

**EVALUATION OF PERMEABILITY AND BIOAVAILABILITY OF A LEAD
ANTILEISHMANIAL COMPOUND BUPARVAQUONE IN ANIMAL
MODELS**

by

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DEDICATION

To Lord Venkateshwara Swamy

and

To my late grand parents Rajanna, Govindappa and Govindamma

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LIST OF ABBREVIATIONS & SYMBOLS

ANOVA	=	Analysis of variance
AUC	=	Area under curve
BALB/c	=	Albino Laboratory Breed Strain of Mice
BCS	=	Biopharmaceutical classification system
BSA	=	Bovine serum albumin
BPQ	=	Buparvaquone
CL	=	Cutaneous leishmaniasis
Caco-2	=	Cell line: human colon carcinoma cells
CV	=	Coefficient of variation
DMSO	=	Dimethyl sulfoxide
FDA	=	Food and Drug Administration
HPLC	=	High performance liquid chromatography
IAM	=	Immobilized Artificial Membrane
IV	=	Intravenous
LOQ	=	Limit of quantification
MDR	=	Multidrug resistance
MRP	=	Multidrug resistance associated protein
MDCK	=	Madin-Darby canine kidney cells
mg	=	Milligram
MW	=	Molecular weight
MCL	=	Mucocutaneous leishmaniasis
NTDs	=	Neglected tropical diseases
NWF	=	Net water flux
PAMPA	=	Parallel artificial membrane permeation assay
P-gp	=	P-glycoprotein
PBS	=	Phosphate buffer saline
PEG	=	Polyethylene glycol
RD	=	Relative difference
RE	=	Relative error

RPM	=	Rotation per minute
RSD	=	Relative standard deviation
SPIP	=	Single pass intestinal perfusion technique
SD	=	Standard deviation
SEDDS	=	Self-emulsifying drug delivery systems
SMEDDS	=	Self-microemulsifying drug delivery systems
SIF	=	Simulated intestinal fluid
SGF	=	Simulated gastric fluid
SPSS	=	Statistical procedures for social science
SPE	=	Solid phase extraction
TDR	=	Training in tropical diseases
TEER	=	Transepithelial electrical resistance
UV	=	Ultra violet
v/v	=	Volume in volume
VL	=	Visceral leishmaniasis
WHO	=	World Health Organization
w/w	=	Weight in weight

**KAJIAN KETELAPAN DAN BIOKEPEROLEHAN BUPARVAQUONE,
SUATU SEBATIAN PELOPOR ANTILEISMANIAL DALAM MODEL
HAIWAN**

ABSTRAK

Leishmaniasis merupakan suatu kumpulan penyakit parasit yang disebabkan oleh genus *Leishmania* yang kini adalah endemik di lapan puluh lapan negara. Penyakit ini wujud dalam dua bentuk utama, iaitu leishmaniasis kulit dan visera. Sebatian antimoni pentavalensi merupakan kaedah yang paling umum digunakan untuk mengubati semua penyakit jenis ini. Kajian *in vitro* dan *in vivo* menunjukkan buparvaquone (BPQ) sebagai suatu sebatian yang berpotensi untuk digunakan terhadap jangkitan *Leishmania donovi*. Dalam membangunkan formulasi oral BPQ, penentuan ciri-ciri biofarmaseutikal seperti keterlarutan dan ketelapan drug perlu dikaji terlebih dahulu. Kajian kestabilan di peringkat awal menunjukkan bahawa sampel BPQ yang dilarutkan dalam metanol dan asetonitril membentuk sebatian degradasi yang belum dikenalpasti apabila didedahkan kepada cahaya. Kadar degradasi juga didapati meningkat apabila didedahkan kepada cahaya ultra lembayung. Kajian keterlarutan BPQ dalam pelbagai jenis larutan penimbal piawai (pH 1.2 hingga 7.5) telah dikaji dan didapati keterlarutan tertinggi tidak melebihi 210 ng/ml. BPQ didapati stabil dalam bendalir gastrik dan usus simulasi, di mana degradasi adalah kurang dari 5%. Keputusan ini mencadangkan bahawa BPQ akan tetap stabil dalam saluran perut usus sebelum penyerapan berlaku. Ketelapan atenolol and propranolol telah dikaji dalam model tikus *in situ* pada kepekatan DMSO yang berbeza (1-5%). Tiada perubahan signifikan ($p > 0.05$) ke atas nilai purata petunjuk-petunjuk ketelapan tersebut. Nilai yang didapati ini adalah sejajar dengan nilai ketelapan yang dilaporkan sebelum ini. Keputusan kajian ini menunjukkan integriti

membran tisu usus tikus tidak dipengaruhi oleh kehadiran DMSO sehingga sebanyak 5%. Drug yang berketerlarutan rendah dalam air sering memberi masalah dalam kajian ketelapan kerana keterlarutan yang rendah dan penyerapan tidak spesifik menjadi punca kepada anggaran ketelapan yang tidak tepat. Oleh kerana BPQ adalah suatu sebatian yang berketerlarutan rendah dalam air, maka kajian ketelapan dilakukan dengan kehadiran 5% DMSO sebagai pelarut bersama. Nilai ketelapan purata bagi BPQ adalah 0.912×10^{-4} cm/s dan ini menunjukkan bahawa ia adalah sebatian jenis kelas II, di mana ia adalah berketelapan tinggi walaupun berketerlarutan rendah berbanding kepada Sistem Klasifikasi Biofarmaseutikal (SKB). Sistem Penyampaian Drug Swa-Mikroemulsifikasi (SPDSM) merupakan suatu formulasi oral yang boleh digunakan untuk drug kelas II SKB untuk meningkatkan keterlarutan dan biokeperolehan. Perkembangan dan penyaringan formulasi oral SPDSM telah dilakukan berdasarkan kepada keputusan kajian keterlarutan pelbagai eksipien, gambarajah fasa untuk penentuan kawasan swa-mikroemulsifikasi, analisis saiz butiran dan masa emulsifikasi. Pelarutan *in vitro* dan kajian farmakokinetik telah dilakukan ke atas formulasi yang optimum yang terdiri daripada Capryol 90 (9.82 %w/w) dan campuran Cremophor EL/Labrasol (4:1) (88.40 %w/w) dan BPQ 1.78 %w/w. Formulasi ini menghasilkan saiz butiran yang terkecil berukuran 18 nm. Kaedah analisis fasa terbalik HPLC-UV yang berbeza telah diperkembangkan dan disahsahkan untuk penentuan BPQ dalam pelbagai matriks biologi. Kajian biokeperolehan BPQ telah dilakukan dalam arnab jantan putih New Zealand. Parameter farmakokinetik telah ditentukan untuk kedua-dua intravena dan formulasi oral bagi BPQ dan nilai-nilai C_{\max} , T_{\max} dan $AUC_{(0-36)}$ adalah 999.83 ng/ml, 0.25 jam, 2872.71 ng.jam/ml dan 171.74 ng/ml, 7.33 jam, 3456.38 ng.jam/ml masing-masing. Biokeperolehan mutlak adalah 40.10 %.

EVALUATION OF PERMEABILITY AND BIOAVAILABILITY OF A LEAD ANTILEISHMANIAL COMPOUND BUPARVAQUONE IN ANIMAL MODELS

ABSTRACT

Leishmaniasis is a group of parasitic disease caused by the protozoan parasites of the genus *Leishmania* and is currently endemic in 88 countries. This disease occurs in two major forms, cutaneous and visceral leishmaniasis. Pentavalent antimonials are the most widely used therapy for all forms of the disease. In *in vitro* and *in vivo* studies buparvaquone (BPQ) has been found to be a promising lead compound for the treatment of *Leishmania donovani* infections. The successful development of oral drug delivery formulation for BPQ requires determination of the biopharmaceutical properties mainly solubility and permeability. In preliminary stability studies, the test samples of BPQ in methanol and acetonitrile form unknown degradation compounds when exposed to the light and the rate of degradation was increased in the presence of UV light. BPQ solubility studies were carried out in various standard buffer solutions (pH 1.2 to 7.5) and the solubility was found to be not more than 210 ng/ml. BPQ was stable in both simulated gastric and intestinal fluids with < 5% degradation. These results suggest that BPQ would be stable in the gastrointestinal tract prior to absorption. Permeability of atenolol and propranolol were carried out in rat *in situ* model at various concentrations of dimethyl sulfoxide (DMSO, 1-5%). There was no significant alteration ($p>0.05$) in the mean values of the permeability markers. The values agreed with previously reported permeability values. The results indicate that the membrane integrity of the rat intestinal tissues was not affected by the presence of up to 5% DMSO. Poorly water soluble drugs often cause difficulties in permeability studies due to their poor solubility limitation and non-specific

adsorption which lead to inaccurate estimation of permeability. Since BPQ is a poorly water soluble compound the permeability study was carried out in the presence of 5% DMSO as a co-solvent. The mean permeability value for BPQ was 0.912×10^{-4} cm/s indicating that it is a class II compound which is a highly permeable and poorly water soluble compound according to the biopharmaceutical classification system (BCS). Self-Microemulsifying drug delivery system (SMEDDS) is a feasible oral formulation approach for BCS class II drugs since it increases their solubility and bioavailability. Oral SMEDDS formulation development and screening was done based on results obtained from solubility in various excipients, phase diagrams for identifying microemulsification region, droplet size analysis and emulsification time. *In vitro* dissolution and pharmacokinetic studies were performed with the optimized formulation which is composed of Capryol 90 (9.82% w/w) and mixture of Cremophor EL/Labrasol (4:1) (88.40% w/w) and BPQ (1.78% w/w). This formulation yielded the smallest droplet size of 18 nm. Different RP-HPLC-UV analytical methods were developed and validated for determining BPQ in the various biological matrices. Bioavailability study of BPQ was conducted in healthy white New Zealand male rabbits. Pharmacokinetic parameters were calculated for both i.v and the optimized oral formulation of BPQ and the mean C_{max} , T_{max} and $AUC_{(0-36)}$ were 999.83 ng/ml, 0.25 hour, 2872.71 ng.hour/ml and 171.74 ng/ml, 7.33 hour, 3456.38 ng.hour/ml respectively. Absolute bioavailability was found to be 40.10%.

CHAPTER 1

INTRODUCTION

Tropical diseases are infectious diseases that occur uniquely in tropical and subtropical regions. In the Special Programme for Research and Training in Tropical Diseases of the World Health Organization (WHO/TDR), various international/national bodies and philanthropic foundations focus on neglected infectious diseases that mainly affect poor and marginalized populations in the developing regions of America, Asia, and Africa. Neglected Tropical Diseases (NTDs) cause approximately 534,000 deaths annually. These include bacterial, protozoan and helminth tropical infectious diseases such as leishmaniasis, African trypanosomiasis, dengue fever, malaria, schistosomiasis, tuberculosis, chagas disease, leprosy, lymphatic filariasis and onchocerciasis. Although leprosy, tuberculosis, cholera and yellow fever do not occur in the tropics their highest incidence in the tropics justify their inclusion in tropical diseases (Nwaka and Hudson, 2006; Hotez et al., 2007).

Some tropical diseases are very rare but they may occur in sudden epidemics, such as the ebola hemorrhagic fever, marburg haemorrhagic fever and lassa fever. There are hundreds of different tropical diseases which are less known such as oropouche fever, lobomycosis, west nile disease, labrea fever, rocio disease, mapucho hemorrhagic fever, trachoma, guinea worm disease and chikungunya (Tropical disease, 2008).

1.1 Leishmaniasis

1.1.1 Epidemiology

Leishmaniasis is a major widespread parasitic disease and is caused by the protozoan haemoflagellates of the genus *Leishmania*. There are at least 20 species of leishmania which are transmitted by 30 species of sandflies and each may cause disease specific to the species and the host response. It is endemic in many parts of the world and the geographical distribution of the disease is shown in Figure 1.1. There are several forms of the disease named by their clinical presentation including cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL). Each of these forms of disease is caused by different species of sandflies found in different regions of the world. Table 1.1 shows the common species that cause leishmaniasis in different geographical locations. Old World CL in humans is associated with members of *L. aethiopica*, *L. major*, and *L. tropica* complex while *L. mexicana* and *L. braziliensis* complex transmit New World CL. VL is caused by *L. donovani* and *L. infantum* in the Old World regions while *L. chagasi* is primarily responsible for visceral disease in the New World (Herwaldt, 1999; Bailey and Lockwood, 2007; Davies *et al.*, 2003).

The disease is prevalent worldwide and threatens 350 million men, women and children, affecting 88 countries of which 72 are developing countries and 13 of them are among the least developed. High occurrence of VL is found in 65 countries and 90% of VL cases occur in poor rural and suburban areas of 5 countries namely Bangladesh, India, Nepal, Sudan and Brazil. Majority of CL cases occur in 7 countries namely Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria

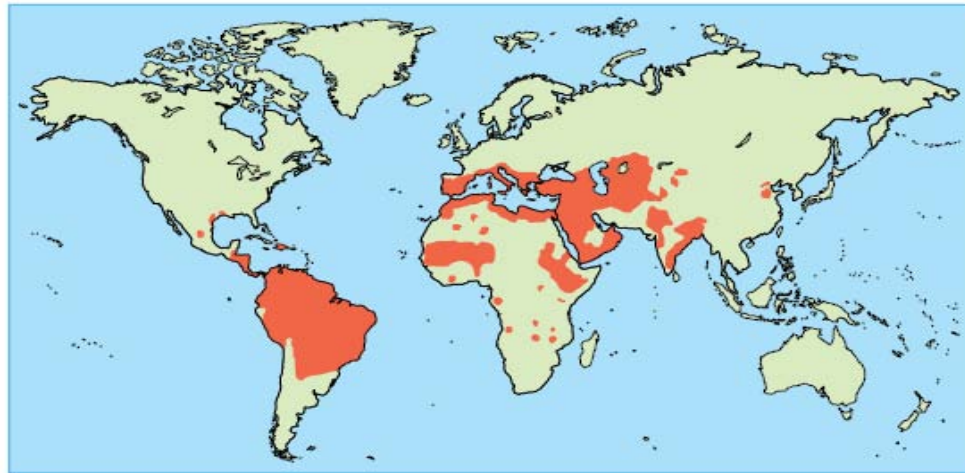


Figure 1.1 Global distribution of *leishmaniasis* (Davies *et al.*, 2003).

Table 1.1 Leishmaniasis disease patterns and common organisms in different geographical locations.

Clinical disease	Leishmaniasis species	Geographical location
Cutaneous leishmaniasis	<i>L. tropica</i> complex: <i>L. tropica</i> , <i>L. aethiopica</i> and <i>L. major</i> .	Old World
	<i>L. mexicana</i> complex: <i>L. Mexicana</i> , <i>L. pifanoi</i> , <i>L. amazonensis</i> , <i>L. garnhami</i> and <i>L. venezuelensis</i> .	New World
	<i>L. braziliensis</i> complex: <i>L. peruviana</i> , <i>L. guyanensis</i> , <i>L. panamensis</i> , <i>L. lainsoni</i> and <i>L. colombiensis</i> .	New World
	<i>L. infantum</i>	Old World
	<i>L. chagasi</i>	New World
Visceral leishmaniasis	<i>L. donovani</i> complex: <i>L. donovani</i> , <i>L. infantum</i>	Old World
	<i>L. chagasi</i>	New World
	<i>L. tropica</i>	Old World
	<i>L. amazonensis</i>	New World
Mucocutaneous leishmaniasis	<i>L. braziliensis</i> complex: <i>L. braziliensis</i> , <i>L. guyanensis</i> and <i>L. panamensis</i>	New World
	<i>L. Mexicana</i>	New World
	<i>L. tropica</i> and <i>L. major</i>	Old World

Old World: N.Africa, South Asia, Middle East, Tunisia Ethiopia, Kenya, India, Sudan, Bangladesh, China, Central and West Asia, Central and South America, Mexico, Belize, Guatemala and Ecuador.

New World: Venezuela, Brazil, Peru, French Guiana, Guyana, Surinam, Panama and Costa Rica

and 90% of MCL occur in Bolivia, Brazil and Peru (Bailey and Lockwood, 2007; Piscopa and Mallia, 2006).

1.1.2 Vector transmission

The leishmania species belongs to the class *Kinetoplastida* and family *Trypanosomatida*. The sandflies (2-3 mm long) which are the vectors that transmit the disease are from genus *Phlebotomus* in the Old World and *Lutzomyia* and *Psychodopygus* in the New World. Sandflies are relatively weak, noiseless fliers; they rest in dark and moist places and are typically more active in the evening and night. These vectors are nocturnal by nature and it is the females that prey on blood. The parasites are introduced into the host (human) through the bite of an infected female sandfly (Herwaldt, 1999).

1.1.3 Life cycle

During the life cycle, the parasite exists in two forms; amastigote form in human and promastigote form in sand fly. The various stages of the leishmania life cycle is shown in Figure 1.2. The female sandflies inject the infective stage metacyclic promastigotes (elongated motile and with an anterior flagellum) during blood meals (1). Metacyclic promastigotes that reach the puncture wound are phagocytized by macrophages (2) and undergoes transformation into amastigotes (3). Amastigotes (2-3 μm in diameter) multiply by binary fission in the macrophages and proceed to infect other mononuclear phagocytic cells (4). These amastigotes will affect different tissues of the body, depending on which leishmaniasis species is involved. These differing tissue specificities cause the different clinical manifestations of the various

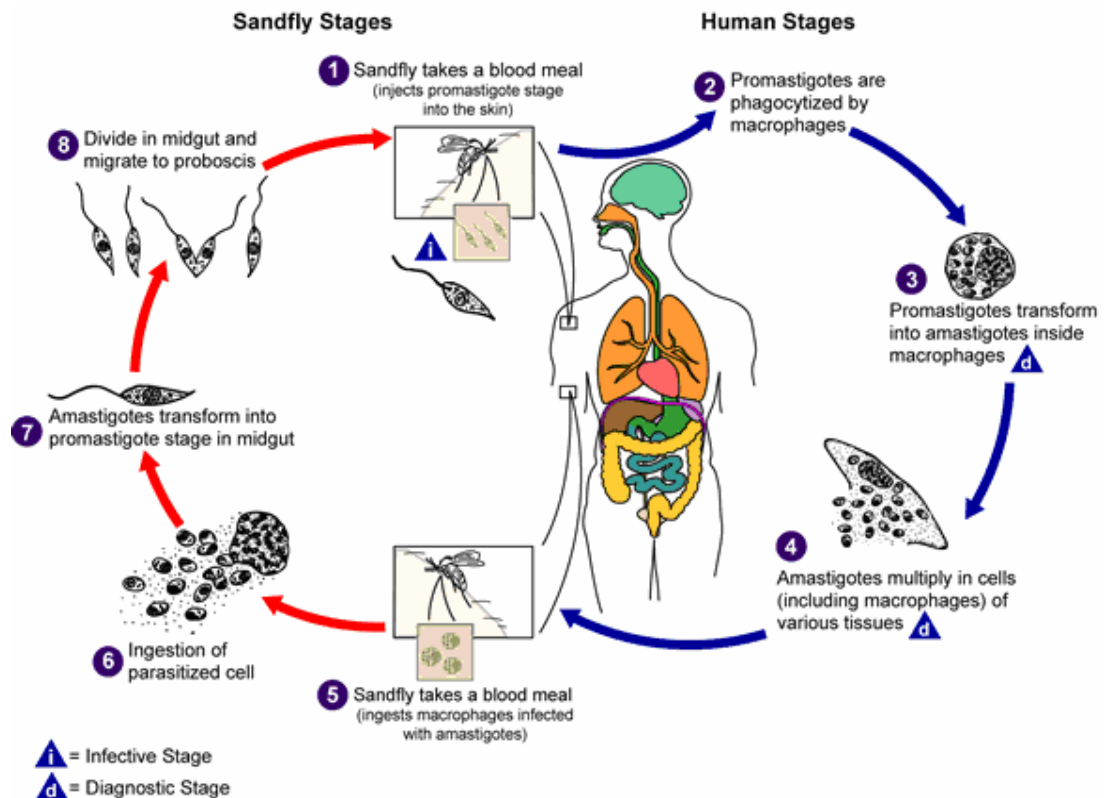


Figure 1.2 *Leishmania* life cycle (Source: <http://en.wikipedia.org>).

forms of leishmaniasis. Sandflies become infected during blood meals from an infected host when they ingest macrophages infected with amastigotes (5, 6). In the sandfly midgut, the parasites differentiate into promastigotes (7), which multiply, differentiate into metacyclic promastigotes and migrate to the pharynx and proboscis (8) (Leishmaniasis, 2007; Chappuis *et al.*, 2007).

1.1.4 Clinical manifestations

1.1.4 (a) Visceral leishmaniasis or Kala-azar

VL comprises a broad range of manifestations of infection. The clinical symptoms are characterized by fever, weakness, night sweats and weight loss and these symptoms continue over weeks to months. Lymphadenopathy, hyperpigmentation

and hepatosplenomegaly (spleen much larger than the liver) are of common occurrence in VL. Anaemia, leucopenia, or thrombocytopenia, and hypergammaglobulinaemia are also frequently manifested symptoms. Untreated or progressing disease can produce profound cachexia, multisystem disease, bleeding from thrombocytopenia, susceptibility to secondary infections and death may rapidly follow (Chappuis *et al.*, 2007; Singh *et al.*, 2006).

1.1.4 (b) Cutaneous and mucocutaneous leishmaniasis

Cutaneous forms of the disease normally produce skin ulcers on the exposed parts of the body such as face, arms and legs. It starts as a papule at the site of a bite, increasing in size, causing ulcer. It may take 3-18 months to heal in typical cases. The incubation period lasts from 2 weeks to months and cases up to 3 years have been reported in Old World CL. In New World CL, the incubation period is usually 2-8 weeks (Piscopa and Mallia, 2006). In the Old World CL most of the lesions appear in the form of nodules, papules or nodule ulcers, but most New World CL may begin with ulcerative lesions. In MCL the lesions affect the mucocutaneous membranes of the nose, mouth, and throat cavities causing partial or total destruction of the surrounding tissues (Bailey and Lockwood, 2007; Desjux, 2004). The incubation period for MCL is 1-3 months, but it may occur many years after the initial cutaneous ulcer has healed. Mucosal type occurs in South American cases of CL (espundia). This causes difficulty in eating and an increased risk for secondary infection, which carries considerable mortality (Piscopa and Mallia, 2006).

1.1.5 Diagnosis

1.1.5 (a) Visceral leishmaniasis

Diagnosis of VL is routinely based on microscopic detection of amastigotes in smears of tissue aspirates or biopsy samples. An aspirate or biopsy of the bone marrow is frequently the tissue of choice, with sensitivities in the 55-97% range. The gold standard for diagnosing VL is parasite identification in tissue smears with splenic aspirate being more sensitive than bone marrow or lymph node aspirates. Lymph node aspirate smears (sensitivity 60%) or biopsy, and splenic aspirates (sensitivity 97%) may also be taken for diagnosis. The parasite may also be cultured from microscopy negative tissue samples on special media such as Novy, McNeal or Nicolle medium or inoculated into animals such as hamsters (Singh *et al.*, 2006). Direct Agglutination Test (DAT) is commonly used diagnosis for VL and it is easy to use in the field. DAT has been shown to be sensitive and quite specific (sensitivity of 91-100% and a specificity of 72-100%). However its use is limited due to the non availability of standardized antigen and adaptability especially in the rural centres. Immunochromatographic rapid strip testing of blood from a finger prick for leishmanial rK39 antibody has been used successfully in the field of serodiagnosis with a sensitivity of 90-100% in patients showing symptoms. Leishmania DNA can be detected in the tissue aspirates and peripheral blood using polymerase chain reaction (PCR), with sensitivities in the range of 70-93% in peripheral blood. PCR and *in situ* hybridisation have been used to amplify DNA sequences of the parasite in biopsy material and is highly sensitive for the diagnosis of VL and can be used to monitor therapeutic response (Piscopo and Mallia, 2006; Chappuis *et al.*, 2007; Singh *et al.*, 2006). A new commercial latex agglutination test (KATEX) was used for preliminary testing to detect the leishmanial antigen in patient's urine with

sensitivity in the range of 68-100% and 100% specificity. However, this test is still under the preliminary phase evaluation and its commercialization is still far from reality (Singh *et al.*, 2006; Davies *et al.*, 2003).

1.1.5 (b) Cutaneous leishmaniasis

Tissue aspirate, scraping and fresh skin biopsies are employed frequently to obtain samples. Microscopical examinations of skin scrapings or biopsy specimens taken from the edge of lesions are used for the diagnosis of CL. This method is rapid and economical but has limited sensitivity, especially for chronic lesions. Monoclonal antibodies are used for identification, but a direct analysis of clinical specimens is better achieved using PCR. Antibody detection is less sensitive due to the lack of significant antibody production in CL. Other means of diagnosing CL include the Montenegro (leishmanin) skin test, which detects specific cutaneous delayed-type hypersensitivity. It involves intradermal injection of *L.mexicana* antigen, and monitoring for a local reaction. Limitations of this test include its inability to distinguish between current and past infection, as well as report of false positivity in other skin infections (Piscopo and Mallia, 2006; Bailey and Lockwood, 2007).

1.1.6 Current chemotherapy for cutaneous and visceral leishmaniasis

Currently available drugs which are used for treatment of leishmaniasis include meglumine antimonite (Glucantime, Aventis), sodium stibogluconate (Pentostam, Glaxo SmithKline), amphotericin B(Bristol-Myers Squibb), amphotericin B lipid formulation (AmBiosome®, Gilead) and pentamidine (Aventis).

1.1.6 (a) Pentavalent antimonials

For the past 50 years, treatment for both CL and VL was mainly dependent on pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Figure 1.3). The antimonials were introduced in 1945 and they are effective for the treatment of some forms of leishmaniasis. In recent years it has been found that there is an increase in the parasite resistance for these drugs. These antimonials are less effective against VL and MCL in the Bihar state of India because of poor pharmacokinetics and variation in the species sensitivity, all these factors limiting the drugs usefulness. The standard therapy for both VL and CL involves daily intravenous injections for 20 days of sodium stibogluconate or meglumine antimoniate at a dose of 5 mg on first day and increased to maximum total dose of 850 mg/day (Fernando *et al.*, 2001; Croft and Coombs, 2003; Ouellette *et al.*, 2004).

1.1.6 (b) Pentamidine

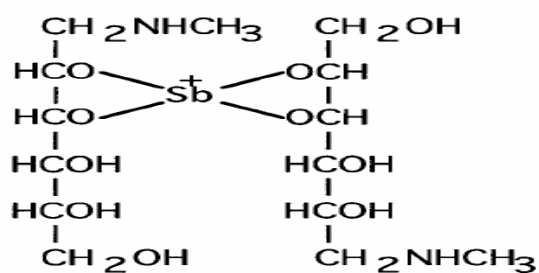
Pentamidine isethionate (Figure 1.3) is an antiprotozoal agent introduced in 1952 and is used as a second-line antileishmanial drug for both CL and VL when antimonials are resistant to the parasite. The effective treatment for CL was found to be at the dose of 2 mg/kg administered intravenously on alternate days for 14 days. The treatment for VL which produces a cure rate of 70% requires a dose of 4 mg/kg on alternate days for a total of 40 days. However pentamidine has limitations in its use as an antileishmanial drug due to considerable toxicity such as hypotension, hypoglycemia, and nephrotoxicity. (Fernando *et al.*, 2001; Ouellette *et al.*, 2004).

1.1.6 (c) Amphotericin B

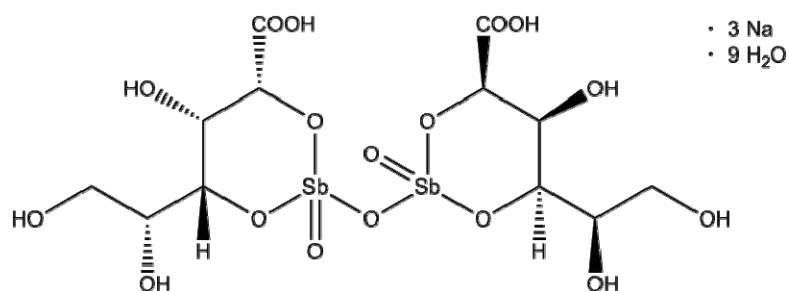
Amphotericin B (Figure 1.3) is an antifungal agent recommended as a second-line drug for the treatment of VL, CL and MCL. Amphotericin B was administered at a dose of 0.75-1.0 mg/kg on alternate days for 30 days. Amphotericin B usage is limited by its narrow therapeutic index and the side effects are fever, hypotension, liver failure and blood dyscrasias. (Singh *et al.*, 2006). However the efficacy of Amphotericin B has been improved by the development of a liposomal formulation which is rapidly effective and non toxic and has been approved by FDA for treatment of VL in adults. It is given by IV at 3 mg/kg/day for 30 days for children with drug resistance VL (Croft *et al.*, 2005; Kafetzis *et al.*, 2005; Murray, 2004).

1.1.6 (d) Other drugs and physical treatment methods

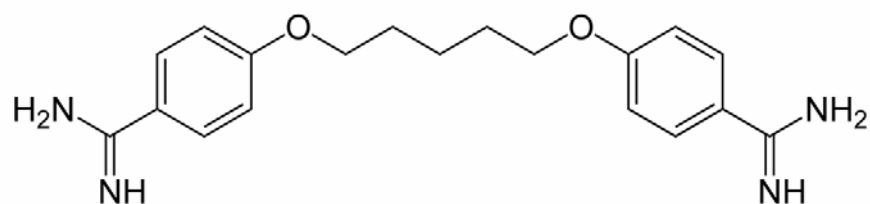
12% methybenzethonium chloride in soft paraffin is used for CL and the duration of application depends on the strain of parasite. Oral medications such as dapsone, allopurinol, ketaconazole, levamisole and rifampicin are also used for the treatment of CL. The dose of dapsone, allopurinol, ketaconazole, levamisole and rifampicin is 1-2 mg/kg/day for six weeks, 20 mg/kg/day for 15 days, 10 mg/kg in three divided doses for 4 weeks, 150 mg/day on two successive days each week for 8 weeks and 10 mg/kg for 6 -12 weeks respectively. However these drugs have side effects such as hepatocellular changes, allergic reactions, vomiting, nausea and abdominal pain. In case of rifampicin, the body fluids become red. Allopurinol and ketaconazole or both in combination are also used for the treatment of VL (Fernando *et al.*, 2001).



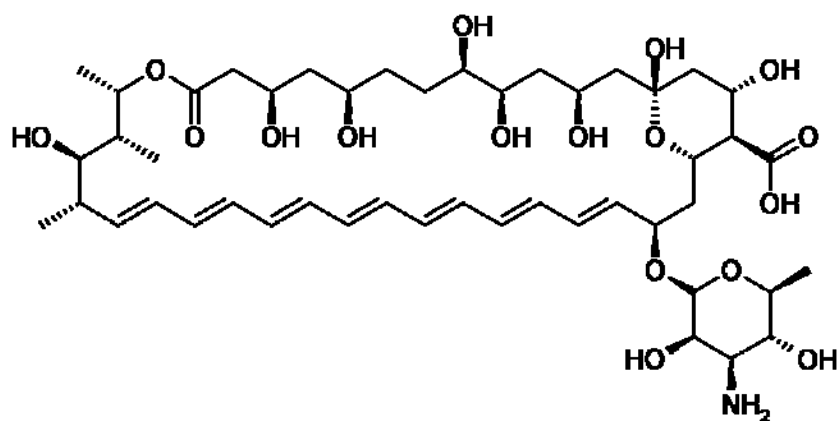
(a)



(b)



(c)



(d)

Figure 1.3 Chemical structures of (a) meglumine antimoniate, (b) sodium stibogluconate, (c) pentamidine and (d) amphotericin B.

Physical treatments like cryotherapy and thermotherapy have been used for the treatment of cutaneous lesions for duration of 4 weeks with a success rate from 77% to 100%. However their use is limited because of availability of equipment, secondary infections, slower healing of large scars and poor compliance (Bailey and Lockwood, 2007).

1.1.7 Immunotherapy

Active VL causes a defect in the immune system by causing a deficiency in interferon- γ production in the body. In this case, rational therapeutic approach of treatment would involve the stimulation of the immune system. For AIDS patients with VL, pentavalent antimoniate and interferon- γ with a dose of 100-400 $\mu\text{g}/\text{m}^2$ body surface/day is recommended for a course of 10-40 days. However the treatment is still in its early stages and requires further development. (Fernando *et al.*, 2001).

1.1.8 Drugs in development

1.1.8 (a) Paramomycin

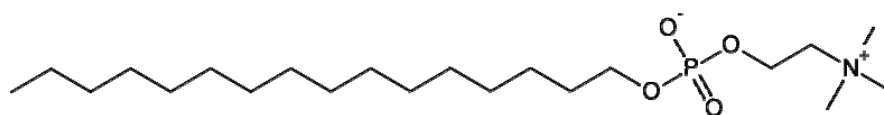
Paramomycin is a non toxic aminoglycoside aminocyclitol antibiotic which is an effective for the treatment of both VL and CL and is currently in clinical trials. A suitable monotherapy regimen of paramomycin sulphate is 16 mg/kg, administered intramuscularly for 21 days (Singh *et al.*, 2006). An ointment with 15% paramomycin and 0.5% gentamicin has been shown to be 100% effective for CL in BABL/c mice (Davis and Kedzierski, 2005).

1.1.8 (b) Miltefosine

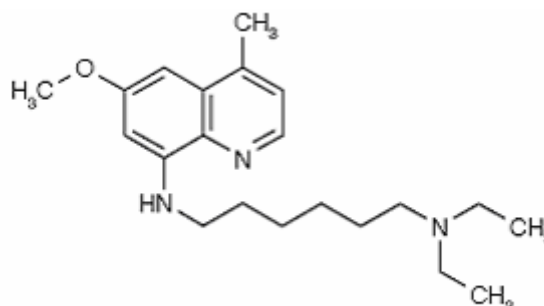
Miltefosine (Figure 1.4) is an alkylphosphocholine originally developed as an anticancer agent. Miltefosine has shown efficacy in several *in vitro* and *in vivo* experimental models as an effective oral drug for the treatment of VL. These findings led to clinical trials and registration of miltefosine for oral treatment of VL in India in March 2002 and for CL in Colombia in 2005. In phase 3 trials miltefosine cured 94 % of VL patients with a dose of 2.5 mg/kg for 28 days. Cutaneous lesions were healed with topical application of 6% miltefosine ointment for 2-5 weeks (Croft and Engel, 2006; Croft and Coombs, 2003). A study was conducted in VL male patients in India to evaluate the patients' fertility and results showed that there were no clinically relevant changes on patients' fertility. The major limitation of miltefosine is teratogenicity and is therefore not recommended for women in child-bearing age. (Sindermann and Engel, 2006).

1.1.8 (c) Sitamaquine

Sitamaquine (Figure 1.4) is a primaquine analogue (8-aminoquinolene) and was developed as an alternative oral drug by Walter Reed Army Institute of Research/Glaxo SmithKline. An initial phase I/II study showed 67% cure rate in patients treated with a dose of 2 mg/kg/day for a duration of 28 days in Brazil and 92% cure rate when treated with 1.7 mg/kg daily for 28 days in Kenya for VL (Davis and Kedzierski, 2005; Dietze *et al.*, 2001). Sitamaquine has mild toxicity and causes methemoglobinaemia (Croft *et al.*, 2006).



(a)



(b)

Figure 1.4 Chemical structures of (a) miltefosine and (b) sitamaquine.

1.1.9 Buparvaquone

Buparvaquone (BPQ), 4-hydroxy-3- [(4-tert-butylcyclohexyl) methyl] naphthalene-1, 2-dione (Figure 1.5) is a second-generation hydroxynaphthoquinone related to parvaquone. It has novel features that make it a promising compound for the therapy and prophylaxis of all forms of theileriosis. In *in vivo* studies BPQ showed approximately twenty times more activity than parvaquone and it has been tested widely against *Theileria annulata*, *T. parva* and *T. sergenti* both in laboratory studies and in field trials. In an acute oral toxicological study in rat, the observed LD₅₀ value for BPQ was more than 8000 mg/kg which showed BPQ has low toxicity (McHardy, 1988). Currently BPQ has been formulated as a solution for intramuscular injection (Butalex, Coopers Animal Health). The Butalex injection is a safe, convenient and an additional alternative drug product to the existing antitheilerial products (McHardy *et al.*, 1985).

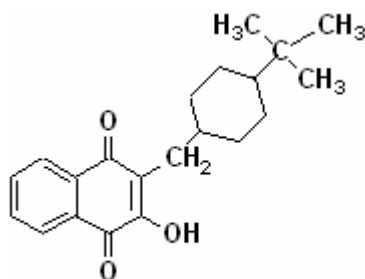


Figure 1.5 Chemical structure of buparvaquone (BPQ).

As part of a programme to find new drugs for treatment of leishmaniasis, a series of hydroxynaphthoquinones were tested against *Leishmania donovani* *in vitro* and *in vivo*. *In vitro* experiments showed BPQ to be 100 fold more active against *L. donovani* amastigotes than other hydroxynaphthoquinones with ED₅₀ value of 0.005 μ M. However, *in vivo* experiments performed in BABL/c mice model with oral and subcutaneous routes with a dose of 100 mg/kg BPQ for five days resulted in suppression of only 35 to 62% liver amastigotes. This low activity was considered to be due to its poor distribution from the site of injection and poor oral bioavailability due to insufficient absorption (Croft *et al.*, 1992). Butalex was injected intramuscularly to seven dogs which were symptomatic and parasitologically positive for canine visceral leishmaniasis with 4 doses of 5 mg/kg over a 12 day period. Only two dogs showed minor clinical improvement and as a consequence it was concluded that BPQ was ineffective for the treatment of symptomatic visceral leishmaniasis (Vexenat *et al.*, 1998).

BPQ has very poor aqueous solubility (<0.03 μ g/ml), high lipophilicity (at pH 3.0 Log D -7.02) and a pKa value of 5.70. The low solubility and high lipophilicity are the most probable reasons for the low *in vivo* activity of BPQ against leishmaniasis in oral drug delivery. Furthermore BPQ metabolizes more actively with mouse and

dog hepatic microsomes in the presence of NADPH when compared to human hepatic microsomes. There is no reported pathway available in literature on the metabolism of BPQ (Mantyla *et al.*, 2004a; Croft *et al.*, 1992).

A pharmacokinetic study has been reported for BPQ and parvaquone in cattle in which BPQ was administered into the neck muscles at a dose of 2.5 mg/kg. There was no significant variation in absorption and distribution rates for both drugs but a wide variation was observed in the mean plasma concentrations for BPQ (50 to 280 ng/ml). The average elimination half life was 26.44 hours for BPQ and there was no intravenous data for calculating bioavailability (Kinabo and Bogan, 1988).

1.1.10 Buparvaquone-oxime derivatives

Mantyla *et al.* (2004a) reported that BPQ was active in *in vitro* studies against both amastigotes and promastigotes in low nanomolar range but *in vivo* results were disappointing. This finding led to the synthesis of various oxime derivatives of BPQ as novel antileishmania compounds. The buparvaquone oxime containing C=NOH group undergoes a CYP-450 dependent oxidative cleavage to BPQ (Figure 1.6). Buparvaquone oxime has better aqueous solubility (5.42 µg/ml) when compared to BPQ and other oxime derivatives such as buparvaquone-O-methyloxime and O-methly-buparvaquone oxime. Buparvaquone oxime derivatives have shown low *in vitro* activity against intracellular leishmania donovani amastigotes when compared to BPQ. The lower activity of these buparvaquone oxime compounds is most probably due to their slow conversion to BPQ during the experiment.

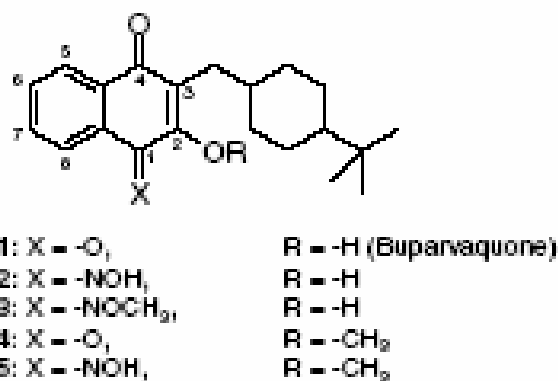


Figure 1.6 Chemical structures of buparvaquone oxime derivatives.

1.1.11 Water soluble prodrugs of buparvaquone-oxime and buparvaquone-O-methyloxime

The phosphate prodrug approach is a widely used strategy to improve aqueous solubility of drugs containing hydroxyl group. Thus researchers combined the oxime and phosphate prodrug strategies for improving the aqueous solubility of BPQ. Novel water soluble phosphate prodrugs of buparvaquone-oxime and buparvaquone-O-methyloxime were synthesized and all the prodrugs have good aqueous solubility (Figures 1.7 and 1.8). The most promising prodrug is 3-(*trans*-4-*tert*-butyl-cyclohexylmethyl)-2-hydroxy-[1,4]naphthoquinone-1-(*O*-phosphonooxymethyl)oxime or (Phosphonooxymethyl-buparvaquone-oxime) (Figure 1.7 b). It has good aqueous solubility (4.2 mg/ml) and chemical stability over the pH range of 3.0-7.4 and rapidly releases the parent buparvaquone oxime via chemical or enzymatic bioconversion by alkaline phosphatases ($t_{1/2}$ 4.1 min) (Figure 1.9). Parent buparvaquone-oxime has been suggested to be able to efficiently permeate through the intestinal wall and form BPQ after oxidative metabolism (Mantyla *et al.*, 2004b).

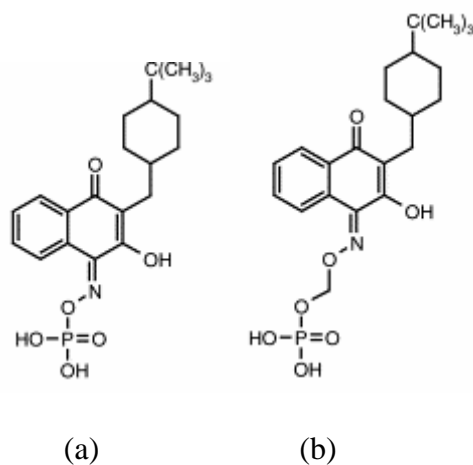


Figure 1.7 Chemical structure of water soluble prodrugs of BPQ (a) 3-(*trans*-4-*tert*-butyl-cyclohexylmethyl)-2-hydroxy-[1, 4] naphthoquinone-1-(*O*-phosphono oxime) and (b) 3-(*trans*-4-*tert*-butyl-cyclohexylmethyl)-2-hydroxy-[1,4] naphthoquinone-1-(*O*-phosphon ooxymethyloxime).

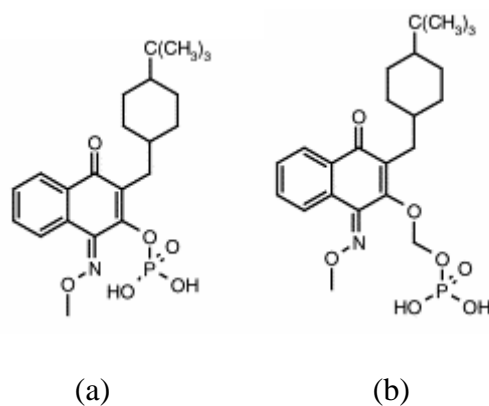


Figure 1.8 Chemical structure of water soluble prodrugs of BPQ (a) 3-(*trans*-4-*tert*-butyl-cyclohexylmethyl)-2-phosphono-[1,4]naphthoquinone-1-(*O*-methyloxime) and (b) 3-(*trans*-4-*tert*-butyl-cyclohexylmethyl)-2-phosphonoox ymethoxy - [1,4]naphthoquinone-1-(*O*-methyloxime).

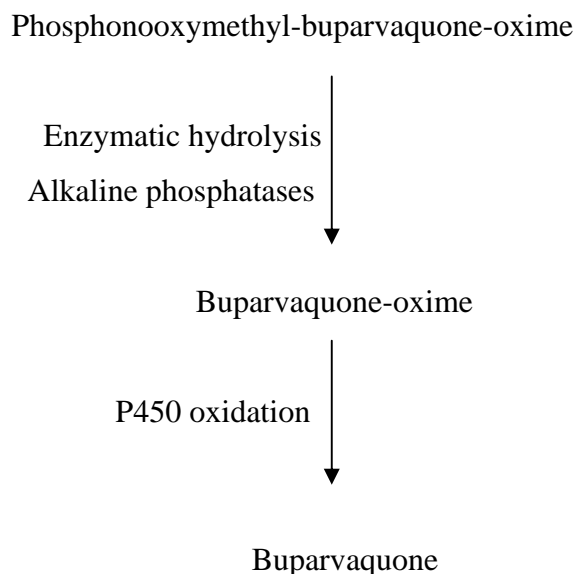


Figure 1.9 Schematic flow chart for formation of BPQ from buparvaquone-oxime phosphate prodrugs.

1.1.12 Buparvaquone water soluble phosphate prodrugs

Prodrugs release the parent drug during or after absorption by enzymatic or chemical hydrolysis. However a phosphate group that is directly attached to a hydroxyl group is not always cleaved enzymatically due to steric hindrance. Therefore researchers focussed on designing enzymatically labile phosphate prodrugs which release the drug as BPQ by hydrolysis. Aqueous solubility of the prodrugs buparvaquone-3-phosphate and 3-phosphonooxymethyl-buparvaquone is more than 3.5 mg/ml over the pH range of 3.0-7.4 (Figure 1.10). These phosphate prodrugs are sufficiently stable in the gastrointestinal tract before their absorption and rapidly hydrolyze ($t_{1/2}$ =1.2 and 3.8 min) to the parent compound in the presence of alkaline phosphatase (Mantyla *et al.*, 2004).

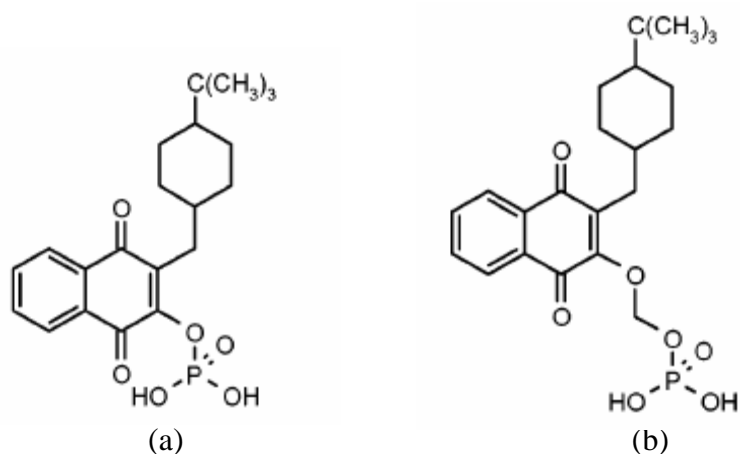


Figure 1.10 Chemical structures of water soluble phosphate prodrugs of BPQ: (a) buparvaquone-3-phosphate and (b) 3-phosphonooxymethyl-buparvaquone

In *in vitro* activity studies phosphate prodrugs have been shown to have potent activity in the nanomolar range against the various species of the promastigotes and amastigotes due to their conversion to BPQ. Of these two phosphate prodrugs 3-phosphonooxymethyl-buparvaquone is the more potent antileishmanial compound. Human skin permeation of BPQ can also be significantly improved by using phosphate prodrugs. 3-phosphonooxymethyl-buparvaquone was shown to be rapidly converted to the parent drug during skin permeation studies (Mantyla *et al.*, 2004; Garnier *et al.*, 2007).

BPQ and phosphate prodrugs were evaluated by *in vivo* studies for both VL and CL. BPQ (hydrous gel and water-in-oil emulsion formulations) and phosphate prodrugs (anhydrous gel formulation) significantly reduce the parasite burden and lesion size when compared to the untreated control for CL. These findings suggest that BPQ and phosphate prodrugs are effective for the treatment of CL. In visceral model BPQ was prepared as a solution in isopropyl myristate and polyethylene glycol 400 and the

phosphate prodrugs were prepared in 50 mM sodium acetate buffer (pH 5.0). Prodrugs caused a significant decrease in the liver parasite burden when compared to BPQ. These results suggest that BPQ and phosphate prodrugs need to be investigated further for the treatment of VL (Garnier *et al.*, 2007a).

1.2 Solubility and gastrointestinal stability

In the drug discovery process, the lead candidates must have proper physicochemical properties in order to have a better chance of success in the development. Solubility, pKa (ionization constant) and lipophilicity are the most fundamental physicochemical properties of a drug candidate and the determination of these parameters are essential in both *in silico* and *in vitro* models. Based on Biopharmaceutical Classification System (BCS), the drug bioavailability mainly depends on the aqueous solubility and the ability of molecule to permeate through the biological membrane (Figure 1.11). Many pharmacologically active compounds fail to become pharmaceutical drugs because of poor bioavailability and unacceptable physicochemical properties. As a consequence, solubility and permeability have been incorporated into drug discovery programs (Delaney, 2005; Huuskonen, 2001).

BCS recommends determining the solubility of drug substances under physiological pH conditions. The experiments should be carried out at 37 ± 1 °C in aqueous media with a pH range of 1.2 to 7.5. To obtain accurate solubility data, the pH solubility profile of the compound should be determined based on its pKa. Solubility should be determined at pH= pKa, pH= pKa +1, pH= pKa -1, pH 1 and 7.5 (USFDA, 2000).

<p>Class I</p> <p>High Solubility</p> <p>High Permeability</p>	<p>Class II</p> <p>Low Solubility</p> <p>High Permeability</p>
<p>Class III</p> <p>High Solubility</p> <p>Low Permeability</p>	<p>Class IV</p> <p>Low Solubility</p> <p>Low Permeability</p>

Figure 1.11 Biopharmaceutics Classification System.

Prior to permeability studies, it is necessary to determine the stability of the drug in gastric and intestinal (G.I) fluids. These results will assist in obtaining accurate permeability values. When conducting permeability studies in *in vivo* or *in situ* models there is possibility for degradation of the drug in the G.I fluids prior to absorption which will give rise to erroneous permeability values. Hence it is necessary to determine the stability of the drug in both gastric (pH 1.2) and intestinal (pH 6.8) conditions.

USFDA, (2000) recommends conducting G.I stability by incubating the drug solution in these fluids at 37° C for a period that is representative of *in vivo* drug contact with these fluids. The experiments should be conducted for 1 hour in gastric fluids and for 3 hours in intestinal fluids.

1.3 Intestinal permeability

A major objective in the pharmaceutical industry is to develop new drugs with good oral bioavailability. The new chemical entities are first selected on the basis of their

pharmacological activity followed by sequential profiling to assess the solubility, absorption, distribution, metabolism, elimination and toxicity for lead selection and optimization (Trapani *et al.*, 2004; Li, 2004).

Drug bioavailability mainly depends on the aqueous solubility and the ability of the molecule to permeate through the biological membrane. Hence, the extent of *in vivo* absorption of the drug can be assessed based on solubility and *in vitro* permeability. Thus determination of intestinal permeability is one essential requirement for assessing the oral bioavailability of a new drug candidate at the early drug discovery stage (Volpe, 2008).

Absorption is defined as the movement of unchanged drug from the site of administration into the systemic circulation. The small intestine, which consists of duodenum, jejunum and ileum is the most important site for drug absorption in the gastrointestinal tract. The small intestine permits the absorption of organic and inorganic chemicals/nutrients such as sugars, amino acids, lipids and vitamins and but prevents the entry of xenobiotics and digestive enzymes (Venkateshwarlu, 2004).

Intestinal membrane is not a unicellular structure but it is a group of unicellular membranes parallel to one another. For drugs and nutrients to be absorbed into the systemic circulation they must penetrate the intestinal mucosa. The intestinal mucosa consists of three layers: muscularis mucosa, lamina propria and epithelial cell layer. A basement membrane is present below the epithelial cell layer and lamina propria lies between the basement membrane and muscularis mucosa, which contains connective tissue, blood and lymph vessels (Figure 1.12). The epithelial cell layer which

possesses kerkrings, villi and microvilli provides a large surface area for drug absorption (Venkateshwarlu, 2004; Hiremath, 2000).

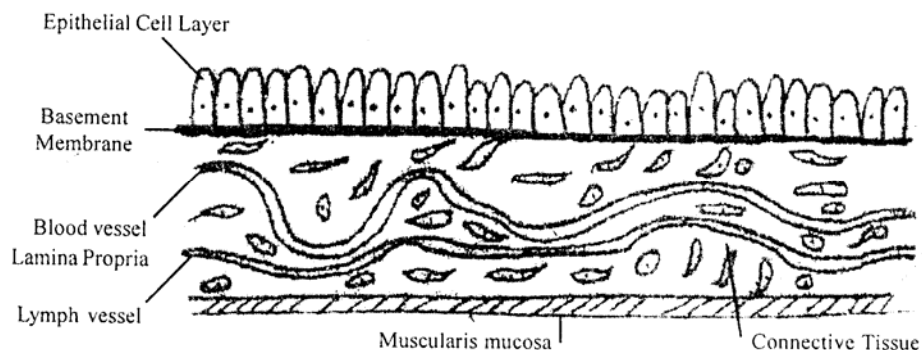


Figure 1.12 Anatomical details of intestinal mucosa (Source: Venkateshwarlu, 2004).

Drug molecules penetrate most of the barriers in the intestine and reach the lamina propria. From this site, molecules either may diffuse through the blood stream or penetrate the central lacteal and enter the lymph. Most of the drugs reach the systemic circulation via the blood stream of the capillary network in the villi. The villi are highly and rapidly perfused by the blood stream with the blood flow rate of approximately 500 to 1000 times greater than lymph flow rate. Therefore the lymphatic system will account for only a small fraction of total amount of drug absorbed compared to the systemic circulation. The capillary and lymphatic vessels are permeable to most low molecular weight and lipid-soluble compounds. However capillary membrane is a more substantial barrier than the central lacteal for the permeation of large molecules (Venkateshwarlu, 2004; Hiremath, 2000).

1.3.1 Permeability pathways

For orally administered drugs to exert pharmacological activity, they must dissolve and permeate through several cellular membranes in the intestinal wall in order to