanalysis ultimately in increasing the throughput of generation of PK data.

SECTION 2

Malaria: A Review

1.2.1 Introduction

Malaria is the world's most important parasitic infection, ranking among the major health and developmental challenges for the poor countries of the world. It is one of the major infectious diseases in the world today despite years of efforts first to eradicate it and subsequently to reduce its impact on mortality and morbidity. The precise determination of malaria-related mortality is difficult because the symptoms of the disease are non-specific and most of the deaths occur at home [41]. *Plasmodium falciparum,* the most virulent of the four *Plasmodium* species, is potentially life threatening, increasing in prevalence and becoming even more resistant to in use drugs. Approximately 500 million people are affected annually and about three million, mostly children die of *P. falciparum* each year [42-44]. In areas of endemic malaria, the most common clinical presentation is that of uncomplicated infection with prompt recovery after treatment [45]. However, in non-immune individuals, malaria may be present in its most severe forms [46]. Although malaria remains an important health problem in some parts of Asia and South America, its main impact is in Sub-Sahara African region where 90% deaths occur due to malaria [47]. These converge to result in a range of outcomes, from an asymptomatic infection to severe disease and death [48].

1.2.2 Epidemilogy, Burden and Economic Consequences

Malaria is widely distributed throughout the tropics. Human malaria is caused by four species of obligate intracellular protozoa of the genus *Plasmodium. P. falciparum* predominates in Africa, French Guiena, Surinam, parts of Asia and New Guiena. *P. vivax* prevails in Latin America, Turkey, the Indian subcontinent and china. *P. malariae* is widely distributed but much less common than either of the former species while *P. ovale* occurs mainly in Africa. The epidemiological species of malaria, mainly determined by the climate and the ecology of the mosquito vectors, influence immunity in the population. Thus, at one extreme in the forest zones of west Africa, children are infected in the second six months of life, parasitemia reaches 80-100% in the childhood, disease occurs throughout the year, and death occur mainly in the first five years of life. In contrast, the disease is seasonal in the dry Savannah of Africa and mortality is spread over a wide range. At the other extreme, the disease may be epidemic as in parts of North India and Thailand, and affect all ages. Malaria-endemic countries are among the poorest in the world. Malaria has both short-term

and long-term health costs and economic consequences. In addition to traditional measures of morbidity and mortality, disease burden in these countries can be quantified through disability-adjusted life years (DALYs) [49, 50] effect on health systems, and socioeconomic impact, but these estimates also suffer from the general lack of high-quality data. In 2000, malaria was estimated to be the cause for the loss of nearly 45 million DALYs (13% of all infectious diseases). In addition to the more routinely measured costs associated with malaria, such as the cost of prevention activities, lost workdays for both patients and care givers, and treatment seeking and medication, malaria-related costs in endemic countries also include those associated with suffering, retarded physical and cognitive development in children and consequently poor educational performance, related malnutrition, anemia, and potential increases in vulnerability to other diseases [51–53]. Malaria incapacitates the labour force, lowers educational achievement, discourages tourism and business investment, and reduces opportunities for specialization both within the household and for the economy as a whole. Slow economic growth prevents improvements in living standards and places a serious constraint on countries' ability to fund and maintain malaria-control efforts, thereby creating a vicious cycle of high disease prevalence and low economic growth.

1.2.3 Life Cycle of Malarial Parasite

Although malaria can be transmitted by transfusion of infected blood, humans are infected more commonly by sporozoites injected by the bite of female *Anopheles* mosquito. The life cycle of the malarial parasite is complex and is basically divided into asymptomatic, pre or exoerythrocytic stage of infection and erythrocytic phase (Figure 1.2.1) [54].

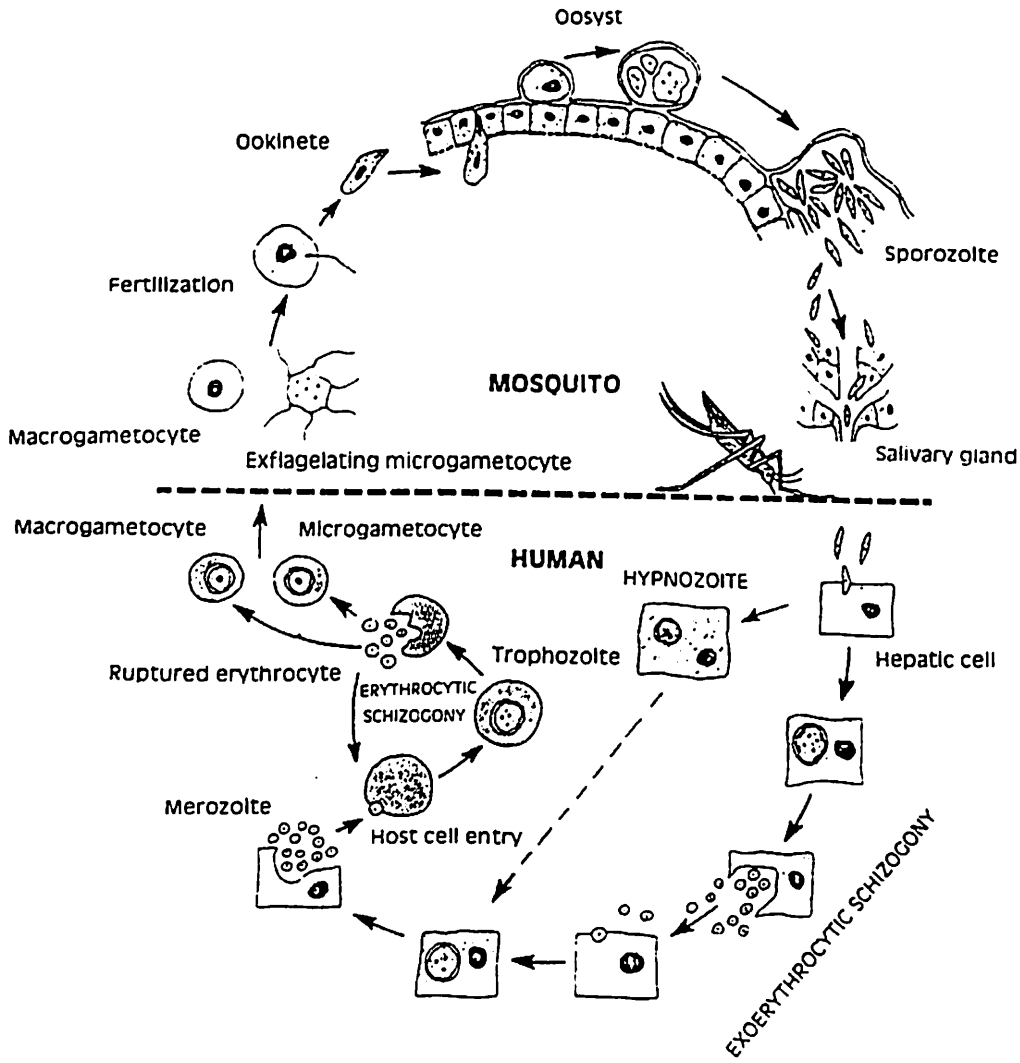

Figure 1.2.1 Life cycle of a malarial parasite

The sporozoites injected through the bite of female *Anopheles* mosquito circulate for a short time in the blood stream and then invade liver cells, where they develop into exoerythrocytic schizonts during the next 5-15 days depending upon the plasmodium species. *P. vivax, P. ovale* and *P. cyanomolgi* have a dormant stage, the hypnozoite [55, 56], that may remain in liver for weeks or many years before the development of erythrocytic schizogony. This results in relapse of infection. *P. falciparum* and *P. malariae* have no persistent phase. An exoerythrocytic schizont contains 10000 to 30000 merozoites, which are released and invade the RBCs. Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocytic membrane with ligands on the surface of the merozoites. In erythrocytes, most parasites undergo asexual development from young ring forms to trophozoites and finally to mature schizonts (erythrocytic schizogony). The parasite modifies its host cell in several ways to enhance its survival. The erythrocyte

containing the segmented schizont eventually ruptures, each releasing about 6-24 merozoites depending upon various species. It is this process which produces febrile clinical attack. The periodicity of parasitemia and febrile clinical manifestations depend on the timing of schizogony of a generation of erythrocytic parasites. Erythrocytic schizogony takes about 48 h in case of *P. vivax, P. falciparum* and, *P. ovale* and thus, these species cause tertian malaria. In *P. malariae* infection, schizogony takes about 72 h resulting in malarial attacks on days 1 and 4 or 'quartan malaria'. In the course of these events, some merozoites invade erythrocytes, become differentiated into sexual forms, which are macro-gametocytes (female) and micro-gametocytes (male). The duration of gametocytogony is assumed to be approximately 4-10 days depending on the *Plasmodium* species. Mature macro-gametocytes taken into the midgut of the *Anophelus* mosquito escape from the erythrocyte to form gametes. This is followed by exflagellation of microgametocytes and fertilization of female gametocyte in the gut wall as an oocyst. Between 7-15 days post infection and ambient temperature, a single oocyst forms more than 1000 sporozoites. The motile sporozoites migrate into the salivary glands and when an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again.

Each *Plasmodium* species has distinguishing morphological features in a blood smear with different symptoms of the illness caused by it [57]:

- *P. falciparum* cause malignant tertian malaria, the most dangerous form of human malaria. By invading erythrocytes of any age, this species can produce fulminating infections in non-immune persons that, if untreated, may rapidly cause death.
- *P. vivax* cause benign tertian malaria and produces mild clinical attacks than those described above. It has a low mortality rate in untreated adults and is characterized by relapses caused by latent tissue forms.
- *P. ovale* causes malarial infection with a periodicity and relapse similar to those of *P. vivax* but it is milder and more rapidly cured.
- *P. malariae* cause quartan malaria that is common in localized areas of tropics.

1.2.4 Control Strategies for Malaria

Although treatment continues to be the main defense against malaria, disease prevention (appropriate case management) is assuming a more important role.

Antimalarials are classified according to the phase of life cycle of the parasite they affect. According to this classification antimalarials can be classified into four major classes:

- Tissue Schizontocides for causal prophylaxis (Proguanil): These agents act on the primary tissue forms of plasmodia within the liver that are destined within a month or less to initiate the erythrocytic stage of infection thus preventing invasion of erythrocytes and further infection.
- Tissue Schizontocides for anti relapse activity (8-Aminoquinolones namely Primaquine): These compounds act on the latent tissue forms of *P.vivax* and *P. ovale* remaining after the primary hepatic forms have been released into the circulation.
- Blood Schizontocides used for clinical and suppressive cure: Chloroquine, quinine, Quinidine, Mefloquine, Halofantrine, artemisinin and related compounds.
- Gametocidal (Primaquine): These agents act against sexual erythrocytic forms of plasmodia, thereby preventing transmission of malaria to mosquitoes.
- Sporontocidal (Pyrimethamine): Such drugs inhibit the transmission of malaria by preventing or inhibiting the formation of malarial oocysts and sporozoites in infected mosquitoes.

Major antimalarial drugs can also be classified chemically as under [58]:

- *4-Amino Quinolines*: Chloroquine, Amodiaquine, Hydroxychloroquine
- *8-Amino Quinolines*: Primaquine
- *Acridine*: Mepacrine
- *Cinchona alkaoloids*: Quinine and its derivatives
- *Quinolinemethanol*: Mefloquine
- *Biguanides*: Proguanil, Cycloguanil
- *Diaminopyridines*: Pyrimethamine, Trimethoprim
- *Sulphonamides*: Sulphadoxine, Sulphalene, Dapsone
- *Antibiotics*: Tetracyclines, Clindamycin and erythromycin
- *Newer Drugs*: Halofantrine, Artemisinin and its analogues

1.2.4.1 Prophylaxis and Treatment of Malaria

Prophylactic use

(i) Chemoprophylaxis, which implies that the drug is used prior to tissue or blood infection with the aim of preventing either the infection or its clinical manifestations.

(ii) Absolute Prevention implying destruction of the inoculated sporozoites before they have invaded tissue cells.

(iii) Causal Prophylaxis meaning the prevention of the erythrocyte infection by destruction of the pre-erythrocytic forms of the parasite, directly deriving from the sporozoites. Drugs acting as prophylactic should be able to destroy the pre-erythrocytic stages before merozoites are liberated into the blood stream. The main implication of causal prophylaxis is that the infection must be eliminated at an early stage and within a short time.

(iv) Clinical prophylaxis, which implies the prevention of clinical symptoms by early destruction of erythrocyte parasites. It often consists of 'suppression', which permits the continued existence of exoerythrocytic forms, or of some erythrocytic forms, enabling subsequent multiplication of the parasite after discontinuation of the drug.

1.2.4.2 Therapeutic (Treatment and Curative) Use

(i) Acute Attack

The chemotherapy has to be done against asexual erythrocytic stages of the parasite by means of a suitable blood schizontocide. Treatment of the attack may result in clinical (temporary) cure, denoting relief of symptoms, in elimination of asexual parasitemia without complete elimination of the infection, or in radical (permanent) cure, indicating complete disappearance of the infecting parasites.

(ii) Radical treatment

This implies treatment adequate to achieve complete elimination of the infection so that relapses do not occur after the treatment is terminated. In *P. falciparum* infections, the radical cure may be obtained by blood schizontocides while in case of *P.vivax* and *P.ovale* infections, it is only clinical cure.

(iii) *Antirelapse Treatment*

Relapse refers to the renewed and parasitological manifestations of malaria following a primary attack with an interval greater than the natural periodicity of the blood infection. This treatment is designed to prevent the occurrences of long-term relapses (six months). The deployment, therefore, particularly of primaquine was seen as a possible solution. Its mode of use varied, but it was particularly beneficial in the areas with *P.vivax* possessing a long incubation period and where attacks of malaria could be expected in spring or summer.

(iv) *Suppressive treatment*

This implies action against asexual erythrocytic parasites, resulting in suppression of clinical symptoms and parasitaemia. This may be given either for prophylactic or therapeutic purposes.

(v) *Suppressive cure*

This denotes that clinical and parasitological manifestations of the infection have been continuously suppressed by regular drug administration for a long enough period for the infection to die out.

1.2.4.3 Prevention of Infection

After World War II, widespread use of Dichloro, diphenyl, trichloroethane (DDT) coupled with the covering and draining of breeding grounds resulted in a substantial reduction in mosquito populations and, together with effective treatment, eradicated malaria in southern Europe, Russia, and parts of Asia. Although substantial successes were achieved in subtropical regions, control of malaria in the tropics proved far more challenging. The effectiveness of the control effort was undermined through a combination of difficult access to health facilities, the deterioration of health infrastructures, and the gradual development of insecticide resistance. As a consequence, plans for eradication of malaria through vector control had to be abandoned in the late 1960s. Malaria-prevention efforts have since shifted toward more appropriate local protection methods, focusing on partial control of breeding grounds and, in particular, on the use of insecticide-treated mosquito nets, which both reduce the number of infective bites for a given mosquito population and have important mass insecticidal effects. Deployment of impregnated bed nets in China and Africa has been successful in reducing malaria morbidity, mortality, or both [59, 60] although the resulting decrease in naturally occurring immunity may

limit this effect in the medium term. Resistance of the vectors to insecticides is generally increasing. Moreover, even in areas where the benefits are substantial and bed nets have been deployed through national programmes, community uptake has been disappointing. The reasons need to be examined and lessons learned to improve sustainability. The effectiveness of insecticide-treated mosquito nets varies with the rate of malaria transmission; the nets do not work well in many areas of low and unstable transmission, where malaria vectors bite in the early evening and morning [61].

1.2.4.4 Vaccines

With an increase in insecticide and antimalarial-drug resistance, the development of a malaria vaccine carries huge expectations. As a reflection of these uncertainties, there has recently been a move away from animal models and an emphasis on clinical trials [62]. Vaccines can target different stages of the parasite cycle, each with a distinct antigenic repertoire [63]. The pre-erythrocytic stage (sporozoite and liver-stage) vaccines are those best supported financially, perhaps because there is potential market in the more developed countries (armed forces, tourists, short-term visitors such as business people and field researchers). Immunization with sporozoites can produce protective immunity, but the experimental means of conferring protection, multiple repeated exposures to bites of hundreds of irradiated mosquitoes is not practicable, and efforts to produce the same degree of protection with vaccines based on sporozoite proteins have so far failed [64-66]. Asexual blood-stage vaccines aim at reproducing the situation that occurs in adults in hyper endemic areas: predominantly antibody-mediated protection acquired through repeated exposure to infection. A gamete-stage vaccine aims to prevent mosquitoes that are feeding on an infected individual from acquiring and transmitting the parasite. However, vaccine research over the past three decades has been characterized by lack of funding, a serious underestimation of the complexity of the parasite, faith in technology above scientific understanding, lack of appropriate models, and above all a lack of adequate knowledge about the immune mechanisms underlying protection.

1.2.4.5 Chemotherapy of Malaria

For decades, malaria chemotherapy has relied on a handful of drugs, each with its own pharmacological limitations, of which parasite resistance has been the most damaging. We are now in a position where the only affordable drugs for use in resource-poor settings, such as chloroquine and sulfadoxine–pyrimethamine are of limited benefit across much of the world. This problem was predicted more than a decade ago, at a time when there appeared to be no easy solution. Fortunately, the intervening period has seen significant advances, which should result in a rapid increase in the number of available (and affordable) antimalarials. The progress in our understanding of the mechanisms of action and resistance to traditional drugs, the emergence of the artemisinins as one of the most important antimalarial class and the recently completed genome projects have provided the scientific community with a wealth of data, upon which to mount effective drug discovery and development programmes. Coincidental with the biological advances have been engagement of the pharmaceutical industry and the establishment of organizations such as the Medicines for Malaria Venture (MMV) which have provided these other key elements in the search for new antimalarials. There have been several classes of drugs, which have been used in chemotherapy of malaria and are elaborated below.

1.2.4.5.1 Cinchona Alkaloids

Recognition of the medicinal value of cinchona bark as a febrifuge preceded by more than two centuries before the identification of *Plasmodium* species as the cause of malaria. The bark contains a mixture of many alkaloids of which quinine is the most abundant and easily extracted [67]. While quinidine, the dextrorotatory diastereoisomer of quinine has significantly greater antimalarial potency than quinine *in vitro* and *in vivo*, the latter is considerably more abundant in nature and therefore, was historically chosen as the preferred alkaloid for antimalarial use [68]. The other alkaloids of cinchona are less abundant and of lower potency.

Mechanism of Action and Spectrum of Activity

Despite the long history of interest in quinine as an antimalarial drug, its mechanism of action remains unknown. It is highly active as a blood schizontocide against all forms of human malaria and with administration for 7-10 days can produce a radical cure in most *P. falciparum* but not in *P. vivax* infections. It is devoid of

activity against sporozoites or exoerythrocytic stages and thus not suitable for causal prophylaxis. It has been shown to bind to plasmodial DNA [69]. Another hypothesis relates interaction of quinine with hemin to form lipid soluble substances, which cause lysis of biological membrane [70, 71]. It has got strong activity against asexual blood forms and is also active against early stages of gametocytes of *P.vivax, P. malariae* and *P. ovale.*

Pharmacokinetics, Metabolism and Adverse Effects

Following oral administration, quinine is rapidly and thoroughly absorbed [72] with peak plasma levels reaching 1-3 h after an oral dose. It has a relatively small volume of distribution and a plasma half-life of 11-18 h [73]. The drug is not significantly concentrated in the erythrocytes but the erythrocytic to plasma quinine is significantly increased in severely ill patients [74, 75]. Quinine is metabolized in the liver to 1-hydroxy quinoline and 6-hydroxy quinuclidine derivatives, which are eliminated by the kidneys. A significant difference in the plasma half-life has been observed between infected and uninfected individuals, patients with malaria showing a significant prolongation of the elimination of parent drug [73, 74, 76]. Delayed elimination of quinine has been shown to be related to the hepatic dysfunction associated with fever [74]. Apparent clinical antagonism between quinine and chloroquine has been commented upon as radical cure rates in similar group of patients were seen to be lower when two drugs were given simultaneously than when quinine was given alone [77]. A common syndrome of characteristic side effects known as 'Cinchonism' occurs in many patients taking quinine. Giddiness, light-headedness, transient hearing loss, tinnitus, amaurosis and blurred vision may appear at the therapeutic blood concentrations of 5-10 μg/ml. The other less frequent but serious side effects of quinine include urticaria, asthma, fever, pruritis, thrombocytopenia, hemolysis and edema of the eyelids, mucus membrane and lungs. Hypoglycemia is a frequent complication of *P. falciparum* malaria and may be aggravated by the administration of quinine, which apparently stimulates insulin secretion [78]. Quinine is excreted in breast milk but the amount ingested by infant is insignificant.

1.2.4.5.2 4-Aminoquinolines

The two world wars were the most powerful stimuli for the development of the synthetic antimalarial compounds. Chloroquine was synthesized as early as 1934, but studies were stopped early in its development on the grounds that it was too toxic. Sontoquine, an analogue of chloroquine was produced later in an attempt to eliminate the toxic side effects of chloroquine, and was at the stage of field testing when the Second World War began in 1939. The pharmacology and antimalarial activity of chloroquine were evaluated in volunteer studies following which the compound became recognized as the antimalarial drug of choice throughout the world [79, 80]. It is of historical interest to consider mepacrine, a 9-aminoacridine, in the evolution of the 4-aminoquinolines. While no longer in use today, mepacrine, introduced in Germany in 1932, was the first synthetic antimalarial drug with blood schizontocidal activity. It was more potent than quinine as a blood schizontocide for suppressive therapy or prophylaxis of *P. falciparum* and *P. vivax* malaria [81]. It did not, however, prevent recurrence of *P. vivax* malaria [82]. Toxic effects of quinacrine included gastrointestinal intolerance during initial therapy in most cases, central nervous system disturbances and a potentially exfoliating dermatitis [83]. Its most remembered side effect was its disturbing propensity for causing reversible yellow discoloration of the skin. It does, like quinine and chloroquine, effectively bind to DNA in high concentrations [84]. Moreover, mepacrine-resistant *P. falciparum* malaria is cross resistant with chloroquine and other 4- aminoquinolines but not with quinine.

Mechanism of Action and Spectrum of Activity

The 4-aminoquinolines appear to exert their antimalarial activity primarily through their effect on the digestion of hemoglobin. The structural resemblance of mepacrine and chloroquine suggests a similar mode of action for the two drugs. They have a fast and prominent effect on hemoglobin containing digestive vesicles of the parasite, first detectable by the fusion and sequestration of adjacent vesicles and their contained malaria pigment into a large autophagic vacuole. The pigment is then extruded into the cytoplasm of the host cell. It is claimed that chloroquine binds with hematin, a transient breakdown product of hemoglobin within the parasite, to produce a hemolytic complex, which disrupts the parasite host membrane, thus resulting in parasite death [70]. The *in vitro* potency of the two 4-aminoquinolines, chloroquine

and amodiaquine, is similar when evaluated against chloroquine-susceptible isolates *P. falciparum*. However, amodiaquine appears significantly more potent than chloroquine against less susceptible isolates, i.e. the two drugs are incompletely cross-resistant. This difference in susceptibility of resistant isolates was based on short-term *in vitro* 'macro' cultures [85, 86]. The observation was confirmed *in vivo* in monkeys experimentally infected with *P. falciparum* [87]. These agents have no activity against sporozoite and primary erythrocytic schizonts but are highly active against the blood forms of *P. vivax, P. ovale, P. malariae,* and susceptible strains of *P. falciparum*. They are reputed to kill early stage gametocytes (protogametocytes) of *P. falciparum,* while hemoglobin digestion is still taking place, but are inactive against mature forms. The compounds eliminate gametocytes, along with asexual blood forms, of *P. vivax, P. ovale* and *P. malariae*. These drugs have no activity against the persisting tissue stages of the relapsing human malarias caused by, *P. vivax* and *P. ovale*.

Pharmacokinetics, Metabolism and Adverse Effects

In the recent past, a number of studies have been done which have contributed appreciably to an understanding of the pharmacokinetics of chloroquine. It is quickly and almost completely absorbed following an oral dose. It has been shown that, following a single oral dose of 300 mg chloroquine in healthy volunteers; peak plasma concentrations of 56-102 ng/ml were reached in 1-6 h, with a bioavailability of 75% [88]. When chloroquine is administered intravenously, there is a real danger of reaching toxic levels. The use of intramuscular chloroquine may be presumed to be associated with similar danger although there are very few recent data from human studies in this regard. However, in rabbits, it has been shown that the peak plasma concentration attained after intramuscular administration of 10 mg chloroquine per kg was approximately twice that following oral administration of the same dose, and occurred 30 minutes following injection [89]. Given the expanding distribution and increasing level of resistance to these drugs, treatment of severe or complicated malaria (including patients unable to tolerate oral medications) should be initiated using intravenous quinine. Despite the relative resistance of *P. falciparum* in parts of South-East Asia, quinine can almost always be relied upon to cause clinical improvement and reduction in parasitemia. Once absorbed, chloroquine is widely distributed throughout the body, and accumulates in the tissues, particularly in the

spleen, kidneys, lungs, heart and liver [90-92]. It has a volume of distribution in man of 111-285 L/kg [88]. Other studies have confirmed a special affinity for the melanin-containing tissues of the skin and eye [93-96]. Red cell concentrations of chloroquine have consistently been shown to be higher than plasma concentrations, particularly in cells harboring *P. falciparum* parasites. In infected children, the red cell/plasma ratio was found to be 21, and this ratio fell to 5.3 after four days, when parasitemia had been completely eliminated [96]. Exceptionally high ratios have been reported for erythrocytes infected with *P. knowlesi* and *P. berghei* [97-99]. Leukocytes and platelets also concentrate the drug, to an even higher degree than red cells. and this explains the fact that chloroquine levels in serum are usually higher than in plasma, since during the clotting process chloroquine is eluted from these elements into the serum [100]. In view of the high degree of tissue binding of chloroquine, plasma protein binding is less than would be expected (50%), primarily to plasma proteins other than albumin. Chloroquine is extensively metabolized and the main metabolite being desethylchloroquine which has been reported to have antimalarial activity against chloroquine-sensitive falciparum parasites similar to that of the parent drug [101]. Desethylchloroquine has the same tissue distribution as chloroquine, but individual variations in distribution may be related to the incidence of certain drug side effects. Pruritus is frequently associated with chloroquine administration in African subjects. It has been shown that the ratio of unchanged chloroquine to desethylchloroquine in the skin is related to the presence of itching. Subjects complaining of pruritus are likely to have a higher parent drug/metabolite ratio than those who do not despite similar plasma concentrations [102]. Chloroquine is eliminated very slowly and disappears from the various tissues at different rates. Erythrocyte and plasma concentrations decline in parallel. However, the other tissue stores behave as separate compartments, with slower elimination rate constants than the plasma. Since there is constant redistribution between tissue compartments and plasma over weeks or even months, estimation of plasma half-life will vary, depending on the duration of sampling. This is the reason why literature contains wide variations of plasma half-life, between two and a half days when sampling continues for seven days to ten days when sampling is prolonged for 56 days. Amodiaquine, another drug in this category was rapidly metabolized after an oral dose, desethylamodiaquine being the major metabolite [103]. Moreover, unchanged amodiaquine disappears from the blood within half an hour of administration, but the

desethyl metabolite may still be detected 21 days later [104]. Chloroquine is eliminated from the body mainly by way of the kidney, and is detectable in the urine for 120 days after dose. The value for renal clearance (400-450 ml/min) is higher than the glomerular filtration rate, suggesting that the drug is probably excreted both by glomerular filtration and tubular secretion. Patients with renal insufficiency eliminate the drug more slowly than normal subjects [105].

The adverse effects related to chloroquine are rare and mild. Nausea and vomiting may occur if the drug is taken on an empty stomach and headache and difficulty in visual accommodation have been reported, accompanying the full therapeutic regimen. Other side effects include photoallergic dermatitis, pigmentation of the skin, leukopenia, bleaching of the hair and, very rarely, aplastic disorders. The side effects associated with the therapeutic administration of amodiaquine are similar to those of chloroquine. However, agranulocytosis has been found to be associated with the prophylactic use of amodiaquine, and there is a relatively high frequency of this serious adverse reaction, which obviously precludes chemoprophylaxis with amodiaquine [106, 107]. For curative treatment of *P. malariae* and susceptible *P. falciparum,* and for termination of the acute attack of *P. vivax* and *P. ovale,* chloroquine and amodiaqine are usually given orally in a three-day course.

1.2.4.5.3 Mefloquine

During the Second World War, the United States of America initiated a programme designed to identify and test chemical compounds with potential antimalarial activity. From the 16000 chemicals screened, those belonging to the quinoline methanol group were found to be among the most promising, and one drug in particular, designated SN 10275, showed a high degree of activity. This drug was developed for human administration, and initially administered to five volunteers with induced *P. vivax* malaria. All five were clinically cured, and this compound was considered to be a promising lead but later in the course of study of this drug, phototoxicity was found to be associated with its use. Meanwhile, other quinoline methanols had been synthesized and one drug selected for advanced testing from this class of compounds was WR 142490, which was later named mefloquine.

Mechanism of Action and Spectrum of Activity

Relatively little is known about the mode of action of mefloquine, although it is assumed to be similar to that of quinine, the 4-aminoquinolines and mepacrine. It has been shown that mefloquine, like quinine, modifies the ultra structure of the malaria pigment. Following treatment, the pigment, which is produced, is abnormally translucent, and then it is seen to disappear entirely [108]. Mefloquine has also been shown to associate strongly with membrane phospholipids [109], which is an important feature of its activity, although it's major mode of action is probably involved with parasite hemoglobin digestion. Furthermore, like other blood schizontocides mefloquine binds to DNA, but with less affinity than quinine, chloroquine or mepacrine [110]. Mefloquine is presumed to have no activity against sporozoites but has an action against gametocytes of *P. falciparum* in their early stages of development while they are still actively digesting hemoglobin. Mefloquine kills all gametocytes stages of *P. vivax, P. ovale,* and *P. malariae.*

Pharmacokinetics, Metabolism and Adverse Effects

Mefloquine is rapidly absorbed, the apparent half-life of absorption being 0.36-2.0 h [111-112]. The decline of plasma concentration is very gradual and prolonged, resulting in a calculated half-life of elimination of 15-33 days. Nearly all the mefloquine in the plasma is bound to protein but the concentration in erythrocytes is 170% that in the plasma. Approximately half of the intra-erythrocytic mefloquine is bound to the cell membrane [113]. The drug is widely distributed in tissue compartments and only a small amount of unchanged drug is eliminated in the urine while the majority is eliminated after biotransformation. Within only four hours after administration, significant amounts of the main metabolite (2, 8-trifluoromethyl-4-quinoline carboxylic acid) can be detected in the plasma, and within a few days the concentration of this metabolite exceeds that of the parent drug. Plasma levels of mefloquine increase gradually when repeated weekly doses are administered, and the steady-state level is attained after only a few months. Following six months of administration, the elimination half-life of mefloquine was found to range from 17 to 35 days identical to that following single-dose administration [112], indicating that no enzyme induction or inhibition occurs under prolonged treatment. The side effects recorded have generally been mild and self-limiting, and have included dizziness, nausea, vomiting, abdominal pain and loose bowel movements. Neuropsychiatric

symptoms, generally including feelings of, paranoia, and occasionally involving hallucinations, have been observed in patients.

1.2.4.5.4 Antimetabolites of the Folate Pathway

Drugs affecting parasite folate metabolism, including the biguanides, proguanil, chlorproguanil and the diaminopyridines (pyrimethamine) have been in clinical use since the mid-1940s. The discovery of proguanil stimulated further work in compounds that compete with folic and folinic acid in parasite metabolism. In 1951, a joint American and British team developed pyrimethamine, which proved to be highly active against human malaria. Pyrimethamine was found to be greatly superior to proguanil in its activity against the blood forms of the human malarias, and its persistence in the body was determined to be very long. However, early in its use, it was observed that parasite resistance to pyrimethamine developed quite rapidly, both in the laboratory and in the field, and that pyrimethamine resistant asexual erythrocytic forms were usually cross-resistant to proguanil. The fact that sulfonamides and sulfones had antimalarial activity was discovered during their development for the therapy of bacterial infections prior to the Second World War. However, when the appearance of chloroquine resistance made an intense search for new preparations imperative, these drugs were re-evaluated. The long-acting sulfonamides, sulfadoxine and sulfalene, in combination with pyrimethamine, proved to be very effective against chloroquine-resistant *P. falciparum*, and the association of the sulfone, dapsone with proguanil was found to be an active prophylactic regimen.

Mechanism of Action and Spectrum of Activity

Cycloguanil (the metabolite and active principle of proguanil), pyrimethamine and trimethoprim are competitive inhibitors of the enzyme tetrahydrofolate dehydrogenase. The mammalian host uses a similar enzyme, but of lower molecular weight, in order to produce tetrahydrofolate. Pyrimethamine and cycloguanil have an affinity for the parasite enzyme one thousand times higher than that of the mammalian enzyme. Sulfonamides and sulfones compete with para amino benzoic acid (PABA) for the active site on the enzyme dihydropteroate synthase. This system, using PABA, would eventually lead to the formation of dihydrofolate. In mammalian cells, dihydrofolate is obtained by the reduction of dietary folic acid. This accounts for the specificity of sulfonamides and sulfones for microorganisms, and their relative safety

in mammalian systems. The fact that these two types of antimetabolites act at different but sequential steps in the parasite's folic acid cycle may explain, in part, their potentiating synergy when they are used in combination. Drugs of this class act on growing parasites, and are therefore inactive against hypnozoites (which are dormant) and against mature gametocytes. Pyrimethamine and the biguanides, in sensitive strains, are also known to be active against the developing mosquito stages of the parasite (sporogonic cycle), and therefore can theoretically play a role in interrupting transmission. This class of compounds has no apparent action on gametocytes. Cycloguanil and chlorcycloguanil, the active metabolites of proguanil and chlorproguanil, are known to be active; pyrimethamine is presumed to be active, whereas the sulfonamides and sulfones are apparently inactive against erythrocytic schizonts. All compounds are inactive against mature gametocytes, but may have some effect on early, developing forms. There is evidence that sulfonamides stimulate the production of gametocytes [114, 115]. These compounds apparently do not show any activity against hypnozoites.

Pharmacokinetics, Metabolism and Adverse Effects

Biguanides

Proguanil is rapidly absorbed, and the peak plasma concentration is attained approximately 4 h after administration of an oral dose [116]. It is concentrated in red cells by a factor of 4-8 over plasma levels. Analysis of human autopsy material shows that higher concentrations are found in kidney and liver than in plasma. The plasma elimination half-life is about 15 h, and approximately 40% of an oral dose may be recovered unchanged from the urine within 48 h, 7-12% from the feces and the remainder is excreted as the active metabolite, cycloguanil [117]. Proguanil has been found to be extremely safe in man at the usual prophylactic adult dose of 100-200 mg daily. Gastrointestinal symptoms in patients with renal complications have been reported in association with doses of 0.8-1.0 g daily, but were completely reversible when the drug was discontinued.

Pyrimethamine

Pyrimethamine is rapidly and almost completely absorbed following oral administration, and peak plasma levels are attained between 2 and 6 h after dosing. The drug is extensively bound to plasma protein, and is moderately concentrated in

various tissues (in monkeys particularly in lung, liver, spleen, kidney and brain). Pyrimethamine is extensively metabolized and at least sixteen metabolites have been identified, the major being an N-oxide. The drug has a long elimination half-life, in the order of 80-100 h [118, 119]. The majority (85%) is eliminated in the urine [120] and 3-5% may be recovered from the feces. In view of the prolonged elimination half-life of pyrimethamine, symptoms related to accumulation of the drug should be expected when it is administered at shorter dose intervals. Pyrimethamine is also known to inhibit the enzyme histamine N-methyl transferase, which is responsible for the metabolism of histamine in several mammalian tissues and therefore CNS accumulation of histamine may be responsible for the occurrence of convulsions following massive overdose. The effect on histamine metabolism may also account for the incidence of postural hypotension following malaria treatment with pyrimethamine, either alone or in combination with sulfonamides.

Sulfonamides

The sulfonamides are readily and completely absorbed following oral administration and maximum plasma levels are achieved 3-6 h after dosing. Generally, less than 10% of the administered dose is metabolized, approximately 5% to the acetyl derivative. Sulfonamides are extensively protein-bound in the plasma, mainly to albumin. Sulfonamides may displace other protein-bound compounds, and this has clinical significance in neonatal hyperbilirubinemia, where bilirubin displaced by sulfonamides may result in kernicterus. The half-life of sulfadoxine has been observed to range between 79 and 200 h in a group of eight volunteers. A study of sulfalene indicated that red cell concentrations were in the order of 40% those of plasma concentrations, while with sulfadoxine, the red cell concentration was the same as the plasma level [121, 122]. It is observed that approximately 90% of administered sulfonamide is eliminated in the urine.

The most frequent and serious side effects associated with sulfonamide use involve the skin and mucous membranes. Mucocutaneous reactions may range from mild, self-limited rashes through vesicular exanthemata to bullous, exfoliative reactions including the Stevens-Johnson syndrome and toxic epidermal necrolysis, both of which may be fatal in 10-20% of cases.

Sulfones

Dapsone is thought to be nearly completely absorbed following oral administration, and peak plasma concentrations are produced in 3-6 h. Protein binding is approximately 80%, and plasma half-life ranges between 21 to 30 h. Red cell and plasma concentrations are nearly identical. The drug is metabolized, mainly to mono-N-acetyl dapsone. Approximately 90% of the administered drug is eliminated in the urine, mainly in the form of metabolites; the rest is excreted in the feces.

The major toxic side effects of sulfones are hematological and are reported even in association with relatively small doses. Methemoglobinemia may be observed at dapsone doses routinely used for malaria prophylaxis, and may be potentiated by concomitant administration of other antimalarials, e.g. chloroquine and primaquine. The most serious side effect related to dapsone is agranulocytosis [123-126].

1.2.4.5.5 8-Amino Quinolines

The first synthetic 8-aminoquinoline antimalarial was pamaquine and it soon became apparent that, although it was active against avian plasmodia, its blood schizontocidal action against the human malarias was much less, and its toxicity was considerable. Pamaquine reduced the relapse rate of *P. vivax* malaria reawakened interest in this compound prior to the Second World War [127]. During the remarkably productive antimalarial research initiative of the US Army during the Second World War, a series of 8- aminoquinolines were synthesized and screened. From this effort, three compounds were felt to be promising: pentaquine, isopentaquine and primaquine which had an anti-relapse effect on *P. vivax* superior to that of pamaquine and, of the three, primaquine had the least toxicity. Primaquine still remains the most effective available compound for the elimination of the hypnozoites of the relapsing malarias, and for anti-gametocyte therapy in *P. falciparum* malaria.

Mechanism of Action and Spectrum of Activity

The mechanism of action of primaquine is not well understood, but research in rodent and simian models indicates that the site of action involves the plasmodial mitochondria. Studies of the metabolism of pamaquine have suggested that

metabolites of drugs of this class may mimic ubiquinone and reduced ubiquinone, substances found in the mitochondrial electron transport chain. Primaquine has not been shown to have any activity against sporozoites. Primaquine is active against primary exoerythrocytic schizonts but at doses which may be expected to be toxic. Primaquine is not used as a prophylactic agent or blood schizontocide. It is highly active against gametocytes of all species of human malaria parasites and against hypnozoites of *P. vivax,* and, presumably, *P. ovale.*

Pharmacokinetics, Metabolism and Adverse Effects

It is observed that following a single oral dose of 45 mg; peak plasma levels of 153 ng/ml are attained in 2-3 h, with a bioavailability of 72-76%, indicating rapid and relatively high absorption. Primaquine is rapidly metabolized in the liver and the parent drug disappears quickly from the blood, having a half-life of only 5-6 h. The main metabolite, carboxy primaquine, has a peak blood level ten times that of primaquine, and at 24 h following administration of primaquine, this metabolite persists at 60% of the peak level. Studies in monkeys have indicated that primaquine and its metabolites are concentrated in red cells. Although toxic symptoms are rare when primaquine is given at the usual doses, the administration of increased doses may be accompanied by gastrointestinal complaints (anorexia, nausea, epigastric distress, vomiting, abdominal pain and cramping) and vague symptoms such as weakness and uneasiness in the chest. The more severe side effects of the higher doses of the 8-aminoquinolines are related to their effect on the formed elements of the blood and bone marrow. Leukopenia, anemia, suppression of myeloid activity and methemoglobinemia may occur which results in cyanosis. Withdrawal of primaquine will result in the disappearance of symptoms within 24-72 h. Individuals deficient in the enzyme Glucose 6 phosphate dehydrogenase (G6PD) may suffer severe hemolysis following the ingestion of primaquine. Defects in some of the other enzymes related to the pentose-phosphate shunt may also render erythrocytes hypersensitive to hemolysis by the 8-aminoquinolines [128]. For use as a gametocytocide in *P. falciparum,* primaquine is effective at a single dose of 30-45 mg. For anti-relapse therapy of *P. vivax* and *P. ovale,* the usual dose is 15 mg daily for 14 days except in patients with G6PD deficiency of the Mediterranean B and certain Asian variants. These patients may be treated using an intermittent regimen

with weekly doses of 45 mg primaquine for 8 weeks, which should be administered under strict medical supervision.

1.2.4.5.6 Newer Drugs: Halofantrine

A potential phenanthrene replacement drug for mefloquine, halofantrine, has demonstrated treatment efficacy in regions where mefloquine is not effective [129, 1300]. It has got blood schizontocidal activity comparable to mefloquine. It is effective against *P. falciparum* resistant to chloroquine, pyrimethamine-sulphadoxine and mefloquine. It has got poor absorption and low bioavailability [131]. The plasma half-life of the drug is one day but that of its active metabolite is 3 days. Recent reports on the cardio toxicity of this drug at higher doses have prompted closer scrutiny of its pharmacokinetics and efforts to evaluate the efficacy of its metabolite with more efficacy and less cardio toxicity [132].

1.2.4.5.7 Newer Drugs: Artemisinin Derivatives

Artemisinin (Quinghauso) is a sesquiterpene lactone, which has shown marked antimalarial activity against *P. falciparum* [133]. It is obtained from a plant that is a traditional source of remedy in china '*Artemisia annua*'. A number of artemisinin derivatives e.g. arteether, artemether, artesunate, artelinic acid have been developed or are under development. The mechanism of action of artemisinin drugs is thought to be iron dependent free radical generation [134, 135]. The potential for fatal neurotoxicity in animal models with artemisinin derivatives, especially the oil soluble derivatives arteether and artemether. A dose dependent brain stem neuropathology has been described in rats treated with repeated arteether doses [136]. Arteether, the oil soluble ethyl ether derivative of artemisinin, has been shown to be a potent erythrocytic schizontocidal agent and is metabolized to dihydroartemisinin (DHA). *In vitro* and *in vivo* experiments showed that DHA is as efficacious as arteether as an antimalarial but possess the potential for neurotoxicity at higher doses. Arteether has been useful in hundreds of patients for the treatment of severe and complicated malaria. Because of their short half-lives, artemisinin derivatives are expected to be used with a sequential combination of drugs such as mefloquine [137]. Artesunate, a water-soluble artemisinin derivative, is currently being formulated as rectal suppository and is being evaluated in a rural health care setting where the access to parentral drugs is limited. Artenilic acid, is being developed by Walter Reed Army

Institute for oral treatment of uncomplicated malaria. This drug has a lower potential for neurotoxicity than arteether [138] and has an advantage that it can be formulated as sodium salt for intravenous injection to treat severe and complicated disease. Artelinic acid is metabolized to dihydroartemisinin, a potentially potent neurotoxin, to a much lower extent than arteether, and newer analogues, which do not undergo metabolism to dihydroartemisinin, are also undergoing development. Second and third generation artemisinin like compounds, specially synthetic trioxanes and tetraoxanes, collectively referred to as "Malperox" candidates, are also being pursued, [139-140].

1.2.4.5.8 Antibiotics

It was reported in 1945 that penicillin had no antimalarial activity [141], but shortly thereafter, in 1949, chlortetracycline was shown to be effective against human and avian malaria parasites. As the problem of resistance to chloroquine emerged and spread, interest in alternative antimalarials, including the antibiotics, was revived. It has been shown that tetracycline had a marked effect against both pre-erythrocytic and erythrocytic schizonts. All of these reports emphasized the need for a fast-acting schizontocide, such as quinine, in conjunction with the slow-acting tetracyclines, which, when used alone, take approximately 48 h to manifest activity. Clindamycin, like tetracycline, is an efficient blood schizontocidal drug with relatively slow action, which requires a fast-acting drug such as quinine to control the infection. Concurrent treatment with quinine is associated with marked toxicity, but sequential treatment with quinine followed by clindamycin seems to be well tolerated. Erythromycin, which has weak antimalarial activity, shows potentiating synergism with chloroquine against chloroquine-resistant strains of rodent malaria *in vivo* and *P. falciparum in vitro*.

Mechanism of Action and Spectrum of Activity

In bacteria, the tetracyclines, clindamycin and erythromycin are all considered to act against ribosomal protein synthesis, and it appears likely that they affect the plasmodia through mitochondria, which are presumed to have similar ribosomal makeup. This could account for their slow activity, since mitochondrial replication may be restricted to a limited part of the cell cycle. It has been suggested that the potentiating synergism between chloroquine and erythromycin in rodent malaria

results from an increase in permeability of the mitochondrion for the antibiotic, caused by chloroquine [142]. In volunteer studies, the tetracyclines were shown to be active against the pre-erythrocytic liver forms of *P. falciparum* even when administration began as long as four days after the infective bite. Their activity against primary liver forms of other species of human malaria have not been adequately studied. The tetracyclines and clindamycin are active against the asexual blood forms of *P. falciparum*, including isolates, which are resistant to the 4-aminoquinolines and to DHFR inhibitors, but their activity is slow. Tetracyclines have no apparent activity against gametocytes of *P. falciparum*. Neither the tetracyclines nor clindamycin appear to provide radical cure of the relapsing malarias.

Pharmacokinetics, Metabolism and Adverse Effects

The absorption of the tetracyclines is variable, ranging from 30 to 80%, depending on the preparation, dose and whether or not the stomach is empty at the time of drug administration. Tetracycline is better absorbed than chlortetracycline or oxytetracycline. Higher doses of these antibiotics have lesser absorption as compared to lower doses. Moreover, food in the stomach decreases their absorption. The absorption of the tetracyclines is inhibited by di- and tri-valent cations, such as calcium, aluminum, magnesium and iron. For this reason, tetracyclines should not be given with milk or antacids, and medications containing the above-mentioned cations should not be given within two hours of the tetracycline dose. The gastric intolerance of tetracyclines, however, occasionally requires that they should be administered with a light meal, despite these considerations. The tetracyclines are widely distributed throughout body tissues and fluids. Pharmacokinetic studies of clindamycin in man [143] indicate that the drug is well absorbed following oral administration, and peak serum concentrations are achieved within 45 min of administration. The average half-life is 2.4 h, with a range of 1.5-3.5 h, after single doses of 250 mg. It is widely distributed in the tissues and drug is highly metabolized in the liver and much of it is excreted in the bile. The tetracyclines should not generally be used in pregnant women and in children less than eight years of age, since they may produce ossification disorders and discoloration of developing teeth [144]. The most common side effects of tetracycline administration are gastrointestinal including epigastric distress, abdominal discomfort, nausea, vomiting and diarrhea. Acute, severe pseudomembraneous colitis may occur in conjunction with clindamycin therapy. It

has been postulated that this condition may be due to alteration of the intestinal flora with resulting overgrowth of *Clostridium difficile*. This organism produces a toxin, which has been incriminated in the causation of the colitis [145]. Skin rashes have been reported in about 10% of patients receiving clindamycin. The tetracyclines are never used alone for the therapy of malaria, but are administered in association with a fast-acting schizontocide such as quinine.

1.2.4.6 Mechanisms of Drug Resistance

1.2.4.6.1 Resistance to Quinine and Mefloquine

It has been shown that resistant parasites lower the concentration of this drug by increasing their efflux in an ATP dependent manner. As quinine and this new drug mefloquine are similar both in their structure and mode of action, quinine resistance may be a potential threat to use of mefloquine. This raises a question of common or overlapping mechanisms of resistance responsible for intrinsic or acquired resistance to mefloquine and its structurally related antimalarials [146].

1.2.4.6.2 Resistance to 4-aminoquinolines

The late 1950s saw the emergence of resistance to *P. falciparum* to the antimalarial drug, chloroquine. In contrast to that of pyrimethamine, resistance to chloroquine in human malaria species is restricted to *P. falciparum*. All other human species remain so far fully susceptible to this and other 4-aminoquinolones. It is suggested that the high level of resistance was probably due to an accumulation of mutations in each parasite at different loci, each conferring a low level of resistance since intermediate resistant clones [147]. Chloroquine resistant rodent malarial parasites have been shown to have many unique biological features. It has been shown that trophozoites of highly chloroquine resistant cell lines failed to produce haemetin [148]. This lead to the suggestion that the lack of haematin in chloroquine resistant malarial parasites was a result of a more efficient proteolytic activity of these parasites compared to the chloroquine sensitive species [149]. Red cells infected with chloroquine resistance strains accumulate less chloroquine than those infected with comparable chloroquine susceptible strains. Moreover calcium channel blockers that reduce chloroquine efflux can particularly restore the susceptibility of resistant parasites to chloroquine [150, 151].

1.2.4.6.3 Resistance to Dihydrofolate Reductase Inhibitors

It is hypothesized that resistance has a Mendelian basis of inheritance with recombinant forms only being obtained after cross-fertilization of the gametes in the mosquito. The pyrimethamine resistant mutants that have been obtained show various degree of cross-resistance to sulphonamides and sulphones and vary in their requirement of PABA. Resistance may be due to various mechanisms like alterations in enzyme, alterations in the transport of the drug across the membrane and gene amplification i.e., an increase in number of genes which result in larger amounts of the enzyme in resistant cells [152]. It has also been shown that pyrimethamine resistance in rodent malarias is associated with an increase in level of dihydro folate reductase within the parasite, a decrease in enzyme affinity for the substrate and a decrease in its sensitivity to inhibition by pyrimethamine.

1.2.4.6.4 Resistance to Sulfonamides and Sulphones

Lines of plasmodium resistant to sulfonamides or sulphones may be developed in the laboratory either by exposing the parasites to single heavy or repeated sub-curative doses of the drug or by withholding the cofactor PABA. In either case it appears that the parasite must survive by bypassing the step at which PABA is incorporated into dihydropteroate. It is possible that it may be able to do this by utilizing the host cell folates. Resistance to these compounds is however, a stable character which is carried through all the stages of the life cycle when the parasite is cyclically transmitted [153].

1.2.4.6.5 Resistance to Primaquine

Resistance of asexual intra-erythrocytic parasites to primaquine is of somewhat academic interest since this is the only compound which is clinically used for radical cure against *P. vivax* and as a gametocytocide against *P. falciparum*. It seems possible therefore that one mechanism of resistance to this compound could be the generation of increased number of these organelles to compensate for drug effect. The biochemical basis of primaquine resistance is still unknown.

1.2.5 Drug Associations and Fixed Combinations

Associations and combinations of antimalarial drugs may be used for the purpose of enhancing the activity in the treatment of individual infections, particularly

Pharmacokinetic Studies of Bulaquine, A Potent Antirelapse Antimalarial Agent

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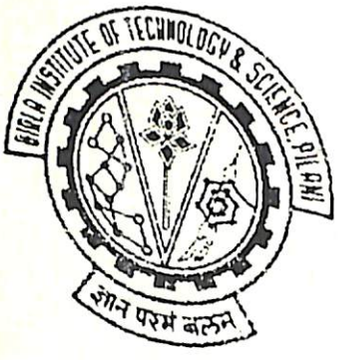
Under the Supervision
of **Dr. R.C. Gupta**



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where drug resistance is a problem and in an attempt to delay the appearance of resistance to one or both partners in an association, when a drug is to be used widely in a malaria-endemic area. Drug associations may be additive, potentiating or complementary. Additive associations are considered to be those in which the combined effect of the constituents equals the sum of their individual activities. These are usually drugs, which act on the same stage of the parasite, but have different modes of action, e.g. chloroquine and pyrimethamine. Potentiating combinations are those in which the combined effect of the components clearly exceeds the sum of activities of the individual components. The latter may act at sequential points in a parasite metabolic pathway. Such activity is seen when drugs acting against the enzyme tetrahydrofolate dehydrogenase (dihydrofolate reductase), e.g. pyrimethamine, are used together with drugs inhibiting dihydropteroate synthase, e.g. the sulfonamides and sulfones. Complementary associations may act against different stages in the life cycle, e.g. chloroquine (which eliminates blood forms of *P. vivax*), given together with a course of primaquine (which acts against the latent liver forms, or hypnozoites, of this parasite).

1.2.6 Future Perspectives

Past approaches aimed at drug discovery at the Walter Reed Army Institute and WHO programmes have evolved empirical screening; screening of plant extracts from traditional medicines; and synthesis of analogues of compounds known to have antimicrobial, antitumour, antimalarial activity and that selectively target specific parasite enzymes. Primary evaluations begin *in vitro* with parasite cultures and in simple rodent models. Candidates, which show promise, are evaluated in secondary and tertiary test systems. Few Compounds are eventually selected for clinical evaluation because of problems with economical synthesis, formulation, stability, toxicity, or lack of sufficient activity during advanced testing or ultimately in cases of human malaria.

With the early discovery of quinine and Quinghaosu, traditional medicinal approaches have demonstrated historical success in antimalarial drug development, and a number of established programmes are pursuing new traditional medicine leads [154]. New approaches involving structure based drug design and molecular modeling have been initiated at Walter Reed Army Institute and represent significant enhancement in the drug discovery process. There is renewed emphasis on drug

discovery and new programmes are based on the most current tenets of using biotechnology for structure based drug design [155, 156]. Molecular biology techniques are used to identify and validate novel therapeutic targets in the parasite. Target genes are cloned and expressed, recombinant protein are used for target based high throughput drug screening assays, and crystallized protein is used for molecular drug design. This new programme is based on established paradigms in the pharmaceutical industry for structure based drug design. Current progress in the malaria genome sequence project and availability of expressed gene sequence tags will soon provide a plethora of potential targets for drug interdiction. A number of drug candidates are currently being prioritized for transition to advanced development. Using Lead-directed synthesis, molecular modeling and structure based drug design, these efforts will result in the discovery of safe and effective new drugs that will circumvent the elusive mechanism by which malaria parasite develop resistance. Multiple drug resistance in *P. falciparum* malaria will continue to cause special problems for targeting the blood stages of malaria. There must also be increased emphasis on developing drugs with true casual prophylactic or radical curative properties before blood stages emerge and cause clinical disease. The search for resistance modulated drugs in resistant malaria parasites shares common goals and interests with resistance modulated drugs developed for tumour cells. Certain malarial drugs are also effective against parasitic infections with large commercial implications [157]. The global solution to the current malaria problem is to maintain a necessary critical mass of investigators and clinical centers focused on and committed to discovering and developing new drugs.

SECTION 3

Research Envisaged

1.3 Research Envisaged

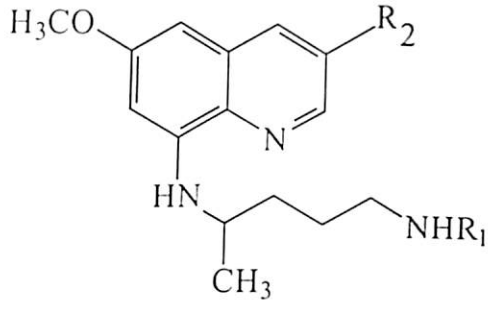
The chemotherapeutic agents of the 8-amino quinoline group are known to have tissue schizontocidal activity (causal prophylactic and radical curative) as well as gametocidal activity against malaria. Of the known compounds only primaquine (PQ) is in clinical use as an antirelapse drug against *P. vivax* [158]. The therapeutic application of primaquine is limited because of the adverse side effects which include methemoglobinemia and cyanosis, hemolytic anemia in G6PD deficient individuals, hepàtotoxicity, gastrointestinal distress, epigastric discomfort, anorexia, nausea, vomiting, headache, etc [159]. The drug is contraindicated in pregnancy and not recommended for infants [160]. Efforts to develop safer and less toxic antirelapse drug continue [161]. Attempts have been made to synthesize and evaluate analogs of PQ either by quinoline ring substitutions or side chain modifications, in the search for the compound that would be more effective or less toxic [162, 163].

In quest of search for newer, safer and potent antimalarial Central Drug Research Institute (CDRI) has developed Bulaquine (BQ). BQ [CDRI compound 80/53, 3-[1-[4-[(6-methoxy-8-quinolinyl) amino] pentylamino] ethylidene] dihydro-2(3H) furanone, an analogue of PQ, is a potent anti-malarial agent. It is safer than PQ and causes only $1/3^{rd}$ as much as methemoglobinemia [164-166]. It has shown curative and casual prophylactic activities against sporozite-induced *P. cyanomolgi* infection in rhesus monkeys and is also safe in subacute toxicity studies in rats and rhesus monkeys with no teratogenic action [167, 168].

A report on HPLC-UV determination of BQ and PQ in rat plasma has been published [169]. The reported method required some modifications under present laboratory conditions. Therefore, a new LC-UV method was developed and validated for the simultaneous detection of BQ and PQ in rabbit plasma for application to pharmacokinetic studies. With this method, BQ and PQ levels could not be detected for sufficient period and thus a more sensitive and selective method using LC-MS-MS was developed for generation of realistic PK parameters.

Considering the promising results of BQ at the pharmacological level, its pre clinical pharmacokinetic studies (*in vitro* and *in vivo*) were planned in various species so as to determine its bioavailability and study the species similarities and differences and promote the drug for further development. As a requisite of drug development process, the studies on systemic bioavailability, protein binding, and whole blood partitioning studies were undertaken in rats, rabbit and monkeys. Urinary and fecal

excretion studies were planned in rats to establish the amount of unchanged BQ and PQ excreted in urine and feces. These studies are detailed separately in chapters 3 and 4. The structures of BQ, PQ and their internal standards 3-bromo bulaquine (3-BBQ) and 3-bromo primaquine (3-BPQ) are given in Figure 1.3.1.



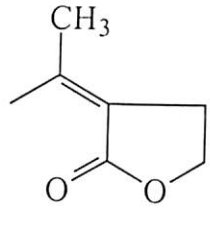
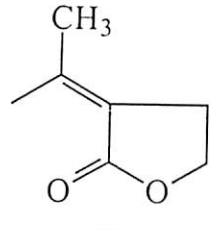
Compound	*R_1*	*R_2*
BQ		H
PQ	H	H
3-BBQ		Br
3-BPQ	H	Br

Figure 1.3.1 Chemical structures of BQ, PQ, 3-BBQ and 3-BPQ

References:

1. Thompson, W.L., 1996. Thompson addresses survival of the pharmaceutical enterprise, *AAPS Newletter*, 5.
2. Fueng, M. Thornton, A., Mybeck, K., Wu, J.H., Hornbuckle, K. and Muniz, E., 2001. Evaluation of the characteristics of safety withdrawals of prescription drugs from world wide pharmaceutical markets-1960 to 1999, *Drug Information J.*, 35: 293-317.
3. Theil, F.P., Guentert, T.W., Haddad, S. and Poulin, P., 2003. Utility of physiologically based pharmacokinetic models to drug development and rational drug discovery candidate selection, *Toxicol. Lett.*, 138: 29-49.
4. Rowland, M. and Tozer, T.N., 1996. Clinical pharmacokinetics: concepts and applications. 3rd edition, Waverly Pvt. Ltd., 1-7.
5. Kaplan, S.A., 1989. Commentary to a phased approach to drug development, *J. Pharmacokin. Biopharm.*, 17: 521-523.
6. Michne, W. F., 1996. Hit-to-lead chemistry: a key element in new lead generation, *Pharm. News*, 3:19-21.
7. Olah, T.V., McLoughlin, D.A. and Gilbert, J.D., 1997. The simultaneous determination of mixtures of drug candidate by liquid chromatography/ atmospheric pressure chemical ionization mass spectrometry as an *in vivo* drug screening procedure, *Rap. Commun. Mass Spectrom.*, 11: 17-23.
8. Bryant, M.S, Korfmacher, W.A., Wang, S.Y., Nardo, C., Nomeir, A.A, Lin, C.C., 1997. Pharmacokinetics screening for the selection of new drug discovery candidates is greatly enhanced through the liquid chromatography atmospheric pressure ionization mass spectrometry, *J. Chrom.*, 777: 61-66.
9. Berman, J., Halm, K., Adkinsons, K. and Shaffer, J., 1977. Simultaneous pharmacokinetic screening of a mixture of compound in the dog using API-LC/MS/MS analysis for increased throughput, *J. Med. Chem.*, 40: 827-829.
10. Gan, L.S.S. and Thakker, D.R., 1997. Application of the caco-2 model in the design and development of orally active drug: Elucidation of biochemical and physical barrier posed by intestinal epithelium, *Adv. Drug Deliev. Rev.*, 23: 77-98.
11. Artursson, P., 2002. Prediction of drug absorption: Caco-2 and beyond, Book of abstract, PAMPA Conference, San Francisco, CA, 14-17 July.

12. Mandagere, K.A., 2002. Graphical model for estimating oral bioavailability of drug in humans and other species from their Caco-2 permeability and *in vitro* liver enzyme metabolic stability rats. *J. Med. Chem.*, 45: 304-311.

13. Stewart, B.H., Chan, O.H, Lu, R.H., Reyner, E.L., Schmid, H.L., Hamilton, H.W., Steinvaugh, B.A. and Taylor, M.D., 1995. Comparison of intestinal permeability determined in multiple *in vitro* and *in situ* models: Relationship to absorption in humans, *Pharm. Res.*, 12: 693-699.

14. Delie, F. and Rubas, W., 1997. A human colonic cell line sharing similarities with enterocytes as a model to examine oral advantages and limitations of the Caco-2 model, *Crit. Rev. Therap. Drug Carrier Sys.*, 14: 221-286.

15. Artursson, P, Palm, K. and Luthman, K., 1996. Caco-2 mono layer in experimental and theoretical prediction of drug transport, *Adv. Drug Deliev. Rev.*, 22: 67-84.

16. Waterbeemd, V., Camenisch, G., Folkers, G. and Raevsky, O.A., 1996. Estimation of Caco-2 cell permeability using calculated molecular descriptors, *Quant. Str. Act. Rel.*, 15: 480-490.

17. Camenisch, G., Folkers, G. and Waterbeemd, V., 1997. Comparison of passive drug transport through Caco-2 cells and artificial membrane, *Int. J. Pharm.*, 147: 61-70.

18. Yang, C.Y., Cai, S.J., Liu, H.L. and Pidgeon, C., 1997. Immobilised artificial membrane screens for drug membrane interaction, *Adv. Drug Deliev. Rev.*, 23: 229-256.

19. Korfmacher, W.A., 1999. Development of an automated mass spectrometry system for the quantitative analysis of liver microsomal incubation samples: a tool for rapid screening of new compounds for metabolic stability, *Rapid Commun Mass Spectrom.*, 13: 901-907.

20. Rourick, R.A., Volk, K.J., Klohr, S.E., Spears, T., Kerns, E.H. and Lee, M.S., 1996. Predictive strategy for the rapid structural elucidation of drug degradants, *J. Pharm. Biopharm. Anal.*, 14: 1743-1752.

21. Wang, G., Hsieh, Y., Lau, Y., Chang, K.L., Korfmacher, W.A. and White, R.E., 2001. Semi automated determination of plasma stability of drug discovery compounds using liquid chromatography tandem mass spectrometry, *J. Chrom. B.*, 780: 451-457.

22. Grass, G.M., 1997. Simulation models to predict oral drug absorption from *in vitro* data, *Adv. Drug Deliev. Rev.*, 23: 199-219.

23. Navia, M. and Chaturvedi, P.R., 1996. Design principles for orally bioavailable drugs, *Drug Discov. Today,* 1: 179-189.

24. Waterbeemd, V., 2000. Intestinal permeability: prediction from theory, In: Oral absorption: prediction and assessment, Marcel Dekker, Inc. New York, 31-49.

25. Gan, L.S.L. and Thakker, D.R., 1997. Applications of Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium, *Adv. Drug Del. Rev.*, 23: 179-189.

26. Amidon, G.L., Lennernas, H., Shah, V.P. and Crison, J.R., 1995. A theoretical basis for a biopharmaceutics drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability, *Pharm. Res,,* 12: 413-429.

27. Leppert, P.S. and Fix, J.A., 1994. Use of averted intestinal rings for *in vitro* examination of oral absorption potential. *J. Pharm.Sci,.* 8: 976-981.

28. Kim, D.C., Burton, P.S. and Borchardt, R.T., 1993. A correlation between the permeability characteristics of a series of peptides using an *in vitro* cell culture model (Caco-2) and those using an in situ perfused rat ileum model of the intestinal mucosa. *Pharm. Res,* 10: 1710-1714.

29. Hidalgo, I.J., 1996. Cultured epithelial cell models, In: Models for assessing drug absorption and metabolism, Plenum Press, New York., 35-50.

30. Artursson, P. and Karisson, J., 1991. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial cells (caco-2), *Biochem. Biophy. Res. Comm.,* 14: 880-885.

31. Yee, S. 1997. *In vitro* permeability across caco-2 cells (colonic) can predict *in vivo* (small intestinal) absorption in man-fact or myth, *Pharm. Res.,* 14: 763-766.

32. Pidgeon, C., Ong, S., Liu, H., Qiu, X., Pidgeon, M., Dantzig, A.H., Munroe, J., Hornback, W.J., Kasher, J.S. and Glunz, L., 1995. IAM chromatography: an *in vitro* screen for predicting drug membrane permeability, *J. Med. Chem.,* 38: 590-594.

33. Kansy, M., Senner, F. and Gubernator, K., 1998. Physiochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption process, *J. Med. Chem.,* 41: 1007-1010.

34. White, R.E., 2000. High throughput screening in drug metabolism and pharmacokinetic support of drug discovery, *Ann. Rev. Pharmacol. Toxicol.*, 40: 133-157.

35. Dedrick, R.J., 1973. Animal scale up, *J. Pharmacokin. Biopharm.*, 1: 435-461.

36. Boxenbaum, H., 1982. Interspecies scaling, allometry, physiological time, and ground plan of pharmacokinetics, J. *Pharmacokin. Biopharm.*, 10: 201-227.

37. Sawada, Y., Hauano, M., Sugiyama, Y. and Iga, T., 1984. Prediction of the disposition of β-lactum antibiotics in humans from pharmacokinetic parameters in animals, *J. Pharmacokin. Biopharm.*, 12: 241-261.

38. Ito, K., Iwatsubo, T., Kanamitsu, S., Nakajima, Y. and Sujiyama, Y., 1998. Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism together with binding and transport, *Ann. Rev. Pharmacol. Toxicol.*, 38: 461-499.

39. Obach, R.S., J.G, Liston, T.E., Silber, B.M., Jones, B.C., MacIntyre, F., Rance, D.J. and Wastall, P., 1997. The prediction of human pharmacokinetic parameters from preclinical and *in vivo* metabolism data, *J. Pharm.Exp.Therap.*, 283: 46-58.

40. Kostiainen, R., Kotiaho, T., Kuuranne, T. And Auriola, S., 2003. Liquid chromatography/atmospheric pressure ionization-mass spectrometry in drug metabolism studies, *J.,Mass Spectrom.*, 38: 357-372.

41. Guerin, P.J., Olliaro, P., Nosten, F., Druilhe, P., Laxminarayan, R., Binca, F., Kilama, W.L., Ford, N., and White, N.J., 2002. Malaria: current status of control, diagnosis. Treatment, and a proposed agenda for research and development, *The Lancet,* 2: 564-573.

42. Olliaro, P., Cattani, J. and Wirth, D., 1996. Malaria, the submerged disease, *JAMA*, 275: 230-233.

43. Cook, G.C., 1992. Malaria: an under diagnosed and often neglected medical emergency, *Aust. N. Z. J. Med.*, 22: 69-80.

44. Greenwood, B.M., 1987. Mortality and morbidity from malaria among children in a rural area of Gambia, West Africa, *Trans. Roy. Soc. Trop. Med. Hyg.*, 81: 478-486.

45. White, N.J., 1996. The treatment of malaria, *New Engl. J. Med.*, 335: 800-806.

46. Waller, D., Krishna, S., Crawley, J., Miller, R., Nosten, F. and Chapman, D., 1995. Clinical features and outcome of severe malaria in Gambian children, *Clin. Infect. Dis.*, 21: 577-587.

47. Greenwood, B. and Mutabingwa, T., 2002. Malaria in 2002, *Nature*, 415: 670-672.

48. Miller, L.H., Baruch, D.I., Marsh, K. and Doumbo, O.K., 2002. The pathogenic basis of malaria, *Nature*, 415: 673-679.

49. Gallup, J.L. and Sachs, J.D., 2001. The economic burden of malaria, *Am. J. Trop. Med. Hyg.*, 64: 85–96.

50. Murray, C.J. and Lopez, A.D., 1996. Evidence-based healthpolicy–lessons from the global burden of disease study, *Science*, 274: 740–743.

51. Murphy, S.C. and Breman, J.G., 2001. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy, *Am. J. Trop. Med. Hyg.*, 64: 57–67.

52. Holding, P.A. and Snow, R.W., 2001. Impact of *Plasmodium falciparum* malaria on performance and learning: review of the evidence. *Am. J. Trop. Med. Hyg.*, 64: 68–75.

53. Breman, J.G., Egan, A. and Keusch, G.T., 2001. The intolerable burden of malaria: a new look at the numbers, *Am. J. Trop. Med. Hyg.*, 64: iv–vii.

54. Hisashi, F. and Masamichi, A., 2002. The malaria parasite and its life cycle, *Mal. Immun. Chem. Immun.*, 80: 1-26.

55. Krotoski, W.A., Collins, W.E., Bray, R.S., Garnham, P.C.C., Cogswell, F.B. and Gwadz, R., 1982. Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection, *Am. J. Trop. Med. Hyg.*, 31: 1291–1293.

56. Krotoski, W.A., Garnham, P.C.C., Bray, R.S., Krotoski, D.M., Killick-Kendrik, R. and Draper, C.C., 1982. Observation on early and late post sporozoite tissue stages in primate malaria, *Am. J. Trop. Med. Hyg.*, 31: 24-35.

57. Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W., Gilman, A.G., 1996. Goodman and Gilman's 'The pharmacological basis of therapeutics', 9th edition, Mc Graw-Hill publications, New York: 965-986.

58. Tripathi, K.D., 1995. Essential of Medical Pharmacology, 3rd edition, Jaypee brother's medical publishers (P) Ltd., New Delhi, 723-735.

59. Aikins, M.K., Fox-Rushby, J. and Alessandro, L., 1998. The Gambian National Impregnated Bednet Programme: costs, consequences and net cost-effectiveness, *Soc. Sci. Med.*, 46: 181–191.

60. Utzinger, J., Tozan, Y. and Singer, B.H., 2001. Efficacy and cost effectiveness of environmental management for malaria control. *Trop. Med. Int. Health.*, 6: 677–687.

61. Kroeger, A., Gonzlez, M. and Ordenez- Gonzalea, J., 1999. Insecticide-treated materials for malaria control in Latin America: to use or not to use?, *Trans. R. Soc. Trop. Med. Hyg.*, 93: 565–570.

62. Richie, T.L. and Saul, A., 2002. Progress and challenges for malaria vaccines, *Nature*, 415: 694–701.

63. Facer, C.A. and Tanner, M., 1997. Clinical trials of malaria vaccines: progress and prospects, *Adv. Parasitol.*, 39: 1–68.

64. Hoffman, S.L., Goh, L.M. and Luke, T.C., 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites, *J. Infect. Dis.*, 185: 1155–1164.

65. Brown, A.E., Singharaj, Y. and Wevester, H.K., 1994. Safety, immunogenicity and limited efficacy study of a recombinant *Plasmodium falciparum* circumsporozoite vaccine in Thai soldiers, *Vaccine*, 12: 102–108.

66. Kester, K.E., McKinney, D.A. and Tornieparth, N., 2001. Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental *Plasmodium falciparum* malaria, *J. Infect. Dis.*, 183: 640–647.

67. Woodward, R.B. and Doering, W.E., 1945. The total synthesis of quinine, *J.Am. Chem. Soc.*, 67: 760-784.

68. White, N.J., Looareesuwan, S., Warrell, D.A., Chongsuphajaisiddhi, T., Bunnag, D. and Harinasuta, T., 1981. Quinidine in falciparum malaria, *Lancet*, 2: 1069-1079.

69. Hahn, F.E., O'Brien, R.L., Ciak, J., Allison, J.L. and Olenick, J.G., 1966. Studies on mode of action of chloroquine, quinacrine and quinine and chroquine resistance, *Military Med. (Supp)*, 131: 1071-1089.

70. Fitch, C.D., Chevli, R., Banyal, H.S., Phillips, G., Pfaller, M.A. and Krogstad, D.J., 1982. Lysis of *P. falciparum* by ferriprotoporphyrin IX and a chloroquine-ferriprotoporphyrin complex, *Antimicrob. Ag. Chemother.*, 21: 819-822.

71. Warhurst, D.C., 1981. The quinine-haemin interaction and its relationship to antimalarial activity, *Biochem. Pharmacol.*, 30: 3323-3327.

72. Taggart, J.V. Earle, D.P., Berliner, R.W., Zubrod, C.G., Welch, W.J, Wise, N.B., Schroeder, E.F., London, I.M. and Shannon, J.A., 1948. Studies on chemotherapy of human malarias. III. The physiological disposition and antimalarial activity of the cinchona alkaloids, *J. Clin. Invest.(Supp)*, 27: 80-86.

73. White, N.J., Looareesuwan, S., Warrell, D.A., Warrel, M.J., Bunnag, D. and Harinasuta, T., 1982. Quinine pharmacokinetics and toxicity in cerebral and uncomplicated falciparum malaria, *Am. J. Med.*, 73: 564-572.

74. Trenholme, G. M., Williams, R.L., Rieckmann, K.H., Fischer, H. and Carson, P.E., 1976. Quinine disposition during malaria and during induced fever, *Clin. Pharmacol. Therap.*, 19: 459-467.

75. White, N.J., Looareesuwan, S., Warrell, D.A., Warrel, M.J., Chanthavanich, P., Bunnag, D. and Harinasuta, T., 1983. A quinine loading dose in cerebral malaria, *Am. J. Trop. Med. Hyg.*, 32: 1-5.

76. Chongsuphajaisiddhi, T., Savchareon, A. and Attanth, P., 1981. *In vivo* and *in vitro* sensitivity of falciparum malaria to quinine in Thai children, *Ann. Trop. Paed.*, 1: 21-26.

77. Hall, A.P., 1973. Quinine and chloroquine antagonism in falciparum malaria (correspondence), *Trans. Roy. Soc.Trop. Med. Hyg.*, 67: 425.

78. White, N.J., Looareesuwan, S., Warrell, D.A., Warrel, M.J., Krishna, S., Williomson, D.H. and Turner, R.C., 1983. Severe hypoglycemia and hyperinsulinemia in falciparum malaria, *New Eng. J. Med.*, 309: 61-66.

79. Berliner, R.W., Earle, D.P., Taggart, J.V., Zubrod, C.G., Welch, W. J., Conan, N.J., Bauman, P., Scudder, S.T. and Shannon, J., 1948. Studies on the chemotherapy of the human malarias. VI. The physiological disposition, antimalarial activity and toxicity of several derivatives of 4-aminoquinoline, *J. Clin. Invest. (Supp)*, 27: 98-107.

80. Most, H., London, I.M., Kane, C.A., Lavietes, P.H., Schroeder, E.F. and Hayman, J.M., 1946. Chloroquine for treatment of acute attacks of vivax malaria, *J. Am. Med. Assoc.*, 131: 963-967.

81. Findlay, G.M., 1947. The toxicity of mepacrine in man, *Trop. Dis. Bull.*, 44: 763-779.

82. Baker, B.M. and Platt, D., 1947. Vivax relapse rates following continued Atabrine suppressive medication: Observations on malaria in an infantry regiment, *Bull. John Hopkins Hospital*, 81: 295-304.

83. Findlay, G.M., Markson, J.L. and Holden, J.R., 1944. Investigations in the chemotherapy of malaria in West Africa, III. Further investigations on treatment with quinine and mepacrine, *Ann. Trop. Med. Parasitol.*, 38: 201- 204.

84. Ciak, J. and Hahn, F.E., 1967. Quinacrine (Atebrin): Mode of action, *Science*, 156: 655-656.

85. Rieckmann, K.H., Powell, R.D., McNamara, J.V., Willerson, D., Kass, L., Frischer, H. and Carson, P.E., 1971. Effects of tetracycline against chloroquine-resistant and chloroquine sensitive *P. falciparum*., *Am. J. Trop. Med. Hyg.*, 20: 811-815.

86. Siddiqui, W.A., Schnell, J.V. and Geiman, Q.M., 1972. A model *in vitro* system to test the susceptibility of human malarial parasites to antimalarial drugs, *Am. J. Trop. Med. Hyg.*, 21: 392-399.

87. Schmidt, L.H., Vaughan, D., Muller, D., Crosby, R. and Hamilton, R., 1977. Activities of various 4-aminoquinolines against infections with chloroquine-resistant strains of *P. falciparum*, *Antimicrob. Ag. Chemother.*, 11: 826-843.

88. Gustafsson, L.L., Walker, O., Alvan, G., Beermann, B., Estevez, F., Gleisner, L., Lindstrom, B. and Sjoqvist, F., 1983. The disposition of chloroquine in man after single intravenous and oral doses, *Br. J. Clin. Pharmacol.*, 15: 471-479.

89. Salako. L.A. and Adelusi, S.A., 1983. Plasma, whole blood and red blood cell kinetics of chloroquine after oral and parenteral administration in rabbits. *Arch. Internat. Pharmacodyn. Therap.*, 261: 4-15.

90. Mc Chesney, E.W., Fasco, M.J. and Banks, W.F., 1957. The metabolism of chloroquine in man during and after repeated oral doses, *J. Pharmacol. Exp. Therap.*, 158: 482-493.

91. McChesney, E.W. and McAuliff, J.P., 1961. Laboratory studies of the 4-aminoquinoline antimalarials, I; Some biochemical characteristics of chloroquine, hydroxychloroquine and SN-7718, *Antibiot. Chemother.*, 11: 800-810.

92. McChesney E.W., Conwey W.D., Banks, W.F., Rogers, J.F. and Shekosky, J.M., 1966. Studies of the metabolism of some compounds of the 4-amino-7-chloroquinoline series, *J. Pharmacol. Exp. Therap.*, 151: 482-493.

93. Larsson, B. and Tjalve, H., 1979. Studies on the mechanism of drug binding to melanin, *Biochem. Pharmacol.*, 28: 1181-1187.

94. Adelusi, S.A. and Salako, L.A., 1982. Protein binding of chloroquine in the presence of aspirin, *Br. J. Clin. Pharmacol.*, 13: 451-452.

95. Adelusi, S.A., Dawodu, A.H. and Salako, L.A., 1982. Kinetics of the uptake and elimination of chloroquine in children with malaria, *Br. J. Clin. Pharmacol.*, 14: 483-487.

96. Stepien, K.B. and Wilczak, T., 1982. Studies of the mechanism of chloroquine binding to synthetic DOPA-melanin, *Biochem. Pharmacol.*, 31: 3359-3365.

97. Polet, H. and Barr, C.F., 1968. Chloroquine and dihydroquinine. *In vitro* studies of their antimalarial effect upon *P. knowlesi*, *J. Pharmacol. Exp. Therap.*, 164: 380-386.

98. Macomber, P.B., O'Brien, R.L. and Hahn, F.E., 1966. Chloroquine: Physiological basis of drug resistance in *P. berghei*, *Science*, 152: 1374-1375.

99. Fitch, C.D., 1969. Chloroquine resistance in malaria: A deficiency of chloroquine binding, *Proc. National Acad. Sci.*, 64: 1181-1187.

100. Bergqvist, Y. and Domeij-Nyberg, B., 1983. Distribution of chloroquine and its metabolite desethyl-chloroquine in human blood cells and its implication for the quantitative determination of these compounds in serum and plasma, *J. Chrom.*, 272: 137-148.

101. Aderounmu, A.F. and Fleckenstein, L., 1983. Pharmacokinetics of chloroquine diphosphate in the dog, *J. Pharmacol. Exp. Therap.*, 226: 633-639.

102. Olatunde, I.A., 1971. Chloroquine concentrations in the skin of rabbits and man, *Br. J. Pharmacol.*, 43: 334-340.

103. Alving, A.S., Eichelberger, L.C., Craige, B., Jones, R., Whorteon, C.M., Pullman, T.N., 1948. Studies on the chronic toxicity of chloroquine SN-7618, *J. Clin. Invest.*, 27: 60-65.

104. Salako, L.A. and Idowu, O.R., 1985. Failure to detect amodiaquine in the blood after oral dose, *Br. J. Clin. Pharmacol.*, 20: 307-311.

105. Salako, L.A., Walker, O. and Iyun, A.O., 1984. Pharmacokinetics of chloroquine in renal insufficiency, *African Med. Sci.*, 13: 177-182.

106. Hatton, C.S.R., Bunch, C., Peto, T.E.A., Pasvol, G., Russell, S.J., Singer, C.R.J., Edwards, G. and Winstanley, P., 1986. Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria, *Lancet*, 1: 411-414.

107. Neftel, K.A., Woodtly, W., Schmid, M., Frick, P.G. and Fehr, J., 1986. Amodiaquine induced agranulocytosis and liver damage, *Br. Med. J.*, 292: 721-723.

108. Davies, E.E., Warhurst, D.C. and Peters, W., 1975. The chemotherapy of rodent malaria. XXI. Action of quinine and WR 122,455 (a 9-phenanthrene methanol) on the fine structure of *P. berghei* in mouse blood, *Ann. Trop. Med. Parasitol.*, 69: 147-153.

109. Chevli, R. and Fitch, C.D., 1982. The antimalarial drug mefloquine binds to membrane phospholipids, *Antimicrob. Ag. Chemother.* 21: 581- 586.

110. Davidson, M.W., Griggs, B.G., Boykin, D.W. and Wilson, W.D., 1975. Mefloquine, a clinically useful quinolinemethanol antimalarial which does not significantly bind to DNA, *Nature*, 254: 632-634.

111. Desjardins, R.E., Pamplin, C.L., Von Bredow, J., Barry, K.G. and Canfield, C.J., 1979. Kinetics of a new antimalarial, mefloquine, *Clin. Pharmacol. Therap.*, 26: 372-379.

112. Schwartz, D.E., Eckert, G., Hartmann, D., Weber, B., Richard-Lenoble, D., Ekue, J.M. and Gentilini, M., 1982. Single dose kinetics of mefloquine in man. Plasma levels of the unchanged drug and of one of its metabolites, *Chemotherapy*, 28: 70-84.

113. Mu, J.Y., Israili, Z.H. and Dayton, P.G., 1975. Studies of the disposition and metabolism of mefloquine HCl (WR 142,409) a quinoline-methanol antimalarial in the rat, *Drug Metab. Dispos.* 3: 198-210.

114. Harinasuta, T., Viravan, C. and Reid, H.A., 1967. Sulphorimethoxine in chloroquine-resistant falciparum malaria in Thailand, *Lancet*, 1: 1117-1119.

115. Powell, R.D., Degowin, R.L. and McNamara, J.V., 1967. Clinical experience with sulphadiazine and pyrimethamine in the treatment of persons experimentally infected with chloroquine-resistant *Plasmodium falciparum*, *Ann. Trop. Med. Parasitol.*, 61: 396-408.

116. Maegraith, B.G., Tottey, M., Adams, A.R.D., Andrews, W.H.H. and King, J.D., 1946. The absorption and excretion of Paludrine in the human subject, *Ann. Trop. Med. Parasitol.*, 40: 493-506.

117. Ritschel, W.A., Hammer, G.V. and Thompson, G.A., 1978. Pharmacokinetics of antimalarials and proposals for dosage regimens, *Int. J. Clin. Pharmacol. Biopharm.*, 16: 395-401.

118. Jones, C.R. and Ovenell S.M., 1979. Determination of plasma concentrations of dapsone, monoacetyl dapsone and pyrimethamine in human subjects dosed with Maloprim, *J. Chrom.*, 163: 179-185.

119. Ahmad, R.A. and Rogers, H.J., 1980. Pharmacokinetics and protein binding interactions of dapsone and pyrimethamine, *Br. J. Clin. Pharmacol.*, 10: 519-524.

120. Smith, C.C. and Schmidt, L.H., 1963. Observations on the absorption of pyrimethamine from the gastrointestinal tract, *Exp. Parasitol.*, 13: 178-185.

121. Trenholme, G.M., Williams, R.L., Frisher, H., Carson, P.E. and Riekmann K.H., 1975. Host failure in treatment of malaria with sulfalene and pyrimethamine, *Ann. Int. Med.*, 82: 219-223.

122. Berneis, K. and Boguth, W., 1976. Distribution of sulfonamides and sulfonamide potentiators between red blood cells, proteins and aqueous phases of the blood of different species, *Chemother.*, 22: 390-409.

123. Ognibene, A.J., 1970. Agranulocytosis due to dapsone, *Ann. Int. Med.*, 72: 521-524.

124. Catalano, P.M., 1971. Dapsone agranulocytosis, *Arch. Dermatol.*, 104: 675.

125. Stickland, J.F. and Hurdle, A.D.F., 1970. Agranulocytosis probably due to dapsone in an infantry soldier, *Med. J. Aus.*, 1: 959-960.

126. Smithurst, B.A., Robertson, I. and Naughton, M.A., 1971. Dapsone-induced agranulocytosis complicated by gram-negative septicemia, *Med. J. Aus.*, 1: 537-539.

127. Sinton, J.A. and Bird, W., 1928. Studies in malaria with special reference to treatment. Plasmoquine in the treatment of malaria, *Indian J. Mal. Res.*, 16: 159-177.

128. Carson, P.E. and Frischer, H., 1966. Glucose-6-phosphate dehydrogenase deficiency and related disorders of the pentose phosphate pathway, *Am. J. Med.*, 41: 744-761.

129. Shanks, G.D., 1991. Halofantrine for the treatment of mefloquine chemoprophylaxis failures in *Plasmodium falciparum* infections, *Am. J. Trop. Hyg.*, 488-491.

130. Terkuile, F.O., Dolan, G., Nosten, F., Edstein, M.D., Luxemburger, C., Phaipun, L., Chongsuphajaisiddhi, T., Webster, H.K. and White, N.J., 1993. Halofantrine

versus mefloquine in treatment of multidrug-resistance falciparum malaria, *Lancet*, 341: 1044-1049.

131. Krishna, S., Terkuile, F., Supanaranond, S., Pukrittayakamee, S., Teja-Isavadharm, P., Kyle, D. and White, N., 1993. Pharmacokinetics, efficacy and toxicity of parentral halofantrine in uncomplicated malaria, *Br. J. Clin. Pharmacol.*, 36: 585-591.

132. Nosten, F., Ter, K.F., Luxemburger, C., Woodrow, C., Kyle, D.E., Chongsuphajaisiddhi, T. and White, N.J., 1993. Cardiac effects of anti malarial treatment with halofantrine, *Lancet*, 341: 1054-1056.

133. Li G.Q., Guo, X.B., Jin, R., Wang, Z.C. and Jian, H.X., 1982. Clinical studies on treatment of cerebral malaria with Qinghaosu and its derivatives. *J. Tradit. Chin. Med.* 2: 125-130.

134. Meschnik, S.R., Yang, Y.Z., Lama, V., Kuypers, F., Kamchon-wongpaisan, S. and Yuthavong, Y., 1993. Iron dependent free radical generation from the antimalarial agent artemisinin (Quinghaosu), *Antimicrob. Ag. Chemother.*, 37: 1108-1114.

135. Parker, F.D., Wesche, D. and Brewer, T., 1994. Does iron have a role in dihydroartemisinin-induced *in vitro* neurotoxicity, *Am. J. Trop. Med. Hyg.*, 51: 260.

136. Genovese, R.D., Newman, D., Petras, J. and Brewer, T., 1998. Dose-dependent brain stem neuropathology following repeated arteether administration in rats, *Brain Res. Bull.*, 49: 199-202.

137. Looareesuwan, S., Wilairatana, P., Viravan, C., Vanijanonta, S., Pitisuttithum, P. and Kyle, D.E., 1997. Open randomized trail of oral artemether alone and a sequential combination with mefloquine for acute uncomplicated falciparum malaria, *Am. J. Trop. Med. Hyg.*, 56: 613-617.

138. Lin, A.J., Klayman, D.L. and Milhous, W.K., 1987. Antimalarial activity of new water soluble dihydroartemisinin derivatives, *J. Med. Chem.*, 30: 2147-2150.

139. Grigorov, M., Weber, J., Tronchet, J.M., Jefford, C.W., Milhous, W.K. and Maric, D., 1997. A QSAR study of the anti malarial activity of some synthetic 1,2,4 trioxanes, *J. Chem. Info. Comput. Sci.*, 37: 124-130.

140. Posner, G., Chong, H., Gerena, L. and Milhous, W., 1995. Synthetic and antimalarial activities of structurally simplified 1,2,4 trioxanes related to artemisinin, *Heteroatom. Chem.*, 103-105.

141. Hindle, J.A., Rose, A.S., Trevett, L.D. and Prout, C., 1945. The effect of penicillin on inoculation malaria, A negative report, *New Eng. J. Med.*, 232: 133-136.

142. Warhurst, D.C., 1977. Chloroquine-erythromycin potentiation in *P. berghei*, *Ann. Trop. Med. Parasitol.*, 71: 383.

143. Wagner, J.G., Novak, E., Patel, N.C. Chidester, C.G. and Lummis, W.L., 1968. Absorption, excretion and half-life of clindamycin in normal adult males, *Am. J. Med. Sci.*, 256: 25-37.

144. Weyman, J., 1965. Tetracyclines and the teeth, *Practitioner*, 195: 661-665.

145. Bartlett, J.G., Chang, T.W., Taylor, N.S. and Onderdonk, A.B., 1979. Colitis induced by *Clostridium difficile*, *Rev. Infect. Dis.*, 1: 370-378.

146. Charet, P., Dei-Cas, E., Prensier, G., Moreau, S., Slomianny, C. and Vernes, A., 1985. Plasmodium resistance to chloroquine: A new hypothesis, *Trans. Roy. Soc. Trop. Med. Hyg.*, 79: 140.

147. Padua, R.A., 1981. *Plasmodium chabudi*: genetics of resistance to chloroquine, *Exp. Parasitol.*, 52: 419-426.

148. Peters, W., 1965. Drug resistance in *Plasmodium berghei*, *Exp. Parasitol.*, 17: 80-89.

149. Aissi, E., Charet, P., Bouquelet, S. and Biguet, J., 1983. Endoprotease in *P. Yoelli nigeriensis*, *Comparative Biochem. Physiol.*, 74: 559-566.

150. Martin, S.K., Oduolla, A.M.J. and Milhous, W.K., 1987. Reversal of cholroquine resistance in *P. falciparum* by verapamil, *Science*, 235: 899-901.

151. Krogstad, D.J., Gluzman, I.Y., Kyle, D.E., Oduolla, A.M.J., Martin, S.K., Milhous, W.K. and Schlesinger, E.H., 1987. Efflux of chloroquine from *P.falciparum*: Mechanism of chloroquine resistance, *Science*, 238: 1283-1285.

152. Schimke, R.T., 1980. Gene amplification and drug resistance, *Scientific Am.*, 243: 60-69.

153. Peters, W., 1971. The chemotherapy of rodent malaria. XIV. The action of some sulphonamides alone or with folic acid inhibitors against malaria vector and parasites. Part IV. The response of normal and drug resistant strains of *P.Berghei*, *Ann. Trop. Med. Parasitol.*, 65: 124-129.

154. Angerhofer, C., Koing, G., Wright, A., Sticher, O., Milhous, W., Cordell, G., Farnsworth, N. and Pezzuto, J., 1992. Selective screening of natural products: a

source of the discovery of novel antimalarial compounds, *Adv. Nat. Prod. Chem.*, Harward Academic Publishers, Switzerland, 311-329.

155. Dunn, C., 1996. The structure of lactate dehydrogenase from *P. falciparum* reveals a new target for antimalarial design, *Nat. Struc. Biol.*, 3: 912-915.

156. Ridely, R.J., 1997. Plasmodium drug discovery and development- an industrial perspective, *Exp. Parasitol.*, 87: 293-304.

157. Schmatz, D. and Schaffer, J., 1991. Antiparasite agents, *Annu. Reports Med. Chem.*, 26: 161-170.

158. Covell, G., Coatney, G.R., Field, J.W. and Singh, J., Chemotherapy of malaria, 1st edition, Monograph series No-27, World Health Organization, Geneva.

159. Clyde, D.F., 1981. Clincal problems associated with the use of primaquine as a tissue schizontocidal and gametocytocidal drug, *Bull. WHO*, 5: 391-395.

160. Bruce-Chwatt, L.J., Black, R.H., Canfield, C.J., Clyde, D.F., Peters, W. and Wernsdorfer, W.H., 1981. Chemotherapy of malaria. 2nd edition, Monograph series No-27, World Health Organization, Geneva.

161. Wernsdorfer, W.H. and Trigg, P.I., 1987. Primaquine: Pharmacokinetics, metabolism, toxicity and activity, John Wiley and Sons, New York.

162. Sweeney, T.R., Peters, W. and Richards, W.H.G., 1984. Handbook of experimental pharmacology, Springer-verlag, New York.

163. Schmidt, L.H., 1983 Relationships between chemical structure of 8-aminoquinolones and their capacity for radical cure of infections with *P.cynomolgi* in rhesus monkeys, *Antimicrob. Ag. Chemother.*, 2: 615-652.

164. Bhat, B., Seth, M. and Bhaduri, A.P., 1984. Recent development in 8-aminoquinoline antimalarials, *Prog. Drug Res.*, 28:197-231.

165. Bhat, B. and Bhaduri, A.P., 1985. Possible antimalarial agents: synthesis of 6-methoxy-8-substituted-aminoquinolines, *Ind. J. Chem.*, 24B: 419-23.

166. Puri, S.K., Srivastava, R., Pandey, V.C., Sethi, N. and Gupta, G.P., 1989. Methemoglobin toxicity and hematological studies on malaria anti-relapse compound CDRI-80/53 in dogs, *Am. J. Trop. Med. Hyg.*, 41:638-42.

167. Dutta, G.P., Puri, S.K., Bhaduri, A.P. and Seth, M., 1989. Radical curative activity of a new 8-aminoquinoline derivative (CDRI compound 80/53) against *Plasmodium cyanomolgi* B in monkeys, *Am. J. Trop. Med. Hyg.*, 41:635-637.

168. Sethi, N., Srivastava, S., Singh, R.K. and Puri, S.K., Long-term toxicity studies with a synthetic antirelapse antimalarial compound CDRI-80/53 in rats and monkeys, *Ind. J. Parasitol.*, 17:15-28.

169. Paliwal, J.K., Gupta, R.C. and Grover, P.K., 1993. Simultaneous determination of CDRI compound 80/53 and its metabolite in serum by high performance liquid chromatography, *J. Chrom. B.*, 616: 155-160.

CHAPTER 2

BIOANALYTICAL METHOD (S): DEVELOPMENT AND VALIDATION

2.1 Introduction

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples are the key determinants in generating reproducible and reliable data which in turn play a significant role in evaluation and interpretation of bioavailability, bioequivalence and PK data [1]. The objective of validation of an analytical procedure is to demonstrate that it is suitable for the intended purpose and is essential to employ well characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. There have been many reports, which discuss the validation of bioanalytical methods in detail highlighting the major points and criteria employed [2-6].

2.2 Bioanalytical Method Validation

The concentration-time data of a drug or its metabolites in biological samples are the basis of all PK endeavors [7]. The most commonly used bioanalytical techniques employ selective separation of the drug and its metabolites from biomatrix followed by simultaneous estimation of different components. HPLC coupled with UV or fluorimetric detection forms significant inroads into the therapeutic drug monitoring and PK studies [8]. The popularity of the technique is largely due to the accurate and precise estimation, specificity, sensitivity, and versatility in the variety of modes of operation, mobile phase, extensive library of column packings and detection systems available for analysis. The rational selection and development of an assay method require special knowledge about the drug to be analyzed in terms of its physicochemical properties like, solubility, pKa, Log P, stability at various pH, absorption, fluorimetric, electrochemical characteristics and ionization and fragmentation characteristics in case of mass spectrometric analysis [8]. All these parameters coupled to liquid chromatography with appropriate selection of stationary and mobile phase along with a suitable extraction solvent is a prerequisite in development of any bioanalytical method. Although, for the past 20 years the technique of HPLC with UV or fluorescence detection has been the mainstay for providing the above information. However, there is a significant need to provide shorter turn around periods for the determination of drug and metabolite concentrations in biological samples from drug discovery and clinical studies. With the advent of mass spectrometry (MS) which is renowned for its sensitivity and selectivity, analytical methods based on combined liquid chromatography and tandem

mass spectrometry (LC-MS-MS) can rapidly establish and determine low level of drugs and their metabolites in biological fluids [9]. Development of the atmospheric pressure ion sources, such as electrospray and atmospheric pressure chemical ionization has added liquid chromatography-mass spectrometry (LC-MS) to the list of present day's bioanalytical techniques, which have become indispensable for rapid and sensitive determination of pharmaceuticals in biological fluids. LC-MS-MS analysis offers high specificity and speed, good precision and accuracy, a wide dynamic range, high sensitivity and applicability to almost every type of compound. Though there are some prerequisites, which are to be considered while developing a quantitative liquid chromatographic mass spectrometric method of which choice of buffer, organic solvents, pH, sample preparation and matrix suppression are of prime importance.

It is recognized that analytical methods and techniques are constantly undergoing changes, improvements and in many instances they are at the cutting edge of the technology [10]. Each analytical technique has its own characteristics, which vary from analyte to analyte, and moreover the appropriateness of the technique may be influenced by the ultimate objective of the study. Despite these widespread changes in technology, the need for clearly defined validation criteria for bioanalytical method intended for analysis of drug and/or metabolite(s) in each separate biological matrix still remains. It is accepted that during the course of a typical drug development programme, a defined bioanalytical method will undergo many modifications, which are required to support specific studies and will require different levels of validation to demonstrate continually the validity of an assay's performance [10].

2.3 Components of Bioanalytical Method Validation

There is a general agreement that at least the following parameters should be evaluated for quantitative procedures: selectivity, calibration model, freeze-thaw stability, accuracy, precision, and limit of quantification. Additional parameters, which might have to be evaluated, include limit of detection, recovery, reproducibility and ruggedness/robustness [11-17].

2.3.1 *Calibration Model, Linearity and Range*

An important aspect to be considered in assay validation is the appropriate calibration model, to define the concentration of the analytes in mobile phase and biological matrices versus the detector response. The current practice to select the model is by analyzing the replicate samples of calibration standards, prior to validation, and treat the data through different transformations and weighting schemes. Previous experiences show that the calibration model should be chosen only after analyzing all validation samples so that one does not over-/under-estimate the concentrations of the analytes in unknown samples during PK studies [18]. The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte(s) in the sample. Furthermore, it is stated that for the establishment of linearity at least 5-8 concentration levels should be studied for linear and may be more for nonlinear relationships [9]. The range of analytical procedure is the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

2.3.2 *Specificity or Selectivity*

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these include impurities, degradents, matrix, etc. The requirements established by conference report demands to analyze at least six different sources of blank matrices to establish specificity [6]. There are two points of view on when a method should be regarded to be selective; one way to establish method selectivity is to prove lack of response in the drug free matrix and the other is based on the assumption that small interferences can be accepted as long as precision and bias remains within certain acceptable limits. Whereas, the selectivity experiments for the first approach can be performed during a pre-validation phase, those with the second approach are usually performed together with the precision and accuracy experiments during the main validation phase. In the

case of LC-MS-MS based procedures, it is essential that appropriate steps be taken to ensure the lack of matrix effect(s) throughout the application of the method, especially if the nature of the matrix changes from that used during initial method validation.

2.3.3 *Limit of Quantitation and Detection*

The limit of detection (LOD) of an individual analytical procedure is the lowest amount of the analyte in the sample, which can be detected but not necessarily quantitated as an exact value. In terms of (signal to noise) S/N ratio, a value of greater than or equal to 3 has been used to define LOD. A sample concentration which shows an S/N ratio ≥ 10 and simultaneously be estimated with desired level of precision and accuracy (< 20% deviation at lowest concentration) is known as limit of quantification (LOQ).

2.3.4 *Accuracy and Precision*

Accuracy is defined as the nearness of the calculated concentration to the spiked one while precision deals with the repetitiveness of the calculated concentration. It is established at a minimum of three concentrations, in replicates, of the quality control (QC) samples (low, medium and high). Intra- and inter-batch accuracy is determined by calculating the %bias from the theoretical concentration using the following equation:

$$\%\text{Bias} = \frac{\text{Observed concentration - Nominal concentration}}{\text{Nominal concentration}} \text{x} 100$$

Inter- and intra-batch precision in terms of relative standard deviation (% RSD) is obtained by subjecting the data to one way analysis of variance (ANOVA). The acceptance criteria for the mean value of accuracy should be within ± 15% of the theoretical value except at LOQ, where it should not deviate by more than ± 20%. The precision around the mean value should not exceed 15% coefficient of variation (% CV), except for LOQ where it should not exceed 20%.

2.3.5 *Extraction Recovery*

Extraction Recovery is calculated (at the concentration of the QC samples) by the comparison of the analyte response after sample work up with the response of the solution containing the analyte at the theoretical maximum concentration.

2.3.6 *Stability Studies*

Stability is defined as the chemical stability of an analyte in a given matrix under specific conditions for given period of time. Long-term stability of the analyte in the sample matrix should be established under the storage conditions. As samples are often frozen and thawed as in case of reanalysis, the stability of the analyte during several freeze-thaw cycles should be evaluated and is to be done minimum at two levels in triplicate [13-15].

2.3.7 *Robustness*

The robustness of the analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In case of liquid chromatography examples of typical variations are: influence of variation of pH in the mobile phase, influence in variation of the mobile phase compositions, different columns, temperature, flow rate, etc.

Acquisition of analytical data using chromatographic methods has evolved from mechanical and electronic integrators to computers with sophisticated algorithms to identify the beginning and the end of chromatographic peaks and to draw appropriate baselines. These computerized data systems vary from simple personal computers to very large laboratory information management systems (LIMS) [19].

The development and validation of assay method for simultaneous determination of BQ and PQ was necessary to generate the pre-clinical data for BQ as a potential antimalarial candidate. Thus, an HPLC-UV assay method in rabbit plasma was developed and validated [20]. Furthermore, a highly sensitive and selective LC-MS-MS method for simultaneous determination of BQ and PQ was also developed and validated in monkey plasma [21], which was further partially validated [10] in rat and rabbit plasma; rat, rabbit and monkey whole blood; rat urine and feces for application to various *in vivo* and *in vitro* studies.

2.4 Experimental

2.4.1 Materials

Pure reference standard of BQ was obtained from Pharmaceutics Division of CDRI, Lucknow, India. PQ diphosphate and dimethyl octylamine (DMOA) were procured from Aldrich chemicals (USA). 3-BPQ diphosphate was obtained as a generous gift from Prof. James D. McChesney of the University of Mississippi, USA and 3-BBQ was obtained from Chemical Technology Division of CDRI. Hexane and ethyl acetate, HPLC-grade, UltimAR™, were obtained from Mallinckrodt Baker Inc., Kentucky, USA. Acetonitrile (ACN, HPLC-grade), GR grade ammonia solution, glacial acetic acid and ammonium acetate were obtained from E Merck Ltd., Mumbai, India. Isopropyl alcohol (IPA, HPLC-grade) was procured from Thomas Bakers (Chemicals) Limited, Mumbai, India. AR grade sodium chloride (NaCl) was procured from s.d. Fine Chemicals Ltd, Mumbai, India. Purified water was obtained from MilliQ system. Heparin sodium for injection (Beparine®, 5000 IU/ml; Biological Evans Ltd., Hyderabad, India) was appropriately diluted with saline and was used to collect whole blood.

Drug-free heparinised rat, rabbit and monkey whole blood was obtained from young, healthy male *Sprague Dawley* rats, rabbits and rhesus monkeys, respectively, housed in the Laboratory Animal Services Division of CDRI. Drug-free heparinised plasma pool of all the respective species was obtained by centrifuging the whole blood at 2000 rpm for 10 min and separating the supernatant (plasma). The plasma was separately pooled and stored at -60°C. Drug-free rat urine and feces pool was generated separately from the male *Sprague Dawley* rats appropriately housed in metabolic cages.

2.4.2 Liquid Chromatographic and Instrumentation Conditions

2.4.2.1 HPLC-UV (System I)

The HPLC system consisted of a LC-10ATvp pump with SCL 10Avp system controller (Shimadzu, Japan) equipped with flow control valve system FCV-10ALvp (Shimadzu, Japan). A Model 7125i syringe loading injector (Rheodyne, Cotati, USA) fitted with a fixed 50 µl loop was used to inject the samples. The chromatographic separation was carried out on an Spheri-5 cyano column (5 µm, 220 x 4.6 mm, id) coupled with a guard column packed with the same material (5 µm, 30 x 4.6 mm, id) (Pierce Chemical Co., Rockford, IL, USA). The optimum separation of BQ, PQ and

the internal standard (3-BPQ) from the endogenous plasma component was achieved using the gradient elution at a flow rate of 1.0 ml/min [20]. The eluents were monitored using an SPD-10Avp UV-VIS detector set at 261 nm and the chromatograms were integrated using Class-VP software (Shimadzu, Japan). The chromatography was carried out at ambient temperature and HPLC system was equilibrated for approximately 30 min at before the commencement for analysis.

2.4.2.2 HPLC-MS-MS (System II)

A Jasco PU980 intelligent pump was used to deliver a premixed mobile phase (ACN: 20 mM ammonium acetate buffer, pH 6) 50:50 %v/v, at a flow rate of 1 ml/min. The mobile phase was degassed for 20 min in an ultrasonic bath (Bransonic Cleaning Equipment Company, Shelton, USA) prior to the analysis. Chromatographic separations were achieved on two spheri-5 cyano columns (each 5 µm, 30 mm x 4.6 mm i.d.) connected in series using a stainless steel connector. The samples were injected through manual injector model 7125 syringe loading injector (Rheodyne, Cotati, USA) fitted with a fixed 20 µl loop. Automated data acquisition was triggered using contact closure signals of the manual injector. The effluent from the column was split such that one-tenth was injected onto the ESI interface. The Micromass Quattro II (Micromass, Manchester, U.K.) Triple Quadrupole Mass Spectrometer was operated using a standard ESI source in positive ion mode. Data acquisition and analysis were performed using Mass Lynx (version 3.3) software.

For optimization of MS parameters, approximately equimolar solutions of each analyte were prepared in the HPLC mobile phase. Nitrogen gas was used as both nebulizing gas (10 L/h) and as drying gas (250 L/h). Cone voltages were optimized for each analyte by performing full scan acquisitions. The source temperature was set at 80°C, and the ESI capillary at 3.5 kv. LC-SIR (selective ion recording)-MS optimizations were performed by constant infusion of the mixture of analytes using infusion pump (Model 6, Harvard Apparatus, USA). The cone voltages, capillary voltage, nebulizing and dry gas conditions were kept at default values. The dwell time and inter-channel delay were 1.0 and 0.02 sec, respectively, with a span of 0.2 Da. SIR masses used for quantification were m/z 370, 260, 448 and 338 for BQ, PQ, 3-BBQ and 3-BPQ, respectively. The optimized cone voltages for the respective analytes were set for the MS-MS experiments with argon as the collision gas at a pressure of 2.5×10^{-3} millibars. Collision energies (CEs) for

fragmentation of precursor to product ions were optimized by constant infusion. The CEs for each of the analytes was optimized to obtain the most intense precursor to product ion transitions. MS-MS acquisition was performed by setting the mass of the analytes with an appropriate scan range.

For the optimization of LC-MRM (multiple reaction monitoring)-MS-MS, the source settings obtained above were utilized. Temperature, nebulizing and drying gas conditions were optimized by repetitive on column injections of the analytes using the optimized LC method. The sum of the responses obtained for the two intense transitions for both BQ and PQ were considered for quantitation and method validation [21].

2.4.3 Mobile Phase Conditions

2.4.3.1 HPLC-UV (System I)

Ammonium acetate buffer (50 mM, pH 6.0) was prepared by dissolving 3.85 g ammonium acetate in 1 L MilliQ water and the pH was adjusted to 6.0 with ammonia solution. The buffer was filtered through a 0.22 µ filter paper before use. ACN was mixed with buffer in the ratio 65:35 %v/v with 0.1% DMOA (organic modifier) to prepare solvent A while ammonium acetate buffer (50 mM, pH 6.0) was solvent B. The gradient started with pump supplying solvents A and B in the ratio 55 and 45%, respectively. The concentration of the mobile phase component in solvent A increased linearly to 90% within 15 min followed by stabilization for 2 min and then decreased to 55% in 19 min and then stabilization of 5 min with a total run time of 24 min. The solvents were degassed in an ultrasonic bath (Bransonic, CY, USA) for 20 minutes prior to chromatography. Reconstitution solution was prepared by mixing ammonium acetate buffer (50 mM, pH 7.0) with ACN in the ratio 50:50 % v/v.

2.4.3.2 HPLC-MS-MS (System II)

Ammonium acetate buffer (20 mM, pH 6.0) was prepared by dissolving 1.5 g ammonium acetate in 1 litre MilliQ water and adjusting the pH to 6.0 with ammonia solution. The buffer was filtered through a 0.22 µ filter paper before use. The mobile phase was prepared by mixing ACN and buffer in the ratio 50: 50 %v/v. Reconstitution solution was prepared by mixing ACN and ammonium acetate buffer (20 mM, pH 7) in the ratio 50:50 %v/v.

2.4.4 Method Development and Validation in Rabbit Plasma using System I (Method A)

To generate preclinical PK data in experimental animals for a drug, development and validation of a technique for its bioanalysis is a prerequisite. In most instances, blood (serum or plasma) and urine are the biological fluids that are investigated for drugs. Therefore, to accomplish the primary objective a selective, sensitive, accurate and precise HPLC assay method for simultaneous quantitative determination of BQ and PQ was developed and validated in rabbit plasma. An internal standard (IS), 3-BPQ was used to achieve better accuracy and precision in the assay method. The concentration-peak area ratios of both the analytes with IS was subjected to least-square regression with and without intercept using Microsoft Excel software (Version 5.0). The concentrations of the analytes from QC and test samples were interpolated from the standard curve. Mean ± SD was calculated from the data and %CV was calculated from the ratio of SD to mean. %RSD was calculated by subjecting the data to ANOVA.

2.4.4.1 Stock Solutions and Analytical Standards.

A 100 µg/ml stock solution of BQ (SS-1) was prepared by dissolving 5 mg of the compound in 50 ml ACN containing DMOA (0.1%, v/v). DMOA was found to be a necessary component in stock solution of BQ and in extraction solvent so as to prevent the conversion of BQ to PQ [22]. Individual stock solutions containing 100 µg/ml of PQ (SS-2) and 3-BPQ were prepared by dissolving appropriate amounts in MilliQ water. Working dilutions of 3-BPQ (20 and 50 µg/ml) were prepared in ACN. Varying volumes of SS were diluted with CAN containing DMOA to prepare working dilutions. The combined analytical standards of BQ, PQ and IS were prepared by using the working dilution of the analytes. A detailed scheme for the preparation of working stocks (WS) and analytical standards (AS) is given in Tables 2.1a and 2.1b, respectively. The concentration of 3-BPQ is AS and CS was 2500 and 1000 ng/ml respectively.

Table 2.1a Preparation of working stock solutions in method A

Working Stock solution		Volume of SS (µl) to be diluted		Volume (ml) of ACN (0.25% DMOA) q.s.
Code	Conc. (ng/ml) of BQ and PQ	SS-1 (BQ)	SS-2 (PQ)	
WS 1	400	100	100	25
WS 2	800	200	200	25
WS 3	2000	200	200	10
WS 4	4000	400	400	10
WS 5	8000	800	800	10
WS 6	20000	2000	2000	10
WS 7	40000	4000	4000	10

Table 2.1b Preparation of analytical standards in method A

Code	Conc. (ng/ml) of BQ and PQ	Volume of WS to be diluted	Volume (µl) of 3-BPQ (50 µg/ml) to be diluted	Volume q.s. with reconstitution solution
AS 1	50	1.25 ml WS 1	500	10 ml
AS 2	100	1.25 ml WS 2	500	10 ml
AS 3	250	1.25 ml WS 3	500	10 ml
AS 4	500	1.25 ml WS 4	500	10 ml
AS 5	1000	1.25 ml WS 5	500	10 ml
AS 6	2500	1.25 ml WS 6	500	10 ml

2.4.4.2 Calibration Standards and Quality Control Samples

Calibration standards were prepared by spiking Blank rabbit plasma (BRbP) with combined working stock solutions containing BQ and PQ such that the volume of the organic phase did not exceed 2.5%. Quality control samples at three different levels of BQ and PQ, low (QCL, 20 ng/ml), medium (QCM, 200 ng/ml) and high (QCH, 1000 ng/ml) were also prepared by spiking with combined working stock solutions into BRbP. The detailed scheme of the preparation of calibration and QC samples are described in Tables 2.2a and 2.2b respectively.

Table 2.2a Preparation of calibration standards in method A

Code	Conc. (ng/ml) of BQ and PQ	Volume of WS to be diluted	Volume (ml) of BRbP used
CS 1	20	25 µl WS 2	1.0
CS 2	50	25 µl WS 3	1.0
CS 3	100	25 µl WS 4	1.0
CS 4	200	25 µl WS 5	1.0
CS 5	500	25 µl WS 6	1.0
CS 6	1000	25 µl WS 7	1.0

Table 2.2b Preparation of quality control samples in method A

Code	Conc. (ng/ml) of BQ and PQ	Volume of WS to be diluted	Volume (ml) of BRbP used
QCL	20	25 µl WS 2	1.0
QCM	200	25 µl WS 5	1.0
QCH	1000	25 µl WS 7	1.0

2.4.4.3 Sample Preparation

A simple and efficient liquid-liquid extraction with 2 x 3.0 ml of extraction solvent [n-hexane: IPA, 98:02% (v/v) with DMOA (0.1% v/v)] was used to isolate the analytes from rabbit plasma. Aliquots of blank or spiked rabbit plasma (0.5 ml) in a 10 ml glass tubes was basified with 50 µl of potassium hydroxide (1 M) followed by addition of 25 µl IS (20 µg/ml) to spiked samples. The samples were vortex-mixed with 3.0 ml of extraction solvent for 1 min and centrifuged at 2000 rpm for 10 min. The organic layer was transferred to conical test tube by snap freezing the aqueous layer in liquid nitrogen. The aqueous phase, after thawing, was again extracted with 3.0 ml extraction solvent and the combined organic phases were evaporated to dryness under reduced pressure in speed vac concentrator (Savant Instruments Inc, USA). The dry residue was reconstituted in 200 µl of reconstitution solution, vortex-mixed, centrifuged and the supernatant was injected onto the HPLC system.

2.4.4.4 Method Validation

A validation protocol was prepared and all the criteria commonly employed for the validation of the HPLC methods were assessed. The method was validated in terms of linearity, specificity, sensitivity, recovery, intra-and Inter-batch accuracy, precision and freeze-thaw stability studies in spiked plasma samples. Accuracy, precision and recovery were assessed at three QC levels in triplicates for three different days.

2.4.5 Method Development and Validation in Monkey Plasma using System II (Method B)

It was deemed necessary to develop a more sensitive and selective method for simultaneous determination of BQ and PQ with low plasma processing volume and shorter run times to increase the throughput. Therefore, a more sensitive, selective, accurate and precise LC-MS-MS for simultaneous quantitative determination of BQ and PQ was developed and validated in monkey plasma to generate the PK parameters in primates. 3-BBQ and 3-BPQ, the structural analogues of BQ and PQ respectively, were used as internal standards to account for the variations due to matrix effect, extraction variability and instrument performance.

2.4.5.1 Stock Solutions and Analytical Standards

Individual stock solutions of BQ (100 µg/ml) and 3-BBQ (100 µg/ml) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in appropriate volumes of ACN containing 0.1% DMOA. Stock solutions of PQ (100 µg/ml) and 3-BPQ (100 µg/ml) were prepared in MilliQ water.

Mixed working stock (MWS) solutions of BQ and PQ, MWS 1 (BQ, 5 µg/ml and PQ, 10 µg/ml) and MWS 2 (BQ, 16 µg/ml and PQ, 40 µg/ml) were prepared by transferring appropriate volumes of stock solutions to 10-ml volumetric flasks and making up the volume with ACN. MWS 1 and MWS 2 were used for the preparation of analytical standards and calibration standards, respectively. Combined working stock solutions of both ISs, CWIS 1 (3-BBQ, 5µg/ml and 3-BPQ, 20 µg/ml) and CWIS 2 (3-BBQ, 2 µg/ml and 3-BPQ, 8 µg/ml) were prepared in ACN from the respective stock solutions by appropriate dilutions. The detailed scheme for the preparation of analytical standards by serial dilution is described in Table 2.3.

Table 2.3 Preparation of analytical standards in method B

Code	Conc. (ng/ml)		Volume of Standard Solution to be diluted	Volume of Reconstitution solution q.s. (ml)
	BQ	PQ		
AS 1	0.98	1.95	1 ml of AS 2	1
AS 2	1.95	3.90	1 ml of AS 3	1
AS 3	3.90	7.81	1 ml of AS 4	1
AS 4	7.81	15.62	1 ml of AS 5	1
AS 5	15.62	31.25	1 ml of AS 6	1
AS 6	31.25	62.5	1 ml of AS 7	1
AS 7	62.5	125	1 ml of AS 8	1
AS 8	125	250	1 ml of AS 9	1
AS 9	250	500	1 ml of AS 10	1
AS 10	500	1000	200 µl of MWS1	1.8

50 µl CWIS 1 was spiked to 1.0 ml of each analytical standard to achieve a concentration of 250 and 1000 ng/ml for 3-BBQ and 3-BPQ respectively.

2.4.5.2 Calibration Standards and Quality Control Samples

Calibration standards were prepared by serial dilution method. First, CS 9 was prepared by spiking 1.0 ml blank monkey plasma (BMP) with 25 µl of MWS 2. Quality control samples at five different concentration levels were prepared which were used in assessment of recovery, accuracy and precision. The elaborate scheme of the preparation of calibration and QC samples are described in Tables 2.4a and 2.4b respectively.

Table 2.4a Preparation of calibration standards in method B

Code	Dilution scheme	Conc. (ng/ml)	
		BQ	PQ
CS 1	100 µl BMP + 100 µl CS 2	1.56	3.90
CS 2	100 µl BMP + 100 µl CS 3	3.12	7.81
CS 3	100 µl BMP + 100 µl CS 4	6.25	15.62
CS 4	100 µl BMP + 100 µl CS 5	12.5	31.25
CS 5	100 µl BMP + 100 µl CS 6	25	62.5
CS 6	100 µl BMP + 100 µl CS 7	50	125
CS 7	100 µl BMP + 100 µl CS 8	100	250
CS 8	100 µl BMP + 100 µl CS 9	200	500
CS 9	25 µl of MWS 2 + 1.0 ml BMP	400	1000

Table 2.4b. Preparation of quality control samples in method B

QC Sample	Conc. (ng/ml)		Dilution
	BQ	PQ	
L 1	1.5625	3.90625	200 µl QCL 2 + 200 µl BMP
L 2	3.125	7.8125	100 µl QCM 1 + 300 µl BMP
M 1	12.5	31.25	100 µl QCM 2 + 300 µl BMP
M 2	50	125	75 µl QCH + 525 µl BMP
H	400	1000	25 µl MWS 2 + 1 ml BMP

2.4.5.3 Sample Preparation

Aliquots (100 µl) of plasma (blank or spiked) were transferred into 5 ml tubes and basified with ammonia solution. CWIS 2 (12.5 µl) was added to spiked sample and vortex-mixed. Extraction solvent [1.0 ml; n-hexane: ethyl acetate: DMOA (90: 10: 0.05, %v/v)] was added and each tube was vortex-mixed for 1 min and then centrifuged at 2000 rpm for 10 min. The aqueous layer was snap-frozen in liquid nitrogen and the organic layer was transferred to another tube. The extraction was repeated and the combined organic layers were evaporated to dryness using Savant speed vac concentrator. The residue was reconstituted in 150 µl of reconstitution

solution, vortex-mixed and centrifuged. The supernatant was injected onto the LC-MS system.

2.4.5.4 Method Validation

The method was validated in terms of, linearity, specificity, sensitivity, recovery, intra- and inter- batch accuracy and precision and freeze-thaw stability. Accuracy, precision and recovery were determined at five different quality control concentrations levels in triplicate for five days. Both BQ and PQ gave two prominent product ions during MS-MS experiments and are detailed in section 2.5.2. The sum of the responses obtained for the two intense transitions for both BQ and PQ were considered in method validation. The final MRM conditions optimized for the validation of the method are given in Table 2.9.

2.4.6 Method Validation in Rat Plasma (Method C) and Rabbit Plasma (Method D) using System II

Pharmacokinetic parameters of BQ and PQ were to be generated in rats and rabbits also and therefore the HPLC-MS-MS method developed and validated in monkey plasma was partially validated using rat and rabbit plasma and then employed for pharmacokinetic studies. Preparation of stock solutions, analytical standards, calibration standards, quality control samples and sample preparation; method validation criteria and parameters were same as described in section 2.4.5. The method was partially validated in terms of linearity, sensitivity, specificity, recovery, intra-batch accuracy and precision at all QC concentrations. Accuracy, precision and recovery were carried out at five QC levels in triplicate for one day.

2.4.7 Method Validation in Whole Blood of Rat (Method E), Rabbit (Method F), and Monkey (Method G) using System II

The HPLC-MS-MS method for simultaneous determination of BQ and PQ was also partially validated in whole blood of rat, rabbit and monkey for whole blood uptake studies of BQ. Preparation of stock solutions, analytical standards, calibration standards, quality control samples and sample preparation, method validation criteria and parameters were same as described in section 2.4.5. The method was partially validated separately in rat, rabbit and monkey whole blood in terms of linearity,

specificity, sensitivity, recovery, intra-batch accuracy and precision. The accuracy, precision and recovery was calculated using five QCs in triplicate for one day.

2.4.8 Method Development and Validation in Rat Urine (Method H) using System II

The HPLC-MS-MS method for simultaneous determination of BQ and PQ was partially validated in rat urine and was applied to study excretion of BQ in rats.

2.4.8.1 Stock Solutions and Analytical Standards

Individual standard stock solutions of BQ, PQ, 3-BBQ and 3-BPQ were prepared as described in section 2.4.5. Mixed working stock solutions of BQ and PQ, MWS 3 (BQ, 2.5 µg/ml and PQ, 5 µg/ml) and MWS 4 (BQ, 20 µg/ml and PQ, 40 µg/ml) were prepared by transferring appropriate volumes of stock solutions to a 10-ml volumetric flask and making up the volume with ACN. MWS 3 and MWS 4 were used in the preparation of analytical standards and calibration standards, respectively. Combined working stock solutions of ISs, CWIS 3 (3-BBQ, 2 µg/ml and 3-BPQ, 10 µg/ml) and CWIS 4 (3-BBQ, 0.8 µg/ml and 3-BPQ, 4 µg/ml) were prepared in ACN from their respective stock solutions by appropriate dilutions. The detailed scheme for the preparation of analytical standards by serial dilution is described in Table 2.5.

Table 2.5 Preparation of analytical standards in method H

Code	Conc. (ng/ml)		Volume of Standard Solution to be diluted	Volume of Reconstitution solution q.s. (ml)
	BQ	PQ		
AS 1	1.95	3.90	1 ml of AS 2	1
AS 2	3.90	7.81	1 ml of AS 3	1
AS 3	7.81	15.62	1 ml of AS 4	1
AS 4	15.62	31.25	1 ml of AS 5	1
AS 5	31.25	62.5	1 ml of AS 6	1
AS 6	62.5	125	1 ml of AS 7	1
AS 7	125	250	1 ml of AS 8	1
AS 8	250	500	200 µl MWS 3	2

50 µl CWIS 3 was spiked to 1.0 ml of each analytical standard to achieve a concentration of 100 and 500 ng/ml for 3-BBQ and 3-BPQ, respectively.

2.4.8.2 Calibration Standards and Quality Control Samples

Calibration standards were prepared with serial dilution such that the volume of the organic phase did not exceed 2.5% in blank rat urine (BRU). Quality control samples at five different concentration levels were prepared which were used in assessment of recovery, accuracy and precision. The detailed scheme of the preparation of calibration and QC samples are described in Tables 2.6a and 2.6b respectively.

Table 2.6a Preparation of calibration standards in method H

Code	Dilution scheme	Conc. (ng/ml)	
		BQ	PQ
CS 1	100 µl BRU + 100 µl CS 2	3.90	7.81
CS 2	100 µl BRU + 100 µl CS 3	7.81	15.62
CS 3	100 µl BRU + 100 µl CS 4	15.62	31.25
CS 4	100 µl BRU + 100 µl CS 5	31.25	62.5
CS 5	100 µl BRU + 100 µl CS 6	62.5	125
CS 6	100 µl BRU + 100 µl CS 7	125	250
CS 7	100 µl BRU + 100 µl CS 8	250	500
CS 8	25 µl of MWS 4 + 1.0 ml BRU	500	1000

Table 2.6b Preparation of quality control samples in method H

QC Sample	Conc. (ng/ml)		Dilution Scheme
	BQ	PQ	
L 1	3.9	7.8	100 µl QCL 2 + 100 µl BRU
L 2	7.8	15.6	50 µl QCM + 350 µl BRU
Medium (M)	62.5	125	50 µl QCH + 350 µl BRU
High (H)	500	1000	25 µl MWS 4+ 1 ml BRU

2.4.8.3 Sample Preparation

Aliquots (50 µl) of urine samples (blank or spiked) were transferred in 5 ml tubes and 50 µl of MilliQ water was added to it. The resulting solution was basified with ammonia solution. CWIS 4 (12.5 µL) was added to spiked samples and vortex-mixed. Extraction solvent [1.5 ml; n-hexane: ethyl acetate: DMOA (90: 10: 0.05, %v/v)] was added and each tube was vortex-mixed for 1 min and then centrifuged at 2000 rpm for 10 min. The aqueous layer was frozen in liquid nitrogen and the organic layer was transferred to another tube. The extraction was repeated and the combined organic layers were evaporated to dryness using Savant vacuum concentrator. The residue was reconstituted in 100 µl of reconstitution solution, vortex-mixed, centrifuged and the supernatant was injected onto the LC-MS system.

2.4.8.4 Method Validation

The method was partially validated for a single day in terms of linearity, specificity, sensitivity, recovery, intra-batch accuracy and precision. The validation was carried out at above mentioned four different quality control concentration levels of BQ and PQ in triplicate.

2.4.9 Method Development and Validation in Rat Feces (Method J) using System II

Method was developed and validated for simultaneous determination of BQ and PQ in rat feces and then applied for the excretion studies of BQ in rats.

2.4.9.1 Stock Solutions and Analytical Standards.

Individual standard stock solutions of BQ, PQ, 3-BBQ and 3-BPQ were prepared as described in section 2.4.5. Mixed working stock solutions of BQ and PQ, WSF 6 (BQ, 5 µg/ml and PQ, 10 µg/ml) was prepared by appropriate dilutions of stock solutions with ACN. CWIS 5 (3-BBQ, 2 µg/ml and 3-BPQ, 4 µg/ml) and CWIS 6 (3-BBQ, 16 µg/ml and 3-BPQ, 32 µg/ml) were prepared by appropriate dilutions of stock solutions with ACN. The detailed scheme of preparation of working stock solutions and analytical standards is given in Tables 2.7a and 2.7b, respectively.

Table 2.7a Preparation of working stock solutions in method J

Code	Conc. (µg/ml)		Volume of Standard Solution to be diluted	Volume of ACN q.s. (ml)
	BQ	PQ		
WSF 1	0.15	0.31	1 ml of WSF 2	1
WSF 2	0.31	0.62	1 ml of WSF 3	1
WSF 3	0.62	1.25	1 ml of WSF 4	1
WSF 4	1.25	2.5	1 ml of WSF 5	1
WSF 5	2.5	5	1 ml of WSF 6	1

Table 2.7b Preparation of analytical standards in method J

Code	Conc. (ng/ml)		Volume of Standard Solution to be diluted	Volume of Reconstitution solution q.s. (ml)
	BQ	PQ		
ASF 1	0.98	1.95	1 ml of ASF 2	1
ASF 2	1.95	3.90	1 ml of ASF 3	1
ASF 3	3.90	7.81	1 ml of ASF 4	1
ASF 4	7.81	15.62	1 ml of ASF 5	1
ASF 5	15.62	31.25	1 ml of ASF 6	1
ASF 6	31.25	62.5	200 µl of WS 2	1.8

50 µl of CWIS 5 was added to 1.0 ml of each analytical standard to obtain a concentration of 100 and 200 ng/ml for 3-BBQ and 3-BPQ, respectively.

2.4.9.2 Calibration Standards and Quality Control Samples

Calibration standards were prepared by individually spiking drug-free dry feces powder with working stock solutions in 5 ml glass tubes. These samples were prepared immediately before the analysis. The scheme for preparation of calibration standards and quality control samples is elaborated in Tables 2.8a and 2.8b, respectively.

Table 2.8a Preparation of calibration standards in method J

Code	Spiking scheme	Conc. (ng/50 mg)	
		BQ	PQ
CS 1	25 µl WSF 1 to 50 mg of dry feces	3.9	7.81
CS 2	25 µl WSF 2 to 50 mg of dry feces	7.81	15.62
CS 3	25 µl WSF 3 to 50 mg of dry feces	15.62	31.25
CS 4	25 µl WSF 4 to 50 mg of dry feces	31.25	62.5
CS 5	25 µl WSF 5 to 50 mg of dry feces	62.5	125
CS 6	25 µl WSF 6 to 50 mg of dry feces	125	250

Table 2.8b Preparation of quality control samples in method J

Code	Spiking scheme	Conc. (ng/50 mg)	
		BQ	PQ
Low	25 µl WSF 1 to 50 mg of dry feces	3.9	7.81
Medium	25 µl WSF 4 to 50 mg of dry feces	31.25	62.5
High	25 µl WSF 6 to 50 mg of dry feces	125	250

2.4.9.3 Sample Preparation

Each CS (50 mg) was spiked with 25 µl of CWIS 6. To prepare blank, drug free rat feces was spiked with 25 µl ACN. These samples were tapped gently to uniformly wet the powder and kept for 10 min at room temperature. To this, 2.0 ml of ACN was added and vortex-mixed for 2 min followed by centrifugation at 3000 rpm for 10 min. The clear supernatant was diluted with ammonium acetate buffer (20 mM, pH 7.0) in 1:1 ratio and injected onto LC-MS-MS system.

2.4.9.4 Method Validation

The method was validated in terms of linearity, specificity, sensitivity, recovery, and intra- and inter-batch accuracy and precision. These studies were carried out in triplicate for three days at the above-mentioned QC levels.

2.5 RESULTS AND DISCUSSION

2.5.1 Chromatographic Conditions

The decision to develop and validate the methods in different matrices and various sample sizes depends upon the volume of sample collection during PK studies and proper resolution of the metabolites from their parent drug and/or endogenous components.

Two HPLC methods have been reported for determination of BQ in biological fluids [22, 23]. The first method coupled with UV detection determines BQ with a quantitation limit of 25 ng/ml [22]. The method could not be reproduced under present laboratory conditions, as the resolution of BQ, PQ and the rabbit plasma impurities were highly dependent on RP-18 column of different makes. Moreover, the peak performance of BQ was found to be unsatisfactory on various RP-18 columns. The second method selectively determined only BQ by using non-aqueous mobile phase and fluorescence detection [23]. This method was not able to determine PQ as it is non-fluorescent in nature. Moreover, an increase in the proportion of water or methanol in the mobile phase decreased the peak response of BQ due to fluorescence quenching. BQ is unstable in commonly used organic solvents and acidic conditions. Under these conditions, BQ is converted into PQ. The addition of DMOA to the solvents results in lesser conversion [22]. Therefore, the solutions of BQ were prepared in solvents containing DMOA (0.1%, v/v). Development of the method was initiated using reversed phase liquid chromatography with RP-18 column (5 µ, 100 x 4.6 mm, id) and isocratic elution using a mobile phase containing phosphate buffer. BQ eluted as a broad peak (t_R, >15 min) even with 80% ACN in mobile phase and PQ and 3-BPQ did not resolve. Therefore, phosphate buffer was replaced with ammonium acetate buffer, which marginally improved the peak response of BQ while PQ and 3-BPQ were still unresolved. This followed the use of RP-18 columns of different length and make (Pierce, Phenomenex and Discovery). For better resolution and sensitivity, varying molarities of buffer, ACN composition and gradient conditions with different RP-18 columns were tried which did not yield any improvement. This prompted the change of column from RP-18 to cyano column. The cyano columns of different makes (Pierce and Phenomenex) and isocratic elution with mobile phase containing ammonium acetate buffer showed good peak response for BQ but PQ and 3-BPQ were still not resolved. Elution was tried with 10, 20, and 50 mM ammonium acetate buffer in combination with ACN. It was

seen that increasing molarity of the buffer drastically affected the elution and peak shape of PQ and 3-BPQ while BQ was not much affected. Low molarities of buffer (10-40 mM) caused delayed elution and broad peaks for PQ and 3-BPQ. A molarity of 50 mM ammonium acetate buffer was found to be optimum for elution of all the analytes. The pH was optimized and kept near to neutral (pH 6.0) as BQ is unstable at lower pH and is converted to PQ. Among the various modifications tried, the low-pressure binary gradient and mobile phase (section 2.4.3.1) resulted in optimal resolution and good peak responses with no endogenous interference in the elution region of the analytes [20].

The LC-MS-MS methods have some prerequisite in the use of buffer salts and solvents. Since non-volatile salts result in the contamination of the electrospray interface and suppression of ionization, the optimization of mobile phase for LC-MS methods has limited choice of buffer salts and solvents. In practice, majority of the mobile phases used for LC-MS contain water with methanol or ACN, ammonium acetate/formate with pH adjusted with ammonium hydroxide, acetic acid or formic acid solution. Moreover, the organic modifiers used in reverse phase chromatography coupled to UV for better peak performance often results in suppression of the analyte ionization. The chromatographic conditions used in LC-UV method [20] for the detection with a lower molarity of ammonium acetate buffer was found to be optimum for LC-MS-MS method. DMOA was removed from mobile phase and molarity of the ammonium acetate was reduced to 10 mM for carrying the method to ESI-MS. Initially, the method was started with 50% ACN in 10 mM ammonium acetate buffer (pH 6) at a flow rate of 1.0 ml/min which gave broad and late eluting peaks for PQ and 3-BPQ. There was no effect on the retention times of PQ and 3-BPQ on increasing concentration of ACN but increase in buffer strength from 10 to 20 mM caused a considerable decrease in retention times, better peak shape and sensitivity for PQ and 3-BPQ. Liquid chromatography was performed on two-spheri cyano columns connected in series with first one serving as a guard column for the next, hence providing longer lifespan for the main column on repeated injections. Moreover, shorter run times and increased sensitivity increased the throughput of the method. The final LC conditions were optimized to ACN: ammonium acetate buffer (20 mM, pH 6.0) (50:50 %v/v) at 1.0 ml/min. Under these conditions, BQ, PQ and 3-BPQ eluted at 1.7, 4.5 and 6.1 min, respectively.

2.5.2 Detection Systems: Selection and Optimization

All the analytes, BQ, PQ and 3-BPQ, showed maximum UV absorption at a wavelength of 261 nm while only BQ showed fluorescence under non-aqueous conditions. BQ also showed absorption at 302 nm but owing to its low sensitivity, this wavelength was not considered for method development. Thus, the UV detection was performed at 261 nm, which enabled quantification of both BQ and PQ with an LOQ of 20 ng/ml.

Ionization and fragmentation efficiency are the two main parameters, which determines the quantitation limit of a specific compound by tandem mass spectrometric detection. The mobile phase containing ACN-ammonium acetate buffer was used to obtain electro spray response. In positive ion mode, the analytes were found to ionize as protonated species $[M+H]^+$ at m/z 370, 260 and 338 for BQ, PQ and 3-BPQ, respectively. 3-BPQ was used as an internal standard for both BQ and PQ. DMOA was found to be a necessary component in stock solution of BQ and in extraction solvent to prevent the conversion of BQ to PQ by providing basic conditions to the solution [22]. The DMOA concentration in these standards injected on to LC-MS were negligible and did not cause any suppression in the ionization of the analytes. The cone voltage optimization by constant infusion yielded best results at 40, 34 and 32V for BQ, PQ and 3-BPQ, respectively. Initially, SIR mode was applied utilizing the above optimized cone voltages but significant interference was observed in the elution regions of the analytes by endogenous impurities of plasma. Thus, MRM was explored, which is known for its higher sensitivity and selectivity as compared to SIR. MS-MS optimization was carried out by constant infusion of the analyte solution in the mobile phase and the product ions were generated through fragmentation of the molecular ions by collision-activated dissociation (CAD) using argon as collision gas. Collision energy and pressure of collision gas were also optimized to 20 eV and 2.5×10^{-3} millibars respectively which gave most intense product ions for all the analytes. The product ions were chosen based on their significance in the MS-MS spectra. The product ion spectra of $[M+H]^+$ ion of BQ, PQ and 3-BPQ showed intense fragments at 196, 86 and 86, respectively. MS-MS spectra for BQ, PQ and 3-BPQ are shown in Figure 2.1 (i). MRM conditions were set based on MS-MS analysis using the most prominent and stable product ions. The transition for BQ, PQ and 3-BPQ were 370 > 196, 260 > 86 and 338 > 86, respectively. Moreover, during MS-MS analysis a second product ion was found,

which was prominent but less intense as compared to the optimized product ions for BQ and PQ. The secondary product ions for BQ and PQ under optimized MS-MS conditions were m/z 243 and 175, respectively. Further, the two transitions (BQ, 370 > 196 and 370 > 243; PQ, 260 > 86 and 260 > 175) were monitored in a single function and the sum of the responses of the two individual transitions was considered for quantitation for both the analytes. This resulted in a two-fold increase in the sensitivity of the analytes. The corresponding MRM conditions are summarized in Table 2.9. Pilot calibration curves of BQ and PQ revealed that the peak area ratio of PQ with 3-BPQ varied linearly while that of BQ gave non-linear response with increasing number of injections. This could be attributed to dissimilar ionization and fragmentation of BQ and 3-BPQ. To overcome this issue, structurally similar analogue of BQ was explored. 3-BBQ exhibited ionization and fragmentation pattern similar to BQ and was therefore chosen as its internal standard. MS-MS of 3-BBQ gave intense protonated product ion at m/z 196 at optimized cone voltage and collision energy of 30 V and 20 eV respectively. Product ion spectra for 3-BBQ are shown in Figure 2.1 (i). The proposed MS-MS fragmentation pattern for BQ, PQ, 3-BBQ and 3-BPQ is given in Figure 2.1 (ii). Under the LC optimized conditions, 3-BBQ eluted at 2.01 min. Though BQ and 3-BBQ were not properly resolved still the specificity of MRM enabled detection of both of them as separate entities on different channels. The temperature, nebulizing and curtain gas conditions for the maximum sensitivity of the analytes were optimized by on column injections of the mixture of analytes. This method was validated rigorously for a period of five days.

Table 2.9 MRM Conditions for BQ, PQ, 3-BBQ and 3-BPQ

Analyte	Function	Channel	Precursor ion [m/z]	Product ion [m/z]	CV (V)	CE (eV)	Dwell time (s)
BQ	MRM of two mass pairs	1	370	196	40	20	1.0
		2	370	243			
PQ	MRM of two mass pairs	1	260	86	30	20	1.0
		2	260	175			
3-BBQ	MRM of two mass pairs	1	448	196	30	20	1.0
3-BPQ	MRM of two mass pairs	1	338	86	32	20	1.0

2.5.3 Sample Preparation and Matrix Suppression

For System I, the extraction of the analytes from the spiked rabbit plasma was initially tried with diethyl ether (with 0.1% DMOA), which yielded endogenous impurities in the elution zone of analytes. As an alternative, hexane with 0.1 % DMOA was employed as the extraction solvent. With this extraction solvent, the recovery of BQ was > 90% whereas the recoveries of PQ and 3-BPQ were 15-20%. Therefore, it was decided to increase the polarity of extraction solvent to enhance the recoveries of PQ and IS. Hence, extraction using various combinations of hexane: isopropanol and hexane: ethyl acetate with 0.1% DMOA were tried. The extraction with hexane: ethyl acetate exhibited interference of endogenous impurities in the elution zone of the analytes. Thus, various combinations of hexane: isopropanol with 0.1% DMOA were tried of which 2% isopropanol in hexane with 0.1% DMOA gave best results. Single extraction with 3.0 ml of extraction solvent resulted in 70 and 30% recoveries for BQ and PQ, respectively. Therefore, double extraction with this solvent system was carried out which gave enriched and consistent recoveries of the analytes.

Mass spectrometry, though very selective and sensitive may be adversely affected by ionization suppression by other components present in the biomatrix. The matrix components co-eluting with the analytes, although precluded from the MRM detection, may still suppress the ionization of the analytes and thereby decreasing their sensitivity [24]. Evaluation and elimination of biomatrix effects should form a critical and essential part of any LC-MS-MS method development. The extraction solvent (hexane: isopropanol: DMOA:: 98: 2: 0.1 %v/v) optimized for HPLC-UV method was tried for LC-MS-MS. Although, recoveries of PQ and 3-BPQ were consistent (50-55%) but extraction solvent caused significant suppression of ionization of both BQ and 3-BBQ probably due to the extracted plasma endogenous impurities. Ethyl acetate having almost same polarity as IPA was tried as its substitute in the extraction solvent. Of various combinations of hexane and ethyl acetate tried, hexane: ethyl acetate (90:10) with 0.05% DMOA gave best results in terms of recovery. With this extraction solvent recoveries of BQ, 3-BBQ and PQ, 3-BPQ were more than 90 and 50%, respectively. Matrix suppression of the analytes was minimized but loss in sensitivity was noticed after 25-30 injections. To eliminate this effect it was decided to reconstitute the dry residue after extraction into 150 µl, which is 1.5 times the volume of plasma extracted, without compromising on the

LOQ of BQ and PQ. These changes eliminated the matrix suppression problem. Moreover, diluted but cleaner samples were injected on to HPLC ensuring longer life for the analytical column.

The above extraction solvent was tried for the sample preparation of rat urine samples. But it posed the problem of gel formation while vortexing. Therefore the urine samples were diluted in 1:1 ratio with MilliQ water before extraction keeping the sample clean procedure similar to plasma samples as described in section 2.4.5.3. In case of rat feces, protein precipitation technique was used with ACN as the precipitating agent. Precipitation with 2.0 ml ACN gave clean chromatographs with no interferences in any of the channels with a recovery of > 90% and 40% for BQ and PQ respectively. The inclusion of some aqueous content in the precipitating solvent (ACN: MilliQ water:: 90:10 %v/v) resulted in increase in recovery of PQ to 60-70% but interference was observed in the elution zone of BQ affecting its quantitation. Thus, precipitation was carried out with ACN alone, followed by centrifugation and dilution of the supernatant with ammonium acetate buffer (20 mM, pH 6.0).

2.5.4 Validation parameters

2.5.4.1 Linearity, Sensitivity and Specificity

2.5.4.1.1 Method A

Linearity of the method was confirmed over the concentration range of 20 - 1000 ng/ml for both BQ and PQ with 3-BPQ as internal standard using 0.5 ml plasma samples. The calibration model was best described by $y = mx+c$ ($r > 0.99$) for both BQ and PQ. Regression equations for BQ and PQ were $y = 0.0030\ x + (-0.006)$ and $y = 0.0026\ x + 0.0069$ respectively. LOD for BQ and PQ was 10 ng/ml while their LOQ was 20 ng/ml. Percent CV was within ±20% at LOQ and ±15% at all other concentrations. The method was found to be specific as no endogenous interference was observed in the elution zone of the analytes. Six individual sources of rabbit plasma were checked for any interference in the regions of analytes. The retention times for BQ, PQ and 3-BPQ were 8.0 ± 1.0, 11.0 ± 0.75 and 13.5 ± 0.50 min, respectively with an overall chromatographic run time of 24 min. Typical chromatograms of an analytical standard containing BQ and PQ (each 250 ng/ml) and 3-BPQ (2500 ng/ml), drug-free rabbit plasma, rabbit plasma containing BQ and PQ (200 ng/ml) and IS (2500 ng/ml) are depicted in Figure 2.2.

2.5.4.1.2 Method B

The method was found to be linear over a dynamic range of 1.56-400 ng/ml and 3.90-100 ng/ml for BQ and PQ, respectively in monkey plasma. The best fit for the calibration curve with best regression coefficient and minimum residuals for both BQ and PQ could be achieved by a linear equation of $y = mx + c$ with a 1/x weighting factor. Regression equations for BQ and PQ were $y = 0.018\ x + (0.004)$ and $y = 0.0038\ x + 0.0019$ respectively. LOD for BQ and PQ was 0.5 and 2 ng/ml respectively, whereas LOQ of 1.56 and 3.91 ng/ml for BQ and PQ respectively was established. Percent CV was within ±20% at LOQ and ±15% at all other concentrations. The method was highly specific and selective with no interferences of endogenous substances of the matrices. Eight individual lots of monkey plasma were checked for any interference in the regions of analytes. Representative chromatograms of extracted BMP fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BMP are shown in Figure 2.3. Thus, there was an increase of 13 and 5 folds sensitivity for BQ and PQ as compared to the HPLC-UV method while the plasma processing volume decreased from 0.5 to 0.1 ml thus increasing the overall sensitivity of the method

2.5.4.1.3 Method C and D

The methods were specific for BQ and PQ and there was no interference from the endogenous impurities of either rat or rabbit plasma. Representative chromatograms of extracted BRP fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BRP are shown in Figure 2.4. Representative chromatograms of extracted BRbP fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BRbP are shown in Figure 2.5. LOD, LOQ, linearity range, calibration model for both BQ and PQ were same as discussed in Method B (2.5.4.1.2). Regression equations for BQ and PQ for Method C were $y = 0.023\ x + (0.009)$ and $y = 0.0036\ x + 0.004$ while for Method D were $y = 0.006\ x + (0.006)$ and $y = 0.0018\ x + 0.0023$, respectively.

2.5.4.1.4 Method E, F and G

The endogenous impurities of rat, rabbit or monkey whole blood did not show any interference indicating that the method was specific. Representative chromatograms of extracted blank rat, rabbit and monkey whole blood fortified with

BQ, PQ, 3-BBQ and 3-BPQ overlaid with their respective blank are shown in Figures 2.6, 2.7 and 2.8, respectively. LOD, LOQ, linearity range, calibration model of BQ and PQ were same as discussed in Method B (2.5.4.1.2). Regression equations for BQ and PQ in Method E were $y = 0.024\ x + (0.004)$ and $y = 0.0019\ x + (0.004)$ respectively. Regression equations for BQ and PQ for Method F were $y = 0.032\ x + (0.026)$ and $y = 0.003\ x + 0.0123$ while for Method G were $y = 0.019\ x + (0.004)$ and $y = 0.0019\ x + 0.0049$ respectively.

2.5.4.1.5 Method H

The selectivity of the method for BQ and PQ was demonstrated by non-interferences in the elution region of analytes by urinary endogenous impurities. Representative chromatograms of extracted BRU fortified with BQ, PQ, 3-BBQ, 3-BPQ overlaid with extracted BRU are shown in Figure 2.9. The method was linear over a range of 3.9-500 ng/ml for BQ and 7.8-1000 ng/ml for PQ. LOD and LOQ for BQ were 2.0 and 3.9 ng/ml while for PQ was 4.0 and 7.8 ng/ml respectively with 50 µl of urine. The calibration model was same as described in Method B and % CV was within ±20% at LOQ and ±15% at all other concentrations. $y = 0.032\ x + (-0.009)$ and $y = 0.010\ x + (0.005)$ were the regression equations for BQ and PQ, respectively.

2.5.4.1.6 Method J

The method was found to be specific with no interferences in the regions of interest with fecal endogenous impurities. Representative chromatograms of precipitated feces fortified with BQ, PQ, 3-BBQ, and 3-BPQ overlaid with its blank is shown in Figure 2.10. The method showed excellent linearity over a calibration range of 0.078 to 2.5 and 0.156 to 5 ng/mg of BQ and PQ respectively. LOD for BQ and PQ was 0.156 and 0.312 ng/mg while their LOQ was 0.078 and 0.156 ng/mg of feces, respectively. The calibration model was $y = mx + c$ with $1/x^2$ weighing scheme for BQ and PQ and % CV was within ±20% at LOQ and ±15% at all other concentrations. Regression equations for BQ and PQ were $y = 0.223\ x + (0.013)$ and $y = 0.094\ x + (-0.004)$, respectively.

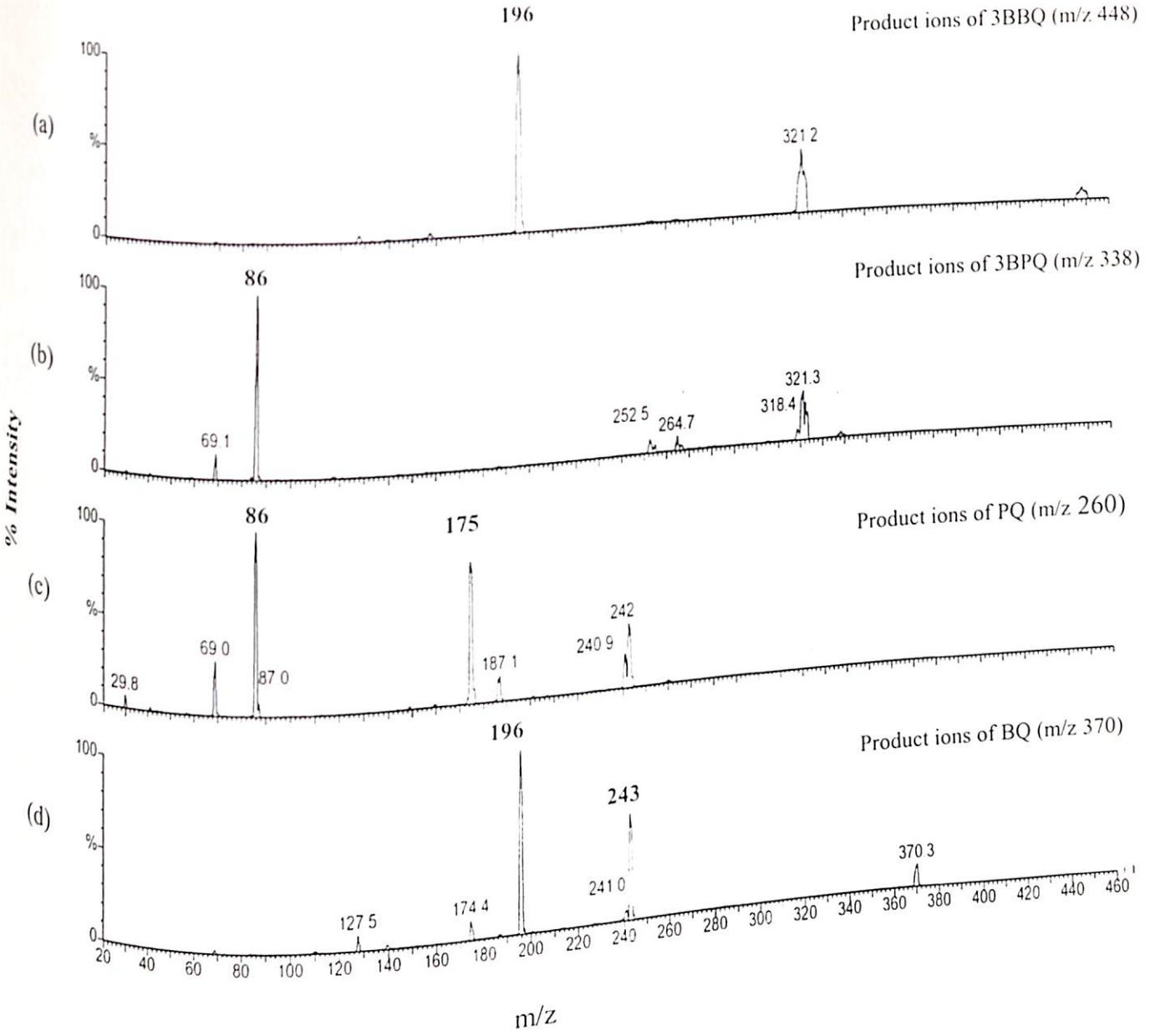


Figure 2.1(i) MS-MS spectra of (a) 3-BBQ, (b) 3-BPQ, (c) PQ, and (d) BQ, showing prominent precursor to product ion transitions in positive ion mode

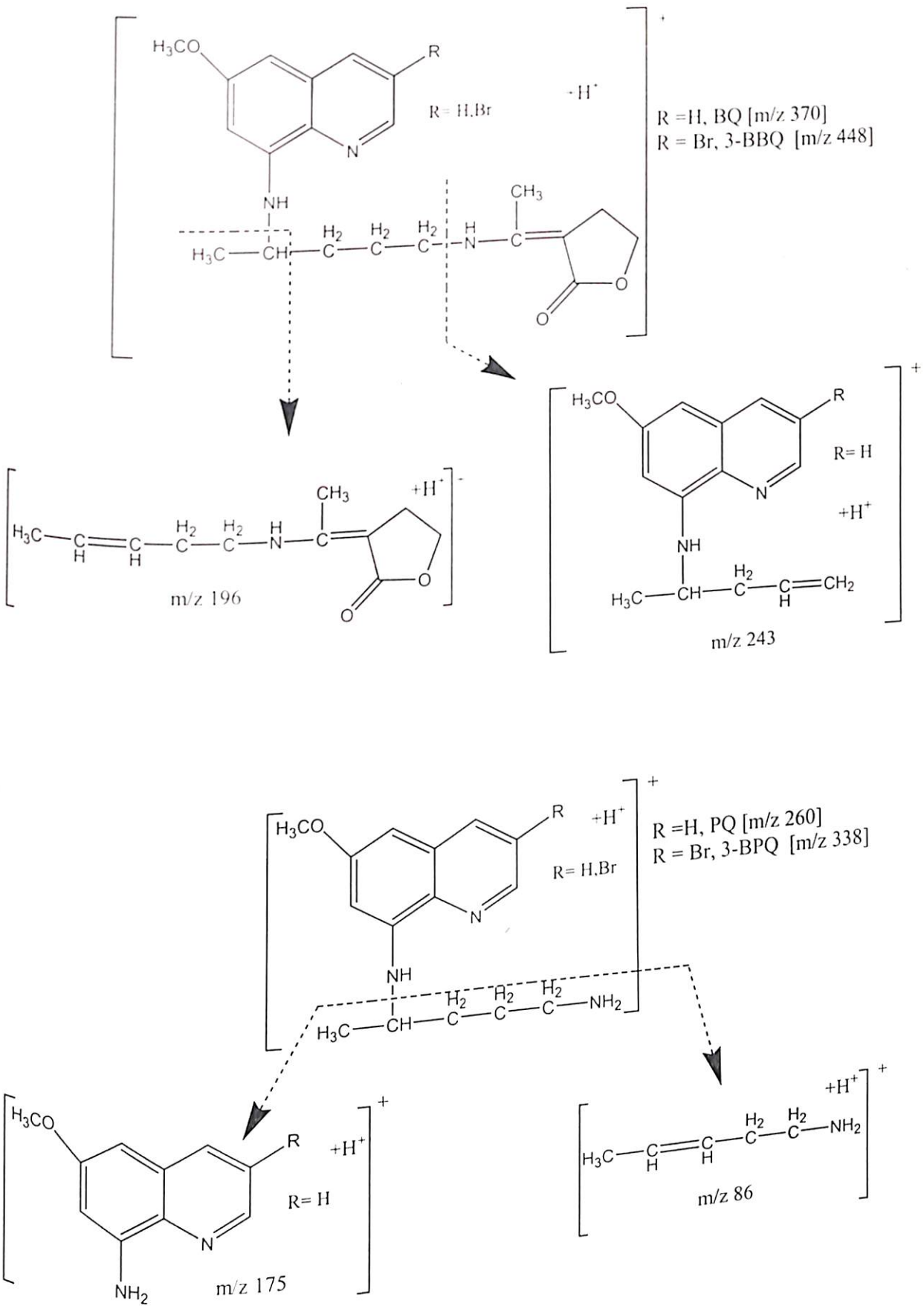


Figure 2.1 (ii) Proposed MS-MS fragmentation pattern of BQ, PQ, 3-BBQ and 3-BPQ in positive ion mode

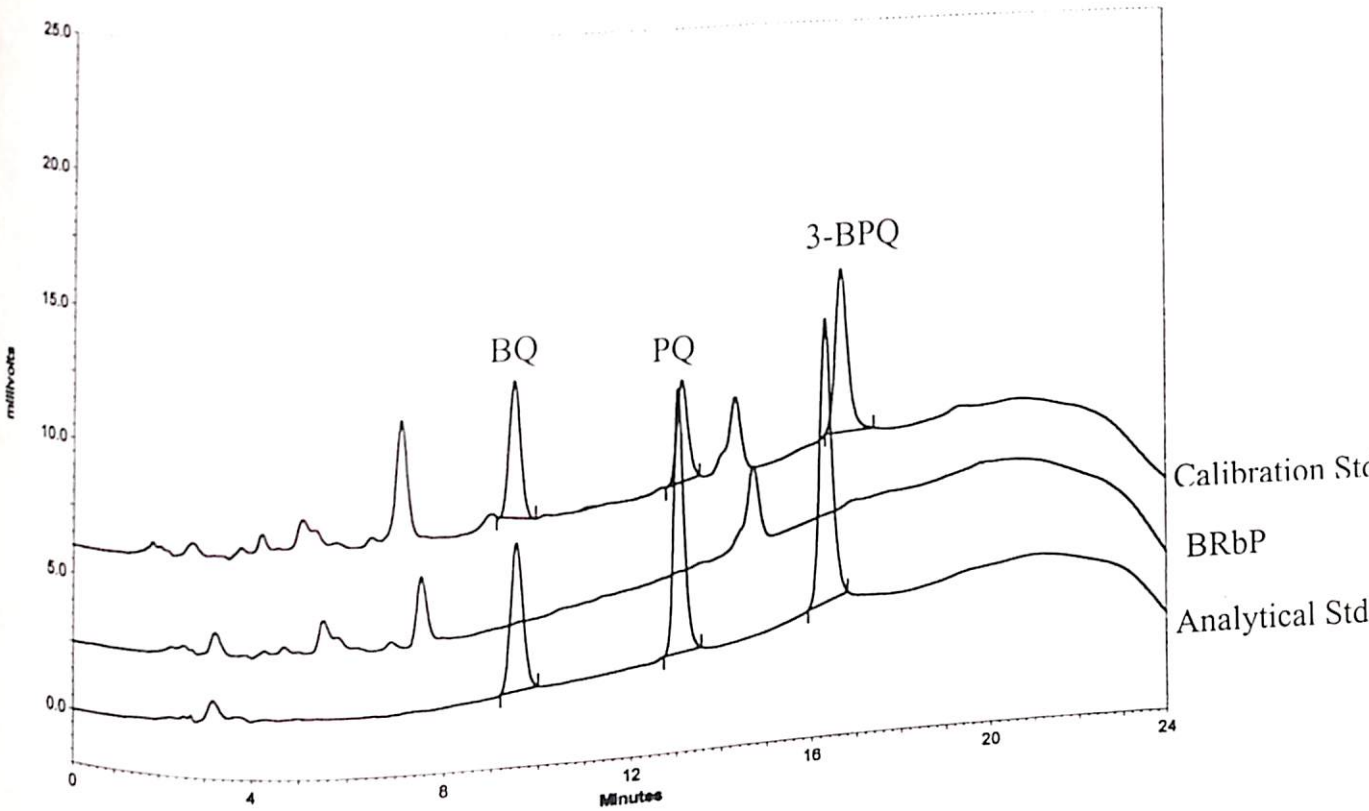


Figure 2.2 Typical chromatograms of an analytical standard containing BQ and PQ (each 250 ng/ml) and 3-BPQ (2500 ng/ml), drug-free rabbit plasma, rabbit plasma containing BQ and PQ (200 ng/ml) and IS (1000 ng/ml)

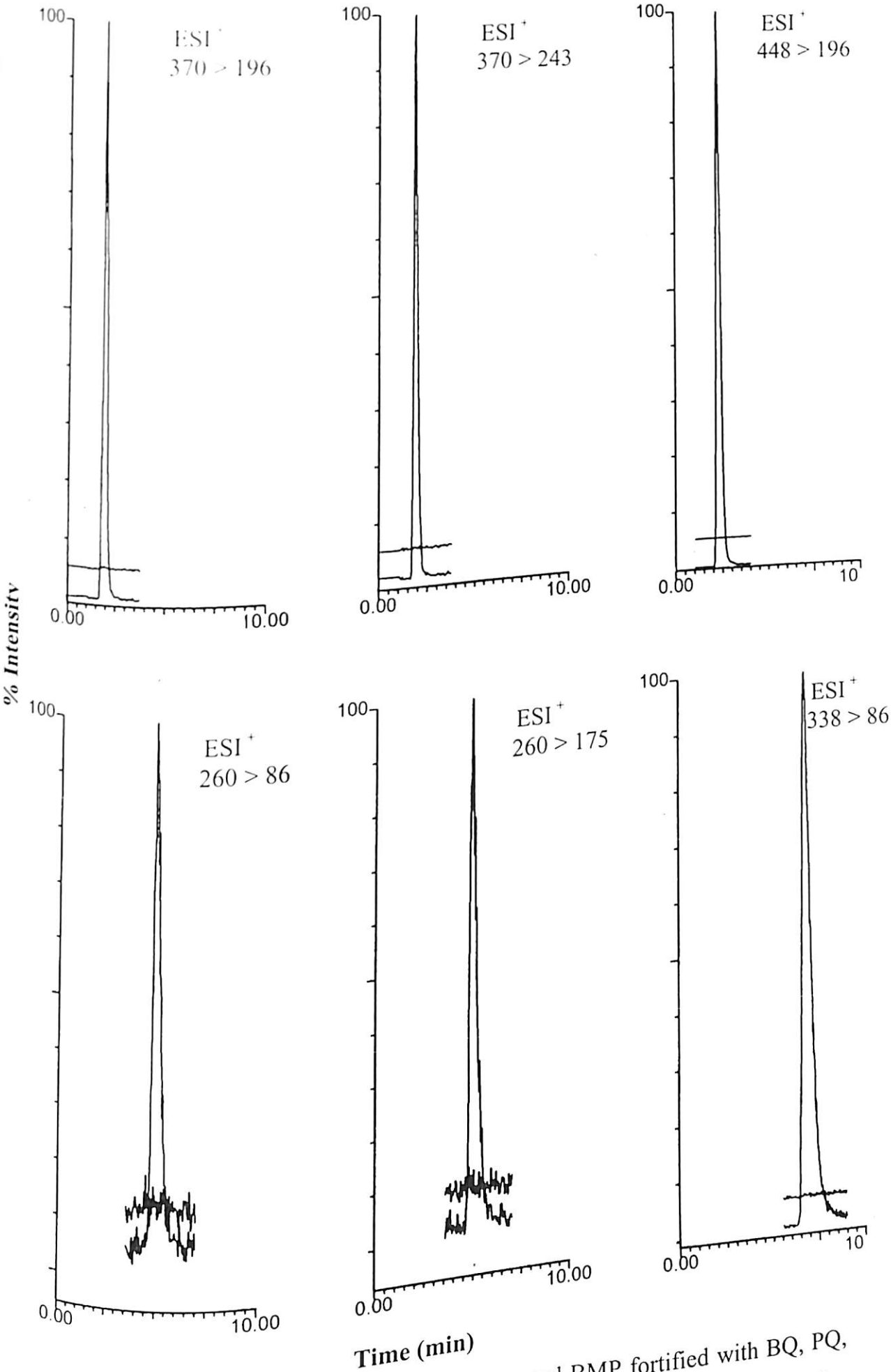


Figure 2.3 Representative chromatograms of extracted BMP fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BMP at LOQ of BQ and PQ

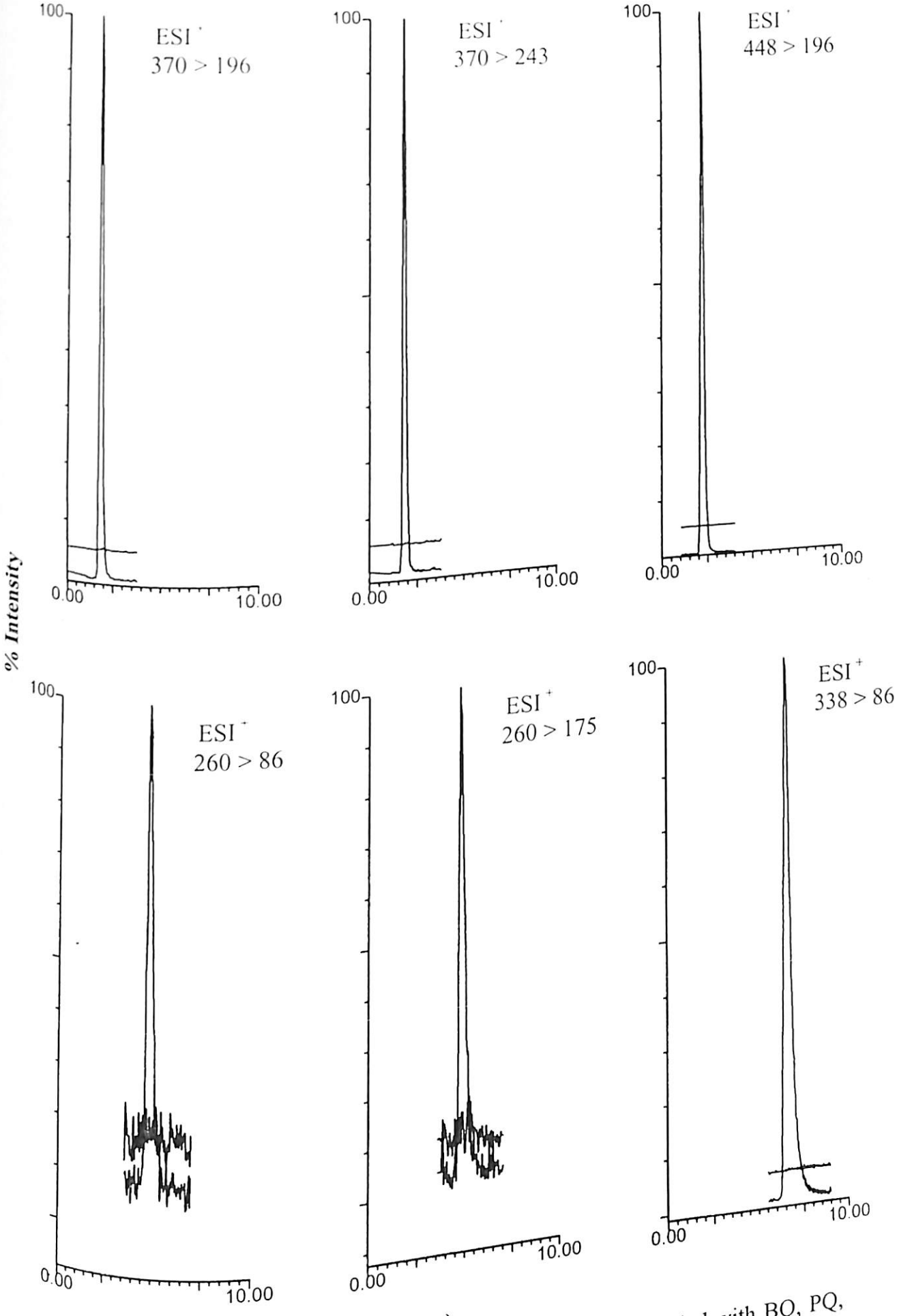


Figure 2.4 Representative chromatograms of extracted BRP fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BRP at LOQ of BQ and PQ

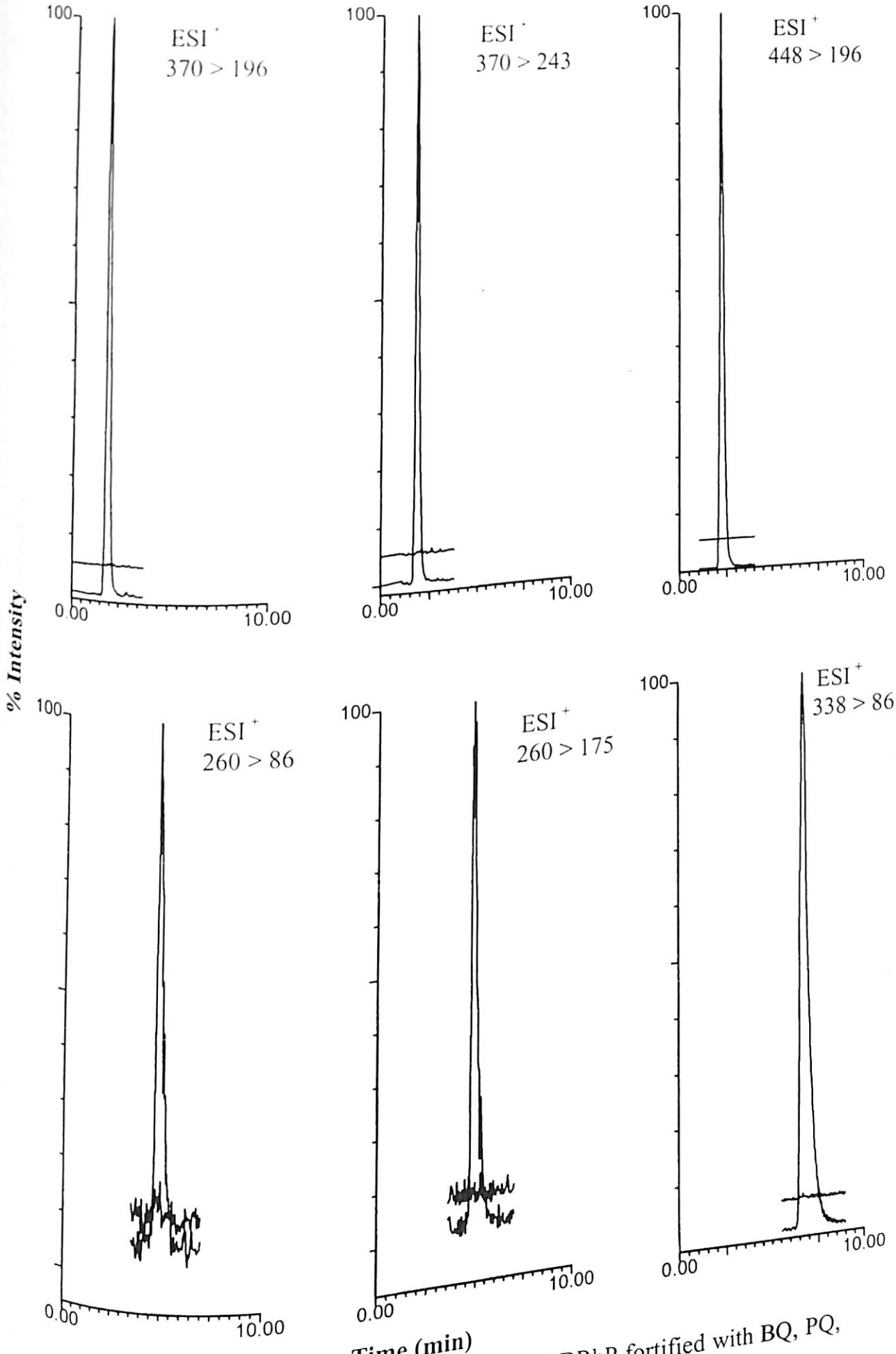


Figure 2.5 Representative chromatograms of extracted BRbP fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BRbP at LOQ of BQ and PQ

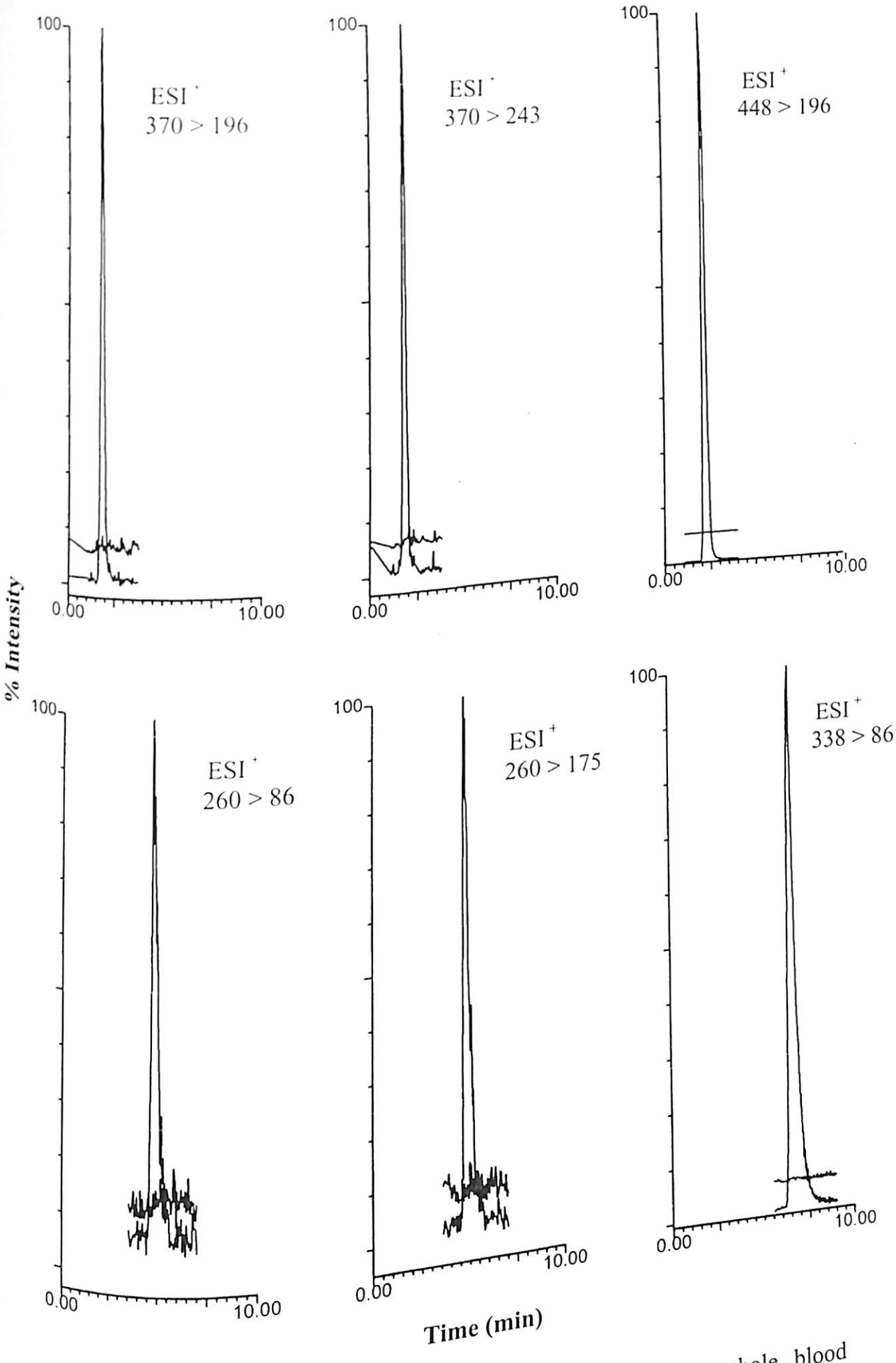


Figure 2.6 Representative chromatograms of extracted blank rat whole blood fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted blank rat whole blood at LOQ of BQ and PQ

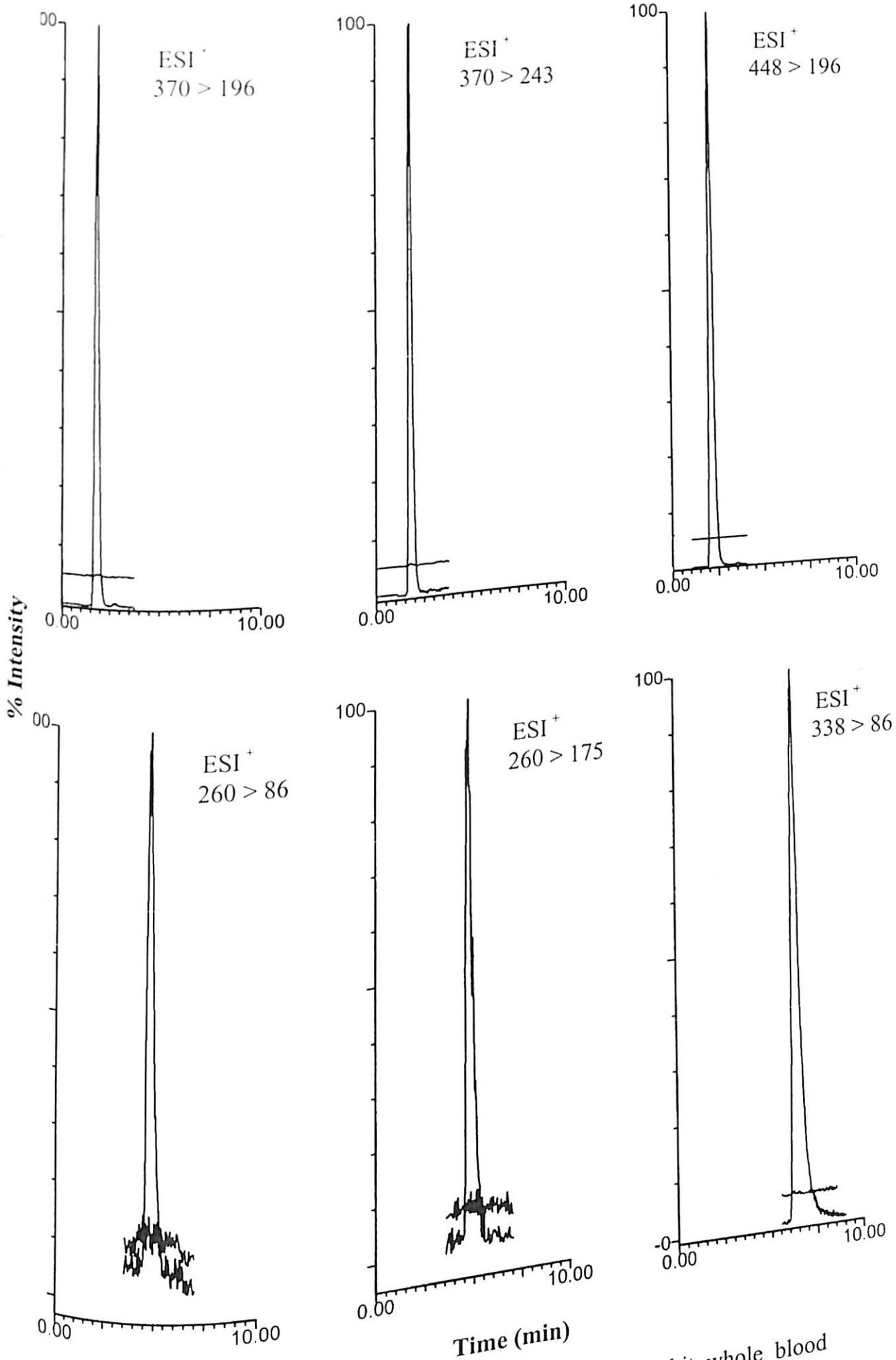


Figure 2.7 Representative chromatograms of extracted blank rabbit whole blood fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted blank rabbit whole blood at LOQ of BQ and PQ

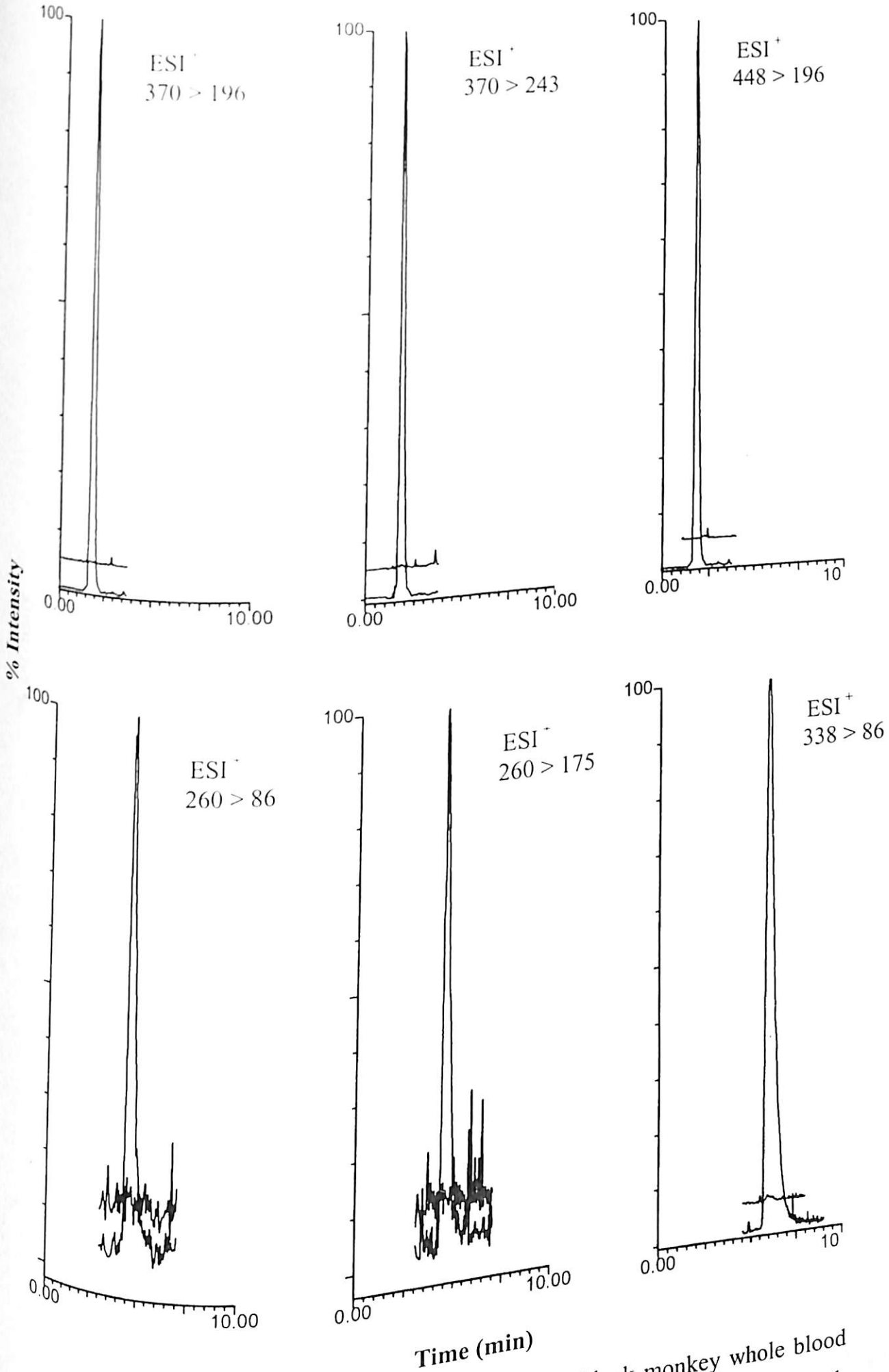


Figure 2.8 Representative chromatograms of extracted blank monkey whole blood fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted blank monkey whole blood at LOQ of BQ and PQ

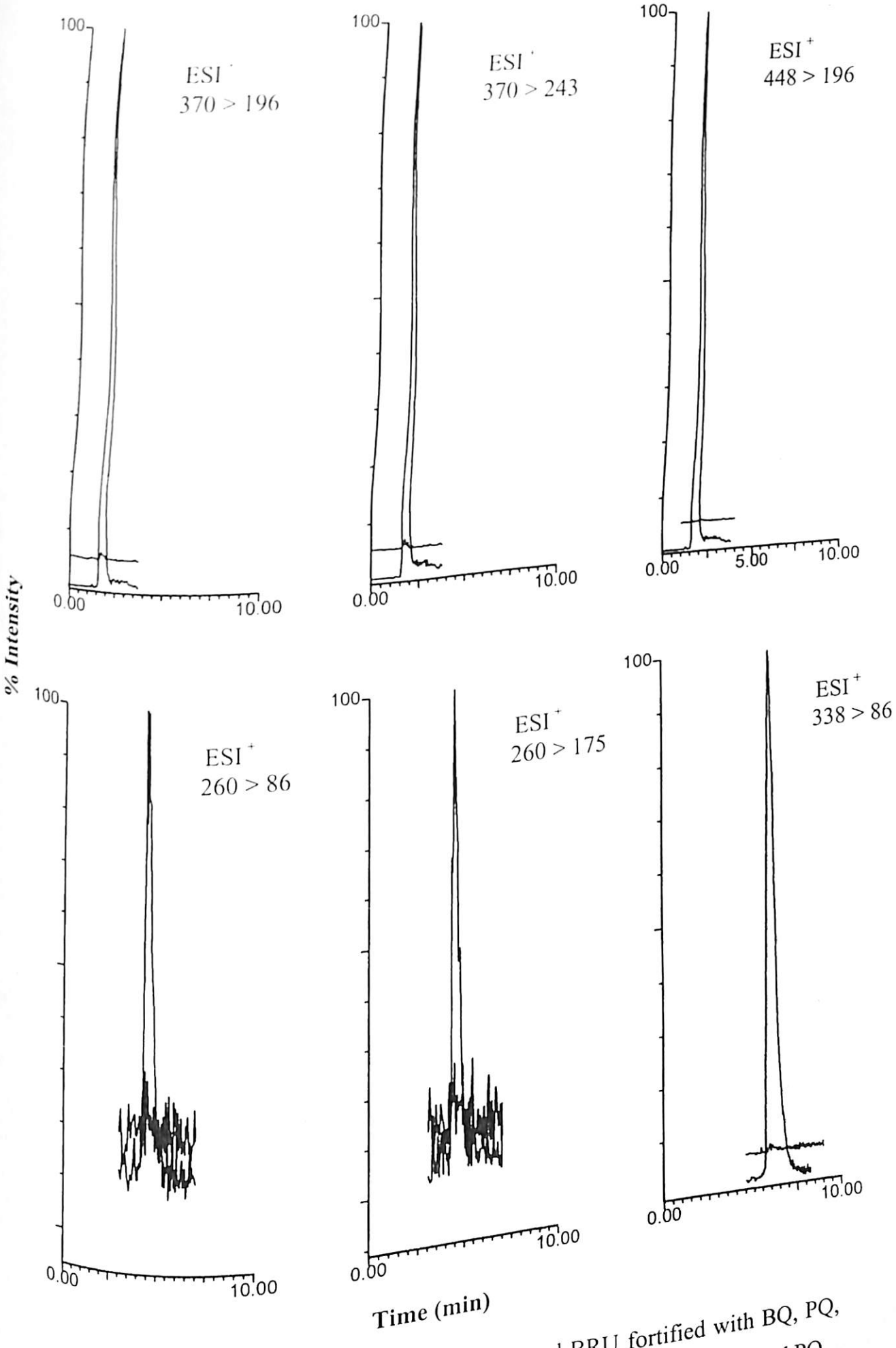


Figure 2.9 Representative chromatograms of extracted BRU fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted BRU at LOQ of BQ and PQ

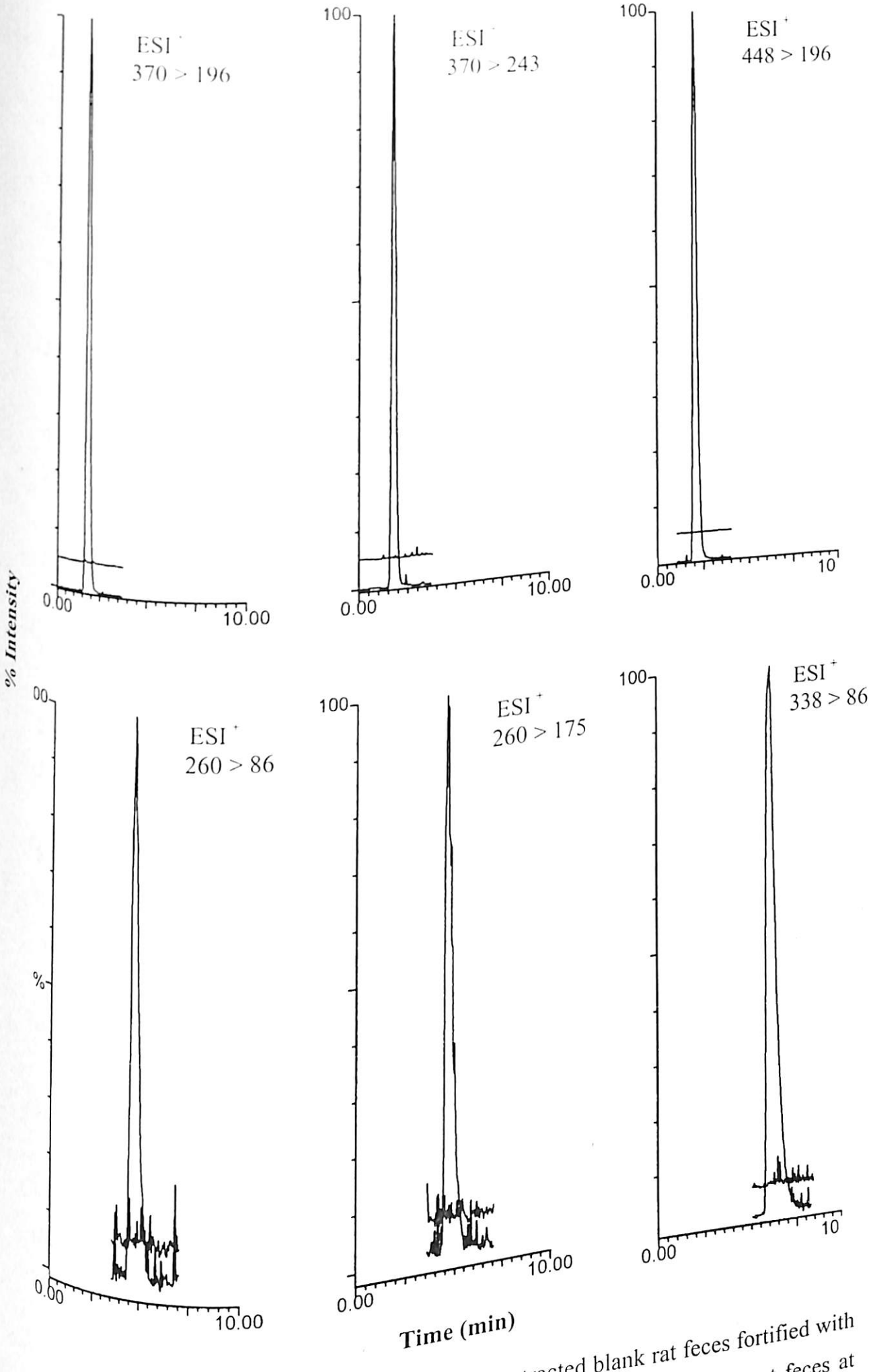


Figure 2.10 Representative chromatograms of extracted blank rat feces fortified with BQ, PQ, 3-BBQ and 3-BPQ overlaid with extracted blank rat feces at LOQ of BQ and PQ

2.5.4.2 Recovery

2.5.4.2.1 Method A and B

The recoveries of BQ and PQ at all QC levels from the biomatrix were calculated in triplicate each day and were found to be consistent, ≥ 90 and 50% for BQ and PQ, respectively [20, 21]. The % absolute recoveries for method A (n=3) and method B (n=5) are represented as mean ± SD in Table 2.10.

2.5.4.2.2 Method C, D, E, F, G

The recoveries for BQ and PQ in all these methods were calculated at five different levels in triplicate for one day and are represented as Mean ± SD in Table 2.11.

2.5.4.2.3 Method H and J

The mean ± SD absolute recoveries from rat urine (n=1) and feces (n=3) are given in Tables 2.12a and 2.12b, respectively.

2.5.4.3 Accuracy and Precision

2.5.4.3.1 Method A and Method B

The overall accuracy (% bias) and precision (%RSD) at the QC levels for Method A (n=3) and Method B (n=5) are presented in Table 2.13. The results show that the bioanalytical method is accurate (±20% difference from nominal concentration at the LOQ and ±15% at all other concentrations) and the precision is within the acceptance limits of 20% at LOQ and 15% at all other concentration levels studied [10].

2.5.4.3.2 Method C,D, E, F and G

These methods were partially validated separately using QCs in triplicates and intra-batch accuracy and precision are listed in Tables 2.14a and 2.14b. The results show that the methods were accurate and precise as variations were within the acceptable limits [10].

2.5.4.3.3 Method H and J

Method H was partially validated for one day at four QC levels in triplicate. Method J in feces was fully validated for three days at three QC levels in triplicate. The results demonstrate that variations were within acceptable limits (Tables 2.15a and 2.15b) [10].

Table 2.10 Mean ± SD recoveries of BQ and PQ from spiked plasma in method A and B

Method	Analyte	Code	Conc. (ng/ml)	% Recovery (Mean ± SD)
A	BQ	QCL	20	91.5 ± 5
		QCM	200	93.4 ± 2
		QCH	1000	92 ± 3.2
	PQ	QCL	20	52.6 ± 4.9
		QCM	200	51.2 ± 2
		QCH	1000	52.4 ± 2.4
B	BQ	L 1	1.56	100 ± 15
		L 2	3.12	103 ± 11
		M 1	12.5	100 ± 5.4
		M 2	50.0	101 ± 4.5
		H	400	103 ± 9.0
	PQ	L 1	3.91	60.8 ± 5.5
		L 2	7.81	57.0 ± 4.0
		M 1	31.25	58.6 ± 6.2
		M 2	125	56.5 ± 4.0
		H	1000	55.7 ± 7.9

Table 2.11 Mean ± SD recoveries of BQ and PQ in methods C, D, E, F and G

Analyte	Code	Conc. (ng/ml)	% Recovery (Mean ± SD)				
			Method				
			C	D	E	F	G
BQ	L 1	1.56	110±3	79±4.6	42±2.3	47±3.3	49±2.1
	L 2	3.12	115±8	76.3±2.4	40±6.3	46±6.3	45±3.2
	M 1	12.5	102±2.4	81.9±7.1	48±1.2	45±1.4	46±2.0
	M 2	50.0	108±3	79.4±2.4	49±4.0	42±2.6	40±3.6
	H	400	113±3.7	78.9±6.9	44±2.0	43±4.6	43±6.5
PQ	L 1	3.91	70±0.65	57.3±6.6	46±6.2	49±3.2	44±7.2
	L 2	7.81	64.6±3.2	54.5±1.7	42±1.2	51±5.6	49±6.3
	M 1	31.25	65±3.8	49.2±1.74	40±3.9	52±6.4	52±6.4
	M 2	125	60.9±1.3	52.1±0.21	41±5.2	40±1.6	48±1.2
	H	1000	59.3±0.81	52.9±0.71	39±2.0	48±6.2	49±5.4

Table 2.12a Mean ± SD recoveries of BQ and PQ from rat urine

Method	Analyte	Code	Conc. (ng/ml)	% Recovery (Mean ± SD)
H	BQ	L 1	3.9	53.8±0.88
		L 2	7.81	58.9±0.75
		M	62.5	53.5±0.85
		H	500	58.9±0.77
	PQ	L 1	7.8	65.9±7.0
		L 2	15.6	68.3±0.1.3
		M	125	66.3±0.61
		H	1000	70.0±0.20

Table 2.12b Mean ± SD recoveries of BQ and PQ from rat feces

Method	Analyte	Code	Conc. (ng/50 mg)	% Recovery (Mean ± SD)
J	BQ	L	3.9	113.3±3
		M	31.25	112.3±1.0
		H	125	106.4±3
	PQ	L	7.8	33±3.3
		M	62.5	48±3
		H	250	42±4.8

Table 2.13 Intra- and inter-batch accuracy and precision for QC samples in method A and method B

Method	Analyte	Code	Conc. (ng/ml)	% Bias		% RSD	
				Intra batch	Inter batch	Intra batch	Inter batch
A	BQ	QCL	20	13.0	15.7	3.4	11.0
		QCM	200	10.5	8.6	1.8	10.5
		QCH	1000	2.3	5.4	3.0	11.6
	PQ	QCL	20	-14.2	-13.6	7.6	15.1
		QCM	200	5.6	7.1	3.1	11.5
		QCH	1000	8.8	8.6	3.2	12.4
B	BQ	L 1	1.56	12.8	3.2	10.2	18.1
		L 2	3.12	7.8	4.8	3.0	14.0
		M 1	12.5	-1.1	-2.3	4.2	10.2
		M 2	50.0	-2.9	-0.5	2.7	12.8
		H	400	-1.5	-3.0	5.2	6.8
	PQ	L 1	3.91	-0.2	2.0	2.8	7.5
		L 2	7.81	-3.3	-1.6	2.8	5.2
		M 1	31.25	-1.6	-3.2	3.9	7.5
		M 2	125	-2.9	1.2	4.4	14.4
		H	1000	-5.7	-7.7	4.6	12.4

Table 2.14a Intra-batch accuracy for BQ and PQ in methods C, D, E, F and G

Analyte	Code	Conc. (ng/ml)	% Bias (Intra-batch)				
			Method				
			C	D	E	F	G
BQ	L 1	1.56	-14.3	19.6	-13.6	-14.3	12.6
	L 2	3.12	-7.4	5.0	2.4	6.8	9.5
	M 1	12.5	-2.4	1.9	2.6	-2.6	-5.8
	M 2	50.0	1.11	-3.4	9.4	2.1	-4.3
	H	400	0.6	1.0	14.5	10.3	11.2
PQ	L 1	3.91	-11.6	7.2	-15.3	-12.6	-11.2
	L 2	7.81	-1.6	7.6	12.2	2	3.4
	M 1	31.25	-4.5	1.2	3.2	6.3	5.4
	M 2	125	-1.8	8.5	-2.4	-3.6	6.2
	H	1000	0.2	10.5	-2.6	-6.8	-7.4

Table 2.14b Intra-batch precision for BQ and PQ in methods C, D, E, F and G

Analyte	Code	Conc. (ng/ml)	% RSD (Intra-batch)				
			Method				
			C	D	E	F	G
BQ	L 1	1.56	3.13	3.09	2.6	12.5	11.2
	L 2	3.12	0.81	2.57	1.3	2.6	6.3
	M 1	12.5	2.5	8.45	2.6	1.3	5.4
	M 2	50.0	1.83	3.09	6.5	9.2	4.3
	H	400	3.73	3.64	12.3	3.5	4.5
PQ	L 1	3.91	1.27	13.06	2.0	9.9	10.2
	L 2	7.81	5.87	3.32	6.3	5.8	11.3
	M 1	31.25	6.0	3.59	4.6	1.6	2.5
	M 2	125	2.2	0.40	9.2	4.3	5.6
	H	1000	1.4	1.35	2.6	2.4	7.6

Table 2.15a Intra-batch accuracy and precision in method H

Method	Analyte	Code	Conc. (ng/ml)	% Bias (Accuracy)	% RSD (Precision)
				Intra batch	Intra batch
H	BQ	L 1	3.9	13.19	1.38
		L 2	7.81	12.19	1.17
		M	62.5	-5.03	1.57
		H	500	3.51	1.30
	PQ	L 1	7.8	3.34	9.86
		L 2	15.6	2.98	2.70
		M	125	-3.60	0.92
		H	1000	-1.42	0.29

Table 2.15b Intra- and inter-batch accuracy and precision in method J

Method	Analyte	Code	Conc. (ng/50 mg)	% Bias		% RSD	
				Intra	Inter	Intra	Inter
H	BQ	L	3.9	1.68	-4.03	2.4	13.8
		M	31.25	0.69	1.63	0.9	4.3
		H	125	-5.16	-5.95	2.3	4.74
	PQ	L	7.8	4.5	8.4	5.7	19.15
		M	62.5	14.7	14.8	5.9	7.9
		H	250	0.7	1.4	4.9	4.5

2.5.4.4 Stability Studies

(i) **Method A and B**

To ascertain the stability of BQ and PQ, freeze-thaw stability studies were performed for methods A and B [20, 21]. To assess the freeze-thaw assay stability of BQ and PQ, the QC samples at low, medium and high concentrations in triplicate for four f-t assay studies were prepared. The freeze-thaw stability data for PQ could not be generated in HPLC-UV method due to some analytical problems but it was studied for BQ and PQ for three complete freeze-thaw cycles (over a period of 7 days) using method B which was applied in various *in vitro* and *in vivo* studies. For Method B, four sets of QC samples at QCL1, QCM2 and QCH in duplicate were prepared. One

set comprising of duplicate samples at each concentration level was assayed on the day of preparation (no freeze-thaw cycle). The remaining three sets were stored frozen at -60°C and analysed after one two and three freeze-thaw cycles. Thawing was achieved by keeping the sample tubes unassisted at ambient temperature for 30 minutes. The results obtained after analysis on day of preparation were taken as standard (100%) and the subsequent results were compared with the standard and expressed as percent deviation. The % deviation observed after 1st, 2nd, and 3rd freeze -thaw in case of Method A and B is given in Table 2.16.

Table 2.16 Freeze-thaw stability of BQ in methods A and B expressed as % deviation

Conc. (ng/ml)	f-t 1	f-t 2	f-t 3
Method-A		**BQ**	
20	-8.1	-9.5	-6.3
200	-1.0	-0.6	4.2
1000	-1.0	2.8	6.5
Method-B		**BQ**	
1.56	-4.0	6.4	7.4
50	9.1	-1.8	7.9
400	1.5	1.0	0.1
Method-B		**PQ**	
3.91	-19.9	18.6	14.8
125	-1.5	-14.0	-7.9
1000	1.3	-5.7	-5.8

2.6 Conclusions

Bioanalytical method validation is a tool, which ensures the reliability and authenticity of the assay data and pharmacokinetic studies. The methods were sensitive and highly selective for the analytes in the presence of endogenous impurities of the biomatrices. Methods for simultaneous determination of BQ and PQ were developed in monkey plasma and then partially validated in rat and rabbit plasma. The applicability of the method was further extended by partially validating in rat, rabbit and monkey whole blood separately. The LOQ of BQ and PQ in all these methods was 1.56 and 3.91, respectively. Method H and J were developed and

validated in rat urine and feces for application to excretion studies in rats. The LOQ of BQ and PQ in method H was 3.9 and 7.8 ng/ml respectively while in method J it was 0.078 and 0.156 ng/mg of feces respectively. Intra- and inter-batch accuracy and precision were within acceptable limits and recoveries were consistent over the entire calibration range in all the methods. The LC-MS-MS methods were more sensitive and specific with lower sample processing volumes as compared to LC-UV method were used for the analysis of BQ and PQ to generate realistic PK parameters. These methods validated in different matrices were applied for various *in vitro* and *in vivo* pharmacokinetic studies.

References

1. Shah, V.P., Midha, K.K., Dighe, S., McGilveray, I.J., Skelly, J.P., Yacobi, A., Layloff, T., Viswanathan, C.T., Cook, C.E., McDowall, R.D., Pittman, K.A. and Spector, S., 1991. Analytical method validation: Bioavailability, bioequivalence and pharmacokinetic studies, *Eur. J. Drug Metab. Pharmacokin.*, 16 (4): 249-255.
2. Lang, J.R. and Bolton, S., 1991. A compression method validation strategy for bioanalytical application in the pharmaceutical industries-1. Experimental considerations, *J. Pharm. Biomed. Anal.*, 9: 357-361.
3. Lang, J.R. and Bolton, S., 1991. A compression method validation strategy for bioanalytical application in the pharmaceutical industries-2. Statistical considerations, *J. Pharm. Biomed. Anal.*, 9: 435-442.
4. Buick A.R., Doig, M.V., Jeal, S.C., Land, G.S. and McDowall, R.D., 1990. Method validation in bioanalytical laboratories, *J. Pharm. Biomed. Anal.*, 8: 629-637.
5. Hartmann, C., Massarrt, D.L. and Mc Dowall, R.D., 1994. An analysis of Washington Conference report on bioanalytical validation, *J. Pharm. Biomed. Anal.*, 12: 1337-1345.
6. Shah, V. P., Midha, K.K., Dighe, S., McGilveray, I.J., Skelly, J.P., Yacobi, A., Layloff, T., Viswanathan, C.T., Cook, C.E., McDowall, R.D., Pittman, K.A. and Spector, S., 1992. Analytical method validation: Bioavailability, bioequivalence and pharmacokinetic studies, *Pharm. Res.*, 9: 588-592.
7. Peng, G.W. and Chiou, W.L., 1990. Analysis of drugs and other toxic substances in biological samples for pharmacokinetic studies, *J. Chrom. B.*, 591: 3-50.
8. Krstulvic, A.M. and Brown, P.R., 1982. Reversed phase high performance liquid chromatography, John Wiley and sons, New York.
9. Clarke, N.J., Rindgen, D., Korfmacher, W.A. and Cox, K.A., 2001. Systematic LC-MS metabolite identification in drug discovery, *Anal. Chem., 73*: 430-442.
10. Shah, V.P., Midha, K.K., Findlay, J.W., Hill, H.N., Hulse, J.D., Megilveray, I.J., McKay, G., Miller, K.J., Patnaik, R.N., Powell, M.L., Tonelli, A., Viswanathan, C.T. and Yacobi, A., 2000. Bioanalytical method validation-a revisit with a decade of progress, *Pharm. Res.*, 17: 1551-1557.

11. Bressolle, F., Bromet, P.M. and Audran, M., 1996. Validation of liquid chromatographic and gas chromatographic methods. Application to pharmacokinetics, *J. Chrom. B.*, 686: 3-10.

12. Causon, R., 1997. Validation of chromatographic methods in biomedical analysis. Viewpoint and Discussion, *J. Chrom. B.*, 689: 175-180.

13. Dadgar, D. and Burnett, P.E., 1995. Issues in evaluation of bioanalytical method selectivity and drug stability, *J. Pharm. Biomed. Anal.*, 13: 89-97.

14. Dadgar, D., Burnett, P.E., Choc, M.G., Gallicano, K., and Hooper, J.W., 1995. Issues in evaluation of bioanalytical method selectivity and drug stability, *J. Pharm. Biomed. Anal.*, 14: 23-31.

15. Hartmann, C., Smeyers-verbeke, I., Massart, D.L. and McDowall R.D., 1998. Validation of Bioanalytical chromatographic methods, *J. Pharm. Biomed. Anal.*, 17: 193-218.

16. Linder, W. and Wainer, I.W., 1998. Requirements for initial assay validation in journal of chromatography B (editorial), *J.Chrom. B.*, 707: 1-2.

17. Wieling, J., Hendriks, G., Tamminga, W.J., Henpenius, J., Mensink, C.K., Oosterhuis, B. and Jonkman, J.H., 1996. Rational experimental design for bioanalytical method validation- Illustration using an assay method for total captopril in plasma, *J. Chrom. A.*, 730: 381-394.

18. Nagaraja, N.V., Paliwal, J.K. and Gupta, R.C., 1999. Choosing the calibration model in assay validation, *J. Pharm. Biomed. Anal.* 20: 433-438.

19. Clayton, C.A., 1980. Guidelines for data acquisition and data quality evaluation, *Anal. Chem.*, 52: 2242.

20. Lal, J., Mehrotra, N. and Gupta, R.C., 2003. Analysis and pharmacokinetics of bulaquine and its major metabolite primaquine in rabbits using LC-UV method- a pilot study, *J. Pharm. Biomed. Anal.*, 32: 141-150.

21. Mehrotra, N., Rajanikanth M., Lal, J., Madhusudanan, K.P. and Gupta, R.C., 2003. Liquid chromatography- tandem mass spectrometric assay with a novel method of quantitation for the simultaneous determination of bulaquine and its metabolite, primaquine, in monkey plasma, *J. Chrom. B.*, 793: 253-263.

22. Paliwal, J.K., Gupta, R.C. and Grover, P.K., 1993. Simultaneous determination of CDRI compound 80/53 and its metabolite in serum by high performance liquid chromatography, J. *Chrom. B.*, 616: 155-160.

23. Paliwal, J.K. and Gupta, R.C., 1993. A rapid and sensitive high performance chromatographic assay for the new antimalarial 80/53 in serum with novel sample clean up method and its pharmacokinetics in rabbits, *J. Pharm. Biomed. Anal.*, 17: 775-783.

24. Fu, I., Woolf, E.J. and Matuszewski, B.K., 1998. Effect of sample matrix on the determination of indinavir in human urine by HPLC with turbo ion spray tandem mass spectrometric assay, *J. Pharm. Biomed. Anal.*, 18: 347.

CHAPTER 3

IN VIVO STUDIES

SECTION 1

Oral and i.v. Pharmacokinetic Studies in Various Species

3.1.1 Introduction

Pharmacokinetic studies gain importance from the fact that the rate and the extent of absorption will result in the pharmacological effect of desired magnitude and response. This attribute of the drug is referred to as its physiological availability, biologic availability or simply bioavailability. An orally administered drug must be absorbed from the gastrointestinal tract to an extent and at a rate that will result in circulating drug levels sufficient to elicit a pharmacological response of desired magnitude and duration. There are numerous factors involved in oral bioavailability of a particular therapeutic agent, like delivery to intestine (gastric emptying, pH, presence of food), absorption from the lumen (dissolution, lipophilicity, particle size, active uptake), intestinal metabolism (phase I and/ or phase II enzymes), active extrusion (drug efflux pumps), and subsequent hepatic first-pass extraction [1, 2]. The oral and intravenous routes are widely used to assess the absolute systemic bioavailability of the compound [3, 4]. Moreover, multiple sites of drug administration have been used to estimate the relative contribution of the gut, liver and lungs as site of metabolism [5, 6].

PK studies in rats, rabbits and monkeys are described in this chapter. PK parameters of BQ determined after oral and i.v. administration to rats, rabbits and monkeys also enabled to highlight the species similarities/differences.

3.1.2 Oral and i.v. Pharmacokinetic Study in Rats

3.1.2.1 Experimental

3.1.2.1.1 Materials

Pure reference standard of BQ was obtained from the Pharmaceutics Division of CDRI. K_2HPO_4 and Xylene (sulphur free) were procured from s.d. Fine Chemicals, Mumbai, India. Propylene glycol (PG) and orthophosphoric acid were purchased from E-Merck India Ltd, Mumbai. Commercially available ethanol was distilled in the laboratory and used for the preparation of formulation. Heparin sodium injection IP (5000 IU/ml) was procured from Biological Evans, India. Purified water was obtained from MilliQ system. Drug-free rat plasma pool was generated from male *Sprague Dawley* rats provided by Laboratory Animal Services Division of the institute.

3.1.2.1.2 Animals

The pharmacokinetic studies of BQ were carried out in young and healthy male albino *Sprague Dawley* rats (n=3 per time point) weighing 250 ± 25 g. The animals were obtained from Laboratory Animal Services Division of CDRI, and housed in plastic cages in standard laboratory conditions with a regular 12 h day-night cycle. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feed, Chandigarh, India) and water were allowed *ad libitum*. The rats were acclimatized to this environment for at least 2 days before conducting the pharmacokinetic studies. The study protocol was approved by local ethical committee (Reg. No. 34/1999/CPCSEA) of the institute. All experiments, euthanasia and disposal of carcass were executed in accordance with the guidelines laid by local ethical committee of the institute for animal experiments.

3.1.2.1.3 Buffer solution

K_2HPO_4 buffer solution (20 mM, pH 7.0) was prepared by dissolving 174.2 mg of K_2HPO_4 in 50 ml MilliQ water and adjusting the pH to 7.0 with 20 % orthophosphoric acid.

3.1.2.1.4 Drug Formulation

Solution formulation of BQ (5 mg/ml) was prepared by dissolving BQ in mixture of PG, ethanol and K_2HPO_4 buffer in the ratio 35:30:35 %v/v respectively. Accurately weighed amount of BQ was transferred into a test tube. To this PG was added and mixture was sonicated followed by addition of ethanol. The sonication was continued till a clear solution was obtained and then buffer was added while vortexing. The formulation was analysed to determine the drug content and its stability. Freshly prepared formulation was used for the studies.

3.1.2.1.5 Dosing

Overnight (12-16 h) fasted rats were dosed orally at 20 mg/kg by oral gavage using oral feeding needle such that each 250 g rat received 1.0 ml of the formulation. For i.v. PK study, formulation was administered to rats (0.25 ml/250 g rat) using a 1.0 ml tuberculin syringe (fitted with 26 gauge needle) via tail vein at 5 mg/kg. The tail was first dilated with warm water followed by xylene.

3.1.2.1.6 Sampling Schedule

Blood samples were withdrawn at 5 min, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24 and 48 h post dose in clean and heparinised glass tubes. Plasma was separated by centrifugation at 1500 rpm for 10 min at 4°C within 1 h of sampling and was stored at –60°C until analysis. Two blood samples were withdrawn from each animal. An initial 0.6 ml blood sample was withdrawn by cardiac puncture with 24G needle under light ether anesthesia and the second blood sample was withdrawn from inferior vena cava.

3.1.2.1.7 Sample Analysis

The concentrations of BQ and PQ were estimated using Method C described in section 2.4.6.

3.1.2.1.8 Data Treatment

Concentration-time data of BQ and its metabolite PQ after oral and i.v administration were subjected to non-compartmental analysis using WinNonlin (ver 1.5 software, SCI consultants, USA) to determine the PK parameters. The clearance (CL), volume of distribution (V_d), elimination phase half-life ($t_{1/2}$), and area under curve (AUC) for both the analytes were determined using non-compartmental analysis. The elimination $t_{1/2}$ was determined by providing the elimination data points in the concentration-time data in non-compartmental analysis while C_{max} and T_{max} were directly read from the raw data. The bioavailability ($\%F_{p.o.}$) was determined as follows:

$$(\%\ F_{p.o.}) = \frac{AUC_{p.o.} \times Dose_{i.v.}}{AUC_{i.v.} \times Dose_{p.o.}} \times 100$$

Where p.o. refers to per oral dose.

3.1.3 Oral and i.v. Pharmacokinetic Study in Rabbits

3.1.3.1 Experimental

3.1.3.1.1 Materials

The chemicals and solvents were the same as described in section 3.1.2.1.1. Drug-free rabbit plasma pool was generated from male healthy rabbits provided by Laboratory Animal Services Division of the institute.

3.1.2.1.6 Sampling Schedule

Blood samples were withdrawn at 5 min, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24 and 48 h post dose in clean and heparinised glass tubes. Plasma was separated by centrifugation at 1500 rpm for 10 min at 4°C within 1 h of sampling and was stored at –60°C until analysis. Two blood samples were withdrawn from each animal. An initial 0.6 ml blood sample was withdrawn by cardiac puncture with 24G needle under light ether anesthesia and the second blood sample was withdrawn from inferior vena cava.

3.1.2.1.7 Sample Analysis

The concentrations of BQ and PQ were estimated using Method C described in section 2.4.6.

3.1.2.1.8 Data Treatment

Concentration-time data of BQ and its metabolite PQ after oral and i.v administration were subjected to non-compartmental analysis using WinNonlin (ver 1.5 software, SCI consultants, USA) to determine the PK parameters. The clearance (CL), volume of distribution (V_d), elimination phase half-life ($t_{1/2}$), and area under curve (AUC) for both the analytes were determined using non-compartmental analysis. The elimination $t_{1/2}$ was determined by providing the elimination data points in the concentration-time data in non-compartmental analysis while C_{max} and T_{max} were directly read from the raw data. The bioavailability ($\%F_{p.o.}$) was determined as follows:

$$(\%\ F_{p.o.}) = \frac{AUC_{p.o.} \times Dose_{i.v.}}{AUC_{i.v.} \times Dose_{p.o.}} \times 100$$

Where p.o. refers to per oral dose.

3.1.3 Oral and i.v. Pharmacokinetic Study in Rabbits

3.1.3.1 Experimental

3.1.3.1.1 Materials

The chemicals and solvents were the same as described in section 3.1.2.1.1. Drug-free rabbit plasma pool was generated from male healthy rabbits provided by Laboratory Animal Services Division of the institute.

3.1.3.1.2 Animals

The pharmacokinetic studies of BQ were carried out in young and healthy male rabbits (n=3 per route of administration) weighing 2.5±0.5 kg, obtained from Laboratory Animal Services Division of CDRI. The animals were housed in stainless steel cages in standard laboratory conditions with a regular 12 h day-night cycle. Standard laboratory food was provided with water being allowed *ad libitum*. The rabbits were acclimatized to this environment for at least 2 days before conducting the pharmacokinetic studies. The study protocol was approved by local ethical committee (Reg. No. 34/1999/CPCSEA) of the institute. All experiments, euthanasia and disposal of carcass were executed in accordance with the guidelines by local ethical committee of the institute for animal experiments.

3.1.3.1.3 Buffer solution

The buffer solution was prepared in the manner similar to that described in section 3.1.2.1.3.

3.1.3.1.4 Drug Formulation

The drug formulation was prepared in the same way as described in section 3.1.2.1.4. The formulation was analysed to determine the drug content and its stability. Freshly prepared formulation was used for the studies.

3.1.3.1.5 Dosing

The oral dose was administered after overnight (12-16 h) fasting. Rabbits were dosed orally at of 10 mg/kg by oro-gastric catheter such that each 2.5 kg rabbit received 5 ml of the formulation followed by flushing the catheter with 2.0 ml of vehicle. Intravenous formulation was administered using a 5.0 ml disposable syringe (fitted with 26 G needle) via marginal ear vein at 2.5 mg/kg so that each 2.5 kg rabbit received 1.25 ml of the formulation. The vein was first dilated with warm water followed by xylene.

3.1.3.1.6 Sampling Schedule

Blood samples (0.6 ml/time point) were withdrawn at 5 min, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24 h post dose in clean and heparinised glass tubes. Plasma was

separated by centrifugation at 1500 rpm for 10 minutes at 4°C and was stored at -60°C until analysis.

3.1.3.1.7 Sample Analysis

The concentrations of BQ and PQ in rabbit plasma samples were estimated using Method D described in section 2.4.6.

3.1.3.1.8 Data Treatment

PK Parameters of BQ and PQ were determined by subjecting individual concentration-time data of the three rabbits to non-compartmental analysis and the parameters were expressed as mean ± SEM. The elimination $t_{1/2}$ was determined by providing the elimination data points in the concentration-time data in non-compartmental analysis while C_{max} and T_{max} were directly read from the raw data. The i.v. data was subjected to compartmental analysis. Decision on the suitability of a particular compartment in explaining the data was taken based on correlation coefficient, Akaike information criteria, Schwarz value [7, 8] and the scatter of residuals. The bioavailability ($\%F_{p.o.}$) was determined as described in section 3.1.2.1.8.

3.1.4 Oral and i.v. Pharmacokinetic Study in Monkeys

3.1.4.1 Experimental

3.1.4.1.1 Materials

Chemicals and solvents were the same as described in section 3.1.2.1.1. Drug-free monkey plasma pool was generated from healthy male rhesus monkeys provided by Laboratory Animal Services Division of the institute.

3.1.4.1.2 Animals

The pharmacokinetic studies of BQ was carried out in young and healthy male rhesus monkeys (n=3 per route of administration) weighing 6±1 kg, obtained from Laboratory Animal Services Division of CDRI, Lucknow. All monkeys underwent physical examination and a complete medical history was maintained. Monkeys, which were under medication or treated with any known enzyme inducing or inhibiting drugs within 30 days of the study or suffering from any illness during the study, were excluded from selection.

The animals were housed in stainless steel cages in standard laboratory conditions with a regular 12 h day-night cycle. Standard laboratory food was provided with water being allowed *ad libitum*. The monkeys were acclimatized to these conditions for at least 2 days before conducting the pharmacokinetic studies. An approval of the study protocol was obtained by the local ethical committee (Reg. No. 34/1999/CPCSEA) of the institute. All experiments, euthanasia and disposal of carcass were executed in accordance with the guidelines laid by local ethical committee of the institute for animal experiments.

3.1.4.1.3 Buffer solution

The buffer solution was prepared in the same way as described in section 3.1.2.1.3.

3.1.4.1.4 Drug Formulation

The drug formulation was prepared in the same way as described in section 3.1.2.1.4. The formulation was analysed to determine the drug content and its stability. Freshly prepared formulation was used for the studies.

3.1.4.1.5 Dosing

The oral dose was administered after overnight (12-16 h) fasting. Monkeys were dosed orally at a dose level of 10 mg/kg such that each 6.0 kg monkey received 12.0 ml of the formulation by oro-gastric catheter followed by flushing the catheter with 5.0 ml of vehicle. Intravenous dose was administered to monkeys using a 10.0 ml disposable syringe via cubital vein of forearm at 10 mg/kg dose level.

3.1.4.1.6 Sampling Schedule

Blood samples (0.6 ml/time point) were withdrawn at 2 min, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, 24, 30, 36 and 48 h post dose in clean and heparinised glass tubes. Plasma was separated by centrifugation at 1500 rpm for 10 min at 4°C and was stored at -60°C until analysis.

3.1.4.1.7 Sample Analysis

The concentrations of BQ and PQ were estimated using Method B described in section 2.4.5.

3.1.4.1.8 Data Treatment

PK Parameters of BQ and PQ after oral and i.v. administration were determined by subjecting individual concentration-time data for the three monkeys to non-compartmental analysis and the parameters were expressed as mean ± SEM. The elimination $t_{1/2}$ was determined by providing the elimination data points in the concentration-time data in non-compartmental analysis while C_{max} and T_{max} were directly read from the raw data. The i.v. data was subjected to compartmental analysis. Decision on the suitability of a particular compartment in explaining the data was taken based on correlation coefficient, Akaike information criteria, Schwarz value [7, 8] and the scatter of residuals. The bioavailability ($\%F_{p.o.}$) was determined as described in section 3.1.2.1.8.

3.1.5 Statistical Analysis

The mean ± SEM values for C_{max} 1 (BQ) for rats, rabbits and monkeys were compared using one way ANOVA (Newman-Keuls multiple comparison test) at 95% confidence interval. The PK Parameters (elimination $t_{1/2}$, AUC, V_d and Cl) of BQ in rabbits and monkeys were compared by two tailed t-test at 95% confidence interval after oral dose. The same test was performed for the above parameters post i.v. administration in rabbits and monkeys. One way ANOVA (Newman-Keuls multiple comparison test) at 95% confidence interval was performed to compare AUCs of PQ in rabbits and monkeys after oral as well as i.v. administration. The value of $P > 0.05$ was considered as the criteria for no significant difference.

3.1.6 Results and Discussion

Oral and i.v. Study in Rats

The mean ± SEM plasma concentration-time profile and PK parameters of BQ and PQ following a single 20 mg/kg oral dose is given in Figure 3.1.1 and Table 3.1.1, respectively. The formulation used for the studies was checked for its BQ content and was found stable upto 4 h with <1% conversion into PQ. It is observed that after oral dosing of BQ, its absorption was rapid with C_{max} reaching in 5 min and

BQ could be monitored upto 6 h. The C_{max} of 399±53 ng/ml at as early as 5 min indicate that major distribution is yet to take place. A secondary peak appeared at 2 h, which may be due to enterohepatic recirculation, storage and subsequent release from post absorptive depot site [9-15]. The irregular concentration-time profiles of BQ with absence of absorption phase could not be explained with any standard one- or two-compartmental models as coefficient of variation and residuals were high for the estimated PK parameters. Hence, non-compartmental approach was used for the analysis of concentration-time data and estimation of PK parameters. Total AUC and elimination $t_{1/2}$ were 194 ng.h/ml and 1.08 h, respectively. The V_d was 5.05 L/kg, which is larger than the total body fluids in rats and indicates wide distribution of the drug [16].

PQ, the major metabolite of BQ showed multiple C_{max} and could be detected upto 48 h post BQ dose exhibiting a longer residence of PQ in the body than BQ. The PQ exhibited an irregular concentration-time profile; which may be due to conversion of BQ to PQ at low pH in different segments of GIT, or metabolism by liver or gastric mucosa [17]. The AUC was 4376 ng.h/ml with AUC_{PQ}/AUC_{BQ} ratio of 22.56 indicating substantial conversion of BQ to PQ (Table 3.1.2).

The concentration-time profile and PK parameters of BQ and PQ following i.v. administration (5 mg/kg) are shown in Figure 3.1.2 and Table 3.1.1, respectively. Levels of BQ declined till 1.5 h and suddenly increased to give a C_{max} of 292.35 ± 15.52 ng/ml at 2.0 h, which indicates a possibility of enterohepatic recycling. The BQ concentration-time profile could not be fitted to one or two compartment model due to unconventional i.v. profile with a secondary maximum and therefore non-compartmental analysis was used to fit the data. Elimination $t_{1/2}$, Cl and V_d were 1.04 h, 3.20 L/h/kg and 4.8 L/kg, respectively. The systemic bioavailability was 3.2 %. Following i.v. administration, PQ appeared in the systemic circulation from first sample point onwards and persisted till 8 h. It exhibited a discontinuous concentration-time profile with AUC_{PQ}/AUC_{BQ} ratio of 0.31 indicating lower conversion of BQ into PQ after i.v. administration (Tables 3.1.1 and 3.1.2). The formation of PQ after i.v. administration indicates a possible role of RBCs in the metabolism of BQ in addition to liver. It is evident by the i.v. like profile of PQ that the rate of metabolism is very fast with maximum conversion within 15 min following which there was decrease in concentration of PQ (Table 3.1.2).

Oral and i.v. study in Rabbits

The mean ± SEM plasma concentration-time profile and PK parameters of BQ and PQ following a single 10 mg/kg oral dose is given in Figure 3.1.3 and Table 3.1.1. It is observed that BQ is rapidly absorbed with C_{max} reaching in 5 min without distinct absorption phase and exhibited i.v. like profile for both BQ and PQ. Therefore, the data was subjected to non-compartmental analysis to determine the PK parameters (Table 3.1.1). Elimination $t_{1/2}$ was 1.24 ± 0.03 h and was comparable to the value observed in rats (Table 3.1.1). The Cl and V_d were 1.2 ± 0.06 L/h/kg and 2.1 ± 0.15 L/kg, respectively. These values were lower than those observed in rats. The V_d is higher than the total body water in rabbits indicating wide distribution of BQ [16]. Appearance of PQ started from 5 min and could be detected upto 8 h. The PQ also exhibited i.v. like profile with lack of absorption phase and indicates conversion of BQ to PQ at stomach pH or its metabolism by liver or gastric flora. The ratio (AUC_{PQ}/AUC_{BQ}) was 1.4 and was lower than in rats indicating lower conversion of BQ to PQ in rabbits than in rats (Tables 3.1.2 and 3.1.2).

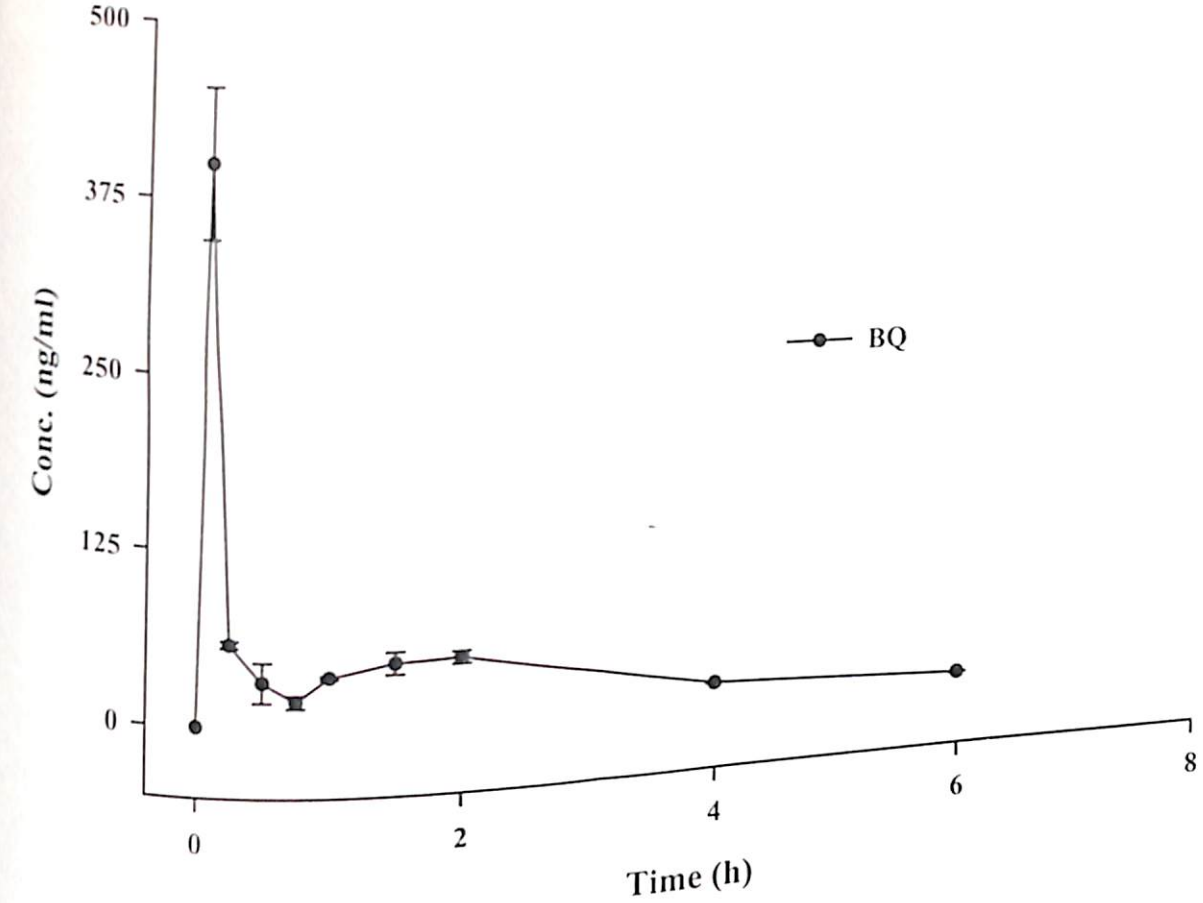
Following 2.5 mg/kg i.v. administration, the concentration-time profile of BQ and PQ are shown in Figure 3.1.4. The data were subjected to non-compartmental analysis to determine the PK parameters and are listed in Table 3.1.1. BQ and PQ exhibited a biphasic decline and the profile was best fitted to a two-compartment model. The PK parameters estimated using both the approaches were comparable and thus model independent approach was used for interspecies comparison (Table 3.1.1). In rabbits, elimination $t_{1/2}$ (1.14 ± 0.03 h), was similar to that observed in rats while V_d (1.82 ± 0.06 L/kg) and Cl (1.1 ± 0.03 L/h/kg) were lower. The value of V_d indicate wide distribution of BQ. The absolute bioavailability of BQ was 5.35 ± 0.003 %. Appearance of PQ started from 5 min and could be detected upto 12 h. The AUC_{PQ}/AUC_{BQ} ratio was 0.08 indicating that the extent of conversion of BQ to PQ was more after oral than after i.v. administration (Table 3.1.2). The formation of PQ after i.v administration indicates a possible role of RBCs in the metabolism of BQ.

Oral and i.v. Study in Monkeys

Following single 10 mg/kg oral dose, the mean ± SEM concentration-time profile and PK parameters of BQ and PQ are given in Figure 3.1.5 and Table 3.1.1, respectively. BQ exhibited a double peak phenomenon with C_{max} (159 ± 24 and 183 ± 46 ng/ml) at 0.25 and 2 h, respectively. BQ was monitored upto 24 h while PQ

could be detected upto 48 h post dose. The irregular concentration-time profiles of BQ and PQ could not be fitted with any standard one- or two-compartmental models. Hence, a non-compartmental approach was used for data analysis. The drug showed elimination $t_{1/2}$ of 3.7 ± 0.1 h which was higher than those observed in rats and rabbits after oral and i.v. administration (Table 3.1.1). The V_d (6.3 ± 0.44 L/kg) which is higher than the total body water in monkeys indicates its wide distribution [16]. Its major metabolite PQ showed C_{max} (383 ± 132 ng/ml) at 12 h after oral administration of BQ. The ratio AUC_{PQ}/AUC_{BQ} indicating conversion of BQ into PQ was 8.81. The value is higher than that in rabbits but lower than that in rats.

The concentration-time profile and PK parameters of BQ and PQ after 10 mg/kg i.v. administration are shown in Figure 3.1.6 and Table 3.1.1 respectively. BQ was monitored upto 18 h and showed a biphasic decline and the data could be best described by two-compartment open model with minimum residuals. But, the data was subjected to non-compartmental analysis to compare the species similarities/difference and are presented in Table 3.1.1. The absolute bioavailability of BQ was 12.0 ± 0.004%. The elimination $t_{1/2}$ and Cl were 1.041 ± 0.09 h and 1.17 ± 0.1 L/h/kg. V_d was 1.74 ± 0.27 L/kg, which is higher than total body fluid (0.6 L/kg) in monkeys indicating wide distribution of drug [16]. The elimination $t_{1/2}$ after i.v. administration was less as compared to oral causing lower V_d after i.v. administration. Its major metabolite PQ was determined upto 24 h after 10 mg/kg i.v. administration of BQ (Figure 3.1.6). The AUC_{PQ}/AUC_{BQ} ratio was 0.6 which is lower than that after oral dose indicating lesser conversion of BQ to PQ after i.v. administration. Also, the conversion of drug to its metabolite was higher in monkeys than that observed in rats and rabbits after i.v. administration (Table 3.1.2).



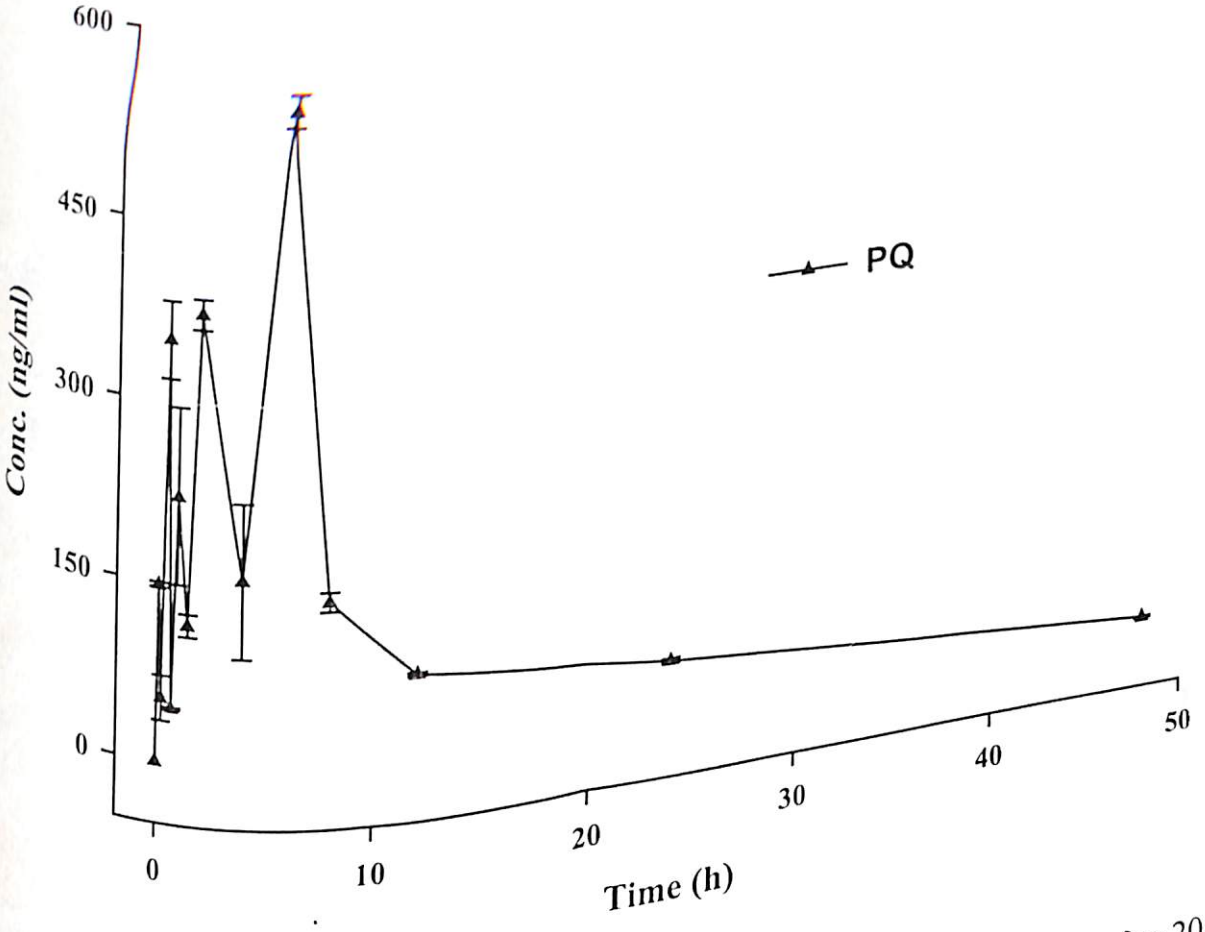


Figure 3.1.1 Mean ± SEM plasma concentration-time profile of BQ and PQ after 20 mg/kg oral dose of BQ in rats (n=3)

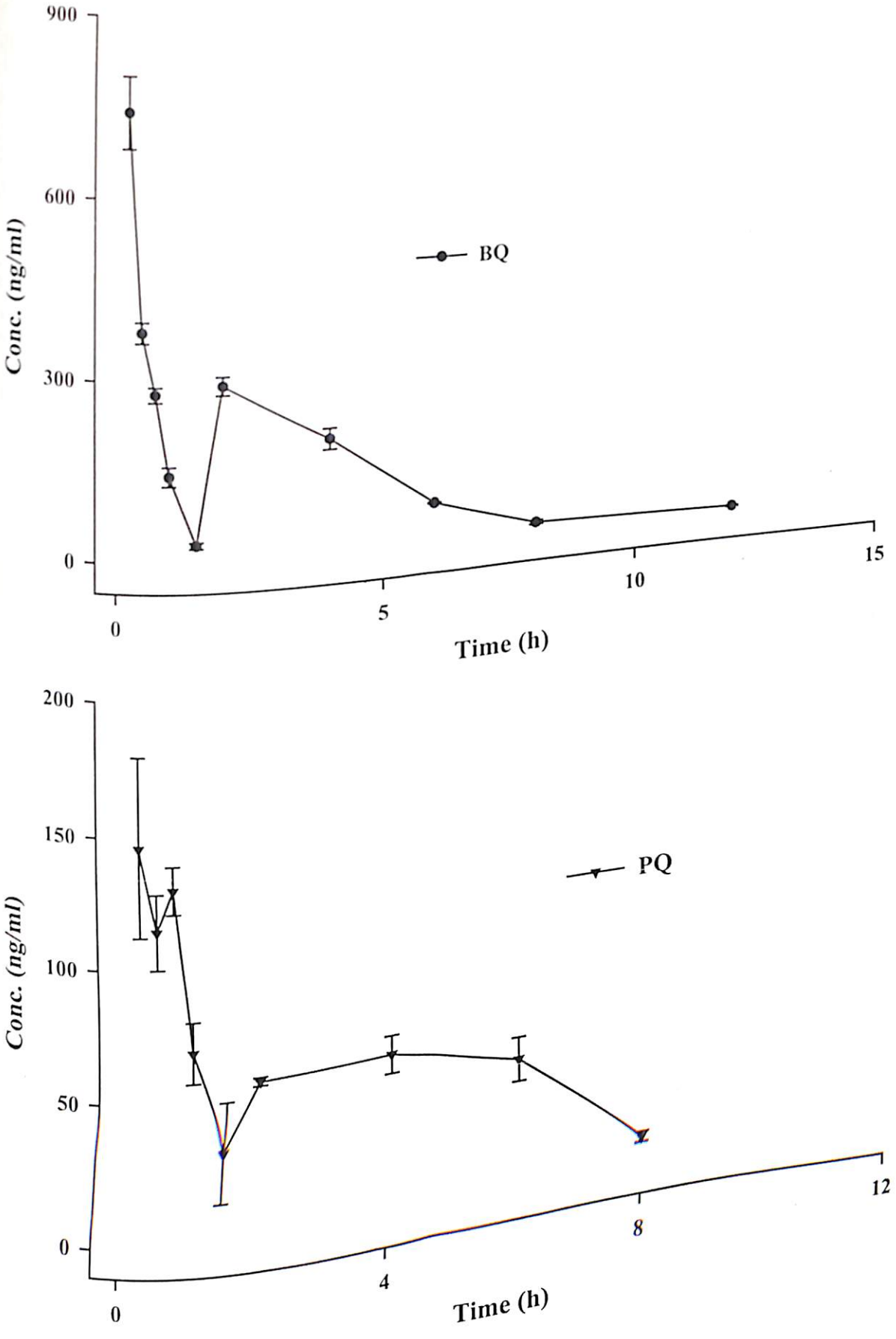


Figure 3.1.2 Mean ± SEM plasma concentration-time profile of BQ and PQ following 5 mg/kg i.v. dose of BQ in rats (n=3)

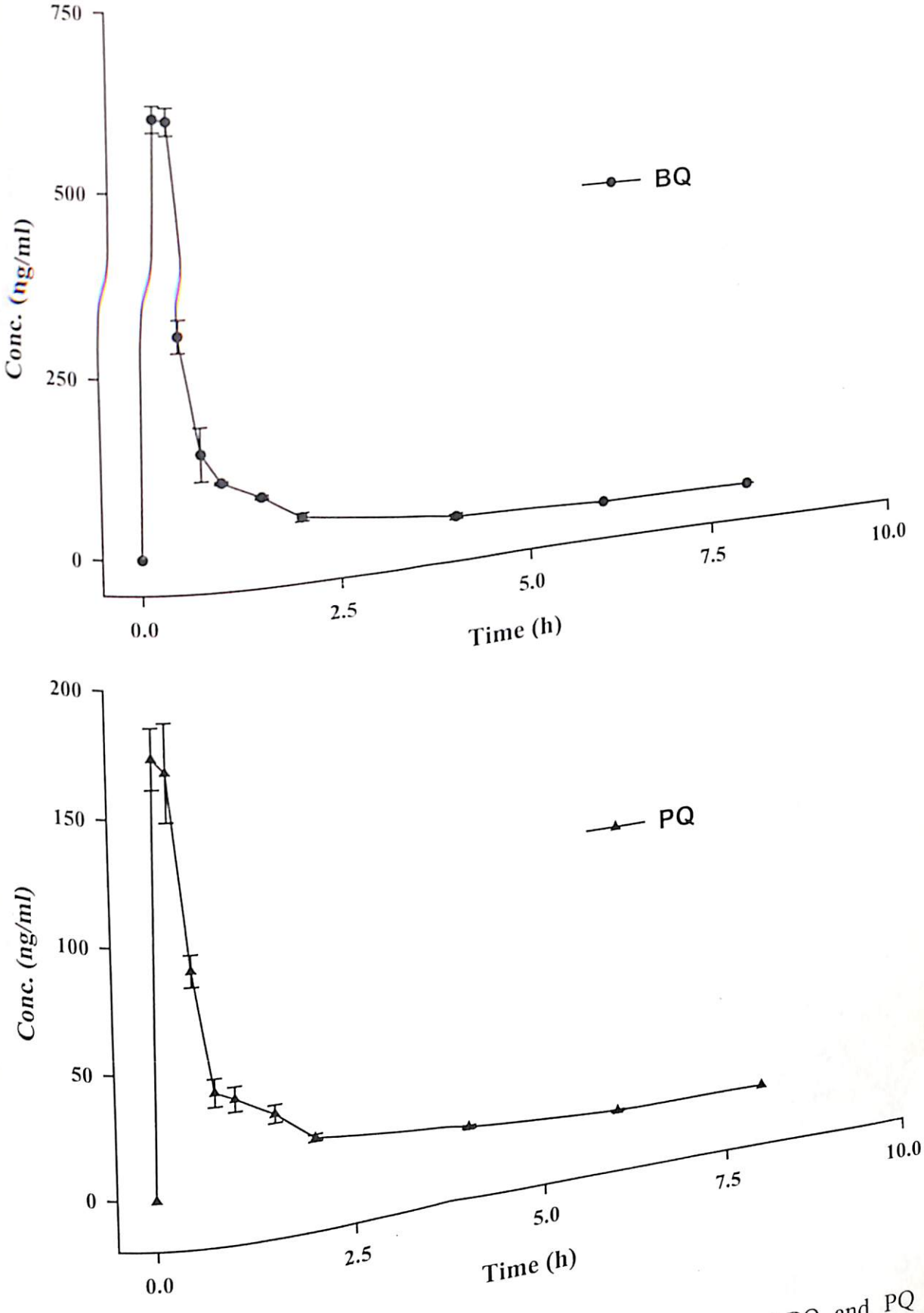
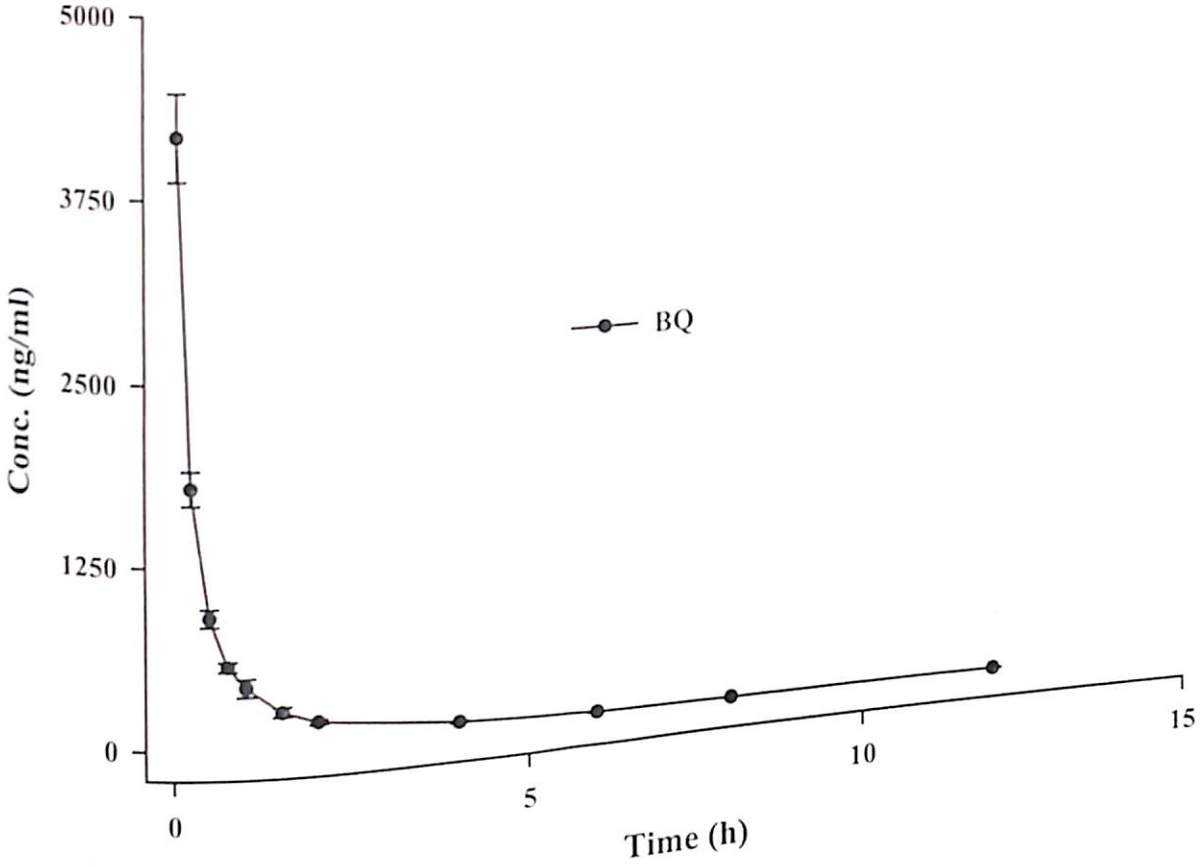


Figure 3.1.3 Mean ± SEM plasma concentration-time profile of BQ and PQ following 10 mg/kg oral dose of BQ in rabbits (n=3)



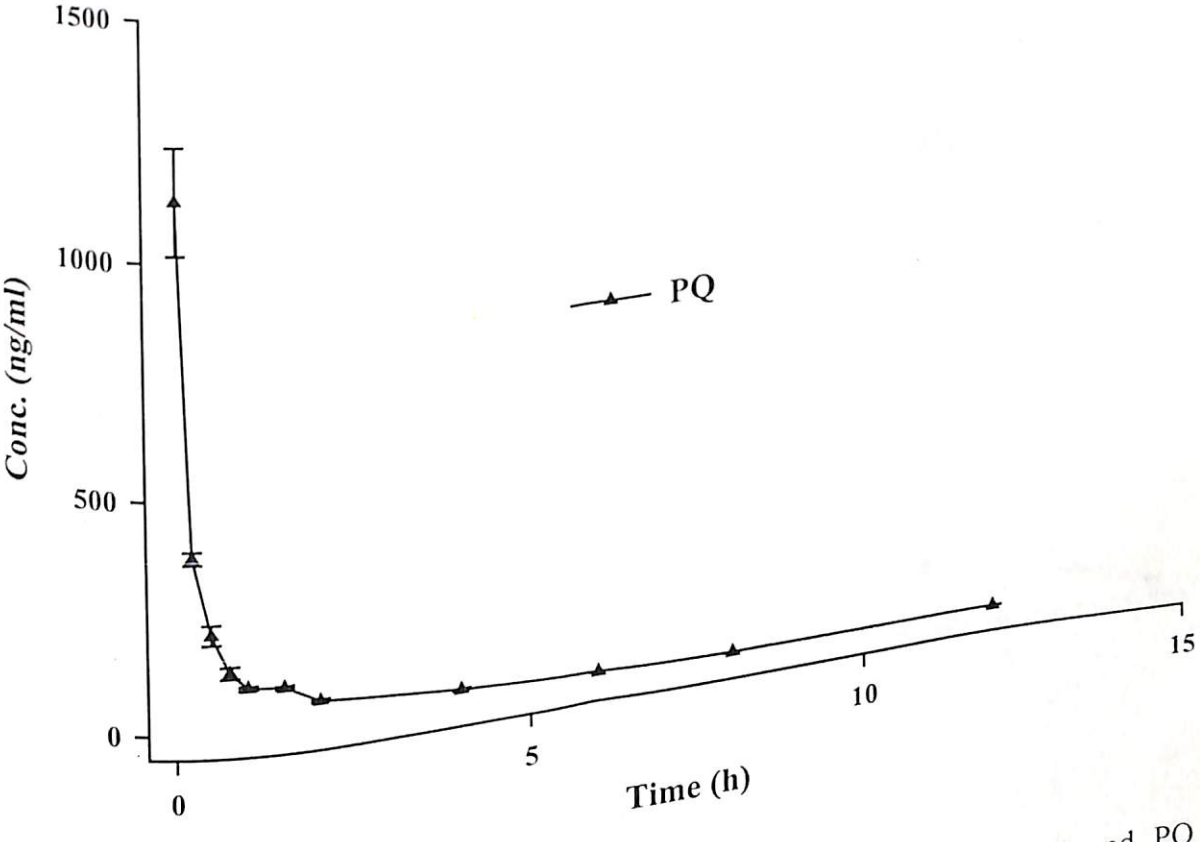
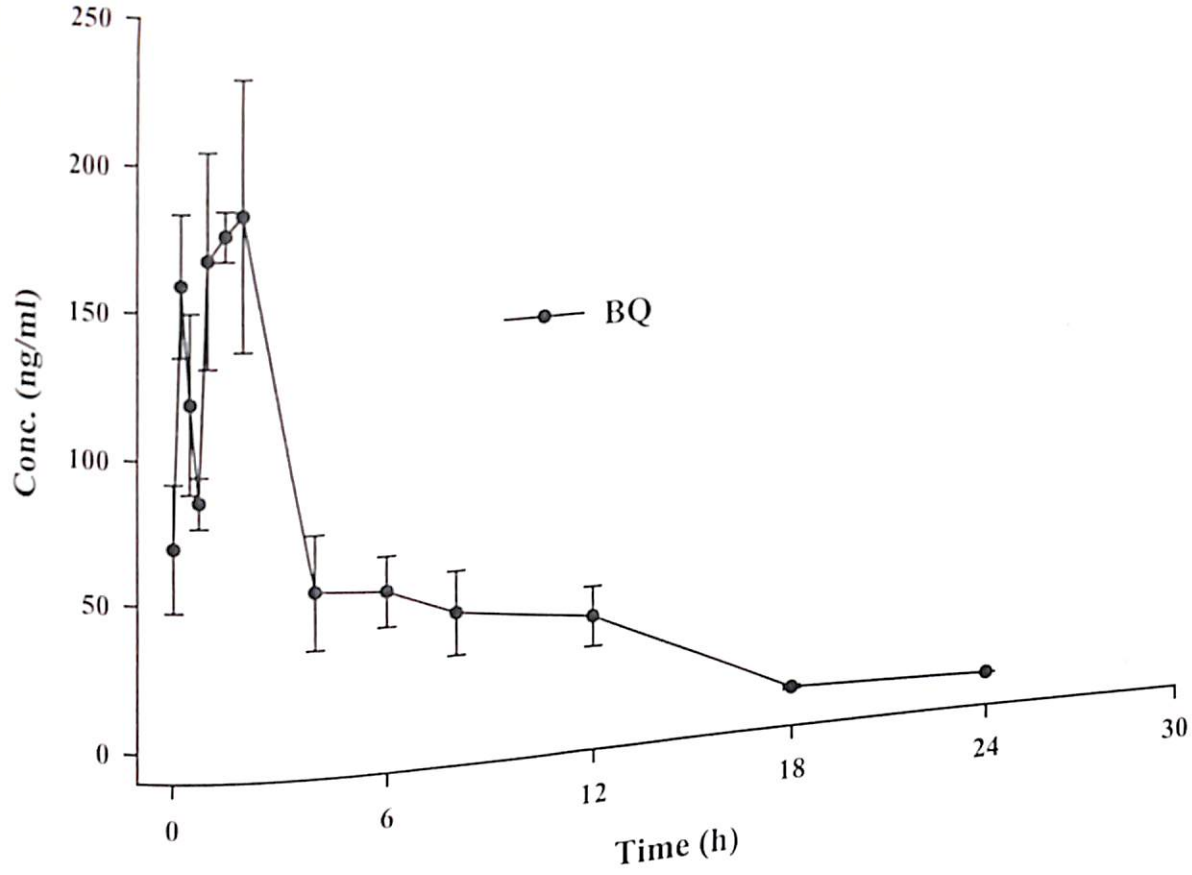


Figure 3.1.4 Mean ± SEM plasma concentration-time profile of BQ and PQ following 2.5 mg/kg i.v. dose of BQ in rabbits (n=3)



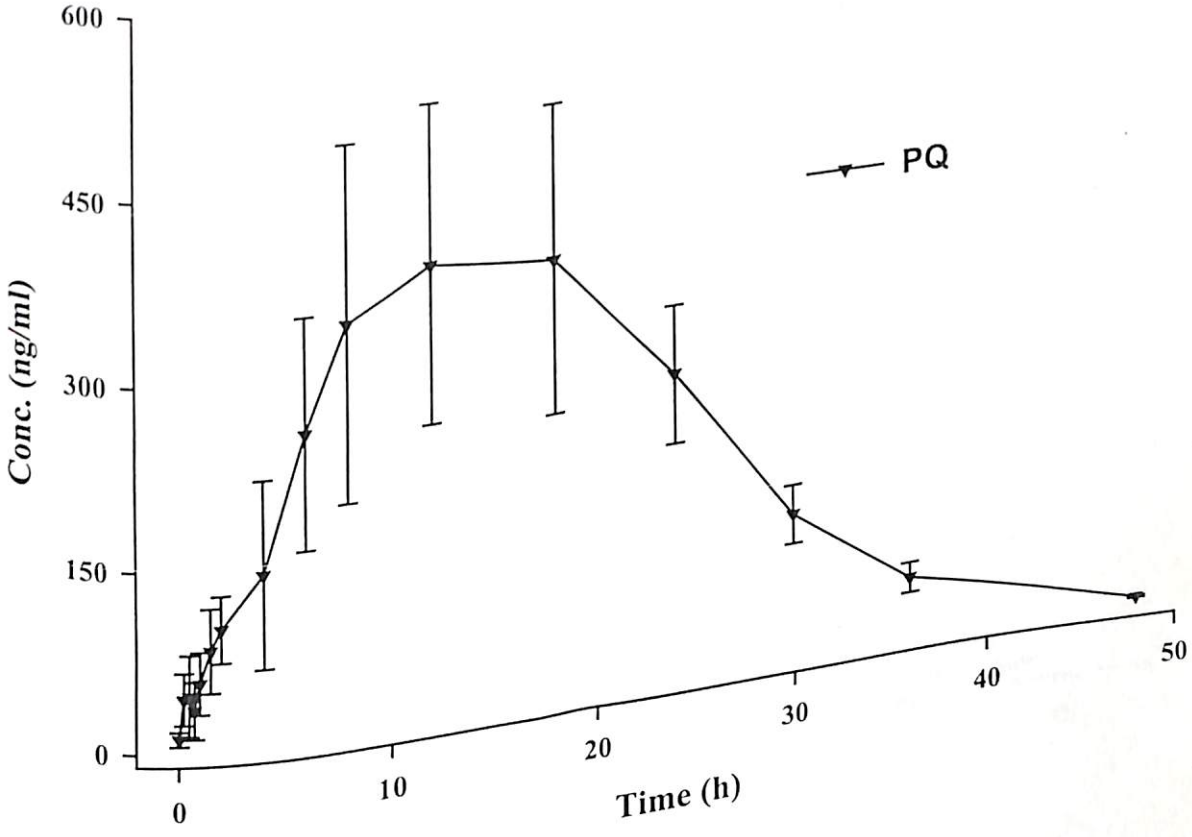
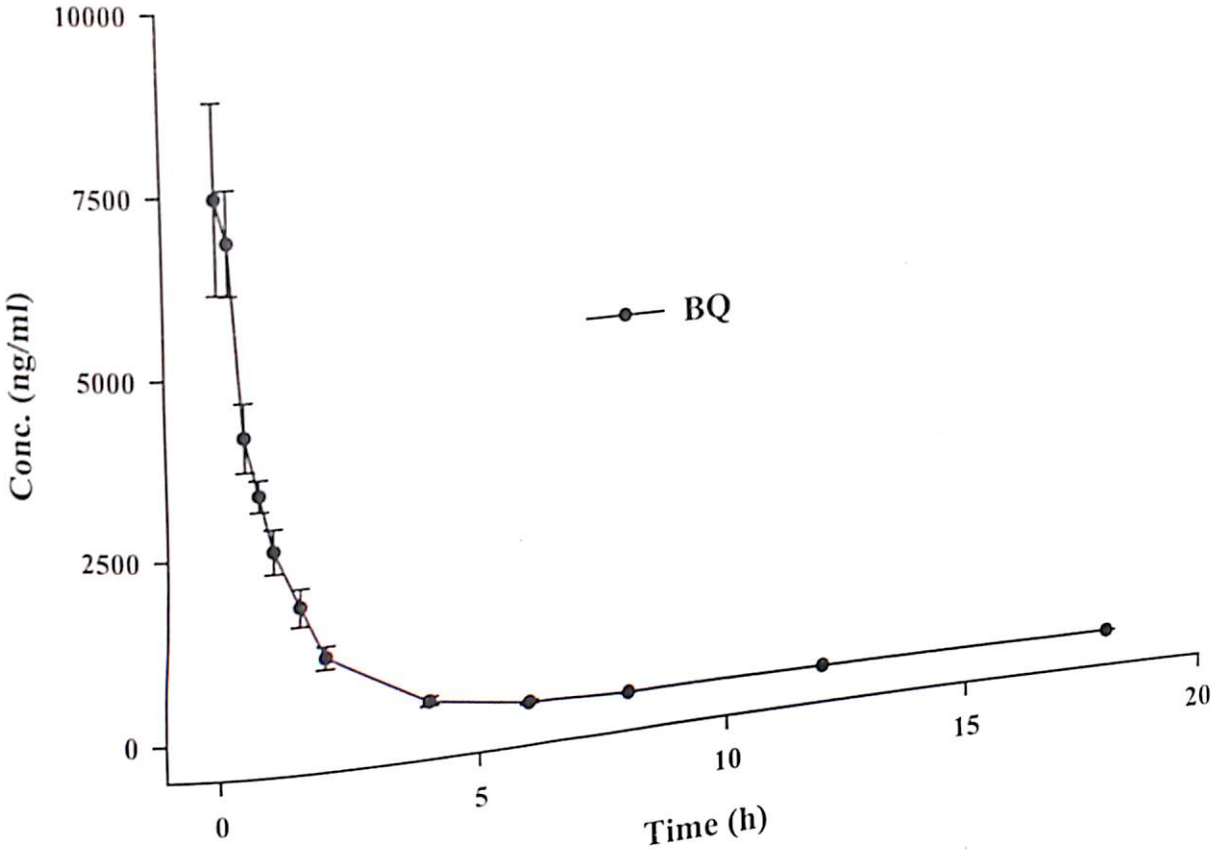


Figure 3.1.5 Mean ± SEM plasma concentration-time profile of BQ and PQ following 10 mg/kg oral dose of BQ in monkeys (n=3)



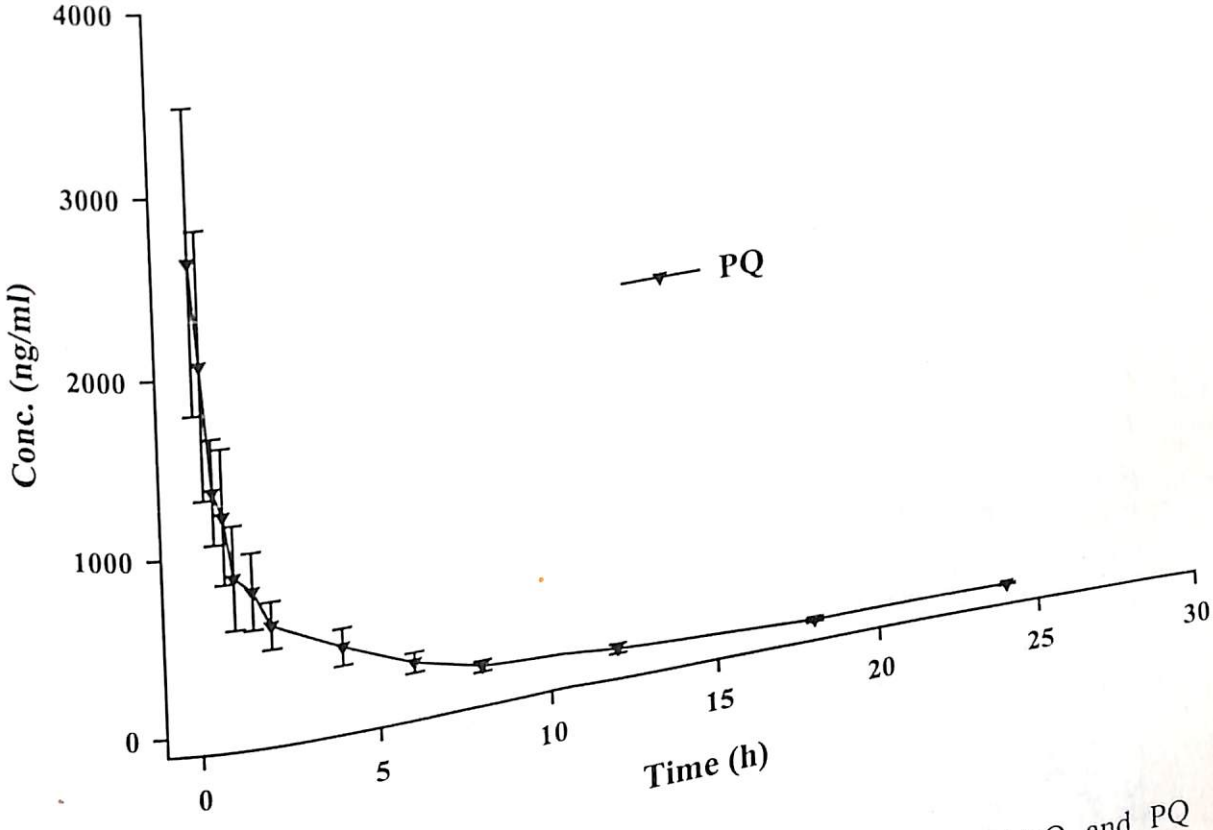


Figure 3.1.6 Mean ± SEM plasma concentration-time profile of BQ and PQ following 10 mg/kg i.v. dose of BQ in monkeys (n=3)

Table 3.1.1 Comparative PK parameters (Mean ± SEM) of BQ and PQ after oral and i.v administration in various species by non-compartmental analysis

Species		Rats (n=3)				Rabbits (n=3)				Monkeys (n=3)			
Parameters		BQ		PQ		BQ		PQ		BQ		PQ	
		Oral	i.v.	Oral	i.v.	Oral	i.v.	Oral	i.v.	Oral	i.v.	Oral	i.v.
C_{max} (ng/ml)	1	399 ± 53	-	Multiple C_{max}	-	603 ± 18	-	174 ± 12	-	159 ± 24	-	383 ± 132	-
	2	46 ± 7.7	-		-	-	-	-	-	183 ± 46	-		-
T_{max} (h)	1	5 min	-	Multiple T_{max}	-	5 min	-	5 min	-	0.25	-		-
	2	2	-		-			-	-	2	-		-
Elimination $t_{1/2}$ (h)		1.08	1.04	-	-	1.24 ± 0.03	1.14 ± 0.03	-	-	3.7 ± 0.1	1.01 ± 0.09		-
AUC (ng h ml^{-1})		194	1554	4376	477	484 ± 25	2270 ± 76	678 ± 28	186 ± 12	1032 ± 67	8603 ± 698	9091 ± 2595	5160 ± 1426
V_d (L/kg)		5.05	4.8	-	-	2.1 ± 0.15	1.82 ± 0.06	-	-	6.3 ± 0.44	1.74 ± 0.27	-	-
Cl (L/h/kg)		3.2	3.2	-	-	1.2 ± 0.06	1.1 ± 0.03	-	-	1.2 ± 0.1	1.17 ± 0.1	-	-
% Absolute Bioavailability of BQ		3.12				5.35 ± 0.003				12.0 ± 0.004			

Table 3.1.2 Extent of conversion of BQ to PQ after oral and i.v. administration in various species

Species	Rat		Rabbit		Monkey	
Route of Administration	Oral	i.v.	Oral	i.v.	Oral	i.v.
AUC_{PQ}/AUC_{BQ}	22.56	0.31	1.4	0.08	8.81	0.60

Comparison of Rat, Rabbit and Monkeys Pharmacokinetics

The statistical comparison of BQ between C_{max} 1 of rats, rabbits and monkeys showed significant difference ($P< 0.05$). The C_{max} of PQ could not be compared due to occurrence of multiple C_{max} in rats. Elimination $t_{1/2}$, AUC and V_d of BQ after oral administration between rabbits and monkeys were found to be significantly different ($P< 0.05$) while there was no significant difference in Cl ($P> 0.05$). The difference between elimination $t_{1/2}$, V_d and Cl of BQ between rabbits and monkeys following i.v administration were insignificant ($P> 0.05$) whereas the AUCs of BQ showed significant difference. The AUCs for PQ between rabbits and monkeys after oral as well as i.v. administration were found to be significantly different ($P< 0.05$). Thus, present pharmacokinetic studies of BQ demonstrate species similarities and differences in rats, rabbit and monkeys.

The absorption of BQ was rapid in rats and rabbits without clear-cut absorption phase whereas monkeys showed double peak phenomenon. This indicates that distribution is still going on in rats and rabbits when C_{max} are attained. One of the factors for rapid absorption may be PG in the formulation, which is a known bioenhancer [18]. The elimination half-life in rats and rabbits were comparable after oral and i.v administration whereas it was higher in monkeys. The Cl in rats was high while it was moderate in rabbits and monkeys, which is expected as the rats being lower animals will clear drug more rapidly than other higher animals on a weight/surface area normalized basis. The conversion is maximum in rats and minimum in rabbits, which may be due to the difference in the constitution of various forms of Cyt P450 enzymes/ gastric pH conditions in various species [19-21]. Possibly, low oral bioavailability of BQ is due to degradation to PQ under acidic pH conditions or precipitation in the GIT, poor permeability or extensive binding [22]. However, BQ to PQ conversion post i.v. administration was lower than that after oral

dosing. Following i.v. administration, the extent of BQ to PQ conversion was in the order of monkey> rat> rabbit. The residence time of PQ was longer than BQ in all the species. It is likely that PQ being the major and active metabolite of BQ is responsible for extended therapeutic effect after BQ administration.

3.1.7 Conclusions

The oral PK of BQ in rats, rabbits and monkeys indicated that, BQ was rapidly absorbed into systemic circulation in all the species. The rapid absorption of BQ may not be true character of the drug as the components of the formulation, alcohol and PG, are known absorption enhancers and may be responsible for the rapid absorption of BQ. All the species showed high V_d and low bioavailability for BQ. The Cl was higher only in case of rats. There is an increase in bioavailability as we move from lower to higher species. BQ is extensively converted to its major metabolite PQ after oral administration in all the species. The conversion was maximum in rats and minimum in rabbits after oral dose while monkeys showed maximum conversion of BQ to PQ after i.v. administration. The residence time of PQ was longer than BQ in rats and monkeys that is probably responsible for extended therapeutic efficacy after BQ administration.

SECTION 2

Excretion Studies in Rats

3.2.1 Introduction

Elimination of xenobiotics occurs by metabolism and excretion. Some drugs are excreted via the biliary route, while others particularly volatile substances, are excreted in the breath. For most drugs, however, excretion occurs predominantly via the kidneys. Metabolism is a major mechanism for elimination of drugs from the the body. Some drugs are eliminated almost entirely unchanged by the kidneys, but these drugs are relatively few. The most common routes of drug metabolism are oxidation, reduction, hydrolysis, and conjugation. Frequently, a drug simultaneously undergoes metabolism by several competitive pathways. The fraction going to each metabolite depends on the relative rates of each of the parallel pathways. Metabolites may undergo further sequential metabolism, e.g., oxidation, reduction, hydrolysis often followed by conjugation reaction.

Excretion studies provide important information regarding the elimination of the drug as unchanged molecule or as its metabolite(s). Compounds that are metabolized in the liver are often excreted via bile into the intestinal tract [23, 24], where they can be reabsorbed into the circulation (enterohepatic recycling) or can be excreted via the feces. The rate and extent of biliary elimination depends on molecular weight, chemical structure, polarity, species, sex and nature of metabolic process [25].

Although excretion can take place through numerous pathways, but the most significant organ for elimination is the kidney [26]. Molecules comprising of small molecular weight (< 2000 g/mole) undergo filtration at the glomerulus. Lipophilic compounds in the glomerulus filtrate normally do not appear in abundance in the urine. Lipophilic molecules tend to be extensively reabsorbed by a passive process. Quantitative collection and analysis of urine, bile, and feces at designate intervals post dose can provide useful information on the rate and extent of excretion of drug and /or metabolites via renal, biliary, or fecal pathways. These studies gain more significance in medical conditions, which demand administration of drugs for longer periods of time during which accumulation of drug and its metabolites may be observed. In the present study, excretion of BQ and PQ were performed in rats after a single 20 mg/kg oral dose of BQ.

3.2.2 Experimental

3.2.2.1 Materials

Chemicals and solvents were same as described in section 3.1.2.1.1. Drug-free rat feces and urine was obtained from healthy male *Sprague Dawley* rats provided by Laboratory Animal Services Division of the institute.

3.2.2.2 Animals

The excretion studies of BQ were carried out in young and healthy male *Sprague Dawley* rats (n=3) weighing 250±25g. The animals were obtained from Laboratory Animal Services Division of CDRI, and housed in the metabolic cages where it was possible to collect urine and feces simultaneously. A regular 12 h day-night cycle was executed with standard pelleted laboratory and water provided *ad libitum*. The rats were acclimatized to this environment for at least 2 days before the commencement of experiment. The dose was administered to rats after an overnight fast (12-16 h). All experiments, euthanasia and disposal of carcass were executed in accordance with the guidelines by local ethical committee (Reg. No. 34/1999/CPCSEA) of the institute for animal experiments.

3.2.2.3 Buffer solution

The buffer and its preparation was same as described in section 3.1.2.1.3.

3.2.2.4 Drug Formulation

Drug formulation and its preparation were same as described in section 3.1.2.1.4

3.2.2.5 Dosing

Rats were dosed orally at 20 mg/kg by oral gavage using oral feeding needle such that each 250 g rat received 1.0 ml of the formulation.

3.2.2.6 Sampling Schedule

Urine and feces samples were collected at 0-6, 6-12, 12-24, 24-36, 36-48 and 48-60 h post dose. Volume of urine samples was measured, centrifuged at 2000 rpm for 10 min at 4°C to remove the debris and clear supernatant was stored at -60°C

pending analysis. Feces samples were allowed to dry at 37°C, weighed, powdered and stored in desiccator until analysis.

3.2.2.7 Sample analysis

Urine and feces samples of rat were assayed for estimation of BQ and PQ using method H (section 2.4.8) and J (section 2.4.9), respectively.

3.2.2.8 Data Treatment

The concentrations of BQ and PQ were read from the calibration standards drawn in the same matrix. Excretion of BQ through urine and feces were expressed in terms of percent of administered dose. Cumulative amount of PQ excreted was calculated and expressed as µg/rat.

3.2.3 Results and Discussion

The amount of BQ and PQ excreted in rats (urine and feces) at different time intervals is given in Table 3.2.1 and 3.2.2 respectively.

Table 3.2.1 Cumulative amount of BQ excreted in rat urine post 20 mg/kg oral dose of BQ

Duration (h)	Amount (µg) excreted		
	Rat 1	Rat 2	Rat 3
Urine			
0-6	0.011	0.021	0.0072
6-12	0.015	0.011	0.0148
12-24	BLOQ	0.007	0.016
24-36	BLOQ	0.009	BLOQ
36-48	BLOQ	BLOQ	BLOQ
48-60	BLOQ	BLOQ	0.038
0-60	0.026	0.048	
Cumulative amount (µg) Mean ± SEM: 0.037 ± 0.006			

Table 3.2.2 Cumulative amount of PQ excreted in rat urine and feces post 20 mg/kg oral dose of BQ

Duration (h)	Amount (µg) excreted		
	Rat 1	Rat 2	Rat 3
Urine			
0-6	18.45	9.55	8.11
6-12	3.18	2.30	9.41
12-24	0.035	1.1	1.03
24-36	0.043	1.0	0.20
36-48	0.006	0.53	
48-60	BLOQ	0.50	0.09
0-60	21.71	15.0	18.84
Cumulative amount (µg) Mean ± SEM: 18.50 ± 1.93			
Feces			
0-6	1.85	1.04	3.79
6-12	1.67	1.1	BLOQ
12-24	0.60	0.97	BLOQ
24-36	BLOQ	0.36	BLOQ
36-48	BLOQ	3.48	BLOQ
48-60	**4.12**	**3.47**	**3.79**
0-60			
Cumulative amount (µg) Mean ± SEM: 3.8 ± 0.17			
BLOQ- Below limit of quantitation			

BQ was excreted in insignificant amount (0.00074 % of administered dose) in urine while it was not detected in feces. The excretion of PQ was higher in urine than in feces. The cumulative amount of unchanged PQ excreted in urine and feces was 0.37 and 0.076% respectively of the administered dose of BQ. Therefore, excretion of BQ in urine was mainly in form of PQ. It was expected as BQ is converted to PQ (polar metabolite) after oral dosing in rats.

3.2.4 Conclusions

The extent of excretion of unchanged BQ and PQ was studied in male *Sprague Dawley* rats following a single 20 mg/kg oral dose of BQ showed that it was not detected in feces while it was present in very low amounts in urine (0.0074% of the administered dose). BQ was eliminated mainly as its metabolite PQ that accounts for 0.446% of the administered dose of BQ. The negligible renal Cl of BQ indicates the role of organs other than kidney in its elimination.

References

1. Rowland, M., 1972. Influence of route of administration on drug availability, J. *Pharm. Sci.*, 1: 70-74.
2. Vincent, J.W., Jefferey, A.S., Chang, Y.C. and Leslie, Z.B., 1998. Role of P-glycoprotein and cytochrome P-450 3A in limiting oral absorption of peptides and peptidominetics, *J. Pharm. Sci.*, 87: 1322-1330.
3. Harris, P.A. and Riegelman, S., 1969. Influence of the route of administration on the area under the plasma-concentration-time curve, J. *Pharm. Sci.*, 58: 71.
4. Gibaldi, M., Boyes, R.N. and Feldman, S., 1971. Influence of the first pass effect on the availability of drugs on oral administration, *J. Pharm. Sci.*, 60: 1338-1340.
5. Lippi, A., Crieuoli, M., Guelfi, M., Santicioli, M. and Maggi, C.I, 1998. Pharmacokinetics of bicyclic peptide tachykinin NK2 receptor antagonist MEN 11420 (Nepadutant) in rats, *Drug. Metab. Dispos.*, 26: 1077-1081.
6. Hasegawa, T., Saneyioshi, M. and Kawaguchi, T., 1996. Intestinal absorption and first pass metabolism of 2, 3,-dideoxynucleotides following oral administration in rats, *Bio. Pharm. Bull.*, 19: 599-603.
7. Schwarz, G., 1978. Estimating the dimension of a model, *Ann.Stat.*, 6: 461-464.
8. Yamaoka, K., Nakagewa, T. and Uno, T., 1978. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations, *J. Pharmcokin. Biopharm.*, 6: 165-175.
9. Pederson, P.V., 1981. Pharmacokinetic analysis by linear systems approach. I. Cimetidine bioavailability and second peak phenomenon, *J. Pharm. Sci.*, 70: 32-38.
10. Suverkrup, R., 1979. Discontinuous absorption process in pharmacokinetic models, *J. Pharm. Sci.*, 68: 1395-1400.
11. Walkenstein, S.S., 1978. Bioavailability of cimetidine in man, *Gastroenterology*, 74:360-365.
12. Oberle, R.L., and Amidon, G.L., 1987. The influence of variable gastric emptying and intestinal transit rates on the plasma curve of cimetidine, an explanation for the double peak phenomenon, *J. Pharmacokin. Biopharm.*, 15: 529-545.

13. Garg, D.C., 1993. Ranitidine bioavailability and kinetics in normal male subjects, *Clin. Pharmacol. Therap.*, 33:445-452.

14. Bergstrom, R.F., Kay, D.R., Harkcom, T.M. and Wagner, J.G., 1981. Pencillamine kinetics in normal subjects. *Clin. Pharmacol. Therap.*, 30: 404-413.

15. Plusquelbece, Y., Campistron, G., Saveris, S., Barre, J. and Jung, J.P., 1987. A double peak phenomenon in the pharmacokinetics of veralipride after oral administration; A double site model for drug absorption, *J. Pharmacokin. Biopharm.* 15: 225-239.

16. Davies, B. and Morris, T., 1993. Physiological parameters in laboratory animals and humans, *Pharm. Res*, 10: 1093-1095.

17. Mummaneni, V. and Jennifer, B. D., 1994. Intestinal uptake of Cimetidine and ranitidine in rat, *Pharm. Res.*, 11: 1599-1603.

18. Gao, P., Guyton, M.E., Huang, T., Bauer, J.M., Stefanski, K.J. and Lu, Q., 2004, Enhanced oral bioavailability of a poorly water soluble drug PNU-91325 by supersaturable formulations, *Drug Dev. Ind. Pharm.*, 30: 221-229.

19. Lin, J.H., 1995. Species similarities and differences in pharmacokinetics, *Drug Metab. Dispos.*, 1008-1021.

20. Stevans, J.C., Shipley, L.A., Cashman, J.R., Vandenbranden, N. and Wroghton, S.A., 1993. Comparison of human and rhesus monkey *in vitro* phase I and phase II hepatic drug metabolic activities, *Drug Metab Dispos.*, 21: 753-760.

21. Zwart, L.L., Rompelberg, C.J.M, Sips, A.J.A.M, Welnik, J. and Engelen Van, J.G.M., 1999. RIVM report 623860010: Anatomical and physiological differences between various species used in studies on pharmacokinetic and toxicology ox xenobiotics, 1-100.

22. Lee, M.G. and Chiou, W.L., 1983. Evaluation for potential causes for the incomplete bioavailability of furosemide: Gastric first pass metabolism. *J. Pharmacokin. Biopharm.*, 11(6): 623-640.

23. Shand, D.G., Rangro, R.E. and Evans, G.H., 1972. The disposition of propranolol. II. Hepatic elimination in the rat, *Pharmacol.*, 8: 344-352.

24. Whitsett, T.L., Dayton, P.G. and McNay, J.L., 1971. The effect of hepatic blood flow on the hepatic removal rate of oxyphenbutazone in the dog, *J. Pharmacol. Exp. Therap.*, 177: 246-255.

25. Klassen, C.D. and Watkins III, J.B., 1984. Mechanisms of bile formation, hepatic uptake and biliary excretion, *Pharmacol. Rev.*, 36: 313-364.

26. Litterst, C.L., Minnaugh, E.G, Reagon, R.L. and Gram, T.E., 1975. Comparison of *in vitro* drug metabolism by lung, liver and kidney of several common laboratory species, *Drug Metab. Dispos.*, 3: 259-265.

CHAPTER 4

IN VITRO STUDIES

SECTION 1

Gastric Stability Studies

4.1.1 Introduction

Stability in gastric juice is of prime importance for the drug intended for oral administration. It is observed that many drugs have low bioavailability as they are degraded in stomach due to low pH (1-2). Simulated gastric fluid (SGF) mimics gastric fluid in terms of acidity and molarity and is a perfect media to determine the stability of the drug candidate *in vitro*. The stability of BQ needs to be evaluated *in vitro* to establish its stability profile in acidic conditions. In the present study, the stability of the drug was studied *in vitro* using SGF at a concentration likely to be present *in vivo*.

4.1.2 Experimental

4.1.2.1 Materials

Pure reference standard of BQ was obtained from the Pharmaceutics Division of CDRI. K_2HPO_4 and NaCl were procured from s.d. Fine Chemicals, Mumbai, India. PG, orthophosphoric acid and Conc. HCl were purchased from E-Merck India Ltd., Mumbai. Commercially available ethanol was further distilled in the laboratory. KCl was procured from Qualigens Ltd., Mumbai, India. Purified water was obtained from MilliQ system. A shaking water bath (Vam 908D, Vam Instruments Pvt. Ltd., India) operated at 37 °C and 50 rpm was used for incubation.

4.1.2.2 Buffer solution

K_2HPO_4 buffer solution (20 mM, pH 7.0) was prepared by dissolving 174.2 mg of K_2HPO_4 in 50 ml MilliQ water and adjusting the pH to 7.0 with 20% orthophosphoric acid.

4.1.2.3 Drug Formulation

Solution formulation of BQ (4 mg/ml) was prepared by dissolving 20 mg of BQ in 5 ml of PG, ethanol and K_2HPO_4 buffer in the ratio 35:30:35 %v/v respectively.

4.1.2.4 Preparation of Simulated Gastric Fluid (SGF)

NaCl (40 mM) and KCl (5 mM) were dissolved in MilliQ water and pH was adjusted to 1.2 with Concentrated HCl. The buffer was filtered through a 0.22 µ filter paper before use.

4.1.2.5 Incubation and sample collection

SGF (10.0 ml) in a 25 ml conical flask was pre-incubated in shaking water bath for 10-15 min at 37 ± 2°C. 250 µl BQ formulation (4 mg/ml) was spiked to pre-incubated SGF to produce a concentration of 0.1 mg/ml and immediately subjected to incubation. 100 µl of the incubation mixture was sampled at 0, 5 min, 0.25, 0.5, 1.0 and 2 h post incubation. The study was carried out for two more occasions at the same concentration of BQ.

4.1.2.6 Sample Analysis

The samples were analysed on the same day of collection by appropriate dilution using the validated HPLC-MS-MS method for simultaneous estimation of BQ and its major metabolite PQ as described in bioanalytical section. The incubation mixture left after 2.0 h was suitably diluted with ACN and then checked for any degradation products against blank using constant infusion (Harvard pump, Model 6) coupled to Micromass Quattro II triple quadrupole mass spectrometer. The solution was infused at 5µl/min using nitrogen as a nebulizing gas.

4.1.2.7 Data Treatment

The gastric stability of BQ was evaluated by measuring the disappearance of the compounds from the incubation mixture. The results of the study were expressed as the percentage of BQ remaining which was calculated using the following equation:

$$\% \text{ BQ remaining} = \frac{[\text{Concentration of BQ after incubation}]}{[\text{Concentration of BQ before incubation}]} \times 100$$

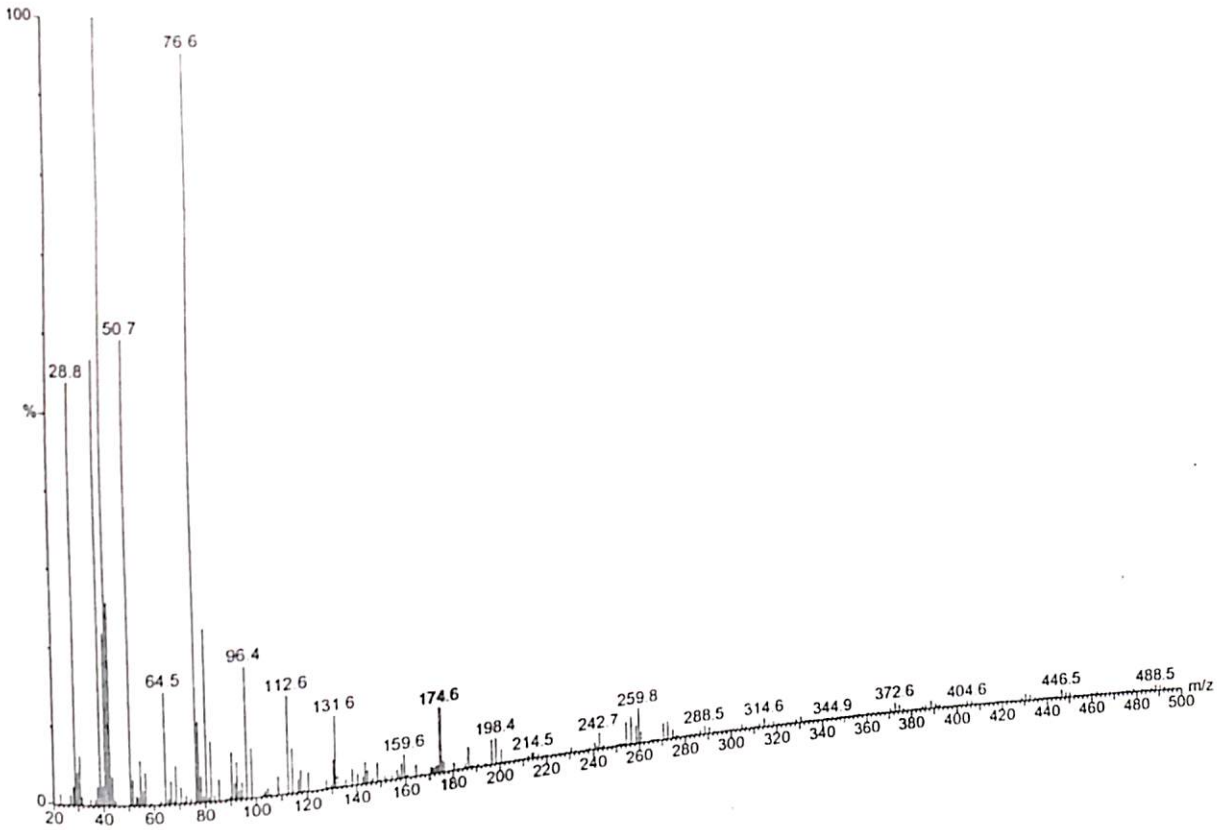
% BQ remaining was plotted against time to obtain K_d: $-K_d/2.303$ = slope, where K_d is the rate of degradation of BQ in SGF.

4.1.3 Results and Discussion

The concentration of BQ was taken to simulate a condition when 25 mg drug will be in contact with 250 ml of gastric fluid in healthy human subjects. The experiment was carried out in triplicate and it was found that BQ was highly unstable in acidic pH and more than 99% of the BQ is degraded as it comes in contact with the SGF. The rate of degradation was very spontaneous and thus a fall in the % BQ remaining versus time could not be obtained. Therefore, K_d could not be calculated.

ESI-LC-MS-MS analysis of the incubate showed that main degradation product was PQ (m/z 260), which accounted for almost 83% of the BQ spiked in SGF. Constant infusion (ESI-MS) of the incubation mixture exhibited the presence of three additional compounds (m/z 132, 175 and 243), which were not detected in the blank incubation medium. MS spectra of blank and spiked incubation mixture are given in Figure 4.1.1. The proposed sequential degradation pathway of BQ to give PQ followed by two other degradation products is given in Figure 4.1.2.

a)



b)

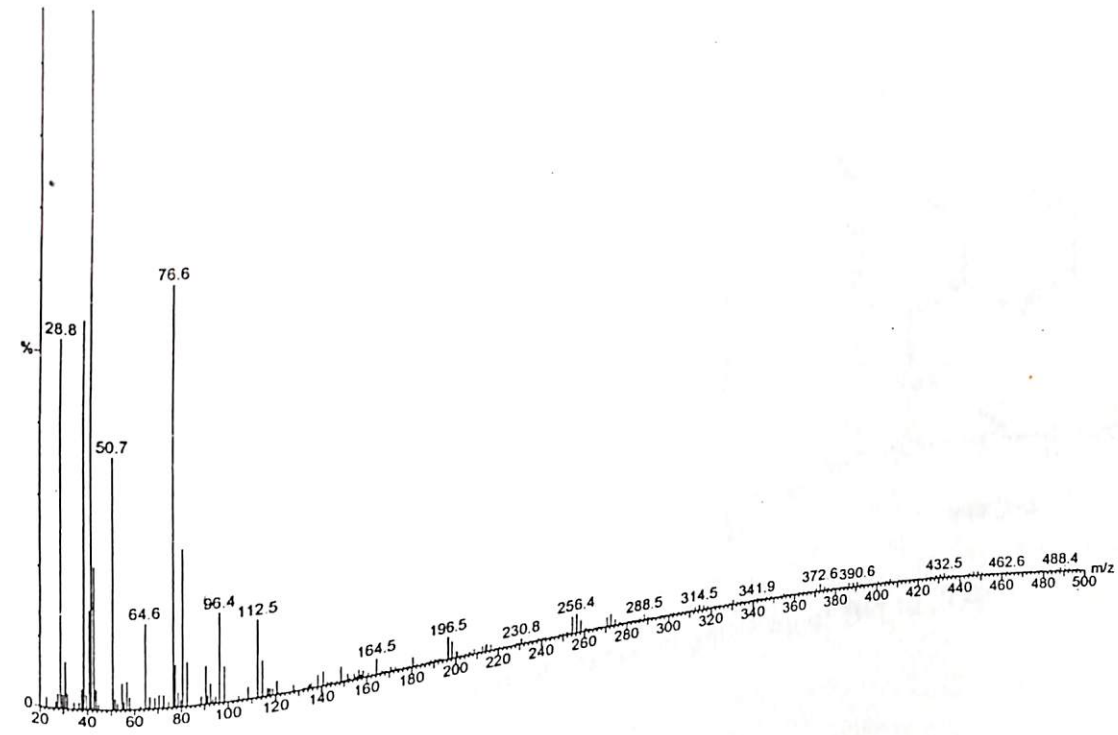


Figure 4.1.1 MS spectra (in positive ion mode) of (a) SGF containing 0.1 mg/ml BQ (b) drug free SGF, each 2.0 h after incubation

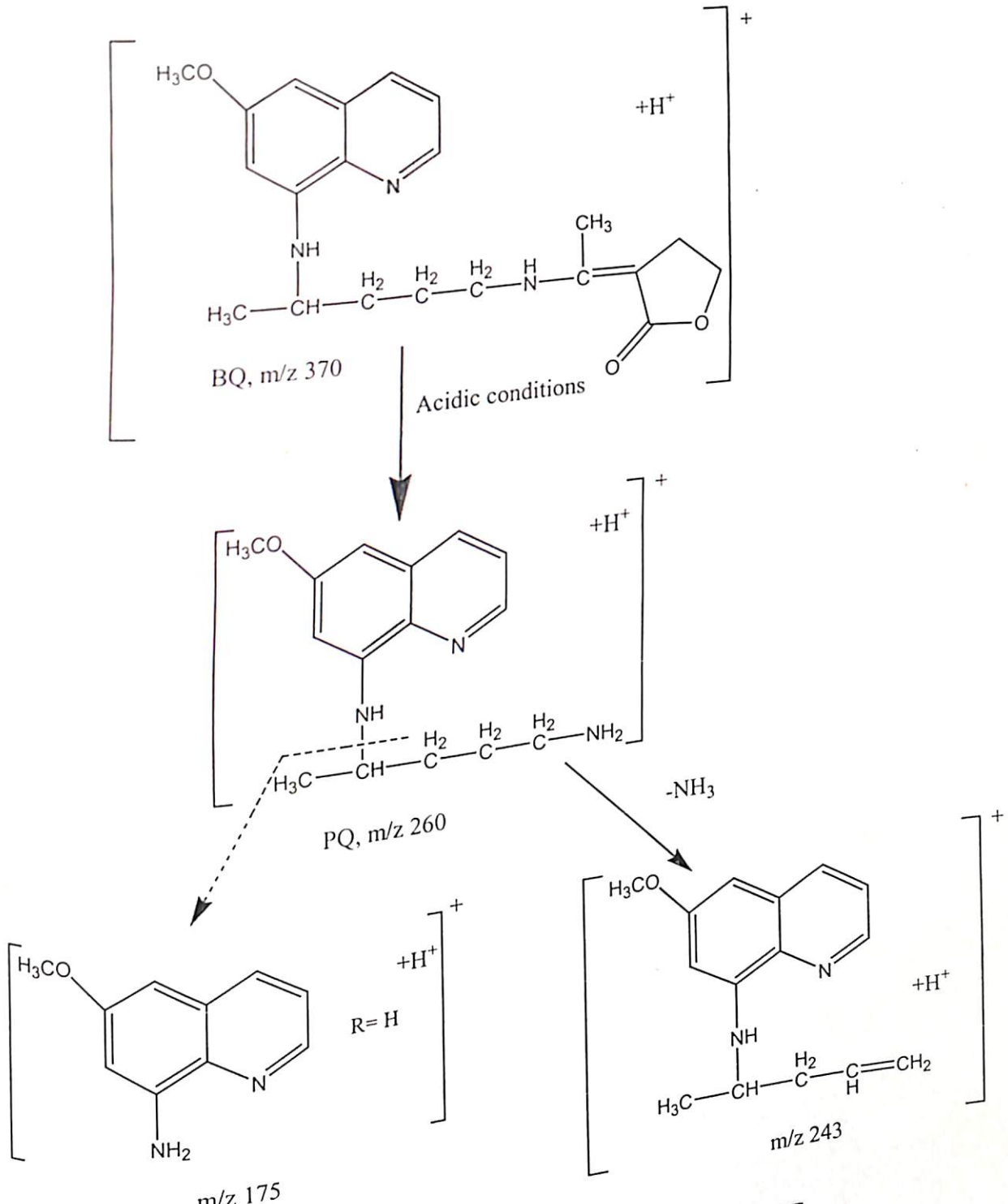


Figure 4.1.2 Proposed sequential degradation pathway of BQ in SGF

4.1.4 Conclusions

The study showed that BQ is highly unstable in acidic pH, which may be the major reason for its very low bioavailability in various species following oral administration. The main degradation product was PQ. Three additional degradation products of BQ were observed which are proposed to be formed from PQ. This indicated sequential degradation of BQ in SGF.

SECTION 2

Plasma Protein Binding Studies

4.2.1 Introduction

Most of the drug molecules bind to plasma proteins in substantial amounts and the unbound concentration can be associated to be more closely related to the therapeutic effect rather than bound concentration. The rate of hepatic metabolism, renal excretion, biomembrane permeation, tissue distribution and steady state distribution volumes are functions of the free drug concentration [1]. Drug molecules are known to circulate in blood in two forms, i.e. red cells, protein or platelet bound and unbound fraction dissolved in plasma [2, 3]. The binding of plasma proteins with drugs is generally reversible and the extent of order of binding is albumin > α_1-acid glycoproteins (AAG) > lipoprotein > globulins. The protein binding varies from as low a 0.1% for caffeine to as high as greater than 95% for warfarin. Albumin having a molecular weight of 65,000 is the most abundant plasma protein with a large drug binding capacity. Both endogenous compounds such as fatty acids, bilirubin, tryptophan as well as majority of anionic and neutral drugs bind to albumin such as warfarin, phenytoin, and valproic acid. More recently basic drugs such as propranolol and tricyclic antidepressants have been shown to bind not only to albumin, but also to other blood proteins such as AAG and lipoproteins [4-5]. There are many factors affecting the protein binding of drugs such as physiochemical characteristics of the drug, concentration of drug in the body and affinity of the drug to the particular binding component.

Protein binding has an important effect on the pharmacokinetics of drugs. Restrictively cleared drugs are those that are removed from the plasma by glomerular filtration through the kidneys or by passive uptake by the liver e.g., theophylline and warfarin [6-7]. Non-restrictively cleared drugs are extracted by active mechanisms in both free and bound forms by the liver or the kidneys e.g., propanolol and lignocaine [8-10]. It is a well-known fact that only the free drug can cross the capillary and cell membrane and reach the site of therapeutic action. Thus, both pharmacokinetic and pharmacodynamic properties of a drug can be influenced by plasma protein binding. The effect of protein binding is more influential on drugs, which are highly protein bound as a slight change in protein binding characteristics of these drugs may drastically increase the free drug concentration in systemic circulation. Thus, a clear understanding of plasma protein binding is essential for safe and proper use of drugs.

Plasma protein binding is usually evaluated *in vitro* and is found to be well correlated to *in vivo* in majority of cases. Reversible protein binding of a drug is of major interest in pharmacokinetics and therapeutics [11] and is often determined by equilibrium dialysis, ultrafiltratrion or ultracentrifugation [12-15]. All these techniques are based on the separation of free drug from bound drug under equilibrium conditions and have their own merits and demerits. These methods are not applicable to drugs, which exhibit non-specific adsorption to various devices or have apparent lipid partitioning into low and very low-density lipoproteins e.g., tirilizad. In the present study, BQ showed non-specific adsorption to the ultracentrifugation device. Therefore, charcoal adsorption assay method was used to determine the extent of protein binding of BQ.

4.2.2 Experimental

4.2.2.1 Materials

Pure reference standard of BQ was obtained from Pharmaceutics Division, CDRI. Dextran coated charcoal was obtained from Sigma Chemicals, USA. DMOA was procured from Aldrich chemicals, USA. Dulbecco's Phosphate buffered saline (DPBS) (Ca and Mg free) was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai. ACN was purchased from E-Merck Ltd., Mumbai, India. Pure water was obtained from MilliQ purification unit, Millipore, USA. Drug-free heparinised rat, rabbit, monkey plasma was obtained from healthy rats, rabbits, monkeys provided by Laboratory Animal Services Division of the institute.

4.2.2.2 Preparation of Dextran Coated Charcoal Suspension

The dextran coated charcoal suspension was prepared by adding 0.66 g of dextran-coated charcoal to 100 ml of DPBS (9.5 g/litre). The mixture was stirred with a magnetic stirrer at room temperature until the charcoal was suspended. This suspension was prepared at least 18 hours before use and stored at 5-10°C for not longer than 30 days. The stored charcoal mixture was re-suspended before use.

4.2.2.3 Preparation of Stock Solutions of BQ

BQ P1 (500 µg/ml) was prepared by appropriately dissolving 5 mg in a 10 ml volumetric flask and making up the volume with ACN (0.1%DMOA). BQ P2 (100

µg/ml) and BQ P3 (20 µg/ml) were prepared from BQ P1 by appropriate dilutions with ACN.

4.2.2.4 Charcoal Adsorption Assay

The protein binding was estimated using the modified charcoal adsorption method reported by Khurana *etal.* [16]. 6.0 ml of plasma was taken and spiked with 60 µl of BQ P3 to produce a concentration of 200 ng/ml. Similarly, two other lots of plasma (6 ml each) were taken and spiked with 60 µl of BQ P2 and BQ P2 to attain a concentration of 1 and 5 µg/ml for BQ. The spiked plasma was allowed to equilibrate for 10 minutes before the start of study. Charcoal suspension (6.0 ml) was transferred into a 30 ml glass tube, centrifuged at 3000 rpm for 15 minutes at 25°C, the supernatant DPBS was carefully decanted off. Spiked plasma (6.0 ml) was then added on to the charcoal pellet under continuous stirring at 37±1°C. Serial samples (400 µl) were withdrawn at 0, 5 min, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 4, 6 and 8 h in 0.6 ml polypropylene microcentrifuge tubes and centrifuged immediately at 11,000 rpm at 37°C. The supernatant was separated and was immediately transferred into 5 ml glass centrifuge tubes and stored at -30°C until analyzed. The study was performed in rat, rabbit and monkey plasma separately, each at three different concentration levels (0.2, 1, 5 µg/ml) in triplicate.

4.2.2.5 Sample analysis

The samples were analyzed by method B (section 2.4.5) C and D (section 2.4.6) for monkey, rat and rabbit plasma, respectively.

4.2.2.6 Protein Estimation

The protein content of the plasma utilized in the experiment was determined by Lowry method [17]. The concentration of the proteins in plasma was determined by the standard curve constructed using bovine serum albumin.

4.2.2.7 Data Treatment

The plasma BQ concentrations were read from the standard curve of BQ in plasma using linear regression analysis on Microsoft Excel and percent drug remaining in plasma was determined by comparison with the initial concentration (prior to charcoal treatment). Percent drug remaining in the supernatant plasma

versus time data was fitted to a two-compartment model with i.v. bolus input using nonlinear regression program using WinNonlin software (Version 1.5) which mimics the events pertaining to charcoal adsorption assay as depicted in Figure 4.2.1. [18].

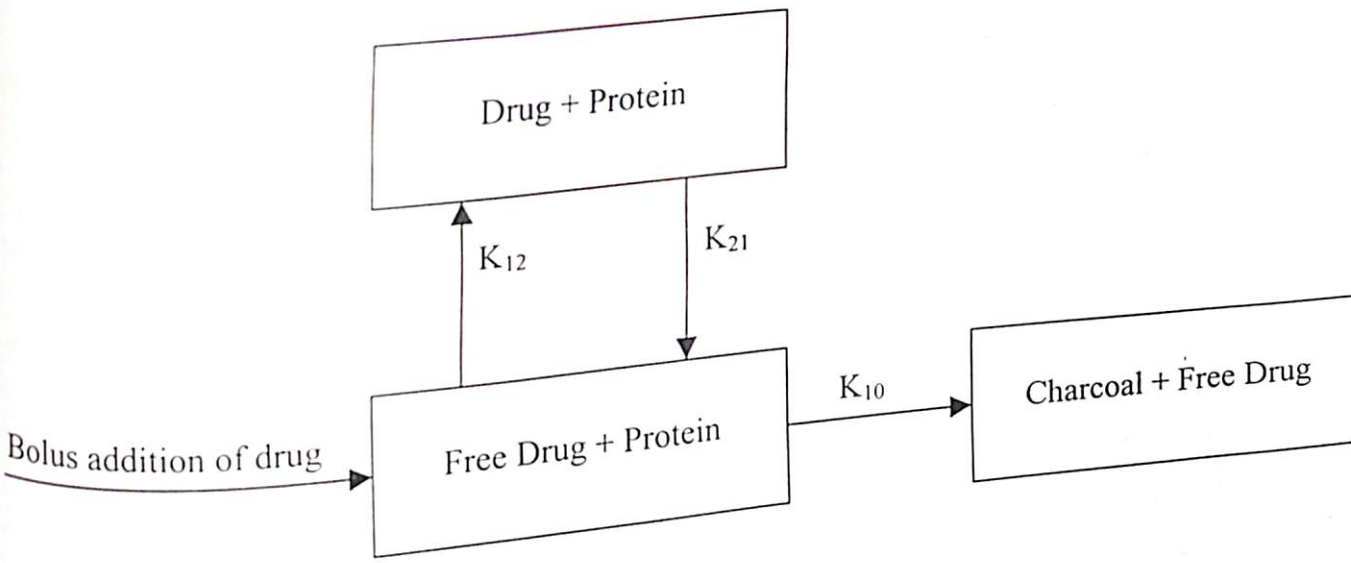


Figure 4.2.1 The hypothetical two-compartment model describing the basis of charcoal adsorption assay for protein binding estimation

The model is described by the following biexponential equation:

$$B(t) = A_1 e^{-\alpha t} + A_2 e^{-\beta t}$$

Where B(t) is % bound at time t, A_1 and A_2 are Y intercepts, α and β are distribution and disposition rate constants for the two phases respectively. The extent of protein binding was given by the C_{max} value (at time t=0). The protein binding at three concentration levels in plasma samples of the same species and at each concentration level between species were compared by one way ANOVA (Newman-Keuls multiple comparison test) at 95 % confidence interval. Mean ± SD values were used for the comparison and $P > 0.05$ was taken as the criteria for no significant difference between the means

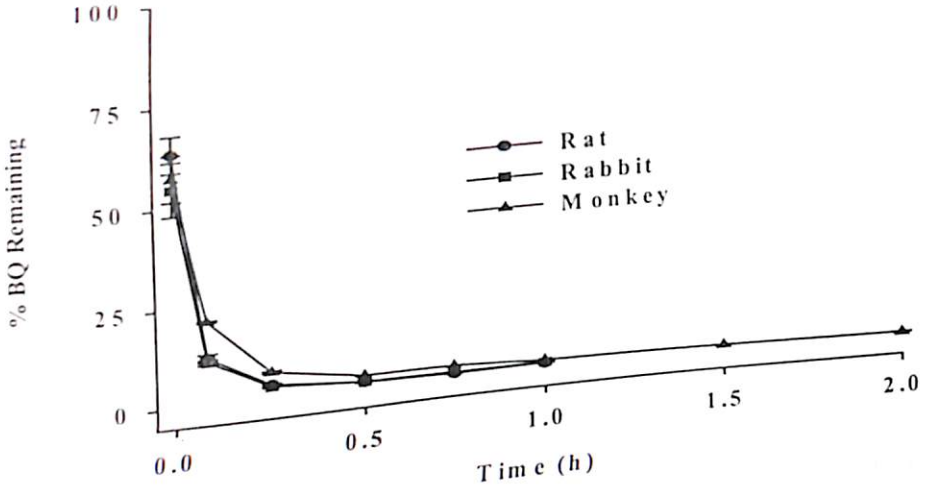
4.2.3 Results and Discussion

The traditional techniques for determination of protein binding could not be used for BQ because of its extensive non-specific adsorption on dialysis cell and various parts of ultrafilteration devices. Ultrafiltrtaion, using *Centifree* micropartition system (Amicon Inc., Baverly, MA, USA), was found to adsorb 70 and 60% of BQ on

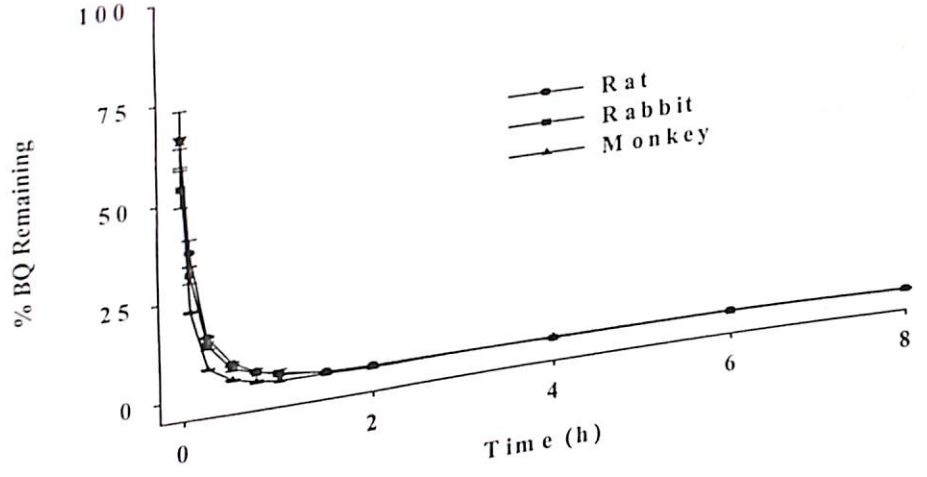
to tubes and cups of *Centifree* device. Therefore, a charcoal adsorption assay [17] specifically designed for these type of compounds was used in the present study. The method is based upon charcoal adsorption kinetics and operates under non-equilibrium conditions. It involves measuring the time course of decline of the concentration of bound drug when the free drug is removed by charcoal adsorption. Association and dissociation of drug with proteins is a dynamic phenomenon with charcoal acting as a sink for free drug removal. The % binding is then estimated from the decline of % drug remaining in the supernatant after the addition of charcoal. Dextran coated charcoal is used since dextran coating effectively prevents the charcoal from adsorbing proteins which will lead to erroneous results [19].

The study was performed at three different concentration levels in triplicate in the three species to see the effect of BQ concentration on its protein binding and at the same time the species similarities or differences in protein binding of BQ. Figure 4.2.2 a, b and c depicts % BQ remaining (mean ± SD, n=3) versus time after the addition of charcoal, at 0.2, 1.0, and 5.0 µg/ml respectively in various species. Percent protein binding at 0.2, 1.0 and 5.0 µg/ml in rat, rabbit and monkey plasma is given in Table 4.2.1. Statistical analysis of the data showed that there was no significant difference ($P > 0.05$) in % protein binding at the three different concentration levels of BQ in rats and rabbits, whereas a significant difference was observed between 1 and 5 µg/ml levels in monkeys ($P < 0.05$) using Newman-Keuls multiple comparison test. Furthermore, there was no significant difference in protein binding of BQ between the species at 0.2 and 5.0 µg/ml concentration levels ($P > 0.05$). However, a significant difference was observed at 1µg/ml between rat versus monkey and rat versus rabbit ($P < 0.05$).

(a)


100
75
50
25
0
% BQ Remaining
Rat
Rabbit
Monkey
0.0
0.5
1.0
1.5
2.0
Time (h)

(b)


100
75
50
25
0
% BQ Remaining
Rat
Rabbit
Monkey
0
2
4
6
8
Time (h)

(c)

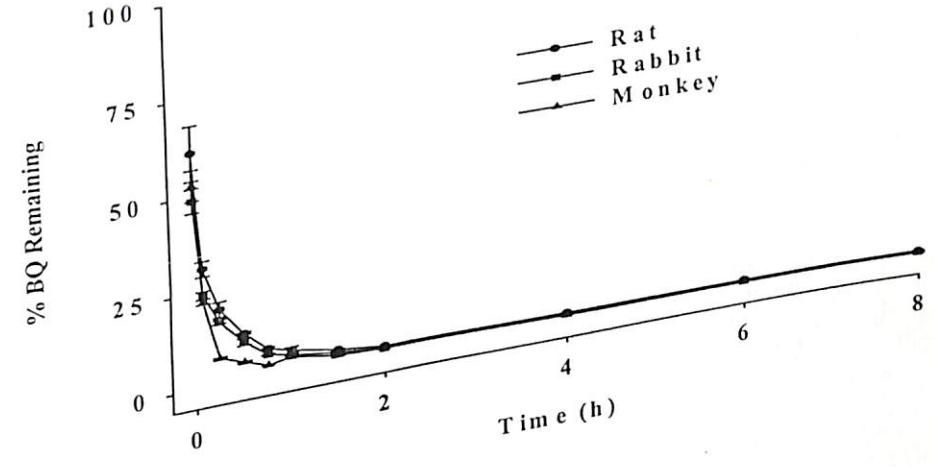

100
75
50
25
0
% BQ Remaining
Rat
Rabbit
Monkey
0
2
4
6
8
Time (h)

Table 4.2.1 % Protein binding (Mean ± SD, n=3) as determined by charcoal adsorption assay

Species (plasma) (n=3)	% Protein binding		
	0.2 µg/ml	1.0 µg/ml	5.0 µg/ml
Rat	63.5±4.5	63.6±6.2	61.1±7.1
Rabbit	54.9±6.7	53.7±4.4	49.0±3.2
Monkey	57.5±5.7	65.2±1.4	52.9±3.9

4.2.4 Conclusions

In the present study, charcoal adsorption method was applied to determine the plasma protein binding of BQ as the dialysis and ultra centrifugation methods were found unsuitable owing to high non-specific adsorption of the drug with the devices. The study at the three concentration levels of BQ in rat, rabbit and monkey plasma showed that BQ binds moderately (55-65%) with plasma proteins.

SECTION 3

Whole Blood Partitioning Studies

4.3.1 Introduction

The significance of studying the kinetics of drug partitioning into RBCs is not fully appreciated, although the importance of routine determination of rate and extent of partitioning of investigational drugs has been stressed [20, 21]. Failure to determine the kinetics of drug in RBCs may be a lost opportunity as its gives knowledge of many attributes of the drug like

- A rational choice of appropriate biological fluid, either whole blood or plasma/serum for assay. The vast majority of drugs are assayed in plasma or serum while for drugs with $K_{wb/p} > 2$, measuring concentrations in whole blood or erythrocytes increases the sensitivity of the method with a given limit of quantification [22-29]. The concept of referencing PK parameters has been applied to inter compartmental clearances and volume of distribution [30, 31]. With the neuroleptic drugs butaperazine, haloperidol, and thioridazine, the RBC concentrations correlate better with therapeutic effect or dose than plasma concentration.
- Effective screening of drugs whose biophase resides in the RBCs, thereby enabling the study of the effects of drugs on RBCs.

Among the cellular constituents of blood, the RBCs represent by far, the largest population both in number and cell size. The RBCs make up more than 99% of the cellular space of blood in humans. Investigations of the RBC partitioning of relatively small organic cationic, anionic, and nonelectrolytic molecules have shown that lipophilicity, molecular size and chiral characteristics are important [32-33]. Lipophillic compounds penetrate the RBC by dissolving into the lipid bilayer membrane [34, 35] while small size hydrophilic compounds enter RBCs through aqueous channels. RBC partitioning by passive diffusion has been reported for organic cationic and anionic drugs as well as for non electrolytes [36-39]. The rate and extent of the RBC uptake can be determined by both *in vitro* and *in vivo* approaches. In the *ex vivo* procedure the drug is administered, a series of samples taken, and following centrifugal separation, the drug concentrations are measured in RBC and plasma. While in the *in vitro* procedure, red cell suspensions in plasma or pH 7.4 buffer are used. The experiments are conducted at normal body temperature. The rate of drug partitioning into RBCs is determined in spiked whole blood or in RBC suspension, which are gently shaken to mimic the *in vivo* situation where drug

distribution occurs by diffusion and convection. Timed samples are taken and rate and extent of RBC partitioning is determined [40]. Apart from the practical considerations that favor the *in vitro* procedure over *in vivo*, there exists several advantages which makes an *in vitro* method suitable than *in vivo* one. With many drugs, the rate of partitioning is fast, and the distribution equilibrium is reached within few seconds to minutes. In these cases, only *in vitro* procedure enables the determination of the rate of partitioning with meaningful results. Moreover, the extent of drug partitioning should be done under steady-state equilibrium conditions and *in vitro* method uses a closed system, equilibrium conditions can easily be established. With rapidly penetrating drugs, quasi steady-state of equilibrium may also be achieved in *in vivo* conditions even though human body represents an open system. However, with more slowly equilibrating drugs this is not the case and the erythrocytic uptake is more difficult to separate from the multiplicity of other kinetic events like tissue distribution and elimination which are occurring simultaneously from the body [41]. BQ being an antimalarial agent may be concentrated in the RBCs. In the present study, *in vitro* procedure of RBC uptake was preferred because of the above mentioned reasons and was correlated with the *in vivo* samples there after.

4.3.2 Experimental

4.3.2.1 Materials

Pure reference standard of BQ was obtained from Pharmaceutics Division, CDRI. DMOA was procured from Aldrich chemicals, USA. ACN and orthophosphoric acid was purchased from E-Merck Ltd, Mumbai, India. Na_2HPO_4, KH_2PO_4 and NaCl were procured from s.d.- Fine chemicals, Mumbai, India. Pure water was obtained from MilliQ purification unit, Millipore, USA. Drug-free heparinised rat, rabbit, monkey whole blood was obtained from healthy rats, rabbits, monkeys provided by Laboratory Animal Services Division of the institute. A shaking water bath (Vam 908D, Vam Instruments Pvt Ltd, India) for incubation was used shaking at 40 rpm and maintained at 37±2 °C.

was taken in a hematocrit tube and

centrifuged at 2000 g for 30 minutes. The hematocrit value (H_C) for the whole blood of each species was calculated by using the formula:

$$H_C = \frac{[\text{Volume of packed cells}]}{[\text{Total volume of blood}]} \times 100$$

4.3.2.3 Preparation of Phosphate Buffered Saline (PBS)

0.345 g Na_2HPO_4, 0.047 g KH_2PO_4 and 2.0 g NaCl were accurately weighed and dissolved in 250 ml MilliQ water. The pH of the solution was adjusted to 7.4 with 20% orthophosphoric acid. The solution was filtered through 0.22 µ Millipore filter before use.

4.3.2.4 Preparation of Stock Solutions of BQ in ACN

BQ 1R (5 µg/ml), BQ 2R (50 µg/ml) and BQ 3R (300 µg/ml) were prepared by appropriately diluting BQ 1P [(500 µg/ml), section 3.2.2.3] with ACN.

4.3.2.5 Preparation of Stock Solutions of BQ in PBS

BQ 4R (0.25 µg/ml), BQ 5R (2.5 µg/ml) and BQ 6R (15 µg/ml) were prepared in PBS by 1:20 dilutions of BQ 1R, 2R and 3R. These solutions were freshly prepared just before the experiment and were used for spiking the whole blood.

4.3.2.6 Incubation and Sample Collection

Fresh heparinised whole blood (5.0 ml) was taken and its H_C was determined as described in section 4.3.2.2. The H_C was adjusted to 0.4-0.5 with PBS, if necessary. It was then pre-incubated in shaking water bath for 10-15 minutes at 37 ± 2°C. 500 µl of BQ 4R was spiked in 5.0 ml whole blood to produce a concentration of 25 ng/ml of BQ. Similarly, two other lots of whole blood (5 ml each) were taken and spiked with 500 µl of BQ 5R and BQ 6R to attain a concentration of 250 and 1500 ng/ml for BQ respectively. Serial samples (600 µl) were withdrawn at 0, 0.25, 0.5 and 1.0 h from the incubation mixture. 200 µl of whole blood was separated while the plasma was obtained from remaining 400 µl of whole blood by centrifugation at 2000 rpm for 10 min. Both whole blood and plasma samples were kept at -30°C until analyzed. The study was performed in rat, rabbit and monkey whole blood separately at three different concentration levels (0.025, 0.25, 1.5 µg/ml) in triplicate in all the three species.

4.3.2.7 Sample Analysis

The plasma samples were analyzed by method B (section 2.4.5) C and D (section 2.4.6) for monkey, rat and rabbit plasma respectively. Method E, F and G as described in section 2.4.7 were used for analyzing rat, rabbit and monkey whole blood samples.

4.3.2.8 Data Treatment

The whole blood uptake partition coefficient ($K_{wb/p}$) for BQ was determined by using the equation,

$$K_{wb/p} = C_{wb}/C_P\ (1\text{-}Hc)$$

Where C_{wb} is the concentration in whole blood, C_P is BQ concentration in plasma and H_C is the hematocrit value. The $K_{wb/p}$ at three concentration levels of the same species and at each concentration level between species were compared by one way ANOVA (Newman-Keuls test) at 95 % confidence interval. Mean ± SD values were used for the comparison and $P > 0.05$ was taken as the criteria for no significant difference between the means.

4.3.3 Results and Discussion

The whole blood uptake of BQ was calculated for three species (rat, rabbit and monkeys) at three concentration levels to determine the effect of BQ blood concentration and to establish species differences in uptake of BQ, if any. The stock solutions were spiked in PBS so as to avoid hemolysis of RBCs thus interfering with the true concentration of BQ in plasma. The stock solutions of BQ in PBS were assayed for its content prior to analysis. The samples were analysed by HPLC-MS-MS method for simultaneous determination of BQ and PQ which was essential to determine degradation of BQ to PQ. It was found that there was 10-15% of conversion of BQ to PQ irrespective of the concentration. The comparative concentration of BQ in plasma samples from incubated whole blood containing 25, 250, and 1500 ng/ml level of BQ in various species is shown in Figures 4.3.1 a, b, and c respectively. The $K_{wb/p}$ values at the three concentration levels in three species are represented in Table 4.3.1.

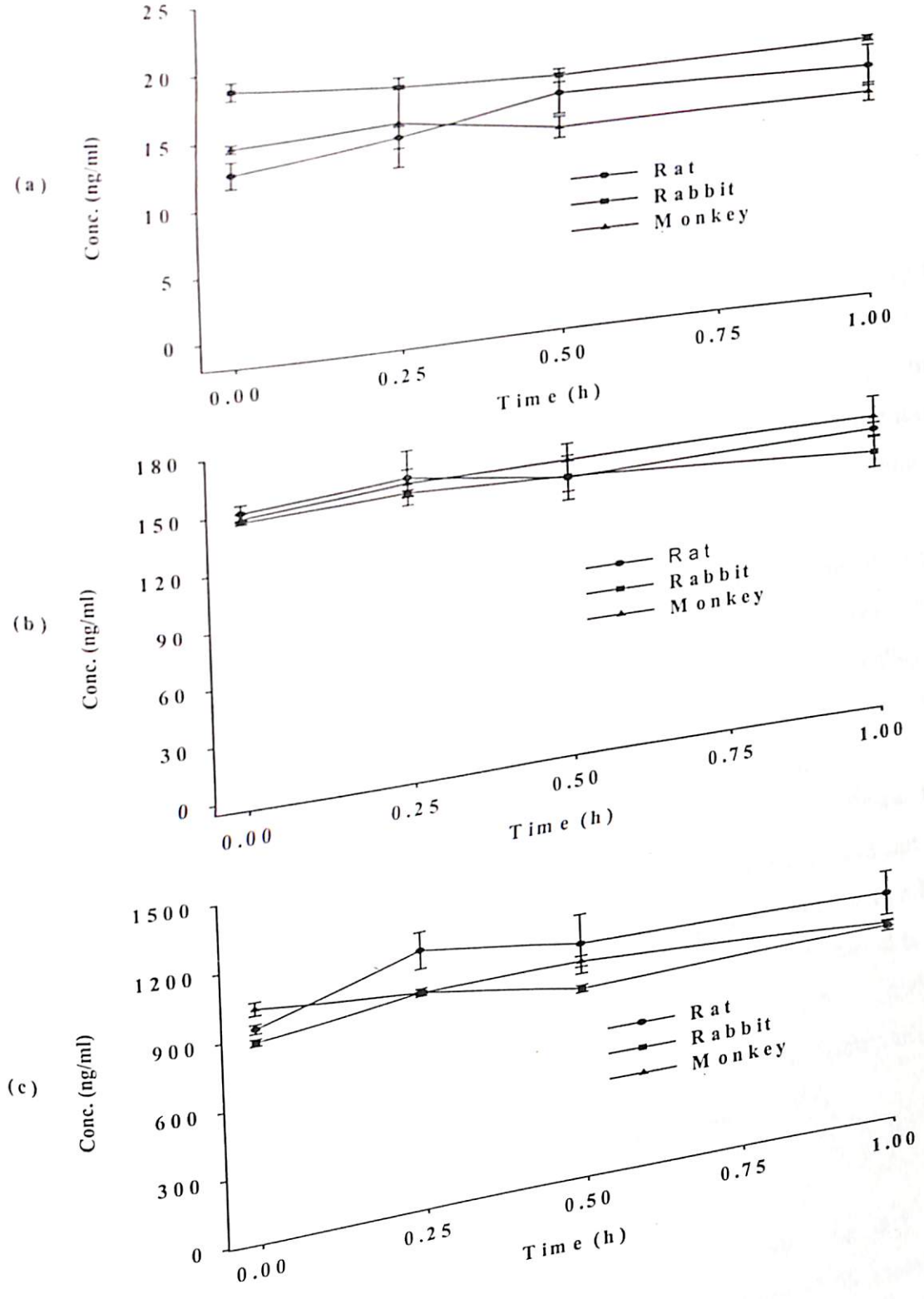


Figure 4.3.1 Mean ± SD concentration of BQ in plasma samples (n=3) from incubated whole blood of rat, rabbit and monkey containing (a) 25, (b) 250, (c) 1500 ng/ml of BQ

Table 4.3.1 $K_{wb/p}$ (Mean ± SD, n=3) as determined by whole blood partitioning studies

Species (n=3)	$K_{wb/p}$		
	25 ng/ml	250 ng/ml	1500 ng/ml
Rat	1.25±0.11	1.33±0.11	1.23±0.07
Rabbit	1.49±0.03	1.42±0.13	1.35±0.09
Monkey	1.26±0.09	1.26±0.11	1.31±0.02

It is evident from the figures that the rate of RBC partitioning is very fast as the amount of BQ after 0 minute till one hour is constant in incubated whole blood samples at all the three concentration level in the species. The $K_{wb/p}$ values indicate 25-35 % uptake by the RBCs in all the concentration levels thus signifying moderate uptake of BQ by the RBCs. Whole blood samples from dosed rats, rabbits and monkey also showed similar $K_{wb/p}$ values.

There was no significant difference ($P > 0.05$) in whole blood uptake of BQ at three different concentration levels in all the three species. However, there existed a significant difference ($P < 0.05$) within species (rat versus rabbit and monkey versus rabbit) in $K_{wb/p}$ at 25 ng/ml level. Furthermore, there was no significant difference ($P > 0.05$) between species at 250 and 1500 ng/ml concentration levels.

In actual terms, whole blood and not plasma or serum flows through capillaries in the body. Therefore, it would appear that whole blood and not plasma or serum is the most appropriate fluid for calculating and interpreting Cl and V_d. In agreement with this rationale, the routine determination of the whole blood to plasma ratio was recently proposed for drugs under development. Though insignificant at this stage, the uptake can be more in diseased stages, as most of the antimalarial drugs are known to have higher RBC uptake in diseased conditions [42, 43]

4.3.4 Conclusions

In the present study, the $K_{wb/p}$ ratios were calculated for BQ at three concentration levels in rat, rabbit and monkey. The $K_{wb/p}$ values (1.23-1.49) indicate a moderate uptake of BQ by the RBCs. The uptake was instantaneous and was not affected by the increasing concentration of BQ.

References

1. Valner, J.J., 1977. Binding of drugs with albumin and plasma proteins, *J. Pharm. Sci.*, 66: 447-465.
2. Weser, J.K. and Sellers, E.M., 1976. Binding of drugs to serum albumin – Part-1. *New Eng. J. Med.*, 294: 311-316.
3. Weser, J.K. and Sellers, E.M., 1976. Binding of drugs to serum albumin – Part-2. *New Eng. J. Med.*, 294: 526-531.
4. Piafsky, K.M., 1980. Disease induced changes in the plasma binding of basic drugs. *Clin. Pharmacokin.*, 5: 246-262.
5. Bickel, M.H., 1975. Binding of chlorpromazine and imipramine to red cells, albumin, lipoproteins and other blood components, *J. Pharm. Pharmacol.*, 27: 733-738.
6. Powell, J.R., Vozeh, S., Hopewell, P., Costello, J., Sheiner, L.B. and Reigelman, S. 1975. Theophylline disposition in acutely ill hospitalized patients, *Am. Rev. Respir. Dis.*, 11: 229-237.
7. O'Reilly, R.A., Welling, P.J., and Wagner, J.G., 1971. Pharmacokinetics of warfarin following intravenous administration in man, *Throm. Diath. Haemorrh.*, 25: 178-186.
8. Pond, S.M. and Tozer, T. N., 1984. First pass elimination, basic concepts and clinical consequences, *Clin. Pharmacokin.*, 9: 1-25.
9. Perucca, E. and Richens, A., 1979. Reduction of oral bioavailability of lignocaine by induction of first pass metabolism in epileptic patients, *Br. J. Clin. Pharmacol.*, 7: 21-31.
10. Evans, G.H. and Shand, D.G., 1973. Disposition of propranolol; V. Drug accumulation and steady state concentrations during chronic oral administration in man, *Clin. Pharmacol. Therap.*, 14: 487-493.
11. Shargel, L. and Yu, A.B.C, 1985. *Applied Pharmaceutics and Biopharmaceutics*, 2nd edition, Appleton-Century Crofts, Norwalk, CT, 197-211.
12. Kurz, J., Trunk, J. and Weitz, B., 1977. Evaluation of methods to determine protein binding of drugs, equilibrium dialysis, ultrafiltration, ultracentrifufation, and gel filteration, *Drug Res.*, 27: 1373-1380.
13. Whitlan, J.B. and Brown, K.F., 1981. Ultarfilteration in serum protein binding determination, *J. Pharm. Sci.*, 70: 146-150.

14. Moll, G.W. and Rosenfeld, R.L., 1978. Some limitations of using equilibrium dialysis to study human serum albumin-testosterone interaction, *J. Clin. Endocrin. Metab.*, 46: 501-503.

15. Bowers, W.F., Fulton, S. and Thompson, J., 1984. Ultrafiltration vs. equilibrium dialysis for determination of free fraction, *Clin. Pharmacokin.*, 9 (supp.1): 49-60.

16. Khurana, M., Paliwal, J.K., Kamboj, V.P., and Gupta, R.C., 1999. Binding of centchroman with human serum as determined by charcoal adsorption method, *Int. J. Pharm.*, 192: 109-114.

17. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the folin phenol reagent, *J. Bio. Cjem.*, 193: 265-272.

18. Yuan, J., Yang, D.C., Berkmeier, J. and Stolzenbach, J., 1995. Determination of protein binding by *in vitro* charcoal adsorption., *J. Pharmacokin. Biopharm.*, 23 (II): 41-55.

19. Herbert, V., Lau, K., Gottileb, C.W. and Bleicher, S.J., 1965. Coated charcoal immunoassay of insulin, *J. Clin. Endocrin. Metab.*, 25: 1375-1384.

20. Lee, M.G., Chen, M.L., Huang, S.M. and Chiou, W.L., 1981. Pharmacokinetics of drugs in blood: Unusual distribution of gentamycin, *Drug Dispos.*, 2: 89-97.

21. Hinderling, P.H., Schmidlin, O., and Seydel, J.K., 1984. Qunatitative relationship between structure and pharmacokinetics of beta adrenoceptor blocking agents in man, *J. Pharmacokin. Biopharm.*, 12: 263-287; 659-666.

22. Fleuren, H.L. and Rossum, V., 1977. Nonlinear relationship between plasma and red blood cell pharmacokinetic of chlorthalidone in man, *J. Pharmacokin. Biopharm*, 5: 359-375.

23. Wallace, S.M. and Riegelman, S., 1977. Uptake of acetozolamide by human erythrocytes, *J. Pharm. Sci.*, 66: 729-731.

24. Bayne, W.F., Tao, T.F., Rogers, G., Chu, L.C. and Theeuwes, F., 1981. Time course and disposition of methazolamide in human plasma and red blood cells, *J. Pharm. Sci.*, 70: 75-80.

25. Rambo, L., Ericsson, O., Alvan, G., Lindstrom, B., Gustafsson, L.L and Sjoqvist, F., 1985. Chloroquine and desethylchoroqunine in plasma, serum and whole blood: Problem in assay and handling samples, *Therap. Drug Monitor.*, 7: 211-215.

26. Winstanley, P., Edwards, G., Orme, M. and Breckenridge, A., 1987. The disposition of amadiaquine in man after oral administration, *Br. J. Clin. Pharmacol.*, 23: 1-7.

27. Jusko, W.J., Piekoszewski, W., Kilntmalm, G.B., Shaefer, M.S., Hebert, M.F, Pipergies, A.A., Lee, C., Schechter, P. and Mekki, Q.A., 1995. Pharmacokinetics of tacrolimus in liver transplant patients, *Clin. Pharmcol. Therap.*, 37: 281-290.

28. Kurokawa, N., Kadobyashi, M., Yamamoto, K., Arakawa, Y., Sawada, M., Takahara, S., Okuyam, A., and Yanaihara, C., 1996. *In vivo* distribution and erythrocyte binding characteristics of cyclosporin in renal transplant patients, *J. Pharm. Pharmacol.*, 48: 553-559.

29. Snoek, E., Jacqmin, P., Van peer, E., Danhof, M., Verdonk, Bells, H., Woestenborghs, R., Crabbe, R., Vangool, R., Dupont, A. and Hitkants, J., 1996. The implication of non-linear red cell partitioning for the pharmacokinetics and pharmacodynamics of the nucleoside inhibitor draflaxine, *Br. J. Clin. Pharmacol.*, 42: 605 –613.

30. Stec, G.P. and Atkinson Jr, A.J., 1981. Analysis of the contribution of permeability and flow to intercompartmental clearance, J. *Pharmacokin. Biopharm,* 9: 167-180.

31. Oedh, Y.K., Wang Z Ruo, T.I., Wang, T., Frederiksen, M.C., Pospisil, P.A. and Atkinson Jr, A.J., 1993. Simultaneous analysis of inulin and N2 urea kinetics in humans, *Clin. Pharmacol. Ther.*, 53 : 419-425.

32. Shaafi, R.I., Gray-Bovo, C.M. and Solomon, A.K., 1971. Permeability of red cell membranes to small hydrophilic and lipophilic solutes, *J. Gen. Physiol*, 50: 238-258.

33. Deuticke, B., 1977. Properties and structural basis for diffusion pathways in the erythrocyte membrane, *Rev. Physiol. Biochem. Pharmacol.*, 78: 1-97.

34. Schanker, L.S., Johnson, J.M. and Jeffrey, J.J., 1961. Rapid passage of organic base in to human red cells, *J. Pharmacol.*, 133: 325-331.

35. Holder, L.B. and Hayes, S.I., 1965. Diffusion of sulfonamides in aqueous buffers into red cells, *Mol. Pharmacol.* 1: 266-279.

36. Shirkey, R.J., Jellett, L.B., Kappatos, D.C., Maling, T.J.B. and McDonald, A., 1985. Distribution of sodium valproate in normal whole blood and in blood from

patients with renal or hepatic disease, *Eur. J. Clin. Pharm. Pharmacol.*, 28: 447-452.

37. Sweeney, K.R., Capron, D.J. and Kramer, P.A., 1988. Effect of salicylate as serum protein binding and red cell uptake of acetazolamide *in vitro*, *J. Pharm. Sci.*, 77: 751-756.

38. Ferrari, V. and Cutler, D.J., 1990. Uptake of chloroquine by human erytrocytes, *Biochem. Pharmacol.*, 39: 753-762.

39. Lin, J. H., Lin, T.H., and Cheng, H., 1992. Uptake of stereoselective binding of enantiomers of MK-927, a potent carbonic anhydrase inhibitor, by human erythrocytes *in vitro*, *Pharm. Res.*, 9: 339-344.

40. Reichel, C., Von, F.M., Brockmeier, D. and Dengler, H.J., 1994. Characterization of cyclosponine uptake in human erythrocytes, *Eur. J. Clin. Pharmacol.*, 46: 417-419.

41. Wallace, S.M. and Riegelman, S., 1977. Uptake of acetazolamide by human erythrocytes, *J. Pharm. Sci.*, 66: 729-73.

42. Trenholme, G.M., Williams, R.L., Rieckmann, K.H., Frischer, H. and Carson, P.E., 1976. Quinine disposition during malaria and during induced fever, *Clin. Pharmacol. Therap.* 19: 459-467.

43. White, N.J., Looareesuwan, S., Warrell, D.A., Warrell, M.J., Chanthavanich, P., Bunnag, D., and Harinusuta, T., 1983. A quinine loading dose in cerebral malaria, *Am. J. Trop. Med. Hyg.*, 32: 1-5.

SUMMARY

partitioning ratio of BQ in various species. The method was also partially validated in rat urine and fully validated in rat feces for excretion studies in rats. All the partially validated methods were accurate and precise within the calibration range.

Oral and i.v. Study in Rats

Following a single 20 mg/kg oral dosing of BQ in rats, its absorption was rapid with C_{max} (399±53 ng/ml) occurring in 5 min and could be monitored upto 6 h post dose. Appearance of a secondary peak at 2 h could be due to enterohepatic recirculation, storage and subsequent release from post absorptive depot site or absorption from different segments of intestine. BQ showed short elimination $t_{1/2}$ (1.08 h) and high clearance (3.2 L/h/kg) and V_d (5.05 L/kg) indicating highly cleared and widely distributing drug. Its major metabolite PQ exhibited an irregular concentration-time profile and was monitored upto 48 h post BQ dose indicating a longer residence of PQ in the body than BQ. A substantial conversion of BQ to PQ (AUC_{PQ}/ AUC_{BQ} = 22.56) may be due to low stomach pH (1-2) and metabolism.

Appearance of a C_{max} of BQ at 2.0 h after 5 mg/kg i.v. dose indicates a strong possibility of enterohepatic recycling. The Cl and V_d were comparable to the values obtained after oral dose. The bioavailability of BQ was 3.12 %. PQ appeared in the systemic circulation after 15 min post i.v. dose and was quantified upto 8 h indicating a possible role of RBCs in the metabolism of BQ. The extent of conversion of BQ to PQ was lower than after oral administration.

Oral and i.v. study in Rabbits

In rabbits, the absorption of BQ was rapid with a C_{max} of 603±18 ng/ml in 5 min and was detected upto 8 h after 10 mg/kg oral dose of BQ. Rabbits also showed short elimination $t_{1/2}$ (1.24 ± 0.03 h) and was comparable to that in rats. The Cl (1.2 ± 0.06 L/h/kg) and V_d (2.1 ± 0.15 L/kg) are indicative of wide distribution. PQ also exhibited an i.v. like concentration-time profile with lack of absorption phase indicating a rapid metabolism of BQ to PQ by liver or gastric flora or instability at stomach pH.

Following 2.5 mg/kg i.v. administration, both BQ and PQ were detected upto 12 h post dose. Elimination $t_{1/2}$, Cl and V_d were comparable to that observed aft oral dose. Cl and V_d were lower than that in rats. The bioavailability of BQ was

± 0.003 %. Its major metabolite PQ was monitored upto 12 h with AUC_{PQ}/ AUC_{BQ} of 0.08 and was lower than that after oral dose.

Oral and i.v. Study in Monkeys

BQ exhibited a double peak phenomenon following 10 mg/kg oral administration with C_{max} (159 ± 24 and 183 ± 46 ng/ml) occurring at 0.25 and 2 h, respectively which may be due to enterohepatic recycling or varying absorption sites in the GIT. The irregular concentration-time profiles of BQ and PQ could not be explained with any standard one- or two-compartmental models. Therefore non-compartmental analysis was performed to estimate the PK parameters. Elimination $t_{1/2}$ (3.7 ± 0.1 h) and V_d (6.3 ± 0.4 L/kg) were higher than in rats and rabbits after both oral administration. Its major metabolite PQ showed C_{max} at 12 h and was detected upto 48 h. The conversion (AUC_{PQ}/ AUC_{BQ}, 8.81) was lower in rats but higher than rabbits after oral dose and indicates the difference in extent of metabolism.

Following i.v. administration, BQ was monitored upto 12 h with elimination $t_{1/2}$ less as compared to oral dose. The bioavailability of BQ was 12.0 % and the extent of conversion of BQ to PQ was lower than that after oral dose.

Comparison of Pharmacokinetics of BQ and PQ in Rats, Rabbits and Monkeys

BQ was rapidly absorbed in rats and rabbits with no distinct absorption phase while in monkeys an absorption phase could be observed with a double peak phenomenon. In all the three species, BQ is extensively converted to PQ after oral administration and the conversion was maximum in rats and minimum in rabbits, which is possibly due to the species difference. The elimination half-life of rats and rabbits was comparable both after oral and i.v administration. Cl was higher in rats than in rabbits and monkeys, which is expected as rats being lower animals, will eliminate drug more rapidly than other higher species when compared on a weight/surface area normalized basis. In all the species it was found that PQ was resident in body for a period longer than BQ. Possibly, PQ being the major active metabolite of BQ might be responsible for extended therapeutic effect. The oral bioavailability of BQ was 3.12, 5.3 and 12% in rats, rabbits and monkeys respectively. It is possible that low bioavailability of BQ may be due to poor permeability and/or aqueous solubility, extensive binding and degradation/ precipitation in the GIT or instability at low pH.

Excretion Studies in Rats

Following 20 mg/kg oral dose in male SD rats (n=3), BQ was insignificantly detected in urine (0.00074% of administered dose) and could not be detected in feces samples till 60 h post dose. The excretion of BQ in urine was mainly in form of its major polar metabolite PQ. The cumulative amount of unchanged PQ excreted in urine and feces were 0.37 and 0.076% respectively of oral dose of BQ.

Stability Studies in SGF

The stability study in SGF showed that BQ is highly unstable in acidic pH as more than 99% of the BQ degraded when it came in contact with SGF. Analysis of samples by LC-MS-MS showed that the main degradation product of BQ was its major metabolite PQ.(m/z 260), which accounts for almost 83% of BQ. MS analysis using constant infusion of the incubation mixture suggested the presence of three additional compounds with m/z 132, 175 and 243, respectively. Thus, degradation at low pH may be the major factor contributing for its low bioavailability.

Protein Binding Studies

The traditional techniques for determination of protein binding could not be used for BQ because of its extensive non-specific adsorption on dialysis cell and various parts of ultrafiltration devices. Therefore, charcoal adsorption assay was used in the present study. The study was performed at three different concentration levels in rats, rabbits and monkeys to see the effect of BQ concentration on its protein binding and at the same time the species similarities or differences in its protein binding. The binding of BQ was found to be moderate (~55-65%).

Whole Blood Uptake Studies

The whole blood uptake of BQ was calculated in whole blood of three species at three concentration levels. It was observed that the rate of whole blood partitioning is very fast as the 0-1 h incubated whole blood samples did not show any variation in BQ concentration. The $K_{wb/p}$ values (1.2 to 1.5) indicate moderate uptake by RBCs in all three species. The $K_{wb/p}$, though insignificant at this stage may be more in diseased

conditions as antimalarial drugs are known to have increased RBC uptake during malaria.

Conclusions

Hence, from the above studies it can be concluded that the absorption of BQ is rapid in all the species with C_{max} reaching within 15 min. Thus, *in vivo* it is converted to its analogue PQ in significant amount, which is different in different species. It has very low bioavailability and is highly unstable in acidic pH, which could be the reason of its very low bioavailability. RBCs may also play a role in metabolism of BQ to PQ. The excretion of BQ in rats is insignificant and is mainly in the form of its metabolite PQ. The protein binding of BQ was found to be moderate (55-65%) in all the three species. The whole blood uptake was lower but instantaneous in rats, rabbits and monkeys.